Respiratory hazard assessment of combined exposure to complete gasoline exhaust and respirable volcanic ash in a multicellular human lung model at the air-liquid interface☆

Ines Tomašek [a, b, *], Claire J. Horwell [a], Christoph Bisig [b], David E. Damby [c], Pierre Comte [d], Jan Czerwinski [d], Alke Petri-Fink [b, e], Martin J.D. Clift [f], Barbara Drasler [b], Barbara Rothen-Rutishauser [b, **]

[a] Institute of Hazard, Risk & Resilience, Department of Earth Sciences, Durham University, Science Labs, Durham, DH1 3LE, United Kingdom
[b] BioNanomaterials group, Adolphe Merkle Institute, University of Fribourg, Chemin des Verdiers 4, 1700, Fribourg, Switzerland
[c] Volcano Science Center, United States Geological Survey, Menlo Park, CA, 94025, United States
[d] Laboratory for IC-Engines and Exhaust Emission Control, Bern University for Applied Sciences, Gwerdtstrasse 25, 2560, Nidau, Switzerland
[e] Chemistry Department, University of Fribourg, Chemin des Musee, 1700, Fribourg, Switzerland
[f] In Vitro Toxicology Group, Swansea University Medical School, Singleton Park Campus, Swansea, SA2 8PP, Wales, United Kingdom

ARTICLE INFO

ABSTRACT

Communities resident in urban areas located near active volcanoes can experience volcanic ash exposures during, and following, an eruption, in addition to sustained exposures to high concentrations of anthropogenic air pollutants (e.g., vehicle exhaust emissions). Inhalation of anthropogenic pollution is known to cause the onset of, or exacerbate, respiratory and cardiovascular diseases. It is further postulated similar exposure to volcanic ash can also affect such disease states. Understanding of the impact of combined exposure of volcanic ash and anthropogenic pollution to human health, however, remains limited.

The aim of this study was to assess the biological impact of combined exposure to respirable volcanic ash (from Soufrière Hills volcano (SHV), Montserrat and Chaitén volcano (ChV), Chile; representing different magmatic compositions and eruption styles) and freshly-generated complete exhaust from a gasoline vehicle. A multicellular human lung model (an epithelial cell-layer composed of A549 alveolar type II-like cells complemented with human blood monocyte-derived macrophages and dendritic cells cultured at the air-liquid interface) was exposed to diluted exhaust (1:10) continuously for 6 h, followed by immediate exposure to the ash as a dry powder ($0.54 \pm 0.19\,\mu g/cm^2$ and $0.39 \pm 0.09\,\mu g/cm^2$ for SHV and ChV ash, respectively). After an 18 h incubation, cells were exposed again for 6 h to diluted exhaust, and a final 18 h incubation (at 37 °C and 5% CO_2). Cell cultures were then assessed for cytotoxic, oxidative stress and (pro-)inflammatory responses.

Results indicate that, at all tested (sub-lethal) concentrations, co-exposures with both ash samples induced no significant expression of genes associated with oxidative stress (HMOX1, NQO1) or production of (pro-)inflammatory markers (IL-1β, IL-8, TNF-α) at the gene and protein levels. In summary, considering the employed experimental conditions, combined exposure of volcanic ash and gasoline vehicle exhaust has a limited short-term biological impact to an advanced lung cell in vitro model.

© 2018 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Abbreviations

ALI	Air-liquid interface
CASP7	Caspase-7 gene
ChV	Chaitén volcano
CO	Carbon monoxide
CO_2	Carbon dioxide
cRPMI	Complete RPMI 1640 cell medium (supplemented with 1% L-Glutamine, 1% Penicillin/Streptomycin and 10% fetal bovine serum)
DEP	Diesel exhaust particles
FAS	FAS receptor gene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase gene
GDI	Gasoline direct injection
GE	Gasoline exhaust
HMOX1	Heme oxygenase 1 gene
IL-1β	Interleukin-1 beta
IL1B	Interleukin-1 beta gene
IL-8	Interleukin 8
IL8	Interleukin 8 gene
LDH	Lactate dehydrogenase
LOI	Loss on ignition
LSM	Laser scanning microscopy
MDDC	Monocyte-derived dendritic cells
MDM	Monocyte-derived macrophages
MDL	Method detection limit
NMHC	Non-methane hydrocarbons
NO_x	Nitrogen oxide(s)
NQO1	NAD(P)H dehydrogenase [quinone] 1 gene
PBS	Phosphate buffered saline
PET	Polyethylene terephthalate
PN	Particle number
PSD	Particle size distribution
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
SEM	Scanning electron microscopy
SHV	Soufrière Hills volcano
SiO_2	Silicon dioxide
THC	Total hydrocarbons
TNF-α	Tumor necrosis factor-alpha
TNFA	Tumor necrosis factor-alpha gene
VA	Volcanic ash
WLTC	Worldwide Light-duty Test Cycle
XRF	X-ray fluorescence

1. Introduction

Communities resident in urban areas located near active volcanoes can experience volcanic ash exposures during, and following, an eruption, in addition to sustained exposures to high concentrations of anthropogenic air pollutants (*e.g.,* vehicle exhaust emissions). Furthermore, ash can be transported over great distances (*e.g.,* as occurred during the 2010 Eyjafjallajökull eruption, Iceland (Gudmundsson et al., 2012)) and may reach distant urban areas, thereby having various impacts on human lives and livelihoods, including potential negative effects upon human health (Barsotti et al., 2010; Horwell and Baxter, 2006; Baxter & Horwell, 2015). Consequently, governments, public health agencies and scientific communities are voicing concerns about the potential health impacts from concomitant exposure to volcanic and anthropogenic emissions (Loughlin et al., 2012), especially whether this may pose a greater respiratory hazard than inhaling respirable volcanic ash and vehicle emissions separately. Hence, it is important to understand the potential hazard of concomitant exposure so that civil protection managers and health agencies can make informed decisions on whether to advise that citizens take action to protect themselves during periods of intense exposure to both urban pollution and volcanic ash (McDonald et al., 2017).

A recent first investigation, by the authors, into the potential effects of volcanic ash exposure combined with exhaust particulate (intending to simulate an urban environment) showed that concomitant exposure of cells to respirable volcanic ash and standardized diesel exhaust particles (DEP (NIST SRM 2975)) can promote a heightened (pro-)inflammatory response *in vitro* (Tomašek et al., 2016). However, the understanding of the respiratory hazard which may result from these combined exposures still remains limited, especially since this first study only considered DEP and not complete exhaust (*i.e.,* the additional gaseous component). Exhaust is a complex mixture that contains particles but, also, condensed and gaseous fractions. These phases can impact lung health (*e.g.,* Reed et al., 2008), but could also interact directly with the ash. This interaction may result in the adsorption of inorganic gases, such as CO_2, CO and NO_x, and volatile organic compounds (*e.g.,* linear and polycyclic aromatic hydrocarbons) onto volcanic ash, potentially altering the ash surface chemical properties and affecting its potential toxicity. Hence, the use of 'complete' exhaust is a critical next step in deducing the hazard posed to populations exposed to volcanic emissions and pollutants within the air.

Inhalation of urban particulate matter is known to cause the onset of, or exacerbate, respiratory and cardiovascular diseases (Pope et al., 2015; Peters et al., 1997; Seaton et al., 1995). Although diesel engines are generally viewed as greater contributors to engine-derived ambient particulate matter (US EPA, 2004), it has been found that vehicles with gasoline engines, which are still the most popular engine type in some European countries (ACEA, 2017), can also emit substantial quantities of soot-like ultrafine particles (diameter < 100 nm) under certain operating conditions (Mathis et al., 2005; Zhang and McMahon, 2012; Banerjee and Christian, 2017). Vehicles with gasoline direct injection (GDI) technology were found to release up to 10^{12} particles/km, exceeding those of current diesel vehicles equipped with filters (Platt et al., 2017; Muñoz et al., 2016; Zhang and McMahon, 2012; Mohr et al., 2006). The effects of emissions, including particulate and gaseous phases (*i.e.,* complete exhaust), produced from gasoline vehicles with various engine technologies have been studied in recent years and some toxic effects, such as oxidative stress and DNA damage in the lungs, have been reported in *in vitro* and *in vivo* studies (Bisig et al., 2015; Mauderly et al., 2014; Reed et al., 2008; McDonald et al., 2007; Lund et al., 2007).

It has been shown that exposure to respirable volcanic ash can exacerbate pre-existing respiratory diseases, such as asthma and bronchitis (Baxter et al., 1981, 1983), and suppress immune function (Monick et al., 2013). Even though there is a high variability in discrete results of *in vitro* and *in vivo* toxicology assessments for volcanic ash (reviewed by Baxter et al., 2014, Hillman et al., 2012, and Horwell and Baxter, 2006), a general view from the studies, to date, is that ash is a low toxicity particle, but various studies have shown that ash can provoke inflammatory reactions in the lungs and (pro-)inflammatory reactions *in vitro* (Damby et al., 2013, 2016, 2018; Horwell et al., 2013; Lee and Richards, 2004). Discerning the components of volcanic ash responsible for any observed toxicity

has been difficult due to compositional variability amongst samples and eruptions. Possible mechanisms identified for ash toxicity involve the presence of reactive surface species, including, but not exclusively, iron, and the corresponding generation of reactive oxygen species (Horwell et al., 2003, 2007), and crystalline silica and its potential to activate the NLRP3 inflammasome (Damby et al., 2018; Baxter et al., 1999). The mechanisms resulting in the (pro-) inflammatory response *in vitro* following combined exposures to ash and DEP (Tomašek et al., 2016) are not yet clear, but may be driven by the individual particle-cell interactions, or possibly particle-particle interactions, which then interact with cells.

