ONLINE DATA SUPPLEMENT 1

Validation of *in vitro* models for smoke exposure of primary human bronchial epithelial cells

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Figure S1. Schematic illustration of Air-Liquid Interface Cigarette Smoke Exposure (ALICE-Smoke) system. Upon the end of differentiation, inserts with phBECs were washed once with pre-warmed HBSS and transferred into the lower exposure chamber. After tightly assembling the lower chamber with the manifold, ALICE-Smoke was placed into incubator chamber set at temperature of 37°C. Along with ALICE-Smoke, a humidifying bottle was put inside the incubator. After lighting up the cigarette, with the flow rate set to 0.6 L/min, the smoke passes through the humidifying bottle, then into the manifold, and next, through individual tubing onto each insert in the lower exposure chamber. Subsequently, the smoke leaves the lower exposure chamber through the outlet tubing and finally, over 95% of the cigarette smoke is deposited onto the quartz filter in the quartz filter chamber. The flow rate is controlled by a flow meter located downstream of the quartz filter and the vacuum pressure is created by a vacuum pump. Figure was created with BioRender.com.

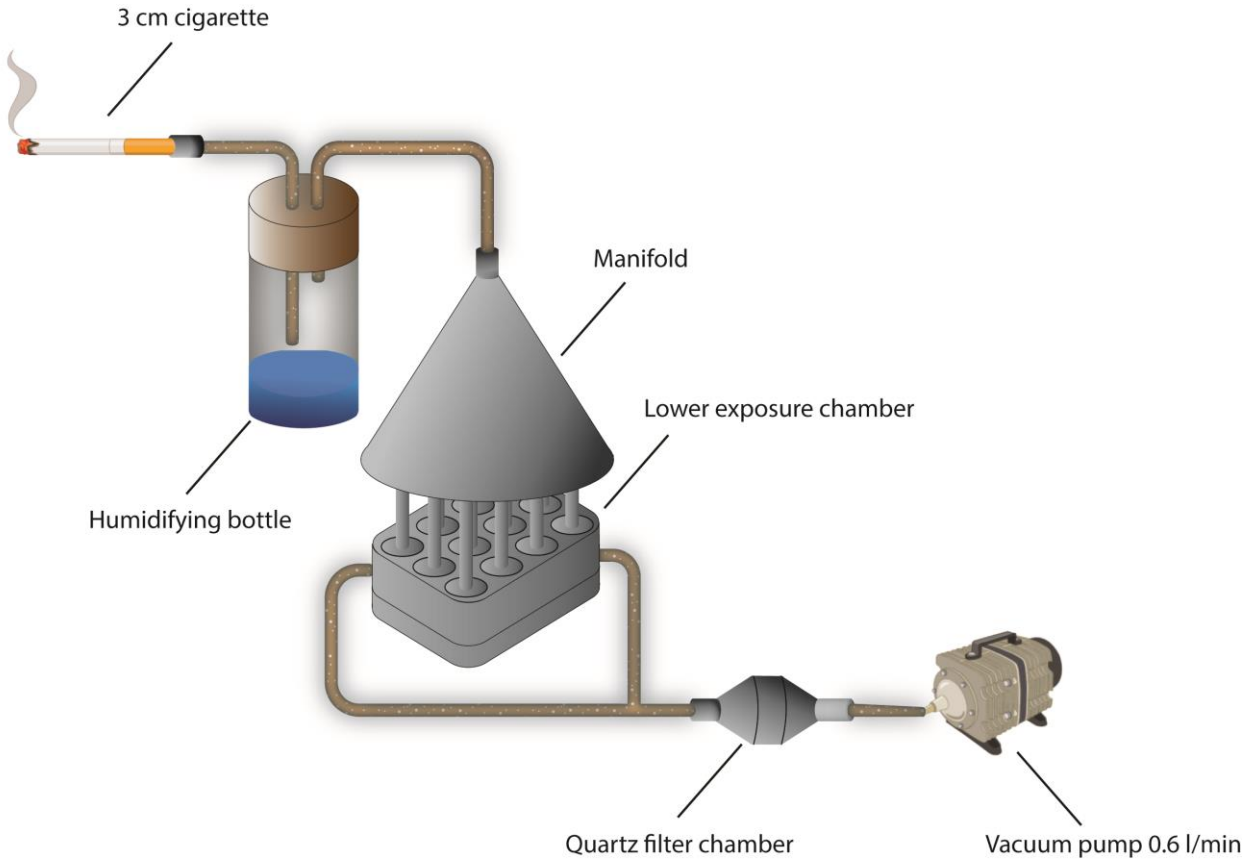


Figure S2. Threshold Cycles (C_t) comparison between AhR-responsive SERGs. C_t values of appropriate genes were pooled together from all experiments (n=25) and are displayed as violin plots. For statistical analysis, Friedman test with Dunn's correction for multiple comparisons was used.

