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Supplementary Material

Biological effects of allergen-nanoparticle conjugates: uptake and immune effects determined on hAELVi cells under submerged vs. air-liquid interface conditions

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Materials and Methods

Chemicals and reagents

Fetal calf serum was obtained from Biochrom (Berlin, Germany), Neutral Red reagent was purchased from Sigma Aldrich (Vienna, Austria) and CellTiterBlue® (CTB) reagent was purchased from Promega (Madison, WI, USA). Rhodamine phalloidin and pHrodo™ Red NHS ester were obtained from Thermo Fischer Scientific (Waltham, MA, USA). Surfactant protein A and Surfactant protein C antibodies were purchased from Bioss Antibodies (Woburn, MA, USA), Surfactant protein B antibody was purchased from antibodies-online (Aachen, Germany). All other reagents were purchased from Sigma Aldrich (Vienna, Austria) and Merck kGaA (Darmstadt, Germany) at analytical grade or higher.

SiO_2 nanoparticles

The mesoporous silica nanoparticles were prepared as previously described (Feinle et al., 2017). Briefly, tetraethyl orthosilicate (TEOS) (22.4 mmol) was and added to H_2O (heated to 80 °C) mixed with cetyltrimethylammonium bromide (CTAB) (2.7 mmol), sodium hydroxide (7 mmol) and ethanol. After 2 h stirring at 80 °C the particles were centrifuged and washed with ethanol. A solution of hydrochloric acid (HCl) in tetrahydofuran (THF) was used to extract the CTAB. The resulting particles were stirred for 12 h under reflux conditions after adding HCl solution in THF. Afterwards the particles were dried at 80 °C.

SiO_2 nanoparticle quantification

For the quantification of the Si content of SiO_2 NPs in solution the previously published blue silicomolybdic assay was used (Coradin et al., 2004) and adapted for microtiter plates. Briefly, the SiO_2 NPs were digested in 0.5 M NaOH at 95 °C for one hour in order to obtain elemental silica. Afterwards the samples were diluted with pure water 1:15 and 320 µl of diluted samples were incubated for 10 min with 30 µl of a solution containing 0.016 M ammonium molybdate tetrahydrate and 0.7 M hydrochloric acid in pure water. Following that 150 µl of a solution containing 0.22 M oxalic acid, 0.019 M 4-methylaminophenol sulphate, 0.033 M anhydrous sodium sulphite and 1.8 M concentrated sulphuric acid pure water was added, and the mixture was incubated for 2 h. The optical density (OD) of the samples was measured at λ = 810 nm. Silicon contents were calculated by comparing OD values from samples with OD values from Si standards with known concentrations.

NP suspension stability analysis

For testing the stability of the SiO_2 NP suspension, NPs were diluted to a final concentration of 100 µg/ml dispersed in a total volume of 5 ml using distilled water, serum-free or serum-containing huAEC media. After the designated time points 200 µl sample was taken from the top 5 mm section of the suspension and the silica content was determined as described above. For dissolution control, 200 µl were taken as described above, and centrifuged at 16.000 x *g* for 30 min. The supernatant was used in the silica assay as described above without digestion in NaOH. The values obtained for the dissolved control were subtracted from the values from the samples.

Protein labelling

The allergen was incubated with a 10-fold molar excess of pHrodo™ Red NHS ester or FITC for 60 min at 37 °C on a shaker protected from light, the labelled protein was then purified using illustra NAP-5 column (GE-Healthcare, Zipf, Austria). To elute the labelled protein, 5 mM sodium phosphate buffer (pH 7.5) was used. The protein content was determined using the Lowry assay which was performed according to the protocol described by Lowry et al. 1951 (Lowry et al., 1951) using purified recombinant Bet v 1 protein as reference. The protein-containing fractions were pooled and stored at 4 °C until further use.

SiO_2 NP Bet v 1 coupling

Bet v 1 was coupled to SiO_2 NPs at a ratio of 5 µg/ml protein to 50 µg/ml SiO_2 NPs. The mixture was couples in milliQ water for at least 6 hours at 4 °C under agitation on a rotational wheel. The ratio was chosen to limit unbound Bet v 1 by overloading the SiO_2 NPs with protein.

