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A Comparative Study of Different *In Vitro* Lung Cell Culture Systems to Assess the Most Beneficial Tool for Screening the Potential Adverse Effects of Carbon Nanotubes

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To determine the potential inhalatory risk posed by carbon nanotubes (CNTs), a tier-based approach beginning with an *in vitro* assessment must be adopted. The purpose of this study therefore was to compare 4 commonly used *in vitro* systems of the human lung (human blood monocyte-derived macrophages [MDM] and monocyte-derived dendritic cells [MDDC], 16HBE14o- epithelial cells, and a sophisticated triple cell co-culture model [TCC-C]) via assessment of the biological impact of different CNTs (single-walled CNTs [SWCNTs] and multi-walled CNTs [MWCNTs]) over 24 h. No significant cytotoxicity was observed with any of the cell types tested, although a significant ($p < .05$), dose-dependent increase in tumor necrosis factor (TNF)-α following SWCNT and MWCNT exposure at concentrations up to 0.02 mg/ml to MDM, MDDC, and the TCC-C was found. The concentration of TNF-α released by the MDM and MDDC was significantly higher ($p < .05$) than the TCC-C. Significant increases ($p < .05$) in interleukin (IL)-8 were also found for both 16HBE14o- epithelial cells and the TCC-C after SWCNTs and MWCNTs exposure up to 0.02 mg/ml. The TCC-C, however, elicited a significantly ($p < .05$) higher IL-8 release than the epithelial cells. The oxidative potential of both SWCNTs and MWCNTs (0.005–0.02 mg/ml) measured by reduced glutathione (GSH) content showed a significant difference ($p < .05$) between each monoculture and the TCC-C. It was concluded that because only the co-culture system could assess each endpoint adequately, that, in comparison with monoculture systems, multicellular systems that take into consideration important cell type-to-cell type interactions could be used as predictive *in vitro* screening tools for determining the potential deleterious effects associated with CNTs.

Key Words: ***in vitro* lung systems; carbon nanotubes; nanotoxicology; oxidative stress; inflammation; risk assessment.**

Carbon nanotubes (CNTs) have unique and novel physico-chemical characteristics (Donaldson *et al.*, 2006), which may be extremely advantageous to a plethora of applications (eg, medicine and sporting equipment) (Robertson, 2004). Despite this, the potential use of CNTs in many of these applications has raised extreme concerns regarding their inevitable human interaction (Maynard, 2007). Recently, understanding the paradox posed by CNTs has gained increased emphasis due to their potential correlation to "asbestos-like" effects (Donaldson *et al.*, 2010; van Berlo *et al.*, 2012). Although the length and stiffness of CNTs clearly play a central role in CNT-associated biological effects (Donaldson *et al.*, 2010; Murphy *et al.*, 2011, 2012), many CNT samples are bundled/entangled and it is these that are most likely to be used (commonly within a polymer matrix; Robert *et al.*, 2012) in their associated application(s) (Robertson, 2004). Therefore, in these forms, CNTs could (potentially) be exposed to humans during their life cycle (either through accidental or occupational exposure) (Donaldson *et al.*, 2006; Nowack *et al.*, 2012).

Exposure routes of CNTs to the human body potentially consist of the epidermis, via the bloodstream and also through the gastrointestinal tract (Oberdörster *et al.*, 2005). Although the extent of CNT exposure to humans via these routes is debateable, especially both skin and bloodstream exposure, inhalation exposure is widely accepted as being the primary route of exposure for aerosolized nanofibers (Mueller *et al.* 2011; Maynard *et al.*, 2004). Many studies have therefore focused upon understanding how CNTs may interact with lung cells (Johnston *et al.*, 2010). Despite this, there is a current lack of knowledge regarding the CNT-lung cell interaction.

This is due to inconsistencies in (1) the concentrations used (Oberdörster, 2010), (2) the specific physicochemical characteristics of the CNT samples (Bouwmeester *et al.*, 2011), (3) the dispersion state (Bihari *et al.*, 2008) and the dispersant used (Gasser *et al.*, 2012; Wick *et al.*, 2007), as well as (4) the biological system and biochemical endpoint tests employed (Clift *et al.*, 2011a,c).

Although numerous *in vivo* studies have focused upon determining CNT-associated effects upon the lung (Johnston *et al.*, 2010), in order to initiate the refinement, reduction and replacement of invasive animal experimentation (Hartung, 2010, 2011) *in vitro* systems have been used to gain an insight into the mechanistic effects of CNTs with lung cells (Brown *et al.*, 2007; Rothen-Rutishauser *et al.*, 2010). The differences between *in vivo* and *in vitro* systems are well documented (Han *et al.*, 2012). Yet, in order to understand NP-cell interactions, as well as the potential risk posed by these materials, it is essential to adopt a tiered approach, beginning with an *in vitro* analytical screening strategy (Hartung, 2010, 2011). Commonly *in vitro* monoculture cell-based systems have been chosen to study the nanofiber-cell interaction. With this approach, however, there are both advantages and disadvantages. Although primary monocultures can provide relative heterogeneous populations, they are not readily available. Cell lines negate such an issue, however, are limited as to their phenotypic differentiation. Furthermore, monocultures do not allow intercellular (cell type-to-cell type) interactions to occur (Rothen-Rutishauser *et al.*, 2008). Therefore, to bridge the extensive divide between *in vitro* monoculture systems and complex *in vivo* models, *in vitro* co-culture models, that enable the important cell-to-cell interplay, pose a valuable and realistic alternative (Rothen-Rutishauser *et al.*, 2008). Many *in vitro* co-culture systems have been highlighted recently, representing reliable and realistic models to study the NP-cell interaction (Alfaro-Moreno *et al.*, 2008; Bhabra *et al.*, 2009; Kasper *et al.*, 2011; Rothen-Rutishauser *et al.*, 2005). Evidence supporting the use of such *in vitro* systems as efficient screening tools for assessing the potential risk of nano-based materials however (ie, CNTs) remains equivocal and limited.

