Supplementary Methods Section

Characterization of welding fume particles by DLS and SEM

Mild steel welding fume (MSWF) dispersion were prepared according to the NANOGENOTOX dispersion protocol, with minor modifications. A 0.5 mg/ml stock was prepared using 0.05% BSA (m/v in H2O) as dispersion media (DM) and sonicated using a probe sonicator at 10% amplitude (Sonifier 450S, Branson Ultrasonics, Danbury, USA) for 9 min. To obtain information on the hydrodynamic size distribution after dispersion, the ZetaSizer Nano ZS (Malvern Instruments Ltd, UK) was used. After sonication, the dispersed particles were transferred to a cuvette and left on the bench for 10 min prior to measurement. ZetaSizer software (Malvern Instruments Ltd, UK) was used to analyze the data. Three independent measurements were performed. Similar measurements were performed after diluting the dispersed MSWF in cell culture media.

MSWF for SEM analysis was prepared as follows: a volume corresponding to 100 µg was taken from a 0.5 mg/ml stock dispersed in 0.05% BSA which was sonicated as described above followed by filtering on a 47 mm Whatman Nuclepore polycarbonate filter with 50 nm pore size. Thereafter, the filter was coated with a thin platinum film in a sputter coater (Cressington 208HR sputter coater, UK). Specimens of 10 x 10 mm were cut from the filter and gently fixed on aluminum specimen stubs with double-sided carbon adhesive discs. The specimens were analyzed with a Hitachi SU 6600 (Ibaraki-ken, Japan) field emission scanning electron microscope (FE-SEM) equipped with a Bruker energy-dispersive X-ray detector. The instrument was operated under the following conditions: accelerating voltage 15 keV and working distance 10 mm. High resolution images of the particles were obtained by acquiring at slow scanning speed.

Estimation of MSWF doses used for cell culture experiments

The doses used for cell culture exposures were kept low to mimic occupational exposure and were calculated following a mathematical calculation modified from Antonini and coworkers (Antonini et al., 2010, 2013) to determine the daily lung burden of a worker working 8 h per day. Incorporated factors were the occupational exposure limit for WF (5 mg/m^3), human minute ventilation volume (20.000 ml/min x E-6 m^3/ml), the exposure duration (8 h/day), the deposition efficiency (set to 20%; (Oberdorster et al., 2005a, 2005b)).

The daily deposited dose was:

5 mg/m^3 x (20.000 ml/min x 10^{-6} m^3/ml) x (8 h x 60 min/h) x 0.20 = 7.2 mg

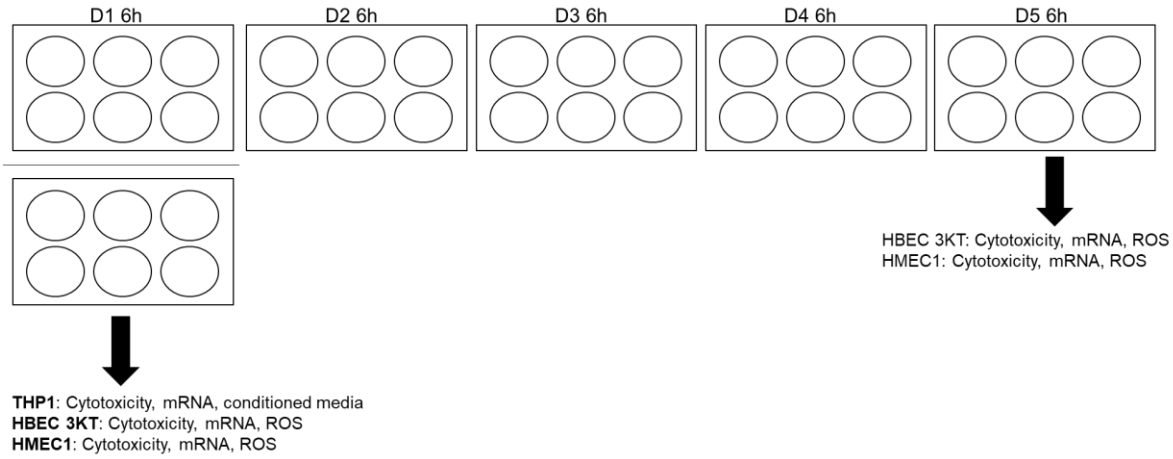
When using the surface area of the alveolar epithelium (human 102 m^2, (Stone et al., 1992)) this leads to a deposited dose of 0.007 µg/cm^2 (7.2 mg/1.020.000 cm^2 = 7.06 x 10^{-6} mg/cm^2 → 0.007 µg/cm^2 corresponding to 0.035 µg/ml in a 6-well plate). This dose was set as 1x and the other doses (5x, 25x and 125x) used in this study were calculated accordingly. Thus, cells were exposed to 0.035, 0.175, 0.875 or 4.375 µg/ml taking into account the surface area of the cell culture dish where 0.035 µg/ml can be directly related to the occupational exposure limit of WF.

Cell culture and exposure to MSWF

Human monocytic THP-1 cells and human microvascular endothelial HMEC-1 cells were kindly provided by Anita Solhaug (Norwegian Veterinary Institute) and Johan Øvrevik (Norwegian Institute of Public Health, Oslo, Norway), respectively, and human bronchial epithelial HBEC-3KT cells were kindly provided by Dr. John D. Minna (Ramirez et al., 2004). THP-1 cells were maintained in RPM1-1640 medium supplemented with 10% FBS, 10mM hepes (pH 7.3) and 1 mM sodium pyruvate. Macrophage differentiation was initiated by 81 nM

PMA for 30 h and experiments were initiated after an additional 16 h resting period. HMEC-1 cells were maintained in MCDB-131 medium supplemented with 10 ng/ml EGF, 1 µg/ml hydrocortisone, 10 mM L-glutamine and 10% FBS. HBEC-3KT cells were cultured on coated cell culture plates in LHC9/RPMI-1640 (1:1) medium. All cells were cultured in presence of 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C.