The aim of the present study was to assess the biological impact of combined exposure to cells of respirable volcanic ash and complete vehicle exhaust. A sophisticated *in vitro* approach, as also used in our previous study (Tomašek et al., 2016), provides a valuable first assessment of potential adverse impacts of such exposures, especially due to a lack of epidemiological studies that consider health effects of ashfall in heavily polluted urban areas.

Volcanic ash samples, from Soufrière Hills volcano, Montserrat, and Chaitén volcano, Chile, were used (to represent different magmatic compositions and eruption styles) in combination with freshly-generated complete gasoline exhaust from a GDI vehicle (containing the particulate, condensed and gaseous fractions). This is the first time that a real, complete exhaust has been used to study combined exposures with volcanic ash. Furthermore, this investigation is the first to evaluate and report on whether the toxicity of either volcanic ash or complete gasoline exhaust are altered by co-exposures, as well as whether the ash (magmatic) composition could influence the outcome of combined exposures *in vitro*.

As with our previous study, a multicellular *in vitro* human lung model, composed of epithelial lung cells (A549 alveolar type II-like cells) and two immune cell types (human blood monocyte-derived macrophages and dendritic cells) (Rothen-Rutishauser et al., 2005, 2008; Blank et al., 2006) cultured at the air-liquid interface (ALI) (Blank et al., 2007) was used. This well-established model has been proven to be suitable for various exposures at the ALI (Fytianos et al., 2016; Müller et al., 2011), thus reflecting, in part, the realistic physiological conditions of a respiratory exposure. Further, human epithelial airway barrier models have previously accompanied hazard assessment studies of products of different engines and/or fuels using a sophisticated, well-characterised exhaust exposure system (Bisig et al., 2015, 2016; Steiner et al., 2013a,b; Müller et al., 2012, 2011, 2010). Here, the multicellular model was directly exposed to gasoline emissions, followed by the addition of respirable volcanic ash and a second gasoline emissions exposure. Subsequent analyses of the cell cultures for cytotoxicity, oxidative stress and (pro-)inflammatory response were undertaken.

2. Materials and methods

2.1. Cell cultures

In vitro experiments were performed using an established multicellular lung model composed of three cell types mimicking the human alveolar epithelial tissue barrier as previously described (Rothen-Rutishauser et al., 2005, 2008; Blank et al., 2007). Briefly, the model consists of a layer of human alveolar type II-like epithelial cells (A549) cultured on polyethylene terephthalate (PET) membrane 6-well inserts (4.2 cm^2 growth area, 3.0 μm pore size; BD Falcon^{TM} Cell Culture Inserts, BD Biosciences, USA) at an initial density of 23.8 x 10^4 cells/cm^2. These were grown for 5 days prior to the addition of immune cells to form a co-culture. Human blood monocytes were isolated from buffy coats provided by the Transfusion Blood Bank (*Blutspendedienst SRK Bern AG*, Switzerland) as described previously (Lehmann et al., 2010) with

the adaptation of using CD14$^+$ magnetic beads (CD14 MicroBeads, *Miltenyi Biotec*, Germany) (Steiner et al., 2012). The monocyte-derived macrophages (MDM) and dendritic cells (MDDC) were added on the apical (1.2 x 10^4 cell/cm^2) and the basal (6.0 x 10^4 cell/cm^2) side of the insert with a A549 layer on the upper side of the insert, respectively. After 24 h incubation under submerged conditions, in complete RPMI 1640 cell medium (cRPMI; *Sigma-Aldrich*, Switzerland; supplemented with 1% ʟ-Glutamine, 1% Penicillin/Streptomycin and 10% fetal bovine serum), the triple-cell co-culture was transferred to the ALI (the cRPMI only present on the basal side of the insert) to habituate for a period of 24 h prior to exposures.

2.2. Volcanic ash sources, preparation and characterisation

Every explosive volcanic eruption generates ash, but particle characteristics (*e.g.*, surface area and reactivity, composition, particle size, leachable elements) and, potentially, toxicity will vary according to the magma composition and eruption style as well as post-eruptive factors such as distance from vent (of deposition), weathering, etc. Two volcanic ash samples were used in this study, to represent different magmatic compositions (andesite and rhyolite; see Section 3.1) and eruption styles. Both samples were chosen because they were erupted, transported and deposited into relatively-clean atmospheres, away from major sources of pollution. Neither was rained on prior to sampling. In addition, both samples have been well characterised for their physical and chemical properties in previous studies (Horwell et al., 2010, 2013; Horwell, 2007). The major elemental oxide composition of bulk samples was determined by X-ray fluorescence (XRF) (Axios-Advanced PW4400 XRF spectrometer, *PANalytical*, The Netherlands) on fused beads prepared from ignited ash powders mixed with a fluxing agent in a 1:5 ratio. Compositional data were recalculated to account for loss on ignition (LOI) (the weight difference between unignited and ignited powders), which also provided confirmation of sample freshness with regards to contamination (*e.g.*, with organic material).

The first ash sample, from the Soufrière Hills volcano (SHV), Montserrat was generated in a dome-collapse event on 12 July 2003 and was collected 4 km from the vent on the day of eruption. The bulk sample's physicochemical characterisation can be found in previous literature under different sample codes, as follows: *Soufrière Hills, Montserrat '03* in Horwell et al. (2007), Soufrière Hills, Montserrat 2003 in Horwell (2007), *Mon12/7/03* in Horwell et al. (2010) and *MBA12/7/03* in Horwell et al. (2013). Briefly, the ash is rich in crystalline silica (~12 wt% of the bulk ash is cristobalite), potentially the most pathogenic of the minerals found in volcanic ash (Baxter et al., 1999), and is considered fine grained for ash, with ~11.5 vol% of the bulk sample being sub-4 μm diameter (Horwell, 2007). This sample was used in the previous study investigating co-exposures with DEP in the same *in vitro* multicellular model (as *MVO12/7/03* in Tomašek et al., 2016).

The second sample was obtained from Chaitén volcano (ChV), in Patagonia, Chile and was deposited from an explosive eruption which occurred on 2 May 2008. The sample was collected 80 km away from the source, in the Patagonian Argentine province of Chubut, on 8 May 2008 (Horwell et al., 2010). This sample contains substantially less crystalline silica (~3 wt% of the bulk ash is cristobalite) than the SHV sample, but is similarly fine-grained, with ~12 vol% sub-4 μm material (as *Chai_03* in Horwell et al., 2010).

A Sioutas Cascade Impactor (*SKC Inc.*, USA) and Leland Legacy sample pump (*SKC Inc.*, USA) attached to a gravitational separation chamber were used to isolate a biologically relevant 'respirable' sub-sample of the bulk ash for use in the *in vitro* exposure model, as previously described (Tomašek et al., 2016). Briefly, bulk ash was introduced into the separation chamber by an airstream

established by the pump at a constant flow rate of 5 L/min. Particles above a theoretical spherical aerodynamic diameter of 5 μm sedimented while the remaining, smaller particles were sampled by the impactor. Size-fractionated samples were recovered and combined for use in characterisation and toxicity assays.

Particle size distributions (PSD) of isolated respirable samples were determined by Mie theory with a laser particle analyser (Beckman-Coulter LS 13 320; *Coulter Corporation,* USA) in water with sonication, with a refractive index set to 1.63 and an absorption coefficient of 0.1 (after Horwell, 2007). Results are presented as the average of three consecutive measurements of the sample. Scanning electron microscopy (SEM) was used to observe particle morphology. Particles were mounted on polycarbonate discs, coated with 30 nm of gold/palladium and imaged on a Hitachi SU-70 FEG SEM (*Hitachi, Ltd.*, Japan) using the secondary electron detector.

2.3. Volcanic ash exposure system

In order to deduce the individual and combined response from the co-culture, respirable ash samples were nebulized as dry powder directly over the cell cultures at the ALI using a dry powder insufflator (Model DP-4; *PennCentury Inc.,* USA). The dry powder insufflator was found suitable for this application in the previous study (Tomašek et al., 2016), where its efficiency in ash administration to cells was evaluated, representing a more realistic approach when compared to studies using pre-suspended ash in submerged cell culture conditions.

The exposure setup was used as described previously (Tomašek et al., 2016). Briefly, the ash was loaded into the sample chamber and then pushed through the device by small pulses of air administered using a 10 mL commercial syringe. The ash was discharged as a cloud over the cell culture plate located below the delivery tube within a closed nebulisation chamber. Two cell culture inserts from the same set (exposed beforehand to the exhaust or filtered air, see section 2.6) were exposed simultaneously to a single ash sample (SHV or ChV). The chamber was made of polystyrene and covered with aluminium foil on the inside to avoid particles being electrostatically attracted and sticking to the chamber walls. The quantification of deposited material was monitored by a quartz crystal microbalance (QCM; *Stanford Research Systems,* USA), also placed within the nebulisation chamber next to the wells, thereby allowing for a reliable estimation of the deposited mass. Calculated from the recorded frequency values before and after deposition of material, the ΔF value (Hz) is converted to deposited mass per area ($\mu g/cm^2$) (Lenz et al., 2009).

