The impact of multi-walled carbon nanotubes with different amount of metallic impurities on immunometabolic parameters in healthy volunteers


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Abstract

The impact of two types of multi-walled carbon nanotubes (MWCNTs) (12–14 nm) with different content of metallic impurities (purified and unpurified nanotubes) on peroxidation processes, the status of immune cells in healthy volunteers and gene expression combined to pathway analysis was studied in vitro. From the study it was shown that the main mechanism of action for both types of MWCNTs is induction of oxidative stress, the intensity of which is directly related to the amount of metallic impurities. Unpurified MWCNTs produced twice as high levels of oxidation than the purified CNTs inducing thus more intense mitochondrial dysfunction. All the above were also verified by gene expression analysis of different human cellular cultures (lung epithelium and keratinoma cells) and the respective pathway analysis; modulation of genes activating the NFkB pathway is associated to inflammatory responses. This may cause a perturbation in the IL-6 signaling pathway in order to regulate inflammatory processes and compensate for apoptotic changes. A plausible hypothesis for the immunological effects observed in vivo, are considered as the result of the synergistic effect of systemic (mediated by cells of the routes of exposure) and local inflammation (blood cells).

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1. Introduction

The evidence accumulated to date suggests that there are health risks associated with occupational exposure to carbon nanoparticles (Shvedova et al., 2005; Poland et al., 2008). Recent studies show that micro- and nano-sized substances may have significant pathological effects on humans (Lim et al., 2012; Golokhvast et al., 2015a,b). The toxic effects of multi-walled carbon nanotubes (MWCNTs) have been studied for several years and adverse responses have been identified in several tissues, including cytotoxicity in keratinocyte cells (Shvedova et al., 2005) and inflammatory and fibrogenic responses in pulmonary tissues (Shvedova et al., 2005). It has been also found that MWCNTs produce a time- and dose-dependent toxic response upon reaching the lungs in sufficient quantity (Helland et al., 2007). Beyond time and dose, chemical functionalization affecting water solubility, dispersibility and agglomeration tendency (Coccini et al., 2010), as well as impurities, amorphous carbon, surface charge, shape, length, and layer numbers (Liu et al., 2013; Lamberti et al., 2015) have been recognized as significant modulators of toxicity. MWCNTs have unique physical and chemical properties (Peng et al., 2008; Dong
they are characterized by high specific surface area and penetration ability, as well as higher activity than micro-sized particles (Nel et al., 2006). The fibrous structure of carbon nanotubes determines their pathogenicity (Donaldson et al., 2013), including mechanical damage in cell membranes and other subcellular structural elements.

Recently the adverse effects of MWCNTs have been observed at the molecular lever through gene expression studies. Snyder-Talkington et al. (2015) compared global multi-walled carbon nanotube (MWCNT)-induced gene expression from human lung epithelial and microvascular endothelial cells in monoculture and co-culture with gene expression from mouse lungs exposed to MWCNT; they identified significant similarities in inflammatory responses between the *in vitro* and *in vivo* responses. Poulsen et al. (2015) identified that pulmonary response to intratracheal exposure to CNT with very different physicochemical properties induced remarkably similar effects on the transcriptome, especially on key processes such as inflammation and acute phase response.

The toxic effects of carbon nanotubes are viewed often as the result of an explosive activation of oxidative processes related to generation of reactive oxygen species (ROS) (Rodriguez-Yanez et al., 2013; Moller et al., 2014). Dong and Ma (2015) showed that exposure to MWCNTs leads to time-dependent intracellular uptake and generation of reactive oxygen species (ROS), while this potential was also highlighted by Funahashi et al. (2015). Thus, we surmise that biological effects of MWCNTs and their potentially harmful health implications should be studied in advance prior to widespread deployment in consumer goods and pharmaceuticals.

It is believed that an important role in the toxicity of MWCNTs is played by metallic impurities, (Kagan et al., 2006; Kuznetsov et al., 2010) which cannot always be removed completely by acids. Significant amounts of metal particles can be mobilized from the carbon nanotubes to the surrounding biological microenvironment. The final effect of this interaction will depend on the components and properties of the cellular and intracellular microenvironment and the physicochemical and toxicological characteristics of the metal particles.

This paper investigates the *in vitro* effects of two types of MWCNTs with different content of metallic impurities on peroxidation processes and on the status of immune cells in healthy volunteers. To better understand the mechanism of effect, modulation of gene expression in human cell lines (A549 human lung cells and HaCaT human keratinocytes) relevant to MWCNTs occupational exposure pathways (inhalation and skin exposure) were also investigated.

### 2. Materials and methods

#### 2.1. Study design

The study aimed at identifying the effects of exposure to purified and unpurified MWCNTs at different levels of biological organization, and at shedding light on the potential mechanism(s) of action. For this purpose, (a) immunological, (b) biochemical, (c) gene expression and (d) biological pathway analysis were carried out, focusing on the mechanisms through which MWCNT exposure induces immunological responses.

#### 2.2. The characteristics of nanotubes

In this work the effects of two types of MWCNTs on peroxidation processes and the status of immune cells in healthy volunteers exposed to a dose level of 1 mg/mL were studied *in vitro*. The MWCNTs studied were synthesized by chemical sedimentation from the gas phase on a catalyst surface (the active Fe–Co component an alloy) in a tubular reactor at 680 °C at Boreskov Institute Catalysis, Siberian Branch of the Russian Academy of Science (Kuznetsov et al., 2010, 2012).

