Toxicity of Scooter Exhaust Emissions

Graduate School for Cellular and Biomedical Sciences

University of Bern

PhD Thesis

Submitted by

Loretta Lina Müller

from Basel

Thesis advisors

Prof. Dr. Peter Gehr and PD Dr. Barbara Rothen-Rutishauser

Institute of Anatomy

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Front page background picture

Schematic overview of the scooter exposure system (published in Muller et al, 2010, Environmental Science & Technology, 44:2632-2638).

Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

For my family.

Jede Antwort wirft zehn neue Fragen auf.

(Christina Brandenberger)

Content

Summary

The number of registered scooters, the small two-wheeled vehicles with a maximal speed of 45 km/h and an engine capacity lower than 50 cm^3, is increasing year by year. Depending on the technology, two-stroke scooters can emit high amounts of carbon monoxide (CO), nitrogen oxides (NO_x), hydrocarbons (HCs), polycyclic aromatic HCs (PAHs) and particulate matter (PM) mainly in the nano-scaled size range. Compared to normal passenger cars, the emissions can be so high that scooters have to be treated as so-called superpolluters.

In a first project the toxicity of different engineered nanoparticles and diesel exhaust particles (DEP) in different cell cultures and especially the differences between mono- and co-cultures was evaluated. It could be shown that the interplay of different lung cell types modulate substantially the oxidative stress and (pro-) inflammatory responses upon DEP exposure (Project 1).

In order to develop a method to evaluate the toxic potential of scooter exhaust emissions, a new exposure system was constructed and established (Project 2). After the removal and the dilution (1:100) of the exhaust sample, the exhaust emissions were CO_2 enriched (5%), humidified (85% relative humidity) and heated (37.5°C). The diluted exhaust emissions passed the exposure chamber where the cell cultures were placed. Mono-cultures of human alveolar epithelial A549 or of human bronchial epithelial 16HBE14o$^-$ cells, as well as a co-culture model of the human epithelial airways consisting of an epithelial cell layer, human monocyte-derived dendritic cells at the basal side and human monocyte-derived macrophages at the apical side were used. The cell cultures were pre-exposed to the air-liquid interface for 24h and then exposed to the exhaust emissions by diffusion processes. In parallel to the exposure to exhaust emissions, cell cultures were exposed to reference air (filtered ambient air, treated similar to the exhaust emissions). In addition a negative control (cells left in the incubator) was included. For the establishment of the exposure system, cell cultures were exposed to the exhaust emissions for one or two hour time periods followed by a post-incubation for zero, four, eight, twelve or twenty-four hours. A two-stroke direct injection Peugeot scooter with "worst case" conditions (normal fuel, army oil, normal oil ratio, dummy muffler) was used. For the cell analysis, the cell morphology and the tight junction arrangement, the cytotoxicity and the (pro-) inflammatory response were measured.

The analysis of the establishment and optimization of the exposure system showed that the triple cell co-cultures with 16HBE14o$^-$ cells exposed to the exhaust emissions during two hours and post-incubated for eight and twenty-four hours presented the highest differences between reference and exposed cells. For all further comparisons of the toxic potential of different vehicles, the previously mentioned settings were used.

In the third project various settings of two different two-stroke scooters (carburetor and direct injection), a four-stroke scooter and a passenger diesel car with and without a diesel particle filter were tested. The applied settings were worst case (same as in the establishment experiments of the exposure system), worst case – filtered (filtering out of the particles after the removal of the exhaust), best case (Aspen fuel, Motorex oil, only 50% oil ratio, oxidative catalyst, wire mesh filter catalyst) and absolute best case (Aspen fuel, Motorex oil, only 50% oil ratio, coated particle filter). The overall toxic potential was highest for carburetor worst case conditions, followed by direct injection worst case, the four-stroke scooter and the passenger diesel car. The technical optimizations for the two-stroke scooters, especially the coated particle filter, reduced the toxic potential to a lower level than for diesel cars. For the passenger diesel car, the particle filter did not reduce the toxic potential. The particle number concentration was found to be the most relevant parameter for the toxic potential.

The newly developed exposure system can be used for standardized testing of different exhaust emissions and for the evaluation of the effects on the toxic potential of single technical optimizations. The toxic potential of two-stroke scooters was higher than of four-stroke scooters and diesel cars and can be reduced with technical optimizations, which should be introduced.

Abbreviations

CO	carbon monoxide
CO_2	carbon dioxide
DEP	diesel exhaust particles
ESI	electron spectroscopic imaging
HC	hydrocarbons
IL-8	Interleukin 8
MDDC	monocyte-derived dendritic cells
MDM	monocyte-derived macrophages
NO_x	nitrogen oxides
NP(s)	nanoparticle(s)
PAH	polycyclic aromatic hydrocarbon
PM	particulate matter
TEM	transmission electron microscopy
TNFα	tumor necrosis factor alpha
TSDI	two-stroke direct injection

1 General Introduction

Humans have always been mobile. In the Stone Age people walked, in the Middle Ages they rode horses and later in the year 1769, when James Watt substantially improved the efficiency of the Newcomen steam machine, the mobility of human beings took a huge step forward: motorized mobility was born. However, with this promising motorization, problems were simultaneously created. The release of exhaust emissions, which are produced mainly by incomplete combustion processes by the motorized vehicles, was found to contaminate the ambient air and to be harmful not only for human health. Leonardo da Vinci (1452-1519, a famous painter, engineer, architect and anatomist) already realized the adverse effects of dust and noted as a comment to a sketched trachea in his Anatomical Notebook “dust is harmful”.

The mobility of human societies is still increasing and to be able to travel at any time to any place is taken as a matter of course. The development of new transport vehicles is a continuing process and will change mobility furthermore in the future. Today the variety of motorized vehicles ranges from electrical bikes to mopeds, scooters, motor-bikes, personal cars, buses, trucks and ships to airplanes.

This PhD thesis focuses on the toxic potential of scootersand diesel car exhaust emissions in order to contribute facts to the discussion about possible measurements to reduce the release of pollutants into the ambient air and therefore reduce the adverse health effects in humans.

1.1 Relevance of Scooter Exhaust Emissions

Scooters, the small two-wheeled vehicles with an engine capacity lower than 50 cm^3 and a maximal speed of 45 km/h, are becoming more and more popular. The number of registered scooters in Switzerland increases year by year and reached the level of about 250’000 in 2008 (source: Swiss Statistics, www.admin.ch). The situation in some Asian countries is even more extreme. For example, in India even by 2003 more than 50 millions scooters were in use (Government of India, 2004). The mobility advantages of scooters in urban areas, as well as their low prices, cause the assumption that the number of scooters is likely to continue to grow.

Different scooter technologies are available on the market. There are carburetor scooters and direct injection scooters, and additionally there is the differentiation

between two- and four-stroke scooters. A two-stroke engine works – like the name indicates - with only two phases in the cycle (http://en.wikipedia.org/wiki/Two-stroke):

(1) During the first phase, the piston is moving up in the cylinder, the air-fuel-mixture is compressed and a spark plug ignites the compressed mixture. Under the piston, the new fresh air-fuel-mixture is sucked into the sealed crankcase.

(2) The second phase is the scavenging phase. The piston moves down in the cylinder and opens the exhaust and transfer ports. The exhaust gas escapes because of higher pressure. The new fresh air-fuel-mixture in the crankcase is compressed due to the moving down of the piston and arrives from the transfer channel in the cylinder, rinsing the rest of exhaust gas. Then the cycle begins again.

A four-stroke engine is more complex and thus also more expensive. The cycle is divided into four phases (http://en.wikipedia.org/wiki/Four-stroke):

(1) Intake stroke: the air-fuel-mixture is sucked into the cylinder.

(2) Compression stroke: is characterized by closed intake and exhaust valves and the returning piston that squeezes the mixture. Before reaching the Top Dead Center the mixture is ignited by a spark plug.

(3) Power stroke: the combustion drives the piston back.

(4) Exhaust stroke: During the last phase the exhaust valve is open and the combustion products are pushed out.

The difference between the carburetor and the direct injection technology is based on the method of the fuel nebulization. While the direct injection technology atomizes the fuel by pumping it with high pressure through a small nozzle directly into the cylinder, the carburetor technology works with low pressure and an intake air stream that rushes through the fuel and adds it to the air stream (http://en.wikipedia.org/wiki/Two-stroke).

These different working principles of the various scooter technologies result in different compositions of the exhaust emissions. Carburetor exhaust emissions contain high amounts of carbon monoxide (CO) and direct injection emissions contain high concentrations of nitrogen oxides (NO_x). For both technologies hydrocarbons (HC), polycyclic aromatic HCs (PAHs) and particulate matter (PM) in the nanoscale range are typically released at high amounts (Czerwinski and

Schramm, 2006; Rijkeboer et al, 2005; Rüdy and Weilenmann, 2006). The exhaust emissions are mostly higher than the emissions of a normal diesel passenger car with Euro 3 standard. Therefore, in certain major cities all over the world, scooters are significant contributors to PM, PAH and CO air pollution (McDonald et al, 2005). Furthermore, scooters are discussed to be so-called superpolluters[1] (McDonald et al, 2005; Siegmann et al, 2008). A reduction of these superpolluters by an improved technology would decrease the amount of air pollution with a relatively low effort.

As the European Union and Switzerland constantly renew the legislation regarding emission limit values, the discussion about an adaptation of the limit values is on going. The current European legislation for scooters (EURO 2 standard, directive 97/24/EC, chapter 5, annex I) includes only limit values for CO (1 g/km) and HC plus NO_x (1.2 g/km), but not for PM or PAHs.

In a previous project at the Bern University of Applied Sciences in Biel, Switzerland, different two-stroke scooter technologies were compared with respect to their exhaust emissions. In addition, the effects of technical optimizations were measured. With the use of different fuels, oils, oil ratios and technical measures, such as wire mesh filter catalyst or particle filters, the emissions could strongly be reduced (Czerwinski et al, 2006; Czerwinski and Schramm, 2005; Czerwinski and Schramm, 2006). This fact awaked the interest regarding how this emission reduction would affect the toxicity of the scooter exhaust.

1.2 The Lung as the Relevant Portal of Entry for Air Pollutants

Every human has to breathe. With every breath more than one million particles are inhaled which result in an amount of about 3000 million inhaled particles per day (reviewed in Gehr et al, 2010). The important question from a biological point of view is now: What happens with this huge amount of inhaled particles in the lung?

The majority of inhaled particles, about 70%, are exhaled again with the next breath. Overall only about 30% of the inhaled particles follow the airflow into the respiratory tract and are deposited in the lung (reviewed in Gehr et al, 2010). Depending on the aerodynamic diameter, particles are deposited in different regions of the respiratory tract (Figure 1).

[1] Superpolluters are defined as a source type which is only present in a small percentage of the total vehicle fleet but contributes strongly to the air pollution (adapted from Siegmann et al, 2008).

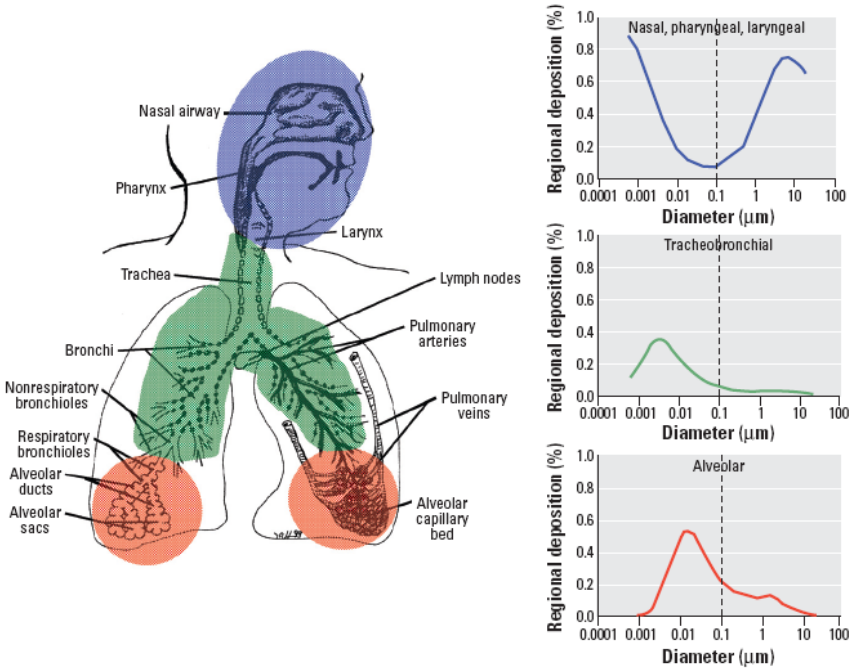

Figure 1. Theoretical fractional deposition of inhaled particles in the different regions of the human respiratory tract during nose breathing depends on the aerodynamic diameter. (Oberdorster et al, 2005)

Particles with an aerodynamic diameter > 10 µm do not enter the lung and are filtered out in the nasopharyngeal region of the respiratory tract. Particles with a diameter < 10 µm can enter the conducting airways. The conducting airways are branched irregularly dichotomously and get thinner towards the peripheral regions of the lung (Ochs and Weibel, 2008). The smallest particles even enter the respiratory region of the lung – where the gas exchange takes place – beginning with the first alveoli appearing at the airway wall and ending in the alveolar region (Weibel, 2009). Not only the diameter of the airway tubes changes but also the airway wall, lined out by an epithelium without any interruptions from the trachea to the alveolar region, shows adaptations to their physiological function (Figure 2). At the level of the trachea and bronchi, the epithelium is pseudostratified with a thick layer of connective tissue and smooth muscles containing glands and capillaries and a columnar epithelial cell layer with goblet cells and a mucus layer on the top. Furthermore cartilage is found in the airways wall to form the shape. Deeper in the lung, in the region of smaller bronchioles, the connective tissue and the smooth muscle layer become thinner and the cartilage is not present anymore. The epithelial cells are now cuboidal and the mucus layer is thinner as well. The mucus layer is covered by a surfactant (surface active agent) film. Finally, in the alveoli, the epithelium becomes extremely thin (Ochs

and Weibel, 2008). The alveolar region is lined out by squamous cells, e.g. the alveolar type I cells, which are interrupted by cuboidal type II epithelial cells (surfactant producing cells). The connective tissue is now reduced to an absolute minimum and the smooth muscle layer has totally disappeared. As the main function of the alveolar region is the exchange of oxygen from the inhaled air and carbon dioxide (CO_2) from the blood, the thickness of the air-blood-barrier is minimized to an approximately 2 µm thin tissue layer, which allows efficient diffusion of the gases (Gehr et al, 1978). In reality, the construction of the airway/alveolar wall is even more complex than shown in Figure 2. It is not only fibroblasts, muscle cells, gland cells, different epithelial and goblet cells which are part of the airway wall, but in total there are more than 40 different cell types, including endothelial cells, nerve cells, lymphoid cells, dendritic cell, and macrophages, with various functions which add to the complexity of the epithelium in the airways (Ochs and Weibel, 2008).

When investigating the inhalation of gases, the situation is different compared to that for particles. Gases are not filtered out by the airway branches. All inhaled gases reach the alveolar region, even though the gases are diluted with the air in the dead space and the concentration reaching the alveoli is lower than the inhaled concentration.

The lung has a huge internal surface area of about 150 m^2 (Gehr et al, 1978) and is in constant and direct contact with the ambient air. Therefore, it is the major portal of entry for gaseous and aerosolized pollutants. Other exposure routes such as the skin and the gastrointestinal tract are not considered in this study.

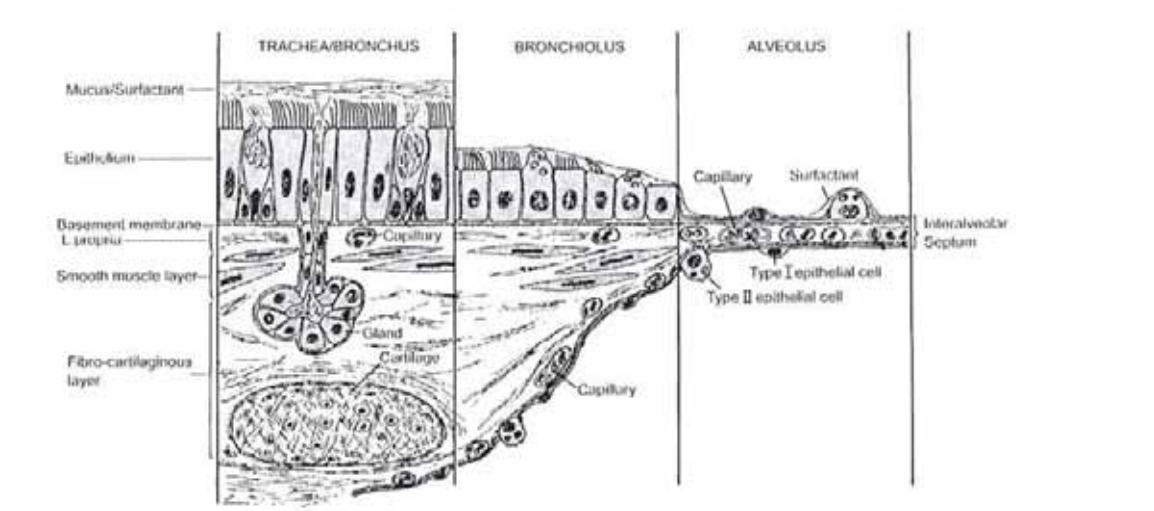

Figure 2. The changing structure of the airway wall at three levels in the respiratory tract (Ochs and Weibel, 2008).

1.3 Particle-Lung Interaction

Exhaust emissions consist of particulate and gaseous compounds. Whereas the contact of the gases with lung structures occurs over normal gas diffusion processes, the interaction of the particulate compounds with the lung is a complex issue. Although a large amount of the inhaled particles are exhaled without deposition, the remaining inhaled particles are conducted to the gas exchange area of the respiratory tract and in this way the particles can come into contact with the airway wall via diffusion processes (Ochs and Weibel, 2008). After being deposited on the airway or alveolar wall, the particles first come in contact with surfactant. Surfactant is located at the air-liquid interface of the liquid lining layer covering the cells at the luminal side of the airway wall. It is composed of around 90% lipids and 10% proteins and its function is to keep the alveoli open, dry and clean (Ochs and Weibel, 2008). Once a particle touches the surfactant, it is displaced into the aqueous lining layer by surface forces (wetting forces) and may interact with the cells (Schurch et al, 1990). After getting in contact with cells of the airway and alveolar wall, particles may be taken up by the cells or may even be translocated across the epithelial cell layer into capillaries where it may reach other organs via the bloodstream (Elder et al, 2006; Kreyling et al, 2002; Mühlfeld et al, 2008).

In order to prevent the human body from unintentionally inhaled particles, the respiratory tract has two main types of defense mechanisms: non-cellular components and cellular components. Non-cellular mechanisms include sneezing, coughing and also the mucociliary clearance in the upper airways (Kilburn, 1968; Mühlfeld et al, 2008). Cell-based defense mechanisms include the continuous epithelium with the tight junctions, adherens junctions and desmosomes to tighten the cell layer (Ochs and Weibel, 2008), professional phagocytic cells (such as macrophages) (Brain, 1988; Lehnert, 1992) and professional antigen-presenting cells (such as dendritic cells) (Holt and Schon-Hegrad, 1987; McWilliam et al, 2000). Although, these defense mechanisms present effective working barriers, particles are able to overcome them. The mechanisms are not yet clear, but particles can probably cross the epithelial layer in a paracellular manner via transcytosis (Oberdorster et al, 2005) or via the cells of the immune system (e.g. macrophages or dendritic cells) (Blank et al, 2007; Blank et al, 2010).

