Human Asthmatic Bronchial Cells are more Susceptible to Sub-Chronic Repeated Exposures of Aerosolized Carbon Nanotubes at Occupationally-Relevant Doses than Healthy Cells

Author list

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ABSTRACT

Although acute pulmonary toxicity of carbon nanotubes (CNTs) has been extensively investigated, the knowledge of potential health effects following chronic occupational exposure is currently limited and based only upon *in vivo* approaches. Our aim was to realistically mimic sub-chronic inhalation of multi-walled CNTs (MWCNTs) *in vitro*, using the Air-Liquid Interface Cell Exposure system (ALICE) for aerosol exposures onto reconstituted human bronchial tissue from healthy and asthmatic donors. The reliability and sensitivity of the system was validated using crystalline quartz (DQ12), which elicited an increased (pro-)inflammatory response, as reported *in vivo*. At the administrated MWCNT doses relevant to human occupational lifetime exposure (10µg/cm² for five weeks of repeated exposures/five days per week) elevated cilia beating frequency (in both epithelial cultures) and mucociliary clearance (in asthmatic cells only) occurred, whereas no cytotoxic reactions or morphological changes were observed. However, chronic MWCNT exposure did induce an evident (pro-)inflammatory and oxidative stress response in both healthy and asthmatic cells. The latter revealed stronger and more durable long-term effects compared to healthy cells, indicating that individuals with asthma may be more susceptible to adverse effects from chronic MWCNT exposure. Our results highlight the power of occupationally-relevant sub-chronic exposures onto human *in vitro* models in nanosafety hazard assessment.

KEYWORDS

In vitro primary lung system; reconstituted healthy and asthma tissue; air-liquid interface; multi-walled carbon nanotubes; sub-chronic repeated exposure; occupational doses; hazard assessment.

The high aspect ratio, excellent strength and good conductivity offered by carbon nanotubes (CNTs) has raised their demand in the global industry market.[1] However, recent studies provided evidence that CNTs can be released during the manufacturing process, and both scientific and public concern has been raised regarding the potential risks to human health.[2,3] Recently, the levels of human occupational exposure were assessed in different departments of a commercial manufacturing facility in the Netherlands, and concentrations up to 41-43μg/m^3 were measured in the production and handling area, whereas in offices and research departments the levels were almost 10 times lower.[4] In addition, Shvedova and colleagues compared the mRNA and ncRNA expression profiles in the blood of exposed workers (with at least six months direct contact with aerosolised MWCNTs) and non-exposed employees of the same production facility. Results revealed significant changes in gene expression associated with cell-cycle regulation, apoptosis and proliferation, with potential pulmonary, cardiovascular and carcinogenic risk in MWCNT-exposed humans.[5]

The extremely high aspect ratio and biopersistence of MWCNTs poses a major concern that fiber inhalation in the workplace might induce unwanted pulmonary effects which resemble the serious effects of micron-sized asbestos fibers.[6] A growing number of animal studies have demonstrated that MWCNT inhalation is a highly potent trigger for the onset of airway injury, inflammation, fibrosis and granuloma formation.[7-9] Once inhaled, MWCNTs can reach the deepest regions of the respiratory tract – the bronchioles-alveolar region – where they are not rapidly degraded but accumulate in the lower lung and distributed to other organs over a period of 90-336 days (d). [10,11]

Most *in vivo* studies do not take into consideration the real concentrations in an occupational setting and as a consequence cannot be extrapolated to human exposure.[12] Since exposure in manufacturing facilities most probably occurs repeatedly over a long timeframe, it is crucial to obtain insight into the pulmonary toxicity of chronic CNT exposure to repeated doses that closely resemble realistic occupational exposure conditions. Many studies addressing possible adverse effects of CNTs were performed on animal-based approaches, however, there is a clear need to circumvent these time-consuming, cost-intensive and ethically problematic *in vivo* studies. In addition, detailed insight into the mechanistic interaction of CNTs with cells (*i.e.* toxicodynamics) is difficult to obtain *in vivo*. Most existing *in vitro* studies focusing on acute or long-term exposures were carried out using relatively high doses and under submerged conditions, despite such exposure conditions having little physiological relevance to the investigation of pulmonary effects since lung cells are exposed to air on their apical surface.[13-16] Consequently, these findings cannot be correlated with the outcomes of *in vivo*

chronic studies, thus illustrating the critical necessity for an advanced *in vitro* strategy to realistically and reliably assess the biological consequences of CNTs following long-term repeated administration.

In the past various reports have indicated the attribution of occupational and environmental factors in the modulation of lung airway diseases. In particular, the role of NPs in exacerbation of pulmonary diseases has been confirmed in environmental studies.[17] A limited number of studies, have also demonstrated that engineered NPs *i.e.* titanium, gold or silica NPs, could have a respiratory exacerbation effect in animal models with pre-existing respiratory diseases *i.e.* asthma.[18,19] Indeed, asthma is a highly prevalent and significant chronic inflammatory airway disease, primarily characterised by bronchial obstruction, affecting 235 million people worldwide.[20] The lack of investigation on the impact of NP inhalation on patients with pulmonary diseases is of importance, considering that workers in NP production facilities are likely to suffer from respiratory illnesses.

To address the aforementioned needs, the aim of the present study was to mimic the chronic inhalation of MWCNTs *in vitro*, by using the Air-Liquid Interface Cell Exposure system (ALICE) system[21] to induce repeated MWCNT aerosol exposure onto primary human bronchial epithelial cells cultured at the air-liquid interface (ALI) at low, realistic doses corresponding to human occupational lifetime exposure (Figure 1). Potential susceptible effects in repeatedly exposed asthmatic cells were also investigated, since it has been shown *in vivo* that CNTs may act as an exacerbating factor in animals with pre-existing lung disorders, particularly asthma.[22,23] However, limited information is still available regarding the impact of CNTs on asthmatic mice models, and to date there has been no *in vitro* report questioning the consequences of MWCNTs exposures in primary lung cell cultures derived from asthmatic donors.

RESULTS AND DISCUSSION

Aerosolisation of MWCNTs and deposition characterization

The ALICE is a well-established aerosolisation system for spherical nanoparticles[21, 24] and was recently demonstrated to be efficient for the aerosolisation of fiber-shaped nanomaterials, such as cellulose nanocrystals and MWCNTs for single and short-term repeated exposures.[25,26] For the current study a stable, well-characterised and well-dispersed MWCNT[27] suspension was aerosolised using the ALICE. For the current work Pluronic F127 has been used as a dispersant since it prevents tube aggregation during the experimental work-procedure and results in well-dispersed suspension allowing an efficient aerosolisation.[25, 27] Pluronic is biocompatible and frequently used in galenics [28], and since it is a non-ionic detergent, it is rapidly displaced by proteins and lipids contained in the surfactant layer on top of the epithelial cultures under experimental conditions, similar to the *in vivo* situation following CNT inhalation.[29] MWCNTs were thoroughly characterised prior to and after the aerosolisation process, as described in previous work.[25, 27, 30] The size distribution of the stock and deposited MWCNTs, as well as all other key physicochemical characteristics of the MWCNT sample are summarized in Supplementary Table 1. Both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) revealed the typical tubular structure of aerosolised MWCNTs (Figure 2a-b, SI Figure 1). Visualisation of deposited MWCNTs following single and several repeated exposures revealed a reproducible, homogenous and dose-dependent deposition. Furthermore, in our earlier study it was shown that the aerosolisation process did not affect the morphology of the deposited tubes, as no MWCNT fracture or damage was observed compared to the fibers in stock suspension.[25] Hence, the ALICE reflects an effective and reproducible approach to study the long-term inhalation of MWCNTs *in vitro*.