The aim of this study, therefore, was to assess the biological impact of CNTs, dispersed in different biologically relevant dispersants, at perceived realistic concentrations, using a series of reliable, efficient, and relative biochemical endpoint tests to assess the applicability of monoculture and co-culture *in vitro* systems in order to determine the most efficient *in vitro* tool to screen, mechanistically, the potential adverse effects posed by CNTs to human health.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Switzerland), unless otherwise stated.

Cell Culture

Monocultures. Monocultures of human monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC) were isolated from human whole blood as previously described in Rothen-Rutishauser *et al.* (2005). In addition, the human bronchial epithelial cell line 16HBE14o- (a generous gift from Dr D. Gruenert [Cardiovascular Research Institute, University of California, San Francisco]) was used as previously described in Blank *et al.* (2007). Both MDM and MDDC monocultures were cultured at a density of 1×10^5 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 cell culture medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (L-G), and 1% penicillin/streptomycin (P/S) for 7 days at 37°C, 5% CO_2. To aid the maturation of the MDM and MDDC monocultures, the growth factors M-CSF (for MDM) and GM-CSF + interleukin (IL)-4 (for MDDC) were added to the culture medium for the maturation period. The 16HBE14o- epithelial cell monocultures were cultured at 0.5×10^6 cells/ml in minimum essential media (MEM) supplemented with 10% FCS, 1% L-G, and 1% P/S for 7 days at 37°C, 5% CO_2.

3D Triple Cell Co-culture Model of the Epithelial Airway Barrier. The 3D *in vitro* triple cell co-culture model (TCC-C) of the epithelial airway barrier, consisting of a layer of 16HBE14o- cells with MDM and MDDC present on the apical (upper section) and basolateral (lower section) sides, respectively, was cultured as previously described and characterized by Rothen-Rutishauser *et al.* (2005, 2008). The cell culture densities of both MDM and MDDC, as well as the 16HBE14o- were kept precisely the same as studied with the monoculture versions of each cell type; MDM and MDDC = 1×10^5 cells/ml in RPMI 1640 supplemented with 10% FCS + 1% L-G + 1% P/S for 7 days at 37°C, 5% CO_2; 16HBE14o- epithelial cells = 0.5×10^6 cells/ml in MEM supplemented with 10% FCS + 1% L-G + 1% P/S for 7 days at 37°C, 5% CO_2. Again, both MDM and MDDC cells were exposed to macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) + IL-4, respectively, in order to promote their maturation state following isolation from human whole blood. It is important to note that although there are over 40 types of cells present within the human alveolar region (Ochs and Weibel, 2008), the present co-culture system takes into consideration 3 important cell types of the alveolar epithelial barrier. Both macrophages, which are professional phagocytes, and dendritic cells, which are essential antigen-presenting cells, are the 2 important immune cells present at the epithelial airway barrier (Nicod, 2007). The 16HBE14o- epithelial cells represent the epithelial layer that is important for the structure and function of the barrier system. All 3 of these cell types are important aspects of lung clearing mechanisms of xenobiotics (Rothen-Rutishauser *et al.*, 2008).

Nanofibers and Positive Control Samples

Single-walled CNTs (SWCNTs) (Yangtze Nanotechnology, China) dispersed in Tween 80 (0.04 mg/ml) and multiwalled CNTs (MWCNTs) (Cheap Tubes Inc.) dispersed in Pluronic F127 (160 ppm) were used as test samples in this study. The dispersion protocol for both SWCNTs and MWCNTs in both Pluronic F127 and Tween 80, respectively, is described in Wick *et al.* (2007) and Thurnherr *et al.* (2009). Both crocidolite asbestos fibers (CAFs) (National Research Institute for Occupational Diseases, Johannesburg, South Africa) and standard diesel exhaust particles (DEPs) from the National Institute of Standards and Technology (SRM No. 2975) were also employed as positive fiber and (nano)particle controls, respectively. Furthermore, because it has been highlighted that the biological impact of CNTs can be attributed to the elemental contaminants (ie, Fe) (Kagan *et al.*, 2006), a SWCNT "pellet" (SWCNTs P) sample, previously described by Wick *et al.* (2007), was also assessed. Briefly, the term "pellet" defines all nonfibrous material of the SWCNT suspension (ie, trace elements). CAFs, DEPs, and SWCNTs P were used as reference materials for the biochemical testing strategy of this study only. All samples were exposed to each different cell culture system at a concentration of 0.005, 0.01, 0.02, 0.03, and 0.04 mg/ml for 24 h at 37°C, 5% CO_2, unless otherwise stated. In addition, to control for the effects of the dispersants used for both SWCNTs and MWCNTs on the biochemical factors investigated because these have also been highlighted as potentially being the driving factor contributing to the (adverse) biological effect of CNTs, both Pluronic F127 and Tween

80 were also assessed independently at concentrations of 50–20 000 ppm and 0.01–0.1 mg/ml, respectively.

Particle Characterization

The length, diameter, chemical content, and endotoxin levels were previously assessed and are reported in Wick *et al.* (2007), Thurnherr *et al.* (2009), and Clift *et al.* (2011b). All key physicochemical characteristics of the SWCNTs and MWCNTs in addition to the SWCNTs P, DEPs, and CAFs used in the present study are summarized in Supplementary Table 1.