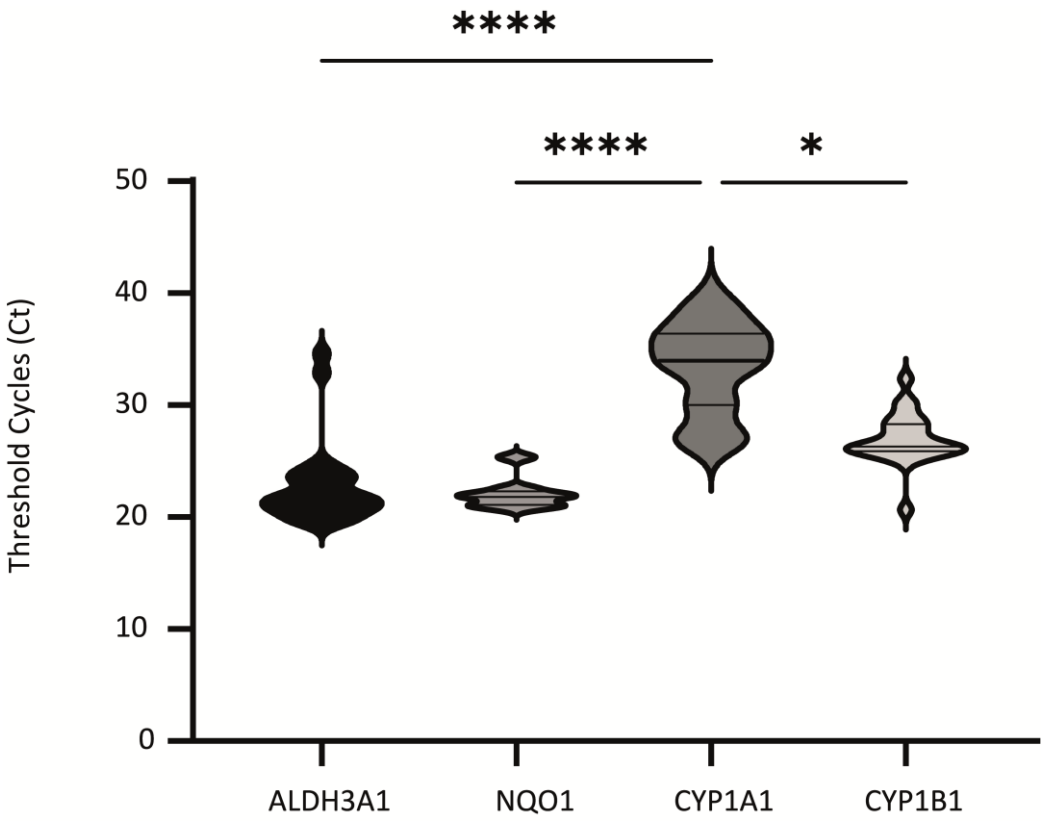


Figure S3. Threshold Cycles (C_t) comparison of basal expression of smoke exposure regulated genes (SERGs) between never-smokers and ex-smokers. C_t values of all SERGs were pooled together from every donor (values from mock-treated fully differentiated phBECs only; n=6, 3 ex-smokers, 3 never-smokers, see also supplementary Table S1). For statistical analysis, one-way ANOVA with Bonferroni correction for multiple comparisons was used. Results are shown as mean ± SD.

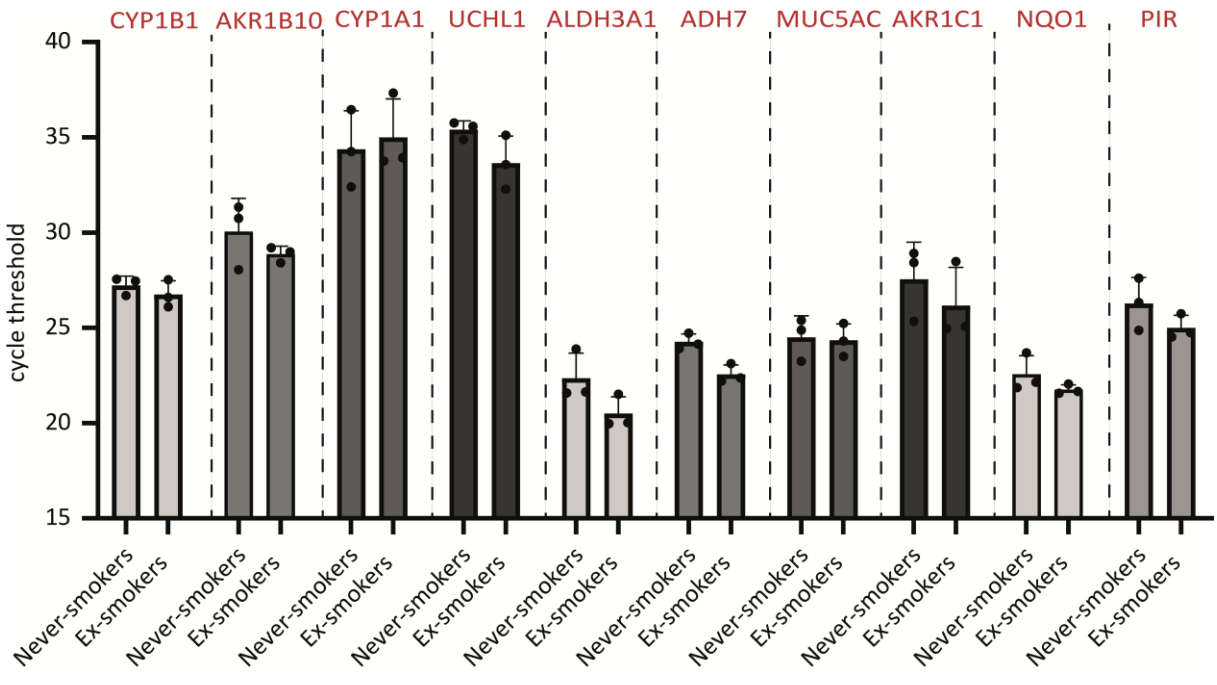


Figure S4. Gravimetric measurements yield the absolute mass of cigarette smoke particulates dissolved in cigarette smoke extract (CSE), allowing for assessment of dose. Samples of ALI (n=7 technical replicates) or BEBM (n=5 technical replicates) medium without supplements and CSE in the same media were pipetted onto quartz filter vessels and placed in a dessicator for drying. The vessels were weighed 3 times before pipetting and 3 times after the filters completely dried up, yielding the abolute weight of cigarette smoke dissolved in the medium. The difference between media and CSE was 0.28 mg and 0.25 mg for ALI and BEGM, respectively. In order to achieve the CSE concentrations, the mass was multiplied by 5, yielding 1.40 mg/ml for PneumaCult-ALI Medium and 1.25 mg/ml for BEGM media. Significance was assessed using two tailed unpaired students' *t*-test (*; $p<0.05$).

