ONLINE SUPPLEMENT

Alveolar macrophage proteinase/antiproteinase expression and lung function/emphysema

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Materials and Methods

Bronchoalveolar lavage

The resected lung or lobe was obtained immediately post-operatively and bronchoalveolar lavage (BAL) was performed, under sterile conditions, within 1 h. A Foley catheter (14 gauge) was inserted into an airway supplying a segment or lobe uninvolved by tumor. Sixty ml of cold saline was instilled and BAL fluid was aspirated back. The tissue was gently massaged to help recover the instilled saline. The BAL procedure was repeated several times for each subject to yield between 240 and 480 ml, and this aspirated BAL fluid was collected into sterile plastic bottles. The BAL fluid was filtered through sterile gauze to remove debris and mucus, and the filtrate was centrifuged (250 *g*, 10 min). Cell viability was consistently >90% as assessed using trypan blue exclusion. Cytospin preparations were made for differential cell counts, using a Shandon II cytocentrifuge (Shandon Southern Products Ltd, Runcorn, Cheshire, UK) and air dried for 1 h at room temperature.

Alveolar macrophage separation and culture

Alveolar macrophages were separated by Ficoll-Hypaque density centrifugation as described previously (E1). Cells were washed twice with RPMI 1640 (Invitrogen, Carlsbad, CA, USA) and resuspended in RPMI 1640 at a final concentration of 10^6 alveolar macrophages/ml. An aliquot of alveolar macrophages was used to determine baseline mRNA levels. Cells were plated onto 6-well plates (Costar, Cambridge, MA, USA) at a density of 4 million cells/well. Following adherence at 37°C in a humidified incubator (95% air, 5% CO_2 vol/vol) for 2 h, cells were washed twice with RPMI 1640 to remove non-adherent cells before stimulation. Fresh macrophage serum-free medium (Invitrogen), supplemented with 2 mM L-glutamine and penicillin-streptomycin (100 U/ml-100 µg/ml; Invitrogen), was added to the adhered cells (>99% alveolar macrophages in all cases). Alveolar macrophages were then cultured for a further 24 h for RNA analysis, or a further 48 h for protein analysis, in the presence of medium alone, medium + lipopolysaccharide (LPS) (2 µg/ml), medium + interleukin (IL)-1β (20 ng/ml), or medium + tumor necrosis factor (TNF)-α (20 ng/ml). LPS (*Escherichia coli*) was from Sigma (St. Louis, MO, USA). Recombinant human IL-1β and TNF-α were purchased from R&D Laboratories (Minneapolis, MN, USA). The choice of cell culture time, stimulants, and stimulant concentrations were based on previous work (E2-E4).

RNA extraction, cDNA synthesis

Total RNA was isolated from alveolar macrophages using RNeasy® Mini Kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). All preparations were treated with RNase-free DNase (Qiagen) to remove genomic DNA, and the quantity of RNA was measured with Quant-iT™ RiboGreen® RNA reagent (Molecular Probes, Inc., Eugene, OR, USA). 0.5 to 1 µg of RNA was reverse transcribed in a total volume of 20 µl in the presence of 200 U SuperScript® RNase H- Reverse Transcriptase (Invitrogen), 40 U RNaseOUT® Recombinant Ribonuclease Inhibitor (Invitrogen), and 0.5 µg Oligo(dT) Primer (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR

The expression study was performed using 384-well plates on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster city, CA, USA) with the 5' to 3' exonuclease TaqMan assay (Applied Biosystems) in order to assess *MMP1*, *MMP9*, *MMP12*, cathepsin L, cathepsin S, *TIMP1*, *TIMP2*, *TIMP3*, and cystatin C expression in alveolar macrophages. Primer and probe sets for this study were made by Applied Biosystems (Table E1). The reactions were performed according to the manufacturer's instructions.

All PCR conditions used cDNA that corresponded to 2 ng mRNA, 0.5 µl of TaqMan Gene Expression Assay (20x) which included primers and a probe, and 5 µl of TaqMan 2x Universal PCR Master Mix (Applied Biosystems) in a final volume of 10 µl. The cycling conditions consisted of an incubation to activate *Taq* DNA polymerase (95°C for 10 min), an amplification and quantification program repeated 50 times (95°C for 15 seconds, 60°C for 60 seconds with a single fluorescence measurement). Each sample was analyzed in triplicate.

The threshold cycle was determined with the use of SDS 2.1 software (Applied Biosystems). We calculated gene expression levels according to the method of Muller *et al*. (E5), i.e. levels were normalized by a calibrator sample (a cDNA sample obtained from non-cultured alveolar macrophages of a GOLD 0 subject who was not involved in this study) and also by a reference gene, guanine nucleotide binding protein, beta polypeptide 2-like 1 (*GNB2L1*), which was the most stably expressed in alveolar macrophages irrespective of disease severity (E6).

Expression change induced by stimulants was defined as the ratio; expression in alveolar macrophages cultured with stimulant / expression in alveolar macrophages cultured without stimulant.

Protein quantification

MMP1 protein levels were determined using a sandwich ELISA developed and standardized in our laboratory. We obtained MMP1 antibodies and purified MMP1 from Chemicon International Inc. (Temecula, CA, USA). In brief, ELISA Immuno-plates (Nalge Nunc International, Rochester, NY) were coated with monoclonal anti-human MMP1 (Catalogue # MAB 1346) in 0.1M $NaHCO_3$ pH 9.6, and incubated overnight at 4°C. The plate was washed 3 times to remove unbound antibodies, and then blocked with 3% bovine serum albumin. Purified MMP1 as standard and culture media samples were added to the wells in duplicate, and incubated for 4 h at room temperature. The wells were then washed 3 times, before the addition of rabbit polyclonal anti-human MMP1 antibody (Catalogue # AB 806), followed by incubation for 2 h at 37°C. The plates were then washed, and goat anti-rabbit antibody conjugated to horseradish peroxidase was used for detection.

Cathepsin L was assayed using ELISA kits (BMS257MST) from Bender MedSystems (Vienna, Austria) following the manufacturer's instructions. In brief, ELISA plates were coated with monoclonal antibody to cathepsin L; after washing and blocking the plate, standards and culture media were added to the wells in duplicate, and after incubation and washing, cathepsin L was detected using cathepsin L antibody linked to horseradish peroxidase. The assay was reproducible and linear up to 50 ng/ml of cathepsin L; samples at a higher concentration were diluted to bring them into the linear range.

MMP12 was assayed using the ELISA technique of Demedst et al. (E7). The coating monoclonal antibody, the biotinylated detecting monoclonal antibody and the assay procedure were obtained from Imgenex Corp (San Diego, Ca., USA), and the assay was standardized using pure recombinant human MMP12 obtained from R&D Systems (Minneapolis, MN, USA).

Standards were done in triplicate and samples were assayed in duplicate. The assay had a usable range of 0 to 60 ng/ml of MMP12.

The ELISA assays for TIMP2 and cystatin C were done using commercially prepared plates and standards prepared by R&D Systems following the manufacturer's procedure; samples were assayed in duplicate.

Measurement of MMP9 activity was performed by gelatin zymography using 8% SDS-polyacrylamide mini-gels incorporating 0.5 mg/mL gelatin as substrate. Molecular weight markers and a standard sample of alveolar macrophage-conditioned medium of known MMP9 activity were run with each gel. The standard sample had been calibrated against a purified MMP9 protein (Chemicon International Inc.). After electrophoresis at 130V for 90 min at 4°C , the gels were washed twice in 5% Triton X-100, and then incubated for 60 min at 37°C in 50mM Tris-HCl buffer pH 7.4 containing 0.2M NaCl and 5mM $CaCl_2$. Then the gels were stained with Coomassie, followed by a destaining solution. For each sample, only one definite band of lysis, corresponding to the molecular weight of MMP9 was visible. The bands of lysis, indicating enzyme activity, were quantified with the FluorChem Imaging System and software (Alpha Innotech Corp, San Leandro, CA, USA). Standard curves were performed periodically to ensure lysis as quantified by this system was in the linear range of the amount of active MMP9 used. Samples which were not within the linear range of this assay were re-assayed at a different dilution if too high or a larger sample volume if too low, so that they were in the linear range.

