Supplementary materials for qPCR

Sample handling

The animal experiment was performed at the Warsaw University of life Sciences (SGGW), Poland, and the RNA isolation and qPCR experiment were performed at the Norwegian Institute of Public Health in Oslo, Norway. The lung tissue samples were flash frozen in liquid nitrogen immediately after BALF sampling as described on page 7 of the submitted manuscript. Samples were kept frozen at -80°C (including transport on dry ice from Warsaw to Oslo) until homogenization and RNA isolation 6-8 months after dissection.

Nucleic acid isolation

RNA isolation was performed according to the protocol from the Allprep DNA/RNA/miRNA Universal kit (Qiagen, Germany). In compliance with the protocol the samples were treated with DNase I at room temperature for 15 minutes.

After RNA isolation, 5 µL of RNA was aliquoted for RNA quality control, whereas the rest of the RNA was immediately frozen at -80°C until reverse transcription could be performed, approximately one week after isolation.

RNA purity and yield were assessed using the NanoDrop spectrophotometer from ThermoFischer Scientific. Information regarding NanoDrop measurements of RNA is listed in Table S1. Nanodrop measurements were performed on aliquots of the isolated RNA.

Reverse transcription

Reverse transcription from RNA to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the kit protocol. RNA from all samples was diluted to 100 ng/µL, and 10 µL of diluted RNA was used for each reverse

transcription reaction. Reaction volumes and composition is listed in Table S2, and cycling conditions are listed in Table S3. The cDNA was immediately stored at -80°C. Optimal cDNA concentration was determined by doing dilution series of pooled cDNA on six (reference) genes as shown in Figure S2.

qPCR target information/qPCR oligonucleotides

The full list of investigated genes with gene symbols (and sequences where available) are listed in Table S4. Primers were purchased from Sigma Aldrich (St. Louis, MO, USA).

qPCR protocol

qPCR reactions were prepared using the Bravo Automated Liquid Handling Platform (Agilent Technologies, USA). To minimize the number of freeze-thaw cycles for the cDNA, cDNA was diluted and transferred in correct concentrations to Hard-Shell® 384-Well PCR Plates (Bio-Rad Laboratories, USA) and frozen at -20°C. For each qPCR reaction, a prepared plate of cDNA as described was thawed at 4°C, while PowerUp™ SYBR® Green Master Mix (ThermoFischer Scientific, USA) was mixed with gene specific primers in a designated hood. Both the cDNA plate and the mastermix plate were centrifuged for 1 minute at 1200 x g and 4°C, and mastermix was added to the cDNA plate using the Bravo Automated Liquid Handling Platform. After preparation, the qPCR reaction plate was sealed and briefly vortexed before centrifugation for 1 minute at 1200 x g and 4°C. The thermocycling conditions were selected based on the Tm of the specific primers; conditions for genes where both primers had Tm >60°C are listed in Table S5, and conditions for genes where one or both primers had Tm <60°C are listed in Table S6.

Reference gene stability was calculated using Normfinder, and the results are listed in Table S7A for the 7 days exposure, and in Table S7B for the 28 days exposure. Reference gene stability compared to the geometric average of the reference genes are shown in Figure S1.

Data analysis

qPCR data was processed using CFX Manager Software v. 3.1 (Bio-Rad Laboratories, USA), and Microsoft Excel 2013. Cq values were determined by single threshold in the CFX manager software. If one of two technical replicates differed by >5 cycles from the other technical replicate, as well as from the biological replicates in the same group, the deviating replicate was treated as an outlier and excluded from the rest of the analysis.