1.4 Impacts of Exhaust Emissions and Diesel Exhaust Particles (DEP) on Human Health

While the comment of Leonardo da Vinci ("dust is harmful") only represented an observation, today we know that he was right. Several epidemiological studies have shown an increased mortality of humans exposed to PM with an aerodynamic diameter < 10 µm (PM_{10}) (Peters et al, 1997; Wichmann et al, 2000). Associations between exposure to PM_{10} and DEP and pulmonary and cardiovascular diseases have been found in different studies and give evidence for involved mechanisms of oxidative stress and (pro-) inflammatory responses (Brunekreef and Holgate, 2002; Pope et al, 2002; Pope et al, 2004; Samet et al, 2000). Furthermore, PM_{10} and DEP have been found to be toxic to lung cells and oxidative stress (Brauner et al, 2007; Xiao et al, 2003) and (pro-) inflammatory responses (Becker et al, 2005; Donaldson et al, 2005) were identified as possible mechanisms for this toxicity. Studies investigating the adverse respiratory effects of different size fractions of ambient particles showed stronger effects for ultrafine particles (< 100 nm in diameter) than for fine particles (< 2.5 µm in diameter) (Peters et al, 1997; Wichmann et al, 2000). This indicates the importance of considering the particle size and not only the particle mass.

In addition to the adverse health effects of PM and DEP, gaseous compounds of exhaust emissions, for example CO or NO_x, are also known to be associated with the development of pulmonary and cardiovascular diseases (Ackermann-Liebrich et al, 1997; Braun-Fahrlander et al, 1997; Ghosh et al, 2010), and oxidative stress and (pro-) inflammatory responses are thought to be involved in the disease development (Brandsma et al, 2008; Chhikara et al, 2009; Lighty et al, 2000; Sevastyanova et al, 2007; Solovey et al, 2010).

1.5 Cell culture models

Various methods exist to analyze possible toxic effects of xenobiotics. (1) Epidemiological studies represent the real exposure for humans, but can only look retrospectively to the past. (2) *In vivo* studies with humans have to use small concentrations, whereas animal studies are often carried out with higher concentrations and the problem of overload situations has to be avoided. Furthermore, because the effects on human beings are of interest, the results have

to be extrapolated from the animals to humans which is not always very simple to perform. For *in vivo* testing with humans and animals, specific ethical qualifications have to be considered. (3) The third category is referred to as *ex vivo* exposures. Whole tissues from animals or from humans can be cultivated *in vitro* and exposed either submerged or, in the case of the lung, at the air-liquid interface directly to exhaust emissions. In the case of submerged exposure, the situation does not represent the real *in vivo* situation in the lung where the tissue is exposed to air. In addition the gaseous compounds can not be included in these studies, and furthermore the particle characteristics may be changed during the process of collection and re-suspension (Teeguarden et al, 2007). Moreover, the particle dosage, which comes in contact with the tissue, can not be precisely determined. Furthermore, depending on the preparation methods, it is not only the tissue parts exposed to exhaust emissions which are also in reality in contact with exhaust emissions, but also other parts, e.g. connective tissue. The handling and the cultivation of the removed tissue is also extremely complex. When animal tissue is used the extrapolation between animals and humans has to be done additionally. (4) The final biological exposure mode is *in vitro* testing. Several culture conditions have been described for lung cell cultures: submerged and direct exposure and mono- and co-culture systems. When the cell cultures, either mono- or co-cultures, are exposed submerged to particles, the same constrictions as mentioned under (3) for the submerged *ex vivo* exposure situations have to be considered. Studies comparing mono- and co-cultures have shown differences in the reactions upon particle exposure (Alfaro-Moreno et al, 2008; Lehmann et al, 2009; Lehmann et al, 2010; Muller et al, 2010b; Rothen-Rutishauser et al, 2008). (5) A fifth method to investigate the possible toxic effects of xenobiotics is the use of cell-free assays (Foucaud et al, 2007; Rothen-Rutishauser et al, 2010). With cell-free assays only short-term effects can be investigated and not long-term effects. Furthermore, the extrapolation from cell free assays to humans is even more complex than from *in vivo* and *in vitro* exposures.

In vitro cell culturing allows studies to be performed at the cellular and subcellular level in a well standardized and well defined environment. In order to test different vehicle emissions and their influence on lung cells *in vitro* mono-cultures and co-cultures offer suitable tools.

Following these considerations, we used the well established and characterized *in vitro* triple cell co-culture model of the airway epithelial barrier (Blank et al, 2007; Rothen-Rutishauser et al, 2005). The model consists of human epithelial cells from a cell line (16HBE14o$^-$ or A549), human blood monocyte-derived dendritic cells (MDDC) at the basal side and human blood monocyte-derived macrophages (MDM) at the apical side of the epithelial cell layer. The model was also characterized for use at the air-liquid interface (Blank et al, 2006; Blank et al, 2007; Brandenberger et al, 2010b).

The here used cell culture model and also other *in vitro* models can not represent the whole complexity of the human airway wall with the connection to the blood and lymph circulation, connective tissue, muscles etc. However it is a valuable tool for screening and for mechanical studies, which tries to consider parts of the complexity.

1.6 Aims

One aim of my PhD study was to complete the work started during my master thesis at the ETH Zurich regarding the differences in the responses of mono- and triple cell co-cultures upon exposure to engineered nanoparticles (NPs) and DEP. This study was performed using monocultures of the alveolar epithelial type II like cell line A549, of MDDC and of MDM, as well as triple cell co-cultures under submersed conditions (Rothen-Rutishauser et al, 2005). The effects of single-walled carbon nanotubes, DEP and titanium dioxide NPs on the production of reactive oxygen species, on the total antioxidant capacity and on the cytokine release (TNFα and IL-8) were investigated. Furthermore, particle entry into the cells was shown by transmission electron microscopy (TEM) (Muller et al, 2010b; 2.1 Project 1).

The main goals of my PhD study were the development and application of an exposure system to evaluate the toxic potential of exhaust emissions in a realistic and applicable way.

In a first step a new exposure system was developed which allows the direct exposure of cell cultures at the air-liquid interface to freshly produced exhaust emissions. The exhaust emission was diluted 1:100, humidified, heated and CO_2 enriched. Different cell cultures, such as monocultures of A549 and 16HBE14o$^-$ epithelial cells, as well as triple cell co-cultures of both epithelial cell lines with MDDC

and MDM, were tested. The establishment of the system was carried out with a two-stroke direct injection (TSDI) scooter of Peugeot with worst case conditions (dummy muffler, normal fuel, Swiss army oil, normal oil ratio). For the estimation of the toxic potential, the endpoints of cytotoxicity and (pro-) inflammatory reactions were considered (Muller et al, 2010a; 2.2 Project 2).

In a second step the toxic potential of different scooters and cars and different technical optimizations were compared. Therefore the TSDI Peugeot scooter, a two-stroke carburetor Peugeot scooter (both with conditions of worst case, worst case – filtered, best case and absolute best case), a Aprilia four-stroke scooter, a diesel car with particle filter and a diesel car without filter were tested (2.3 Project 3).

2 Results

Project 1: Differences in reactions of mono- and co-cultures upon nanoparticle exposure

Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways

Loretta Müller, Michael Riediker, Peter Wick, Martin Mohr, Peter Gehr, and Barbara Rothen-Rutishauser

Published in Journal of the Royal Society Interface 2010, 7:S27-S40

Project 2: Development of an exposure system to estimate the toxic potential of (scooter) exhaust emissions

New Exposure System To Evaluate the Toxicity of (Scooter) Exhaust Emissions in Lung Cells in Vitro

Loretta Müller, Pierre Comte, Jan Czerwinksi, Markus Kasper, Andreas C.R. Mayer, Peter Gehr, Heinz Burtscher, Jean-Paul Morin, Athanasios Konstandopoulos, and Barbara Rothen-Rutishauser

Published in Environmental Science & Technology 2010, 44:2632-2638

Project 3: Comparison of the toxic potential of different vehicles

Toxic Potential of Two- and Four-Stroke Scooter and Diesel Car Exhaust Emissions *In Vitro*.

Loretta Müller, Pierre Comte, Jan Czerwinksi, Markus Kasper, Andreas C.R. Mayer, Peter Gehr, and Barbara Rothen-Rutishauser

Manuscript submitted to Environmental Health Perspectives, June 2010

2.1 Project 1: Differences in reactions of mono- and co-cultures upon nanoparticle exposure

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Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways

Loretta Müller[1,*], Michael Riediker[2], Peter Wick[3], Martin Mohr[4,†], Peter Gehr[1] and Barbara Rothen-Rutishauser[1]

[1]*Institute of Anatomy, Division of Histology, University of Bern, Balzerstrasse 2, 3000 Bern 9, Switzerland*
[2]*Institute for Work and Health, University of Lausanne, Switzerland*
[3]*Laboratory for Materials–Biology Interactions, Empa, Swiss Laboratories for Materials Testing and Research, St Gallen, Switzerland*
[4]*Kantonsschule Frauenfeld, Frauenfeld, Switzerland*

Combustion-derived and manufactured nanoparticles (NPs) are known to provoke oxidative stress and inflammatory responses in human lung cells; therefore, they play an important role during the development of adverse health effects. As the lungs are composed of more than 40 different cell types, it is of particular interest to perform toxicological studies with co-cultures systems, rather than with monocultures of only one cell type, to gain a better understanding of complex cellular reactions upon exposure to toxic substances. Monocultures of A549 human epithelial lung cells, human monocyte-derived macrophages and monocyte-derived dendritic cells (MDDCs) as well as triple cell co-cultures consisting of all three cell types were exposed to combustion-derived NPs (diesel exhaust particles) and to manufactured NPs (titanium dioxide and single-walled carbon nanotubes). The penetration of particles into cells was analysed by transmission electron microscopy. The amount of intracellular reactive oxygen species (ROS), the total antioxidant capacity (TAC) and the production of tumour necrosis factor (TNF)-α and interleukin (IL)-8 were quantified. The results of the monocultures were summed with an adjustment for the number of each single cell type in the triple cell co-culture. All three particle types were found in all cell and culture types. The production of ROS was induced by all particle types in all cell cultures except in monocultures of MDDCs. The TAC and the (pro-) inflammatory reactions were not statistically significantly increased by particle exposure in any of the cell cultures. Interestingly, in the triple cell co-cultures, the TAC and IL-8 concentrations were lower and the TNF-α concentrations were higher than the expected values calculated from the monocultures. The interplay of different lung cell types seems to substantially modulate the oxidative stress and the inflammatory responses after NP exposure.

Keywords: human epithelial airway model; monocultures; triple cell co-cultures; nanoparticles; reactive oxygen species; inflammation

1. INTRODUCTION

Epidemiological studies have shown an association between exposure to particulate matter with a diameter less than or equal to 10 µm (PM_{10}) and adverse health effects such as cardiovascular and cardiopulmonary diseases (Samet *et al.* 2000; Brunekreef & Holgate 2002; Pope *et al.* 2004*b*; Riediker *et al.* 2004). Diesel exhaust particles (DEPs) are an important constituent of PM_{10} and are the main cause of adverse health effects (Lighty *et al.* 2000; Schwartz 2000). Additionally, *in vitro* studies have shown adverse effects of combustion-derived PM_{10} in cultures of different cell types. During

*Author for correspondence (loretta.mueller@ana.unibe.ch).
†Formerly: Laboratory for I.C. Engines, Empa, Swiss Laboratories for Materials Testing and Research, Dübendorf, Switzerland.

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the development of adverse health effects, oxidative stress and inflammatory response play key roles (Brown *et al.* 2001, 2004; Xiao *et al.* 2003; Pope *et al.* 2004*a*; Donaldson *et al.* 2005). Various studies, performed with human lung cells used as monocultures, have shown that particles, especially DEP, can provoke oxidative stress (Xiao *et al.* 2003; Pan *et al.* 2004), induce inflammatory responses (Becker *et al.* 2005) and increase the cell number of human lung epithelium (Bayram *et al.* 2006). Furthermore, it has been shown that there is a link between exposure to diesel soot and lung cancer (Donaldson *et al.* 2005).

Recent studies indicate a specific toxicological effect of inhaled combustion-derived nanoparticles (NPs; diameter less than or equal to 0.1 μm) (Borm & Kreyling 2004). In addition to the generation of NPs produced by combustion processes in large amounts, there are progressively more manufactured NPs (with at least two dimensions less than or equal to 0.1 μm) released into the air, water and soil every year from other sources, such as nanotechnology (Mazzola 2003; Paull *et al.* 2003). Manufactured NPs have also been shown to cause toxicity (Oberdorster *et al.* 2005; Nel *et al.* 2006).

Owing to the direct contact of the lung surface with the inhaled air and the large surface area of the lungs (approx. 150 m^2; Gehr *et al.* (1978)), the effects of inhaled and deposited particles to the lung cells are of particular interest. The surface of the gas exchange area of the lung consists of mainly squamous epithelial cells (type I pneumocytes), which form a tight barrier. These cells are only approximately 0.1 μm in thickness providing a structural lining with a very short diffusion distance for gas exchange between alveolar air and capillary blood. They cover approximately 93 per cent of the alveolar surface, but account for less than 8 per cent of the distal lung cells. Type II epithelial cells are cuboidal in shape and cover approximately 7 per cent of the alveolar surface while making up 16 per cent of the cells in the distal lung (Crapo *et al.* 1982; Stone *et al.* 1992; Ochs & Weibel 2008). In addition to the epithelial cells, there is a population of macrophages (Brain 1988; Lehnert 1992) on the apical side and a population of dendritic cells underneath the airway epithelium (Holt *et al.* 1990). Macrophages are phagocytotic cells, whereas dendritic cells, as the most potent antigen-presenting cells, can initiate the adaptive immune response (Blank *et al.* 2008). Recently, it has been shown that the two immune cell types interact directly with each other as sentinels against fine particulate antigens (Blank *et al.* 2007), or in a paracrine way (Fujii *et al.* 2002). To obtain a more realistic assessment of the effects of nanosized particles on human lung cells, the current study simulated this situation with a co-culture model for the human airway composed of different cell types and not only of monocultures (Tao & Kobzik 2002; Ishii *et al.* 2005; Roggen *et al.* 2006; Alfaro-Moreno *et al.* 2008; Rothen-Rutishauser *et al.* 2008*a*).

The aim of this work was to compare the cellular responses upon particle exposure in monocultures of human epithelial cells (A549 epithelial type II cell line), human monocyte-derived macrophages (MDMs) as well as human monocyte-derived dendritic cells (MDDCs) and in triple cell co-cultures composed of all three cell types (Rothen-Rutishauser *et al.* 2005). By using this cell model, the individual cellular response and the interaction between different cell types could be studied. The study used combustion-derived NPs (DEP) and manufactured NPs (single-walled carbon nanotubes (SWCNTs) and titanium dioxide (TiO_2)). All these NPs have previously been used in our laboratory, and various publications resulted from these experiments (Rothen-Rutishauser *et al.* 2006, 2007*a*, 2008*b*; Wick *et al.* 2007; Helfenstein *et al.* 2008). The intracellular localization of all three particles was assessed by transmission electron microscopy (TEM), and the potential of these particles to induce oxidative stress and an inflammatory reaction was studied in the exposed cell cultures using different cell assay kits. The expected values (such as oxidative stress and inflammatory responses) for the triple cell co-cultures were calculated theoretically from the observed values in the monocultures and compared with the observed values.

2. MATERIAL AND METHODS

2.1. A549 monocultures

The A549 epithelial cell line (Lieber *et al.* 1976) from the American Tissue Type Culture Collection (LGC Prochem, Molsheim, France) was used. The cells (passage numbers 5–38) were handled as described by Rothen-Rutishauser *et al.* (2005). They were maintained in standard tissue culture flasks (25 cm^2, 60 ml, with filter screw cap, sterile; TPP AG, Trasadingen, Switzerland). Cell cultures were kept at 37°C under a 5 per cent CO_2 humidified atmosphere using medium RPMI-1640 (with 25 mM HEPES; Labforce AG, Nuningen, Switzerland) with 10 per cent foetal calf serum (PAA Laboratories, Lucerna–Chem AG, Lucerne, Switzerland), 1 per cent L-glutamine (200 mM stock solution; LabForce AG) and 1 per cent penicillin/streptomycin (10 000 U ml^{-1} penicillin G and 10 000 μg ml^{-1} streptomycin sulphate in 0.85% saline; Gibco BRL, Invitrogen AG, Basel, Switzerland). For splitting and seeding, the cells were detached from the flask with trypsin–EDTA (0.5 g l^{-1} trypsin, 0.2 g l^{-1} EDTA.4Na in Hanks' balanced salt solution (HBSS); Gibco BRL, Invitrogen AG). The cells were counted with a Neubauer counting chamber and diluted to obtain a density of 0.5×10^6 cells ml^{-1}. Two millilitres of the cell suspension was added to cell culture inserts (surface area of 4.2 cm^2, pores with 3.0 μm in diameter, high pore density PET membranes for six-well plates; BD Falcon, BD Biosciences, Basel, Switzerland). The inserts were placed in six-well tissue culture plates (BD Biosciences). Three millilitres of the medium was added individually to the lower chamber of each insert. The medium was changed every 3 days. The cells were kept a total of 7–9 days in culture.

2.2. Monocyte-derived macrophage and monocyte-derived dendritic cell monocultures

The MDMs and MDDCs were obtained from human blood monocytes as described by Rothen-Rutishauser

et al. (2005). First, peripheral blood monocytes were isolated from buffy coats (blood donation service, Bern, Switzerland) by density gradient centrifugation on Ficoll-Plaque (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland). The monocytes were re-suspended in RPMI-1640 with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 10 per cent heat-inactivated human serum (blood donation service). After 2 h in two-chamber slides (Lab-Trek, VWR International AG, Life Science, Lucerne, Switzerland) to allow for adhesion, the non-adherent cells were washed away. The adherent monocytes were cultured in 2 ml RPMI-1640 with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 5 per cent heat-inactivated human serum. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were added to the medium for the generation of MDDCs. For the generation of macrophages, no supplement was added. The monocytes were kept for 7 days in the respective medium to allow for differentiation.

2.3. Triple cell co-cultures

The triple cell co-cultures were prepared as described by Rothen-Rutishauser *et al.* (2005). Briefly, A549 cells were cultured for 7 days on the membranes in inserts. Medium was removed from the upper and lower chambers, and the inserts with the established epithelial cell layer were turned upside down and deposited in sterile Petri dishes (BD Biosciences). During the 7 days in culture, epithelial cells grown in monolayers could traverse the membrane and grow on the bottom side of the membrane. Therefore, the epithelial cells at the bottom side were abraded carefully with a cell scraper. The cell suspension was removed, and the bottom side of the membrane was washed once with RPMI-1640 medium. MDDCs were harvested and 300 µl of the cell suspension was added to the basal side of the inserts turned upside down. The Petri dishes were incubated for 1.5 h before the non-adherent MDDCs were removed and the inserts placed back into the tissue plates with 3 ml of RPMI-1640 supplemented with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 5 per cent heat-inactivated human serum. MDMs were harvested, and 500 µl of the cell suspension was added to the apical surface of the epithelial monolayer. Cells were allowed to attach for 1.5 h, non-adherent cells were washed away and 2 ml of RPMI-1640 supplemented with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 5 per cent human serum was added to the upper chamber. The triple cell co-cultures were kept for 2–24 h at 37°C under a 5 per cent CO_2 humidified atmosphere.

2.4. Particle suspensions and exposures

Particle suspensions were prepared as described in detail by Helfenstein *et al.* (2008). Briefly, SWCNTs produced by arc-discharged evaporation of graphite rods filled with nickel and yttrium powder (diameter approx. 20 nm) were purchased from Yangtze Nanotechnology (Shanghai, China) and handled as described by Wick *et al.* (2007). The SWCNT stock suspensions were then suspended in water containing 40 µg ml^{-1} Tween-80 (Tween-80, cell culture tested, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After centrifugation, two different fractions of SWCNT suspensions were obtained: CNT supernatant (bundles of SWCNTs) and CNT pellet (containing mostly amorphous carbon and catalyst residues such as nickel and yttrium) (Wick *et al.* 2007). These fractions were then added to the cell cultures at a concentration of 30 µg ml^{-1}.