Genotyping

(1) SNP selection

We selected the polymorphisms for MMPs, cathepsins, TIMPs, and cystatin C (shown in Table E2) from literature review of reported polymorphisms that have been previously associated with COPD or with regulation of gene expression; rs1799750 in *MMP1*, D20S838 and rs3918242 in *MMP9*, rs2276109 in *MMP12*, and rs2277698 in *TIMP2* (E8-E12).

In addition, we selected SNPs from HapMap (http://www.hapmap.org/) to identify a haplotype block covering the promoter region of each gene. These SNPs were selected using a tagging algorithm implemented in the Haploview 3.2 software (Whitehead Institute for Biomedical Research, http://www.broad.mit.edu/mpg/haploview), based on genotype data available from HapMap. The definition of haplotype blocks was according to Gabriel et al (E13), and tag SNPs were selected using an LD threshold defined by $r^2 \geq 0.8$ and a minor allele frequency ≥ 0.10. Since we could not find an appropriate haplotype block for *MMP1* and *MMP12*, we selected available SNPs from the HapMap data in the putative promoter regions (-2000 bp to +1 bp from the transcriptional start site), i.e., rs498186 for *MMP1* and rs2276109 for *MMP12*.

Since there were no previously reported SNPs for *TIMP1*, we sequenced 10 individuals for PCR-amplified fragments from -1783 bp to +857 bp from the transcriptional start site which included its promoter region and intron 1 which contains previously reported regulatory elements (E14-16). However, we did not find any SNPs with a minor allele frequency ≥ 0.10.

(2) Genotyping assays

Genomic DNA was isolated from alveolar macrophages using DNeasy Tissue Kit according to the manufacturer's protocol (Qiagen). The SNPs described above and also the α_1-antitrypsin Z allele (E17) were genotyped using the 5' to 3' exonuclease TaqMan assays (Applied Biosystems) (E18), from ABI TaqMan Assays-on-Demand.

Genotyping of rs1799750 in *MMP1* and rs3918242 in *MMP9* was performed by restriction fragment length polymorphism analysis as described previously (E8, E19). For rs1799750, the PCR primers were: forward, 5'-TGACTTTTAAAACATAGTCTATGTTCA-3'; and reverse, 5'-TCTTGGATTGATTTGAGATAAGTCATAGC-3'. After the PCR products were digested with the restriction enzyme *Alu*I (New England BioLabs Inc., Beverly, MA), homozygotes for the 2G allele were represented by a DNA band of 269 bp, whereas homozygotes for the 1G allele were represented by DNA bands of 241 and 28 bp, heterozygotes displayed a combination of both alleles (269, 241, and 28 bp). For rs3918242, an amplicon of 435 bp was generated by PCR using the sense primer 5'-GCCTGGCACATAGTAGGCCC and the antisense primer 5'-CTTCCTAGCCAGCCGGCATC with an annealing temperature of 60°C. After the PCR products were digested with 5 U *Sph*I restriction endonuclease (New England BioLabs Inc.), the digest mixture was electrophoresed, and DNA from individuals with the homozygous C genotype (CC) produced one band at 435 bp; the homozygous T genotype (TT) produced two bands at 188 bp and 247 bp; and the heterozygous genotype (CT) produced all three bands.

GeneScan analysis was used to determine the number of CA repeats in D20S838 of the *MMP9* gene. Thirty-five cycles of PCR were performed using an unlabeled forward primer 5'-GAGGTGGTGTAAGCCCTTTCTCATGC and a fluorescently labeled (TAMRA) reverse primer 5'-ATGGTGAGGGCAGAGGTGTCTGACT, and 0.5 U *Taq* DNA polymerase (Qiagen) with an annealing temperature of 62°C. Following PCR amplification, products were mixed with an internal standard (GeneScan-400 ROX, Applied Biosystems). The alleles were separated on an ABI PRISM 377 slab-gel sequencer (Applied Biosystems) and allele determination was performed using Genotyper 3.7 software (Applied Biosystems). DNA standards containing CA repeats that were identified by direct sequencing were included as positive controls in each experiment. Alleles were defined as: short (less than 16 CA repeats) and long (greater than or equal to 17 CA repeats) (E8). Template-free controls and known genotype controls were included for each genotyping assay.

Computed tomographic analysis

A semi-quantitative emphysema score was assigned to the CT scans by two independent observers for seventy-five of the subjects. The observers graded the extent of emphysema using a six point scale (0: no emphysema and 5: >75% emphysema). A quantitative analysis of the lung parenchyma was performed using a previously described technique (E20, E21). Briefly, the lung parenchyma was segmented from the chest wall and large central blood vessels using a contour-following algorithm. Lung volume was calculated by summing the number of voxels in all slices and multiplying by the voxel volume. The CT density of the lung (g/ml) was estimated from x-ray attenuation of each of the CT voxels (E22, E23). Lung weight and air volume were calculated for the lung volume and density measurements (E20, E21). Emphysema was defined using a density mask cut-off of -950 Hounsfield units (severe emphysema), which are appropriate for this CT acquisition technique (E24), and the proportion of emphysema in the whole lungs was named "quantitative score for emphysema" in this study. CT scans from forty-five cases were available for quantitative analysis.

Correction for multiple comparisons

To correct the results for multiple testing, we used a method that accounts for the correlations between clinical phenotypes (E25). For 5 clinical phenotypes, an effective number of independent phenotypes for correction—4.45—was calculated using the Matrix Spectral Decomposition (matSpD) approach (E26) according to the correlation matrix shown in Table E3. Thus, the threshold of the p-value after correction for, e.g., the number of both genes and phenotypes using this method was 0.05/ (9 × 4.45) = 0.0012. The threshold of the p-value for each table is shown as a note below the tables.

For the analysis of the effect of genetic polymorphisms on gene expression, we used the method described above to calculate an effective number of independent polymorphisms for correction. We first calculated the effective number of independent polymorphisms for each gene based on the LD structure between them. The number of independent SNPs for each gene was summed to derive the appropriate correction factor. The correlation matrices for calculation on MMP1, MMP9, CTSL, CTSS, TIMP2, and TIMP3 are shown in Table E4-a, b, c, d, e, and f, respectively. The effective number of total polymorphisms tested was 14.3 (1.63 (for MMP1) + 2.69 (for MMP9) + 1 (for MMP12) + 2.23 (for CTSL) + 1.36 (for CTSS) + 2.49 (for TIMP2) + 1.87 (for TIMP3) + 1 (for CST3)).

Table S1. Assays for real-time PCR.

Gene	Assay ID	Location of primers		Amplicon size (bp)	Assay location*	mRNA accession number
		Forward	Reverse			
GNB2L1	Hs00272002_m1	Exon 1	Exon 2	66	203	NM_006098.3
MMP1	Hs00233958_m1	Exon 6	Exon 7	133	974	NM_002421.2
MMP9	Hs00234579_m1	Exon 12	Exon 13	54	2026	NM_004994.1
MMP12	Hs00159178_m1	Exon 4	Exon 5	62	640	NM_002426.1
CTSL	Hs00266474_m1	Exon 1	Exon 2	88	334	NM_001912.2
CTSS	Hs00175403_m1	Exon 3	Exon 4	82	385	NM_004079.3
TIMP1	Hs00171558_m1	Exon 4	Exon 5	104	518	NM_003254.1
TIMP2	Hs00234278_m1	Exon 3	Exon 4	73	645	NM_003255.3
TIMP3	Hs00165949_m1	Exon 1	Exon 2	59	1309	NM_000362.3
CST3	Hs00264679_m1	Exon 1	Exon 2	93	321	NM_000099.2

* Assay location is the base where the probe is located in the mRNA whose accession number is shown in this table.

Table S2. Details of the single nucleotide polymorphisms and CA repeat polymorphism used in the study.

