Supplementary material for

Monocytes adhesion induced by multi-walled carbon nanotubes and palmitic acid exposure in endothelial cells and alveolar-endothelial co-cultures

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Supplemental Figures:

Fig. S1. Sketch of the experimental setup of the alveolar-endothelial 3D co-cultures.

Fig. S2. TEER development of the inserts with A549 monolayer for 8 days.

Fig. S3. Permeability of the inserts with or without A549 monolayer.

Fig. S4. Image of monolayer of A549 cells after 7 days

Fig. S5. Cytotoxicity measured by wst-1.

Fig. S6. Proliferation of HUVECs measured by BrdU assay.

Fig. S7. Fluorescence microscopy of the intracellular ROS generation in HUVECs.

Fig. S8. Fluorescence microscopy of THP-1 adhesion to HUVECs.

Fig. S9. Adhesion assay with MWCNT and NAC or IL-6 co-treatment.

Supplemental Fig. S1.

Fig. S1. Experimental setup of the alveolar-endothelial 3D co-cultures. The A549 cells were grown on the BD Falcon cell culture inserts for 7 days before co-culture with HUVECs and THP-1 and THP-1a cells. A549 monolayer was exposed to MWCNTs apically, whereas HUVECs and THP-1 cells were exposed to PA basolaterally.

Supplemental Fig. S2.

Fig. S2. TEER development of the A549 monolayer for 8 days. The TEER of inserts without cells was subtracted. Experiments were done independently four times and the mean±SEM of TEER value was calculated.

Supplemental Fig. S3.

Fig. S3. Percentage of FITC-dextran permeability of the inserts with or without A549 monolayer. Experiments were done independently for four times and the mean $\pm$ SEM of permeability was calculated.

Supplemental Fig. S4.

Fig S4. Overview of monolayer of A549 cells on day 7. 50.000 cells set up in Ibidi microscopy slide and left for 7 days, with media changed every second day. Cells are stained with Hoechst.

Supplemental Fig. S5.

Fig. S5. The cytotoxicity measured by the WST-1 assay after 24 h exposure to MWCNTs. A). Percent WST-1 formation in HUVECs after MWCNTs exposure in medium with or without palmitic acid (PA). B). Percent viability of A549 monocultures after MWCNTs exposure. C). Percent viability of A549 monolayer, A549+THP-1a or THP-1a in the transwell co-culture system after 8 µg/ml MWCNTs exposure. Data represent the percentage of absorbance compared with the unexposed control cells. Bars are means $\pm$ SEM of 4-5 independent experiments. * $P < 0.05$ compared with the unexposed cells.

Fig S5 shows the cytotoxicity of MWCNT exposure as measured by the WST-1 assay. There were only statistically significant single-factor effects of the MWCNT concentration ($P<0.001$) and PA treatment ($P<0.001$) in the HUVEC monocultures. The PA treatment in itself was associated with a modest non-significant 4.6% (95%: 2.4% - 4.6%) decreased WST-1 formation. The exposure to NM400 was associated with decreased WST-1 formation at 8, 16 and 32 $\mu\text{g/ml}$ ($P<0.05$). In the presence of PA, there was only decreased WST-1 formation by NM400 exposure to the highest concentration (32 $\mu\text{g/ml}$; $P<0.001$). The NM402 only decreased the WST-1 formation at the highest concentration (32 $\mu\text{g/ml}$) without the presence of PA ($P<0.01$). Nevertheless, the effect on WST-1 formation in HUVECs was only modest after the treatment of MWCNTs with or without the presence of PA (Fig S5A).

There was a significant single-factor effect on the concentration of MWCNTs in the A549 monocultures ($P<0.001$), whereas there was no difference in response between NM400 and NM402 ($P=0.86$) (Fig S5B). All the concentrations were associated with statistical significantly decreased formation of WST-1, albeit the effect was rather modest. The highest concentration (32 $\mu\text{g/ml}$) was associated with 4.7% (95% CI: 1.2% - 8.2%) and 5.7% (95% CI: 2.1% - 9.2%) decreased WST-1 formation in the cultures that were exposed to NM400 and NM402, respectively.

For the Transwell co-culture system, the exposure did not significantly affect the cytotoxicity of the A549 monolayer or A549+THP-1a co-culture on the inserts assessed by the WST-1 assay ($P>0.05$), whereas both NM400 ($P<0.001$) and NM402 ($P<0.05$) significantly decreased viability of THP-1a cells on the inserts (Fig S5C).

Supplemental Fig. S6.

Fig. S6. The proliferation of HUVECs measured by the BrdU assay after 24 h MWCNT exposure with or without palmitic acid (PA). Data represent the percentage of absorbance compared with the unexposed control cells. Bars are means $\pm$ SEM of three independent experiments.

The proliferation of HUVECs was not affected by MWCNT exposure with or without the presence of PA (Fig S6). We did not attempt to measure the proliferation of A549 cells by MWCNT exposure because it is expected that after long term cultivation, the A549 cells grow to a maximum and do not further proliferate in the Transwell co-culture system during exposure. Furthermore, the THP-1a cells do not proliferate.

Supplemental Fig. S7.

Fig. S7. Representative fluorescence microscope figures showing the intracellular ROS generation of HUVECs after 24 h exposure to 8 $\mu\text{g/ml}$ MWCNTs in the presence or absence of PA. The blue staining by Hoechst indicated the number of cells, and the green staining by DCFH indicated the level of total ROS.

Supplemental Fig. S8.

Fig. S8. Representative fluorescence microscope images showing THP-1 adhesion to the activated HUVECs after 24 h exposure to 8 $\mu\text{g/ml}$ MWCNTs in the presence or absence of PA. The green staining by CellTracker indicated the number of adherent THP-1 cells.

Supplemental Fig. S9.

Fig. S9. Attachment of monocytes to HUVEC cells were measured after HUVECs were exposed to carbon nanotubes in combination with 1mM of NAC or 100pg/ml IL-6. Data are mean of three experiments with each 6 samples and SD. The adhesion is shown as percent change compared to the respective controls without co-exposure.