DMP/O droplet test

The presence of pulmonary surfactant was determined *via* the DMP/O droplet test, which uses the physical property of the DMP/O to form a stable droplet in the presence of surfactant at the surface of the cell layer. DMP/O is a mixture of dimethylphtalate and normal octanol in a 4:1 (v/v) ratio. 4 mg/ml crystal violet was added for optical determination (Schürch et al., 1978). For the test the cells were switched to ALI conditions at least 24 h before the experiment. The test was performed by carefully dropping 20 µl of DMP/O on the cell layer and then taking a picture of the formed drop.

Cell viability

For the CTB assay, which measures the cell metabolism by conversion of resazurin to resorufin, the cells were incubated with 500 µl CTB reagent (1:10 diluted in CCM) for one hour at 37°C. Fluorescence of the supernatant was measured using an excitation wavelength of 560 nm and emission wavelength of 590 nm. Following the CTB assay the cells were washed once with 1 ml PBS and incubated with 500 µl NR reagent (1:80 diluted in CCM) for one hour at 37°C. The NR assay measures the uptake of the NR dye by viable cells. After incubation the supernatant was removed, the cells were washed once with 1 ml PBS and incubated with 500 µl NR extraction solution (50% ethanol absolute, 1% acetic acid, 49% pure water) on an orbital shaker for 10 min at room temperature protected from light. Absorption of extracts was measured at 540 nm with a reference wavelength of 690 nm. Cell viability was calculated as percentage viability compared to control cells (treated with CCM only).

Cell culture for THP-1

THP-1 cells were used as a positive control for the determination of the presence of the Toll-like receptor 4 (TLR4) on the surface of the hAELVi cells. THP-1 cells were seeded into 24-well plates at a density of 1.5×10^5 cells. To differentiate the cells to their macrophage-like state 20 ng of phorbol-12-myristate-13-acetate (PMA) were added to each well directly after seeding. The cells were incubated for differentiation for 48 hours.

Trans-epithelial resistance

The Trans-epithelial resistance (TEER) was measured to determine the epithelial barrier formation of the hAELVi cells over time. Cells were seeded in transparent 24-well ThinCert™ cell culture insert (Greiner, Kremsmünster, Austria) with a pore diameter of 0.4 µm at a density of $5x10^4$ cells per inserts and grown for 23 days. TEER values were monitored daily followed by a change of cell culture medium. The epithelial resistance was measured three times for each transwell using a chopstick electrode STX3 (World Precision Instruments, Sarasota, United States of America) connected to an Epithelial Volt/Ohm meter EVOM2 (World Precision Instruments, Sarasota, United States of America).

Confocal microscopy

After experimental incubation, the cells were washed two times with 500 µl PBS and fixed using 250 µl 4% paraformaldehyde (PFA) for 10 min at RT. Afterwards the cells were rinsed with 500 µl PBS three times for 5 min and then incubated with 250 µl 0.1 % Triton X-100 for 10 min at RT for permeabilization. Following that the cells were washed thrice with 500 µl PBS and then incubated with 250 µl 10% normal goat serum (NGS; #16210-064, Life Technologies, Carlsbad, CA, USA) in 0.1% Triton X-100 for 2 hours at RT to block nonspecific binding sites. Afterwards the cells were washed again three times with 500 µl PBS. For surfactant staining cells were incubated for 2 h at room temperature with 250 µl primary antibody (*i.e.* anti-human surfactant protein A, B, and C diluted 1:200 PBS containing 1% normal goat serum and 0.01% Triton X). The cells were then washed two times with PBS and incubated with 250 µl secondary antibody (diluted 1:200 PBS containing 1% normal goat serum and 0.01% Triton X) for 45 min at room temperature. Next the cells were washed again two times and the actin staining was performed for 30 min at 37 °C in 250 µl staining solution (1:100 rhodamine/phalloidin in PBS containing 1% normal goat serum and 0.01% Triton X). After final washing steps (three times for 5 min), the transwell membranes were cut from the plastic holder using a scalpel and transferred onto a drop of Roti®-Mount FluorCare DAPI (Carl Roth, Karlsruhe, Germany) mounting solution sitting on a microscopy slide. The membrane was then covered by a cover slip and stored at 4 °C, protected from light. For the confocal microscopy FITC was used as label not pHrodo, since due to the permeabilization the acidic pH inside the lysosome was lost and no pHrodo signal can be observed.