Polymorphism	Unique identifier	Location*	Position in gene	Alleles†	Assay	Minor allele frequency			HWE in Caucasians (p value)
						HapMap Caucasians‡	Caucasians	All subjects	
MMP1 -1607	rs1799750	-1607	Promoter	2G/1G	RFLP	0.38	0.54	0.53	1.00
MMP1 -822	rs498186	-822	Promoter	A/C	TaqMan	0.48	0.45	0.44	0.38
MMP9 -2997	rs4810482	-2997	Promoter	T/C	TaqMan	0.38	0.35	0.38	0.64
MMP9 -1812	rs3918241	-1812	Promoter	T/A	TaqMan	0.19	0.17	0.17	1.00
MMP9 -1562	rs3918242	-1562	Promoter	C/T	RFLP	0.19§	0.17	0.17	1.00
MMP9 $(CA)_n$	D20S838	-130 to -90‖	Promoter	$(CA)_n$	Gene Scan	n/a	n/a	n/a	n/a
MMP12 -124	rs2276109	-124	Promoter	A/G	TaqMan	0.11	0.12	0.11	0.35
CTSL -7231	rs3128507	-7231	Promoter	G/A	TaqMan	0.37	0.44	0.37	1.00
CTSL -1656	rs3118869	-1656	Promoter	C/A	TaqMan	0.38§	0.42	0.39	0.27
CTSL 157	rs2274611	157	Intron 1	C/T	TaqMan	0.44	0.41	0.39	0.18
CTSS -12674	rs10888394	-12674	Promoter	C/T	TaqMan	0.37	0.36	0.36	0.82
CTSS -958	rs1136774	-958	Promoter	A/G	TaqMan	0.49	0.49	0.49	0.51
TIMP2 -2803	rs4789932	-2803	Promoter	G/A	TaqMan	0.43	0.41	0.44	0.82
TIMP2 5040	rs7212662	5040	Intron 1	T/G	TaqMan	0.47	0.51	0.50	1.00
TIMP2 54154	rs2277698	54154	Exon 3	G/A	TaqMan	0.1§	0.08	0.10	1.00
TIMP3 -4083	rs1962223	-4083	Promoter	G/C	TaqMan	0.18	0.17	0.20	0.45
TIMP3 -1295	rs9619311	-1295	Promoter	T/C	TaqMan	0.27	0.31	0.28	0.62
CST3 7141	rs6036478	7141	3' UTR	C/A	TaqMan	0.25	0.21	0.19	0.74

* Location from transcriptional start site (bp)

† Minor alleles are shown on the right hand side of the slash

‡Data from National Institute of Environmental Health Sciences Environmental Genome Project (http://egp.gs.washington.edu/) unless otherwise indicated

§ Data from NCBI

|| according to reference (E27)

HWE: Hardy-Weinberg equilibrium

n/a: not applicable

Table S3. Correlation (r value) matrix of the phenotypes.

Phenotype	FEV_1 % predicted	FEV_1 / FVC %	DL_{CO} / VA % predicted	Quantitative score for emphysema	Semiquantitative score for emphysema
FEV_1 % predicted	1	0.4847	0.1701	-0.2613	-0.2832
FEV_1 / FVC %	0.4847	1	0.5233	0.1056	-0.3912
DL_{CO} / VA % predicted	0.1701	0.5233	1	-0.0903	-0.5142
Quantitative score for emphysema	-0.2613	0.1056	-0.0903	1	0.5002
Semiquantitative score for emphysema	-0.2832	-0.3912	-0.5142	0.5002	1

Table S4. Correlation matrices of the genetic polymorphisms.

a) *MMP1*

	rs1799750	rs498186
rs1799750	1.0000	0.6090
rs498186	0.6090	1.0000

b) *MMP9*

	rs4810482	rs3918241	rs3918242	D20S838
rs4810482	1.0000	0.5574	-0.5574	0.7674
rs3918241	0.5574	1.0000	-1.0000	0.4525
rs3918242	-0.5574	-1.0000	1.0000	-0.4525
D20S838	0.7674	0.4525	-0.4525	1.0000

Note: The *MMP9* CA repeat (D20S838) was coded as short or long genotypes.

c) *CTSL*

	rs3128507	rs3118869	rs2274611
rs3128507	1.0000	-0.4872	0.3965
rs3118869	-0.4872	1.0000	-0.8733
rs2274611	0.3965	-0.8733	1.0000

d) *CTSS*

	rs10888394	rs1136774
rs10888394	1.0000	0.8010
rs1136774	0.8010	1.0000

e) *TIMP2*

	rs4789932	rs7212662	rs2277698
rs4789932	1.0000	-0.8326	-0.2190
rs7212662	-0.8326	1.0000	0.1562
rs2277698	-0.2190	0.1562	1.0000

f) *TIMP3*

	rs1962223	rs9619311

rs1962223	1.0000	-0.3577
rs9619311	-0.3577	1.0000

Table S5. Effect of smoking status on basal (uncultured) alveolar macrophage gene expression.

Gene†	Smoking cessation					Pack-years (n=106)		
	Current smoker (n=17)		Former smoker (n=92)		p-value	Regression analysis		
	Mean	SD	Mean	SD		Coefficient	r^2	p-value
MMP1	0.017	0.037	0.016	0.039	0.766	0.000	0.003	0.936
MMP9	0.156	0.115	0.226	0.808	0.180	-0.001	0.003	0.758
MMP12	2.655	1.626	1.746	2.298	0.004	0.001	0.000	0.171
CTSL	0.270	0.096	0.363	0.215	0.099	-0.001	0.010	0.245
CTSS	0.994	0.280	1.071	0.245	0.196	-0.001	0.031	0.039
TIMP1	0.598	0.205	0.566	0.177	0.623	0.000	0.000	0.741
TIMP2	1.120	0.292	1.282	0.271	0.015	-0.002	0.029	0.067
TIMP3	1.353	1.563	1.230	1.989	0.625	0.019	0.069	0.029
CST3	1.002	0.336	1.126	0.358	0.132	-0.002	0.015	0.133

†The expression values were log-transformed to be normally distributed for calculation of the p-values.

Table S6. The associations between genetic polymorphisms and expression of mRNA and protein of proteinases or antiproteinases.

Gene	Polymorphism		mRNA				Protein	
			Basal expression	Expression change				
				with LPS	with IL-1β	with TNF-α	without LPS	with LPS
MMP1	rs1799750	β	0.5366	0.0578	-0.2600	-0.0515	0.2018	0.5158
		p-value	**0.0071**	0.8274	0.0790	0.7614	0.3154	**0.0033**
	rs498186	β	-0.1167	0.1815	0.2131	0.1457	0.1592	-0.2646
		p-value	0.5331	0.4582	0.1147	0.3518	0.3932	0.1164
MMP9	rs4810482	β	-0.2369	-0.0282	-0.0188	0.1465	0.0448	-0.0454
		p-value	0.2051	0.8377	0.7044	0.0696	0.5504	0.5320
	rs3918241	β	-0.3147	-0.0592	-0.0232	-0.0025	0.0683	-0.0256
		p-value	0.1886	0.7356	0.7210	0.9809	0.4738	0.7819
	rs3918242	β	-0.3147	-0.0592	-0.0232	-0.0025	0.0683	-0.0256
		p-value	0.1886	0.7356	0.7210	0.9809	0.4738	0.7819
	D20S838	β	0.1169	0.0872	-0.0129	-0.1058	0.0126	0.0730
		p-value	0.5325	0.5207	0.7918	0.1856	0.8652	0.3105
MMP12	rs2276109	β	0.3323	-0.3371	0.1846	-0.0527	0.0349	0.3653
		p-value	0.2956	0.1686	0.1321	0.7019	0.9262	0.4019
CTSL	rs3128507	β	-0.1052	-0.0266	-0.0651	-0.0056	0.1050	-0.0731
		p-value	0.2019	0.7560	0.3888	0.9346	0.5856	0.6524
	rs3118869	β	0.0257	-0.0432	0.0108	-0.0160	-0.4657	-0.1948
		p-value	0.7556	0.6204	0.8856	0.8194	0.0143	0.2319
	rs2274611	β	0.0233	-0.0369	0.0093	0.0297	-0.6562	-0.3527
		p-value	0.7847	0.6821	0.9048	0.6817	**0.0007**	0.0347
CTSS	rs10888394	β	-0.0252	-0.0447	0.0429	0.0477	n.d.	n.d.