The DEPs (diameter of 15–300 nm with a mode at approx. 60 nm) were collected at a test bench of EMPA (Dübendorf, Switzerland). They were derived from a state-of-the-art heavy-duty engine with six cylinders and a maximum power of 390 kW. The engine lacks any after-treatment systems. This type of engine is typically installed in construction machines. The particle sample was collected at the constant operation mode at 1800 r.p.m. producing a torque of 200 Nm. A small part of the exhaust gas was directed to a dilution tunnel where particle-free air of ambient temperature was added. This aerosol sample diluted by a factor of about 5 was sucked through a glass fibre filter (Pall, T60A20, 70 mm diameter), which retained the particles. The temperature on the filter was approximately 60°C. After conditioning and weighing, the filter was transferred to a sterile 15 ml tube. Sterilized, high-purity water supplemented with 30 mg ml^{-1} Tween-80 was added (2 ml mg^{-1} DEP on the filter). The tube was vortexed, sonicated for 1.5 h and then the filter was removed. To calculate the DEP concentration of the stock solution, a drop of the solution was transferred to a 200-mesh uncoated copper grid and this sample was compared with samples of known DEP concentrations using TEM. The determined concentration of the stock solution was 250 µg ml^{-1}. The DEP suspension was added to the cell cultures at a concentration of 125 µg ml^{-1}.

TiO_2 NPs (titanium (IV) oxide, anatase; Alfa Aesar, Johnson Mathey GmbH, Karlsruhe, Germany; diameter of 20–30 nm) were suspended in sterilized, high-purity water at a concentration of 2.5 mg ml^{-1} TiO_2 and vortexed. TiO_2 NPs were added to the cell cultures at a concentration of 2.5 µg ml^{-1} diluted with serum-free medium.

Before adding the suspensions to the cell cultures, they were sonicated for 2 min and heated to 37°C. For all cultures, the medium in the well or in the upper chamber was removed and 1 ml of the diluted particle suspension was added.

2.5. Transmission electron microscopy and energy-filtered transmission electron microscopy

TEM analysis was performed as described by Rothen-Rutishauser *et al.* (2006). Briefly, cells were fixed with 2.5 per cent glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. The cells were post-fixed with 1 per cent osmium tetroxide in 0.1 M sodium cacodylate buffer and with 0.5 per cent uranyl acetate in 0.05 M maleate buffer. Cells were then dehydrated in a graded series of ethanol and embedded in Epon.

Ultra-thin (less than or equal to 80 nm) sections were cut, transferred onto 200-mesh uncoated copper grids, stained with uranyl acetate, counter-stained with lead citrate and observed with a Philips 300 TEM at 60 kV (FEI Company Philips Electron Optics, Zurich, Switzerland).

For energy-filtered TEM (EFTEM) analysis, the ultra-thin sections were mounted onto 600-mesh uncoated copper grids and observed unstained with a LEO 912 transmission electron microscope (Zeiss, Oberkochen, Germany) using electron energy loss spectroscopy.

2.6. Reactive oxygen species detection

To detect the reactive oxygen species (ROS) in the cells, an ROS detection kit was used (Image-iT LIVE Green Reactive Oxygen Species Detection Kit, Molecular Probes, Invitrogen AG). The kit was applied as described in the manufacturer's protocol. Briefly, intracellular, unspecific ROS were marked with carboxy- H_2DCFDA solution and the cell nuclei with Hoechst 33342 solution. After washing the cells with HBSS (Gibco BRL, Invitrogen AG), the cells were fixed. Medium alone was used as a negative control and with tert-butyl hydroperoxide (TBHP, at a concentration of 100 µM) as a positive control, incubated for the same duration as the samples.

After ROS and cell nuclei labelling had been performed, the cell cultures were fixed by incubating them for 15 min at room temperature in paraformaldehyde (3% in phosphate-buffered saline (PBS; 10 mM, pH 7.4, 130 mM NaCl, Na_2HPO_4, KH_2PO_4)). The paraformaldehyde was removed and PBS was added.

The cells were finally mounted in PBS : glycerol (2 : 1) containing 170 mg ml^{-1} Mowiol 4-88 (Calbiochem, VWR International AG, Life Sciences, Dietikon, Switzerland) on object holders covered with cover slips (Rothen-Rutishauser *et al.* 2005).

The cell culture samples were imaged using a Leitz DMDR fluorescence microscope (Leica Microsystems (Schweiz) AG, Glattbrugg, Switzerland) with an Olympus Digital Camera (C-3000 Zoom, with the objective C3030-ADU). The ROS signal was taken with a standard fluorescein isothiocyanate (FITC) filter. The Hoechst signal of the cell nuclei was imaged with a 4′, 6-diamidino-2-phenylindole (DAPI) filter.

The imaging and the processing were done with analySIS software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and included the overlapping of the single photos taken with the FITC filter and the DAPI filter.

2.7. Total antioxidant capacity

The total antioxidant capacity (TAC) (including vitamins, proteins, lipids, glutathione and uric acid) was detected with the Antioxidant Assay Kit (Cayman Chemical, Chemie Brunschwig AG, Basel, Switzerland) as described in the manufacturer's protocol with some small adaptations. Briefly, the cells were washed three times with PBS, scraped off from the inserts and transferred to Eppendorf tubes. After centrifugation (Eppendorf Minispin Plus, 3000 r.p.m., 10 min, 6°C), the supernatant was removed, 100 µl cold buffer was added and the cell solution was stored on ice. Afterwards, the samples were vortexed for a short time and sonicated for 2 min in ice water. Then the samples were centrifuged for a second time at 12 200 r.p.m. at 6°C for 15 min. The supernatant was finally transferred to another Eppendorf tube and the samples were frozen at −70°C.

The test followed the manufacturer's instructions. In brief, the reagents and the standard solutions were prepared. The prepared kit solutions, metmyoglobin and chromogen, were added to each well of the 96-well plate. As standard solutions, different concentrations of Trolox (a water-soluble tocopherol analogue) were used. The samples were added to the wells. Each sample and standard was measured as a duplicate. At the end, hydrogen peroxide was added as fast as possible with a multichannel pipette. The plate was covered and incubated on a shaker at room temperature. Exactly 5 min after the addition of hydrogen peroxide, the measurement in a microplate reader (Benchmark Plus Microplate Spectrophotometer; BioRad, Hemel Hempstead, UK) was initiated.

The resulting TACs are indicated in Trolox equivalents. High values of TAC represented upregulated antioxidative defence systems, indicating an induction of oxidative stress. The test was repeated twice for each sample.

2.8. Cytokine/chemokine detection

Following particle incubation, the supernatants of the cell cultures out of the two-chamber slides (MDM and MDDC monocultures) or of the upper and lower chamber (A549 monocultures and triple cell co-cultures) were collected separately and stored at −70°C. After centrifugation, the cytokine tumour necrosis factor (TNF)-α and the chemokine IL-8 concentration were quantified by a commercially available DuoSet ELISA Development kit (R&D Systems, catalogue number: DY 210, respectively, DY 208, Oxon, UK) performed according to the manufacturer's recommendations. The assay was repeated twice, each in duplicate. An aliquot of 100 µl of the diluted capture antibody (mouse anti-human TNF-α/IL-8, concentration of 4 µg ml^{-1} PBS) was incubated overnight in a 96-well immunoassay plate (NUNC, MaxiSorp) at room temperature. Differing from the producer's protocol, the plate was blocked with PBS supplemented with 1 per cent bovine serum albumin (BSA), 5 per cent sucrose and 0.05 per cent NaN_3 for 1 h at room temperature. After washing with buffer, supernatants from samples and the standards (0–10 ng ml^{-1} recombinant human TNF-α and 0–2 ng ml^{-1} recombinant human IL-8) were pipetted into the wells and incubated at room temperature for 2 h. After washing, the detection antibody (biotinylated goat anti-human TNF-α/IL-8) diluted in reagent diluent was added. The plate was covered with an adhesive strip and incubated again for 2 h. The anew washing was followed by the addition of horseradish peroxidase-conjugated streptavidin to the plates and incubation for 20 min at room temperature in the dark. Finally,

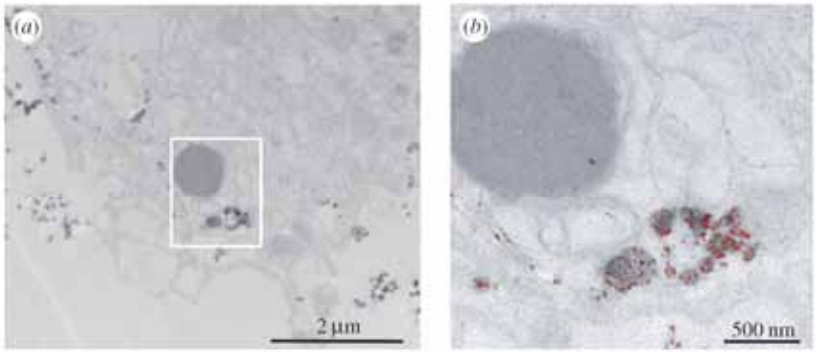

Figure 1. EFTEM analysis of yttrium as a constituent of the CNT pellet catalyst in an epithelial cell. (*a*) Overview of one A549 epithelial cell in the triple cell co-cultures with intracellular CNT pellet. (*b*) EFTEM detection of yttrium (red points).

the substrate solution (tetramethylbenzidine/H_2O_2; R&D Systems, catalogue number: DY 999) was added. After 20 min in darkness, the colour development was stopped by adding 1 M H_2SO_4 and the plate was put on the shaker (differing from the protocol) for 10 min. The absorbance was then read at 450 nm using an ELISA reader (SpectraMax 340 PC or Benchmark Plus Microplate Spectrophotometer). The concentration of the cytokine or chemokine was determined by comparing the absorbance of the samples with standard samples.

2.9. Calculation of the expected triple cell co-culture values

To calculate the expected values for the triple cell co-cultures, the amount of TAC, TNF-α and IL-8 per cell in each type of monoculture was determined (observed values). These values were multiplied by the number of each cell type in the triple cell co-culture model (Blank *et al.* 2007) and summarized to result in expected values.

2.10. Statistical analysis

The data are expressed as mean values with the standard deviation from at least three independent experiments with at least two internal replicates, with each in duplicate. The statistical analysis was performed using Excel for Windows and SigmaStat for Windows (v. 3.10, SigmaSTAT Software, Inc.). Different groups were compared using the ANOVA on ranks test followed by Dunn's test in case of significance. *p*-values less than 0.05 were considered to be statistically significant.

Owing to the variability of cytokine/chemokine and oxidative stress production, the results of the single cultures are presented as percentages of the concentrations of the unexposed control. In order to evaluate the interplay of the different cell types in the real triple cell co-culture, the cytokine and oxidative stress values of the triple cell co-cultures were compared with an expected toxicity value gained from the results of the monocultures. The expected and observed values were compared using the rank-sum test.

3. RESULTS

3.1. Particle penetration into cells

When particles are added to cell cultures as a suspension, it is important to show whether particles are inside the cells or whether they are attached to the cell surface. Therefore, the cells were analysed after particle exposure by EFTEM or conventional TEM. Titanium from the TiO_2 NP as well as yttrium, a residual of the SWCNT, can be identified by EFTEM. TiO_2 NPs were found in all cell types of the triple cell co-cultures (data not shown; see also Rothen-Rutishauser *et al.* (2007*a*)). Yttrium of the CNT pellet suspension was identified in epithelial cells by EFTEM (figure 1). For EFTEM, the sections were not post-treated with uranyl acetate; therefore, the contrast of the cells was not as strong as in conventional TEM. By applying this method, CNT could be identified in the cytoplasm, not membrane bound. Additionally, bundles of the CNT pellet were found in all three cell types of the triple cell co-cultures by conventional TEM (figure 2). DEPs were found by TEM as agglomerates in all cell types of the triple cell co-cultures (figure 3). By conventional TEM, it was not possible to detect single SWCNTs or DEPs. All three particle types were also found in the cells of monocultures (data not shown).

3.2. Qualitative analysis of reactive oxygen species production

After it had been shown that DEPs and the two manufactured NPs penetrated the cells, the potential of these particles to induce oxidative stress in the mono- and triple cell co-cultures was compared. ROS production was induced in all cell culture types after exposure to TBHP, a substance that is known to induce oxidative stress at the concentration used. In epithelial cells and MDM monocultures, ROS production was induced by all particle types (figure 4*a*,*b*). MDDC monocultures showed almost no production of ROS (figure 4*c*). In the triple cell co-cultures, the majority of the cells were ROS positive after exposure to all particle types (figure 4*d*). As far as the particle type is concerned, it can be concluded that CNT pellet and DEPs induced most ROS, followed by TiO_2 NPs, while CNT supernatant produced the least.

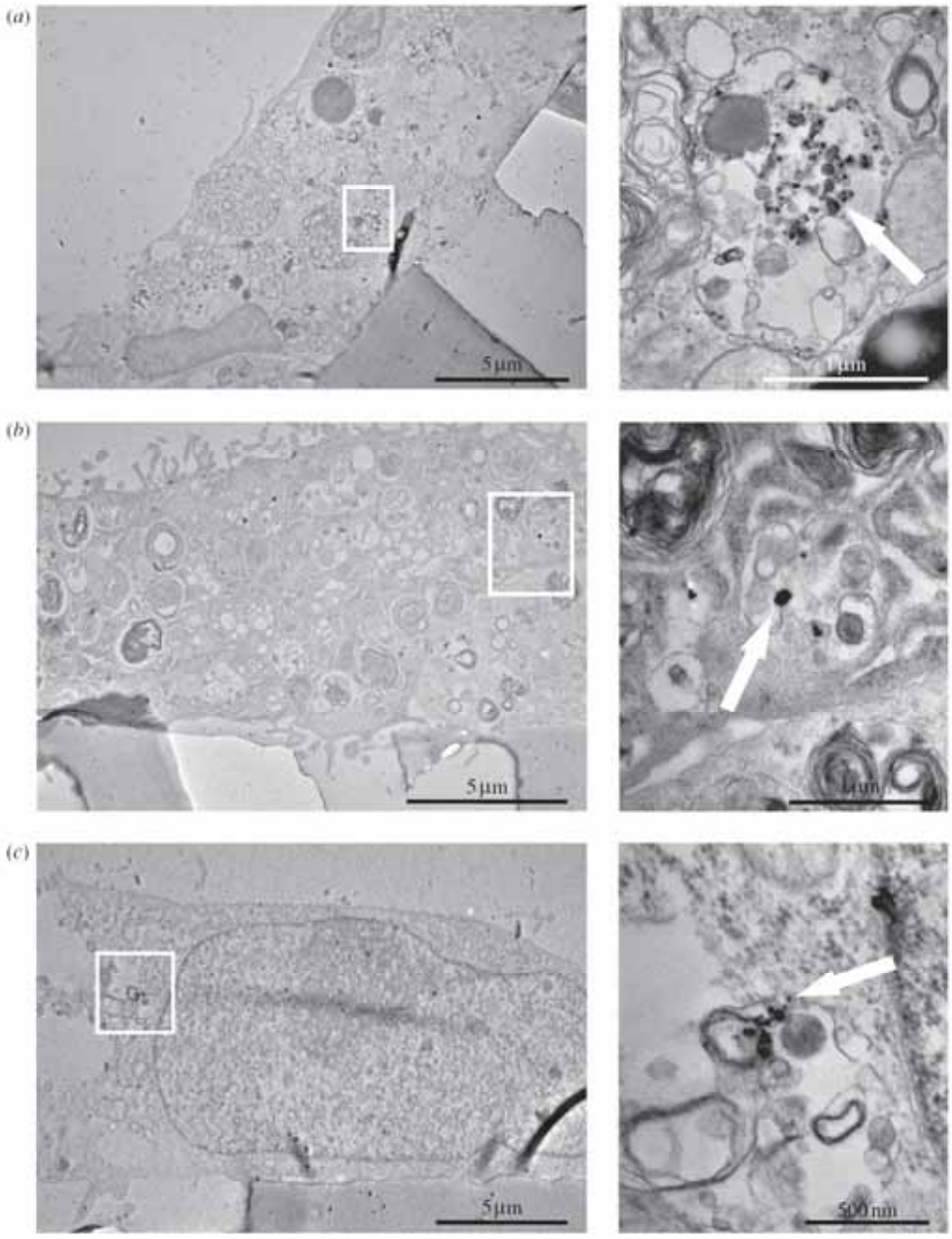

Figure 2. TEM pictures of the triple cell co-cultures with intracellular CNT pellet. Overview of the cell (left) and details of the part in white frame (right). (*a*) A549 epithelial cell, (*b*) MDM and (*c*) MDDC.

3.3. Quantitative analysis of oxidative stress and inflammation reactions

For quantitative analysis of cellular responses, the TAC and the release of TNF-α as well as IL-8 were determined.

3.3.1. A549 monocultures. In the A549 cultures, a TAC level of 0.22 ± 0.07 mM Trolox was detected in the negative control; this value was then taken as 100 per cent. The IL-8 concentration in the control cultures was 16.38 ± 5.9 ng ml^{-1} (100%). Compared with the controls, the A549 monocultures exposed to CNT pellet and DEPs showed an increased TAC value of 124 per cent (s.d. 56%) and 130 per cent (s.d. 51%), respectively, and a moderate increase in IL-8 release of 106 per cent (s.d. 11%) and 110 per cent (s.d. 11%), respectively (figure 5*a*). In the supernatant of A549 monocultures, no TNF-α could be measured in particle-exposed cultures or in the negative or positive control (data not shown).

3.3.2. Monocyte-derived macrophage monocultures. In the MDM control cultures, the TAC level was 0.30 ± 0.09 mM Trolox (100%) and the concentration of IL-8 was 33.10 ± 4.9 ng ml^{-1} (100%). Again no TNF-α

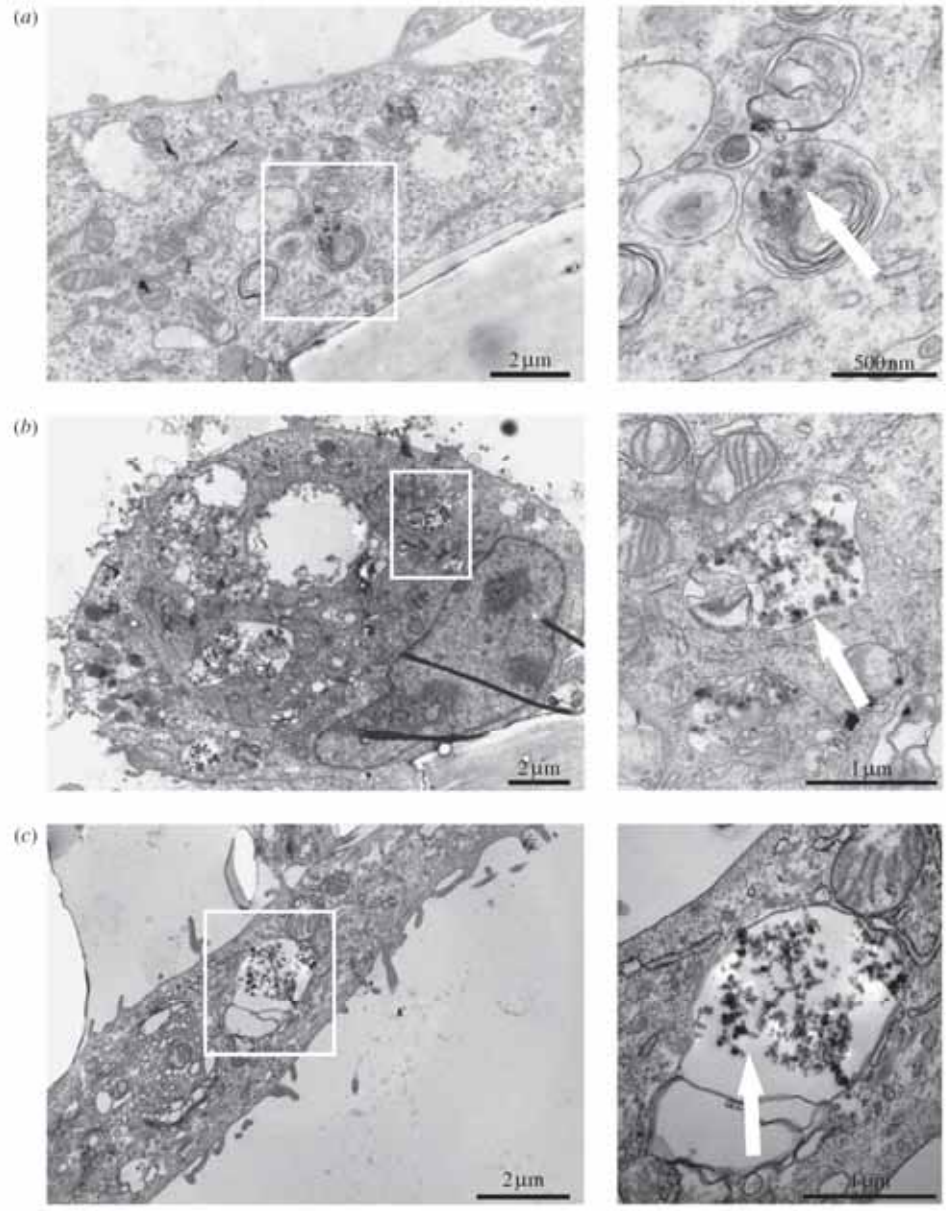

Figure 3. TEM pictures of the triple cell co-cultures with intracellular DEPs. Overview of the cell (left) and details of the part in white frame (right). (*a*) A549 epithelial cell, (*b*) MDM and (*c*) MDDC.

could be detected in any of the cultures (data not shown). The TAC of MDMs exposed to TBHP (75%, s.d. 28%) and TiO_2 (82%, s.d. 31%) was reduced and the TAC of MDMs exposed to DEPs was higher than the negative control (119%, s.d. 44%) (figure 5*b*).