Figure S1

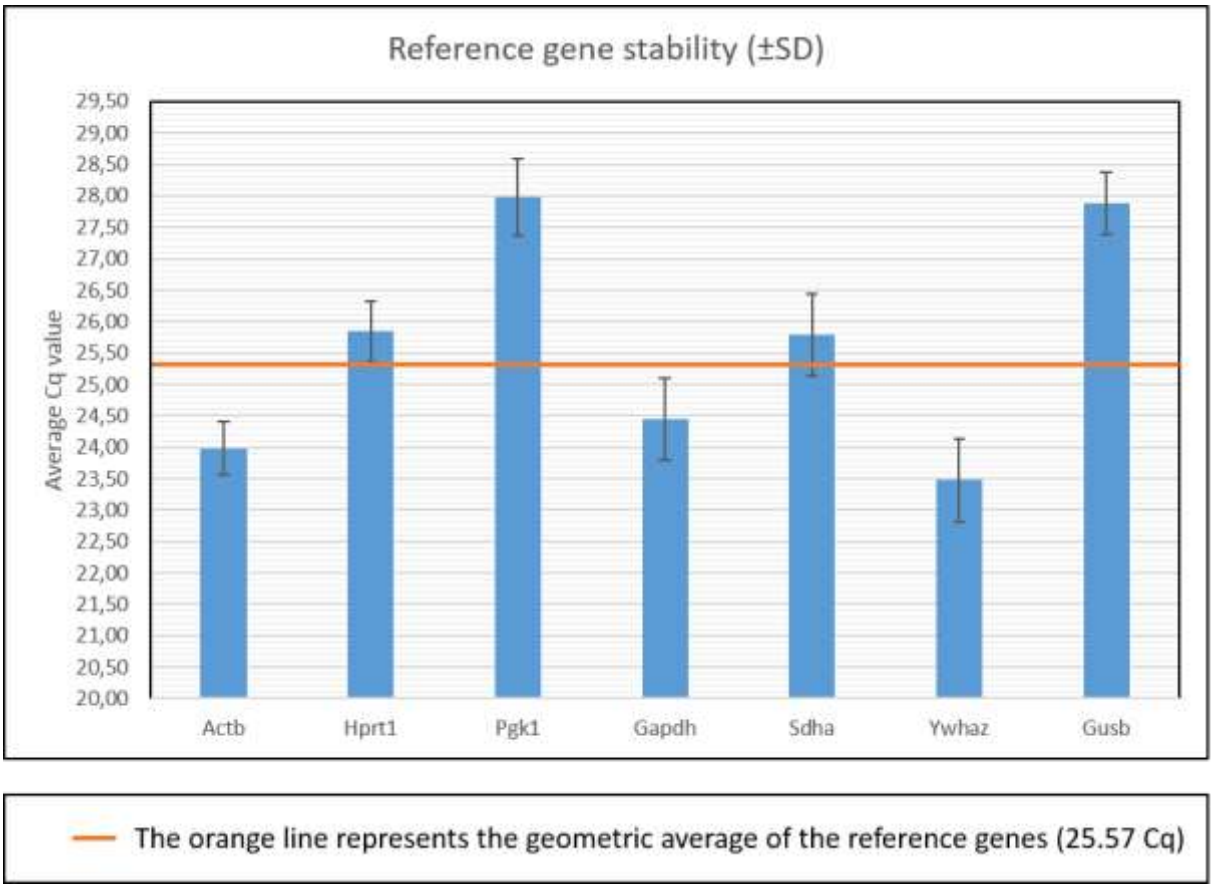

Figure S2

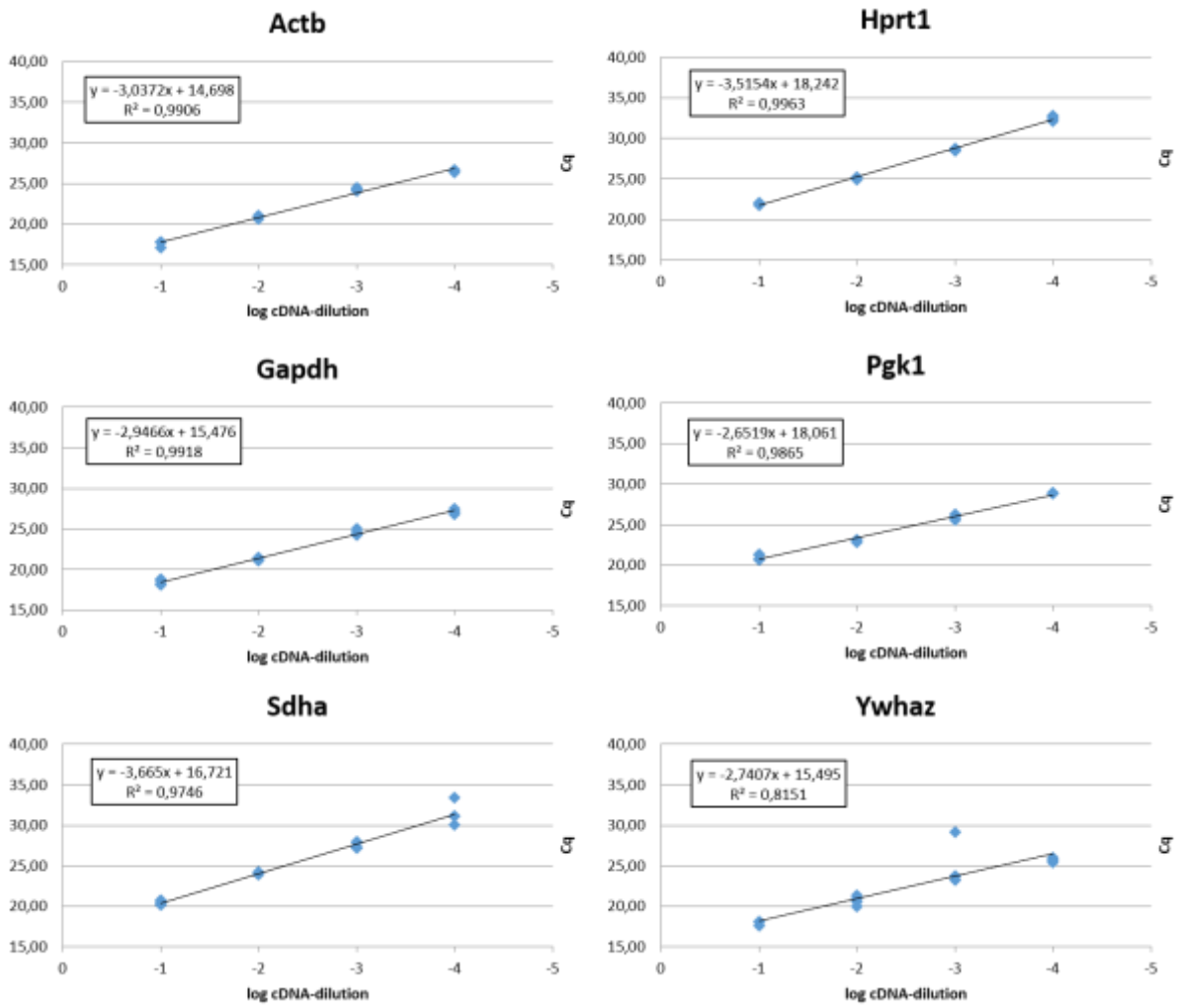

Table S1

	RNA concentration (ng/µL)	RNA OD260/280	RNA OD260/230	Total RNA (µg)	RNA yield (µg RNA/mg tissue)
Average	752,65	2,12	1,68	22,58	1,03
Median	774,85	2,13	1,76	23,25	0,98
SD	216,39	0,03	0,27	6,49	0,33
Min	207,59	2,01	0,77	6,23	0,41
Max	1272,86	2,15	2,02	38,19	2,68

Table S2

Component	**Volume (µL)/Reaction**
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Diluted RNA (100 ng/µL)	10.0
Total volume per Reaction	**20.0**