Gene	Polymorphism		mRNA				Protein	
			Basal expression	Expression change				
				with LPS	with IL-1β	with TNF-α	without LPS	with LPS
		p-value	0.4902	0.3055	0.1080	<u>0.0183</u>	n.d.	n.d.
	rs1136774	β	0.0088	-0.0355	0.0563	0.0454	n.d.	n.d.
		p-value	0.7976	0.3968	<u>0.0285</u>	<u>0.0186</u>	n.d.	n.d.
TIMP2	rs4789932	β	-0.0313	-0.0448	-0.0115	-0.0522	-0.0134	0.1450
		p-value	0.3439	0.5020	0.7119	0.0926	0.9343	0.3957
	rs7212662	β	0.0297	0.0026	0.0051	0.0600	0.2125	-0.0977
		p-value	0.3830	0.9703	0.8800	0.0682	0.1909	0.5693
	rs2277698	β	0.0230	0.1575	0.0373	0.0950	-0.1947	0.4890
		p-value	0.6628	0.1284	0.4544	<u>0.0488</u>	0.4156	<u>0.0492</u>
TIMP3	rs1962223	β	-0.1347	0.1333	0.2157	-0.0578	n.d.	n.d.
		p-value	0.6120	0.5727	0.2156	0.6711	n.d.	n.d.
	rs9619311	β	-0.0292	-0.0057	-0.2883	-0.2063	n.d.	n.d.
		p-value	0.9025	0.9794	0.0816	0.0989	n.d.	n.d.
CST3	rs6036478	β	-0.2776	-0.0717	-0.0283	-0.0755	-0.2724	-0.0944
		p-value	<u>**1×10^{-7}**</u>	0.1686	0.4810	0.0517	<u>0.0295</u>	0.4626

The mRNA and protein expression levels and expression changes were log-transformed because they were not normally distributed. Linear regression was performed on log-transformed values of expression or expression change versus the number of minor alleles for each polymorphism.
The regression analyses were adjusted for age, gender, race, and smoking status (current smoker or not).
MMP9 $(CA)_n$ repeat: less than 16 repeats = small, equal or larger than 17 repeats = large.
Significant results after Nyholt's correction are shown in bold. For example, the threshold of p-value is 0.05/(1.63 [number of polymorphisms] x 4 [conditions]) = 0.0077 for *MMP1* mRNA . The threshold of p-value is 0.05/(1.63 [number of polymorphisms] x 2 [conditions]) = 0.015 for MMP1 protein . Nominally significant values are underlined.
n.d. = not done

Table S7. Linear regression analysis of pulmonary function tests and computed tomography-scan data with mRNA expression at baseline.

Gene	Coefficient of determination (r^2) and p value*	FEV_1% predicted	FEV_1 / FVC %	DL_{CO}/VA %predicted	Semiquantitative score for emphysema	Quantitative score for emphysema
MMP1	r^2	0.2142	0.1047	0.0613	0.0597	0.3578
	p-value	0.6196	0.2537	0.3023	0.0052	0.1197
MMP9	r^2	0.2127	0.0913	0.0544	0.0296	0.2956
	p-value	0.7756	0.9538	0.6792	0.3034	0.2991
MMP12	r^2	0.2565	0.2393	0.2112	0.0261	0.2972
	p-value	0.0265	**8×10^{-5}**	**0.0004**	0.4080	0.2915
CTSL	r^2	0.2258	0.0927	0.0625	0.0232	0.2924
	p-value	0.2198	0.7048	0.3813	0.9107	0.3144
CTSS	r^2	0.2270	0.0913	0.0521	0.0235	0.2479
	p-value	0.2013	0.9901	0.9866	0.7934	0.7195
TIMP1	r^2	0.2122	0.0915	0.0627	0.0371	0.3126
	p-value	0.8681	0.8793	0.3764	0.1065	0.2312
TIMP2	r^2	0.2547	0.0974	0.0521	0.0253	0.3635
	p-value	0.0299	0.4426	0.9560	0.5231	0.1103
TIMP3	r^2	0.2149	0.1272	0.0693	0.0247	0.2666
	p-value	0.5698	0.0602	0.2588	0.5826	0.4823
CST3	r^2	0.2736	0.1242	0.0568	0.0231	0.2749
	p-value	0.0087	0.0723	0.5530	0.9960	0.4170

*From regression analysis of each clinical outcome including age, gender, race, pack-years, and mRNA expression as covariates. r^2 values are for the whole model, p-values are for the mRNA expression level.

FEV_1 % predicted and FEV_1 / FVC % were measured post-bronchodilator. For the semiquantitative emphysema scores an ordinal logistic regression was performed. DL_{CO}/VA%pred and quantitative score for emphysema were log-transformed.

Significant results after correction for multiple comparisons are shown in bold. The p-value threshold is $0.05/ (9 \times 4.45) = 0.0012$.

Nominally significant values are underlined.

Table S8. Linear regression analysis of pulmonary function tests and computed tomography-scan data with changes in mRNA in response to stimuli.

Gene	Stimulus		FEV_1 %predicted	FEV_1 / FVC %	DL_{CO}/VA %predicted	Semiquantitative score for emphysema	Quantitative score for emphysema
MMP1	LPS	r^2	0.1946	0.1111	0.0923	0.0352	0.4006
		p-value	0.9293	0.1306	0.0825	0.2361	0.9401
	IL-1β	r^2	0.2077	0.0872	0.0360	0.0208	0.5097
		p-value	0.2162	0.3445	0.7505	0.5785	0.8971
	TNF-α	r^2	0.1953	0.0929	0.0484	0.0297	0.5146
		p-value	0.7877	0.3984	0.9028	0.4294	0.1038
MMP9	LPS	r^2	0.1983	0.0975	0.0725	0.0272	0.4882
		p-value	0.5506	0.2922	0.2000	0.5421	0.1592
	IL-1β	r^2	0.2170	0.0790	0.0348	0.0220	0.5322
		p-value	0.1256	0.6158	0.8803	0.4941	0.4745
	TNF-α	r^2	0.3150	0.1039	0.0782	0.0245	0.4476
		p-value	<u>0.0005</u>	0.1973	0.1541	0.8543	0.3110
MMP12	LPS	r^2	0.2337	0.1240	0.0515	0.0306	0.4008
		p-value	0.0522	0.0644	0.6365	0.3659	0.9249
	IL-1β	r^2	0.1958	0.0843	0.0347	0.0203	0.5161
		p-value	0.4832	0.4159	0.8822	0.6172	0.6948
	TNF-α	r^2	0.1945	0.0931	0.0590	0.0263	0.4682
		p-value	0.9562	0.3921	0.3935	0.6034	0.2201
CTSL	LPS	r^2	0.1951	0.0846	0.0488	0.0653	0.4004
		p-value	0.8211	0.9108	0.8435	<u>0.0162</u>	0.9785
	IL-1β	r^2	0.1909	0.0792	0.0344	0.0318	0.5245
		p-value	0.7965	0.6067	0.9964	0.1813	0.5604
	TNF-α	r^2	0.1953	0.0886	0.0518	0.0302	0.4353