Purification of MWCNTs from metallic impurities was performed by boiling concentrated hydrochloric acid (HCl) for 8 h. The characteristics of MWCNTs are given in Table 1.

Metallic impurities were determined by X-ray fluorescence spectroscopy (XRF). Analysis was performed using the X-ray fluorescence spectrometer ARL ADVANT’X 3600 (Thermo Scientific, USA) and the data analysis software OXAS V1.5.

Aggregates of nanotubes were photographed using the S-3400N scanning electron microscope (Hitachi, Tokyo, Japan). Deionized water was produced at SGWASSER Ultra Clear TWF/EL-I0N UV plus TM (Siemens, Germany).

#### 2.3. The characteristics of the study participants

The study involved 12 healthy volunteers with an age range from 25 to 30 years (mean age 27 ± 2.1). All volunteers were fully informed about the goals, objectives and procedures of the study; all of them signed an informed consent form and passed the preliminary clinical and laboratory tests. The criteria for exclusion from the study were: pregnancy, chronic disease (as stated in medical records or reported personally), acute illness and/or current drug treatment.

The study was approved by the Committee on Biomedical Ethics of Vladivostok Branch of Far Eastern Scientific Center of Physiology and Pathology of Respiration — Research Institute of Medical Climatology and Rehabilitation Treatment and carried out in accordance with the Helsinki Declaration, as revised in 2013.

Venous blood samples were collected in the early morning following overnight fasting in vacuum EDTA tubes (Vacutainer). The doses of MWCNTs were selected according to the recommended exposure level – 1 μg/m^3 (NIOSH, 2013). Load tests were carried out by adding MWCNTs to blood samples and careful mixing at 370 °C for 1 h via rotamix RM-1 (ELMI, Latvia).

#### 2.4. Cell cultures: A549 and keratinocytes (HaCaT) human cells

Cell culture reagents were obtained from Sigma–Aldrich (Milan, Italy). A549 cells from a human Caucasian lung adenocarcinoma with the alveolar type II phenotype were obtained from ECACC (Sigma–Aldrich, Milan, Italy). The cells were cultured in Dulbecco’s modified Eagle medium (DMEM), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere containing 5% CO2 at 37 °C and grown to 80% confluence. Exposure to purified and unpurified MWCNTs was done on sub-confluent cells. HaCaT cells were cultured in calcium-free DMEM (HyClone # SH3031901), with 10% chelexed FBS (GE MINI # 100–106), 4 mM L-Glutamine (HyClone # SH30343.01), and supplemented with calcium chloride at 0.03 mM or 2.8 mM final concentration. FBS was calcium-depleted by incubation with Chelex 100 resin (BioRad # 142–2832) for 1 h at 4 °C according to the BioRad protocol (Deyriex and Wilson, 2007). The Chelex was subsequently removed using a 50 mL Millipore 0.22 um filter unit system (Millipore # SCGP00525).

### Table 1

The characteristics of multi-walled carbon nanotubes tested in this study.

<table>
<thead>
<tr>
<th>Type</th>
<th>Size (nm)</th>
<th>Treatment</th>
<th>Content of metallic impurities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12–14</td>
<td>Purified</td>
<td>Fe 0.16; Co 0.07; Mg 0.05</td>
</tr>
<tr>
<td>2</td>
<td>12–14</td>
<td>Unpurified</td>
<td>Fe 2.4; Co 1.3; Mg 2.5</td>
</tr>
</tbody>
</table>
2.5. Immunological study

The immunological study included the evaluation of levels of CD3+CD95+, CD3+CD25+, CD3+CD126+ cells using the BD FACS CANTO II flow cytometer (BD Biosciences, USA). All reagents were purchased from BD Biosciences (USA). Mitochondrial membrane potential (MMP) was measured by the MitoProbe™ JC-1 Assay Kit (Life Technologies, USA) using the BD FACS CANTO II flow cytometer (BD Biosciences, USA). MMP was measured in leukocytes (MMPL) and mature CD3+ T lymphocytes (MMP CD3+). The percentage of cells with low MMP was reckoned on the basis of the measurement results.

2.6. Biochemical analyses

Biochemical analyses were used to assess the levels of primary products of lipid peroxidation (LPO) — lipid hydroperoxides (LHP) and the intermediate products of LPO — diene conjugates (DC), ketodienes (KD), conjugated trienes (CT), compounds containing isolated double bonds (CCIDB). The LPO products have different absorption maxima. The LPO products have different ketodienes (KD), conjugated trienes (CT), compounds containing isolated double bonds (CCIDB). The LPO products have different absorption maxima. The LPO products have different ketodienes (KD), conjugated trienes (CT), compounds containing isolated double bonds (CCIDB).