2.4. Vehicle exhaust exposure system

A flex-fuel GDI vehicle with a three-way catalyst was driven on a chassis dynamometer with standard market gasoline (RON 95) and lubrication oil. A dynamic, worldwide light-duty test cycle (WLTC) (UNECE, 2016), representing transient driving in urban, extra-urban, highway and motorway conditions, was driven and repeated for 6 h per day of exposures (10 cycles). The WLTC is the official driving cycle from September 2017 onwards used by the European Union for new vehicle registration (Euro6).

The exhaust exposure experiments were performed at the exhaust gas control station of the Bern University of Applied Sciences in Nidau, Switzerland, as previously described (Bisig et al., 2015; Müller et al., 2010, 2011; Morin et al., 2008). Briefly, the exhaust was diluted 1:10 in filtered air, based on previous work (Steiner et al., 2013a, 2013b) and to enable comparison with previous gasoline exhaust studies (Bisig et al., 2015, 2016), where it was noted that it represents a highly-polluted site (*i.e.,* a high dose

exposure).

The diluted exhaust was pumped through the cell culture exposure chamber with a constant flow of 2 L/min. In the chamber, the exhaust emissions pass above the cell culture plates and diffuse onto the cell cultures. Simultaneously, in a reference chamber, filtered ambient air supplied under identical conditions served as the negative control. The conditions in both chambers were controlled at 37 °C, 85% relative humidity and 5% CO_2.

2.5. Exhaust characterisation

Characterisation of the exhaust was performed in parallel to the exposure experiments, yielding detailed information on the emission sample that the cells were exposed to. Measurements were taken during the initial exposures (n = 2) and repeated exposures (n = 2) (each 10 WLTC cycles) resulting in 4 distinct datasets. The particle number (PN) was measured in the 1:10 diluted exhaust using an engine exhaust condensation particle counter (Model 3790, *TSI Inc.,* USA). The concentrations of carbon monoxide (CO), carbon dioxide (CO_2), total gaseous hydrocarbons (THC), non-methane hydrocarbons (NMHC) and nitrogen oxides (NO_x) were measured using a Horiba MEXA-9400H (*Horiba*, Japan) exhaust gas measuring system in a constant volume sampling tunnel (Horiba CVS-9500 T, *Horiba,* Japan). CO_2 was added to reach 5% for optimal buffering capacity in the cell culture medium. The concentration was controlled with two sensors, right before and after the chamber, and adjusted with a flowmeter, if needed.

2.6. Cell culture exposures

The two-day cell exposure scenario (Fig. 1) was designed to simulate a real-life situation when volcanic ash is introduced to the urban environment (where people are continuously exposed to urban pollution; *e.g.,* gasoline vehicle emissions), resulting in pollutants being concomitantly inhaled. Two sampling time-points were chosen, 24 h and 48 h, to enable observation of the effects after one day of co-exposure (as in the previous study by Tomašek et al., 2016) and, in addition, to account for possible effects following another, repeated exposure to gasoline exhaust.

Daily experimental exposure to diluted (1:10) gasoline exhaust for 6 h was chosen according to earlier studies (Bisig et al., 2015, 2016; Steiner et al., 2013a; Steiner et al., 2012). It is difficult to assess whether this represents real life exposures, but could represent exposure of an urban outdoor worker. The cell-delivered volcanic ash doses fall within the range of the lowest and highest doses used in the previous study (Tomašek et al., 2016). In order to achieve equivalent mass exposure for both ash samples, the QCM was monitored during the exposures until a target dose between 0.4 and 0.5 $\mu g/cm^2$ was reached. There is a lack of dosimetry data for inhalation of ash, or exposure data on ambient air concentrations following volcanic eruptions, making average exposures difficult to constrain and apply *in vitro*, but Tomašek et al. (2016) determined their dose range to be a worst-case scenario. Therefore, these doses may not be realistic for personal exposure and could be considered as a particle over-load relative to a real-life exposure. Overall, the chosen exposure scenario may be considered as a short-term, high-level exposure to both pollutants, individually and when combined. Even though the doses used likely deviate from realistic inhalation exposure, the assessment of cellular responses herein can be seen as a valuable screening of possible (adverse) effects that this specific type of combined exposure may incite which has not previously been considered or investigated.

The multicellular lung model was exposed at the ALI to diluted exhaust (1:10) or filtered air (reference chamber) continuously for
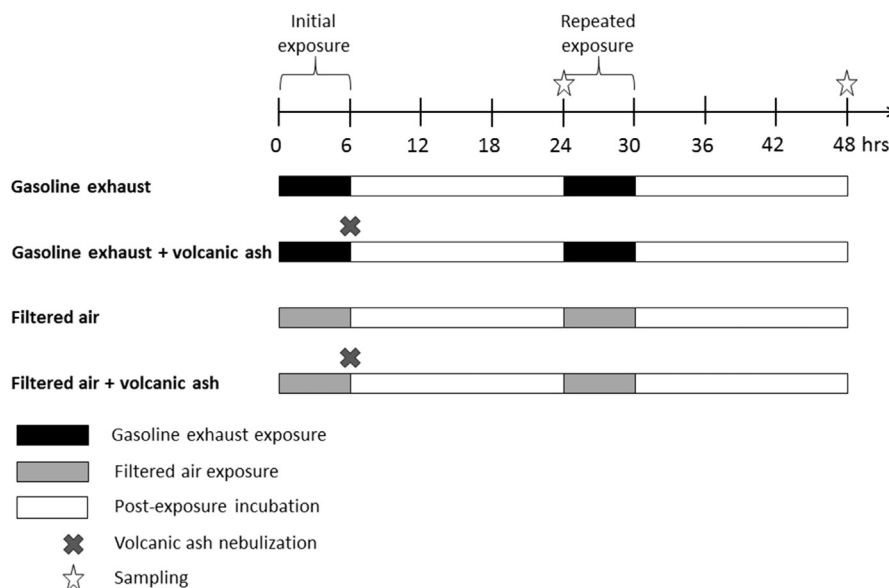
Fig. 1. Schematic of the cell culture exposures at the ALI in the present study. Culture supernatant was sampled at the 24 h time-point, and both the supernatant and insert membranes were sampled at the 48 h time-point. Cell-cultures were maintained under air-liquid interface (ALI) conditions throughout the entire exposures (initial exhaust, volcanic ash, repeated exhaust).

6 h (*i.e.,* the initial exposure), followed by immediate exposure to respirable volcanic ash as described in section 2.3, and then incubated (at 37 °C and 5% CO_2) for 18 h, maintaining the ALI conditions. Subsequently, the supernatants (*i.e.,* cell culture medium) were collected (24 h time-point) from the basal side of the insert and replaced with fresh cell medium. Cells were exposed again for 6 h to diluted exhaust or filtered air (*i.e.,* the repeated exposure), followed by a final 18 h incubation, maintained under ALI conditions. The supernatants were then collected (48 h time-point) (Fig. 1). Each 48-h exposure scenario (exhaust, ash, repeat exhaust) was conducted with two different sets of the multicellular lung model (*i.e.,* with cells from different passage numbers and monocyte isolations), each exposed separately to volcanic ash after the initial exposures, resulting in two replicates (n = 2) per exposure scenario. Two exposure scenarios were conducted over a 4-day period, resulting in 4 experimental replicates in total (n = 4 per ash sample). All data are presented relative to the filtered air (reference) cultures, however, we also included an untreated control (kept in the incubator) to assess the influence of the exposure protocol on cell response (see supplementary information). Collected supernatants were stored at either 4 °C or −80 °C prior to biochemical assays (section 2.7). Insert membranes were split and one half of each replicate's membrane was used for gene expression analysis (section 2.7.4) whilst the other half was fixed and prepared for fluorescent labelling, as described in section 2.7.1.

2.7. Cellular assays and analysis

Cell culture supernatants collected at 24 and 48 h time-points (Fig. 1) were analysed for key/relevant bio-markers of pulmonary-related toxicity *in vitro* (Donaldson et al., 2005). All analyses were performed according to established, standardized protocols as described below.

2.7.1. Cell morphology

Cell membranes collected at the 48 h time-point were fixed with 4% paraformaldehyde (15 min, room temperature), washed and stored in phosphate buffered saline (PBS). Subsequently, they were permeabilised with 0.2% Triton X-100 (*Sigma-Aldrich*, Germany) in

PBS (15—30 min, room temperature). Samples were then stained with the F-actin stain Phalloidin-Rhodamine (*Thermo Fisher Scientific Inc.,* USA) and the nuclei stain 4',6-diamidin-2-phenylindole (DAPI; *Sigma-Aldrich,* Germany) diluted 1:50 and 1:100 in 0.1% BSA in PBS (1—2 h, room temperature), respectively, and then mounted with Glycergel (*DAKO Schweiz AG,* Switzerland) on microscope slides.

Images of the cell membranes were acquired via confocal laser scanning microscopy (LSM; Zeiss 710 Confocal Microscope, *Carl Zeiss,* Switzerland) using a 63x/1.4 NA oil immersion lens. Representative images were processed using the public domain image analysis software ImageJ (http://rsb.info.nih.gov/ij).

2.7.2. Lactate dehydrogenase release

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) into the co-culture supernatant, at both 24 h and 48 h time-points, using a LDH Cytotoxicity Detection Kit (*Roche Applied Science,* Germany) according to the manufacturer's protocol. The test was conducted in duplicate for each experimental replication. Absorbance was determined at 490 nm using a microplate reader (*Bio-Rad,* Switzerland), with a reference wavelength set at 630 nm. The positive assay control was 100 μL of 0.2% Triton X-100 in PBS, applied for 24 h on the apical side of the cell culture insert.