Gene	Stimulus		FEV_1 %predicted	FEV_1 / FVC %	DL_{CO}/VA %predicted	Semiquantitative score for emphysema	Quantitative score for emphysema
		p-value	0.7833	0.5571	0.6217	0.3796	0.3861
CTSS	LPS	r^2	0.2023	0.0926	0.0535	0.0302	0.3567
		p-value	0.2111	0.6748	0.4510	0.2603	0.6343
	IL-1β	r^2	0.1909	0.0759	0.0349	0.0238	0.5975
		p-value	0.7965	0.9275	0.8618	0.4090	0.1480
	TNF-α	r^2	0.2091	0.0848	0.0499	0.0259	0.4154
		p-value	0.2402	0.8720	0.7352	0.6461	0.5735
TIMP1	LPS	r^2	0.2128	0.1445	0.0692	0.0420	0.5239
		p-value	0.1876	0.0219	0.2341	0.1087	0.0892
	IL-1β	r^2	0.1943	0.0827	0.0356	0.0192	0.5665
		p-value	0.5461	0.4632	0.7875	0.7438	0.2523
	TNF-α	r^2	0.1953	0.0981	0.0512	0.0283	0.4872
		p-value	0.7822	0.2806	0.6516	0.4721	0.1617
TIMP2	LPS	r^2	0.2214	0.0852	0.0513	0.0246	0.5071
		p-value	0.1091	0.8083	0.6458	0.8401	0.1173
	IL-1β	r^2	0.1952	0.0803	0.0519	0.0187	0.5447
		p-value	0.5096	0.5529	0.2964	0.8221	0.3725
	TNF-α	r^2	0.1955	0.0865	0.0488	0.0284	0.4606
		p-value	0.7584	0.6778	0.8403	0.4586	0.2497
TIMP3	LPS	r^2	0.1974	0.0959	0.0502	0.0582	0.4029
		p-value	0.6043	0.3253	0.7131	0.0407	0.8175
	IL-1β	r^2	0.1919	0.0769	0.0369	0.0228	0.5417
		p-value	0.6977	0.7632	0.6934	0.4822	0.3944
	TNF-α	r^2	0.1971	0.0862	0.0509	0.0279	0.4233
		p-value	0.6194	0.6995	0.6694	0.5170	0.4850

Gene	Stimulus		FEV_1 %predicted	FEV_1 / FVC %	DL_{CO}/VA %predicted	Semiquantitative score for emphysema	Quantitative score for emphysema
CST3	LPS	r^2	0.1946	0.0846	0.0492	0.0488	0.4006
		p-value	0.9325	0.9236	0.7960	0.0753	0.9491
	IL-1β	r^2	0.2193	0.0957	0.0741	0.0207	0.5547
		p-value	0.1102	0.2121	0.1139	0.5870	0.3102
	TNF-α	r^2	0.2083	0.1110	0.0547	0.0253	0.4354
		p-value	0.2528	0.1314	0.5079	0.7180	0.3850

The mRNA was obtained after 24 hours of culture with the various stimuli, and the change of mRNA from cells cultured with no stimuli was analyzed.

Coefficients of determination (r^2) and p values were calculated from regression analysis of each clinical outcome including age, gender, race, pack-years, and mRNA expression as covariates. r^2 values are for the whole model, p-values are for the mRNA expression level.

FEV_1 % predicted and FEV_1 / FVC % were measured post-bronchodilator. For the semiquantitative emphysema scores an ordinal logistic regression was performed. DL_{CO}/VA % predicted and the quantitative score for emphysema were not normally distributed and were log-transformed prior to analysis.

Significant results after correction for multiple comparisons are shown in bold. The p-value threshold is 0.05/(9 [Number of SNPs] x 3 [Number of conditions] x 4.45 [Number of phenotypes]) = 0.00042. Nominally significant values are underlined.

Table S9. The expression level of the proteinases and antiproteinases in the culture media.

Gene	Without LPS	With LPS	p value (with LPS *vs.* without LPS)*
MMP1	2.7 [0.8-6.1]	79.5 [38.7-128.1]	8×10^{-41}
MMP9	21.5 [14.7-28.3]	31.6 [24.6-42.7]	1×10^{-16}
MMP12	0.6 [0.4-3.1]	3.0 [0.8-9.0]	2×10^{-5}
CTSL	18.7 [8.1-42.9]	23.5 [12.8-51.3]	9×10^{-7}
TIMP2	8.4 [4.8-12.0]	2.9 [1.4-8.4]	3×10^{-5}
CST3	28.9 [17.4-40.6]	26.2 [17.9-37.7]	0.0192

The alveolar macrophages were cultured with or without lipopolysaccharide (LPS) for 48 hours, and the protein concentration in the media was measured. Units are ng/mL.
Median and interquartile range is shown.
*From paired t test of log transformed values.

Table S10. The correlation between the level of expression of mRNA and protein for proteinases and antiproteinases.

Gene	Coefficient of determination (r^2) and p value	Without LPS	With LPS
MMP1	r^2	0.0007	0.4738
	p-value	0.8053	**3×10^{-14}**
MMP9	r^2	0.2738	0.2298
	p-value	**5×10^{-8}**	**9×10^{-7}**
MMP12	r^2	0.3874	0.2294
	p-value	**2×10^{-8}**	**5×10^{-5}**
CTSL	r^2	0.0163	0.0037
	p-value	0.2203	0.5644
TIMP2	r^2	0.0296	0.0059
	p-value	0.1425	0.5189
CST3	r^2	0.0513	0.0357
	p-value	0.0524	0.1092

Correlation between mRNA expression at 24 hrs and protein expression at 48 hrs
The expression levels were log-transformed because of their deviation from normal distribution.
Significant results are shown in bold.

Table S11. The correlation between the fold changes in expression of mRNA and protein for proteinases and antiproteinases.

Gene		Fold change after stimulation:		
		With LPS	With IL-1β	With TNF-α
MMP1	r^2	0.0752	0.0975	0.0363
	p-value	**0.0093**	**0.0040**	0.0925
MMP9	r^2	0.4181	0.1278	0.1165
	p-value	**1×10^{-12}**	**0.0007**	**0.0008**
MMP12	r^2	0.0345	ND	ND
	p-value	0.1417	ND	ND
CTSL	r^2	0.0007	0.0215	0.0004
	p-value	0.7928	0.1775	0.8511
TIMP2	r^2	0.0447	ND	ND
	p-value	0.0744	ND	ND
CST3	r^2	0.0428	ND	ND
	p-value	0.0811	ND	ND

Correlation between mRNA expression at 24 hrs and protein expression at 48 hrs
The changes in expression levels were log-transformed because of their deviation from normal distribution.
ND: not done

Table S12. Linear regression of COPD phenotypes with protein expression of proteinases and antiproteinases.

Gene	Condition	Coefficient of determination (r^2) and p value	FEV_1 % predicted	FEV_1 / FVC %	DL_{CO}/VA %predicted	Semiquantitative score for emphysema	Quantitative score for emphysema
MMP1	without LPS	r^2	0.1832	0.0748	0.0501	0.0210	0.1949
		p-value	0.4938	0.4057	0.9929	0.9209	0.6583
	with LPS	r^2	0.1859	0.0882	0.0891	0.0243	0.30928
		p-value	0.2921	0.1771	0.0936	0.8128	0.1773
MMP9	without LPS	r^2	0.1920	0.0849	0.0619	0.0252	0.2950
		p-value	0.7056	0.8032	0.3956	0.7315	0.2173
	with LPS	r^2	0.1910	0.0918	0.0520	0.0258	0.3078
		p-value	0.8503	0.3994	0.9336	0.6318	0.1811
MMP12	without LPS	r^2	0.2352	0.1291	0.1506	0.0391	0.2924
		p-value	0.4387	0.9462	0.8049	0.2930	0.1860
	with LPS	r^2	0.2247	0.1596	0.2173	0.0408	0.4457
		p-value	0.6091	0.1469	0.0449	0.1540	0.0326
CTSL	without LPS	r^2	0.2163	0.0652	0.0413	0.0132	0.2200
		p-value	0.0447	0.7833	0.6551	0.9821	0.8127
	with LPS	r^2	0.2251	0.0693	0.0418	0.0133	0.2228
		p-value	0.0262	0.5070	0.6292	0.8982	0.7431
TIMP2	without LPS	r^2	0.2345	0.1587	0.1525	0.0270	0.1831
		p-value	0.4646	0.1392	0.6771	0.9883	0.7598