Assessment of the Nanofiber-Cell Interaction

Electron Tomography. To investigate the nanofiber-lung cell interaction, the TCC-C was exposed to SWCNTs, MWCNTs, and CAFs, as a positive control, in suspension at a particle concentration of 0.03 mg/ml for 24 h at 37°C, 5% CO_2. This particle concentration was used because it was found to be the optimal concentration allowing for the visualization of the nanofibers via electron microscopy. Samples were then prepared for transmission electron microscopy (TEM) as previously described by Brandenberger *et al.* (2010). Briefly, ultrathin sections were formed through a series of dehydration steps and cut to 300 nm thick before being mounted onto 100 mesh copper grids. The samples were then screened for a suitable area for electron tomography using a CM12 TEM (FEI Co. Philips Electron Optics, Zurich, Switzerland). Electron tomography was performed as previously reported by Clift *et al.* (2011b). Briefly, tomography imaging was recorded on a selected area with a Tecnai F20 TEM (FEI, Eindhoven, The Netherlands) equipped with a GIF Tridiem energy filter and Ultrascan 1000 CCD camera (Gatan, Pleasanton, California). The tomogram was recorded at a magnification of ×34 000 while performing a continuous tilt angle shift from −70° to +70° with a dual tilt Fischione specimen holder (Fischione Instruments). To correct for the missing wedge (−90° to −70° and +90° to +70°), dual tilt axis acquisition was performed with an angle difference of 90°. Image processing and 3D stack reconstruction was performed with the Inspect 3D software V.3.0. (FEI Company). Further image processing and the reconstruction of the different (nano)fibers was performed using AMIRA 5.2.2 (Visage Imaging, Berlin, Germany) (Clift *et al.*, 2011b).

Cytotoxicity

Lactate dehydrogenase release. The ability for CAFs, DEPs, SWCNTs, MWCNTs, and SWCNTs P, as well as Pluronic F127 and Tween 80 to cause lactate dehydrogenase (LDH) release from MDM, MDDC, and 16HBE14o- monocultures as well as the TCC-C (both upper and lower sections) was measured using the method previously described in Brown *et al.* (2001). As a positive control, 0.2% Triton X-100 diluted in PBS was used. It is important to note that although the LDH assay is principally considered the fundamental test to determine the cytotoxicity of any nanosized material (Clift *et al.*, 2011c), in order to gain an insight into the viability of the cells, further testing is necessary, such as the Annexin V assay via flow cytometry and/or confocal laser scanning microscopy. In the present study, however, the limited cytotoxic levels observed were supported by both conventional light and TEM, which eluded no signs of apoptosis or necrosis within the different cellular systems (Clift *et al.*, 2013).

LDH adsorption. The potential for the LDH enzyme to adsorb to the surface of CAFs, DEPs, SWCNTs, MWCNTs, and SWCNTs P as well as Pluronic F127 and Tween 80 in the lysate of MDM, MDDC, and 16HBE14o- epithelial cell monocultures or the TCC-C, thus eliciting a false negative toxicity was determined by using the protocol previously described by Clift *et al.* (2008). It is important to note that for the TCC-C, both the upper and lower sections were combined in order to assess the adsorption patterns of the system as 1 entity. All data are presented within the Supplementary Information.

Cytokines and Chemokines

Tumor necrosis factor-α. In MDM and MDDC monocultures as well as the TCC-C, the ability for CAFs, DEPs, SWCNTs, MWCNTs, and SWCNTs P as well as Pluronic F127 and Tween 80 to elicit a release of the proinflammatory cytokine tumor necrosis factor (TNF)-α was assessed via the use of an ELISA diagnostic kit (R&D Systems, Switzerland). As a positive control, lipopolysaccharide (LPS) at a concentration of 0.1 mg/ml was used. It is important to note that the 16HBE14o- epithelial cell monocultures were not tested for their TNF-α release because these exact cells do not readily elicit this proinflammatory cytokine, as recently shown by Lehmann *et al.* (2010).

Interleukin-8. In 16HBE14o- epithelial cell monocultures and the TCC-C, the ability for CAFs, DEPs, SWCNTs, MWCNTs, and SWCNTs P as well as Pluronic F127 and Tween 80 to stimulate the release of the (pro)inflammatory chemokine IL-8 was assessed via the use of an ELISA diagnostic kit (R&D Systems, Switzerland). As a positive control, TNF-α at a concentration of 0.1 mg/ml was used for the 16HBE14o- epithelial cell monocultures, whereas LPS at a concentration of 0.1 mg/ml was used for the TCC-C. It is important to note that neither the MDM or the MDDC monocultures were assessed for their IL-8 release because it has previously been shown that these cell types do not readily produce this (pro)inflammatory chemokine (Lane *et al.*, 2002; Müller *et al.* 2010).

Protein adsorption. The potential for the protein of either TNF-α or IL-8 to adsorb to the surface of CAFs, DEPs, SWCNTs, MWCNTs, and SWCNTs P as well as Pluronic F127 and Tween 80, thus eliciting a false negative toxicity, was also assessed. All nano-objects were initially incubated with the specific protein at 10 ng/ml (diluted in PBS) for TNF-α or IL-8, respectively, for 1 h at 37°C, 5% CO_2. Samples were then centrifuged at 2000 rpm to remove all debris prior to being assessed using each specific ELISA test. All data are presented within the Supplementary Information.

Oxidative Stress

Reduced glutathione content. The intracellular reduced glutathione (GSH) content of MDM, MDDC, 16HBE14o-, and TCC-C after exposure to CAFs, DEPs, SWCNTs, MWCNTs, or SWCNTs P at 0.005, 0.01, and 0.02 mg/ml, as well as Pluronic F127 (160 ppm) and Tween 80 (0.04 mg/ml) was determined using a diagnostic kit (Cayman Chemical), as previously described in Steiner *et al.* (2012). It was not possible to gain information pertaining to the oxidative GSH component (GSSG); therefore, all GSH values are presented relative to the protein expression in the sample (GSH:protein [μM/mg]). tert-Butyl hydrogen peroxide at a concentration of 0.04 mg/ml was used as a positive control.

Statistical Analysis

All results are presented as the mean ± SEM. All data sets were observed to be normally distributed (data not shown). Statistical significance was determined via a parametric 1-way ANOVA, followed by, when appropriate, a Tukey's pairwise comparisons *post hoc* test (SPSS, IBM). The result was considered significant if $p \leq .05$.