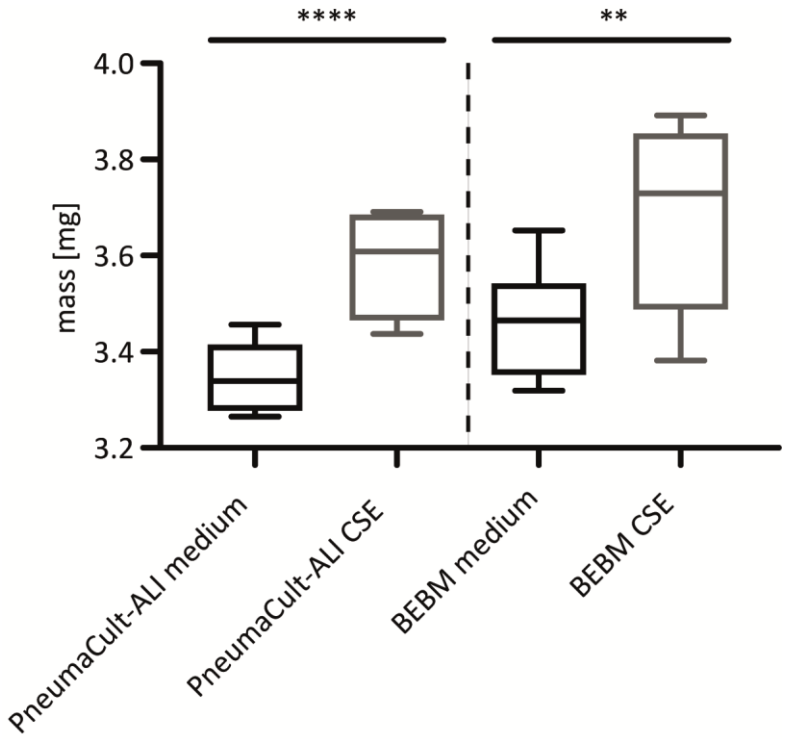


Figure S5. Assessment of cytotoxicity and epithelial integrity upon CS exposure indicates non-toxic doses of CS and CSE were used. (**A**) Lactate dehydrogenase (LDH) release for **Air Liquid Interface Cigarette smoke Exposure** (ALICE-Smoke) and short apical CSE exposure in direct comparison (n=3 donors), acute submerged basal cells CSE (n=3 independent donors) and acute apical CSE exposure, with or without prior starvation (medium with no supplements). All tests were carried out in technical triplicates. (**B**) Measurement of transepithelial electrical resistance (TEER) pre- and post-exposure for ALICE-Smoke in direct comparison with short acute apical CSE. All tests were carried out in technical triplicates, on at least 3 inserts per donor. Basolateral exposure with 5% CSE has been established previously as non-toxic (Schamberger et al., 2014; Schamberger et al., 2015). For TEER measurements in the course of basolateral exposure, please refer to supplemental Figure S7.