Gene	Condition	Coefficient of determination (r^2) and p value	FEV_1 % predicted	FEV_1 / FVC %	DL_{CO}/VA %predicted	Semiquantitative score for emphysema	Quantitative score for emphysema
	with LPS	r^2	0.2573	0.1332	0.1554	0.0273	0.3055
		p-value	0.1200	0.6116	0.5582	0.8370	0.1611
CST3	without LPS	r^2	0.2282	0.1314	0.1507	0.0275	0.2599
		p-value	0.8820	0.7178	0.7929	0.7762	0.2672
	with LPS	r^2	0.2404	0.1304	0.1500	0.0271	0.3741
		p-value	0.3143	0.8199	0.8683	0.8861	0.0754

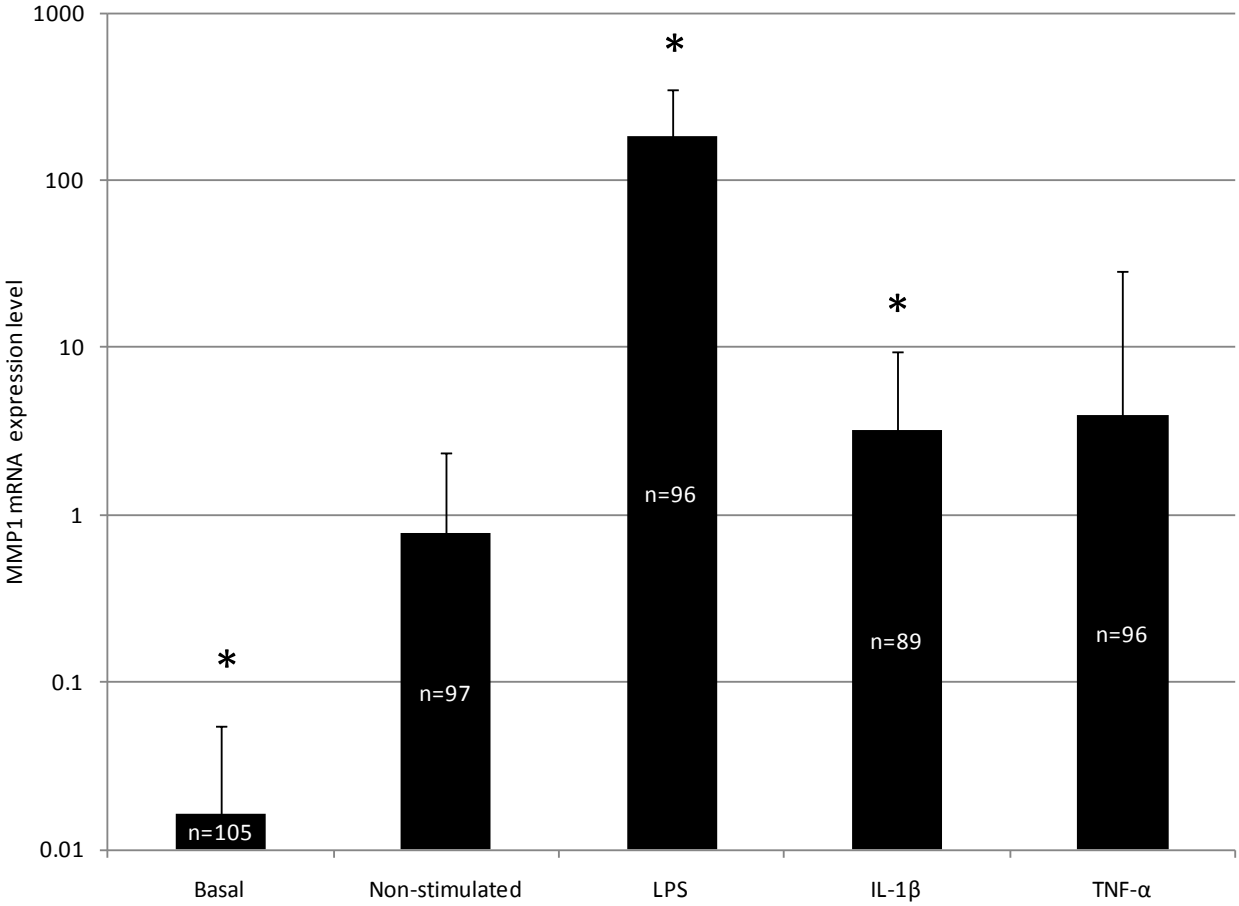
The protein was obtained after 48-hour culture with various stimuli in the media, and the associations between protein concentration and COPD phenotypes were analyzed by regression.

Adjusted by age, gender, race, and pack-years.

FEV_1 % predicted and FEV_1 / FVC % were measured post-bronchodilator. For the semiquantitative emphysema scores an ordinal logistic regression was performed. DL_{CO}/VA % predicted and quantitative score for emphysema deviated from the normal distribution and were log-transformed.

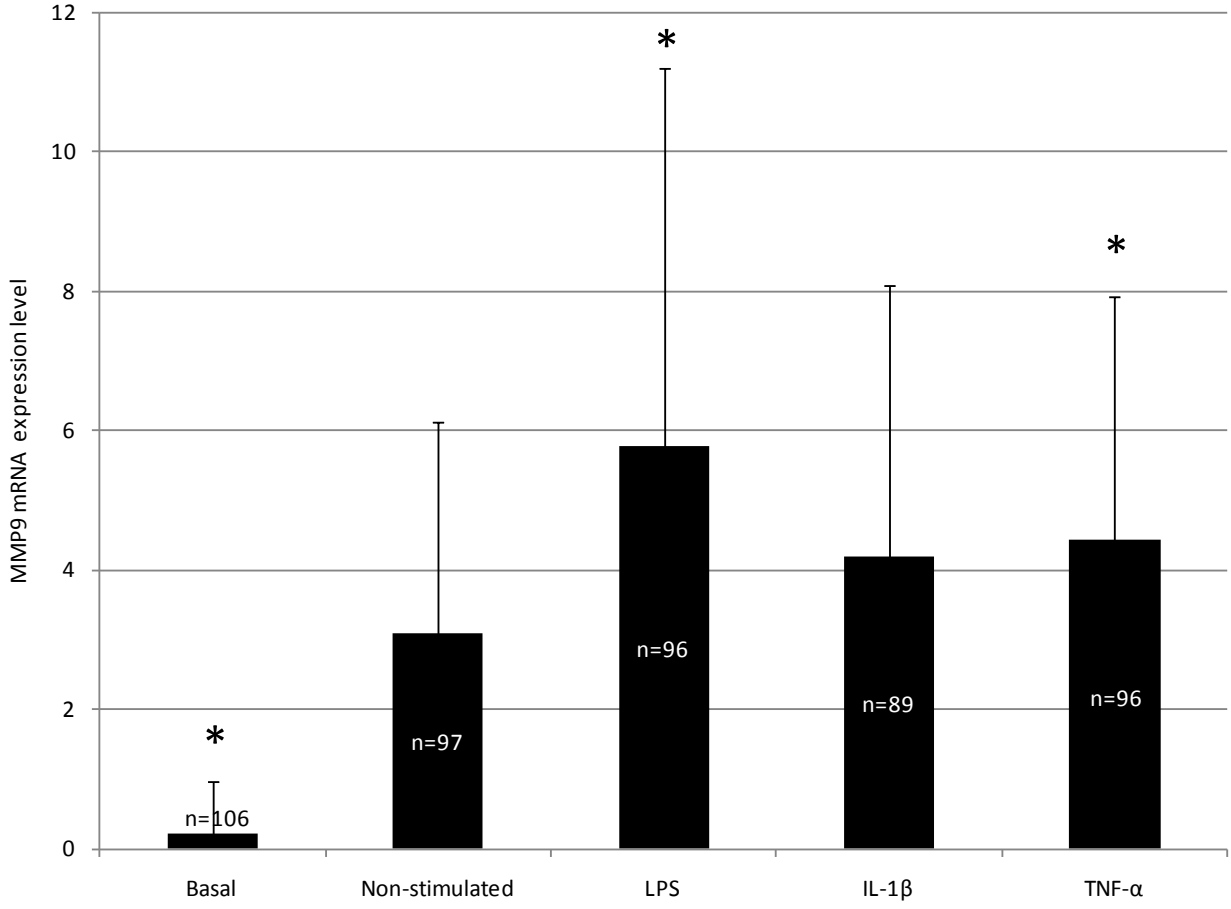
The p-value threshold after Nyholt's correction is 0.05/(6 [Number of SNPs] x 2 [Number of conditions] x 4.45 [Number of phenotypes]) = 0.00094. Nominally significant values are underlined: MMP12 level after stimulation with LPS was inversely related to DLco/VA % predicted and was positively associated with quantitative score for emphysema. CTSL levels without and with LPS stimulation were both positively related to FEV_1 % predicted.

