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Biologic effects of nanoparticle-allergen conjugates: Time-resolved uptake using an *in vitro* lung epithelial co-culture model of A549 and THP-1 cells

BETTINA GROTZ, MARK GEPPERT, ROBERT MILLS-GOODLET, SABINE HOFER, NORBERT HOFSTÄTTER, CLAUDIA ASAM, ANDREA FEINLE, KRISZTINA KOCSIS, THOMAS BERGER, OLIVER DIWALD, NICOLA HÜSING, MICHAEL WALLNER, FATIMA FERREIRA, ALBERT DUSCHL AND MARTIN HIMLY

--- ELECTRONIC SUPPLEMENTARY INFORMATION ---

Supplementary methods:

Detailed description of cytotoxicity assays

LDH assay: After incubation, the cells were washed twice with 1 mL PBS and then lysed for 30 min with 500 µL of 1% Triton X-100 in PBS. 10 µL of each lysate was pipetted into 170 µL LDH buffer (80 mM Tris, 200 mM NaCl, pH = 7.2) in a well of a 96-well microtiter plate and then mixed with 180 µL of LDH reaction mixture (4 mM sodium pyruvate, 0.4 mM NADH, 200 mM NaCl in 80 mM Tris/HCl buffer, pH = 7.2). Immediately, the absorbance at 340 nm was measured and its decrease followed for 10 min. Cell viability was calculated by comparing LDH activities in untreated control cells with cell exposed to TiO_2 or SiO_2 NPs.

Celltiter-Blue assay: After incubation, the cells were washed twice with 1 mL PBS and then incubated with 500 µL Celltiter-Blue (CTB) reagent diluted 1:10 in serum-free cell culture medium (SFM). After 1 h of incubation, the supernatants were collected and their fluorescence was measured (λ_{ex} = 560 nm, λ_{em} = 590 nm). To calculate cell viability, fluorescence of untreated control cells was compared with cells exposed to TiO_2 or SiO_2 NPs.

Neutral Red assay: After the CTB assay, the cells were washed once with 1 mL PBS and then incubated with 500 µL Neutral Red solution diluted 1:80 in SFM. After 1 h of incubation, the Neutral Red solution was aspirated, the cells washed once with 1 mL PBS and then lysed with 500 µL Neutral Red extraction solution (50% Ethanol, 49% H_2O, 1% acetic acid). The absorbance of the extracts was measured at 540 nm (reference wavelength: 690 nm). Absorbance of untreated control cells was compared with cells exposed to TiO_2 or SiO_2 NPs to calculate cell viability.

Antibody staining of cells in A549/THP-1 co-cultures

After growing the co-cultures, the cells were washed twice with 1 mL PBS and then detached by incubation with 100 µL Trypsin/EDTA solution for 5 min and transferred to FACS tubes using PBS. After centrifugation for 5 min at 250 *g*, the cells were washed once with 1 mL PBS and again centrifuged for 5 min at 250 *g*. Cells were resuspended in 200 µL PBS containing the respective antibodies (anti CD11a and anti-CD326; 1:200 diluted) and incubated for 30 minutes at room temperature. Finally the cells were washed once with 1 mL PBS, resuspended in PBS containing 3 mM EDTA and analyzed by flow cytometry.

Simulation of time-resolved nanoparticle delivery based on particle kinetics

Time-resolved nanoparticle delivery to the cellular surface due to gravitational settling and diffusion was simulated by *In vitro* Sedimentation, Diffusion and Dosimetry model (ISDD), version ISDD_GUI_7_30_2015 (Hinderliter *et al.,* 2010. ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for *in vitro* toxicity studies. *Part. Fibre Toxicol.* **7**:36). Delivered dose was calculated for every hour during the maximum applied incubation time of 24 h. Diameter method was selected to calculate agglomerate properties with default values for Fractal Dimensions and Packing Factor.