RESULTS

Nanofiber-Cell Interaction

The lung-cell interactions of the MWCNTs and CAFs with the TCC-C have previously been reported in Clift *et al.* (2011b). Focusing upon the SWCNTs, these CNTs were specifically found within the MDM of the TCC-C after 24 h exposure at 0.03 mg/ml (Fig. 1). Furthermore, the SWCNTs were found to be fragmented and not present within a vesicular body (Fig. 1). Although no clear signs of frustrated phagocytosis were seen with the SWCNTs because their characteristics would not indicate such a biological response, the SWCNTs were, however, found to be protruding from the MDM after 24 h exposure (Fig. 1). For comparison, a negative control image of a MDM is provided in Supplementary Figure 1.

Cytotoxicity

No significant ($p > .05$) cytotoxicity was observed for the SWCNTs, MWCNTs, CAFs, or DEPs in any of the monoculture systems tested (MDM,

MDDC, 16HBE14o- epithelial cells) after 24 h exposure at concentrations ranging from 0.005 to 0.04 mg/ml (Supplementary Figure 2). Both the SWCNTs and MWCNTs were only noncytotoxic ($p > .05$) in the TCC-C up to 0.02 mg/ml. A complete description of the cytotoxic nature of the panel of each CNT and standard samples with each different biological system tested is given in Supplementary Figures 2 and 3.

(Pro)inflammatory Response

TNF-α release. A significant ($p < .05$) dose-dependent increase in TNF-α release was observed from MDM and MDDC following exposure to MWCNTs for 24 h at all concentrations tested (Fig. 2). A similar trend was also found for SWCNTs, CAFs, and DEPs in both MDM and MDDC monocultures at concentrations 0.01–0.04 mg/ml ($p < .05$) (Fig. 2). No significant ($p > .05$) TNF-α release was noted from either MDM or MDDC monocultures after exposure to SWCNTs P (Fig. 2). The latter finding was associated with the increased cytotoxicity previously shown (Supplementary Figure 2). Significant adsorption patterns ($p < .05$) were found with all 5 samples at concentrations of 0.03 and 0.04 mg/ml, suggesting false positive events recorded for both MDM and MDDC at these concentrations (Supplementary Figure 3). Similar results were also observed following exposure of SWCNTs, MWCNTs, CAFs, DEPs, and SWCNTs P to the TCC-C.

Following exposure to the TCC-C (upper section only), CAFS, DEPs, SWCNTs, and MWCNTs all elicited a significant dose-dependent increase in TNF-α release, at similar TNF-α concentrations produced by both MDM and MDDC monocultures, from baseline levels (medium only) to 0.03 mg/ml (Fig. 2). In the lower section of the TCC-C, only the SWCNTs and MWCNTs elicited a dose-dependent increase in TNF-α release up to 0.03 mg/ml. At 0.04 mg/ml, no significant biological effect ($p > .05$) was noted for either SWCNTs or MWCNTs (the TNF-α concentration for both CNTs was shown to decrease from 0.03 mg/ml) (Fig. 2). This was attributed to a significant adsorption ($p < .05$) of the TNF-α protein to the SWCNTs and MWCNTs at 0.04 mg/ml (Supplementary Figure 4). A similar effect was also observed at a concentration of 0.03 mg/ml (Supplementary Figure 4). DEPs and CAFs were both found to stimulate an intermittent significant TNF-α release from 0.005 to 0.04 mg/ml (Fig. 2). Significant adsorption patterns ($p < .05$) at both 0.03 and 0.04 mg/ml for both samples were found (Supplementary Figure 4), additionally due to the variance observed within each sample concentration; these results were not considered significant. Similar to the MDM and MDDC monocultures, no effect was observed for the SWCNTs P at any concentration tested (Fig. 2), although again at both 0.03 and 0.04 mg/ml, a significant ($p < .05$) adsorption was observed for SWCNTs P (Supplementary Figure 4).

IL-8 release. A significant dose-dependent increase in the release of the (pro)inflammatory chemokine IL-8 was observed for CAFs, DEPs, SWCNTs, and MWCNTs in 16HBE14o- monocultures up to 0.02 mg/ml after 24 h suspension exposure (Fig. 3). At concentrations 0.03 and 0.04 mg/ml, similar adsorption patterns with these samples and the IL-8 protein were observed as for the TNF-α protein (Supplementary Figure 5). SWCNTs P showed a significant, dose-dependent increase up to 0.04 mg/ml although this was attributed to the significant cytotoxicity elicited by this nonfibrous fraction of the SWCNT sample (Fig. 3). In the TCC-C, the IL-8 release caused by CAFs, DEPs, SWCNTs, and MWCNTs is significant ($p < .05$), 4-fold lower than that produced by the 16HBE14o- epithelial cells. In the upper and lower sections of TCC-C, a significant dose-dependent increase ($p < .05$) was found for CAFs, DEPs, SWCNTs, and MWCNTs up to 0.04 mg/ml (Fig. 3), although these findings should have careful consideration due to the significant adsorption patterns ($p < .05$) for all 4 of these materials at 0.03 and 0.04 mg/ml (Supplementary Figure 5). No significant effects were found following exposure of SWCNTs P to the TCC-C (in either the upper or lower sections) (Fig. 3).

Reduced GSH Content

Because a significant interference was observed at both 0.03 and 0.04 mg/ml (Supplementary Figures 3 and 4), concentrations ranging from 0.005 to 0.02 mg/ml were used. Both MDM and MDDC monocultures only showed a significant loss ($p < .05$) in intracellular reduced GSH following exposure to MWCNTs at 0.01 and 0.02 mg/ml (Fig. 4). In the 16HBE14o- epithelial monocultures, the DEPs, SWCNTs, and MWCNTs, at all concentrations tested (0.005, 0.01, and 0.02 mg/ml), showed a significant loss ($p < .05$) in reduced GSH content (Fig. 4). CAFs also caused a significant loss in reduced GSH at a 0.02 mg/ml in 16HBE14o- cell monocultures (Fig. 4). Analysis following exposure of CAFs, DEPs, SWCNTs, and MWCNTs to the TCC-C showed a 10-fold, significant increase ($p < .05$) in the intracellular reduced GSH content compared with each monoculture assessed (Fig. 4). Interestingly though, a similar trend for each sample was observed (Fig. 4), with a dose-dependent significant ($p < .05$) loss in reduced GSH content for DEPs, SWCNTs, and MWCNTs from 0.005 to 0.02 mg/ml after 24 h exposure (Fig. 4). Due to the heightened cytotoxicity of the SWCNT P (Supplementary Figure 1), a significant loss in the reduced GSH of MDM, MDDC, 16HBE14o- as well as the TCC-C was recorded.