Table S3

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

Table S4

Gene symbol	Primer (L/R)	Primer sequence (5' → 3')	Tm°	Manufacturer
Actb	L			
Actb	R			
Hprt1	L			
Hprt1	R			
Pgk1	L	CTCATAAGCACAACCGACTTG	62.3	Sigma-Aldrich
Pgk1	R	GAAGCGGGTCGTGATGAG	64.7	Sigma-Aldrich
Gapdh	L	GTAACCAGGCGTCCGATAC	62.0	Sigma-Aldrich
Gapdh	R	TCTCTGCTCCTCCCTGTTC	62.2	Sigma-Aldrich
Sdha	L	CTACAACCACAGCATCAAACTC	61.2	Sigma-Aldrich
Sdha	R	GACTCGTGGCTTTCACTTCT	61.1	Sigma-Aldrich
Ywhaz	L	TCAGTGACAGACTTCATGCAG	61.8	Sigma-Aldrich
Ywhaz	R	ACCCACTCCGGACACAG	62.4	Sigma-Aldrich
Gusb	L			
Gusb	R			
Ptgs2	L	TCCTATGCTCATACATTCACCAC	62.6	Sigma-Aldrich
Ptgs2	R	CTCTTCCGAGCTGTGCTG	62.7	Sigma-Aldrich
Adipoq	L	TGGCGATTTTCTCTTCATTC	61.7	Sigma-Aldrich
Adipoq	R	AGGATTAAGAGGAACAGGAG	56.5	Sigma-Aldrich
Cyp1a1	L	AACCATGACCAGGAACTATG	58.9	Sigma-Aldrich
Cyp1a1	R	AGAATGACCTTCTCACTCAG	55.8	Sigma-Aldrich
Cyp1b1	L	CCACTATTACAGACATCTTTGG	57.7	Sigma-Aldrich
Cyp1b1	R	CATGACGTATGGTAAGTTGG	57.9	Sigma-Aldrich
Il1b	L	CTGGATGCTCTCATCTGGAC	62.5	Sigma-Aldrich
Il1b	R	AACTGTCCCTGAACTCAACTG	60.8	Sigma-Aldrich
Il3	L	CAATCTGAGGAATAGTACCTTG	57.0	Sigma-Aldrich
Il3	R	GATCTTTATTGTAGACACCTGG	56.8	Sigma-Aldrich
Il10	L	ACCCAAGTAACCCTTAAAGTCC	61.0	Sigma-Aldrich
Il10	R	GCTGCCTTCAGTCAAGTGA	61.9	Sigma-Aldrich
Il6	L	CAGAGTCATTCAGAGCAATAC	56.5	Sigma-Aldrich
Il6	R	CTTTCAAGATGAGTTGGATGG	61.1	Sigma-Aldrich
Ccl20	L	CGAGAAATTTTGTGGGTTTC	60.9	Sigma-Aldrich
Ccl20	R	CATCTTCTTGGTTCTTAGGC	58.0	Sigma-Aldrich
Sod1	L			
Sod1	R			
Sod2	L			
Sod2	R			
Sod3	L			
Sod3	R			
Adrb2	L			
Adrb2	R			
Apex1	L			

Table S4 (continued)

Apex1	R			
Cat	L	CAAGTTCCATTACAAGACTGAC	57.8	Sigma-Aldrich
Cat	R	TTAAATGGGAAGGTTTCTGC	60.5	Sigma-Aldrich
Cxcl11	L	CCCCTTTGAACATAACGAAGC	64.4	Sigma-Aldrich
Cxcl11	R	GAAAGATCACCAGAGCCACAG	63.8	Sigma-Aldrich
Cxcl5	L	TGTTTGCTTAACCTTAGCTC	56.3	Sigma-Aldrich
Cxcl5	R	CTTCAACTTAGCTATGACTTCC	56.4	Sigma-Aldrich
Cxcl7	L	GCACACTTCCTTTCCATTCTTC	63.7	Sigma-Aldrich
Cxcl7	R	TGCAGATGTACGAATACACTCTC	60.8	Sigma-Aldrich
Gpx1	L			
Gpx1	R			
Gpx2	L			
Gpx2	R			
Hmox1	L			
Hmox1	R			
Ifna2	L	AGAAGGCTCAAGCTATCC	56.0	Sigma-Aldrich
Ifna2	R	AATGAGTCTAGCAGGGATG	56.7	Sigma-Aldrich
Il11	L	ACATGAACTGTGTTTGTCAC	55.7	Sigma-Aldrich
Il11	R	GGAATTTGTCTCTCATCTGTG	58.7	Sigma-Aldrich
Il23a	L	GTGAAGATGTCCGAGTCCAG	62.4	Sigma-Aldrich
Il23a	R	CAGTGTGGTGATGGTTGTGA	63.4	Sigma-Aldrich
Ppbp	L	CTTCAGACTCAGACCTACATC	55.4	Sigma-Aldrich
Ppbp	R	CTCGTTGTTATCAGGTTTTCC	60.0	Sigma-Aldrich
Serpinb2	L	GTGTGAACTTGAATGAAAGC	57.3	Sigma-Aldrich
Serpinb2	R	TATACGGAAGTTCTAGGATCTG	56.6	Sigma-Aldrich
Thpo	L	GCTTGAGCTTTTGTACCTCTG	60.6	Sigma-Aldrich
Thpo	R	TGTACCTGGGTCCTGAAGC	62.8	Sigma-Aldrich
Tnf	L	GTCTTTGAGATCCATGCCATTG	65.5	Sigma-Aldrich
Tnf	R	AGACCCTCACACTCAGATCA	59.9	Sigma-Aldrich
Tslp	L	AAAAACAGGTTGCTGTCTG	57.9	Sigma-Aldrich
Tslp	R	AAAATGATATGGGAAGATGCC	61.5	Sigma-Aldrich
Cxcl1	L	CGCCATCGGTGCAATCTATC	67.6	Sigma-Aldrich
Cxcl1	R	CGAAGTCATAGCCACACTCAA	62.9	Sigma-Aldrich
Ogg1	L	GATGATGTCACTTATCATGGC	59.4	Sigma-Aldrich
Ogg1	R	TAGCACTGGCACATACATAG	56.3	Sigma-Aldrich
Nthl1	L	GCTTGACTGTGGAAAGTATC	56.3	Sigma-Aldrich
Nthl1	R	GAATTTTACCTTGCTCCTCC	59.2	Sigma-Aldrich