2.7.3. Quantification of (pro-)inflammatory response

The quantity of tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8) and interleukin-1 beta (IL-1β) secreted into the culture medium was assessed in the co-culture supernatants collected at 24 h and 48 h time-points by enzyme-linked immunosorbent assay (ELISA DuoSet Development Kit, *R&D Systems,* USA) according to the manufacturer's protocol. The concentrations were determined spectrophotometrically at 450 nm using a microplate reader (*Bio-Rad,* Switzerland). Analyses were conducted in duplicate for each repetition. The positive assay control was lipopolysaccharide (LPS, from *E-coli* 055:B5 strain ((*Sigma-Aldrich*, Germany), 1 μg/mL) applied as 1.2 mL solution in cRPMI in the bottom compartment of the cell culture insert for 24 h.

2.7.4. Gene expression analysis

Quantification of gene expression at the transcriptional level was performed by real-time reverse-transcription polymerase chain reaction (real-time RT-PCR), as previously described (Bisig et al., 2015). Cell culture membranes sampled at the 48 h time-point were stored in the ribonucleic acid (RNA) protect buffer (RNAprotect® Cell Reagent, *Qiagen*, Germany; diluted in PBS 1:4 (v/v)) prior to the analysis. RNA was isolated with a RNeasy plus kit (*Qiagen*, Germany). Complementary deoxyribonucleic acid (cDNA) was synthetized with the Omniscript RT system (*Qiagen*, Germany), Oligo-dT primer (*Microsynth*, Switzerland) and RNasin Inhibitor (*Promega*, USA). Real-time RT-PCR was performed using SYBR-green (*Applied Biosystems*, USA) on a 7500 Fast Real-Time PCR system (*Applied Biosystems*, USA).

Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the standard (housekeeping) gene and filtered air as the control. The induction of cell death was determined by expression levels of (pro-)apoptotic genes caspase 7 (*CASP7*) and FAS receptor (*FAS*). Heme oxygenase 1 (*HMOX1*) and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*) genes were used to observe the onset of oxidative stress. For assessment of (pro-)inflammatory responses, expression of pro-interleukin-1 beta (*IL1B*) and interleukin-8 (*IL8*) genes were measured.

2.8. Data processing and statistical analysis

All data in the figures are presented as single values and means derived from gasoline exhaust or filtered air exposures (the initial exposures (n = 2) and repeated exposures (n = 2), over 4 days in total); each exposure was performed with two different sets of the multicellular lung model (n = 2; each exposed separately to volcanic ash), leading to 4 repetitions (n = 4).

All statistical analyses were performed using the software Origin (version 9.3, *OriginLab Corporation*, USA). Statistical significance was deduced through the use of a one-way analysis of variance (ANOVA), assuming a normal distribution of the datasets. Subsequent Tukey's post hoc tests were conducted to determine statistical significance between different exposures and the reference exposure (filtered air). The alpha value was set at 0.05.

3. Results

3.1. Volcanic ash characterisation

Particle size analysis of isolated respirable fractions showed that 98% by volume and 84% by volume of particles were sub-10 μm diameter, for ash from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV), respectively (Fig. 2a). The SHV sample consisted of 58% by volume particles with size <4 μm, while ChV contained less with 40% by volume.

The morphology of the particles from both volcanoes, as observed by SEM, was mostly blocky and angular with varying amounts of sub-micron particles adhering to the surfaces of larger particles (Fig. 2b). This is congruent with previous observations of respirable volcanic ash (*e.g.*, Damby et al., 2016, Lahde et al., 2013, Horwell et al., 2013, Hillman et al., 2012, Le Blond et al., 2010), but may not mirror the morphology of larger ash particles, which can differ according to (magmatic) composition.

Bulk oxide elemental data for samples are listed in Table 1 and indicate magmatic composition of the ash samples. The SHV ash was confirmed to be 'andesitic', with an intermediate composition regarding silicon dioxide (SiO_2) content (61.8 wt% SiO_2), while ChV is 'rhyolitic', being comparatively richer in SiO_2 (73.4 wt% SiO_2).

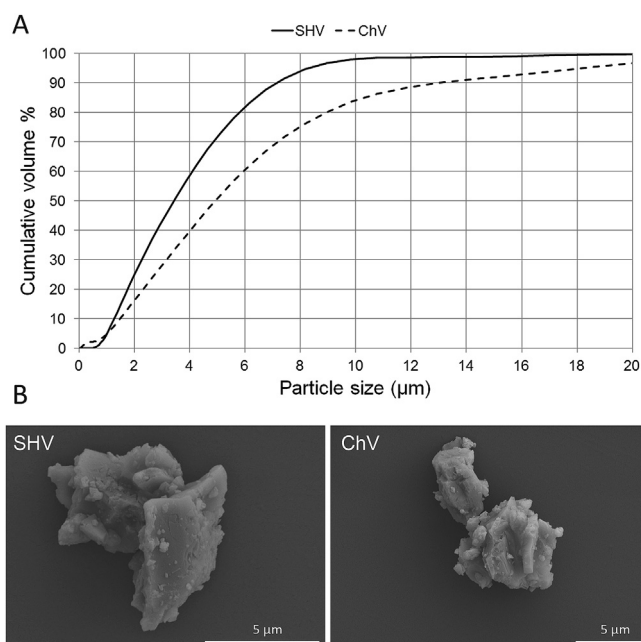**Fig. 2. Volcanic ash characterisation. A)** Particle size distribution (PSD) of the isolated respirable fraction of volcanic ash samples determined by a Beckman Coulter LS 13 320 PSD analyser (*Coulter Corporation*, USA). Data are the mean of n = 3. **B)** Representative scanning electron microscopy images of volcanic ash samples from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV). Images were collected at 10.0 kV and WD 16 mm. Scale bars are 5 μm.

3.1.1. Volcanic ash nebulisation

The average cell-delivered doses of nebulized ash using the dry powder insufflator, as monitored by a QCM, were 0.54 ± 0.19 μg/cm^2 and 0.39 ± 0.09 μg/cm^2 for SHV and ChV ash, respectively (Fig. 3).

3.2. Exhaust characterisation

The composition of the gaseous fraction, comprising carbon monoxide (CO), total hydrocarbons (THC), non-methane hydrocarbons (NMHC), nitrogen oxides (NO_x) and carbon dioxide (CO_2), as well as the average count of produced particles are shown in Table 2.

3.3. Biological endpoints

For the assessed biological endpoints (cytotoxicity, oxidative stress and (pro-)inflammatory mediators, including measurements for both protein production and gene expression), no significant ($p > 0.05$) changes in cell cultures were observed at 24 h or 48 h time-points for any of the experimental exposures, *i.e.,* volcanic ash (VA), gasoline exhaust (GE) and co-exposures (GE + VA). The response of the untreated cells (*i.e.,* incubator control) was lower in comparison to the cells treated with filtered air, albeit not significant (SI Fig. 1). To account for the influence of the potentially stress-inducing airflow as well as for the different baseline levels in the various cultures, the comparison of the effects of cell treatments (VA, GE and GE + VA) was made with the filtered air (reference) exposure.

For all exposure scenarios (VA, GE and GE + VA) at 48 h, LSM imaging revealed a homogenous and confluent epithelial cell layer with no alteration in cell morphology compared to the filtered air (reference) exposure (Fig. 4a). LDH release by the cells following the exposures, for both time-points, showed limited elevation in

Table 1
Bulk chemical compositions of the volcanic ash samples used in the study. Results are presented as component weight percent oxide and recalculated to include loss on ignition (LOI) in the final total.

Sample	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	SO_3	LOI	Total
SHV	61.8	0.5	17.0	6.6	0.2	2.4	6.3	3.7	0.9	0.1	0.1	0.6	100.1
ChV	73.4	0.2	13.9	1.6	0.1	0.4	1.5	4.2	2.9	0.1	0.0	1.1	99.4

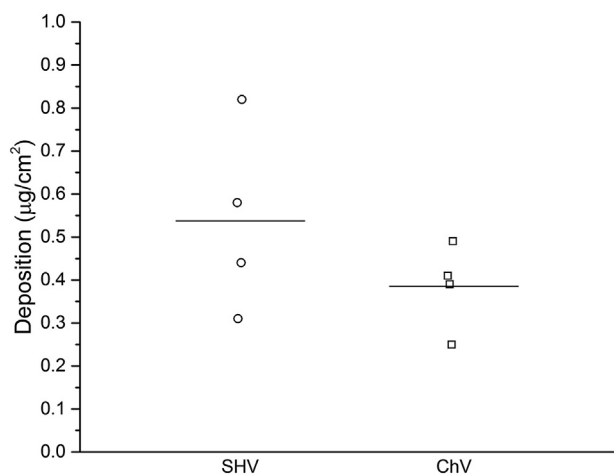**Fig. 3. Deposition of nebulized respirable volcanic ash.** Average mass deposition ($\mu g/cm^2$) of respirable ash from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV) quantified using a QCM, following their dry nebulisation over cell cultures using a dry powder insufflator (DP-4, *Penn Century*, USA). All data are presented as single values and mean (solid line), n = 4.