2.6.1. The assessment of LHP levels in blood plasma

The LHP levels in blood plasma were assessed according to Volchegorsky’s method (Volchegorsky et al., 1989). 6 mL of heptane/ isopropanol mixture (2:1, v/v) were added to 0.2 mL of plasma and then shaken vigorously for 15 min. Then 1 mL of HCI (0.02 M, pH = 2.0) was added, and shaking was repeated. After keeping the sample for 25 min at room temperature, the heptane layer was collected. Light extinction was measured at 233 nm on a BioTek PowerWave XS microplate spectrophotometer (BioTek Instruments, USA). Distilled water was used as control in all of the above processing steps. LHP levels were calculated as shown below:

\[ \text{LHP, arb. unit} = \Delta E_{233} \cdot \text{Vheptane/Vsample} = \Delta E_{233} \cdot 20, \]

where \( \Delta E_{233} = (E_{233} \text{ sample} - E_{233} \text{ control}) \), Vheptane — heptane volume (4 mL), Vsample — sample volume (0.2 mL).

2.6.2. The assessment of the levels of LPO intermediate products in blood

The levels of LPO intermediate products in blood were assessed according to Volchegorsky’s method (Volchegorsky et al., 1989). 3 mL of heptane/isopropanol mixture (1:1, v/v) were added to 0.3 mL of blood with EDTA (or 0.3 mL of 1% EDTA solution in saline as control) and then shaken vigorously for 15 min. The samples were centrifuged at 10,000 g for 10 min. 3 mL of extract were taken into new tubes, and 3 mL of heptane/isopropanol mixture (3:7, v/v) and 1.2 mL of HCl (0.02 M, pH = 2.0) were added. After keeping the sample for 10 min at room temperature, the heptane layer was collected. Light extinction was measured at 220, 232, 278 nm on a BioTek PowerWave XS microplate spectrophotometer (BioTek Instruments, USA). The water-isopropanol extract was dehydrated with 1 g of dry NaCl and water was removed. Light extinction was measured at the same lengths as for the heptane phase.

The LPO product levels were measured in absorbance units on 1 mL of blood and calculated as shown below:

\[ \text{C, absorbance units/mL} = \Delta E \cdot \text{Vphase/Vsample}, \]

where \( \Delta E \) — the difference between the extinctions of the tested and control samples for each wavelength, Vphase — phase volume: heptane layer — 2.4 mL; isopropanol extract — 3.6 mL; Vsample — sample volume (0.3 mL).

2.7. Gene expression analysis

Gene expression profiling offers considerable potential for identifying chemical causer of effects induced in exposures to xenobiotics, and for understanding the mechanistic basis for their phenotypic effects. It entails a series of experimental steps, including RNA extraction and preparation, microarray hybridisation, chemiluminescent signal detection, image acquisition and image analysis of the microarrays, and microarray data analysis (in our case we used the Applied Biosystems Expression System software to extract assay signal and signal to noise values from the microarray images) (Sarigiannis et al., 2009). Microarray data analysis identifies differentially expressed genes, applying different statistical filters to allow for only the statistically and biologically significant probes to be evaluated. The results of microarray analysis were validated using quantitative real-time polymerase chain reaction (PCR).

2.8. Statistical and pathway analysis

All analyses were done in triplicate. Data are presented as mean and 25%–75% interquartile range. Statistica 10.0 (Statsoft) software was used for the statistical analysis. The differences between the groups were analyzed using the Mann—Whitney test. A value of \( p < 0.05 \) was considered significant. Significant probe sets were evaluated for relevance to canonical pathway, molecular function and biological function using the Protein ANalysis Through Evolutionary Relationships (PANTHER) Classification System (http://www.pantherdb.org). Statistically significant over- and under-represented annotation categories were determined by binomial statistics, using the observed number of expressed genes versus the numbers expected by chance within a certain annotation group. Categories with p-values > 10—2 were rejected.

3. Results and discussion

3.1. MWCNT Morphological characteristics

Both types of MWCNTs in dry state were found in aggregate form, with size ranging from 20 to 200 μm (Fig. 1a) and high surface area (Fig. 1b).

The structure of MWCNTs changed in the liquid phase (deionized water). More precisely, the nanotube aggregates were segregated (Fig. 2a and b).

Figs. 1 and 2, as well as the results obtained previously (Golokhvast et al., 2015a,b), indicate that the characteristics of MWCNTs are significantly altered in the liquid phase. Fig. 2b shows that the hydration of MWCNTs is a key process changing the physico-chemical properties of MWCNTs; the latter determine, in turn, their biological effects.

3.2. Biochemical and immunological responses

Our study shows that the MWCNTs with lower content of metallic impurities (Type 1) affected lipid peroxidation parameters inducing pre-inflammatory effects (Table 2).

Oxidative disorders were manifested in the form of increase in the content of the primary products of lipid peroxidation (LPO) and
redistribution of the intermediate products (CCIDB, DC, KD + CT) in neutral lipids and phospholipids of blood cell membranes. Type 1 MWCNTs increased the levels of LHP in blood by 1.8 times (р < 0.05).

The load test by the MWCNTs with the higher amount of metallic impurities (Type 2) on the whole blood caused a significant increase in LHP levels as compared to the purified MWCNTs (1.5 times, р < 0.05) and 2.6 times as compared to the untreated blood (Table 2). This may be due to the high content of metal ions in the unpurified MWCNTs that originate from Fe–Co catalyst and can be washed away by water. Peroxidation induction is consistent with other studies; it is presumably caused by the pro-oxidant action of iron ions (Fe2+), which generate hydroxyl and alcoxy lipid radicals. The initiation of new lipid peroxidation chain reactions leads to an increase in the levels of ketodienes and conjugated trienes.