3.3.3. Monocyte-derived dendritic cell monocultures. A level for TAC of 0.24 ± 0.06 Mm Trolox (100%), a TNF-α concentration of 0.95 ± 0.11 ng ml^{-1} (100%) and a concentration of 21.82 ± 9.09 ng ml^{-1} IL-8 (100%) were measured in the control cultures. The differences induced by the incubation with particles were not statistically significant and were inconsistent (figure 5*c*). TBHP-exposed MDDC monocultures showed increased TAC (115%, s.d. 35%) and TNF-α levels (143%, s.d. 38%), but no enhancement of IL-8. Exposure to TiO_2 induced an increase in TAC (152%, s.d. 23%), TNF-α (124%, s.d. 1.5%) and IL-8 levels (106%, s.d. 9.5%), but the latter only weakly. CNT supernatant increased the TAC level (118%, s.d. 31%) and decreased TNF-α (84%, s.d. 51%) as well as IL-8 levels (75%, s.d. 40%). CNT pellet induced a small increase for all three parameters, such as 113 per cent (s.d. 32%) for TAC, 109 per cent (s.d. 17%) for TNF-α and 106 per cent (s.d. 13%) for IL-8.

3.3.4. Triple cell co-cultures. In the triple cell co-cultures, the control reached a TAC level of

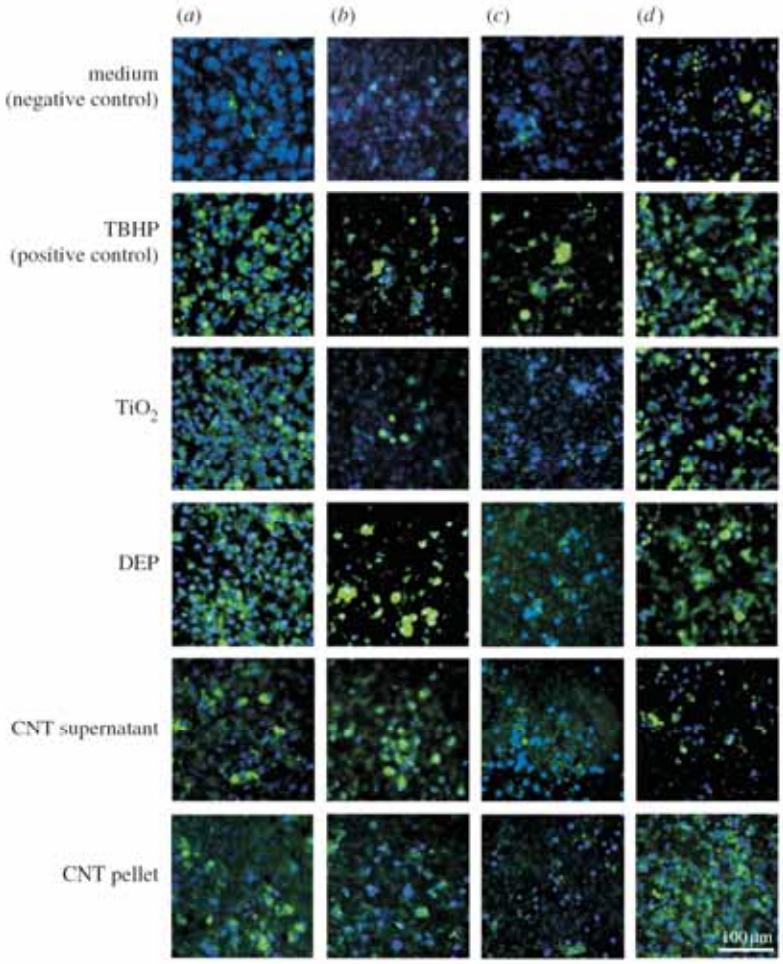

Figure 4. ROS production in cell cultures exposed to different nanosized particles for 24 h. Blue staining shows the cell nuclei and green staining shows the ROS. All signals were compared with the negative control (medium) and the positive control (TBHP). (*a*) A549 epithelial cells. TiO_2, DEP, CNT supernatant and CNT pellet induce a high production of ROS. (*b*) MDM. TiO_2, DEP, CNT supernatant and CNT pellet induce a high production of ROS. (*c*) MDDC. DEP and CNT supernatant induce a high production of ROS. (*d*) Triple cell co-cultures. TiO_2, DEP, CNT supernatant and CNT pellet-exposed cells show a higher production of ROS.

0.22 ± 0.09 mM Trolox (100%), a TNF-α concentration of 0.73 ± 0.55 ng ml^{-1} (100%) and an IL-8 concentration of 27.85 ± 3.84 ng ml^{-1} (100%). The particle-exposed cells showed only small and not statistically significant different values compared with the negative control (figure 5*d*). The TAC levels of TiO_2-exposed cells were statistically significantly higher than the levels of CNT supernatant and in the DEP-exposed cell cultures (figure 5*d*).

3.4. Expected versus observed oxidative stress and cytokine/chemokine concentrations in the triple cell co-cultures

As all three cell types from the monocultures are combined in the triple cell co-culture system, it was hypothesized that all the effects observed in the individual cell type cultures could be summarized in a weighted way, and this should result in the values observed in the co-cultures. In order to test this hypothesis, the summarized values of the monocultures (expected values) were compared with the values of the triple cell co-cultures (observed values).

3.4.1. Reactive oxygen species. As there were only qualitative ROS results, it was not possible to calculate an expected ROS value from the monocultures for the triple cell co-cultures. However, from the fluorescence shown in the micrographs, it seemed that there were no or only small differences between cells in monocultures when compared with cells in triple cell co-cultures.

3.4.2. Total antioxidant capacity. The comparison of expected and observed TAC levels showed statistically significant higher than expected values in cells exposed to medium only, CNT supernatant, CNT pellet and DEPs. For TBHP and TiO_2 exposure, the differences were not statistically significant, but higher (figure 6*a*).

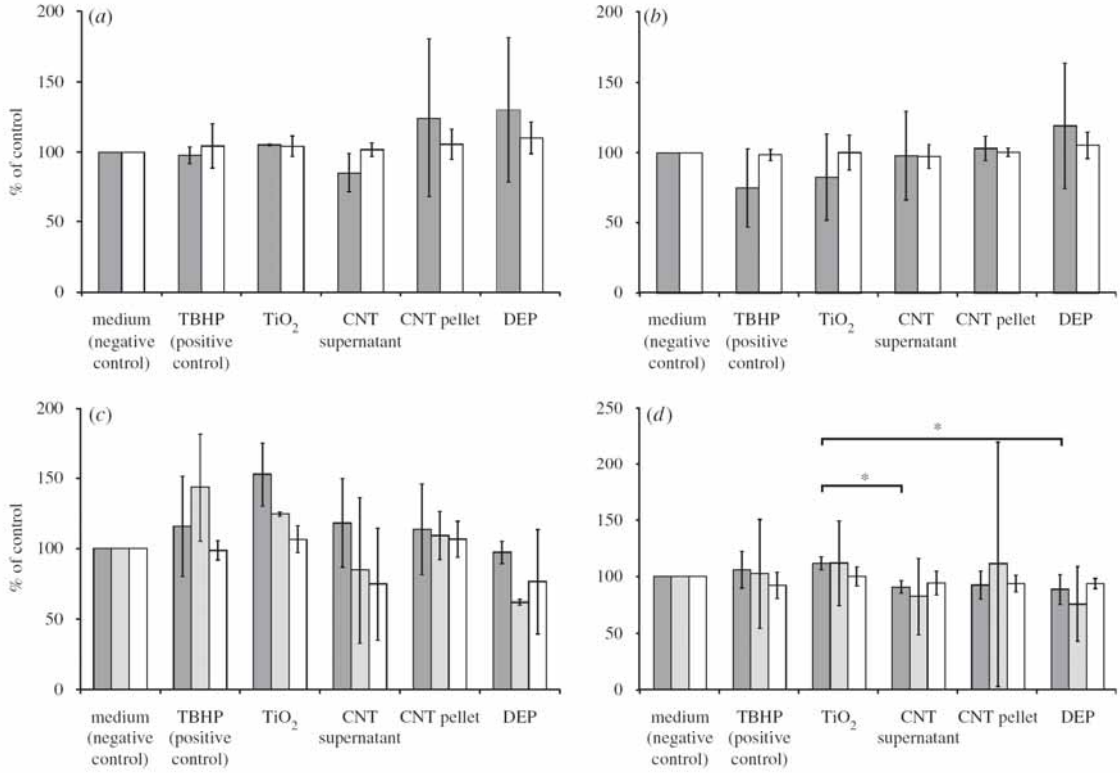

Figure 5. TAC equivalent, TNF-α and IL-8 concentrations in monocultures and triple cell co-cultures following exposure to different nanosized particles for 24 h. All values are expressed as a percentage of control. (*a*) A549 epithelial cell monocultures show no statistically significant enhancement of TAC or IL-8 release compared with the negative control and do not produce TNF-α. (*b*) MDM monocultures produce no TNF-α. For TAC and IL-8, no differences between the negative control and particle exposure are detected. (*c*) In MDDC monocultures, no statistically significant differences between control and particle exposure could be observed. (*d*) The levels in triple cell co-cultures were not enhanced by particle exposure; however, the TAC levels of TiO_2-exposed cells were statistically significantly ($P < 0.05$) higher than the levels in CNT supernatant and DEP-exposed cell cultures. Dark grey, TAC; light grey, TNF-α; unfilled, IL-8.

3.4.3. Tumour necrosis factor-α. All observed TNF-α concentrations in the triple cell co-cultures (observed values) were higher than the expected values calculated from the monocultures, except for the cultures exposed to TiO_2 NPs, although these findings were not statistically significant (figure 6*b*).

3.4.4. Interleukin-8. The observed concentrations of IL-8 in the triple cell co-cultures were, under all conditions, lower than the expected values estimated from the monocultures, but not statistically significant (figure 6*c*).

4. DISCUSSION

Many studies investigating the toxicity of particles in lung cells give a good basis for the evaluation of the toxic potential of particles and, in particular, of NPs (Donaldson *et al.* 2005, 2006; Oberdorster *et al.* 2005). In most studies, cell lines or primary cell cultures were used as monocultures (Amakawa *et al.* 2003; Shvedova *et al.* 2003; Cheng *et al.* 2004; Brunner *et al.* 2006; Kagan *et al.* 2006; Mundandhara *et al.* 2006; Limbach *et al.* 2007; Wick *et al.* 2007; Mitschik *et al.* 2008). However, the histological composition of the lung is not restricted to one single cell type. It is a rather complex network of various cell types (Brain 1988; Holt *et al.* 1990; Lehnert 1992; Nicod 1997). In fact, the lung consists of more than 40 different and highly specialized cell types (Ochs & Weibel 2008). This complexity can never be mimicked by artificial cell cultures, but the use of co-cultures consisting of various cell types realistically mimics the situation in the human lung and gives a more reliable toxicological evaluation than studies with cell monocultures (Roggen *et al.* 2006; Rothen-Rutishauser *et al.* 2008*a*). In the present work, it was found that all the NPs used including TiO_2, DEP and SWCNT can penetrate into epithelial cells, MDMs and MDDCs (figures 1–3). In addition, the differences of cellular responses between monocultures of epithelial cells, MDMs and MDDCs and triple cell co-cultures composed of these three cell types after exposure to different NPs in suspension were investigated and compared.

4.1. Nanoparticle penetration into cells

The penetration of different NPs into human lung cells *in vivo* and *in vitro* has been shown in many studies

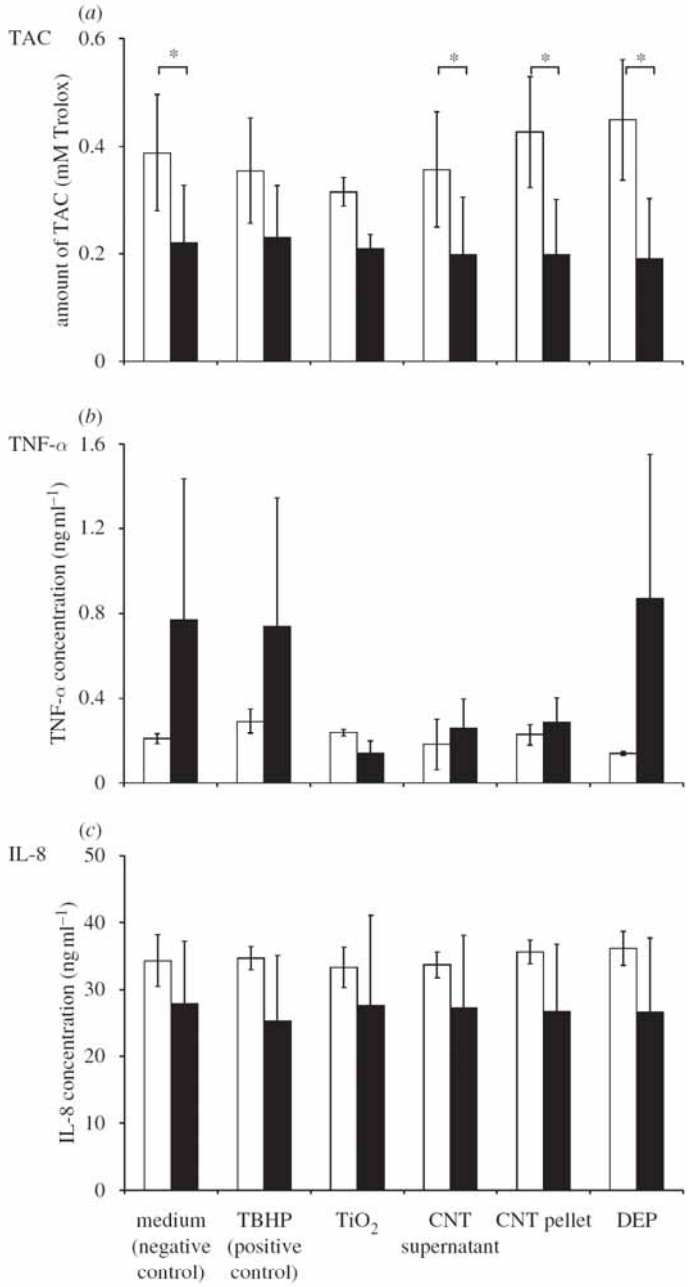

Figure 6. Comparison of the observed versus expected cytokine/chemokine and oxidative stress values in the triple cell co-cultures. The expected co-culture values were calculated by summing up the amounts produced in the monocultures, adjusted for cell number. The expected TAC (*a*) and IL-8 (*c*) values were higher than the observed values. The observed TNF-α (*b*) concentrations are higher for all conditions, but not for TiO_2 NPs. Unfilled bar, expected; filled bar, observed.

(Shvedova *et al.* 2003; Geiser *et al.* 2005; Limbach *et al.* 2005; Wick *et al.* 2007). However, the penetration mechanisms are still not known (Rothen-Rutishauser *et al.* 2007*b*). It is not only endocytic pathways, which all include vesicle formation, that are discussed to account for the translocation of NPs. In many *in vitro* and *in vivo* studies, NPs were found to be non-membrane bound (Kapp *et al.* 2004; Geiser *et al.* 2005; Rothen-Rutishauser *et al.* 2007*a*). This supports the theory of NPs to enter cells by a non-endocytic mechanism that is supported by a study performed with human red blood cells (Rothen-Rutishauser *et al.* 2006).

The current study found TiO_2 NPs in all cell types of the monocultures and in the triple cell co-cultures, which is in agreement with previously published results (Stearns *et al.* 2001; Rothen-Rutishauser *et al.* 2007*a*). The titanium of the TiO_2 NPs has been identified in all three cell types by EFTEM, and the particles have

been detected as agglomerates, which were membrane bound or as small aggregates free in the cytoplasm (Rothen-Rutishauser *et al.* 2007*a*). TiO_2 NPs were also detected by EFTEM in A549 monocultures in another study (Stearns *et al.* 2001).

The identification of SWCNTs in cells is difficult as the tubes are built from a graphene layer that cannot be distinguished from the cellular structures that also consist of carbon. There are studies showing intracellular CNTs by conventional TEM; however, in these studies, only larger aggregates of SWCNTs were detected (Shvedova *et al.* 2003; Worle-Knirsch *et al.* 2006; Pulskamp *et al.* 2007). In the present work, yttrium, a residual of the SWCNTs, was identified—to our current knowledge—for the first time by EFTEM inside the cells (figure 1). Using this method, single SWCNTs or small aggregates could be shown to be free within the cytoplasm. In another study (Davoren *et al.* 2007), intracellular SWCNTs in A549 cells by conventional TEM could not be found, which might be explained by the fact that single SWCNTs or small aggregates cannot be identified by conventional TEM methods.

DEPs were found in every cell type of the triple cell co-cultures, but only as aggregates in vesicles and not as single particles (figure 3). It is not possible to state that there are no single particles inside the cells, because they might not be detectable. The uptake of DEPs by human epithelial cells by formation of vesicles was previously shown in other studies (Boland *et al.* 1999), but, to our knowledge, never in A549 cells. In other studies, the uptake of DEPs by a human fibroblast-mutant Chinese hamster ovary hybrid cell (Bao *et al.* 2007), the internalization of NPs and fine particles in alveolar macrophages (Beck-Speier *et al.* 2005) and in primary cultures of human bronchial epithelial cells (Reibman *et al.* 2002) were presented. As it is not possible to verify DEPs inside cells by elemental analysis (EFTEM), it may not be possible to detect single particles in cells as was done by other researchers using conventional TEM (Bao *et al.* 2007).