Table 2
Average exhaust composition for the flex-fuel GDI vehicle in the WLTC as measured during the experiments (n = 4). SD = standard deviation, CO = carbon monoxide, THC = total hydrocarbons, NMHC = non-methane hydrocarbons, NO_x = nitrogen oxides, CO_2 = carbon dioxide. *Note that the CO_2 concentration applied to the cell culture chamber was adjusted as necessary to 5% CO_2. PN = particle number. All data are shown 1:10 diluted (as applied to the cell cultures).

Exhaust component	Unit	Mean	SD
CO	ppm	27.71	2.98
THC	ppm	6.97	0.44
NMHC	ppm	4.82	0.46
NO_x	ppm	1.76	0.12
CO_2^*	%	0.98	0.01
PN	$\#/cm^3$	1.32E+05	1.65E+04

comparison to the filtered air (Fig. 4b). The positive LDH assay control Triton X-100 showed a significant ($p < 0.05$) increase in LDH content in culture medium, confirming that the biological model used was responsive for the measured endpoint.

For expression of pro-apoptotic genes *FAS* and *CASP7*, none of the exposures showed a statistically significant ($p > 0.05$) outcome relative to filtered air (Fig. 4c). *CASP7* expression was found to be slightly suppressed by VA, GE, and combined exposure to gasoline exhaust and SHV ash (GE + SHV), while only slightly increased in the combined exposure to gasoline exhaust and ChV ash (GE + ChV); these changes, however, are still within the observed biological variation.

As determined via release of specifically chosen (pro-)inflammatory mediators, none of the cell exposures induced a significant ($p > 0.05$) (pro-)inflammatory response (Fig. 5a). In fact, the concentrations of TNF-α and IL-1β in all measured samples were below the method detection limits (MDL; SI Fig. 1). LPS, which served as a positive assay control, significantly ($p < 0.05$) increased the release of IL-8 (Fig. 5a) as well as TNF-α and IL-1β (SI Fig. 2) compared to the filtered air and other cell treatments. Lack of a (pro-)inflammatory response was supported by the findings on a gene level, where the cell exposures did not induce any change in mRNA levels of measured markers, *IL8* and *IL1B*, relative to filtered air (Fig. 5b). In agreement with the protein measurements, none of the treatments induced a detectable upregulation of *TNFA* (data not shown). In the ChV ash-treated cultures, a slight, yet insignificant ($p > 0.05$), increase in expression of both *IL8* and *IL1B* was observed compared to the filtered air (reference) exposure.

Similarly, the expression of investigated genes related to oxidative stress, namely *HMOX1* and *NQO1*, showed no significant ($p > 0.05$) increase after exposure to VA, GE or GE + VA (Fig. 6). Combined exposure to gasoline exhaust and ChV ash (GE + ChV) did, however, result in a slight upregulation of *HMOX1* relative to filtered air, although not significant.

4. Discussion

The purpose of the study was to investigate the potential respiratory hazard of combined exposure to volcanic ash and anthropogenic pollution, through experiments designed to assess the impact to a multicellular lung model of exposure to both volcanic ash and complete gasoline exhaust.

We have found that exposure of cells to gasoline exhaust, alone, does not induce any significant effects on any of the biological endpoints measured. These results are in agreement with research performed by Bisig et al. (2016) using gasoline exhaust, alone, on a multicellular human lung model mimicking the bronchial compartment, under similar experimental conditions. Another study by Bisig et al. (2015), using the same experimental setup but a different car, found that gasoline exhaust induced oxidative stress; however, the particle number measured in the diluted exhaust was up to three orders of magnitude higher than that used by Bisig et al. (2016), and nearly twice as high as the average daily number of particles produced during exposures in the present study (Table 2). Similarly, the concentrations of volatile compounds in Bisig et al. (2015) exceed those measured in Bisig et al. (2016) and the current study. Bisig et al. (2015) found that filtration of the particulate fraction from the exhaust was not sufficient to eliminate the adverse effects *in vitro*, confirming the importance of the volatile compounds in GE-induced toxicity. The toxic effects of gasoline exhaust, particularly after particle filtration, have also been observed with *in vivo* animal studies (Reed et al., 2008; Lund et al., 2007). It was noted, though, that volatile compounds, alone, might react differently with the lung cells than when part of the complete exhaust, where these compounds can adsorb onto the particle surfaces (Steiner et al., 2014, 2016).

The biological sensitivity of the employed multicellular model has been validated in the past through use of positive particulate controls, *e.g.*, crystalline quartz (DQ12) for a (pro-)inflammatory response (Endes et al., 2014; Chortarea et al., 2015). The lack of adverse effects observed following gasoline exhaust exposure in the present study may be explained by important differences in experimental parameters, including the employed cell lines, the
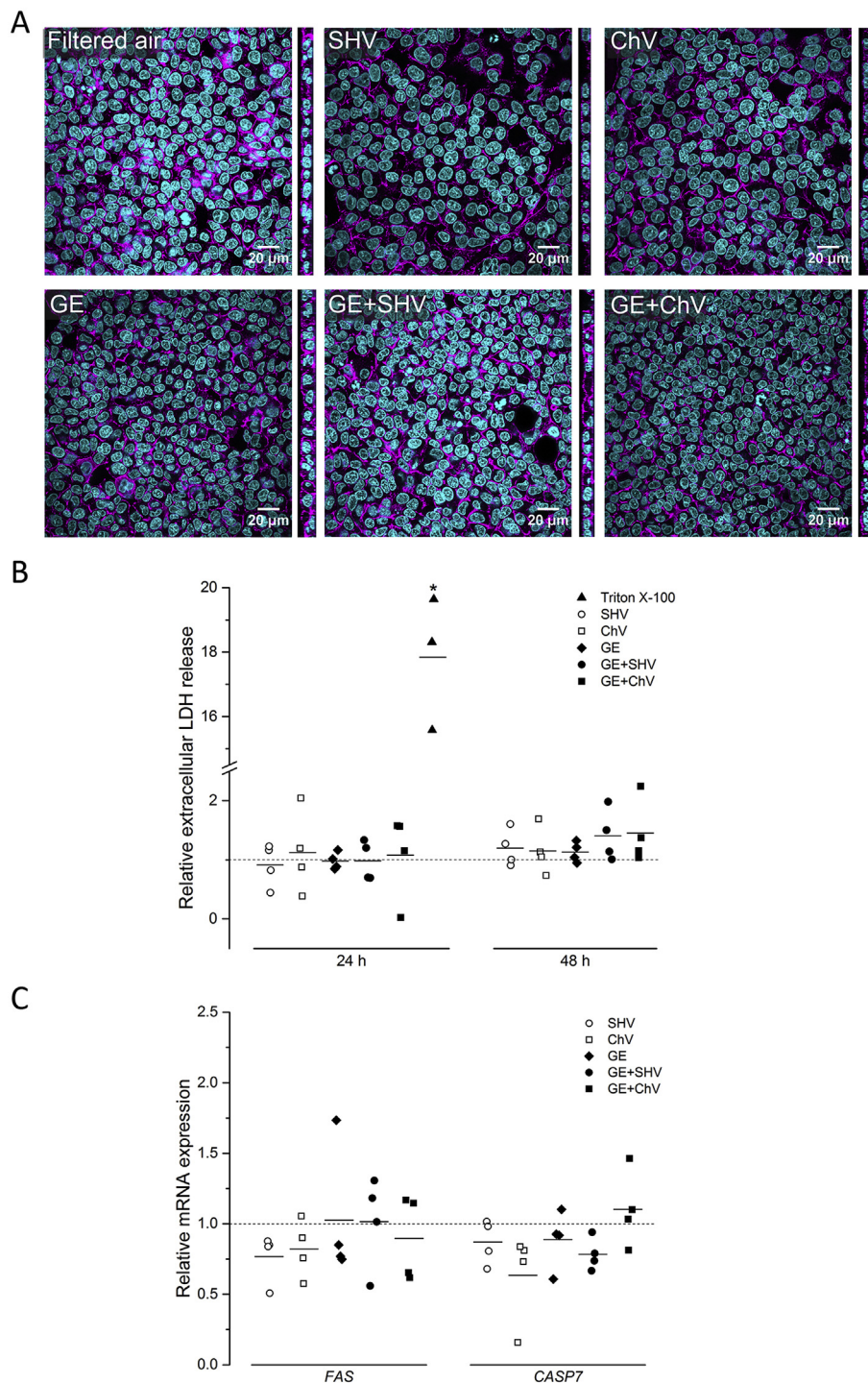
Fig. 4. Cell morphology and cell viability of the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. **A)** Representative confocal LSM images from XY and YZ projections for cultures exposed to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE + SHV), and combined exposure to gasoline exhaust and Chaitén ash (GE + ChV). Cells were stained with Phalloidin-Rhodamine (F-actin cytoskeleton, magenta) and DAPI (cell nuclei, cyan). Scale bars are 20 μm. **B)** Extracellular LDH levels in the culture medium after 24 h and 48 h normalized to filtered air (reference) exposure (dashed line). The positive assay control was 0.2% Triton X-100 in PBS. **C)** Amounts of mRNA of pro-apoptotic genes FAS receptor (*FAS*) and caspase-7 (*CASP7*), 48 h post-exposures, normalized to filtered air (reference) exposure (dashed line). All data are presented as single values and mean (solid line), n = 4; * denotes a significant difference ($p < 0.05$) between the positive control and the other samples tested. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

driving test cycle and vehicle tested and, hence, the lower particle numbers and volatile concentrations (and consequent lower doses) as compared to other studies, and exposure times. In addition, it is known that the response of cultured cells to exposure may vary,