Supplementary figures/tables

Table S1: Replacement of nanoparticle (NP) bound proteins by components of serum-containing cell culture media

NP	protein	protein content on NP (% of untreated)		
		untreated	**1 h**	**24 h**
TiO_2	**GFP**	100 ± 3	40 ± 11	31 ± 11
	Bet v 1.0101	100 ± 8	66 ± 8	53 ± 5
	Bet v 1.0102	100 ± 18	132 ± 25	140 ± 20
SiO_2	**GFP**	100 ± 24	54 ± 11	36 ± 8
	Bet v 1.0101	100 ± 60	76 ± 22	71 ± 21
	Bet v 1.0102	100 ± 51	117 ± 34	54 ± 39
	pHrhodo Bet v 1.0101	100 ± 32	49 ± 18	37 ± 10
	pHrhodo Bet v 1.0102	100 ± 17	49 ± 10	28 ± 25

350 µL TiO_2 or SiO_2 NPs (c = 0.1 mg/mL) were incubated for 24 h with the indicated proteins (c = 0.01 mg/mL) at 4 °C on a rotator. After incubation, the samples were either left untreated, or incubated for 1 or 24 h with serum containing cell culture medium (90% RPMI-1640 supplemented with 10% FCS, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM L-Glutamine). Then, the samples were centrifuged for 30 min at 18000 *g* and the protein contents on the pellets were quantified using SDS-PAGE with subsequent densitometric analysis.