Flow cytometry analysis of TLR4

For investigation of the presence of TLR4 the cells were detached from the 24-well plate using 100 µl of Trypsin/EDTA. Next, 900 µL of PBS was added to each well and the detached cells were transferred into FACS tubes for centrifugation at 250 x *g* for 5 min. Afterwards the supernatant was removed and the cell pellets were washed with 1 mL of PBS followed by an additional centrifugation for 5 min at 250 x *g*. The supernatant was removed and the cells were stained with a FITC-labelled antibody against human TLR4 for 30 min at 4 °C in the dark. After the staining, cells were washed and the pellet was resuspended in 300 µL PBS containing 3 mM EDTA. The flow cytometry analysis was performed on a FACSCanto II (BD Biosciences, San Jose, United States of America) by measuring the median fluorescence intensity of the samples.

mRNA preparation and cDNA transcription

For mRNA analysis, the cells were lysed for 5 min using 500 µl Tri-Reagent (Sigma), transferred into 1.5 ml reaction tubes and frozen at -80 °C until analysis. On the day of RNA isolation, cells were thawed, 100 µl chloroform was added and the tubes were vortexed and incubated on ice for 3 – 5 min. After centrifugation for 15 min at 4 °C and 16 000 x *g*, 200 µl of the supernatant were transferred to a new 1.5 ml reaction tube containing 5 µg linear polyacrylamide. The samples were vortexed and 250 µl isopropanol were added. The tubes were again vortexed and incubated at RT for 10 – 15 min. After centrifugation for 10 min at 16 000 x *g* and 4 °C the isopropanol was removed, and the pellet was washed with 70% EtOH and centrifuged for 5 min at 16 000 x *g* and 4 °C. The supernatant was removed, and the pellet was dried. A volume of 25 µl of RNase-free water was added to the dried pellet to let dissolve for 1.5 hours at 4 °C.

To measure the RNA content the sample was heated to 65 °C for 5 min to break up the loops and then the absorbance was measured at 260 nm and 280 nm.

Between 2 and 4 µg of RNA were used for cDNA generation. The appropriate amount of RNA was transferred to a PCR tube and mixed with RNase-free water, if necessary to achieve a final volume of 20 µL. To the 20 µl RNA 1 µl of Oligo dT-Primer was added and the sample was heated to 65 °C for 7 min. 9 µl of a master mix containing 6.5 µL RT 5x Buffer, 2 µL dNTPs and 0.5 µL RevertAid H Minus M-MulV reverse transcriptase (Thermo Scientific), were added to the RNA and the samples were heated to 42 °C for 80 min. Afterwards the RNA was hydrolysed at 70 °C for 10 min with 0.1 M NaOH. The samples were neutralised with 0.1 M HCl and stored at -20 °C. To calculate the relative gene expression (x) the formula $x = 2^{-\Delta\Delta ct}$ (Livak and Schmittgen, 2001) was used. The melting curve for the PCR products was recorded to monitor the specificity of the PCRs.

Quantitative real time PCR

The sample preparation for the quantitative real time PCR (qPCR) was performed by mixing 1 µl of cDNA with 4 µl of nuclease free H_2O per primer and 0.3 µl of primer mix (forward and reverse primer) and 5 µl iQ SYBR® Green enzyme mix (Bio-Rad Laboratories, Inc., Hercules, USA) per different cDNA. From the cDNA mix 4.7 µl were added to a PCR tube together with 5.3 µl of primer-enzyme mix. For qPCR a Rotorgene 3000 (Corbett Research) was used. The program for the qPCR was 10 min denaturation at 95 °C followed by 40 cycles of 10 sec at 95 °C, 15 sec at 65 °C and 30 sec at 72 °C, followed by a final melting step ramping from 70 to 95 °C in 0.5 °C increments.