especially over time (Poland et al., 2014). The evidence from other studies such as Bisig et al. (2015) suggests that we should not conclude that gasoline exhaust is incapable of inciting a biological response.
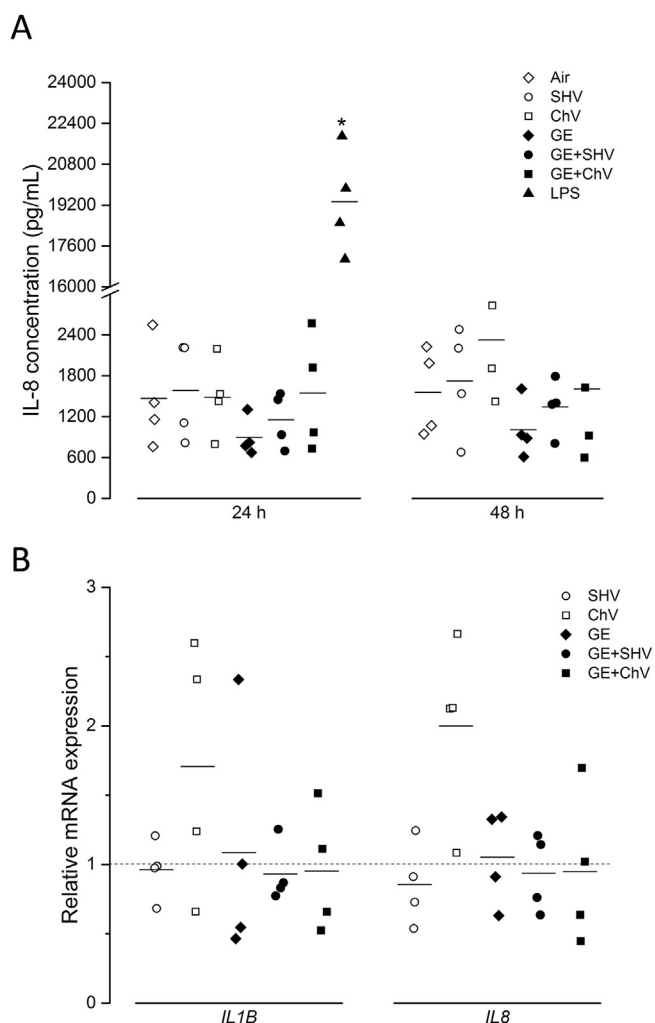
Fig. 6. Oxidative stress response in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. Amounts of mRNA of oxidative stress responsive genes, heme oxygenase 1 (*HMOX1*) and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*), following exposures to (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE + SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE + ChV), normalized to filtered air exposure (dashed line). Data are shown as single values and mean (solid line), n = 4.

Fig. 5. Release of (pro-)inflammatory mediators in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. A) Interleukin-8 (IL-8) release in the culture medium after 24 h and 48 h following exposures to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE + SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE + ChV). The positive assay control was lipopolysaccharide (LPS; 1 μg/mL, 24 h). **B)** Amounts of mRNA of (pro-)inflammation-related genes encoding interleukin-1 beta (*IL1B*) and IL-8 (*IL8*), 48 h post-exposures, normalized to filtered air exposure (a dashed line). All data are shown as single values and mean (solid line), n = 4; * denotes significant difference ($p < 0.05$) between the positive control and the other samples tested.

The two ash types used in this study, erupted from different volcanoes of different magmatic compositions, also did not elicit a significant response in the biological endpoints measured from the multicellular model. Ash samples from these volcanoes have been previously investigated for their toxic potential and also showed limited biological responses (Damby et al., 2016, Horwell et al., 2013, Wilson et al., 2000, Cullen and Searl, 1998 and unpublished data for ChV). The SHV ash has also caused minimal response to the same multicellular model in our previous study (Tomašek et al., 2016). Hence, these data confirm the generally-observed lack of potential of ash to significantly affect healthy lung cell integrity or function including, in this case, initiation of an inflammatory response for the chosen time-points and endpoints.

Given the lack of significant response to gasoline exhaust, alone, and volcanic ash, alone, the finding that the co-exposures did not cause significant adverse effects in the multicellular model is
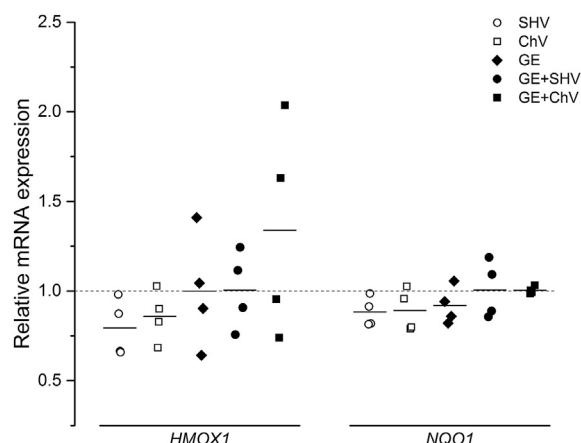
perhaps not surprising, and could indicate that these combined exposures did not generate either an additive or synergistic response. Due to experimental design, the effect of direct ash-volatile interactions (*e.g.*, volatile adsorption) prior to co-culture exposures was unable to be tested here. The absence of alterations in cell morphology, cell viability and oxidative stress state for any combined exposure scenario are in line with our previous *in vitro* study, which showed limited cytotoxic and oxidative potential of SHV ash when exposed concomitantly with DEP (Tomašek et al., 2016). However, the low (pro-)inflammatory response following combined exposures is contrary to the previous findings, where co-exposures of SHV ash and DEP increased release of (pro-)inflammatory mediators TNF-α and IL-8, as well as significantly increased ($p < 0.05$) IL-1β (Tomašek et al., 2016). In Tomašek et al. (2016) it was hypothesized that the observed IL-8 production was driven by DEP, and that volcanic ash, alone, did not result in significant production of TNF-α, although it augmented TNF-α production in the co-exposures. Together with the results from Bisig et al. (2016), who report no upregulation of *TNFA* or *IL8* in response to gasoline exhaust exposure in a bronchial epithelium model, it appears that co-exposures to volcanic ash and gasoline exhaust do not induce inflammation via these pathways, at least at the doses tested here, and using the lung model composed of cells from healthy donors. However, the non-significant increase in *IL8* expression in response to ChV ash remains unexplained, especially since this sample has not been used in cytokine assays previously.

The similar biological responses to both ash samples in combined exposures with gasoline exhaust indicates that, within the parameters of this particular experimental setup, differences in sample composition (*e.g.*, iron content) and mineralogy (*e.g.*, crystalline silica content) did not affect the biological response to co-exposures. Whilst we chose two fairly different ash samples, volcanic ash is a heterogeneous dust, the physicochemical characteristics of which can vary considerably, even during a discrete eruption (Damby et al., 2017; Horwell et al., 2013), and samples from different eruptions have shown differences in toxicity when tested comparatively *in vitro*, previously (Damby et al., 2016; Horwell et al., 2013; Wilson et al., 2000). Hence, a different sample from an individual eruption or a different ash type might incite a different cellular response.

The potential for diesel exhaust, and DEP in particular, to cause adverse respiratory effects is well known (see review by Steiner et al., 2016) while, on the contrary, the toxicity of exhaust from GDI vehicles is still relatively unknown (CCEM, 2016; Muñoz et al., 2016). Given this, we believe that there is a need to conduct further studies to clarify the hazard posed by combined exposures, particularly with a fuel that generates exhaust of likely greater toxicity (*e.g.,* diesel) (Bisig et al., 2016), which was not possible during the time-frame of the current experiments. Future studies that consider the very complex and variable components of ambient urban air would be prudent, as would additional endpoints, such as genotoxicity, that help derive a more comprehensive understanding of the potential hazard. Furthermore, the experimental approach in this study, although performed over a two-day period (as opposed to the commonly-used time-point of 24 h), still represents a short-term exposure scenario. Hence, potential chronic effects that such exposures could elucidate, over a prolonged period, have not been accounted for and need to be investigated.

5. Conclusion

This study provides the first insights into the biological effects caused by exposure to complete gasoline exhaust in the presence or absence of volcanic ash conducting a realistic *in vitro* hazard assessment. The findings show that combined, and individual, gasoline exhaust and volcanic ash exposure at the ALI has limited adverse biological impact to a multicellular lung model *in vitro*, considering the employed experimental conditions and biological endpoints measured (cytotoxicity, oxidative stress and (pro-)inflammatory response at the protein and gene levels).

More detailed investigation of the potential respiratory hazard following such combined exposures in future eruptive events is necessary, especially considering the complexity of the ambient urban air. Additional biological markers should be studied in further experiments *in vitro* and a complete diesel exhaust could also be used.

Conflict of interest

The authors declare no conflict of interest. The authors are responsible for the content of the manuscript.

Consent for publication

All authors have read and approved the manuscript for publication.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.01.115.

References

ACEA, 2017. European Automobile Manufacturers' Association [Online]. http://www.acea.be/. (Accessed 9 August 2017). http://www.acea.be/statistics/tag/category/passenger-car-fleet-by-fuel-type.

Banerjee, T., Christian, R., 2017. On-field and laboratory measurement of nanoparticle emission in the wake of gasoline vehicle. Atmos. Pollut. Res. 8 (6), 1179—1192.