The fatty acids (FA) of neutral lipids and the free FA of plasma are more susceptible to peroxidation by unpurified MWCNTs than the FA of membrane phospholipids. This was evidenced by the increased levels of KD and CT (35%, р < 0.01 and 28%, р < 0.05) and the stable levels of CCIDB and DC. This may be due to reoxidation reactions of low polar neutral lipids on the surface of non-polar MWCNTs that are highly able to adsorb non-polar substances. MWCNTs are also able to accumulate reactive oxygen species (ROS). According to previous studies, (Fenoglio et al., 2006), nanotubes exhibit a high radical adsorption capacity.

The levels of the intermediate peroxidation products in blood phospholipids after the load test with unpurified MWCNTs remained almost unaltered. This can be explained in two ways: first, free plasma phospholipids are not available to become an oxidation substrate for ROS on MWCNTs; furthermore, they cannot be adsorbed on the surface of MWCNTs due to the high polarity of the molecules. Second, membrane phospholipids are only partially available to MWCNTs that are able to easily penetrate cell membrane.

<table>
<thead>
<tr>
<th>LPO products</th>
<th>Before load test n = 12</th>
<th>Load test with MWCNTs, type 1 (purified) n = 12</th>
<th>Load test with MWCNTs, type 2 (unpurified) n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHP, arb. units</td>
<td>2.20 (1.98–2.74)</td>
<td>3.89** (3.40–4.23)</td>
<td>5.73** (4.81–6.83)</td>
</tr>
<tr>
<td>Neutral lipids:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCIDB, absorbance units</td>
<td>3.69 (3.04–4.04)</td>
<td>4.23 (3.08–4.80)</td>
<td>4.32 (3.81–4.56)</td>
</tr>
<tr>
<td>DC, absorbance units</td>
<td>1.94 (1.80–2.24)</td>
<td>2.55 (1.87–2.81)</td>
<td>2.17 (1.82–2.67)</td>
</tr>
<tr>
<td>KD + CT, absorbance units</td>
<td>0.52 (0.33–0.58)</td>
<td>0.70** (0.58–0.78)</td>
<td>0.67* (0.33–0.58)</td>
</tr>
<tr>
<td>Phospholipids:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCIDB, absorbance units</td>
<td>2.10 (1.51–2.73)</td>
<td>1.47** (0.65–1.71)</td>
<td>2.15 (1.14–3.19)</td>
</tr>
<tr>
<td>DC, absorbance units</td>
<td>1.02 (0.79–1.43)</td>
<td>0.68* (0.42–0.90)</td>
<td>1.07 (0.70–1.73)</td>
</tr>
<tr>
<td>KD + CT, absorbance units</td>
<td>0.47 (0.38–0.65)</td>
<td>0.32** (0.27–0.40)</td>
<td>0.53 (0.27–0.40)</td>
</tr>
</tbody>
</table>

Note: Statistically significant differences between groups 1–2, 1–3: * р < 0.05; ** р < 0.01.
Partial purification of MWCNTs from metallic impurities, especially iron (up to 15 times lower after treatment) reduces significantly their ability to generate ROS. In comparison to unpurified MWCNTs, the levels of LHP after exposure to purified MWCNTs increased less. The reduction in the content of the intermediate products of phospholipid peroxidation by purified MWCNTs in comparison to the levels of these intermediates without load test is possibly linked to predominance of MWCNT adsorption over peroxidation of phospholipid fatty acids that could increase the content of intermediate products. The adsorption properties of MWCNTs are enhanced by the release of metal ions in the environment. This confirms our assumption that phospholipid fatty acids show a lesser tendency towards free radical oxidation caused by MWCNTs compared to fatty acids in neutral lipids. Toxicity of carbon nanotubes (CNT) is attributed to their length, metal content, tendency to aggregate/agglomerate and surface chemistry (Johnston et al., 2010). In any way, exposure to CNT results in a concentration dependent cytotoxicity (Cocciini et al., 2010) that is associated with increased oxidative stress (Reddy et al., 2010). The signaling pathways important for the development of CNT toxicity such as the nuclear factor-kappaB (NF-κB), NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, transforming growth factor-beta1 (TGF−β1), mitogen-activated protein kinase (MAPK), and p53 signaling cascades (Dong and Ma, 2015). Both single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) provoke significant increases in the expression levels of pro-inflammatory genes interleukin-1beta (IL-1β) and IL-6 (Qu et al., 2012). Furthermore metallic nanoparticle impurities which remain in CNT from their synthesis even after purification may be responsible for properties of CNTs (Pumera and Iwai, 2009). Despite the fact that in vitro cytotoxicity and genotoxicity are different for various metal-containing nanoparticles, all of the latter induce reactive oxygen species, lipid peroxidation, and depletion of catalase, reduced glutathione, and superoxide dismutase (Rajiv et al., 2015). In our study despite the purification procedures Fe, Co and Mg impurities were detected in both unpurified and purified MWCNTs. It has been previously shown that iron oxide nanoparticles induce cytotoxicity via oxidative stress and lead to biphasic inflammatory responses. Immediately following exposure, the concentrations of pro-inflammatory cytokines, IL-1, tumor necrosis factor-alpha (TNF−α), and IL-6 increased with the dose. Furthermore, nanoparticle exposure markedly increases malondialdehyde concentration, while intracellular reduced glutathione and antioxidant enzyme activities are decreased. Besides pro-inflammatory cytokines, Th1 and Th2-type cytokines also increase (Srivas et al., 2012; Park et al., 2010). Similarly, exposure to MgO nanoparticles produce a significant dose-dependent reduction in blood total antioxidant capacity, superoxide dismutase, and catalase activity levels due to nanoparticle-induced oxidative stress (Kiranmai and Reddy, 2013). Cobalt is the other metallic impurity of our purified as well as unpurified MWCNTs. Actually CoO nanoparticles induced oxidative stress and activated the signaling pathway of TNF−α-Caspase-8-p38-Caspase-3, something that could induce cell death (Chattopadhyay et al., 2015). In particular iron (Fe), and cobalt (Co) undergo redox-cycling reactions. In this case common mechanism is the Fenton reaction which primarily associates with mitochondria, microsomes and peroxisomes and generates the superoxide and the hydroxyl radicals (Valko et al., 2005). Iron particles influence the mitochondrial permeability transition through the mitochondria adenine nucleotide phosphate (NAD(P)H) oxidation due to loading of the mitochondrial antioxidant defense systems and uptake of iron to the mitochondrial matrix via a calcium uniporter (Call et al., 2012). Whereas cobalt induce the phenomenon of mitochondrial permeability transition (MPT) with the opening of the transition pore. These particles cause mitochondrial swelling and proton leakage through the inner mitochondrial membrane. Nevertheless MPT induction is due to generation of the highly damaging hydroxyl radical by Co2+, with the oxidation of sulfhydryl groups, glutathione and pyridine nucleotides (Battaglia et al., 2009). MWCNTs also induce loss of the mitochondrial membrane potential by regulating expression of Bcl-2 family proteins and cause release of cytochrome c from mitochondria to cytosol and induce apoptosis (Ye et al., 2012).