4.2. Oxidative stress: reactive oxygen species production and total antioxidant capacity

In both assays, TBHP, a potent inducer of oxidative stress, was used as a positive control. ROS formation was observed, but the TAC levels were surprisingly low. Since the dose was used according to the company's manual, it might be that the activity in the cell culture medium decreased. Dringen *et al.* (1998) measured a half-time of 24 min for TBHP at 37°C in a similar culture medium to the one currently used. In all cultures, it was observed that exposure to DEPs, TiO_2 NPs and CNT pellet as well as to CNT supernatant induced the production of ROS (figure 4). This was in accordance with other studies for DEPs (Jacobsen *et al.* 2008*a*), TiO_2 (Long *et al.* 2006; Rothen-Rutishauser *et al.* 2008*b*) and CNTs (Pulskamp *et al.* 2007; Jacobsen *et al.* 2008*b*). CNT supernatant induced less ROS-positive cells than the other particle types. This is in agreement with other studies that showed that CNT supernatant suspension is less toxic than CNT pellet in a mesothelioma cell line MSTO-211H (Wick *et al.* 2007) and in RAW 264.7 macrophages (Kagan *et al.* 2006). MDDCs in monocultures seem to react less to all NPs used in the present study than MDMs or epithelial cells. Instead, there is an observable tendency for an upregulation of the TAC. This may suggest that MDDCs are better at adapting their defence systems against ROS production than the other cell types. MDDCs play an important role during the immune response against antigens, and often ROS are involved in a non-pathogenic but physiological way during cellular signalling (Matsue *et al.* 2003). This could be a reason for an optimized defence system against environmental stressors and thus a lower production of ROS and a higher level of TAC in MDDCs when compared with the other cell types.

The triple cell co-cultures showed ROS production at a similar level to the monocultures of epithelial cells, and there is no evidence for a synergistic or alleviative effect by the interplay of the different cell types. Only for the triple cell co-cultures treated with CNT pellet there appears to be more ROS-positive cells than in the monocultures. However, for the ROS production, it was not possible to calculate an expected value owing to the qualitative form of the analysis. When comparing the expected and observed levels of TAC in the triple cell co-cultures, higher expected values than observed values are seen under all exposure conditions (figure 6). The cellular interplay of the different cell types seems to help the cells dealing with oxidative stressors.

4.3. Inflammation reaction: release of tumour necrosis factor-α and interleukin-8

In the current experiments, a higher release of TNF-α and IL-8 into the supernatants owing to particle exposure was not observed in all cultures (figure 5). The differences between control and exposed cells were small and inconsistent. Various studies have also shown that CNTs, TiO_2 or DEPs do not enhance the release of TNF-α and IL-8 (Tao & Kobzik 2002; Lindbom *et al.* 2006; Pulskamp *et al.* 2007; Veranth *et al.* 2007; Rothen-Rutishauser *et al.* 2008*b*). However, there are also contradictory studies that found inflammatory reactions induced by DEPs and TiO_2 particles in epithelial cells, macrophages and dendritic cells (Boland *et al.* 1999; Tao & Kobzik 2002; Lindbom *et al.* 2006; Porter *et al.* 2007; Veranth *et al.* 2007). Finally, it has to be considered that cell cultures, DEP compositions and collection methods as well as the suspension preparations of the other particles used in different studies might vary, and therefore a direct comparison can be difficult.

No detectable increase in TNF-α and IL-8 concentrations could be a consequence of cytokine or chemokine binding by particles, as described in a previous study (Kocbach *et al.* 2008). However, there is no indication for a binding of cytokines to the applied particles, as the present work has used three completely different particle types and the fact that all three NP types bind to the chemokines/cytokines to the same extent seems to be unrealistic.

When comparing the expected and observed TNF-α concentrations in the triple cell co-cultures, higher values in the triple cell co-cultures than expected from the calculations based on the monoculture exposures (figure 6) were observed. As only TNF-α was measured in the monocultures of MDDCs and not in MDMs or epithelial cells, it is assumed that, in the triple cell co-cultures, either the MDDCs stimulate the MDMs or the epithelial cells to produce TNF-α, or the interplay of the cell activates the MDDCs to release more TNF-α. It is thought that, for this modulation, MDDCs play the key role. Since in a previous study with co-cultures of A549 epithelial cells, macrophages (THP-1 cell line) and mast cells (HMC-1 cell line), but without dendritic cells, the opposite observation was seen, namely that the TNF-α concentrations were higher for the expected values than for the observed values (Alfaro-Moreno *et al.* 2008).

Regarding the IL-8 concentrations, the opposite effect was seen. The expected concentrations were higher than the observed concentrations for all conditions, but not to a statistically significant extent. This result is again in contradiction to the results of a previous co-culture study by Alfaro-Moreno *et al.* (2008), who found higher observed IL-8 concentrations than expected. The current result was not expected because it is known that the pro-inflammatory chemokine TNF-α stimulates other cells to release IL-8 (DeForge *et al.* 1992; Nukada *et al.* 2008). Therefore, higher observed values, compared with the expected values calculated from the values of the monocultures, were expected.

4.4. The potential of the three-dimensional epithelial airway model to mimic the interplay of various cell types

Based on the current findings, it is hypothesized that there is a synergistic effect between the different cell types (epithelial cells, MDMs and MDDCs), owing to the interaction of the three cell types that modulate the TAC levels as well as the release of cytokines and chemokines, as was observed in the present study. There is a need for the inclusion of different cell types in cell culture models. Co-culture models are better at simulating the real situation in the lung than monocultures. This is particularly important for toxicological studies including oxidative stress and inflammatory reactions in lung cell culture models upon NP exposure.

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2.2 Project 2: Development of an exposure system to estimate the toxic potential of (scooter) exhaust emissions

New Exposure System To Evaluate the Toxicity of (Scooter) Exhaust Emissions in Lung Cells in Vitro

Loretta Müller, Pierre Comte, Jan Czerwinksi, Markus Kasper, Andreas C.R. Mayer, Peter Gehr, Heinz Burtscher, Jean-Paul Morin, Athanasios Konstandopoulos, and Barbara Rothen-Rutishauser

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New Exposure System To Evaluate the Toxicity of (Scooter) Exhaust Emissions in Lung Cells in Vitro

LORETTA MÜLLER,*,† PIERRE COMTE,‡ JAN CZERWINSKI,‡ MARKUS KASPER,§ ANDREAS C. R. MAYER,|| PETER GEHR,† HEINZ BURTSCHER,⊥ JEAN-PAUL MORIN,# ATHANASIOS KONSTANDOPOULOS,◆ AND BARBARA ROTHEN-RUTISHAUSER†

Institute of Anatomy, Division of Histology, University of Bern, Baltzerstrasse 2, CH-3000 Bern 9, Switzerland, Laboratory for Exhaust Emission Control, Bern University of Applied Sciences, Biel-Bienne, Switzerland, Matter Engineering AG, Nanoparticle Measurement, Wohlen, Switzerland, Technik Thermische Maschinen (TTM), Switzerland, Institute of Aerosol and Sensor Technology, University of Applied Sciences Northwestern Switzerland, Windisch, Switzerland, INSERM, Université de Rouen, France, and APTL, CERTH, Thessaloniki, Greece

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A constantly growing number of scooters produce an increasing amount of potentially harmful emissions. Due to their engine technology, two-stroke scooters emit huge amounts of adverse substances, which can induce adverse pulmonary and cardiovascular health effects. The aim of this study was to develop a system to expose a characterized triple cell coculture model of the human epithelial airway barrier, to freshly produced and characterized total scooter exhaust emissions. In exposure chambers, cell cultures were exposed for 1 and 2 h to 1:100 diluted exhaust emissions and in the reference chamber to filtered ambient air, both controlled at 5% CO_2, 85% relative humidity, and 37 °C. The postexposure time was 0−24 h. Cytotoxicity, used to validate the exposure system, was significantly increased in exposed cell cultures after 8 h postexposure time. (Pro-) inflammatory chemo- and cytokine concentrations in the medium of exposed cells were significantly higher at the 12 h postexposure time point. It was shown that the described exposure system (with 2 h exposure duration, 8 and 24 h postexposure time, dilution of 1:100, flow of 2 L/min as optimal exposure conditions) can be used to evaluate the toxic potential of total exhaust emissions.

Introduction

Scooters, the small two-wheel vehicles with a maximal speed of 45 km/h and an engine capacity of 50 cm^3, are becoming more and more popular. Not only in India, where already more than 50 millions scooters are in use (*1*), but also in Europe, the use of these vehicles is high. In Switzerland, the number of registered scooters is also increasing year by year (from 12,000 in 1990, to 245,000 in 2007; source: Swiss Statistics, www.admin.ch). Due to their low cost and mobility advantages in cities, the number of scooters is likely to continue to grow in the near future.

Comparisons between cars (Euro 3 standard) and different scooters during the warm phase show that two-stroke scooters can emit 10−23 times more carbon monoxide (CO; carburetor technique), 10−171 times more total hydrocarbons (HCs; carburetor and direct injection (DI)), and 3−8 times more nitrogen oxides (NO_x; DI) (*2*). Further high emissions of polycyclic aromatic HCs (PAHs) and very high amounts of particle matter (PM) in the nanoscale range, consisting mainly of incompletely burned lubrication oil products and condensed heavy HCs, are typical for scooter exhaust emissions (*3, 4*). Therefore scooters are significant contributors to PM, PAH, and CO pollution in certain major cities all over the world (*5*). In spite of this fact, the European Union's legislation includes only limit values for CO (1 g/km) and HC plus NO_x (1.2 g/km) but not for PM or PAHs (EURO 2 standard, directive 97/24/EC, chapter 5, annex I).

It has been shown in several studies that PM_{10} (PM with a diameter ≤10 μm) is toxic to lung cells (*6−10*), and in epidemiological studies associations between exposure to PM_{10} and pulmonary and cardiovascular diseases have been found (*11−14*). It was shown that oxidative stress (*7*) and (pro-) inflammatory reactions (*8*) play crucial roles during disease development. Furthermore, there is a link between exposure to diesel soot and lung cancer (*10*). PAHs have been identified as the toxic and carcinogenic part of PM_{10} (*15, 16*). Since the PAH level in scooter emissions was found to be higher than in car exhaust emissions (*17*) and since most of the particles belong to the fraction of combustion-derived nanoparticles, the toxicological potential of scooter emissions is considered to be high.

As the lung is the most relevant organ for investigating exposure to exhaust emissions, a triple cell coculture model of the epithelial airway barrier (*18*) has been used. The model consists of a layer of human epithelial cells, with monocyte-derived macrophages (MDMs) on the apical side and monocyte-derived dendritic cells (MDDCs) on the basal side. The model enables direct exposure of the surface of the lung cells to the exhaust emissions, which allows a more realistic mimicking than it would by sampling the particles and adding them in to submersed cell cultures (*19*).

The toxic potential of the scooter exhaust was compared using two different cell lines for the model of the human epithelial airway barrier: the human bronchial cell line 16HBE14o$^-$ and the human alveolar type II cell line A549. Postexposure analysis included cytotoxicity, (pro-) inflammatory cyto- and chemokine detection, tight junction arrangement, and cell morphology.

The current study is a further development of a project focused on the physical comparison of emissions of various scooter types (carburetor and DI technology) and different technical measurements (such as oxidative catalysts, wire mesh filter catalysts (a kind of particulate trap w/o deNOx purpose), different fuels, and lubricant oils) at the University of Applied Sciences in Biel-Bienne, Switzerland (*20*). For the current study, the aim was to establish an exposure system to compare, in further studies, the toxicological potential of different scooters and the improvements through technical measurements.

* Corresponding author phone: +41 31 631 84 46; e-mail: loretta.mueller@ana.unibe.ch.
† University of Bern.
‡ Bern University of Applied Sciences.
§ Matter Engineering AG, Nanoparticle Measurement.
|| Technik Thermische Maschinen (TTM).
⊥ University of Applied Sciences Northwestern Switzerland.
Université de Rouen.
◆ APTL, CPERI/CERTH & Aristotle University.

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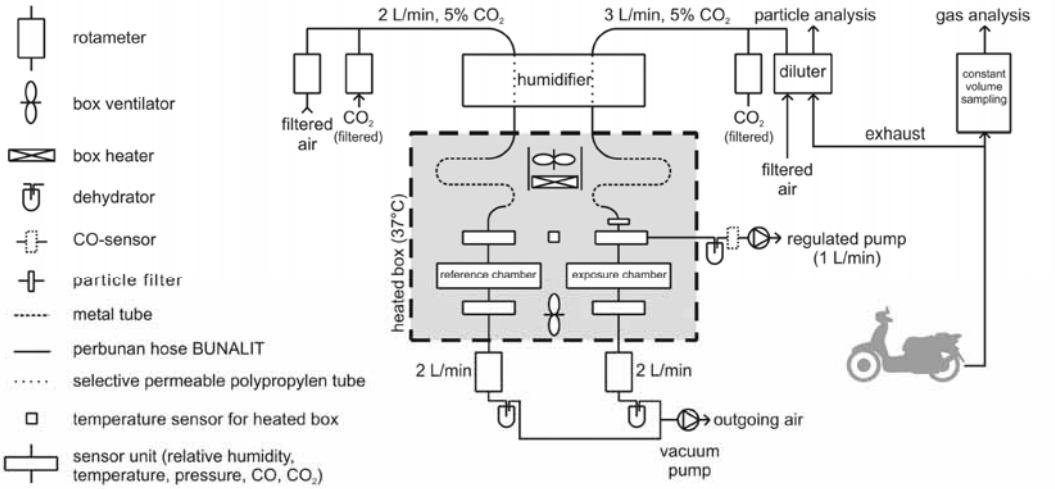

FIGURE 1. Schematic overview of the exposure system. For details about gas and particle analysis see also SI, Table S1.

Materials and Methods

Test Bench and Scooter Description. The scooter was driven on a chassis dynamometer (Supporting Information (SI) Figure S1 and Table S1) at a constant velocity of 40 km/h, an engine rotation speed of 7000 rpm, and with a wheel power of 0.55 kW. The scooter (Peugeot, two-stroke DI (TSDI), details in SI, Table S2) was used with the conditions defined as worst case (dummy muffler (without catalyst or particle filter), Army SAE 50 motor oil (SI, Table S3) with a normal ratio of 100% and unleaded fuel (unleaded 95 Octane; Coop, Switzerland)) in preliminary experiments without cell exposure (*20*). This scooter type and conditions were chosen because of the high particle number produced and therefore providing the best possibilities for the system evaluation. The scooter was warmed up for at least 45 min before starting the exposure to the cell cultures.

Characterization of Exhaust Emissions. For gas analysis, the exhaust emissions were sampled directly from the tail following the EU-regulations (directive 97/24/CE) with constant volume sampling (CVS). Various gas compounds such as HCs, methane (CH_4), NO_x, CO, carbon dioxide (CO_2), and oxygen were measured (details in SI, Table S1).

The characterization of the particulate components was performed using a rotating disk diluter for emissions (Raw Gas Dilution for Aerosols and Gases, Type MD19-2E, Matter Engineering AG, Wohlen, Switzerland). The exhaust emissions were characterized using the size distribution of particles between 10 and 400 nm (scanning mobility particle sizer (SMPS) consisting of a differential mobility analyzer (TSI, DMA model 3081 (long tube)) and a condensation particle counter (TSI, model 3772)) and the active surface of the aerosol particles (diffusion charging particle sensor (LQ1-DC, Matter Engineering)).

Sample Treatment and Cell Exposure System. The flow of the 1:100 diluted exhaust emissions (Figure 1) was controlled at 2 L/min by rotameters (VAF Fluid-Technik GmbH, LVS) and driven by a vacuum pump (SI, Figure S1B; Busch, SV 1010 B). In the whole system, perbunan hose BUNALIT (inner diameter of 4 mm; Angst+Pfister AG, Allschwil, Switzerland) were used. After dilution, filtered CO_2 (medical CO_2, Carbagas, Bern-Liebefeld, Switzerland) was added to a final concentration of $5 \pm 0.2\%$, and the exhaust was humidified to $85 \pm 5\%$ relative humidity (rH) by passing a heated water bath in selective permeable polypropylene tubes (SI, Figure S1B; Accurel PP V8/2 HF, Membrana GmbH, Wuppertal, Germany). Afterward, the exhaust sample was heated in an isolated box (SI, Figure S1A), which was controlled at 37 ± 0.5 °C. Two exposure chambers (SI, Figure S1C (*21*)) had been placed in this box. Before the diluted exhaust sample entered the exposure chamber, the following parameters were measured: temperature (Thermoelement Typ K), rH (Honeywell HIH 4602C; Honeywell Sensing and Control), CO_2 (Vaisala GMT221 Carbocap, 0−10% CO_2 (SN: C4930006, C4930008, C4930009, C4930010)), and CO (Unitronic Figaro TGS5042, 0−10,000 ppm) concentration (SI, Figure S1C). After entering the exposure chamber, the exhaust sample passed above the 6-well plates with the cell cultures on inserts, and the exhaust emissions were allowed to diffuse to the cells (SI, Figure S1D-E). The exhaust emissions were then sucked out at the bottom of the exposure chamber and again temperature, rH, CO_2 and CO concentration, and additionally the pressure difference (Honeywell ASDX001-D4) between before and after the chamber were measured. Afterward, the humidity was removed with dehydrators (Bühler Technologies GmbH, distributed by MBE AG, Wetzikon, AGF-PV-30-S2, PVDF/Glas) from the exhaust sample, which was filtered before being released into the environment.

In parallel to the exposure of cell cultures to diluted scooter exhaust emissions in the exposure chamber, a reference measurement was done in the reference chamber. There the cell cultures were exposed to filtered ambient air (ultra low penetration air filter; Pall, Pallflex T60A20 Æ 47 mm) in a chamber, which was exactly the same as the exposure chamber, and were treated and controlled equal to the scooter exhaust but without measurement of CO.

In addition to exposure and reference cell cultures, a negative control was used to relate the toxicity results to the normal background level. Cell cultures of the same cell passage and isolation were also exposed to the air−liquid interface but were left in the incubator for the same duration like the reference and exposure cells. The toxicity values of these negative controls were taken as a baseline and set at 100%.

Exposure Conditions. The cell cultures (two types of triple cell cocultures, one with 16HBE14o$^-$ and the other with A549 epithelial cells combined with MDMs and MDDCs; for culturing and exposure details see SI) were placed on inserts in 6-well plates in the exposure chambers for 1 and 2 h with a flow of 2 L/min and an exhaust dilution of 1:100. The conditions (temperature, rH, CO_2 and CO concentration, pressure difference) were measured and recorded during the whole exposure (SI, Figure S2). Following the scooter exhaust exposure, cells were placed back into the incubator and were fixed immediately after the exposure (0 h postexposure time), after 4 h, 8 h, 12 h, and 24 h. Supernatants were

TABLE 1. Physical Characterization of the Exhaust Emissions

			scooter exhaust emission
particle number [1/cm³, 10-400nm]			$4.02 \cdot 10^6 \pm 4.75 \cdot 10^4$
mean diameter [nm]			111.08 ± 3.25
surface area [µm²/cm³]			$4.11 \cdot 10^4 \pm 1.07 \cdot 10^4$
particles deposited on TEM grids [1/cm²]	2 h exposure duration	exposure	$11.8 \cdot 10^7 \pm 11.0 \cdot 10^7$
		reference	$4.56 \cdot 10^7 \pm 4.22 \cdot 10^7$
	1 h exposure duration	exposure	$8.63 \cdot 10^7 \pm 8.79 \cdot 10^7$
		reference	$5.81 \cdot 10^7 \pm 7.70 \cdot 10^7$
gas phase emissions		**ambient air**	**scooter exhaust emission**
CO [ppm]		1.17 ± 0.24	40.2 ± 8.9
CO_2 [%]		0.04 ± 0.00	0.124 ± 0.002
HC [ppm]		12.05 ± 8.80	100.2 ± 7.0
NO_x [ppm]		0.05 ± 0.03	13.3 ± 1.8

collected and stored at −70 °C until they were required for the biological investigation.

Control of Particle Deposition and Particle Quantification by Transmission Electron Microscopy (TEM). In parallel to the exposure of cell cultures, at least three copper TEM grids per condition were placed in the 6-well plates to evaluate the number of deposited particles on the cell surface. Therefore the grids were analyzed in a systematic way (SI, Figure S3A) using the 40,000x extension of a Philips 300 TEM at 80 kV (FEI Company Philips Electron Optics, Zurich, Switzerland). One end of the grid hole was searched, a picture was taken, and the next picture was taken ten half turns in the opposite direction, away from the end. This procedure was repeated until the other end of the grid was reached. The pictures (between three and eleven pictures) were printed (SI, Figure S3B−B′), and the particles (from a size of about 10 nm and bigger) were counted manually (between 3.80 particles/picture on blank grids and 9.00 on 2 h exhaust exposed grids). The level of blank grids was taken as background and subtracted from the values.