Figure S1. Mean expression level of *MMP1* under different experimental conditions.



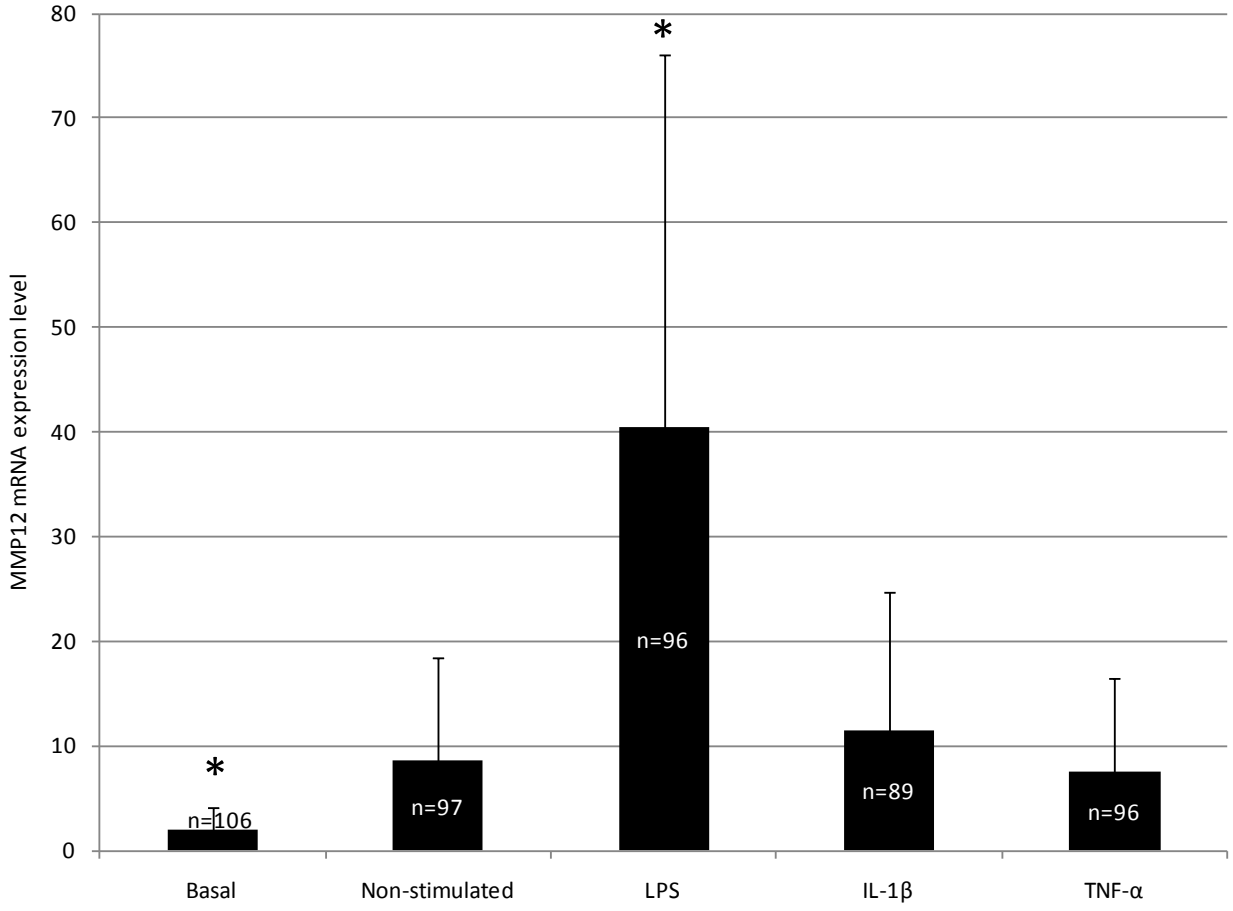
*p<0.0001 *vs*. non-stimulated
Error bars show standard deviation

Figure S2. Mean expression level of *MMP9* under different experimental conditions.



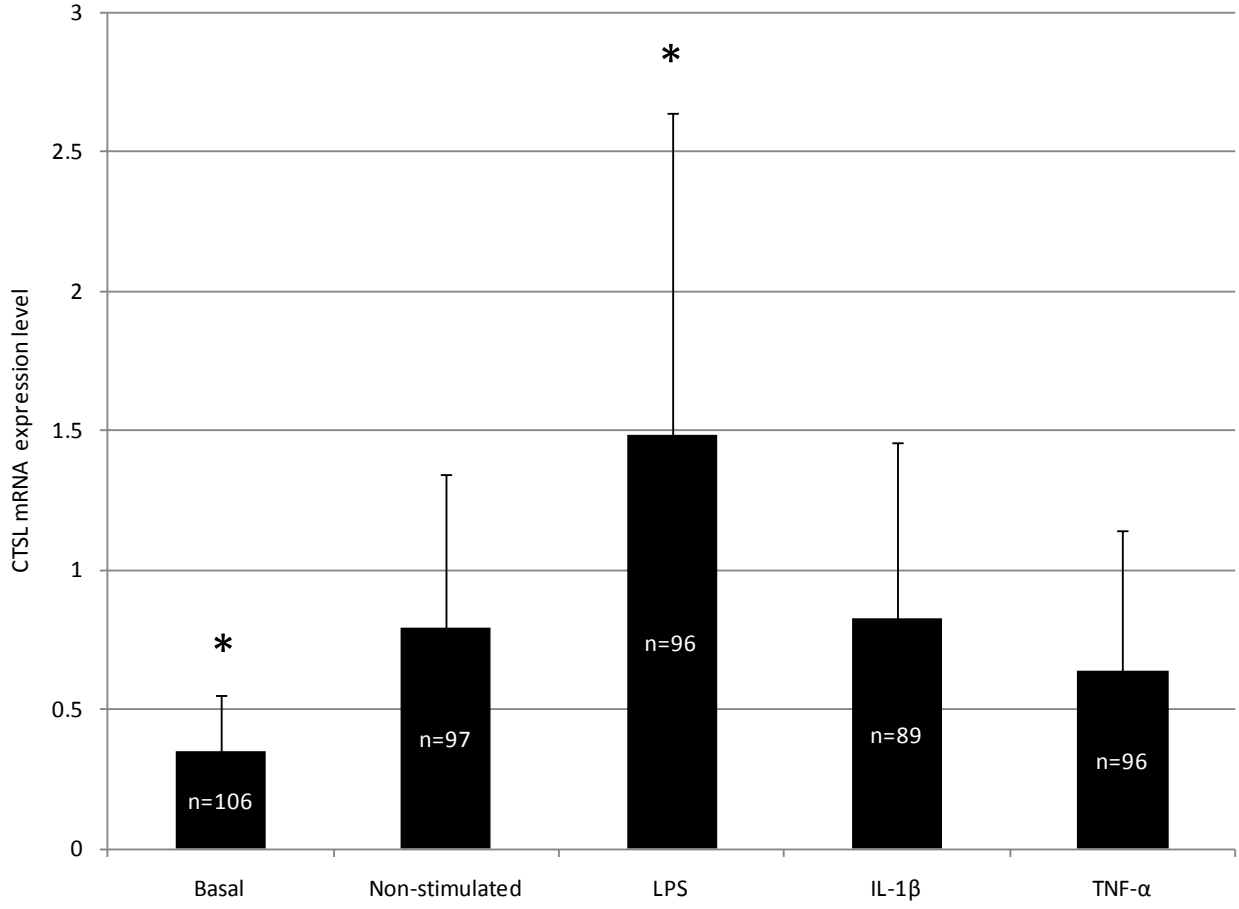
*p<0.0001 *vs.* non-stimulated
Error bars show standard deviation

Figure S3. Mean expression level of *MMP12* under different experimental conditions.