Exposure to MWCNTs with a minimal content of impurities caused an increase in the percentage of immune cells with reduced mitochondrial membrane potential. The number of mature T lymphocytes with reduced potential underwent a more than three-fold increase (Table 3). At the same time, upregulation of the pre-apoptotic CD95 marker on CD3+ cells was observed. Purified MWCNTs activated the early phase of T-cell response and caused an increase in the percentage of the functionally active T lymphocytes expressing interleukin-6 (IL-6 receptor (CD3+CD126+)). The damaging effect of unpurified MWCNTs was manifested in the significant increase in the percentage of lymphocytes and CD3 + cells with reduced mitochondrial membrane potential (MMPL and MMP CD3+ respectively). The increased levels of CD3+CD95+, CD3+CD25+ cells were not significantly different from the levels corresponding to exposure to the purified MWCNTs.

3.3. Gene expression in vitro

Whole genome transcriptomics in the lung epithelium (A549) and keratinocytes (HaCaT), which were selected as in vitro models in this study showed that gene expression significantly differs after exposure to purified and unpurified MWCNTs. 48 h exposure resulted in a significant number of genes differentially expressed compared to the control; approximately 500 genes were expressed differentially in the case of MWCNTs with different levels of impurities. Similar results were found when we analyzed the data isolating the 25 genes involved in the molecular pathway associated to oxidative stress (Table 4). The picture changes slightly in terms of modulated gene expression, when focusing on the genes involved in inflammation mediated by chemokine and cytokine signaling. Table 4 summarizes the data, which show that cells behaved differently when exposed to MWCNTs with different levels of impurities. Both purified and unpurified MWCNTs showed a reduction in the number of genes that had modulated their expression level after 48 h exposure, and the results are summarized in the following table.

The observed effects after 48 h of exposure would seem to indicate the molecular processes that underlie actual cellular toxic response to MWCNT exposure. The persistence of the induction of genes driving chemokine and cytokine-signaling-mediated inflammation showed that further attention would have to be paid to the inflammatory effects of the tested MWCNTs, and in particular the unpurified ones. For this reason, we analyzed the gene expression of the genes involved in the inflammation pathway for the two types of in vitro models we used, i.e. HaCaT keratinocytes and A549 lung epithelial cells. Comparative analysis showed that the modulation in gene expression of A549 cells was much higher than the gene expression of the keratinocytes after 48 h of exposure (Table 5).

3.4. Pathway analysis

Analysis of the effects of different types of MWCNTs on gene expression showed that impurities influence significantly the induction of key toxicity pathways such as inflammation mediated by chemokine and cytokine signaling. In particular, MWCNT induced
the over-expression of chemokine (C–C motif) (Ccl2) and ligand 19 (Ccl19). Both are cytokines involved in immunoregulatory and inflammatory processes. Moreover, unpurified MWCNTs induced the over-expression of chemokine (C–X–C motif) ligand 10 (Cxcl10) and ligand 11 (Cxcl11), interferon gamma-induced proteins, involved in immunoregulation. More importantly, clinical observations have recently connected the abundant presence of these cytokines to hypersensitivity pneumonitis and, to a lesser extent, to lung fibrosis (Simonian et al., 2009). Pathway analysis showed the modulation of genes related to the NFkB pathway, which is more evident for unpurified MWCNTs (Fig. 3).