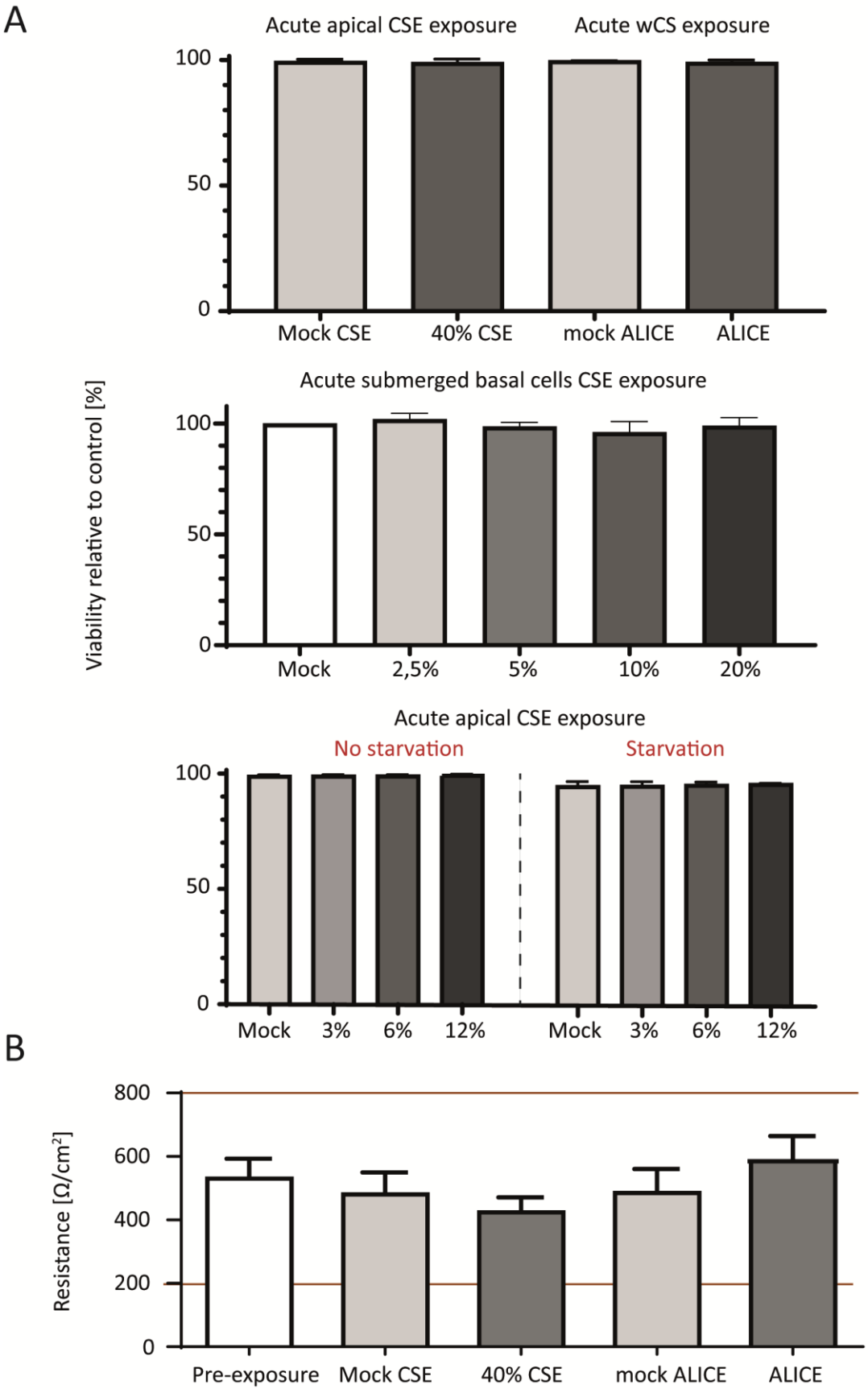
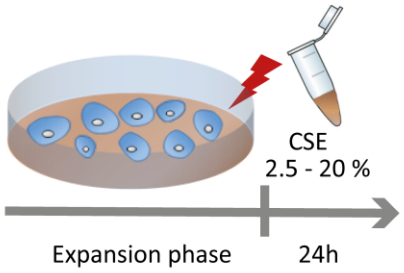
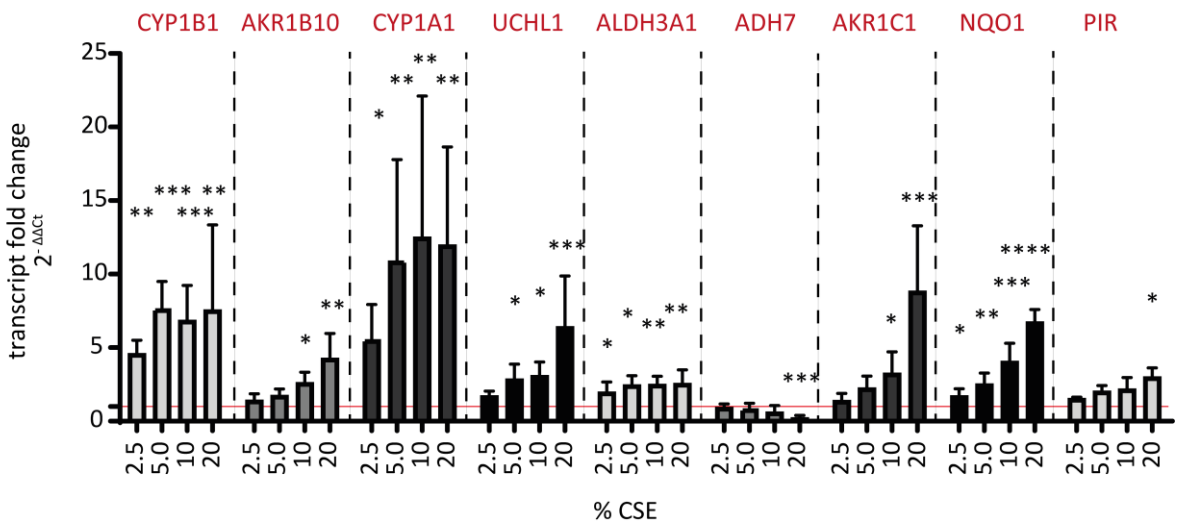

A
Acute apical CSE exposure
Acute wCS exposure
100
50
0
Mock CSE
40% CSE
mock ALICE
ALICE
Acute submerged basal cells CSE exposure
Viability relative to control [%]
100
50
0
Mock
2,5%
5%
10%
20%
Acute apical CSE exposure
No starvation
Starvation
100
50
0
Mock
3%
6%
12%
Mock
3%
6%
12%
B
Resistance [Ω/cm²]
800
600
400
200
0
Pre-exposure
Mock CSE
40% CSE
mock ALICE
ALICE

Figure S6. Acute exposure with cigarette smoke extract (CSE) of submerged basal primary human bronchial epithelial cells (phBECs, here cells of healthy donors purchased from Lonza). (A) Experimental set-up. PhBECs (n=3) were exposed to 0%, 2.5%, 5%, 10% and 20% CSE under submerged conditions for 24 h. **(B)** Results of RT-qPCR are presented as fold change of 9 genes (excluding mucin 5AC (MUC5AC) as a specific goblet cell marker) relative to control normalized to 1 (dotted line). Porphobilinogen deaminase (HMBS) was used as a housekeeper gene. Statistical analysis was assessed by one-way ANOVA followed by Bonferroni correction for multiple comparisons ($p<0.05$). (**C**) Representative Western Blots are shown for 4 assessed genes. β-actin (ACTB) was used as loading control.