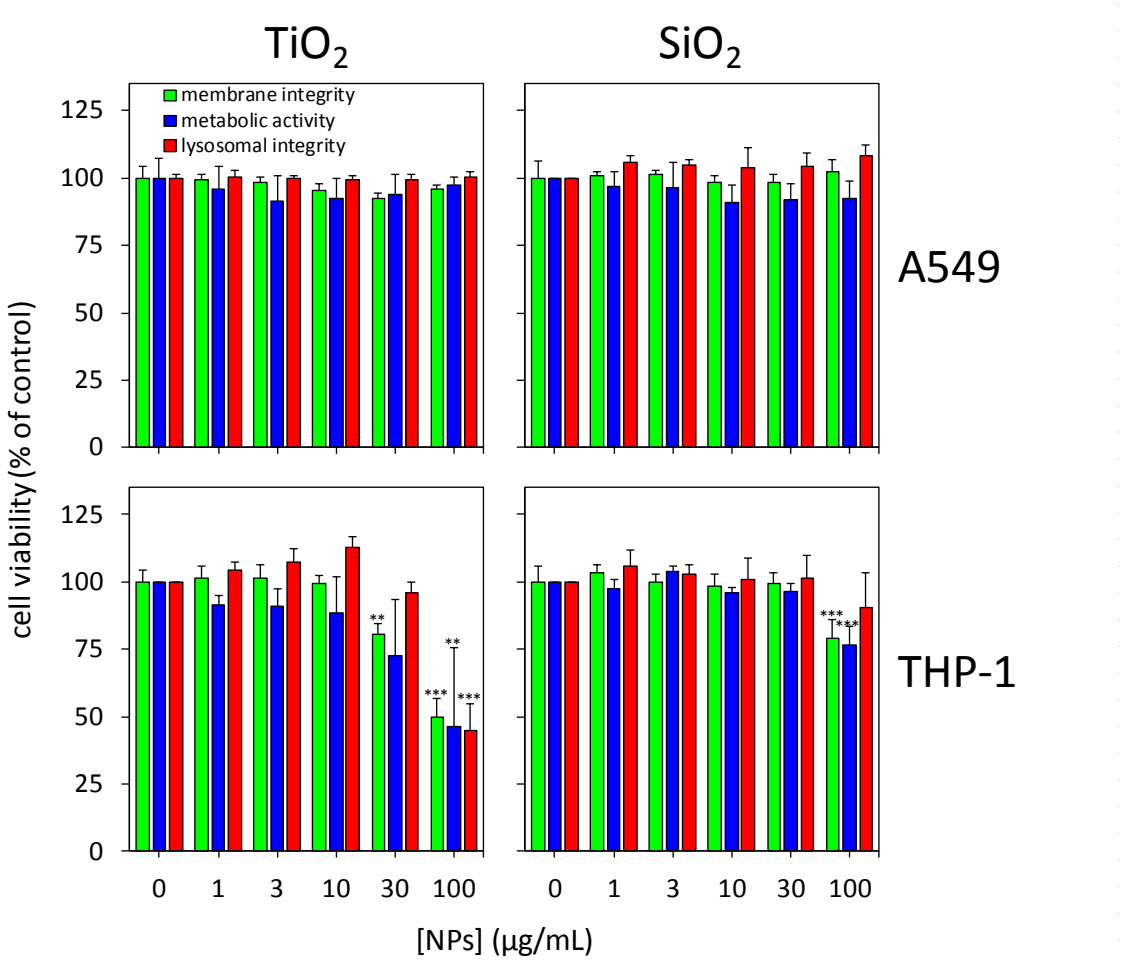

Figure S1: Cytotoxicity of TiO_2 and SiO_2 NPs on A549 and THP-1 cells. The cells were incubated for 24 h with the indicated concentrations of TiO_2 or SiO_2 NPs and the cell viability was determined using the LDH assay (membrane integrity), the CTB assay (metabolic activity) and the Neutral Red assay (lysosomal integrity). The data represent mean values ± SD of three independent experiments. Stars indicate significances of differences between control (without NPs) and NP-exposed conditions, $^{**}p < 0.01$, $^{***}p < 0.001$.