Several methods such as cell culture procedure, isolation of monocytes, triple cell cocultures, cell staining, cytotoxicity assay, detection of cytokine/chemokine, and statistics are described in the Supporting Information.

Results

Physical Characterization of Exhaust Emissions. Table 1 shows the particle number measured by SMPS, the active surface area, and the particles deposited on TEM grids. In the reference air, less than 25 particles/cm³ were measured. The gas concentrations of exhaust emissions and ambient air are shown in Table 1.

After subtracting the value of blank grids ($7.07 \cdot 10^7 \pm 2.35 \cdot 10^7$ particles/cm²), the resulting amount of particles on reference grids probably came from the handling of the grids (transferred into and out of a grid box, placement and removal in the plates, and the exposure chambers). The numbers of deposited particles on reference and exposure grids are shown in Table 1. The differences between reference and exposure were $2.83 \cdot 10^7$ particles/cm² after 1 h exposure and $7.23 \cdot 10^7$ particles/cm² after 2 h exposure. This resulted in a deposition per hour of about $4 \cdot 10^7$ particles/cm².

In order to estimate if the counted amount of particles on the TEM grids was within a realistic range, the theoretical diffusion deposition rate was calculated based on data shown in Table 1: The particle concentration in the chamber was $4.02 \cdot 10^6$ particles/cm³; corresponding to their mean diameter of 111 nm the particles have a diffusional drift velocity of 12.3 cm/h at standard conditions, at which they drift toward the grid to be eventually deposited (*22*). By multiplying the particle density above the grid with the diffusion velocity, we obtain the number of particles deposited per hour as $4.9 \cdot 10^7$ particles/cm² per hour. This value is in good agreement with the actual deposition of about $4 \cdot 10^7$ particles/cm² per hour, calculated from the count of particles on TEM grids. The calculated diffusion velocity (12.3 cm/h) is about 34 times higher than the sedimentation velocity (0.36 cm/h). It can be concluded that particle deposition in the chambers is dominated by diffusion processes.

Distribution of Particles in the Exposure System. In order to determine how many particles from the original scooter exhaust reached the cells, the number of particles on different sampling points in the system was measured: (1) after exhaust dilution, (2) after CO_2 addition and flow control, (3) before and (4) after the exposure chamber. Between sampling points (1) and (2), almost no particle loss (decrease of 6% of total particle number from $3.19 \cdot 10^6$ to $2.99 \cdot 10^6$ particles/cm³) and no change of size distribution (SI, Figure S4) were detected. From (2) to (3), a decrease of 13% (decrease from $2.99 \cdot 10^6$ to $2.60 \cdot 10^6$ particles/cm³) and a minimal shift to smaller particles were measured. From (3) to (4), 50% of the particles stayed in the chamber ($2.60 \cdot 10^6$ particles/cm³ before and $1.29 \cdot 10^6$ particles/cm³ after the chamber) and the distribution shifted to larger particles. As in the lung about 40−50% of inhaled nanoparticles are deposited (*23*), the chamber represents realistic conditions.

Cytoskeleton, Cell Nuclei, and Tight Junction Staining. For controlling the confluence of the cellular layer, cell nuclei, actin cytoskeleton, and occludin tight junctions were stained. In all conditions (control, reference, and exposure) the cell layer was compact, with clearly formed tight junctions between the 16HBE14o⁻ epithelial cells, and no damage could be observed (SI, Figure S5). The same, but with less clearly formed tight junctions, was observed for triple cell cocultures with A549 epithelial cells (SI, Figure S6).

Cytotoxicity and (Pro-) Inflammatory Response. The cytotoxicity was assessed by lactate dehydrogenase (LDH) measurements, and the (pro-) inflammatory responses were assessed by termination of tumor necrosis factor alpha (TNF-α) and interleukin 8 (IL-8) concentrations in the supernatants. In A549 triple cell cocultures, no significant differences between control and exposed cells (Figure 2) were found and neither in 16HBE14o⁻ triple cell cocultures exposed during 1 h. In 16HBE14o⁻ triple cell cocultures, after an exposure of 2 h, significantly more LDH release was found at 8 h postexposure between exposure and reference, exposure and control, and reference and control (Figure 2A). The TNF-α and IL-8 concentrations at 12 h postexposure were statistically, significantly higher in scooter exhaust exposed cells than in the control (Figure 2B−C). Additionally, there was a statistically significant difference between the IL-8 concentration in the reference and the control at 12 h postexposure (Figure 2C).

The absolute values of the negative controls were as follows: 0.05 ng/mL TNF-α in A549 and 0.28 ± 0.11 ng/mL in 16HBE14o⁻ triple cell cocultures, 68.31 ng/mL IL-8 in A549 and 29.64 ± 0.14.68 ng/mL in 16HBE14o⁻ triple cell cocultures.

Discussion

The aim of this study was to develop and evaluate a new exposure system to assess possible adverse effects of scooter exhaust emissions. The exhaust emissions and the conditions within the box were extensively characterized. The system was tested using two different triple cell cocultures (A549 or 16HBE14o⁻ epithelial cells, MDMs and MDDCs) during 1 and 2 h of exposure, combined with various postexposure times (0−24 h).

System and Exposure Unit. The conditions in the exposure and reference chambers in our system were ideal for cell cultures (37 ± 0.5 °C, 5 ± 0.2% CO_2 and 85 ± 5% rH),

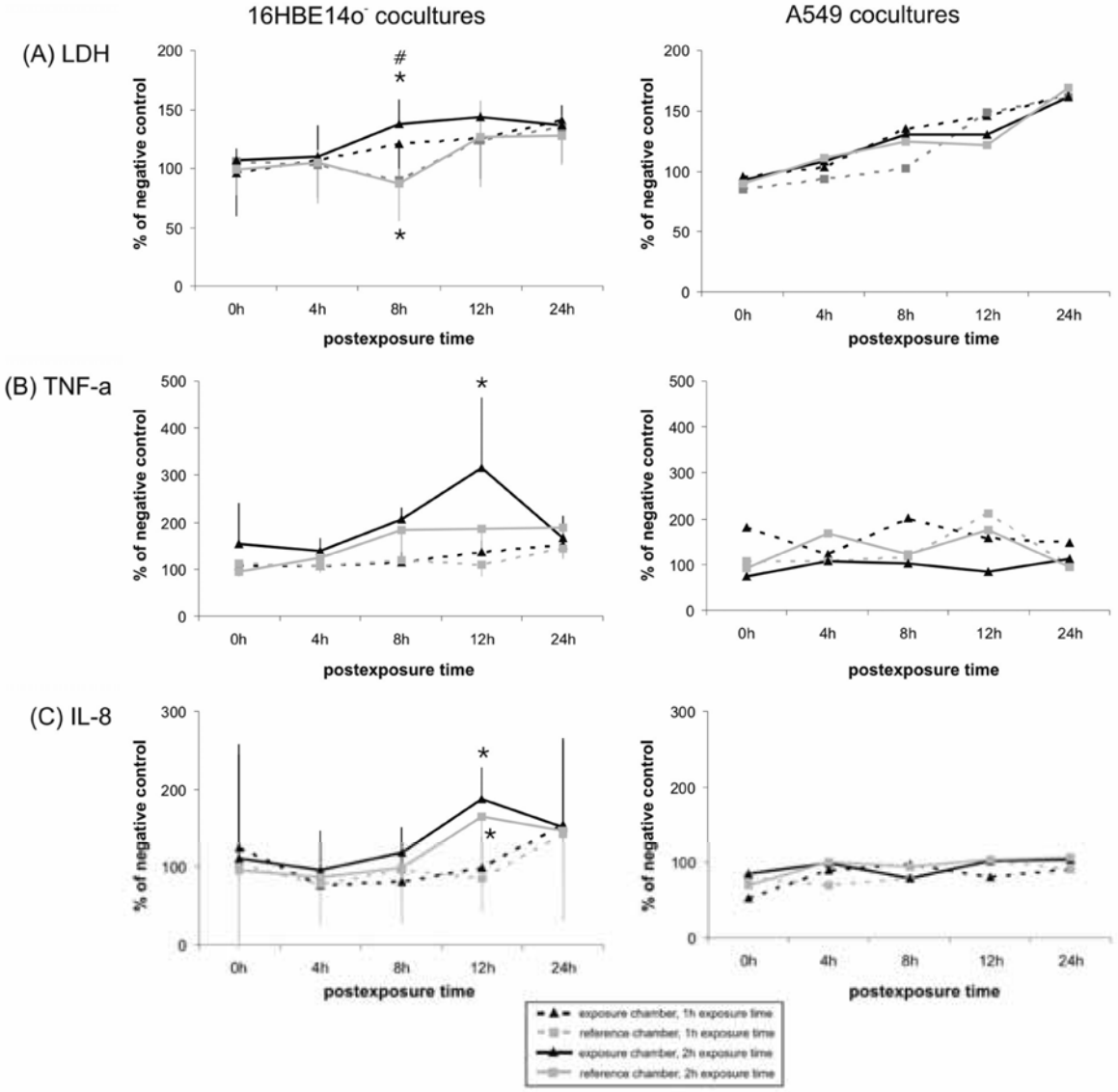

FIGURE 2. Cytotoxicity and (pro-) inflammatory response in different cell culture types after exposure for 1 and 2 h, and 0−24 h postexposure time, owing to scooter exhaust emissions. * means statistically significant difference ($p < 0.05$) compared to control and # compared to reference.

which is not the case in other exposure systems also used for testing the effects of exhaust emissions (Table 2). Some systems are only able to alter the temperature. There are also differences between the various systems, concerning the exposure units. One system used an exposure chamber for one single cell culture (*24*), whereas all the others had chambers for the exposure of more than one cell culture simultaneously. In most studies, the exhaust emission passed parallel to the cell cultures and was not directed vertically to the cells. When considering the situation in the human lung, a parallel flow of the exhaust fumes is realistic for the conducting airways. As the airway cross section is huge in the respiratory part of the lung, the mass flow velocity is slow, and so the contact of inhaled air with cells occurs via diffusion processes (*25*). Unfortunately the behavior of the exhaust emissions, especially the particulate fraction, was not characterized for all systems (*24, 26−28*). Sometimes the exposure unit was a simple box, and no validation of exhaust flow and particle deposition on the cell cultures was done (*26*).

Exposure Duration, Dilution, and Flow Ratio. After a 2 h exposure time, a 2.5-fold increase of deposited particles on exposed cells compared to reference air exposed cells was found (Table 1). During 1 h of exposure only, a 1.5-fold increase was counted. Even the 2 h of exposure was not long, when compared to other air−liquid interface exposure studies, which have exposed 16HBE14o$^-$ monocultures for 1 to 6 h to diesel exhaust emissions (*29*), A549 monocultures for 48 h to different gaseous compounds or cigarette smoke (*27*), and rat lung slices to diesel exhaust emissions for 3 to 6 h (*30, 31*). In another study, A549 monocultures were exposed for only 1 h but to concentrated diesel exhaust emissions (*32*). In comparison with these mentioned studies, the exposure duration chosen in the present study was on the lower end of the usual durations and could be a reason for the weak effects.

However, when considering the biological effects, it is not only the exposure duration but also the dilution of the exhaust that is essential. The dilution ratio was kept constant

TABLE 2. Summary of Various Exposure Systems[a]

source	tested vehicle	exposure unit	dilution	exposure duration	flow	biological sample	controlled conditions	post-exposure time	reference, control	observed biological effects	exhaust characterizations	remarks
Abe et al, 2000 *(26)*	2,300-cc diesel engine	20 x 25 x 6 cm³ chamber	1:8	0-14 h	5 L/min	human bronchial EC (BET-1A)	5% CO_2, 37°C	-	filtered exhaust	cell survival ↓, release of IL-6 →, IL-8 ↑, IL-10 →, TGF-β1 →, mRNA of IL-6 ↑, IL-8 ↑, TGF-β1 ↑, IL-10 →; filtered : lower effects	PN, CO, NO_x, SO_2	no information about PD in the system
Cheng et al, 2003 *(39)*	Diesel and gasoline car	air-cell exposure apparatus with six transwell membranes (no detailed description)	1:10-1:15	1-6 h	400 mL/min	A549	37°C	-	particle-free dilution air	IL-8 in TNF-α primed cells ↑→	PSD	no detailed description of the particle behavior in the system
de Bruijne et al, 2009 *(32)*	Mercedes-Benz model 300SD	electrostatic aerosol in vitro exposure system (EAVES)	No dilution, concentration	1 h	0.95 L/min, total sample volume of 56 L	A549	37°C, 5% CO_2	9 h	EAVES turned off (particle-blind)	release of IL-8 ?, LDH ?	PSD	very detailed characterization of the particle behavior in the system, concentration of particles, no gas effects, short exposure times
Holder et al, 2007; Holder et al, 2008 *(29, 40)*	4-stroke direct injection diesel generator	instrumented environmental chamber (2.2x2.4x 4.6 m³) for exhaust ageing, stainless steel enclosure (7.6x14x21.6 cm³) in incubator for cells	1:100-1:114	0-6 h	1.3 L/min	16HBE14o monocultures	37°C	0-24 h	in incubator	cell viability ↓ release of IL-8 ?	PSD, NO_x, CO, SO_2, PN	particles in the ultrafine size range (<100nm) are deposited less efficiently than larger particles, consideration of exhaust aging, no reference with flow
Knebel et al, 2002 *(24)*	75 horse-power, six cylinder DE	CULTEX System *(41, 42)*	1:10-1:100, undiluted	1 h	8.3 mL/ membrane/min	human bronchial EC line HFBE21	37°C	2 h	clean air	cell viability ↓	CO, NO, NO_2, NO_x, HC, PN	no information about PD, mainly used for gases only
Komori et al, 2008 *(27)*	- (only gases & cigarette smoke)	closed, round gas chamber with an inner volume of 150 mL	-	48 h	collected in polyethylene bag	A549 monocultures	37°C, 0.04% CO_2, humidified	-		biological effects of cigarette smoke in this context not of interest.	not of interest (cigarette smoke)	gas is pre-collected in a bag, unknown particle behavior
Morin et al, 1999; Le Priéur et al 2006; Bion et al, 2002; Morin et al, 2008 *(30, 31, 38, 43)*	5-horsepower Robin DE	rotating, horizontal tubes	1:5	1 h	2 L/min (2.5 chamber volume/min)	slices of rat lung tissue	20% O_2, 5% CO_2, 37°C	1 h, 24 h		K →, ATP ↓, O_2 consumption →, GSH ↓ (1 h pe) ? (24 h pe), Cat →, SOD ↓, Se -GPx ?		several dilutions, filtered/unfiltered in parallel, only usable for lung slices, not for cell cultures
			1:10-1:12	3 h, 6 h						TNF-α ?, IL1-β tissue level →, microvesicles ?, # apoptotic cell nuclei ?		
	21 common rail direct injection turbo-charged intercooled DE			3 h, 6 h						ATP →, GPx ?, GST ?, Catalase ?→?, GSH ↓, SOD ?→?, TNF-α ?→, Nucleosomes ?→, TUNEL ?→, DNA Ladders ?→, 8-OxoG ?→ (low NO_2/NO ratio total exhaust, filtered, high NO_2/NO ratio exhaust)	O_2, CO_2, NO_x, CO, COV, PSD, ELPI	
New, in this paper described system	two-stroke direct injection scooter	round exposure chamber *(21)*	1:100	2 h	2 L/min	triple cell co-culture model of human epithelial airway	37°C, 80% rH, 5% CO_2	0-24 h	clean air, in incubator	LDH ?→, TNF-α ?→, IL-8 ?→ (total exhaust, filtered exhaust)	PSD, surface area, CO, HC, NO_x	control of particle deposition

[a] ↑ increased level, →, no impact, ↓ decreased level. Abbreviations: adenosine-5′-triphosphate (ATP), catalase (Cat), diesel engine (DE), epithelial cells (EC), electrical low pressure impactor (ELPI), glutathione peroxidase (GPx), glutathione (GSH), glutathione S-transferase (GST), hydrocarbons (HC), nitrogen monoxide (NO), nitrogen dioxide (NO_2), nitrogen oxides (NO_x), 8-oxo-2′-deoxyguanosine (8 OxoG), particle distribution (PD), postexposure (pe), particle number (PN), particle size distribution (PSD), sulfur dioxide (SO_2), superoxide dismutase (SOD), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

at 1:100, which is comparable to the dilution ratio between 1:106 and 1:114 in Holder et al. (*29*). In that study with rat lung slices (*31*), the dilution ratio was much lower, between 1:1.2 and 1:10. Again, when considering the real situation on the road, a dilution of 1:100 is realistic for a distance of 2−3 m between the tail pipe of a driving scooter and a person walking on a sidewalk next to the road (*33, 34*). As the effects in this study were not strong, it would be interesting and important to look at a dilution series, either through varying the dilution ratio or the exposure duration. Both are possible with the currently described system, but for a dilution ratio smaller than 1:100 some adaptations with the dilution instruments would be required. A chronic exposure of the cell cultures would also be interesting; however, since the normal lung cell cultures cannot be cultured for several days or weeks at the air−liquid interface, this might not be possible with cell cultures.

Another important point is the flow ratio. It has to be considered in relation to the chamber volume, so that the best factor to compare would be the flow velocity. Due to missing details in previous publications it is not possible to make such a comparison. The flow in the currently described system is 2 L/min and cannot be changed, as the chamber and the regular particle exhaust distribution was developed for a flow rate of about 2 L/min (*21*). Table 2 shows that this flow rate is comparable to that of other studies.

Biological Sample: Cell Culture Type and Postexposure Time. For exhaust emissions, inhalation is the most relevant exposure route. Therefore, triple cell coculture models of the human epithelial airways with A549 and 16HBE14o$^-$ epithelial cells were used to evaluate the toxic potential of scooter exhaust fumes. The A549 triple cell cocultures showed inconsistent or no differences between exposure and references (Figure 2). For further experiments, only cocultures with 16HBE14o$^-$ human bronchial epithelial cells were used. The use of the triple cell coculture model made comparison with other studies difficult, because cell monocultures were mainly used previously, and cocultures have rarely been used. However, studies done with cocultures are of higher biological relevance (*19, 35*) because the human lung alone consists of more than 40 different cell types (*25*), and studies with cocultures provide results different from that of monocultures (*36, 37*).

Biological reactions of cell cultures are time-dependent; cells need time to regulate the RNA level and/or the protein production. Therefore the postexposure time was varied, and various time end points were evaluated. The LDH levels and TNF-α and IL-8 concentrations increased with longer postexposure time not only in exposed but also in reference cell cultures. This showed that even the handling alone and the exposure to a slow air flow have an effect on cytotoxicity and (pro-) inflammatory reactions of the cell cultures. However, the observed effect was stronger in exhaust exposed cells, as could be seen by looking at the statistically significant differences between exhaust fume exposed cells and negative control and reference air exposed cells 8 h postexposure (for LDH) and 12 h postexposure (for TNF-α and IL-8), respectively. The end points of 8 and 24 h postexposure were chosen for future experiments. Also, these experimental characteristics are in the same range as in the often cited study of Holder et al. (*29*), which used 6, 20, or 24 h. In that study, the statistically significant differences for IL-8 were already detected after a postexposure time of 0 to 6 h. However as the exposure duration was 4 to 6 h, the dose reaching the cell cultures was higher and that probably allowed the cells to react faster. The postexposure time of exposed lung slices in the French studies was between 0 (*31*) and 24 h (*30, 38*), also in the same duration range. The currently chosen postexposure time seems to be in the range of that already applied.