4. Discussion

Our results are consistent with other studies (Rodriguez-Yanez et al., 2013) in showing that the main mechanism inducing the MWCNT toxic action, including those with the minimal content of impurities, is oxidative stress. Of note, ROS generation is significantly enhanced in the presence of metal impurities.

The impact of MWCNTs with a minimum content of impurities is expressed by the induction of apoptosis, including dysregulation of receptor-dependent apoptosis of immunocompetent cells. The exact mechanisms of MWCNT-induced apoptosis are not well understood yet. MWCNTs block G1 phase of the cell cycle, affecting related genes to induce the expression of apoptotic genes. This may be due to the induction of redox-sensitive signaling pathways, including pathways mediated by mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinases and extracellular signal-regulated kinases 1 and 2. A number of recent studies have shown that ROS-mediated activation of the p38 MAPK cascade is critical for the observation of adverse effects from exposure to MWCNTs (Eishal et al., 2012; Jiang et al., 2013; Gomes and Florida-James, 2014; Luo et al., 2015). The MAPK signaling pathway plays an important role in the activation of NF-kB, which leads to induction of early response genes, critical for cellular apoptosis. The percentage of cells initiating apoptosis, including effector T cells, increases after exposure to MWCNTs due to the activation of MAPK and NF-kB. Pro-inflammatory effects of oxidative stress are caused by the activation of redox-sensitive transcription factors NFkB and AP-1, which regulate the expression of pro-inflammatory mediators and protective antioxidants. This mechanism hypothesis has been verified by gene expression analysis. NFkB regulates the expression of many genes involved in inflammation, including the pre-inflammatory cytokines IL-1b, TNF-α and IL-6 (Manna et al., 2005; Rossignol and Frye, 2014; Rydman et al., 2014). IL-6 is an early and very important mediator in the development of inflammation. Elevated levels of IL-6 contribute to the maintenance of oxidative stress. Recently obtained data suggest that IL-6 plays also a role in the development of inflammatory response. It was shown that IL-6 induces anti-apoptotic regulators, such as Bcl-2 and Bcl-XL, and therefore protects T cells from apoptosis (Dienz and Rincon, 2009). The classical IL-6 pathway – binding of IL-6 to the membrane receptor IL-6R (CD126) - results in the phosphorylation of the transcription factors STAT1 and STAT3. Modulation of IL-6 via classic IL-6R or trans-signaling pathways determines its effect on effector functions in T cells. Changes in the expression of IL-6R on T cells may be associated with changes in STAT1 signaling, but not STAT3 (Gareth, 2010). The balance between STAT1 and STAT3 in T cells greatly affects the lineage commitment and apoptosis. Recent studies found increased levels of Th2 cytokines (IL-4, IL-5, IL-6) after exposure to MWCNTs (Rydman et al., 2014). However, in the light of the above, we can also assume that the increase in expression of the IL-6R receptor in mature T cells is associated with compensatory anti-apoptotic mechanisms as response to MWCNT exposure.

In inflammation, TNF-α induces mitochondrial permeability transition pore (MPTP) opening, mitochondrial membrane potential loss, and increased cell apoptosis. This was accompanied by upregulation of Fas/Fasl; Bax; and caspase-3, -8, and -9 activation (Gao et al., 2013). NF-kB activation protects the cells against both TNF-α and Fas-mediated apoptosis. TNF-α and Fas recruit similar but non-identical, pathways signaling apoptosis. The MPT is obligatory for TNF- α-induced apoptosis, while in Fas-mediated apoptosis, the MPT accelerates the apoptotic events but is not obligatory (Hatano et al., 2000). The increased mitochondrial production of reactive oxygen species are triggered by overproduction of the mitochondrial reduced form of nicotinamide-adenine dinucleotide (NADH). However, both NADH-driven mitochondrial MPT and up-regulation of the expression of CD95-Fas ligand (FasL)-mediated apoptosis, involving in reactive oxygen species production, operates apoptosis (Sastre et al., 2007). Members of the TNF receptor (TNFR) superfamily and their ligands are crucial regulators of cellular activation and death. One of the three subgroups of TNFR

### Table 3

<table>
<thead>
<tr>
<th>Immunological parameters</th>
<th>Before load test n = 12</th>
<th>Load test with MWCNTs, type 1 (purified) n = 12</th>
<th>Load test with MWCNTs, type 2 (unpurified) n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+CD95+, %</td>
<td>9.2 (8.9–9.4)</td>
<td>14.4* (13.2–15.6)</td>
<td>14.9* (13.6–15.8)</td>
</tr>
<tr>
<td>CD3+CD25+, %</td>
<td>7.4 (6.8–8.1)</td>
<td>12.3* (11.0–12.5)</td>
<td>12.6* (11.5–12.9)</td>
</tr>
<tr>
<td>CD3+CD126+, %</td>
<td>1.2 (1.0–1.3)</td>
<td>1.8* (1.6–1.9)</td>
<td><em>2.4</em>* (2.2–2.8)</td>
</tr>
<tr>
<td>MMPF, %</td>
<td>1.3 (1.1–1.6)</td>
<td>3.1** (2.8–3.5)</td>
<td><em>5.4</em>* (4.8–6.0)</td>
</tr>
<tr>
<td>MMP-C3D3+, %</td>
<td>3.1 (2.9–3.3)</td>
<td>4.6** (4.2–5.1)</td>
<td><em>7.2</em>* (6.8–7.6)</td>
</tr>
</tbody>
</table>

Note: Statistically significant differences between groups 1–2, 1–3 at right, between groups 2–3 at left: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.