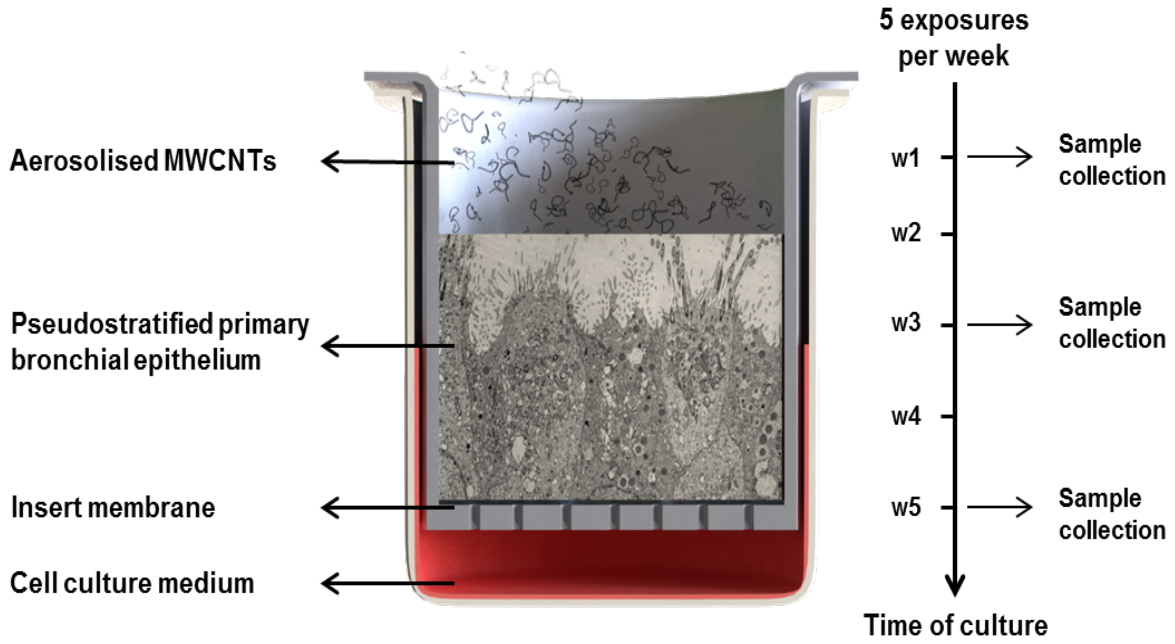
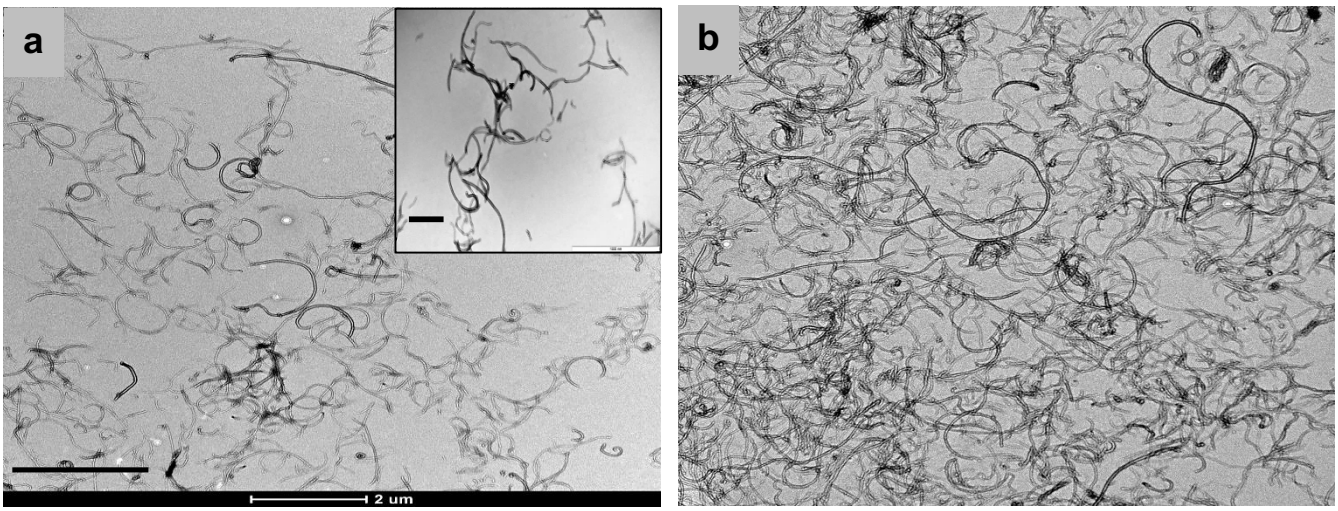


Figure 1: Schematic presentation of the exposure scenario. Cell cultures were exposed for up to five weeks, five days per week to aerosolised MWCNTs using the ALICE system. The cellular response following MWCNT exposure was analyzed at the end of w1, w3 and w5.

Single aerosolisation of 250µg/ml MWCNTs resulted in an average deposited value of 0.39±0.05µg/cm^2. Repeated MWCNTs nebulisations revealed deposited doses of 1.95±0.07, 5.76±0.056 and 9.58±0.054µg/cm^2 for week (w) 1, w3 and w5 time-points, respectively (Figure 2c). The selected deposited dose in our experiments can be considered relevant to a human occupational lifetime exposure. In particular, the alveolar mass retention of a full working lifetime exposure (45 years) to CNTs of different sizes was modelled and calculated to be in the range of 12.4 to 46.5µg/cm^2.[31] Based on these findings, the highest deposited dose in the present study (10µg/cm^2) reached after five weeks of repeated exposure reflects a full working lifetime human exposure to MWCNTs. It is worthwhile to mention, that the calculation in the applied dosimetry model has been based on alveolar mass deposition. However, to the best of our knowledge this is the only existing model to predict realistic CNTs doses upon long-term occupational settings. Although bronchial epithelial surfaces may not receive the same amount of deposited CNTs, however, a large portion of the deposited particles will be cleared by the mucociliary clearance mechanism. Therefore, the applied doses are considered highly realistic.



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Deposition [µg/cm²]	Single Exposure	Week 1	Week 3	Week 5
MWCNTs	0.39 ± 0.05	1.95 ± 0.07	5.76 ± 0.056	9.58 ± 0.054
DQ12	0.23 ± 0.07	1.05 ± 0.10	3.92 ± 0.24	5.59 ± 0.25

Figure 2: MWCNT morphology and deposition upon repeated aerosol exposures. TEM images of deposited MWCNTs (**a**) after single (d1) and (**b**) after five repeated aerosolisations (w1) using the ALICE system (Scale bars: 2µm). Inset shows higher magnification image of aerosolised MWCNTs after single exposure (scale bar: 1µm). (**c**) Average deposition (µg/cm^2) of MWCNTs and DQ12 as measured by QCM at different tested time-points (n=3).

The deposition for the negative control (ultrapure H_2O) was below the detection limit (0.09µg/cm^2) of the integrated Quartz Crystal Microbalance (QCM). Moreover, exposure to aerosolised crystalline quartz, *i.e.* DQ12, demonstrated an average deposited dose of 0.23±0.07µg/cm^2 and also resulted in a dose-dependent increase at repeated exposures.

Cell morphology and functionality

The airway epithelial cells, together with macrophages, constitute the first line of cellular defense against external antigens, *i.e.* inhaled nanomaterials, allergens or chemicals.[32] Undoubtedly, the presence of immune cells *i.e.* primary macrophages and dendritic cells in the culture would enhance the structural resemblance to the native lung state and would further give the possibility to investigate the cellular interplay. However, the short lifetime (up to twelve days from the isolation) of primary immune cells (data not shown) remains a limitation in chronic experimental settings and more research is required to extend the

survival of primary immune cells to allow long-term toxicity testing. Taking into account the difficulties and challenges to simulate a chronic repeated experimental setting *in vitro,* an effective and sensitive methodology was applied for long-term dose-controlled aerosolisation of fiber-based nanomaterials directly at the cells surface resembling the physiological situation in the lungs as realistic as possible. Despite the limitations of the *in vitro* model and although it is impossible to fully represent the complexity of the human respiratory system, it contains important features, that are valuable for inhalation safety assessment studies, sometimes even more than the whole animal experiments. In addition, by using a positive particle control *i.e.* DQ12, we have shown that the model is sensitive to an inflammatory stimulus and also reflects *in vivo* findings.