Supplementary results

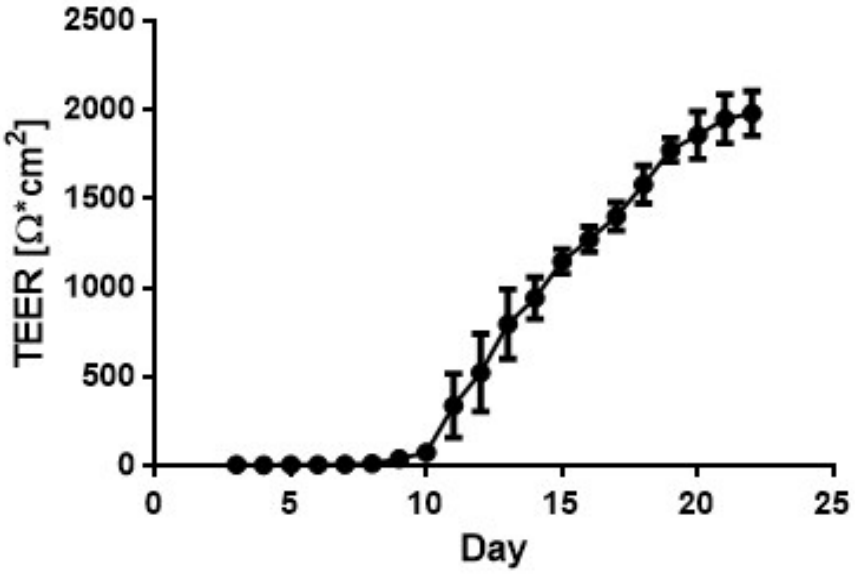

Figure S1: TEER values of hAELVi cells grown for up to 23 days on transwell inserts. Data represent three individual repeats.