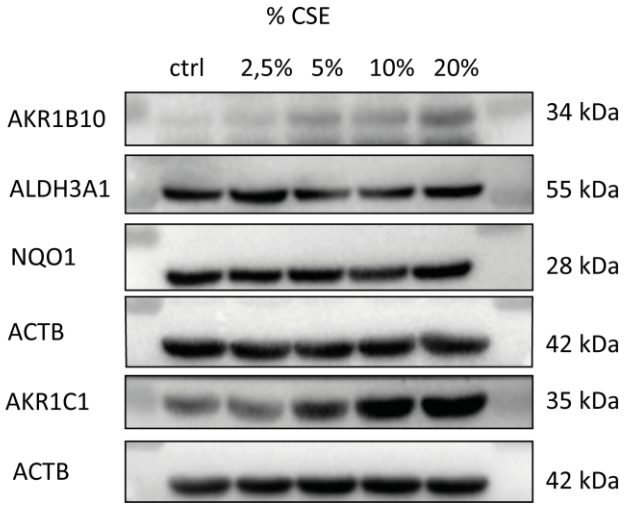
A


CSE
2.5 - 20 %
Expansion phase
24h

B


CYP1B1
AKR1B10
CYP1A1
UCHL1
ALDH3A1
ADH7
AKR1C1
NQO1
PIR
transcript fold change
2-ΔΔCt
% CSE

C


% CSE
ctrl
2,5%
5%
10%
20%
AKR1B10
34 kDa
ALDH3A1
55 kDa
NQO1
28 kDa
ACTB
42 kDa
AKR1C1
35 kDa
ACTB
42 kDa

71

72

Figure S7. Quantification of major cell types and transepithelial electrical resistance (TEER) during chronic basolateral treatment with cigarette smoke extract (CSE) during differentiation, relative to mock (see Figure 3). (**A**) Epithelial barrier integrity assessed by TEER was not significantly altered by CSE. Results shown are derived from n=5 (independent donors) and given as mean ± SD. (**B**) Quantification of all main bronchial epithelial cell types. Results shown are derived from n=4 (independent donors) and given as mean ± SD. For statistical analyses one-way ANOVA test with Bonferroni correction for multiple comparisons was used.