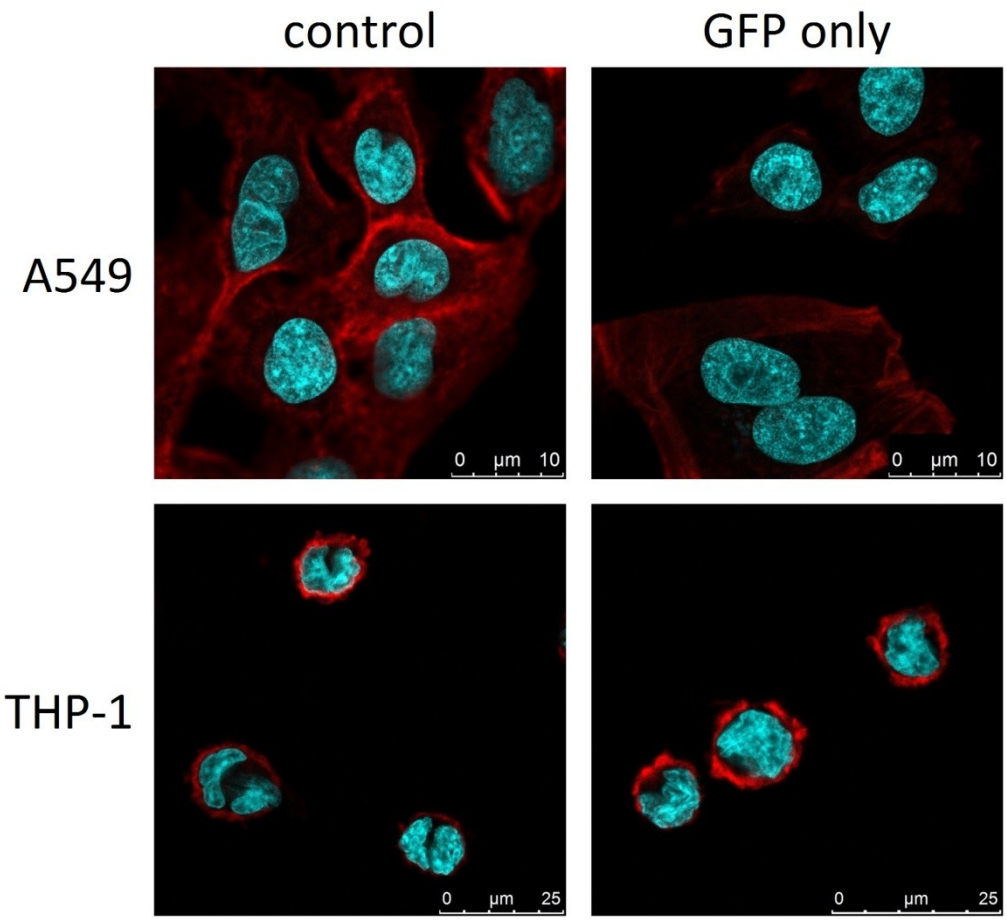

Figure S2: Confocal microscopy images of control cells or cells incubated with GFP only (without TiO_2 or SiO_2 NPs). The cells were incubated for 24 h with serum-free cell culture medium without or with 3 µg/mL recombinant GFP in the absence of TiO_2/SiO_2 NPs. After incubation, the cells were fixed, the actin cytoskeleton was stained in red using rhodamine phalloidin and the nuclei were counterstained in blue using DAPI.

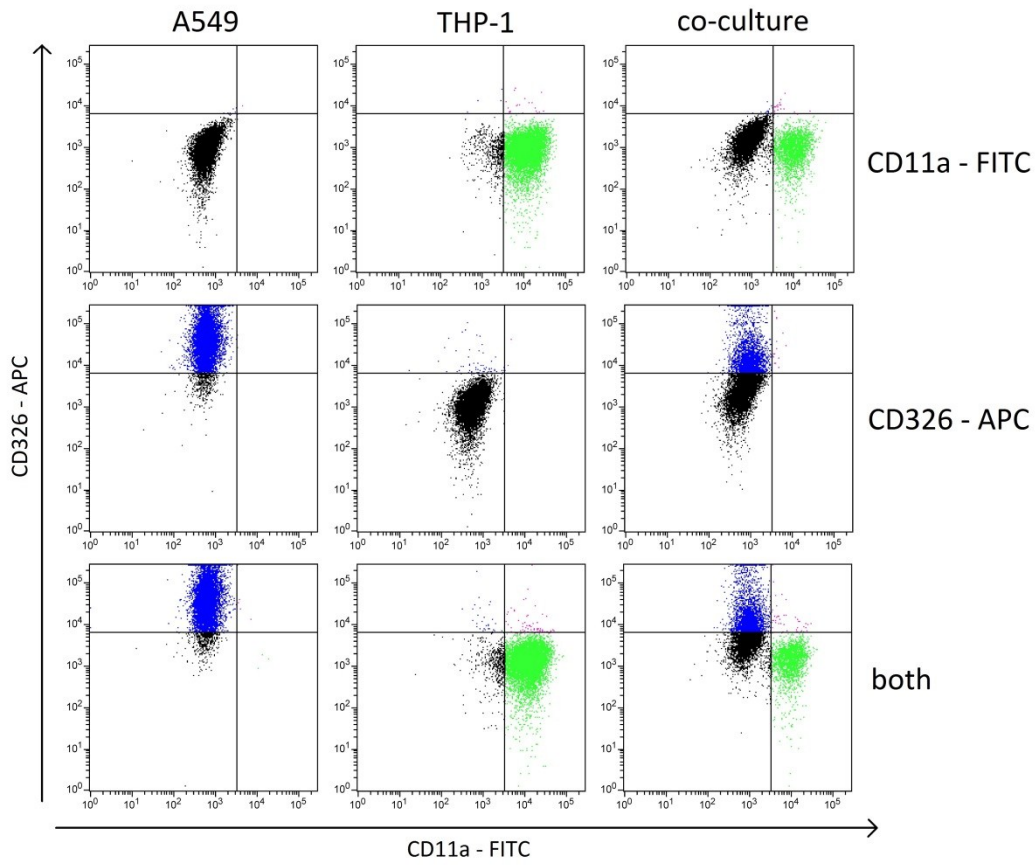

Figure S3: Characterization of A549/THP-1 co-cultures. A549/THP-1 co-cultures were grown as described in "Materials and Methods", stained with antibodies against their specific surface markers (CD11a for THP-1 cells, CD326 for A549 cells) and analyzed by flow cytometry. The data show A549 cells, THP-1 cells or co-cultures stained with CD11a, CD326 or both dyes.