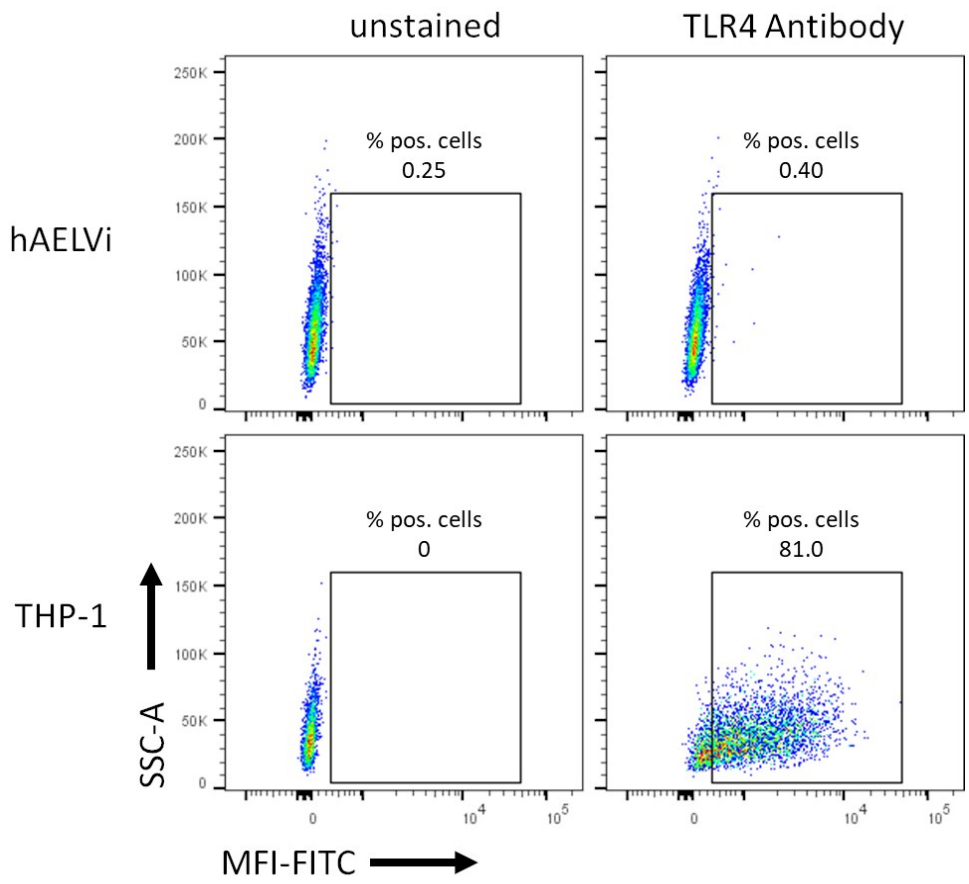

Figure S2: TLR4 surface staining on THP-1 and hAELVi cells. To determine the presence of TLR4 on the surface of hAELVi cells a FITC-labelled antibody against human TLR4 was employed. As positive control, THP-1 cells were used since they express TLR4 on their surface as typical for immune cells. From the flow cytometry data a clear difference between the hAELVi and the THP-1 samples were observed (right panel). hALEVi cells resulted in 0.4 % TLR4 positive with a MFI of 137, while THP-1 were 81 % positive with a MFI of 1527 (Fig. S2).