The crucial role of lung epithelium in initiating respiratory responses was further supported in a recent study, which demonstrated that carbon nanoparticle (NP)-induced inflammation is an epithelial-dependent process, with no macrophage involvement.[33] Indeed, it is well-accepted that the airway epithelium controls the recruitment and activation of immune cells and regulates inflammatory reactions through the release of a vast array of cytokines, that play a central role in the pathogenesis of most pulmonary diseases, including asthma.[34] Notably, an important defense mechanism of the bronchial epithelium is the secretion of mucus that potentially traps the majority of inhaled nanomaterials and microorganisms and eliminates them by the cooperative function of mucociliary clearance (MCC) and cilia beating.[35] In order to simulate *in vivo* lung conditions in the present study, fully differentiated primary bronchial epithelium derived from healthy (no reported lung pathology) or asthmatic donors were used. This cellular model consists of a pseudo-stratified structure of basal, ciliated and mucus-producing cells, therefore possessing an active protective MCC mechanism. In addition, it allows long-term repeated dose toxicity testing.

One of the advantages of human primary cell cultures is that they retain the enzymatic, protein and signaling pathways of the native tissue.[36] Indeed, recent studies provided evidence that reconstituted asthmatic bronchial epithelial cells maintain in culture their intrinsic/epigenetic, if not genetic characteristics, such as the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) activity.[37,38] The production of GM-CSF, a key cytokine involved in asthma pathogenesis is much higher in bronchial epithelial cells derived from biopsies of asthmatic people compared to healthy control tissues.[37] GM-CSF release therefore was assessed to confirm that the employed asthma tissues retain their asthmatic phenotype. As shown in SI Figure 2, asthmatic cultures showed significant GM-CSF release

compared to healthy tissues, thus highlighting their ability to maintain their asthmatic phenotype within the experimental approach.

It should be noted that the use of primary cells from different donors inevitably increased the variation in biological responses across four independent replicates. The standard deviation could not be reduced by increasing the number of experimental repetitions as the variation of four experiments was similar to the n of three, which confirms that the reported variability is most likely an intrinsic characteristic of the obtained data set and not due to a small sample size. However, and importantly, such donor-dependent variation is highly reflective of the heterogeneity in the human population and therefore can be considered to be indicative of a real-world situation.[39]

Conventional light microscopy images of reconstituted human bronchial epithelial cells upon exposure to MWCNTs revealed no signs of modification in cellular morphology, although increased MWCNT accumulation was observed on the cell surface over weeks of exposure (Figure 3a-b, SI Figure3). Furthermore, TEM micrographs of the bronchial epithelial tissue revealed that MWCNTs were internalised by both healthy and asthmatic cultures. In particular, MWCNTs were observed within vesicular structures either as single tubes or as small agglomerates (Figure 3a´-b´).

Importantly, following five weeks of repeated MWCNT exposures, the epithelial cell layer structure was maintained, without ruptures or signs of apoptosis (*i.e.* fragmented cell nuclei or cellular blebbing). Moreover, no observed modifications in tight junctions or cilia formation were noted compared to negative control cultures over the analysed period.

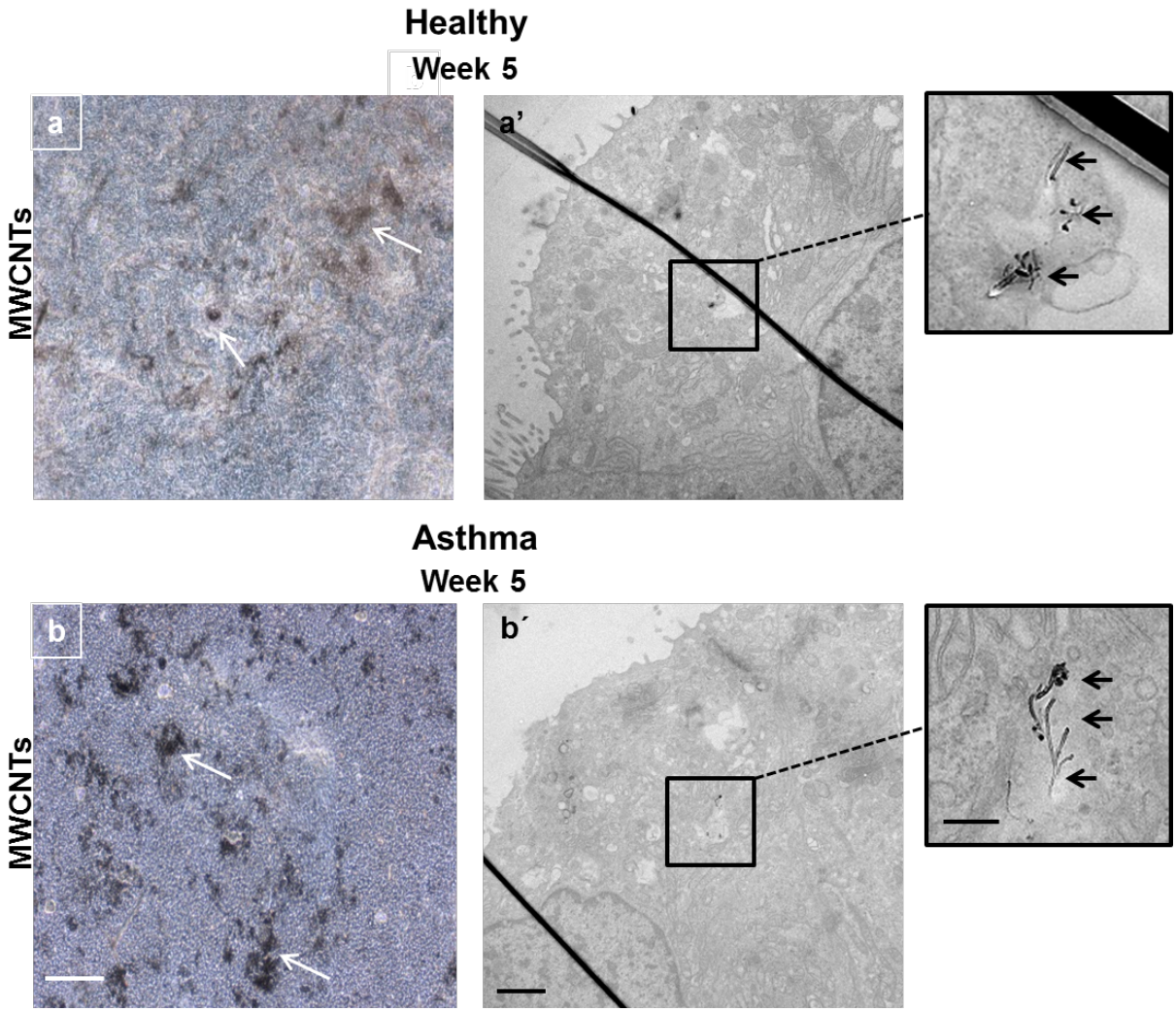

Figure 3: Morphological assessment of healthy and asthma cultures and MWCNT uptake after sub-chronic repeated exposure. Light microscopy images of healthy **(a)** and asthmatic **(b)** cultures at w5. White arrows indicate MWCNTs accumulated in the cells surface (scale bars: 100µm). TEM micrographs of internalised MWCNTs in healthy **(a´)** and asthmatic **(b´)** bronchial epithelial cells at w5 (scale bars: 2µm at lower magnification images and 500nm at higher magnification images). Black arrows indicate the position of MWCNTs.