Table S5

Step	Temperature	Duration	Cycles	Comments
UDG Activation	50°C	2 min	Hold	This thermocycling setup was used for genes where both primers had Tm >60°C
Dual-Lock™ DNA Polymerase	95°C	2 min	Hold	
Denature	95°C	15 sec	40	
Anneal/Extend	60°C	1 min		

Table S6

Step	Temperature	Duration	Cycles	Comments
UDG Activation	50°C	2 min	Hold	This thermocycling setup was used for genes where one or both primers had Tm <60°C Anneal temperature was set <Tm of the primer with the lowest Tm in each pair
Dual-Lock™ DNA Polymerase	95°C	2 min	Hold	
Denature	95°C	15 sec	40	
Anneal	52-60°C	15 sec		
Extend	72°C	1 min		

Table S7A

B20 7 days - Normfinder			
Gene name	**Stability value**		
Actb	0,082		
Hprt1	0,064		
Pgk1	0,050		
Gapdh	0,053		
Sdha	0,071		
Ywhaz	0,080		
Gusb	0,058		
Intragroup variation			
Group identifier	1	2	3
Actb	0,020	0,065	0,018
Hprt1	0,020	0,016	0,014
Pgk1	0,003	0,004	0,022
Gapdh	0,022	0,010	0,006
Sdha	0,009	0,012	0,013
Ywhaz	0,020	0,043	0,011
Gusb	0,006	0,009	0,012
Intergroup variation			
Group identifier	1	2	3
Actb	-0,071	0,055	0,016
Hprt1	-0,009	0,038	-0,030
Pgk1	0,022	-0,013	-0,009
Gapdh	0,020	-0,013	-0,007
Sdha	0,085	-0,046	-0,039
Ywhaz	-0,022	-0,071	0,093
Gusb	-0,026	0,050	-0,024
Best gene	Pgk1		
Stability value	0,050		
Best combination of two genes	Pgk1 and Gusb		
Stability value for best combination of two genes	0,035		

Table S7B

B20 28 days - Normfinder			
Gene name	**Stability value**		
Actb	0,110		
Hprt1	0,051		
Pgk1	0,071		
Gapdh	0,053		
Sdha	0,039		
Ywhaz	0,053		
Gusb	0,039		
Intragroup variation			
Group identifier	1	2	3
Actb	0,103	0,074	0,073
Hprt1	0,009	0,040	0,012
Pgk1	0,047	0,071	0,007
Gapdh	0,010	0,028	0,020
Sdha	0,004	0,017	0,010
Ywhaz	0,025	0,011	0,020
Gusb	0,010	0,005	0,014
Intergroup variation			
Group identifier	1	2	3
Actb	0,028	-0,118	0,090
Hprt1	-0,010	-0,028	0,038
Pgk1	0,003	0,040	-0,043
Gapdh	-0,031	0,066	-0,035
Sdha	-0,014	0,053	-0,039
Ywhaz	-0,011	0,050	-0,039
Gusb	0,035	-0,064	0,028
Best gene	Sdha		
Stability value	0,039		
Best combination of two genes	Sdha and Gusb		
Stability value for best combination of two genes	0,028		