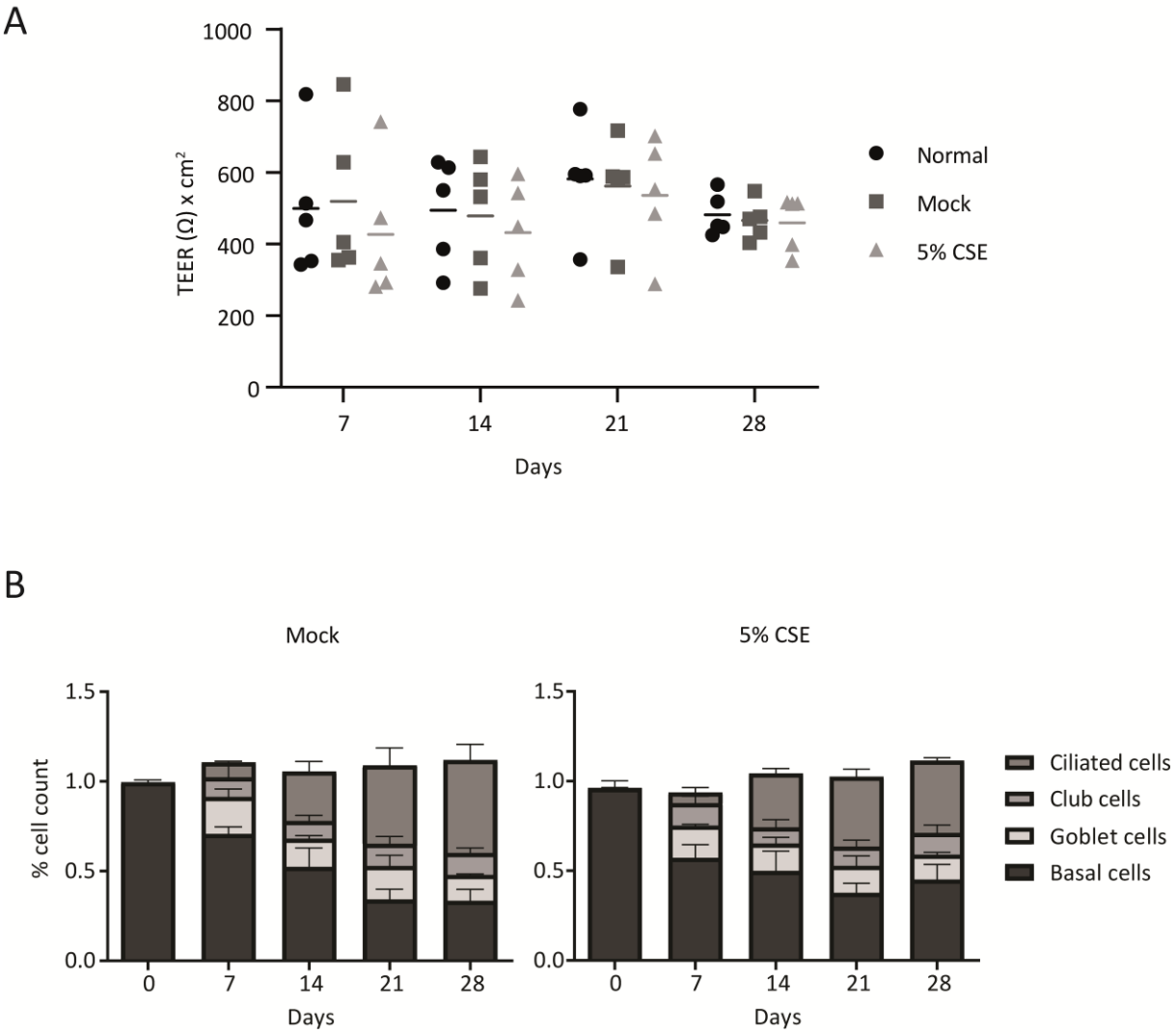


Figure S8. Acute apical cigarette smoke extract (CSE) exposure including starvation prior to treatment. (**A**) Experimental set-up. At the end of differentiation, phBECs (n=4) were exposed apically to 200µl of 0% 3%, 6%, 12% CSE for 24 h and then cells were collected for mRNA and protein analysis. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a reference gene. (**B**) Results of RT-qPCR are presented as fold change of 10 genes relative to control normalized to 1 (dotted line). Statistical analysis was assessed by one-way ANOVA test with Bonferroni correction for multiple comparisons ($p<0.05$). (**C**) Representative Western Blots are shown for 4 assessed genes. β-actin (ACTB) was used as loading control. Results shown are based on n= 4 (independent donors) and given as mean ± SD.

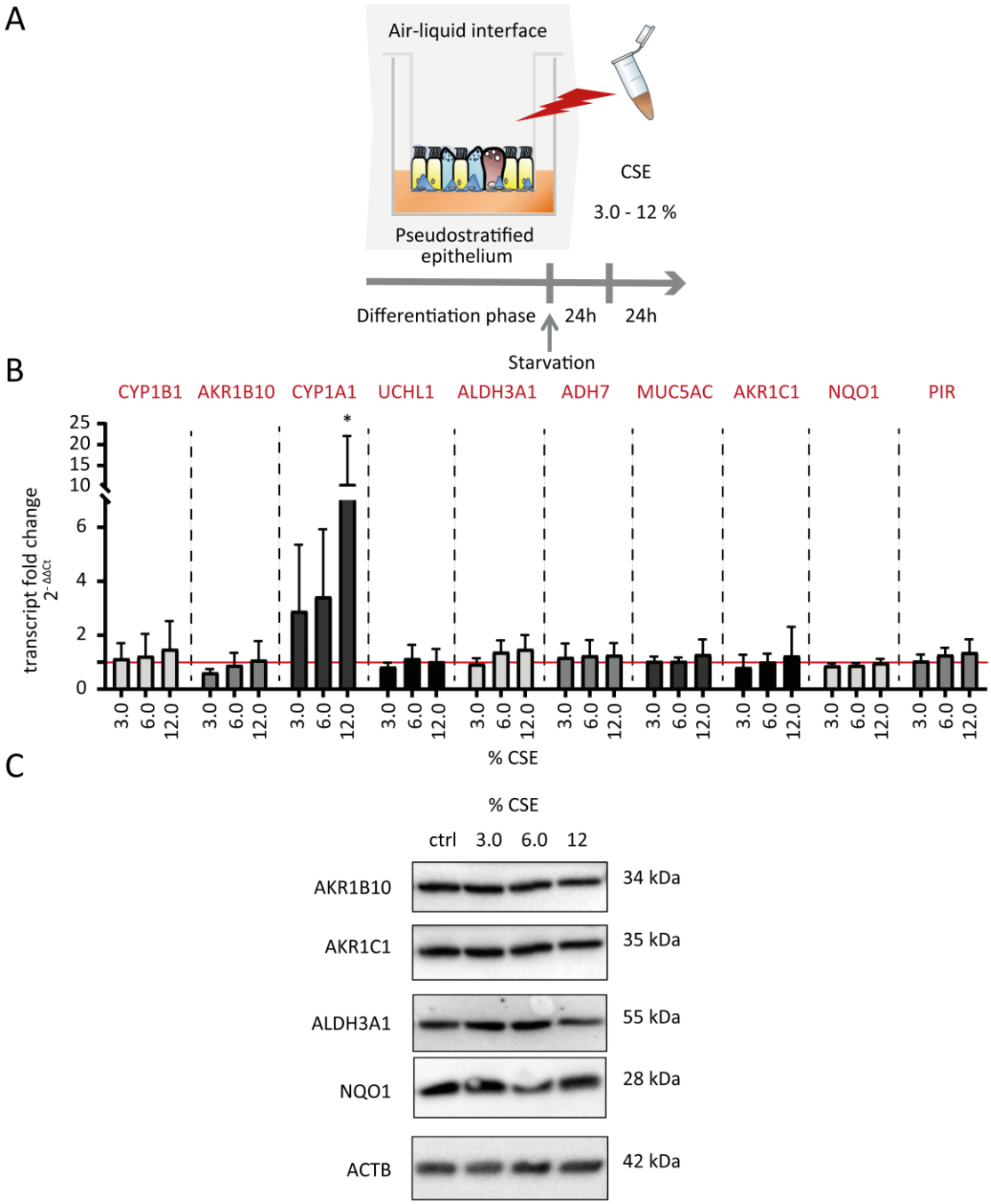

A
Air-liquid interface
CSE
3.0 - 12 %
Pseudostratified epithelium
Differentiation phase
24h
24h
Starvation
B
CYP1B1
AKR1B10
CYP1A1
UCHL1
ALDH3A1
ADH7
MUC5AC
AKR1C1
NQO1
PIR
transcript fold change
2^-ΔΔCt
25
20
15
10
6
4
2
0
*
3.0
6.0
12.0
% CSE
C
% CSE
ctrl
3.0
6.0
12
AKR1B10
34 kDa
AKR1C1
35 kDa
ALDH3A1
55 kDa
NQO1
28 kDa
ACTB
42 kDa