DISCUSSION

The aim of this study was to compare 4 commonly used *in vitro* lung cell cultures to determine which may be most apt in the hazard assessment of nanomaterials. The findings of this comparison are summarized in Table 1.

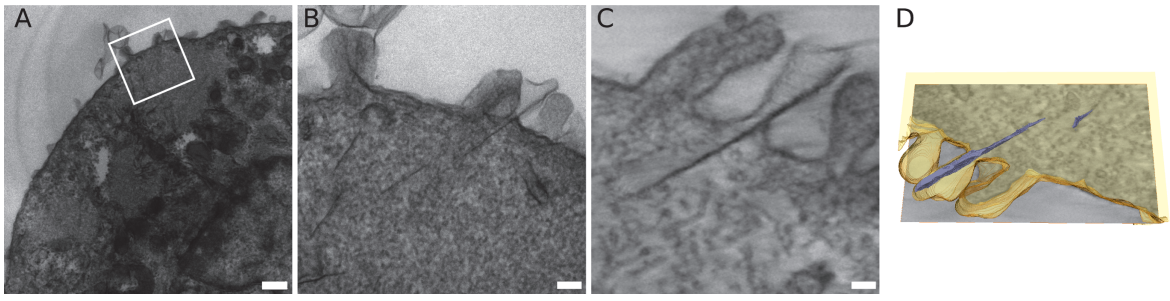


FIG. 1. Electron tomography still images of the triple cell co-culture system (TCC-C) after exposure to single-walled carbon nanotubes (SWCNTs) after submerged culture exposure at 0.03 mg/ml for 24 h at 37°C, 5% CO_2. Image (A) is a still 2D image of SWCNTs present in the monocyte-derived macrophages of the TCC-C, whereas image (B) is the projected image (inset) of image (A). Image (C) shows representative tomogram slices of image (B), whereas image (D) shows the rendered 3D electron tomogram (SWCNTs are colored blue, whereas the cell membrane is yellow). Scale bar in image (A) represents 1 μm. Scale bar in images (B and C) represents 0.2 μm.

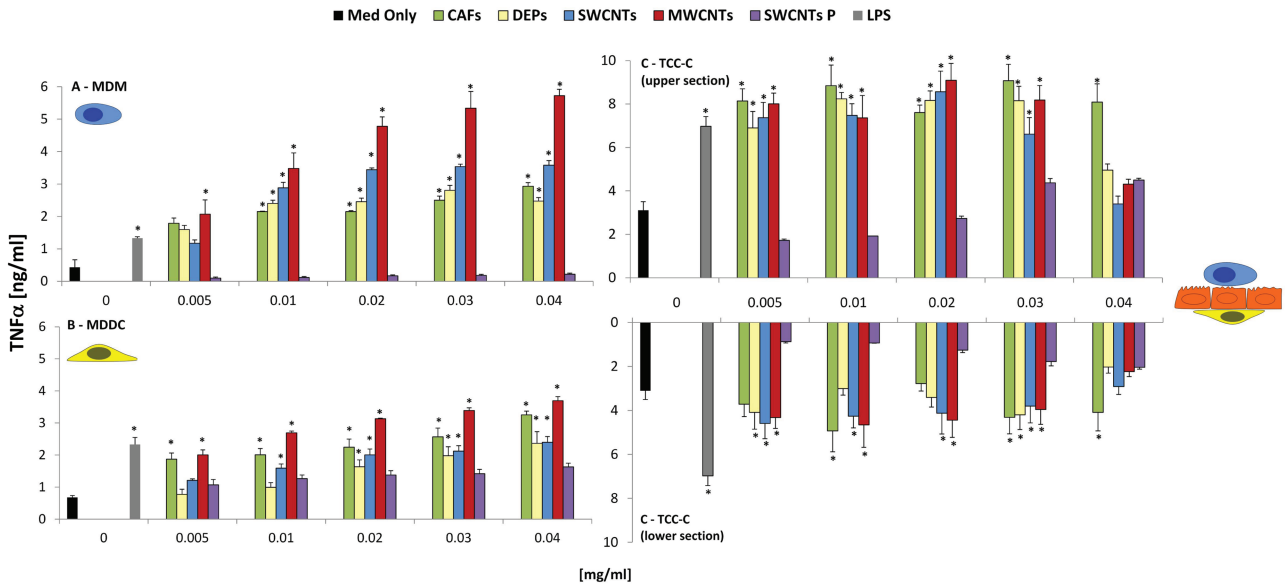


FIG. 2. Release of the proinflammatory cytokine tumor necrosis factor-α from (A) monocyte-derived macrophages (MDM), (B) monocyte-derived dendritic cells (MDDC), and (C) 3D triple cell co-culture model (TCC-C) of the epithelial airway barrier (upper and lower sections represented on the graph) following exposure to crocidolite asbestos fibers (CAFs), diesel exhaust particles (DEPs), single-walled carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs), and single-walled carbon nanotube "pellet" (SWCNTs P) at 0.005, 0.01, 0.02, 0.03, and 0.04 mg/ml after 24 h at 37°C, 5% CO_2 ($n = 3$). Data presented are the mean ± SEM. Lipopolysaccharide at a concentration of 0.1 mg/ml was employed as a positive control (solid grey bar). Cell culture media only (Med Only) represents the negative control (solid black bar). *Relates to a significant difference from baseline ($p < .05$).