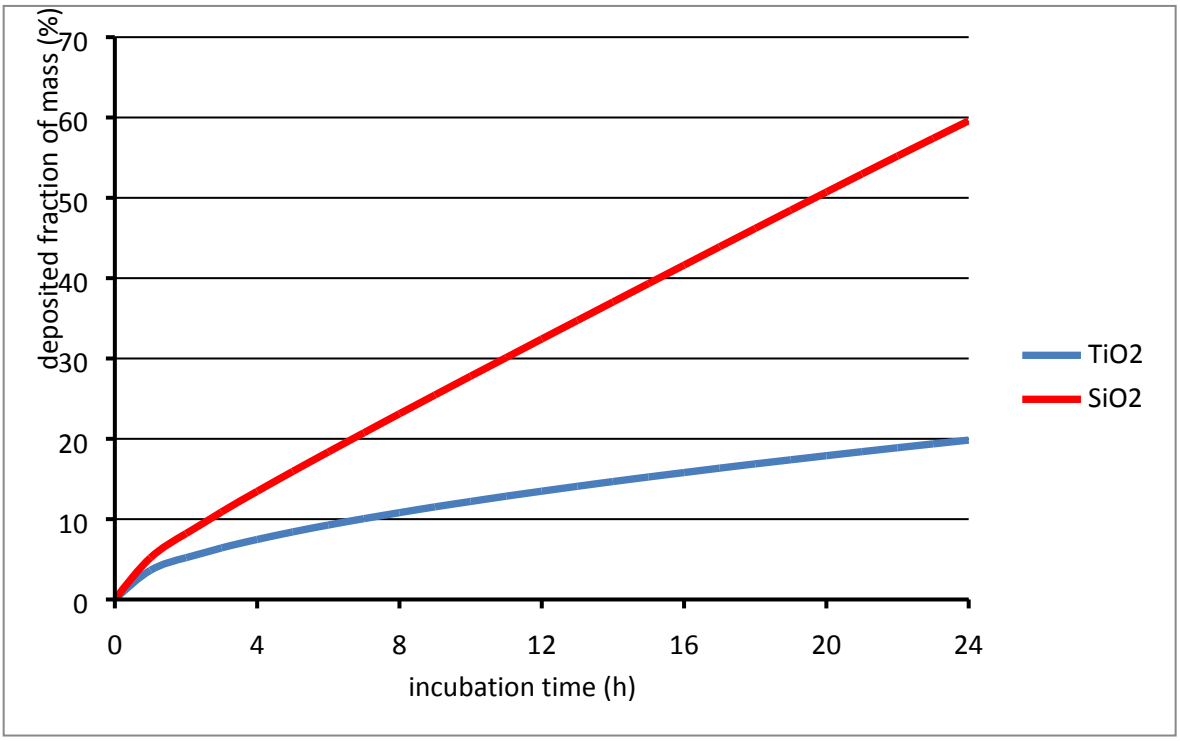

Figure S4: Time-resolved nanoparticle target cell delivery based on particle kinetics. Delivered dose in mass % was calculated from administered dose (30 µg/mL) by simulation of particle gravitational settling and diffusion by means of ISDD model. Time points were taken every hour from 1 h up to 24 h incubation. Mean diameter measured from TEM images was applied for *particle diameter* (TiO_2: 12 nm / SiO_2: 136 nm). *Agglomerate diameter* was taken from dynamic light scattering measurement of hydrodynamic particle diameter (mean values of intensity-weighted distribution) in RPMI+10% FCS media (TiO_2: 257 nm / SiO_2: 209 nm).