Figure S9. Immunofluorescent stainings with no co-expression of smoke exposure regulated genes (SERGs) with cell-type-specific markers. Representative immunofluorescent stainings (n=3) of primary human bronchial epithelial cells (phBECs) exposed to whole cigarette smoke (wCS) revealed no co-expression of AKR1C1 with acetylated tubulin, specific marker for ciliated cells, and NQO1, PIR and UCHL1 were not colocalized with uteroglobin (CC10), a club cell-specific marker. None of the SERGs were expressed in goblet cells, demonstrated by costainings with mucin 5AC (MUC5AC). To provide better overview of the stainings, pictures were enlarged by 25%**.** Scale bars, 50µm.

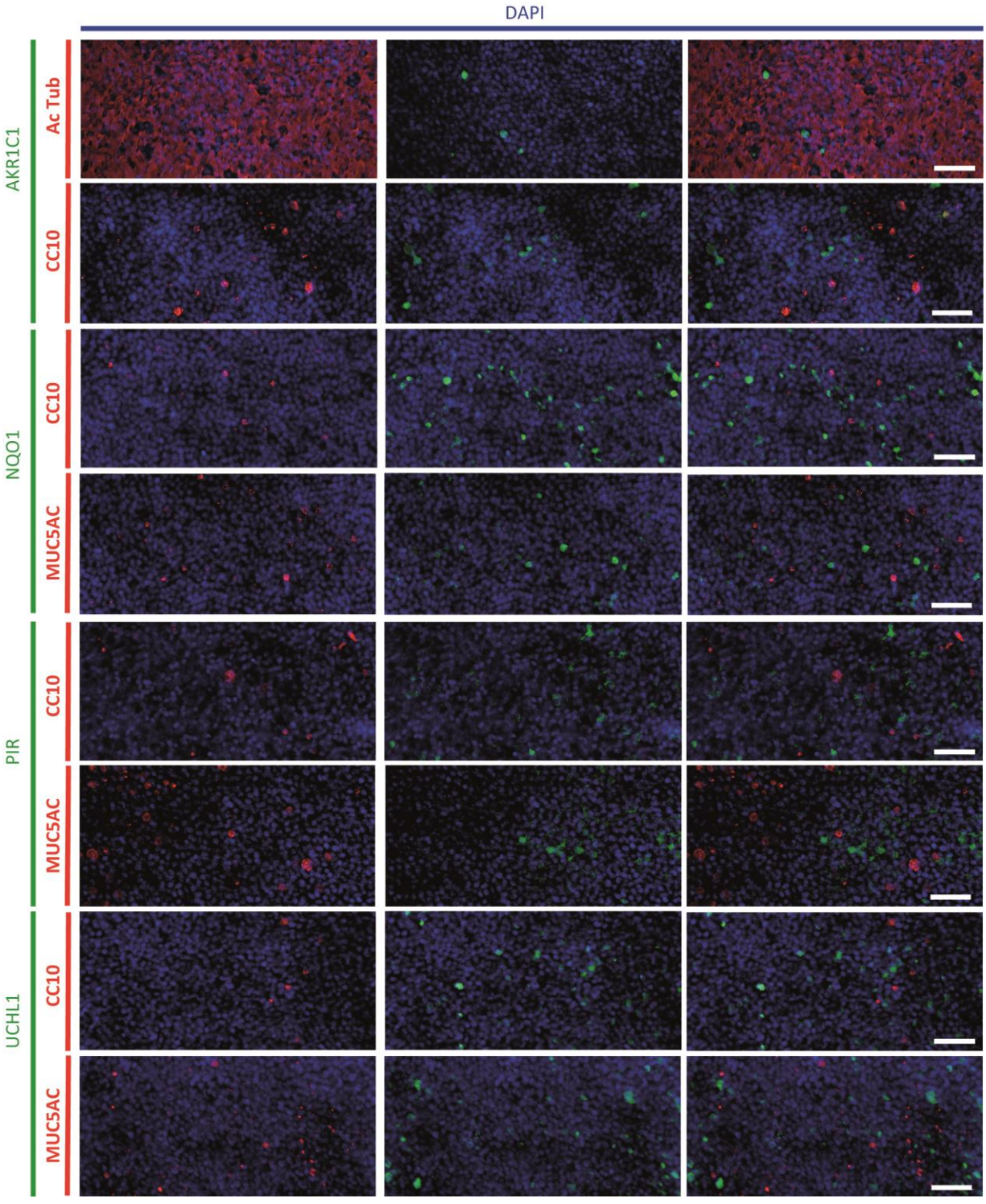


Table S1. General donor characteristics and information on smoking status.