Outlook. Following the here described evaluation of the new exposure system, the toxic potential of different two-stroke scooter types (DI, carburetor) with various technical measurements (oxidative catalyst, particle filter, different fuels, lubricant oils), of four-stroke scooters and of a diesel passenger car with and without particle filter are planned to be compared. The toxic potential of different vehicles should be comparable, when looking at the differences shown between exposure and reference. Additionally, it would be possible to expand the measurements to other emission types, such as emissions from modern wood heating. Our system allows the validation of the hazard potential of engine emissions in an experimental setup and can support policy makers to promote or to discourage the use of technologies or fuels at a very early stage in the development.

Acknowledgments

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Supporting Information Available

Details about technical equipment and biological procedures and assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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2.2.1 Supporting Information to Project 2

New Exposure System To Evaluate the Toxicity of (Scooter) Exhaust Emissions in Lung Cells in Vitro – Supporting Information

Loretta Müller, Pierre Comte, Jan Czerwinksi, Markus Kasper, Andreas C.R. Mayer, Peter Gehr, Heinz Burtscher, Jean-Paul Morin, Athanasios Konstandopoulos, and Barbara Rothen-Rutishauser

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A New Exposure System to Evaluate the Toxicity of (Scooter) Exhaust Emissions in Lung Cells in vitro.

Loretta Müller*[,1], Pierre Comte[2], Jan Czerwinski[2], Markus Kasper[3], Andreas C.R. Mayer[4], Peter Gehr[1], Heinz Burtscher[5], Jean-Paul Morin[6], Athanasios Konstandopoulos[7], and Barbara Rothen-Rutishauser[1]

* Corresponding author phone: +41 31 631 84 46; e-mail: loretta.mueller@ana.unibe.ch

[1] Institute of Anatomy, Division of Histology, University of Bern, Baltzerstrasse 2, CH-3000 Bern 9, Switzerland

[2] Laboratory for Exhaust Emission Control, Bern University of Applied Sciences, Biel-Bienne, Switzerland

[3] Matter Engineering AG, Nanoparticle Measurement, Wohlen, Switzerland

[4] Technik Thermische Maschinen (TTM), Switzerland

[5] Institute of Aerosol and Sensor Technology, University of Applied Sciences Northwestern Switzerland, Windisch, Switzerland

[6] INSERM, Université de Rouen, France

[7] APTL, CERTH, Thessaloniki, Greece

Supporting Information

Number of pages: 14

Number of figures: 6

Number of tables: 3

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Materials and Methods

16HBE14o cell cultures

Human bronchial epithelial cells (cell line 16HBE14o, passage p2.43-p2.78, kindly received from Dr. Gruenert, University of California, San Francisco) were kept at 37°C in a 5% CO_2 humidified atmosphere using medium MEM 1x (with Earle's Salts, without L-Glutamine; Gibco BRL Life Technologies Invitrogen AG, Basel, Switzerland) supplemented with 10% fetal calf serum (PAA Laboratories, Lucerna-Chem AG, Lucerne, Switzerland), 1% L-Glutamine (200mM stock solution; LabForce AG, Nuningen, Switzerland) and 1% penicillin/streptomycin (10'000 units/ml penicillin G and 10'000 µg/ml streptomycin sulfate in 0.85% saline; Gibco BRL, Invitrogen AG). The cells were maintained in standard tissue culture flasks (25 or 75 cm^2, with filter screw cap, sterile; TPP AG, Trasadingen, Switzerland), treated with fibronectin coating solution containing 10% albumin from bovine serum (1 mg/ml, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), 1% bovine collagen Type I (3 mg/ml, BD Biosciences, Basel, Switzerland) and 1% human fibronectin (1 mg/ml, BD Biosciences) in basal medium eagle (product number B1522, Sigma-Aldrich Chemie GmbH). For splitting and seeding, they were removed from the flasks surface with trypsin – ethylene-diamine-tetra-acetic acid (EDTA; 0.5 g/l of trypsin and 0.2 g/l of EDTA•4Na in Hanks' Balanced Salt Solution; Gibco BRL, Invitrogen AG) and counted with a Neubauer counting chamber. For experiments, cells were seeded at a density of $1x10^6$ cell/insert (surface area of 4.2 cm^2, pores with 3.0 µm in diameter, high pore density PET membranes for 6-well plates; BD Falcon, BD Biosciences) and the inserts placed in 6-well tissue culture plates (BD Biosciences). In the lower chamber 3 ml and in the upper chamber 2 ml of medium were added. A change of medium was done after three days in culture.

A549 cell cultures

Human epithelial lung cells (cell line A549 Lieber et al. 1976), passage p90-p95, from American Tissue Type Culture Collection, LGC Prochem, Molsheim, France) were handled almost the same way as 16HBE14o. The only difference is the basic medium used, Roswell Park Memorial Institute medium (RPMI) 1640 (with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); Labforce AG) was different and the plates/inserts were uncoated.

Isolation of monocytes

The monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (MDDCs) were obtained from human blood monocytes as previously described (*1*). Briefly, peripheral blood monocytes were isolated from Buffycoat (blood donation service, Bern, Switzerland) by density gradient centrifugation on Ficoll-Plaque (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland). The monocytes were resuspended in RPMI 1640 with 1% L-Glutamine, 1% penicillin/streptomycin and 10% human serum (blood donation service, Bern, Switzerland). After 1.5 h in 6-well plates, to allow for adhesion, the medium was removed together with nonadherent cells. The adherent monocytes were cultured for 7-10 days with medium RPMI 1640 with 1% L-Glutamine, 1% penicillin/streptomycin and 5% of human serum. For the development of MDDC, Granulocyte monocyte colony stimulating factor (50 ng/ml; R&D systems, Oxon, UK) and interleukin 4 (34 ng/ml; Sigma-Aldrich Chemie GmbH) were added to the medium.

Triple cell co-cultures

The triple cell co-cultures were prepared as described previously (*1, 2*). Briefly, after culturing the 16HBE14o for 5 days on inserts, the MDDCs were removed, by scraping, and resuspended and 300 µl were added for 1.5 h on the basal side of the inserts, turned upside down. Non-

adherent MDDCs were removed, the inserts placed back in the plates with fresh medium (RPMI 1640 with 1% L-Glutamine, 1% penicillin/streptomycin and 5% human serum). MDMs were scraped and resuspended and 500 µl were added to the apical surface of the epithelial monolayer. After 1.5 h, the medium with non-adherent MDMs was removed and 2 ml fresh medium (RPMI 1640 with 1% L-Glutamine, 1% penicillin/streptomycin and 5% human serum) was added to the upper chamber.

The day after the preparation, the medium was removed and in the lower chamber 1 ml of fresh medium (RPMI 1640 with 1% L-Glutamine, 1% penicillin/streptomycin and 5% human serum) was added to supply the cells through the membrane. The volume was reduced to 1 ml to decrease the liquid pressure from the lower chamber. The co-cultures were exposed to air to be ready for exposure the following day.

Cytoskeleton, cell nuclei and tight junction staining

Cells were fixed with 4% paraformaldehyde during 15 minutes on ice. The staining of actin cytoskeleton and tight junctions was done as previously described (*1*). Briefly, after washing once with phosphate buffered saline (PBS; 10 mM, pH 7.4, 130 mM NaCl, Na_2HPO_4, KH_2PO_4) and storing in PBS, the cells were treated for 5 minutes with 0.1 M glycine (Cat. No. 50049, BioUltra, Sigma Aldrich Chemie GmbH) in PBS at 4°C and permeabilized in 0.2% Triton®X-100 (Fluka BioChemika, Sigma-Aldrich Chemie GmbH) in PBS for 15 minutes at room temperature. After washing with PBS, the cells were incubated at room temperature for 45 minutes with the first antibody (rabbit anti-human occludin (71–1500, Zymed, P. H. Stehelin & Cie AG, Switzerland), diluted 1:50 in PBS), washed with PBS three times and incubated for 45 minutes with the second antibody (goat anti-rabbit cyanine-5 (AP187S, Chemicon, VWR International AG, Life Sciences, Dietikon, Switzerland), diluted 1:50 in PBS) together with phalloidin rhodamine (R-415, Molecu-

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lar Probes, Invitrogen AG; diluted 1:100) and with 4',6-diamidino-2-phenylindole (DAPI; Bio-Chemika, for fluorescence, Sigma Aldrich Chemie GmbH, 1 mg/10ml, diluted 1:100). The cell cultures were finally mounted in PBS : glycerol (2 : 1) containing 170 mg/ml Mowiol 4-88 (Calbiochem, VWR International AG) on object holders, covered with coverslips.

Cytotoxicity

In order to determine cell death, the release of lactate dehydrogenase (LDH) from necrotic cells was measured. The LDH content in the supernatants was measured using the Cytotoxicity Detection Kit (Cat. No. 11 644 793 001, Roche Applied Science, Rotkreuz, Switzerland) according to supplier's manual. Briefly, 100 µl supernatant of cell cultures was transferred to a 96-well plate (NUNC, MaxiSorp; Fisher Scientific AG, Wohlen, Switzerland), solution 1 and solution 2 were mixed in the correct ratio and added to the wells. After 15 minutes, the absorbance (excitation of 490 nm, reference wavelength of 630 nm) was measured using a spectrophotometer (Benchmark Plus Microplate Spectrophotometer, BioRad, Hempel Hempstead, UK). Cytotoxicity was expressed as percentage of LDH content of the negative control.

Cytokine/chemokine detection

Concentrations of TNF-α and IL-8 were detected using ELISA Kits (Cat. No. DY210 and DY208, respectively, DuoSet ELISA Development kit, R&D Systems, Oxon, UK) following manufacturer's recommendations. Briefly, the supernatant of cell cultures was removed, stored at -70°C and centrifuged (3000rcf, 10 minutes). The 96-well plate was coated over night with capture antibody, washed three times, blocked for 1 h with blocking buffer, washed again and then incubated with samples and standard for 2 h. For detection of TNF-α, the samples were used undiluted, and for IL-8 detection the samples were diluted 1:10. The highest standard concentration

was in both cases 5 ng/ml. After another washing step, detection antibody was added and incubated for 2 h. Washing and 20 minutes of incubation with streptavidin-HRP was followed by another washing step and incubation with the substrate solution for 20 minutes. After stop solution (1 M H_2SO_4 solution) addition, the plate was placed for 2 minutes on a shaker and then the absorbance was measured with a spectrophotometer (Benchmark Plus Microplate Spectrophotometer, BioRad) using 450 nm as excitation and 570 nm as wavelength correction.

Statistics

Data are expressed as mean values with standard deviation (three independent experiments). The statistical analysis was performed using Excel for Windows and SigmaStat for Windows (Version 3.10, SigmaSTAT Software, Inc.). Many groups were compared using ANOVA On Rank test, followed by Dunn's test in the case of significance. Significance of only two groups was tested using Mann-Whitney U test. $p < 0.05$ was considered to be significant.

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Supporting Figures

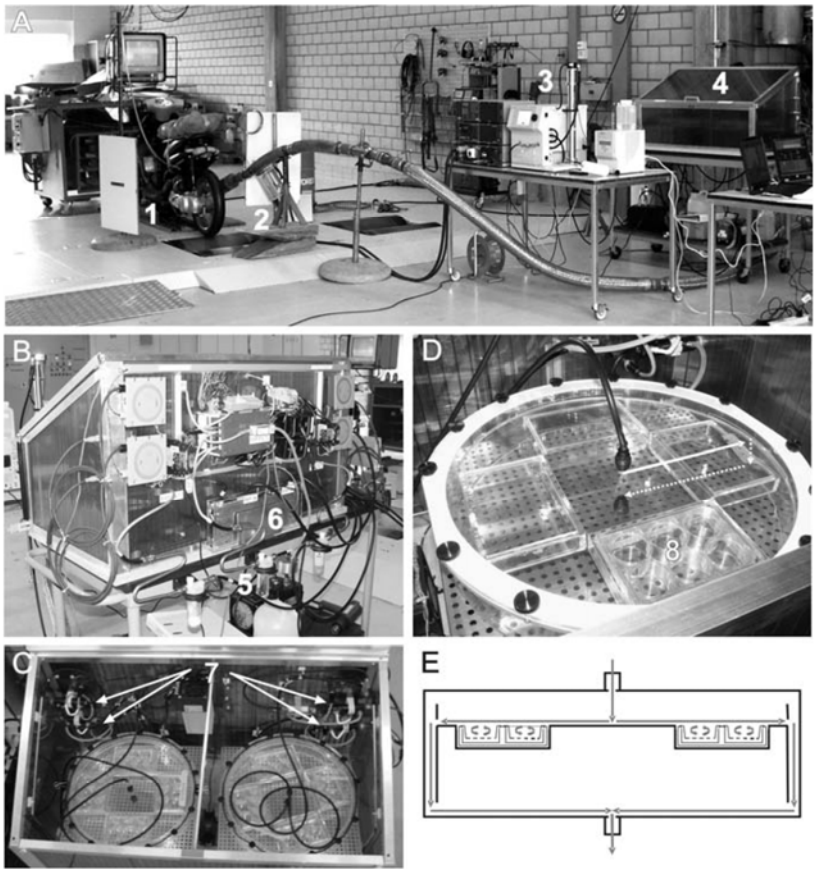

Figure S1. (A) Overview of the exposure system with the scooter on the chassis dynamometer (1), the dilution unit (2), the measuring instruments (3) and the exposure box (4). (B) Overview of the exposure box with the vacuum pump at the end of the system (5) and the heated water chamber which passed by the exhaust emissions in semi-permeable tubes for humidification (6). (C) Interior view of the exposure box with the exposure (right) and reference (left) chambers for the exposure of cell cultures and the measurement units (7) controlling the conditions (temperature, pressure, relative humidity, CO_2 and CO concentration) in the chamber. (D) Details of the exposure chamber with space for four 6-well plates (8) and white arrows showing the flow direction. (E) Schematic lateral view of the exposure chamber with the flow pattern (grey straight arrows) and the diffusion processes (grey twisted arrows).

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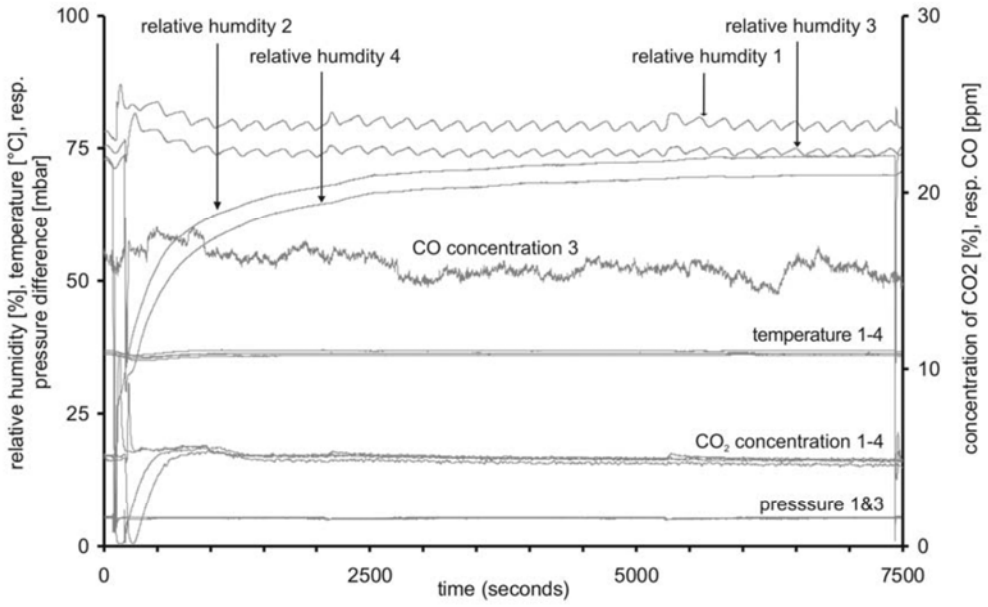

Figure S2. Measurement of the conditions before and after the chamber to control the environment for the cell cultures. Measurement point 1 is before the exposure chamber, point 2 after the exposure chamber, point 3 before the reference chamber and point 4 after the reference chamber.

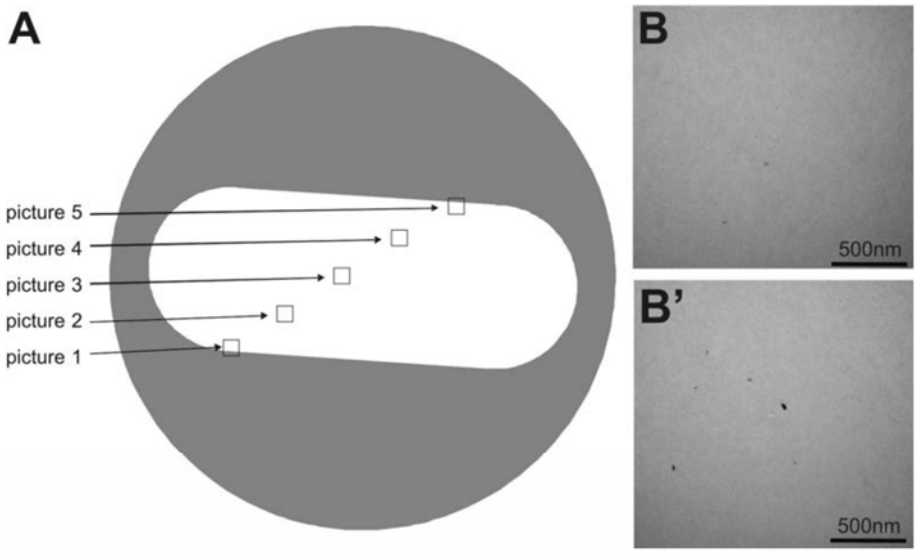

Figure S3. Example of particles on TEM grids. (A) draft of the systematically picture sampling, (B) picture of a TEM grid exposed to reference air and (B') exposed to exhaust emissions.

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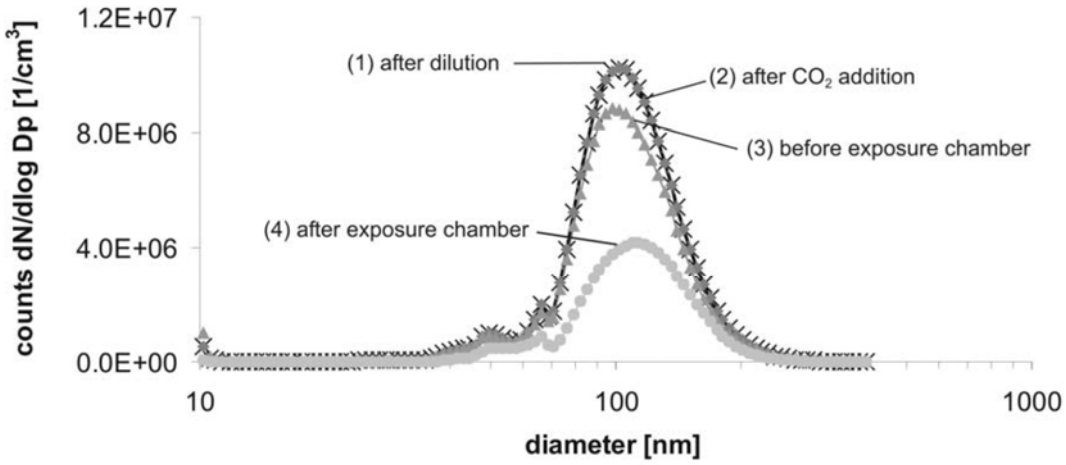

Figure S4. Size distribution of particles at different sampling points in the exposure system measured with SMPS.