No alterations to the cytoskeleton and nuclei were evident in Laser Scanning Microscopy (LSM) images in either healthy or asthmatic cultures following repeated exposures to MWCNTs for up to five weeks when compared to control cultures (Figure 4a, SI Figure 4).
No induction of cytotoxic reactions was reported for either healthy or asthmatic cells after MWCNT exposures at w1 and w3 (Figure 4b), supporting the microscopy data. However, a slight but insignificant increase (p=0.07) in lactate dehydrogenase (LDH) release was

observed in MWCNT-exposed asthmatic cells at w5. These findings support those recently reported in our previous study, where the effects of the same MWCNT type were investigated, upon short-term aerosol exposure into a 3D *in vitro* model of the human epithelial tissue barrier (A549 alveolar type II epithelial cells combined with two immune cell types).[25] Thurnherr *et al.* compared the long-term effects of exposure to MWCNT suspensions to their acute effects, using the A549 cell line, demonstrating that long-term accumulation of CNTs did not influence either cell viability or morphology.[13] In line with our findings, Ryman-Rasmussen *et al.* observed no changes in LDH release in both healthy and allergic (ovalbumin (OVA)-sensitized) asthmatic mice following a 6h MWCNT inhalation, although increased inflammation and fibrosis was identified.[40] No significant cytotoxicity was observed for cells exposed to aerosolised Pluronic or DQ12 at any of the tested time-points.

The ciliary mechanism of the bronchial epithelium following five weeks of repeated exposures was assessed by MCC and cilia beating frequency (CBF) analysis. The velocity of MCC in MWCNT-exposed healthy cells at w5 did not show any difference to those of the negative controls. Nevertheless, a significant increase ($p<0.05$) was measured in MWCNT-treated asthma cultures at the same time-point (Figure 4c). In addition, long-term exposure to MWCNTs resulted in significant CBF increase in both healthy and asthmatic cells, when compared to control cultures (Figure 4d). Alterations of this self-defense mechanism can contribute to the pathogenesis of serious lung diseases *e.g.* cystic fibrosis and primary ciliary dyskinesia.[41] A number of studies also hypothesized that different stress factors or physicochemical properties of NPs can result in increased CBF and MCC.[42-44] Of note, comparing the impact of a five week MWCNT exposure in MCC and CBF activity between asthma and healthy cultures, no significant effect was shown for MCC. CBF however, was found significantly elevated in asthma tissues, giving a first indication of the potential adverse effects of chronic MWCNT exposure.

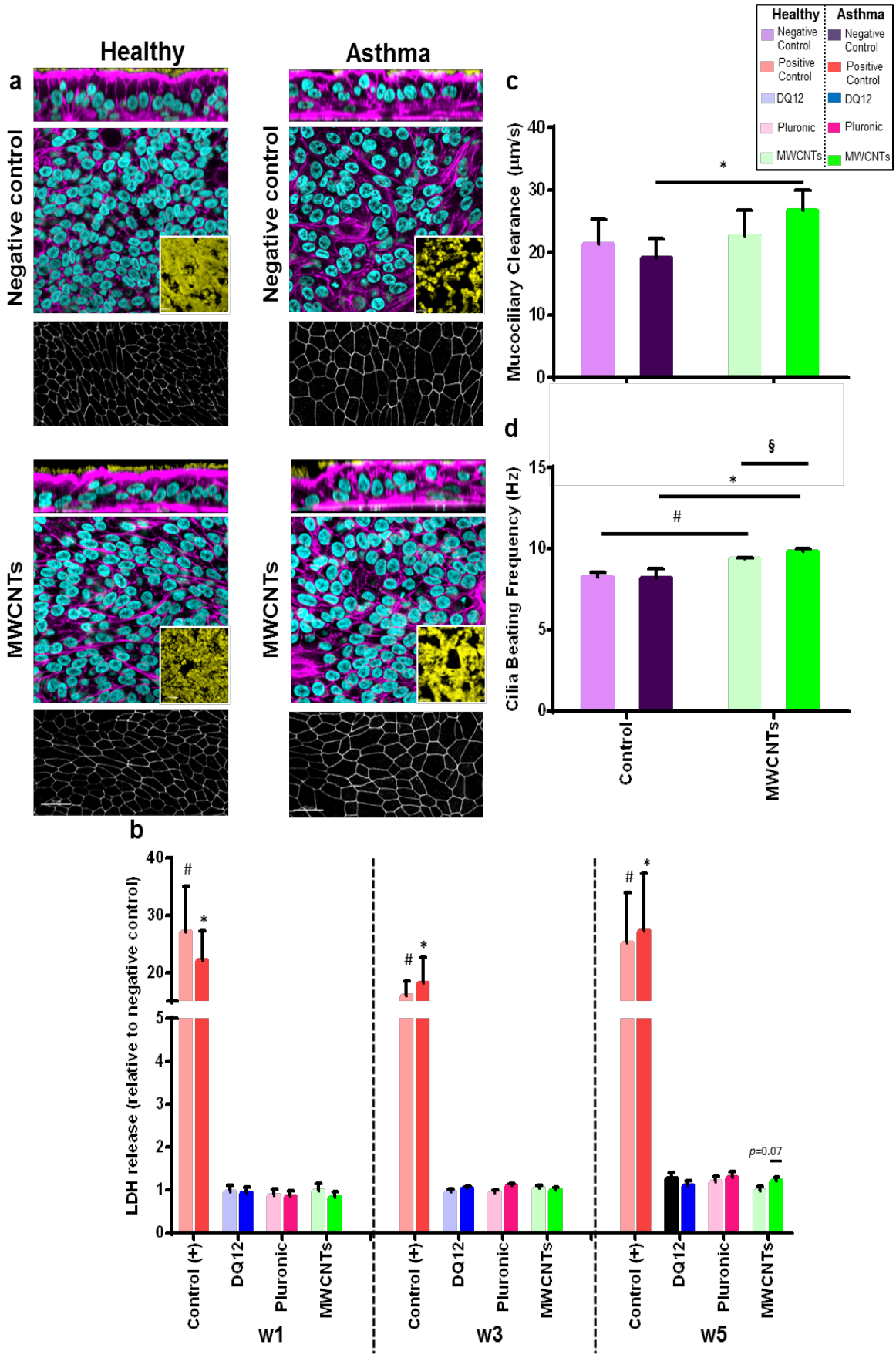
a
Healthy
Asthma
Negative control
MWCNTs
c
Healthy
Asthma
Negative Control
Positive Control
DQ12
Pluronic
MWCNTs
Mucociliary Clearance (µm/s)
d
Cilia Beating Frequency (Hz)
Control
MWCNTs
b
LDH release (relative to negative control)
Control (+)
DQ12
Pluronic
MWCNTs
W1
W3
W5
p=0.07

Figure 4: Cellular morphology, cytotoxicity and cellular functionality following sub-chronic repeated MWCNT exposure in healthy and asthma cells. (**a**) Confocal LSM images of healthy and asthmatic human bronchial epithelial cells exposed repeatedly for five weeks to aerosolised MWCNTs (scale bars: 20μm). Magenta color shows F-actin (cytoskeleton), blue color shows DNA (cell nuclei), yellow represents cilia and white the tight junctions. (**b**) Cytotoxicity as estimated by quantification of LDH release in cell culture medium after repeated MWCNT administration for up to five weeks (data shown relative to negative controls, n=4). 0.2% Triton X-100 was used as positive control. (**c**) Velocity of mucociliary clearance and (**d**) cilia beating frequency measurements in healthy and asthmatic cells at w5 (n=3). Values were considered significant if $p<0.05$. Data are presented as the mean ± standard error of the mean (SEM). # represents a significant increase ($p < 0.05$) in healthy cultures compared to the negative control. * indicates statistical significance in asthmatic cells compared to the negative control ($p < 0.05$). § shows a statistical significant response ($p < 0.05$) of asthma cultures, when compared to healthy cultures at w5.