### Table 4

<table>
<thead>
<tr>
<th>Oxidative stress pathway</th>
<th>Chemokine/cytokine signaling-mediated inflammation pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCNT (purified)</td>
<td>MWCNT (unpurified)</td>
</tr>
<tr>
<td>MWCNT (purified)</td>
<td>MWCNT (unpurified)</td>
</tr>
<tr>
<td>HaCaT</td>
<td>7</td>
</tr>
<tr>
<td>A549</td>
<td>19</td>
</tr>
</tbody>
</table>

### Table 5

Comparison of modulated gene expression in the chemokine/cytokine signaling-mediated inflammation pathway between HaCaT and A549 cells after 48 h of exposure.

<table>
<thead>
<tr>
<th>Cell model</th>
<th>MWCNT (purified)</th>
<th>MWCNT (unpurified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaCaT</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>A549</td>
<td>15</td>
<td>29</td>
</tr>
</tbody>
</table>
family is defined as TNFR-1 (TNFRSF1) or CD95; they are essential for direct induction of cell death (Paulsen and Janssen, 2011). Membrane-bound CD95L is essential for triggering cytotoxic activity, whereas soluble CD95L primarily promotes non-apoptotic activities (O’Reilly et al., 2009). CD95 co-ligation on primary T cell activation has dose-dependent effects. CD95 is capable of transducing non-apoptotic costimulatory signals in T cell receptor (TCR)/CD3-stimulated naïve T cells, low doses augment TCR-induced activation and proliferation. Thus CD95 can act as a silencer or enhancer of primary T cell activation (Paulsen and Janssen, 2011). Furthermore, high density CD95L, CD95-costimulated T cells display inefficient nuclear translocation of transcription factors including the nuclear factor of activated T cells, NF-κB and the activator protein-1 (AP-1) (Jun/c-Fos), reduced Ca²⁺-mobilization and decreased MAPK and caspase activation. Subsequently, TCR induced cytokine production and upregulation of activation markers become impaired. Down-regulation of activation markers and reduced secretion of several cytokines including IL-2R, IL-2 (CD25), interferon-gamma (IFN-γ) or TNF-α may occur (Strauss et al., 2009). In this context CD95 is a dual-function receptor that exerts pro- or anti-apoptotic effects depending on the cellular context, the state of activation, the signal threshold and the mode of ligation. CD95 engagement modulates TCR/CD3-driven signaling pathways in resting T lymphocytes in a dose-dependent manner. While high doses of immobilized CD95 agonists silence T cells, lower concentrations augment activation and proliferation. The levels of CD95L on antigen-presenting cells (APCs), neighboring T cells regulate inhibitory or co-stimulatory CD95 signaling, which in turn is crucial for fine-tuning of primary T-cell activation (Paulsen and Janssen, 2011). A strong CD95 stimulation initiates death, whereas CD95 stimulation below a certain threshold level triggered cellular Fas-associated protein with death domain-like IL-1β-converting enzyme-inhibitory protein (cFLIP) dependent survival associated with MAPK and NF-κB activation. Fas (CD95) and TNF-R1, activate the caspase cascade and result in lymphocyte death by apoptosis. Anti-apoptotic proteins, FLIP (also known as FLICE/caspase-8 inhibitory protein) expression is tightly regulated in T cells and might be involved in the control of both T-cell activation and death (Thome and Tschopp, 2001). The CD95 death-inducing signaling complex (DISC) is formed at the cell membrane upon stimulation with low concentrations of agonistic anti-CD95 (APO-1/Fas) monoclonal antibodies; however, activation of procaspase-8 at the DISC is blocked due to high cellular FLICE-inhibitory protein recruitment into the DISC. Death signaling does not occur upon CD95 stimulation at low (threshold) anti-APO-1 concentrations (Lavrik et al., 2007). The formation of the CD95 DISC is the initial step of CD95 signaling. Activation of procaspase-8 at the DISC leads to the induction of CD95 (APO-1/Fas) is a member of the death receptor-mediated apoptosis. The activation of procaspase-8 is blocked by cellular FLICE-inhibitory proteins (c-FLIP) (Lavrik and Krammer, 2012). In our study, regarding the increase in the CD25 expressing CD3⁺ T cells, CD95-stimulation was not strong enough to trigger apoptotic signaling. However, low level of stimulation was in favor of the activation of anti-apoptotic pathway and IL-2 synthesis.