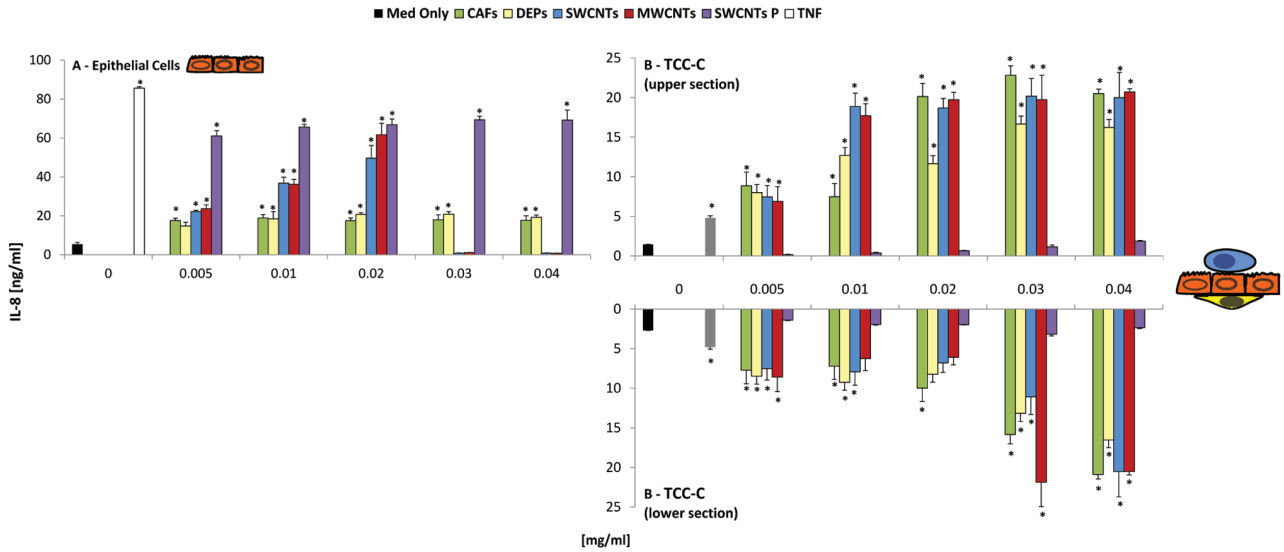


FIG. 3. Release of the (pro)inflammatory chemokine interleukin-8 from (A) 16HBE14o- epithelial cells and (B) 3D triple cell co-culture model (TCC-C) of the epithelial airway barrier (upper and lower sections represented on the graph) following exposure to crocidolite asbestos fibers (CAFs), diesel exhaust particles (DEPs), single-walled carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs), and single-walled carbon nanotube "pellet" (SWCNTs P) at 0.005, 0.01, 0.02, 0.03, and 0.04 mg/ml after 24 h at 37°C, 5% CO_2 ($n = 3$). Data presented are the mean ± SEM. Both tumor necrosis factor (16HBE14o- cells) (solid white bar) and lipopolysaccharide (TCC-C) (solid grey bar) at concentrations of 0.1 mg/ml were employed as positive controls. Cell culture media only (Med Only) represents the negative control (solid black bar). *Relates to a significant difference from baseline ($p < .05$).

Observation that the SWCNTs, as well as the MWCNTs and CAFs (Clift *et al.*, 2011b) only interacted with the MDM of the TCC-C suggests that the macrophages are performing their primary function following a xenobiotic insult. It is important to note, however, that although none of the (nano)fibers were observed to be located within the epithelial layer or the MDDC of the co-culture system, it does not discount the possibility that they were present or interacting with these cell types of the