Cell responses

In order to test the applicability of the presented *in vitro* lung system, the repeated administration of aerosolised DQ12 in human bronchial epithelial cells was assessed, since we have shown previously that this material can induce (pro-)inflammatory reactions in a 3D human epithelial tissue model upon acute and short-term exposures.[25,26] Repeated exposure to DQ12 caused a pronounced increase in *HMOX-1* gene levels (Figure 5a) at all tested time-points for both culture types, with the highest activity being observed at week 3. In addition, the *SOD-2* gene was highly expressed in DQ12-exposed healthy cells at all time-points (Figure 5b), while only a moderate increase was detected in asthmatic cultures. Importantly, induction of (pro-)inflammatory response was demonstrated in both healthy and asthma cultures. In healthy cells, all tested (pro-)inflammatory markers were increased even after week 1. Chronic repeated exposure to DQ12 for five weeks elicited a pronounced secretion of all tested cytokines for both healthy and asthma cultures when compared to control cultures. In particular, asthmatic cells revealed a significant response ($p<0.05$) in TGF-β compared to negative control (SI Figure 5d). Multiple animal studies confirmed the inflammatory properties of DQ12.[45] Indeed, DQ12-exposed animals have been observed to express severe inflammatory effects following either 14d or 90d of exposure respectively.[46,47] Therefore, the ability of DQ12 to trigger (pro-)inflammatory reactions at both gene and protein level

highlights the effectiveness of the employed *in vitro* chronic strategy. Notably, the highest cytokine expression in DQ12-exposed cells was observed at w3 followed by a decrease over the last week, indicating a possible cell threshold or recovery from the repetitive challenge with DQ12. This finding is supported by a recent *in vivo* study where intratracheal administration of DQ12 in mice lead to increased acute inflammation but limited sub-chronic inflammation following three months of exposure.[48]

In vitro studies have reported oxidative stress in human lung epithelial cells upon exposure to MWCNTs.[13, 49] Therefore, the possible induction of oxidative-stress-related biomarkers was examined in chronically MWCNT-exposed cultures. In fact, an increase in *HMOX-1* expression levels was detected over the weeks of MWCNT exposure at all tested time-points in healthy as well as in asthmatic cells, compared to the negative control (Figure 5a). At w1 and w5 a significant *HMOX-1* gene expression increase in asthmatic cells (18.14±10.9-fold and 22.6±10.8-fold respectively) was observed, which was not only significant compared to those of control cells, but also significantly higher than for MWCNT-exposed healthy cultures. Focusing on *SOD-2*, repeated healthy tissue exposure to MWCNTs did not change the expression profile of this oxidative stress marker at w1. However, a slight increase (3.73±2.2) was observed at w3, followed by a strong increase (79.2±45.1, p=0.07) at w5 although not statistically significant (Figure 5b). Moreover, higher *SOD-2* response was obtained at all tested time points in exposed asthmatic cultures, with the highest activity reported at w5 (104.7±74.6, significant p<0.05). As shown for the *HMOX-1* gene, diseased cells showed stronger *SOD-2* effects compared to healthy cultures at w5. Overall, long-term MWCNT exposure resulted in an augmented oxidative stress response which was considerably stronger during the final week of exposure in both normal and diseased cultures. Similarly, a notable decrease in intracellular glutathione levels (GSH) was observed during our earlier study after short-term repeated exposure to the same material.[25] In particular, oxidative stress has been proposed as the possible underlying mechanism by which CNTs exacerbate asthma in animal studies.[22,23, 50] Indeed, the observed oxidative stress response was more pronounced in asthmatic tissues, thus further supporting the *in vivo* observations.

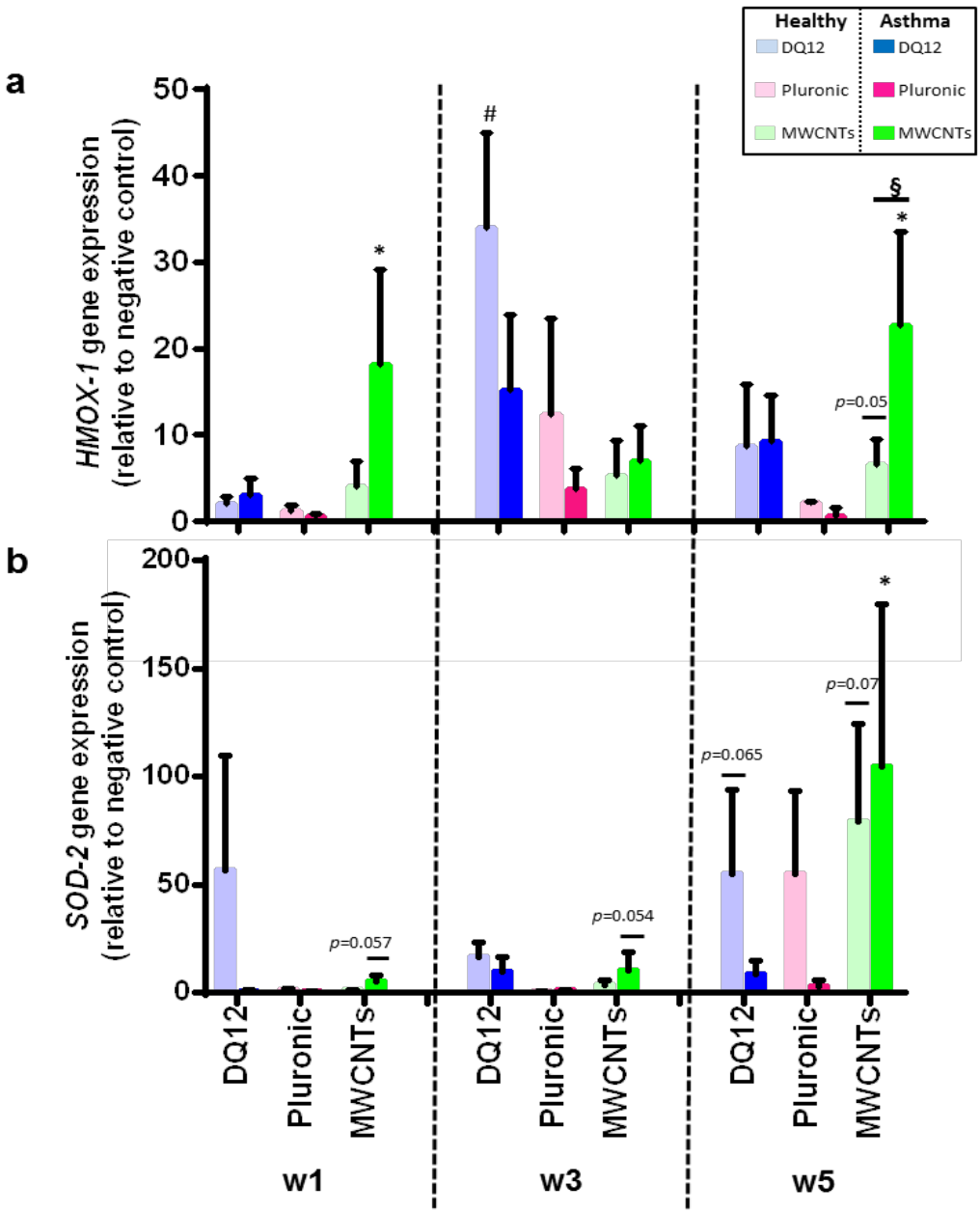

Figure 5: Cellular response to oxidative stress upon long-term exposure to MWCNTs. Real-time RT-PCR on (**a**) *HMOX*-1 and (**b**) *SOD-2* gene expression levels (n=4). Values were considered significant if $p<0.05$. Data are presented as the mean ± standard error of the mean (SEM). # represents a significant increase ($p < 0.05$) in healthy cultures compared to the negative control. * indicates statistical significance in asthmatic cells compared to the negative control ($p < 0.05$). § shows a statistical significant response ($p < 0.05$) of asthma cultures, when compared to healthy cultures at w5.