Cells were exposed to MSWF for 6 h intervals over a five day period (6 h exposure each day for five days (D1-D5)). Control cells were exposed only to dispersion media. For analysis of cytotoxicity, 4000 cells/well (HMEC-1 and HBEC-3KT) and $2x10^4$ cells/well (differentiated THP1) were seeded in 96-well plates. Cell viability was assessed at day one and day five of exposure by Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich), according to the manufacturer's instructions.



THP-1 conditioned medium was collected from THP-1 cells exposed to 0, 0.035, and 4.375 µg/ml MSWF for 6 h. Shortly, the cells were seeded at $1,2x10^7$ cells/10 cm^2 plate in the presence of PMA and differentiated as previously described. The cells were then exposed for MSWF and the medium was centrifuged at 6000 RPM for 10 min at 4°C and stored for later conditioned media experiments. For assessment of effects of conditioned media on ROS in HMEC-1 and HBEC-1 cells, $3x10^5$ cells/well were seeded in mixed media (original medium/original THP-1 medium, 1:1). The following day the cells were changed to conditioned medium (original

medium/THP-1 conditioned medium, 1:1) and incubated for 6 h or 24 h, where after the cells were used for ROS analysis.

Confocal microscopy

HBEC-3KT and HMEC-1 cells were cultured on cover slips in 6-well plates and exposed to the highest dose of MSWF for five days. After fixation with 4% paraformaldehyde (PFA), Hoechst (Sigma-Aldrich) was added for visualization of the cell nuclei. After mounting with Mowiol, a laser scanning microscope (LSM 710, Zeiss) was used to visualize the cells, cell nuclei and MSWF particles with 20x magnification. Representative photographs were taken with an AxioCam camera (Zeiss).

Gene expression analysis

Briefly, $1x10^5$ cells/well HMEC-1 and HBEC-3KT cells were seeded per well in 6-well plates, however, for assessment of expression in HBEC-3KT cells at day five the amount was reduced to $5x10^4$ cells/well. THP-1 cells were seeded at a concentration of $2x10^6$ cells/well. Total RNA was isolated, DNAse treated and concentrations and quality were assessed by Nanodrop 2000 spectrophotometer and Qubit fluorometric measurement. Expression of 40 genes involved in inflammation, fibrosis and endothelial activation was analyzed by a custom RT2 gene array, according to the manufacturer's instructions (Qiagen). In addition, expression of *ICAM-1*, *VCAM-1*, and *SELE* was assessed on a StepOne Real-Time PCR system (Applied Biosystems) using Perfecta SYBR Green FastMix, ROX (Quanta Biosciences). Changes in gene expression were analyzed using the DDCT method and data was normalized to the geometric mean of the housekeeping genes *PP1A*, *RPLP0* and *UBC* (for array data) and to the reference gene *ACTB* (for assessment of single assays).

Measurement of intracellular ROS

Intracellular ROS levels were measured using dichlorodihydrofluorescein (DCF) fluorescence. 2'7'-dichlorodihydrofluorescein diacetate (DCFH/DA) (Sigma-Aldrich) is a cell-permeable compound that yields a fluorescent product when oxidized by ROS. HMEC-1 and HBEC-3KT cells were seeded in 6-well plates, allowed to attach for two days and exposed to WF 6 h per day up to five days. After 6 h exposure to WF on day 1 and day 5 the medium was removed and medium containing 100 µM DCFH/DA (Sigma-Aldrich) was added to the cells which were then incubated at 37°C for 1 h. For the experiments with conditioned media from THP-1 cells, ROS was measured after 6 and 24 h incubation with the conditioned media. Positive and negative controls were included in each experiment. Cells were then washed with 1x PBS and incubated with 2% TritonX-100 in PBS on ice for 5 min. Cells were collected in Eppendorf tubes by scraping and then sonicated on a VialTweeter (UIS250V, Hielscher Ultrasonics GmbH) for 2 x 5 sec at maximum amplitude. Thereafter tubes were centrifuged at 15.000 RPM for 10 min at 4°C and supernatant was transferred to new Eppendorf tubes. Each sample was applied in triplicate to a 96-well plate and fluorescence was measured using SpectraMax i3 (Molecular Devices) upon excitation of 488 nm. Finally, the protein concentration of each sample was measured using Bradford assay and DCF fluorescence was calculated relative to the protein content of each sample.

Migration analysis

HMEC-1 and HBEC-3KT cells were seeded in conditioned medium at the concentration of $6x10^4$ cells/well on ImageLock 96-well plates (Essen BioScience), coated with 100 ng/µl matrigel (Corning, Corning, NY, USA). Cells were allowed to attach and after 18 h, a wound

was made in the cell monolayer using the wound maker pin tool (Essen BioScience). For analyses of cell migration the cells were washed and conditioned medium was added to the wells. Cell migration was monitored for 48 h by live-cell imaging using IncuCyte ZOOM (Essen BioScience). Analyses were performed in three independent experiments with six to eight replicates in each.

References

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