Barsotti, S., Andronico, D., Neri, A., Del Carlo, P., Baxter, P., Aspinall, W., Hincks, T., 2010. Quantitative assessment of volcanic ash hazards for health and infrastructure at Mt. Etna (Italy) by numerical simulation. J. Volcanol. Geoth. Res. 192, 85—96.

Baxter, P.J., Horwell, C.J., 2015. Impacts of eruptions on human health. In: The Encyclopedia of Volcanoes, second ed., pp. 1035—1047.

Baxter, P.J., Bonadonna, C., Dupree, R., Hards, V.L., Kohn, S.C., Murphy, M.D., Nichols, A., Nicholson, R.A., Norton, G., Searl, A., Sparks, R.S.J., Vickers, B.P., 1999. Cristobalite in volcanic ash of the Soufriere Hills volcano, Montserrat, British West Indies. Science 283, 1142—1145.

Baxter, P.J., Ing, R., Falk, H., French, J., Stein, G.F., Bernstein, R.S., Merchant, J.A., Allard, J., 1981. Mount St Helens eruptions, May 18 to June 12, 1980: an overview of the acute health impact. Jama 246 (22), 2585—2589.

Baxter, P.J., Ing, R., Falk, H., Plikaytis, B., 1983. Mount St. Helens eruptions: the acute respiratory effects of volcanic ash in a North American community. Arch. Environ. Health 38, 138—143.

Baxter, P.J., Searl, A.S., Cowie, H.A., Jarvis, D., Horwell, C.J., 2014. Evaluating the respiratory health risks of volcanic ash at the eruption of the Soufriere Hills Volcano, Montserrat, 1995 to 2010. Geol. Soc. Lond. Mem. 39 (1), 407—425.

Bisig, C., Roth, M., Müller, L., Comte, P., Heeb, N., Mayer, A., Czerwinski, J., Petri-FINk, A., Rothen-Rutishauser, B., 2016. Hazard identification of exhausts from gasoline-ethanol fuel blends using a multi-cellular human lung model. Environ. Res. 151, 789—796.

Bisig, C., Steiner, S., Comte, P., Czerwinski, J., Mayer, A., Petri-Fink, A., Rothen-Rutishauser, B., 2015. Biological effects in lung cells in vitro of exhaust aerosols from a gasoline passenger car with and without particle filter. Emiss. Contr. Sci. Technol. 1, 237—246.

Blank, F., Rothen-Rutishauser, B., Gehr, P., 2007. Dendritic cells and macrophages form a transepithelial network against foreign particulate antigens. Am. J. Respir. Cell Mol. Biol. 36, 669—677.

Blank, F., Rothen-Rutishauser, B.M., Schurch, S., Gehr, P., 2006. An optimized in vitro model of the respiratory tract wall to study particle cell interactions. J. Aerosol Med. 19 (3), 392—405.

CCEM, 2016. GasOMeP. Gasoline vehicle emission control for organic, metallic and particulate non-legislative pollutants. Annu. Act. Rep. 57—59.

Chortarea, S., Clift, M.J.D., Vanhecke, D., Endes, C., Wick, P., Petri-Fink, A., Rothen-Rutishauser, B., 2015. Repeated exposure to carbon nanotube-based aerosols does not affect the functional properties of a 3D human epithelial airway model. Nanotoxicology 9 (8), 983—993.

Cullen, R.T., Searl, A., 1998. Preliminary Toxicological Hazard Assessment of Montserrat Volcanic Ash: In vitro Cytotoxicity. Institute of Occupational Medicine, Edinburgh, p. 13. P752/200.

Damby, D.E., Horwell, C.J., Baxter, P.J., Delmelle, P., Donaldson, K., Dunster, C., Fubini, B., Murphy, F.A., Nattrass, C., Sweeney, S., Tetley, T.D., Tomatis, M., 2013. The respiratory health hazard of tephra from the 2010 Centennial eruption of Merapi with implications for occupational mining of deposits. J. Volcanol. Geoth. Res. 261, 376—387.

Damby, D.E., Horwell, C.J., Baxter, P.J., Kueppers, U., Schnurr, M., Dingwell, D.B., Duewell, P., 2018. Volcanic ash activates the NLRP3 inflammasome in murine and human macrophages. Front. Immunol. 8, 2000.

Damby, D.E., Horwell, C.J., Larsen, G., Thordarson, T., Tomatis, M., Fubini, B., Donaldson, K., 2017. Assessment of the potential respiratory hazard of volcanic ash from future Icelandic eruptions: a study of archived basaltic to rhyolitic ash samples. Environ. Health 16 (1), 98.

Damby, D.E., Murphy, F.A., Horwell, C.J., Raftis, J., Donaldson, K., 2016. The in vitro respiratory toxicity of cristobalite-bearing volcanic ash. Environ. Res. 145, 74—84.

Donaldson, K., Tran, L., Jimenez, L.A., Duffin, R., Newby, D.E., Mills, N., Macnee, W., Stone, V., 2005. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part. Fibre Toxicol. 2 (1), 10.

Endes, C., Schmid, O., Kinnear, C., Mueller, S., Camarero-Espinosa, S., Vanhecke, D., Foster, E.J., Petri-Fink, A., Rothen-Rutishauser, B., Weder, C., Clift, M.J., 2014. An in vitro testing strategy towards mimicking the inhalation of high aspect ratio nanoparticles. Part. Fibre Toxicol. 11 (1).

Fytianos, K., Drasler, B., Blank, F., Von Garnier, C., Seydoux, E., Rodriguez-Lorenzo, L., Petri-Fink, A., Rothen-Rutishauser, B., 2016. Current in vitro approaches to assess nanoparticle interactions with lung cells. Nanomedicine 11, 2457–2469.

Gudmundsson, M.T., Thordarson, T., Höskuldsson, Á., Larsen, G., Björnsson, H., Prata, F.J., Oddsson, B., Magnússon, E., Högnadóttir, T., Petersen, G.N., Hayward, C.L., Stevenson, J.A., Jonsdottir, I., 2012. Ash generation and distribution from the April-May 2010 eruption of Eyjafjallajökull, Iceland. Sci. Rep. 2, 572.

Hillman, S.E., Horwell, C.J., Densmore, A.L., Damby, D.E., Fubini, B., Ishimine, Y., Tomatis, M., 2012. Sakurajima volcano: a physico-chemical study of the health consequences of long-term exposure to volcanic ash. Bull. Volcanol. 74, 913–930.

Horwell, C.J., 2007. Grain-size analysis of volcanic ash for the rapid assessment of respiratory health hazard. J. Environ. Monit. 9, 1107–1115.

Horwell, C.J., Baxter, P.J., 2006. The respiratory health hazards of volcanic ash: a review for volcanic risk mitigation. Bull. Volcanol. 69, 1–24.

Horwell, C.J., Baxter, P.J., Hillman, S.E., Calkins, J.A., Damby, D.E., Delmelle, P., Donaldson, K., Dunster, C., Fubini, B., Kelly, F.J., Le Blond, J.S., Livi, K.J.T., Murphy, F., Nattrass, C., Sweeney, S., Tetley, T.D., Thordarson, T., Tomatis, M., 2013. Physicochemical and toxicological profiling of ash from the 2010 and 2011 eruptions of Eyjafjallajökull and Grimsvotn volcanoes, Iceland using a rapid respiratory hazard assessment protocol. Environ. Res. 127, 63–73.

Horwell, C.J., Fenoglio, I., Fublni, B., 2007. Iron-induced hydroxyl radical generation from basaltic volcanic ash. Earth Planet Sci. Lett. 261, 662–669.

Horwell, C.J., Fenoglio, I., Ragnarsdottir, K.V., Sparks, R.S.J., Fubini, B., 2003. Surface reactivity of volcanic ash from the eruption of Soufriere Hills volcano, Montserrat, West Indies with implications for health hazards. Environ. Res. 93, 202–215.

Horwell, C.J., Le Blond, J.S., Michnowicz, S.A.K., Cressey, G., 2010. Cristobalite in a rhyolitic lava dome: evolution of ash hazard. Bull. Volcanol. 72, 249–253.

Lahde, A., Gudmundsdottir, S.S., Joutsensaari, J., Tapper, U., Ruusunen, J., Ihalainen, M., Karhunen, T., Torvela, T., Jokiniemi, J., Jarvinen, K., Gislason, S.R., Briem, H., Gizurarson, S., 2013. In vitro evaluation of pulmonary deposition of airborne volcanic ash. Atmos. Environ. 70, 18–27.

Le Blond, J.S., Horwell, C.J., Baxter, P.J., Michnowicz, S.A.K., Tomatis, M., Fubini, B., Delmelle, P., Dunster, C., Patia, H., 2010. Mineralogical analyses and in vitro screening tests for the rapid evaluation of the health hazard of volcanic ash at Rabaul volcano, Papua New Guinea. Bull. Volcanol. 72, 1077–1092.

Lee, S.H., Richards, R.J., 2004. Montserrat volcanic ash induces lymph node granuloma and delayed lung inflammation. Toxicology 195, 155–165.

Lehmann, A., Brandenberger, C., Blank, F., Gehr, P., Rothen-Rutishauser, B., 2010. A 3D model of the human epithelial airway barrier. Meth. Bioeng.: Alter. Technol. Animal Test. 239–260.

Lenz, A.G., Karg, E., Lentner, B., Dittrich, V., Brandenberger, C., Rothen-Rutishauser, B., Schulz, H., Ferron, G.A., Schmid, O., 2009. A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. Part. Fibre Toxicol. 6 (1), 32.