Analysis of -the *IL-8* expression, a critical airway epithelial-derived (pro-)inflammatory chemokine primarily implicated in acute inflammation and accumulation of neutrophils in

inflammatory diseases[51], revealed an increase in both healthy and asthmatic MWCNT-exposed cells (Figure 6a). In particular, after the final experimental week a notable increase was shown for healthy tissues (9.2±1.99; p=0.06), while asthma cultures reported the highest *IL-8* activity (26.4±7.9). The results obtained for the protein level reflect a similar pattern. Healthy as well as asthmatic cells exposed repeatedly to MWCNTs showed an increase in secreted IL-8 at all exposure times, although not significant compared to control cultures (SI Figure 5a). IL-6 is considered a key inflammatory marker (in both acute and chronic inflammation) with a potentially pivotal role in the pathogenesis and exacerbation of several lung diseases and in particular in asthma.[52] In fact, recent findings have shown a correlation between IL-6 and human asthma, indicating an active role of IL-6 in the asthmatic altered bronchial lung function.[53] An evident *IL-6* gene induction, as well as release of the protein, was measured after five weeks of exposure in both healthy and asthmatic epithelium, although for both cell types this effect was not statistically significant (p<0.05) (Figure 6b, SI Figure 5b). Similar observations were obtained for *IP-10* and *TGF-β* gene expression after repeated MWCNT exposure. At w5, healthy and asthmatic cultures reported significant expression levels for *IP-10* as well as pronounced effects for *TGF-β*. Of interest, increased IP10 production has been associated with airway inflammation and hyper-responsiveness, resulting in asthma-related effects in animal studies.[54] In addition, recent evidence have shown that enhanced TGF-β activity may contribute markedly to airway epithelial tissue damage, mucus hyper-secretion and airway remodeling, all key characteristics of a diseased *i.e.* asthma lung phenotype.[55,56] Protein levels for both cytokines showed an elevated, but insignificant release at the tested time-points in comparison to the negative controls, independent of the culture type. Although the cytokine and chemokine protein levels reflect a similar trend with the gene expression analysis, the increased observed transcriptional gene activity resulted in a lower cytokine secretion which could potentially be explained by the time-frame required between the gene transcription and the proteins synthesis/secretion, by post-transcriptional regulations or by possible protein degradation.

Regarding the potential impact of the dispersant in the cellular system, it is worth noting that although some effects were observed in cultures exposed to Pluronic F127, *i.e.* elevated expression of oxidative stress markers as well as induction of (pro-)inflammatory cytokines, the response was not as strong as in MWCNT-exposed cells, indicating that the effects observed following MWCNT exposure are mainly relevant to the MWCNTs themselves and not due to the surfactant coating.

Shvedova *et al.* reported significant changes in the expression profiles of crucial biomarkers (including elevated *IL-6* and *TGF-β*) associated with pulmonary and cardiovascular risk in blood samples from exposed workers in a MWCNT production facility.[5] The (pro-)inflammatory potential of long-term MWCNT exposure was assessed by evaluating the activity of classical toxicological indicators (IL-8, IL-6, IP-10 and TGF-β) that are well-known for their ability to initiate (pro-)inflammatory and (pro-)fibrotic responses and further activate other involved (pro-)inflammatory mediators.[32, 52] Repeated occupationally relevant MWCNT exposure elicited an enhanced, sustained and concentration-dependent (pro-)inflammatory reaction in both healthy and asthmatic exposed cultures, with the highest activity observed in the final week. Acute *in vitro* deposition of MWCNTs onto the MucilAir bronchial epithelial tissue resulted in similar observations, such as induction of various (pro-)inflammatory cytokines and ROS production, with absence of cytotoxic effects.[57] Increased *IL-6* expression was also demonstrated in mice repeatedly exposed to MWCNTs at doses relevant to 7.6 and 76 years of occupational exposure.[3] In accordance with our findings, induction of IL-6, TGF-β and IFN-γ (activator of IP-10) was reported in healthy and OVA-sensitized asthmatic mice upon exposure to single-walled CNTs (SWCNTs) and MWCNTs.[22, 40, 50]

By assessing pivotal (pro)-inflammatory parameters, it has been possible to gain a clear indication of the inflammatory potential of MWCNTs in a more realistic and advanced *in vitro* cellular and exposure system, under the presented chronic occupational test conditions. The substantial and duration dependent (pro-)inflammatory response can be the result of ongoing inflammation in the tissues.

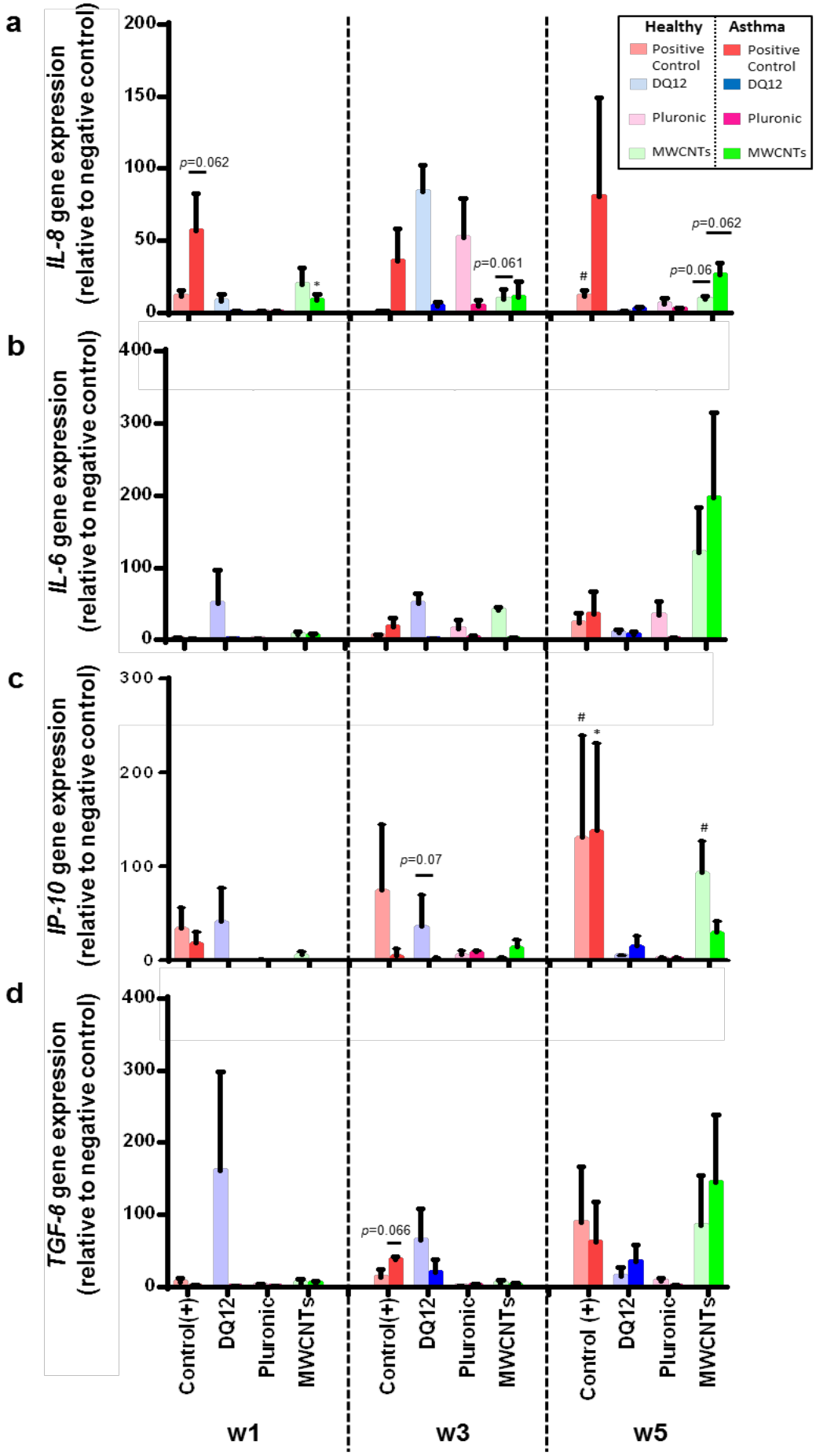
a
b
c
d
IL-8 gene expression (relative to negative control)
IL-6 gene expression (relative to negative control)
IP-10 gene expression (relative to negative control)
TGF-β gene expression (relative to negative control)
Healthy
Positive Control
DQ12
Pluronic
MWCNTs
Asthma
Positive Control
DQ12
Pluronic
MWCNTs
p=0.062
p=0.061
p=0.06
p=0.062
p=0.07
p=0.066
Control(+)
DQ12
Pluronic
MWCNTs
Control (+)
w1
w3
w5