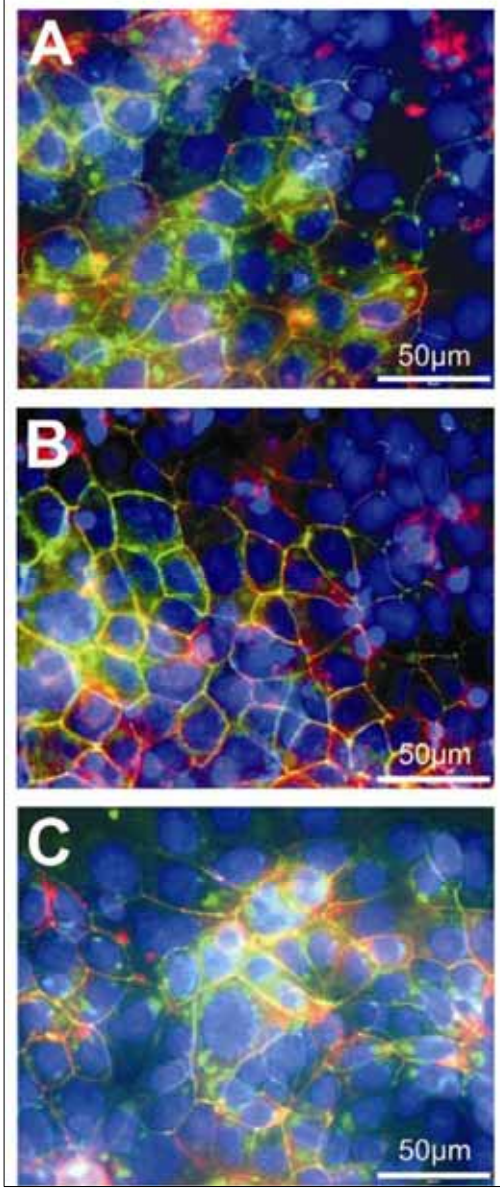

Figure S5. Fluorescence pictures of 16HBE14o epithelial cell layer. (A) control, (B) reference and (C) exposed cells. Cell nuclei are visualized in blue, actin cytoskeleton in red and occludin tight junctions in green.

S9

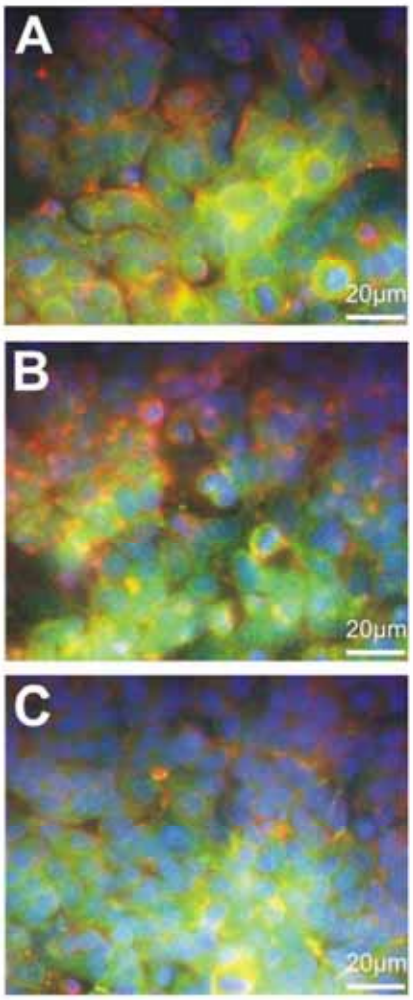

Figure S6. Fluorescence pictures of A549 cell layer. (A) control, (B) reference and (C) exposed cells. Cell nuclei are visualized in blue, actin cytoskeleton in red and occludin tight junctions in green.

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Supporting Tables

Table S1. Technical details of the test bench and exhaust emission characterization as it is done routinely following international regulations.

company and location	University of Applied Sciences, Biel-Bienne; CH-2560 Nidau		
chassis dyno	maker and type	Schenck, 500 GS 60	
	power capacity	brake power: 60 kW; driving power: 56 kW	
	maximum speed	200 km/h	
	roller diameter	502 mm	
capable drive system	front and rear wheel drive, two-rollers		
inertia weight	mechanical	140 kg – 3350 kg	
	electrical	yes	
climate controlling of chassis dyno cell		yes [20°C - 30°C]	
constant volume sampling (CVS)	maker and type	Horiba, 9500 T (PDP)	
	maximum flow	13.3 m^3/min	
	tunnel dimension	length: 4300 mm, diameter: 323 mm	
CVS gas analyzer	maker and type	Horiba, Mexa 9400 H	
	maximum flow	13.3 m^3/min	
	THC [range] [ppm]	flame ionization detector	10; 100; 500; 2000
	HC [ppm]	non dispersive infrared (NDIR)	100; 250; 1000
	CH_4 [ppm]	NDIR with catalyst	10; 100; 1000; 10000
	NO_x [ppm]	chemiluminescent detector	10; 50; 200; 2000
	CO_{low} [ppm]	NDIR	50; 250; 1000; 2500
	CO_{high} [%]	NDIR	1; 3

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	CO_2 [%]	NDIR	2; 8; 16
	O_2 [%]	magneto-pneumatic analyzer	5; 10; 25

Table S2. Technical details of the used scooter.

vehicle identification	Peugeot Looxor TSDI
model year	2002
transmission number of gears	variomat
kilometers at beginning	1400
engine type	two-stroke
engine displacement	49.1 cm^3
number of cylinders	1
cooling	air forced
rated power	3.6 kW
rated speed	7800 rpm
idling speed	1700 rpm
maximal vehicle speed	45 km/h
weight empty	94 kg
mixture preparation	direct injection with automatic oil pump

Table S3. Physical and chemical analysis of the lubricant oil Army SAE50.

parameter	condition	unit	limit value	test method
visual nature	visual		lucent, bright	visual
density	20°C	kg/m³	max. 910	DIN 51757

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kinetic viscosity	100°C	mm²/s	12.7	DIN 51562/1
	40°C	mm²/s	154	DIN 51562/1
viscosity index		-	65	ISO 2909
pour point		°C	max. -24	ISO 3016
flash point	Cleveland Open Cup	°C	min. 240	ISO 2592
evaporation loss	1h/250°C	g/100g	max. 7.0	NOACK volatility test
foaming characteristics	foam volume at 24°C (sequence 1)	ml	max. 30/0	American Society for Testing and Materials (ASTM d 892
	foam volume at 93.5°C (sequence 2)	ml	max. 70/0	
high pressure characteristics	FZG gear test rig a/8.3/90	strength level	min. 11	DIN 51354
sulfate ash	775°C	g/100g	min. 0.5	DIN 51575
residual coke	Conradson	g/100g	max. 0.85	DIN 51551
sulfur		g/100g	1.25	X-ray fluorescence/ inductively coupled plasma optical emission spectrometry
trace elements	silver (Ag)	mg/kg	< limit of detection (LOD)	inductively coupled plasma optical emission spectrometry
	aluminum (Al)	mg/kg	< LOD	
	boron (B)	mg/kg	< LOD	
	barium (Ba)	mg/kg	93	
	calcium (Ca)	mg/kg	1122	

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cadmium (Cd)	mg/kg	< LOD
chrome (Cr)	mg/kg	< LOD
cupper (Cu)	mg/kg	< LOD
iron (Fe)	mg/kg	1
magnesium (Mg)	mg/kg	1
manganese (Mn)	mg/kg	< LOD
molybdenum (Mo)	mg/kg	< LOD
nickel (Ni)	mg/kg	< LOD
sodium (Na)	mg/kg	22
phosphor (P)	mg/kg	553
lead (Pb)	mg/kg	1
silicon (Si)	mg/kg	10
tin (Sn)	mg/kg	< LOD
titan (Ti)	mg/kg	< LOD
vanadium (V)	mg/kg	< LOD
zinc (Zn)	mg/kg	466

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(2) Blank, F.; Rothen-Rutishauser, B. M.; Schurch, S.; Gehr, P. An optimized in vitro model of the respiratory tract wall to study particle cell interactions. *J.Aerosol Med.* **2006,** *19*, 392-405.

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2.3 Project 3 - Comparison of the toxic potential of different vehicles

Toxic Potential of Two- and Four-Stroke Scooter and Diesel Car Exhaust Emissions *In Vitro*.

Loretta Müller, Pierre Comte, Jan Czerwinksi, Markus Kasper, Andreas C.R. Mayer, Peter Gehr, and Barbara Rothen-Rutishauser

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3 Discussion of the Findings

This PhD study mainly aimed to develop a new exposure system to directly expose lung cell cultures to total exhaust emissions at the air-liquid interface and the evaluation of the toxic potential of different vehicle emissions. In addition we investigated the differences in reactions of cell mono-cultures and triple cell co-cultures upon NP exposure.

This new exposure system allows the direct exposure of cell cultures to total exhaust emissions. The direct exposure of cell cultures *in vitro* mimics the situation in the lung as realistically as possible. The flow pattern of diffusion processes especially reflects the *in vivo* situation (Ochs and Weibel, 2008). Additionally the use of the triple cell co-culture model of the airway epithelial barrier offers a more realistic model than mono-cultures of one cell type alone (Alfaro-Moreno et al, 2008; Muller et al, 2010b; Rothen-Rutishauser et al, 2008). Compared to the *in vivo* situation in the lung even the use of three different cell types (epithelial cells, macrophages and dendritic cells) combined in the co-culture model only represents part of the complexity. However, the co-culture model can not consider all of the different cell types (over 40) in the lung (Ochs and Weibel, 2008), the blood circulation, the lymph circulation or the interplay of various organs. The whole complexity of the human lung could only be included by using *in vivo* testing of animals or humans. With the application of *in vivo* exposure of animals at least the complexity of a whole organism can be considered, but the problem of the extrapolation from animals to humans still exists. The actual experimental design and the cell culture model used need to be adapted to investigate chronic exposure as it occurs in the reality. This is a general disadvantage of *in vitro* cell cultures exposed at the air-liquid interface. Further experiments will use cell cultures to co-expose them to exhaust emissions and to other stressors, such as the influenza virus. Furthermore, it allows the testing of single technical optimizations, which would be not possible with epidemiological tools. This testing can be carried out even before the technical optimization is on the market and can be treated as a pre-test and evaluation of the effects on the toxic potential. Industrial players may use such testing as a sales argument and for their marketing.

Comparing the new exposure system with other exposure systems described in the literature (Table 2 in Muller et al, 2010a), the probably most important conclusion is

the need for more standardization. As the systems use not the same conditions, different biological samples and also variable technical settings of the vehicles, it is just not possible to compare the results of different publications.

Only very few and quite old studies about the toxicity of scooter exhaust emissions are published. Zhou and colleagues showed the mutagenicity of scooter exhaust PM (Zhou and Ye, 1997) and an activation of oncogenes in human diploid cells strain cells (Zhou and Ye, 1998b). Further they could show the effects of lubricant on the mutagenicity (Zhou and Ye, 1998a). Studies using cell cultures or *in vivo* studies about the toxic effects of scooter exhaust emissions do not exist and therefore the results can not be compared to the current research literature.

The presented PhD thesis showed adverse effects of exhaust emissions in a model of the human airway epithelial barrier and a reduction of the toxic potential when technical optimizations, such as better fuel, better oil or particle filters, were introduced. Following the precautionary principle with this knowledge and the knowledge of adverse health effects due to the single components of the exhaust (e.g. CO, HC, NO_x, particles) (Ackermann-Liebrich et al, 1997; Becker et al, 2005; Braun-Fahrlander et al, 1997; Brauner et al, 2007; Donaldson et al, 2005; Ghosh et al, 2010; Pan et al, 2004; Xiao et al, 2003), there is a high importance of a (further) reduction of the exhaust emissions. For a more precise analysis about which of the exhaust emission compounds should be focused on, further studies are necessary. Out of the here presented results it can only be concluded that the particle number concentration is the most relevant parameter and should be reduced as much as possible.

Effects of particles can be induced by two different ways: Either particles enter the cells and interact with cell-internal structures or particles attach to the cell surface and activate receptors inducing cell signaling. Further it has to be differentiated if the effect is induced by the particle itself or by substances adsorbed to the particle surface and released either in the cell or close to the cell surface (Limbach et al, 2007; Oberdorster et al, 2005). In order to distinguish between the two ways, the number of particles localized intracellular should have to be determined. However, for exhaust particles it is very difficult and refers to a real challenge. Their sizes are in the nanoscaled size range and therefore need to be visualized by TEM. Only bigger agglomerates could be detected inside cells, however, since this occurs only in suspension experiments in the here presented experimental setup, i.e. air-liquid

exposed cells, no such agglomerates could be detected. It has to be assumed that smaller agglomerates or single particles entered the cells, but as they are not very electron dense and have no well defined shapes, they could only be identified clearly including the methods of elemental analysis (Brandenberger et al, 2010a). Element analysis by the mean of electron spectroscopic imaging (ESI) is applicable when the elemental composition of the particles is known. This requirement is not fulfilled for exhaust particles. They have no constant chemical composition and a previous chemical element analysis of the particles would be needed. With this information ESI could be performed and theoretically particles could be identified intracellular. As an elemental analysis of exhaust particles is a time-consuming and expensive issue, this was not possible to do within this study and would be an additional interesting topic for an ongoing study.

Finally, it can be concluded that the newly developed exposure system is a tool for testing the toxic potential of different vehicle's exhaust emissions in a human epithelial airway model, as well as testing the effects of single technical optimizations (e.g. particle filter, better fuel and oil). Further, statistically significant differences between cell cultures exposed to exhaust emission and such exposed to reference air were found. Between the toxic potential of different vehicles or set-ups only few statistically significant differences could be shown. Probable reasons for that fact can be the short exposure time (2 h), the high dilution of the exhaust emissions (1:100) and the low number of experimental repetitions (n=3). The most relevant exhaust parameter which might mainly be responsible for the toxic potential is the particle number concentration, which should be reduced as much as possible.

In summary, it can be concluded that a new exposure system to investigate the effects of (scooter) exhaust emissions in cell cultures *in vitro* could be developed and established. Furthermore, it was applied to compare the effects of exhaust emissions of different vehicles and set-ups. The implementation of modern particle filters in two-stroke scooters was shown to reduce the toxic potential. Overall, the particle number concentration was found to have a statistically significant effect on the toxic potential and should be reduced as much as possible. Concerning the cell cultures, it was shown that the interplay of different co-cultured cell types (epithelial cells, MDM and MDDC) alters the oxidative stress and (pro-) inflammatory responses upon NP exposure and can mimic the reality in the airways better than mono-cultures.

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Curriculum Vitae

Loretta Lina Müller

Personal Data

Address:	Mörsbergerstr. 48
	CH-4057 Basel, Switzerland
Email:	lorettamueller@gmx.ch
Date of birth:	21.06.1982
Place of birth:	Basel, Switzerland
Nationality:	Swiss

photo by andrea lehmann

Education

Since July 2007	PhD study in Cellular and Biomedical Sciences Group of Peter Gehr, Institute of Anatomy, Faculty of Medicine, University of Bern, Switzerland
Since October 2005	Study of Behavioral Sciences ETH Zurich, Switzerland
April - October 2006	Diploma thesis ("Masters" thesis equivalent) *"Nanoparticle-related Oxidative Stress on Human Lung Cells: The Effects of Carbon Nanotubes, Diesel Exhaust Particles and Titanium Dioxide Nanoparticles on the Cell Types A549, AM, DC and Triple Cell Co-Cultures"* Group of Peter Gehr, Institute of Anatomy, Faculty of Medicine, University of Bern, Switzerland
October 2001 - October 2006	Diploma study in Environmental Sciences (aquatic systems and biomedicine) ETH Zurich, Switzerland
June 2001	University entrance qualification ("Matura") Gymnasium Bäumlihof, Basel, Switzerland

Assistance in courses during PhD

Tutorial supervision on problem based learning (PBL) for medical students (1st year), Institute of Anatomy, University of Bern

Teaching assistance in microscopic histological courses for medical students (1st and 2nd year), Institute of Anatomy, University of Bern

Assistance for Histology Exams for medical students (1st and 2nd year), Institute of Anatomy, University of Bern

Grant

Swiss National Foundation fellowship for prospective researchers: personal two year founding for the project *Effects of Diesel Exhaust Emissions in Co-Cultures mimicking Human Nasal Mucosa and the Impact on Influenza Infections* in the group of Prof. Ilona Jaspers at the University of North Carolina, North Carolina, USA

Awards

Award for highly skilled persons in the field of scientific research (2008) from a foundation in Liechtenstein for my diploma thesis *"Nanoparticle-related Oxidative Stress on Human Lung Cells: The Effects of Carbon Nanotubes, Diesel Exhaust Particles and Titanium Dioxide Nanoparticles on the Cell Types A549, AM, DC and Triple Cell Co-Cultures"*

Berner Umwelt-Forschungspreis, Anerkennungspreis (2007) for my diploma thesis *"Nanoparticle-related Oxidative Stress on Human Lung Cells: The Effects of Carbon Nanotubes, Diesel Exhaust Particles and Titanium Dioxide Nanoparticles on the Cell Types A549, AM, DC and Triple Cell Co-Cultures"*

"Schweizer Jugend forscht" award (2001) (grade "very good") for my work *"Exposure to carbon monoxide (CO) during the course of the day and in special situations"* and a special price to participate in the International Summer Science Institute at the Weizmann Institute of Science in Rehovot, Israel.

Memberships

European Respiratory Society (ERS)

Swiss Society for Optics and Microscopy (SSOM)

Swiss Society for Anatomy, Histology and Embryology (SAGHE)

Publications in peer reviewed journals

Helfenstein M, Miragoli M, Rohr S, Müller L, Wick P, Mohr M, Gehr P and Rothen-Rutishauser B: *Effects of combustion-derived ultrafine particles and manufactured nanoparticles on heart cells in vitro*. Toxicology 253(253), 70-78 (2008)

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Conference abstracts

Müller L, Riediker M, Wick P, Mohr M, Gehr P and Rothen-Rutishauser B: Nanoparticle-related oxidative stress in human lung cells: the effects of carbon nanotubes, diesel exhaust particles and titanium dioxide nanoparticles.

Poster presentation at the 11th ETH Conference on Combustion Generated Nanoparticles, Zurich, Switzerland (2007).

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P, Burtscher H, Morin J P and Rothen-Rutishauser B: A new exposure system to evaluate the toxicity of scooter emissions in lung cells in vitro.

Oral presentation at the 12th ETH Conference on Combustion Generated Nanoparticles, Zurich, Switzerland (2008).

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P, Burtscher H, Morin J P and Rothen-Rutishauser B: A new exposure system to evaluate the toxicity of scooter emissions in lung cells in vitro.

Poster presentation at Nanotoxicology - 2nd International Conference, Zurich, Switzerland (2008).

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P, Burtscher H, Morin J P and Rothen-Rutishauser B: A new exposure system to evaluate the toxicity of scooter emissions in lung cells in vitro.

Poster presentation at the Tag der Klinischen Forschung, DKF, University of Bern (2008)

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P and Rothen-Rutishauser B: Cytotoxicity and inflammatory potential of two stroke scooter exhaust in lung cells in vitro.

Poster Presentation at the Graduate School Symposium, University of Bern (2009)

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P, Burtscher H, Morin J P and Rothen-Rutishauser B: Cytotoxicity and inflammatory potential of two stroke scooter exhaust in lung cells in vitro.

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Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P and Rothen-Rutishauser B: Cytotoxicity and inflammatory potential of two stroke scooter exhaust in lung cells in vitro.

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Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P and Rothen-Rutishauser B: Cytotoxicity and inflammatory potential of two stroke scooter exhaust in lung cells in vitro.

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Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P and Rothen-Rutishauser B: Cytotoxicity and inflammatory potential of two stroke scooter exhaust in lung cells in vitro.

Poster presentation at the Graduate School Symposium, University of Bern (2010)

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P and Rothen-Rutishauser B: Scooter Nanoaerosol Health-Effect Research or simply Toxicity of Scooter Emissions

Oral Presentation at the 1st VERT-Forum, Dübendorf, Switzerland (2010)

Müller L, Comte P, Czerwinski J, Kasper M, Mayer A C R, Gehr P and Rothen-Rutishauser B: Higher toxic potential of 2-stroke scooter exhaust emissions compared to 4-stroke scooter and diesel car emissions.

Poster presentation at the American Thoracic Society (ATS) International Conference, New Orleans, USA (2010).

This abstract was accepted as a "late breaking abstract".

Declaration of Originality

Last name, first name: **Müller, Loretta Lina**

Matriculation number: **01-922-558**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

Bern, 29.06.2010