Donor No.	Age	Gender	Smoking status	Smoking cessation period	Pack years	Experiment
1	73	M	Ex-smoker	>20 years	21-40	1, 2, 3, 4, 5, 6
2	80	W	Ex-smoker	>20 years	21-40	1, 2, 3, 4, 5, 6
3	66	W	Ex-smoker	10-20 years	21-40	1, 2, 3, 6
4	72	W	Never-smoker	n/a	n/a	4, 5, 6
5	80	M	Never-smoker	n/a	n/a	2, 3
6	74	W	Never-smoker	n/a	n/a	1, 2, 3, 4, 5, 6

List of experiments:

1 – Exposure of basal phBECs under submerged conditions

2 – Chronic basolateral exposure of differentiated phBECs

3 – Acute basolateral exposure of differentiated phBECs

4 – Acute apical exposure of differentiated phBECs with CSE

5 – Acute apical exposure of differentiated phBECs with CSE with 24h starvation

6 – Acute apical exposure of differentiated phBECs with wCS

Table S2. Primer list for qRT-PCR. Primers were synthesized by Eurofins.

Target	Forward primer (5'-3')	Reverse primer (5'-3')
AKR1C1	CTGCAGAGGTTCCTAAAA	CCTGCTCCTCATTATTGTA
PIR	GTAAGGATGGTGTGACAGTT	GTCCACCCTTTAGGGATA
CYP1B1	GCCACTATCACTGACATCT	CAGGATACCTGGTGAAGA
ADH7	GAGTGACTACAGTGAAACCA	CCAGTAATATCGCTCCTAAT
NQO1	AAGGACATCACAGGTAAACT	GAACTGGAATATCACAAGGT
CYP1A1	CTTGGACCTCTTTGGAGCTG	CGAAGGAAGAGTGTCGGAAG
IL8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT
ALDH3A1	CCTGACTACATCCTCTGTG	CCCGTAGAACTCTTTCAGT
MUC5AC	AGCAGGGTCCTCATGAAGGTGGAT	AATGAGGACCCCAGACTGGCTGAA
UCHL1	CTGAAGGGACAAGAAGTTAG	ACTGATCCATCCTCAAATC
AKR1B10	GTGTTGCAATCCTCTCAT	GGACATGAGTGGAGGTAGT
MUC5B	GCTGGAGCTGGATCCCAAAT	CTGGCGTTGTGGGCATAGA
WDR89	AGTACGTTCCATCCCAGCAATCC	AGGCCATCAGATGAACCTGAGACT
GADPH	TGACCTCAACTACATGGTTTACATG	TTGATTTTGGAGGGATCTCG
DHX8	TGACCCAGAGAAGTGGGAGA	ATCTCAAGGTCCTCATCTTCTTCA
HPRT	AAGGACCCCACGAAGTGTTG	GGCTTTGTATTTTGCTTTTCCA

Table S3. Primary antibodies used in Western Blot (WB) and Immunofluorescence (IF) analysis.

Target	Antibody, clone	Ref. number	RRID	Provider	Dillutions	
					WB	IF
NQO1	Rabbit, polyclonal	ab34173	AB_2251526	Abcam, Berlin, Germany	1:1000	1:1000
AKR1B10	mouse, monoclonal	SAB1405200	AB_10739476	Sigma Aldrich, St. Louis, USA	1:1000	
AKR1C1	rabbit, polyclonal	HPA068265	AB_2685966	Atlas Antibodies, Stockholm, Sweden	1:500	1:100
ALDH3A1	sheep, polyclonal	AF6705	AB_2847428	Rndsystems, Minneapolis, USA	1:800	
PIR	Rabbit, polyclonal	HPA000697	AB_1079621	Atlas Antibodies, Stockholm, Sweden		1:100
UCHL1	Rabbit, polyclonal	HPA005993	AB_1858560	Atlas Antibodies, Stockholm, Sweden		1:100
MUC5AC	Mouse, monoclonal	ab3649	AB_2146844	Abcam, Berlin, Germany		1:250
p63	Mouse, monoclonal	ab735	AB_305870	Abcam, Berlin, Germany		1:100
CC10	Mouse, monoclonal	Sc365992	AB_10915481	Santa Cruz, Dallas, USA		1:300
Acetylated tubulin	Mouse, monoclonal	T7451	AB_609894	Sigma Aldrich, St. Louis, USA		1:100
ACTB	HRP-conjugated anti-ACTB antibody, mouse, monoclonal	A3854	AB_262011	Sigma Aldrich, St. Louis, USA	1:40000	

Table S4. Secondary antibodies used in Western Blot (WB) and Immunofluorescence (IF) analysis.

Antibody, clone	Target	Ref. number	RRID	Provider	Dilution	Application
Rabbit Anti-Sheep IgG H&L (HRP), polyclonal	Sheep	Ab6747	AB_955453	Abcam, Berlin, Germany	1:30000	WB
Amersham ECL Rabbit IgG, HRP-linked F(ab')$_2$ fragment (from donkey), monoclonal	Rabbit	NA934	AB_772206	GE Healthcare Chicago, USA	1:50000	WB
Amersham ECL Mouse IgG, HRP-linked whole Ab (from sheep), monoclonal	Mouse	NA931	AB_772210	GE Healthcare Chicago, USA	1:50000	WB
Donkey anti-ms (red 568), polyclonal	Mouse	A10037	AB_2534013	Thermofischer	1:500	IF
Goat anti-rb (green 488), polyclonal	Rabbit	A32731	AB_2633280	Thermofischer	1:500	IF