Figure 6: (Pro-)inflammatory response in cells exposed repeatedly to MWCNTs for up to five weeks. Gene expression levels of (**a**) *IL-8*, (**b**) *IL-6* (**c**) *IP-10* and (**d**) *TGF-β* at w1, w3 and w5 time-points (n=4). TNF-α (1μg/ml) was used as positive control for *IL-8* and *IL-6* induction, and IFN-γ (1μg/ml) for *IP-10* and *TGF-β*, respectively. Values were considered significant if $p<0.05$. Data are presented as the mean ± standard error of the mean (SEM). # represents a significant increase ($p < 0.05$) in healthy cultures compared to the negative control.* indicates statistical significance in asthmatic cells compared to the negative control ($p < 0.05$).

The effects of multiple functional, oxidative stress and (pro-)inflammatory parameters from asthma patients was shown to be greater than the effects on non-asthmatic. Given the importance of the assessed biological markers in the pathogenesis and exacerbation of asthma, our findings suggest a possible predisposition of asthmatic individuals to be more sensitive to the effects of chronic occupational MWCNT exposure. The exact mechanisms underlying the MWCNTs-induced chronic effects on the function and expression of mediators of reconstituted primary bronchial epithelium on asthmatic and non-asthmatic subjects however are not entirely clear.

It is worth noting that experimental animals do not naturally develop asthma, therefore the disease has to be artificially induced by an antigen/allergen challenge, which sometimes does not portray the truest of pictures regarding human lung disease.[39] Thus, an additional advantage of our alternative strategy is the use of human cells derived from asthmatic individuals.

Overall, considering that the mechanisms of asthma are complex and that various factors are involved in the development and provocation of the disease, the findings provide a valuable indication that asthma cultures are probably more susceptible to MWCNTs in a long-term scenario. Taken together the observed findings, results revealed potential health risks associated with human occupational lifetime exposure to inhaled MWCNTs.

CONCLUSIONS

Due to the inevitable human exposure to aerosolised CNTs, mainly during their production and handling, it is imperative to investigate potential adverse effects following long-term, repeated occupational exposures. In the present study we have proven the reliability of an air-liquid exposure system for aerosol exposure onto normal and diseased human primary cells, providing an effective platform to investigate possible risks after chronic nanofibre exposure, therefore presenting a more realistic and valid alternative to mimic the chronic inhalatory hazard of nanomaterials *in vitro*. Chronic MWCNT exposure elicited a duration-dependent (pro-)inflammatory and oxidative stress response as well as a significant alteration of the mucociliary clearance mechanism in both healthy and asthmatic cultures, under the presented experimental conditions. The latter revealing stronger and more durable long-term effects compared to healthy cells, indicating that individuals with asthma may be more prone to adverse effects from MWCNT exposure compared to non-asthmatic populations. In conclusion, the present study clearly indicates that there is an urgent need to develop more reliable and relevant predictive *in vitro* systems to assess adverse effects of sub-chronic exposed NMs in healthy and susceptible persons.

MATERIALS AND METHODS

Chemical and Reagents

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

Human Reconstituted Bronchial Epithelium

Fully differentiated primary human bronchial epithelial cells (MucilAir^{TM}) obtained from bronchial biopsies from non-smoking, healthy (without respiratory pathologies) and asthmatic donors were purchased from Epithelix (Epithelix Sàrl, Geneva, Switzerland) and cultured according to supplier's instructions. Briefly, cells were cultured at the ALI, in 24-well Transwell® inserts (6.5mm diameter, 0.4 µm pore size, Corning Incorporated, Massachusetts, USA) with MucilAir serum-free culture medium (0.7ml) (Epithelix Sàrl, Geneva, Switzerland; supplemented with 5µg/ml Posaconazole (Sigma-Aldrich, Switzerland)), on the basal side. Cell cultures were maintained at 37ºC, 5% CO_2 for a period up to six weeks (*i.e.* one week prior to experimentation; up to five weeks of exposure). Cell culture media was changed every 2-3d. The apical side was washed with MucilAir medium at the end of each week, to remove mucus, surface dead cells and non-internalised MWCNTs, as recommended

by the supplier. The tissue isolation was conducted according to the declaration of Helsinki on biomedical research (Hong Kong amendment, 1989), and received approval from local ethics commission.

Characterisation of asthmatic cultures

In order to demonstrate that asthma tissues preserve the asthmatic phenotype the release of GM-CSF was assessed in both negative control healthy and asthmatic tissues at w1, using the commercially available DuoSet ELISA Development Kit (R&D Systems, Switzerland) and following the supplier's protocol.

MWCNTs and positive controls

In order to obtain a well-dispersed and stable suspension, MWCNTs (Cheaptubes Inc., USA) were dispersed in Pluronic F127 (160ppm) as previously described.[27] MWCNT stock suspension was thoroughly characterised by Thurnherr *et al.* and Clift *et al.* in terms of morphology, length, diameter, metal impurities (%wt) and endotoxin level.[27, 30] The size distribution (length and width) of these MWCNTs following aerosolisation was reported previously.[25]

To monitor the biological effects of the dispersant, cell cultures exposed to aerosolised Pluronic F127 (at 160ppm) were tested with all biochemical endpoints used. In addition, aerosolised crystalline Dörentruper Quartz (DQ12; ≤ 5 µm[58], at 0.2µg/cm²) was employed as a positive (pro-)inflammatory particle control.[46]

Air-Liquid Interface Cell Exposure System (ALICE)

Aerosolisation of MWCNTs was performed using the ALICE system[21], as reported by Chortarea *et al*.[25] Briefly, the exposure system consists of a nebuliser, an exposure and an incubation chamber (connected to an air-flow system that provides optimum humidity and temperature conditions required for cell cultivation) as well as a QCM (operated at 5 MHz, detection limit: 0.09µg/cm^2, Stanford Research Systems, GMP SA, Renens, Switzerland) for online measurements of the deposited dose. For each aerosolisation, 1ml of well-dispersed MWCNT suspension (250µg/ml) with 500µM NaCl (NAAPREP® physiological saline, GlaxoSmithKline, France) was added to the nebuliser (customized eFlow nebuliser system, PARI Pharma GmbH, Germany). The vibrating perforated membrane of the nebulizer then generates the aerosol, which is transported into the exposure chamber. Inside the chamber, it gently deposits the aerosolised MWCNT suspension onto cells that are maintained at the ALI.

The selected flow rate (5L/min) is ideal for the aerosol to sufficiently mix to all sides of the chamber, hence resulting in uniform droplet deposition.