IL-6 also inhibits Fas-mediated cell injury through upregulation of Bcl-2, Bcl-xL, and FLIP proteins. IL-6 may function to maintain a critical level of these specific antiapoptotic factors. These factors allow the cells to maintain an appropriate threshold to prevent activation of a death program. Inactivation of caspase 8 terminates the Fas apoptotic pathway proximally, prior to mitochondrial depolarization. FLIP can be processed by caspase 8 and binds tightly to and inactivates caspase 8. FLIP also bind to Fas (CD95) associated

Fig. 3. Inflammation mediated by chemokine and cytokine signaling pathway: comparison of results after exposure to purified MWCNTs (in red) and unpurified MWCNTs (in blue) and; in yellow are marked the genes the expression of which is modulated after both treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
protein with death domain (FADD) and procaspase 8 (FLICE) and inhibits apoptosis by all death receptors (Fig. 4) that require FADD and caspase 8 for signal transduction (Kovalovich et al., 2001). IL6 is a pleiotropic cytokine that has long been described as having both pro and anti-inflammatory properties (Gadient and Patterson, 1999; Jones et al., 2001). On target cells, IL6 acts by binding to a specific cognate receptor (IL6R), which triggers gp130 and leads to the activation of the Jak/STAT signaling pathway and in particular the activation of STAT3 (Peters et al., 1998). The receptor complex mediating the biological activities of IL-6 consists of two distinct membrane-bound glycoproteins, a cognate receptor subunit (IL-6R, CD126) and a signal-transducing element (gp130, CD130). Expression of the trans-membrane-spanning gp130 is found in almost all organs (Saito et al., 1992). Interleukin 6 signaling is facilitated through the homodimerization of gp130 to the ligand–receptor complex. Intracellular signaling is subsequently triggered via activation of gp130-associated cytoplasmic tyrosine kinases (JAK1, JAK2, and TYK2) and phosphorylation of STAT1 and STAT3 (Murakami et al., 1993; Gerhartz et al., 1996). Binding of IL-6 to its receptor induces the homodimerization of two gp130 molecules, thereby transducing the signal into the cell (Murakami et al., 1993). Three members of the Janus tyrosine kinase family, JAK1, JAK2, and TYK2, are found to be constitutively associated with gp130 and are activated and autophosphorylated after IL-6 stimulation (Lutticken et al., 1994; Stahl et al., 1994). Unlike the ubiquitously expressed gp130, the cellular distribution of IL6R is limited to a few cell types, including monocytes, neutrophils, and some T cells and B cells; however, IL6R also exists in a soluble form (slIL6R) that upon binding to IL6 stimulates cells via direct interaction with gp130. Binding to the membrane-bound IL-6 receptor (mIL-6R, CD126) causes the recruitment of two gp130 co-receptor molecules (CD130) and the activation of intracellular signaling cascades via gp130. This classical pathway is mainly limited to neutrophils, monocytes/macrophages and certain other leukocyte populations, which express IL-6R on their surface (Rose-John et al., 2007). Upon its secretion, IL6 binds to its receptor (IL6Ra or CD126). CD126 encompasses a short cytoplasmic domain that interacts with the ubiquitously expressed second transducer gp130. Binding of IL6 to CD126 and their association with gp130 triggers the activation of JAK, which phosphorylate STAT3, thus modulating the expression of a broad spectrum of downstream target genes (Heinrich et al., 2003). Thus, the increased percentage of CD3+CD126+ cells may suppress the apoptotic signaling via alteration of the CD95 expression.

From the overall results of our multi-level analysis, it seems that the key biological process resulting from exposure with both purified and unpurified MWCNTs is NFkB activation. We are also aware that each cell type provides its own unique signaling signature (Snyder-Talkington et al., 2015); thus, testing different cell lines and types results in different gene expression patterns (Tilton et al., 2014). In this way a closer representation of the in vivo signaling environment is captured by in vitro models. Inhalation and skin are the dominant routes of exposure to MWCNTs. Considering that epithelial cells make up the inner lining of the lung and the keratinocytes of the skin, they both represent a plausible first point of contact with MWCNT during inhalation and skin exposure respectively and a potential source of cellular signaling (Snyder-Talkington et al., 2015). The use of lung epithelium and keratinocytes for in vitro testing has shown that inflammation is mediated before MWCNTs actually translocate into systemic circulation, targeting the related blood cells. Based on the combined biochemical and transcriptomic responses in our study, the synergistic effect of systemic inflammation mediated in exposure barriers (skin and lung epithelium) and local inflammation mediated in blood cells, forms a plausible hypothesis for immunological in vivo responses upon exposure to MWCNTs.

5. Conclusions

Our study showed that the main mechanism of effect for both types of MWCNTs is oxidative stress induction. Its intensity is directly related to the amount of metallic impurities in the MWCNTs. The subsequent development of inflammatory responses and enhanced peroxidation cause damage to mitochondria in immunocompetent cells. Unpurified MWCNTs generate more oxidation reactions than the purified CNTs, inducing significant mitochondrial dysfunction. Transcriptome analysis showed that activation of the NFkB pathway is the key biological process.

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**Fig. 4.** Up-regulation of anti-apoptotic pathway in the presence of purified and unpurified multiwalled carbon nanotubes.
initiating a cascade of effects. This may cause a perturbation of the IL-6 pathway that aims to regulate the inflammatory processes and compensate apoptotic changes. Overall, the immunological responses related to MWCNT exposure are considered as the result of the synergistic effect of systemic (mediated by cells of the routes of exposure) and local inflammation (blood cells).

Acknowledgments

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