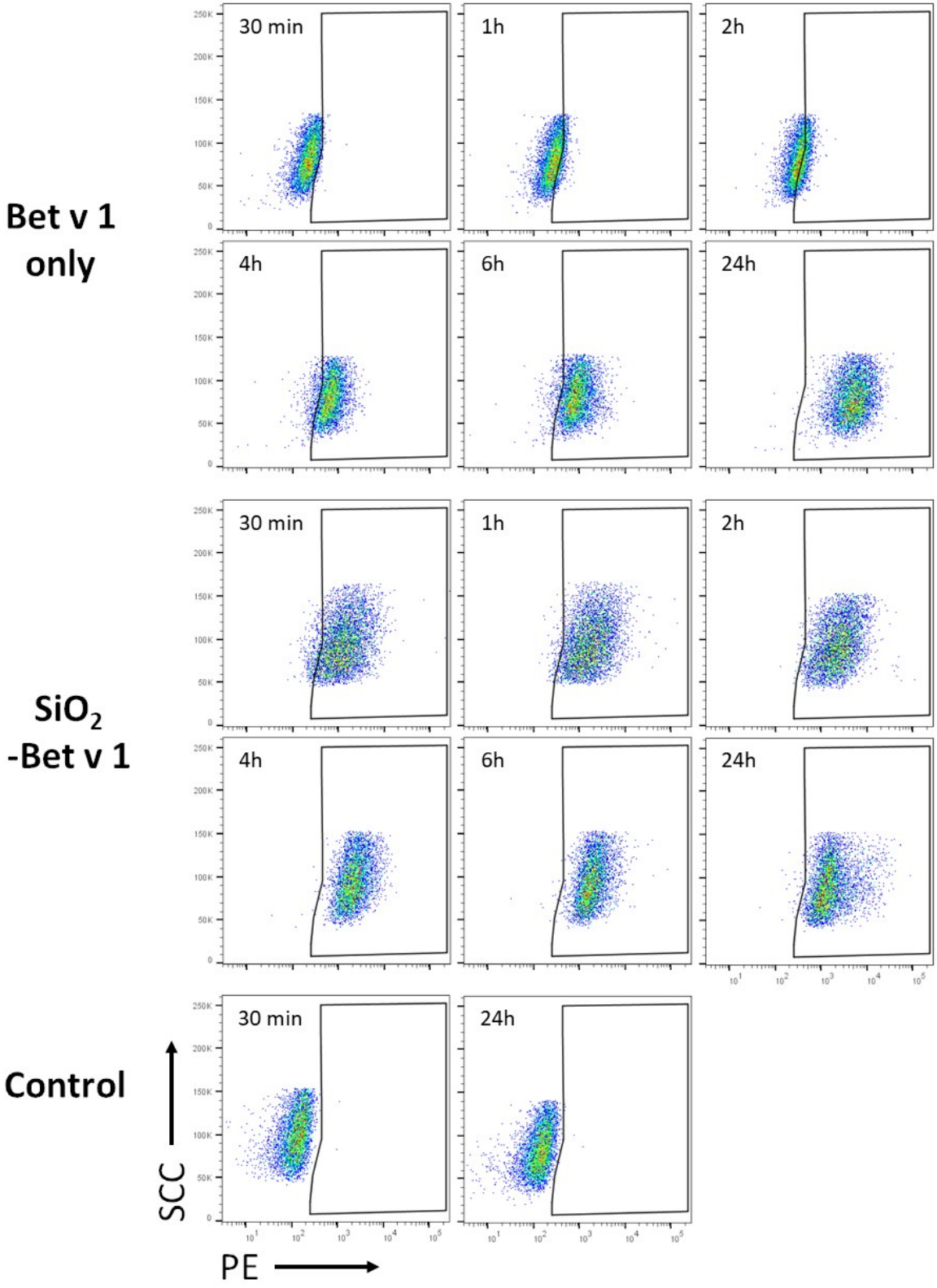

Figure S3: Time-resolved uptake of free Bet v 1-pHrodo and SiO_2 NP-bound Bet v 1-pHrodo measured using flow cytometry for cells that were seeded onto transwell inserts and exposed under submerged conditions. Dot plots of hAELVi cells, control: cells without NPs or Bet v 1, Bet v 1 only: cells incubated with labelled Bet v 1 only, SiO_2 Bet v 1: cells incubated with NP-bound labelled allergen.

Table S1: Primer sequences for real time PCR

Target	Sequence
IL-1α fwd.	5’- AAGCAAAGGGGTGAATAAATGAACCAA -3’
IL-1α rev.	5’- GTGGCTACAAGTGCGTCGTCAAAAC -3’
IL-1β fwd.	5’- GTACCTGAGCTCGCCAGTGA -3’
IL-1β rev.	5’- TCGGAGATTCGTAGCTGGATG -3’
IL-8 fwd.	5’- CCAGGAAGAAACCACCGGAAG -3’
IL-8 rev.	5’- TGGTCCACTCTCAATCACTCTCAG -3’
IL-25 fwd.	5'- GGAGCGACCCAGATTAGGTGAGGA -3'
IL-25 rev.	5'- CTAGGGGAGGCACAGGCACAGT -3'
IL-37 fwd.	5' - CCCAAGCCTCCCCACCATGAATTTT - 3'
IL-37 rev.	5' - GGGCGTATGTAGTTTTTATCTGGAACTGCTATGAG - 3'
TSLP fwd.	5’- TATGAGTGGGACCAAAAGTACCGAGTTC -3’
TSLP rev.	5’- TCCGAATAGCCTGGGCACCAGATA -3’
TNF-α fwd.	5’-TTGAGGGTTTGCTACAACATGGG-3’
TNF-α Rev.	5’-GCTGCACTTTGGAGTGATCG-3’

Table S2: Uptake inhibitors

Inhibitor	**Mechanism/Inhibition**	**Pre-incubation time**	**Concentration (in well)**	**Viability of control**	**Ref.**
Chlorpromazin hydrochloride (CPZ)	Clathrin	30 min	5 µg/ml	87.7 ± 6.3 %	(Hsiao et al., 2014)
Methyl-β-cyclodextrin (MβCD)	Clathrin/Calveolin	30 min	6.65 mg/ml	41.6 ± 7.4 %	(Kuhn et al., 2014)
Cytochalasin D (CytoD)	Phagocytosis/Macropinocytosis	90 min	10 µM	74.7 ± 6.0 %	(Kuhn et al., 2014)
Nocodazol (Noco)	Macropinocytosis	30 min	12.5 µg/ml	76.0 ± 6.6 %	(Bannunah et al., 2014)

References

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