Exposure experiments

To address the potential biological impact of a lifetime of occupational MWCNT exposure on healthy and asthmatic cell cultures, cells were exposed for up to five weeks, five days per week (from Monday to Friday) to aerosolised MWCNTs using the ALICE system. The cellular response following MWCNT exposure was analyzed at three defined time-points; after w1, w3 and w5 (Figure 1).

Characterisation of MWCNT deposition

Material deposition was quantified by the incorporated QCM. Briefly, the deposited MWCNT dose was determined by the linear decrease in the resonance frequency of the vibrating piezoelectric crystal, due to increasing deposited mass. The differences in the frequency of the QCM, prior to and after aerosolisation were recorded and subsequently calculated to determine the mass per surface area (µg/cm²), as previously reported by Lenz *et al*.[21]

To examine the morphology of the deposited MWCNT aerosols, TEM copper grids or SEM holders were exposed to aerosolised particles using the ALICE system. Representative images of deposited MWCNTs after a single exposure (d1) and five exposures (w1) were captured using TEM (Fei Technai Spirit (Oregon, USA)), operating at 120kV. Images were recorded with a Veleta CCD camera (Olympus, Japan). SEM samples were coated with a 4nm gold layer to improve electrical conductivity. Images were taken with a Tescan Mira3 LM FE (Czech Republic) at 4kV, working distance of 6mm and secondary electron mode.

Interaction of MWCNTs with human bronchial epithelial cells

The MWCNT-lung cell interaction following repeated exposures at w1, w3 and w5, was investigated by TEM. Sample preparation was performed as previously described.[59] Briefly, the exposed cells were fixed with 2.5% glutaraldehyde in HEPES buffer, then post-fixed with 1% osmium tetroxide and stained with 0.5% uranyl acetate. Following dehydration in a graded ethanol series, samples were embedded in epon. Embedded cells were cut into ultrathin sections (50-80nm), mounted on copper grids and finally stained with uranyl acetate and lead citrate. Images were recorded using TEM (Fei Technai Spirit (Oregon, USA)), operating at 120 kV with a Veleta CCD camera (Olympus, Japan).

Cytotoxicity

As an indicator of cell membrane damage[60], the release of LDH into the supernatant was assessed using a LDH cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's protocol. Each sample was tested in triplicate on four independent occasions (n=4) and evaluated against the negative control. Quantification of LDH activity was performed photometrically by measuring at 490nm (reference wavelength at 630nm). For positive controls, cell cultures were exposed apically to 0.2% Triton X-100 in PBS for 24h.

Cell morphology

Cell cultures were fixed for 15min in 3% paraformaldehyde (PFA) at room temperature and treated with 0.1 M glycine in PBS for another 15min. Subsequently, fixed cells were permeabilised in 0.2% Triton X-100 in PBS for 15min at room temperature. Antibodies were diluted in 0.3% Triton X-100 and 1% BSA in PBS. Mouse anti-alpha tubulin (Sigma-Aldrich, Switzerland; 1:100 dilution) was used to stain the epithelial cilia and rabbit anti-zonula occludins (ZO)-3 (Sigma-Aldrich, Switzerland; 1:50 dilution) for staining the tight junctions. Secondary antibodies were goat anti-mouse Alexa 488 (Abcam, Cambridge, UK; 1:200 dilution) and goat anti-rabbit Dylight 650 (Millipore, Darmstadt, Germany; 1:200 dilution) respectively. F-actin cytoskeleton was labelled with Phalloidin rhodamine (R-415; Molecular Probes, Life Technologies Europe B.V., Zug, Switzerland; 1:50 dilution, whilst the nucleus was stained with DAPI (1µg/ml; Sigma Aldrich, Switzerland). Samples were incubated with primary and then secondary antibodies for 2h at room temperature. Following incubation, the samples were embedded in Glycergel (DAKO Schweiz AG, Baar, Switzerland). Cell visualisation was performed using an inverted LSM 710 (Axio Observer.Z1, Carl Zeiss, Germany). Image processing was achieved by using the 3D restoration software IMARIS (Bitplane AG, Zurich, Switzerland). Additionally, the morphology of the bronchial epithelium was also observed using a conventional light microscope (AE200, Motic, Switzerland) containing a digital camera (Nikon, Switzerland).

Cilia beating frequency (CBF)

Cell cultures (at the w5 time point only) were visualized using an Axiovert 200M microscope (Carl Zeiss, Germany) connected to a Sony XCD V60 Firewire camera. A total 256 images of the cultures were recorded per insert and CBF was then calculated using Cilia FA software as

described by Smith *et al*.[61] Each condition was tested in triplicate on four independent occasions (n=4).

Mucociliary Clearance (MCC)

For the MCC measurements, 30µm polysterene microbeads (Sigma-Aldrich, Switzerland) were added to the apical surface of the epithelial cells (w5). Cells were monitored using a high speed acquisition camera (Sony) connected to an Axiovert 200M microscope (Carl Zeiss, Germany). The movement of the micron-sized beads were captured on 60-second movies (4 movies per insert). Tracking analysis was performed using imaging software Image Pro Plus (Mediacy, Rockville, USA) in order to calculate the velocity of the bead clearance. An average number of 300 beads were tracked per insert. Samples were tested in triplicate.

Gene expression analysis of oxidative stress and (pro-)inflammatory markers

Following w1, w3 and w5, the insert membranes were transferred into RNA-Protect cell reagent (Qiagen AG, Hombrechtikon, Switzerland) and stored at 4°C until further processing. RNA was isolated using the RNeasy plus kit (Qiagen) according to manufacturer's manual and RNA concentration was determined by a NanoDrop 2000 (Thermo Scientific, Witec AG, Littau, Switzerland). The reverse transcriptase reactions were performed with the Omniscript RT system and Oligo dT primers (Qiagen) as described by Bisig *et al*.[62] Real-time PCR was carried out using the 7500 Fast Real-Time PCR system, (Applied Biosystems, Life Technologies Europe B.V., Zug, Switzerland) using Fast SYBR Green Master Mix (Applied Biosystems) as a reporter dye. The relative expression levels were subsequently calculated using the $^{\Delta\Delta}$Ct method as described by Schmittgen *et al*.[63] Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* acted as the internal standard gene. Oxidative stress markers *HMOX-1* and *SOD-2* were assessed for oxidative stress. To examine (pro-)inflammatory responses, the *IL-8*, *IL-6*, *IP-10* and *TGF-β* genes were evaluated. The primer sequences for all tested genes can be found in supporting information (Supplementary Table 2).

(Pro-)inflammatory cytokine secretion

The (pro-)inflammatory response was also investigated by quantifying the amount of the (pro-)inflammatory mediators IL-8, IL-6, IP-10 and TGF-β by using the commercially available DuoSet ELISA Development Kit (R&D Systems, Switzerland) according to the supplier's manual. Cell cultures treated apically with Tumor Necrosis Factor-α (TNF-α; 1µg/ml;

Immunotools, Germany) and Interferon-γ (IFN-γ; 1µg/ml; Immunotools, Germany) for 24h served as positive controls for the induction of a (pro-)inflammatory response.

Statistical analysis

All data are presented as the mean±standard error of the mean (SEM). Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, USA). An independent two-sided Student's *t* test was performed. Values were considered significant if $p<0.05$. All endpoints were evaluated at four different repetitions (n=4), except MCC and CBF measurements (n=3).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

SEM images, additional phase-contrast, LSM images and ELISA results (SI Figures 1-5). Detailed physicochemical characteristics of MWCNTs; list of gene sequences and summary of biological effects in cell cultures (SI Tables 1-3).

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AUTHOR CONTRIBUTION

S.C. performed all experiments. H.B. performed the SEM images and assisted in experimental work. B.R.R. designed and supervised the project. All authors contributed to the design of the study, the discussions and manuscript preparation.

DECLARATION OF INTEREST

The authors would like to express no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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