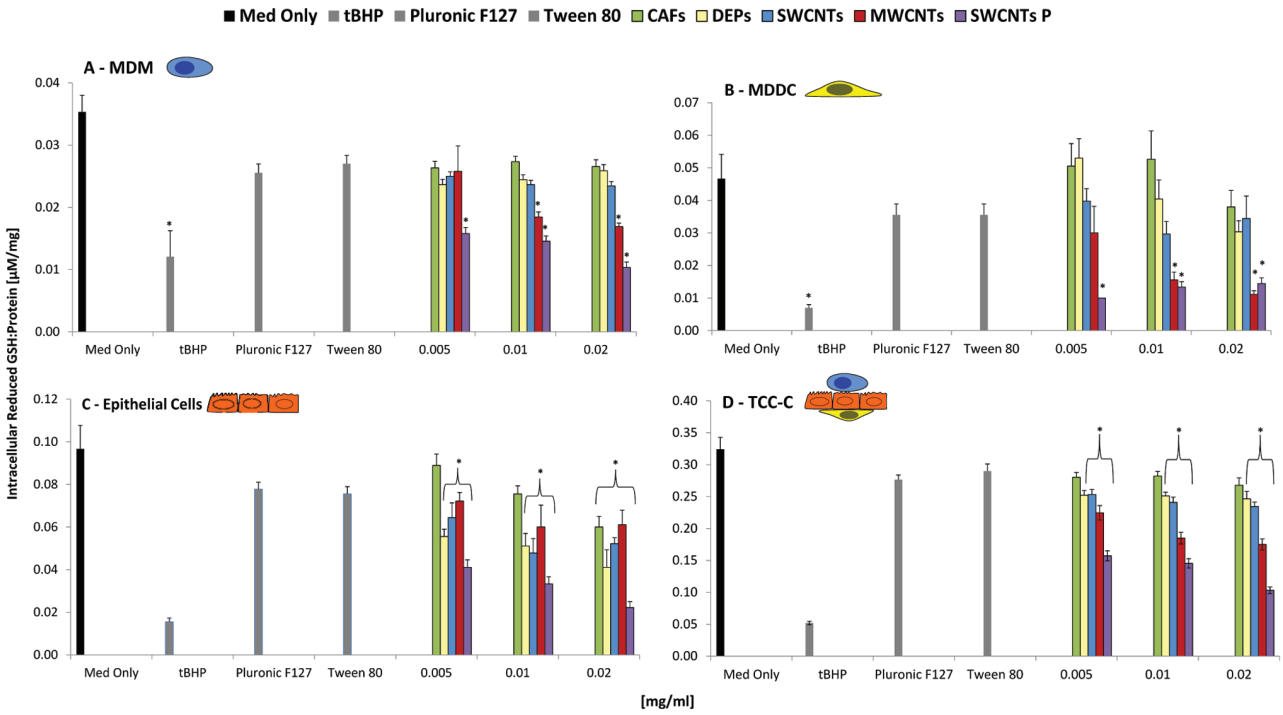


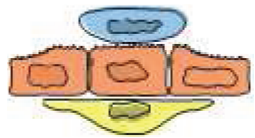
FIG. 4. Reduced glutathione (GSH) content for (A) human blood monocyte-derived macrophages (MDM), (B) human blood monocyte-derived dendritic cells (MDDC), (C) 16HBE14o- epithelial cells, and (D) 3D triple cell co-culture model (TCC-C) of the epithelial airway barrier (upper and lower sections represented on the graph) following exposure to crocidolite asbestos fibers (CAFs), diesel exhaust particles (DEPs), single-walled carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs), and single-walled carbon nanotube "pellet" (SWCNTs P) at 0.005, 0.01, and 0.02 mg/ml after 24 h at 37°C, 5% CO_2 ($n = 3$). Effects of Pluronic F127 (160 ppm) and Tween 80 (0.04 mg/ml) are also shown in graphs (A and D). Data presented are the mean ± SEM. tBHP, at a concentration of 0.04 mg/ml, was employed as a positive control. Cell culture media only (Med Only) represents the negative control. *Relates to a significant difference from baseline ($p < .05$).

TCC-C at this time point. Of interest is the observation that the interaction of the SWCNTs and MWCNTs differed with the MDM. SWCNTs showed 2 "bundles" within the cytosol of the MDM, suggesting a possible, preferential entry route into the MDM via a nonendocytotic mechanism. The MWCNTs, however, were found to be present within a vesicular body (Clift *et al.*, 2011b). Furthermore, the presence of proposed SWCNT "fragments" in the cytosol of the cell suggests that there is a possibility that they have undergone degradation by intracellular enzymes (Kagan *et al.*, 2010). This aspect requires further research. Despite the differences in the interaction observed, in the present study, the overall biochemical response was not found to be significantly different between the 2 different CNT types, suggesting a limited influence of the specific CNT-cell "interaction" as regards their cellular effects.

Observation of the biochemical response of each *in vitro* system following exposure to the SWCNTs and MWCNTs, in addition to the CAFs and DEPs tested, found that in all *in vitro* culture systems, no significant cytotoxicity ($p > .05$) occurred. These findings support those previously reported when comparing the effects of cellulose nanowhiskers to MWCNTs at concentrations up to 0.03 mg/ml using the same 4 *in vitro* systems (Clift *et al.*, 2011b). It was further shown that despite the SWCNTs and the MWCNTs being dispersed using different surfactants (Tween 80 and Pluronic F127, respectively) that no cytotoxic effect was evident in either MDM, MDDC, 16HBE14o- epithelial monocultures or the TCC-C, and therefore supporting that, depending on the concentration used, surfactants may be advantageous in obtaining a well-dispersed and characterized CNT sample (Wick *et al.*, 2007). The lack of any cytotoxic response also supports Thurnherr *et al.* (2009) who reported the same MWCNTs used in the present study to cause no apoptosis or necrosis in the Jurkat A3 human leukemic T cell line after 24 h exposure to 0.03 mg/ml.

The SWCNTs pellet sample showed a significant LDH release over 24 h at the highest concentrations in all 4 different cell culture systems. These findings support those of Kagan *et al.* (2006) who reported that the catalyst metals used to produce CNTs (ie, Fe) are directly responsible for the adverse cellular effects noted and that any biological effects observed are not due to a fibrous effect (Kagan *et al.*, 2006). Thus, the present study highlights that if a correct and specific dispersion method is used (Wick *et al.*, 2007), then the proposed "cytotoxic" component of the CNT sample (ie, contaminant metals) (Kagan *et al.*, 2006) can be extracted and allow for a thorough investigation of CNT effects upon normal cellular homeostasis (ie, the effects noted for the SWCNTs can be attributed to their fibrous characteristics).

TABLE 1
A Summary of the Biological Response of Human Blood MDM, Human Blood MDDC, 16HBE14o- Epithelial Cells, and the 3D TCC-C of the Epithelial Airway Barrier (Response Is Designated Between "Upper" and "Lower" Sections Where Appropriate) After Suspension Exposure to SWCNTs and MWCNTs

In Vitro System	MDM Monocultures 		MDDC Monocultures 		16HBE14o- Monocultures 		TCC-C 	
Nanofiber (mg/ml)	SWCNTs	MWCNTs	SWCNTs	MWCNTs	SWCNTs	MWCNTs	SWCNTs	MWCNTs
Cytotoxicity (LDH release)	—	—	—	—	—	—	—	—
TNF-α ELISA	−[0.005] ++[0.01–0.02]	++[0.005–0.02]	−[0.005] ++[0.01–0.02]	++[0.005–0.02]	N/A	N/A	++[0.005–0.02] (Upper + Lower)	++[0.005–0.02] (Upper + Lower)
IL-8 ELISA	N/A	N/A	N/A	N/A	++[0.005–0.02]	++[0.005–0.02]	++[0.005] (Upper + Lower) ++[0.01–0.02] (Upper) −[0.01–0.02] (Lower)	++[0.005–0.02] (Upper + Lower) ++[0.01–0.02] (Upper) −[0.01–0.02] (Lower)
GSH content	−[0.05–0.02]	−[0.005] ++[0.01–0.02]	−[0.05–0.02]	−[0.005] ++[0.01–0.02]	++[0.005–0.02]	++[0.005–0.02]	++[0.005–0.02]	++[0.005–0.02]

Effects corresponding to after 24 h exposure CNT exposure at 37°C, 5% CO_2 to 0.005, 0.01, and 0.02 mg/ml are highlighted due to the assay interference observed (Supplementary Information). The response measured from each *in vitro* system for each different biochemical assay used (LDH release; proinflammatory cytokine TNF-α or inflammatory chemokine IL-8 release; intracellular reduced GSH content) is indicated as either (1) no effect from baseline levels (negative control [cell culture medium only]) (symbol denoted as "−") or (2) considered biologically significant ($p < .05$) (symbol denoted as "++"). The effect at each concentration measured is given. N/A refers to "not applicable" (due to the nonspecific nature of the endpoint to the cell type tested).