Loughlin, S.C., Aspinall, W.P.A., Vye-Brown, C., Baxter, P.J., Braban, C., Hort, M., Schmidt, A., Thordarson, T., Witham, C., 2012. Large-magnitude Fissure Eruptions in Iceland: Source Characterisation. BGS Open File Report, OR/12/098.

Lund, A.K., Knuckles, T.L., Akata, C.O., Shohet, R., Mcdonald, J.D., Gigliotti, A., Seagrave, J.C., Campen, M.J., 2007. Gasoline exhaust emissions induce vascular remodeling pathways involved in atherosclerosis. Toxicol. Sci. 95, 485–494.

Mathis, U., Mohr, M., Forss, A.-M., 2005. Comprehensive particle characterization of modern gasoline and diesel passenger cars at low ambient temperatures. Atmos. Environ. 39, 107–117.

Mauderly, J.L., Barrett, E., Day, K., Gigliotti, A., Mcdonald, J., Harrod, K., Lund, A., Reed, M., Seagrave, J., Campen, M., 2014. The National Environmental Respiratory Center (NERC) experiment in multi-pollutant air quality health research: II. Comparison of responses to diesel and gasoline engine exhausts, hardwood smoke and simulated downwind coal emissions. Inhal. Toxicol. 26, 651–667.

McDonald, F., Horwell, C.J., Ugarte, C., Kamanyire, R., Wecker, R., Dominelli, L., Loh, M., 2017. Ethical Considerations of Recommending or Distributing Facemasks for Community Protection from Ambient Air Pollution Events. Talk presented (by M) at IHRR/DWD conference (*The Impact of Hazard, Risk and Disasters on Societies*), Durham University, United Kingdom, September 2017.

McDonald, J.D., Reed, M.D., Campen, M.J., Barrett, E.G., Seagrave, J., Mauderly, J.L., 2007. Health effects of inhaled gasoline engine emissions. Inhal. Toxicol. 19, 107–116.

Mohr, M., Forss, A.-M., Lehmann, U., 2006. Particle emissions from diesel passenger cars equipped with a particle trap in comparison to other technologies. Environ. Sci. Technol. 40, 2375–2383.

Monick, M.M., Baltrusaitis, J., Powers, L.S., Borcherding, J.A., Caraballo, J.C., Mudunkotuwa, I., Peate, D.W., Walters, K., Thompson, J.M., Grassian, V.H., 2013. Effects of Eyjafjallajökull volcanic ash on innate immune system responses and bacterial growth in vitro. Environm. Health Perspect. 121 (691).

Morin, J.-P., Hasson, V., Fall, M., Papaioanou, E., Preterre, D., Gouriou, F., Keravec, V., Konstandopoulos, A., Dionnet, F., 2008. Prevalidation of in vitro continuous flow exposure systems as alternatives to in vivo inhalation safety evaluation experimentations: outcome from MAAPHRI-PCRD5 research program. Exp. Toxicol. Pathol. 60, 195–205.

Müller, L., Comte, P., Czerwinski, J., Kasper, M., Mayer, A.C.R., Gehr, P., Burtscher, H., Morin, J.-P., Konstandopoulos, A., Rothen-Rutishauser, B., 2010. New exposure system to evaluate the toxicity of (scooter) exhaust emissions in lung cells in vitro. Environ. Sci. Technol. 44, 2632–2638.

Müller, L., Comte, P., Czerwinski, J., Kasper, M., Mayer, A.C.R., Schmid, A., Rosinus, L., Clift, M.J.D., Steiner, S., Gehr, P., Rothen-Rutishauser, B., 2012. Investigating the potential for different scooter and car exhaust emissions to cause cytotoxic and (pro-)inflammatory responses to a 3D in vitro model of the human epithelial airway. Toxicol. Environ. Chem. 94, 164–180.

Müller, L., Gasser, M., Raemy, D.O., Herzog, F., Brandenberger, C., Schmid, O., Gehr, P., Rothen-Rutishauser, B., Clift, M.J., 2011. Realistic exposure methods for investigating the interaction of nanoparticles with the lung at the air-liquid interface in vitro. Insciences J 1 (1), 30–64.

Muñoz, M., Heeb, N.V., Haag, R., Honegger, P., Zeyer, K., Mohn, J., Comte, P., Czerwinski, J., 2016. Bioethanol blending reduces nanoparticle, PAH, and Alkyl- and Nitro-PAH emissions and the genotoxic potential of exhaust from a gasoline direct injection flex-fuel vehicle. Environ. Sci. Technol. 50, 11853–11861.

Peters, A., Wichmann, H.E., Tuch, T., Heinrich, J., Heyder, J., 1997. Respiratory effects are associated with the number of ultrafine particles. Am. J. Respir. Crit. Care Med. 155, 1376–1383.

Platt, S.M., El Haddad, I., Pieber, S.M., Zardini, A.A., Suarez-Bertoa, R., Clairotte, M., Daellenbach, K.R., Huang, R.J., Slowik, J.G., Hellebust, S., Temime-Roussel, B., 2017. Gasoline cars produce more carbonaceous particulate matter than modern filter-equipped diesel cars. Sci. Rep. 7 (1), 4926.

Poland, C.A., Miller, M.R., Duffin, R., Cassee, F., 2014. The elephant in the room: reproducibility in toxicology. Part. Fibre Toxicol. 11 (42).

Pope 3rd, C.A., Turner, M.C., Burnett, R.T., Jerrett, M., Gapstur, S.M., Diver, W.R., Krewski, D., Brook, R.D., 2015. Relationships between fine particulate air pollution, cardiometabolic disorders, and cardiovascular mortality. Circ. Res. 116 (1), 108–115.

Reed, M., Barrett, E., Campen, M., Divine, K., Gigliotti, A., Mcdonald, J., Seagrave, J., Mauderly, J., Seilkop, S., Swenberg, J.A., 2008. Health effects of subchronic inhalation exposure to gasoline engine exhaust. Inhal. Toxicol. 20, 1125–1143.

Rothen-Rutishauser, B., Mueller, L., Blank, F., Brandenberger, C., Muehlfeld, C., Gehr, P., 2008. A newly developed in vitro model of the human epithelial airway barrier to study the toxic potential of nanoparticles. Altex-Alternativen Zu Tierexperimenten 25, 191–196.

Rothen-Rutishauser, B.M., Kiama, S.G., Gehr, P., 2005. A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am. J. Respir. Cell Mol. Biol. 32, 281–289.

Seaton, A., Godden, D., Macnee, W., Donaldson, K., 1995. Particulate air pollution and acute health effects. Lancet 345, 176–178.

Steiner, S., Bisig, C., Petri-Fink, A., Rothen-Rutishauser, B., 2016. Diesel exhaust: current knowledge of adverse effects and underlying cellular mechanisms. Arch. Toxicol. 90, 1541–1553.

Steiner, S., Czerwinski, J., Comte, P., Mueller, L.L., Heeb, N.V., Mayer, A., PEtri-Fink, A., Rothen-Rutishauser, B., 2013a. Reduction in (pro-)inflammatory responses of lung cells exposed in vitro to diesel exhaust treated with a non-catalyzed diesel particle filter. Atmos. Environ. 81, 117–124.

Steiner, S., Czerwinski, J., Comte, P., Popovicheva, O., Kireeva, E., Mueller, L., Heeb, N., Mayer, A., Fink, A., Rothern-Rutishauser, B., 2013b. Comparison of the toxicity of diesel exhaust produced by bio- and fossil diesel combustion in human lung cells in vitro. Atmos. Environ. 81, 380–388.

Steiner, S., Heeb, N.V., Czerwinski, J., Comte, P., Mayer, A., Petri-Fink, A., Rothen-Rutishauser, B., 2014. Test-methods on the test-bench: a comparison of complete exhaust and exhaust particle extracts for genotoxicity/mutagenicity assessment. Environ.Sci. Technol. 48, 5237–5244.

Steiner, S., Mueller, L., Popovicheva, O.B., Raemy, D.O., Czerwinski, J., Comte, P., Mayer, A., Gehr, P., Rothen-Rutishauser, B., Clift, M.J.D., 2012. Cerium dioxide nanoparticles can interfere with the associated cellular mechanistic response to diesel exhaust exposure. Toxicol. Lett. 214, 218–225.

Tomašek, I., Horwell, C.J., Damby, D.E., Barošová, H., Geers, C., Petri-Fink, A., Rothen-Rutishauser, B., Clift, M.J., 2016. Combined exposure of diesel exhaust particles and respirable Soufrière Hills volcanic ash causes a (pro-)inflammatory response in an in vitro multicellular epithelial tissue barrier model. Part. Fibre Toxicol. 13 (1), 67.

UNECE, 2016. Global Technical Regulation No. 15. http://www.unece.org/trans/main/wp29/wp29wgs/wp29gen/wp29globregistry.html.

U.S. EPA, 2004. Air Quality Criteria for Particulate Matter (Final Report, 2004). U.S. Environmental Protection Agency, Washington, DC. EPA 600/P-99/002aF-bF.

Wilson, M.R., Stone, V., Cullen, R.T., Searl, A., Maynard, R.L., Donaldson, K., 2000. In vitro toxicology of respirable Montserrat volcanic ash. Occup. Environ. Med. 57, 727–733.

Zhang, S., McMahon, W., 2012. Particulate emissions for LEV II light-duty gasoline direct injection vehicles. SAE Int. J. Fuel. Lubr. 5, 637–646.