The findings that both SWCNTs and MWCNTs can cause a (pro)inflammogenic response *in vitro* (TNF-α and IL-8 release) support previous literature using cells representing the lung *in vitro* (Brown *et al.*, 2007; Donaldson *et al.*, 2006; Johnston *et al.*, 2010). It is important to note the cell-specific proinflammogenic assessment performed in the present study. In regard to the lack of TNF-α analysis performed on the 16HBE14o- cells, as described in the Materials and Methods section, these cells, as with epithelial cells in general, do not readily produce this proinflammatory cytokine. This has previously been shown in another comparison study of the TCCC and with its respective monocultures following exposure to iron oxide hybrid nanoparticles (Lehmann *et al.* (2010). Similar results were also found in another study by Müller *et al.* (2010), where SWCNTs were shown not to produce a detectable level of the proinflammatory chemokine IL-8 from either MDM or MDDC.

The (pro)inflammogenic effects suggestive of mediation via oxidative stress (loss in GSH) confirm that CNTs can be highly reactive *in vitro* (Rothen-Rutishauser *et al.*, 2010). Similar findings were also found for both particle and fiber controls (DEPs [found to be present within each cell type of the TCC-C; Müller *et al.*, 2010] and CAFs [causing frustrated phagocytosis in the MDM of the TCC-C; Clift *et al.*, 2011b]), further suggesting that the biochemical response recorded is not dependent upon the specific cellular "interaction." Furthermore, the findings that both SWCNTs and MWCNTs can elicit similar biochemical reactions as both DEPs and CAFs, both human class 1 carcinogens as recognized by the International Agency for Research on Cancer, highlight the potential hazardous nature that CNTs may pose toward human health (Donaldson *et al.*, 2010).

In respect to which *in vitro* system is most apt for hazard assessment of nanomaterials, Müller *et al.* (2010) showed that the SWCNTs, as used in the present study, cause no significant difference between mono- and co-cultures following 24 h exposure up to 0.03 mg/ml when assessing markers of oxidative stress (eg, the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate [H_2DCFDA]) and (pro)inflammatory cytokine release (TNF-α and IL-8). Although Müller *et al.* (2010) investigated A549 epithelial cells and not 16HBE14o-, it is due to high variability shown by Müller *et al.* (2010) in all data presented that no significant differences were observed. A recent study by Gasser *et al.* (2012), however, in which different surface charged MWCNTs, either coated with or without lung surfactant (Curosurf), were assessed in regard to their interaction with MDM and the TCC-C (using 16HBE14o- cells), did report significant differences between mono- and co-cultures. Interestingly, it was shown that the trend in the decrease of the intracellular thiol GSH was a significant 100×-fold difference between each respective *in vitro* system (GSH decrease = TCC-C 10× > MDM), evident for all the different surface charged MWCNTs. It was also shown that the TNF-α response between MDM and the TCC-C was 3× decreased in the TCC-C than in the MDM (Gasser *et al.*, 2012). The trends shown by Gasser *et al.* (2012), at least those of the GSH analysis, are comparable with the present study. Although, considering the findings of Müller *et al.* (2010), as well as those of Lehmann *et al.* (2010) and Clift *et al.* (2011b), in which iron oxide NPs and cellulose nanowhiskers, respectively, were shown to cause significant differences in the response (eg, proinflammatory) between mono- and co-cultures, it is apparent that the biochemical endpoint and exposure period tested are essential determinants regarding the resultant effect between the different *in vitro* systems. Yet, none of these studies categorically show that *in vitro* co-cultures are better, or worse, than monocultures, and vice versa. Although the advantages and disadvantages of both types of *in vitro* systems must be weighed (Rothen-Rutishauser *et al.*, 2008), this argument does not allow for a clear conclusion to be met. Comparison with the *in vivo* response is therefore essential to gain an understanding of which *in vitro* system may elicit a similar effect. Although it is possible to compare the CNT effects of the present study with the *in vivo* literature (eg, Johnston *et al.*, 2010), this comparison is futile due to the many, innate differences such as the characteristics of the samples used and the exposure methods employed, highlighting a further important issue of specificity in comparing the biological response of nanomaterials across different systems. Therefore, a clear, defined, comparative *in vitro* (mono- and co-cultures) versus *in vivo* study must be conducted that considers these aspects in addition to many others (eg, standardized concentrations [doses], exposure methods, and times) in order to define which *in vitro* system is optimal in assessing the (potential) hazard posed by nanomaterials (eg, CNTs).

In conclusion, the findings presented from the current study show that there are significant differences between the biochemical responses monitored between mono- and co-culture *in vitro* systems that are used to mimic the human lung. It is not possible to state from these findings alone that either mono- or co-cultures are prevalent over the other in determining the (potential) hazard posed by nanomaterials. Although it is possible to highlight that while monocultures suffice to determine a simple live/dead assessment following nanomaterial exposure, multicellular systems additionally provide the ability to determine the mechanistic, molecular pathology of nanomaterials *in vitro* because they take into consideration the important cell-to-cell interplay as occurs *in vivo*. Therefore, by adopting the approach of using multicellular systems instead of monocultures, it might be possible to truly undertake an adequate *in vitro* study that may holistically assess the (potential) risk of nanomaterials and that may be sufficient enough to refine, reduce, and replace animal experimentation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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