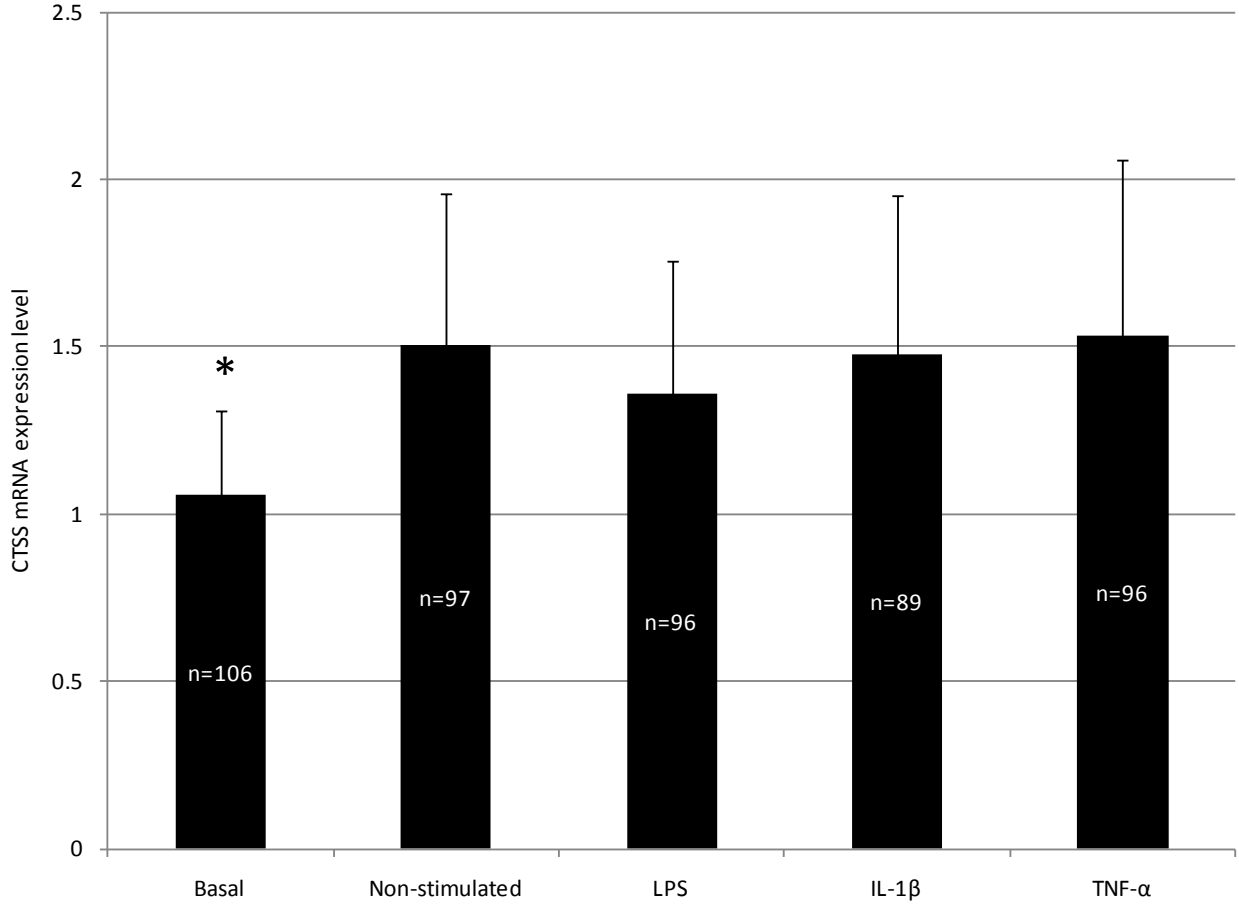
*p<0.0001 *vs*. non-stimulated
Error bars show standard deviation

Figure S4. Mean expression level of *CTSL* under different experimental conditions.



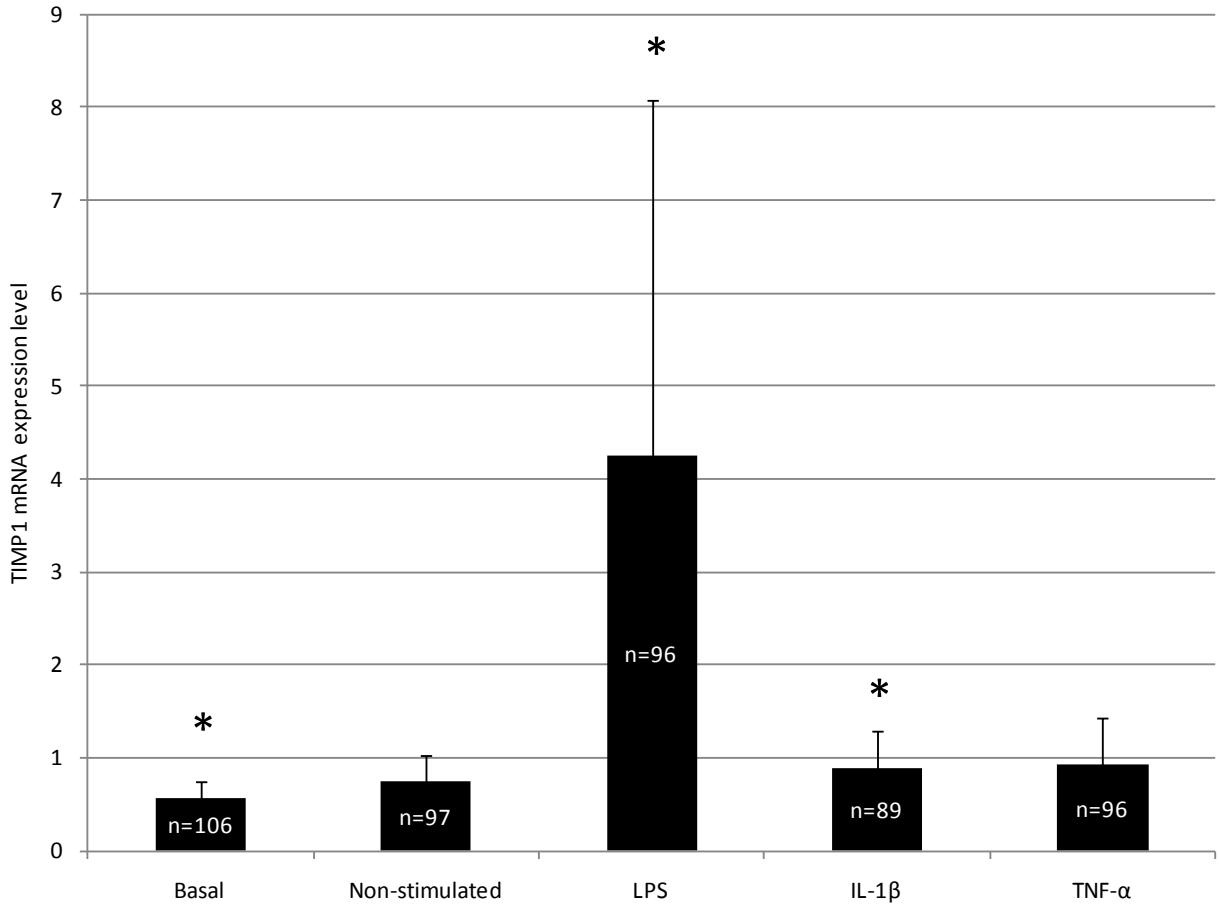
*p<0.0001 *vs.* non-stimulated
Error bars show standard deviation

Figure S5. Mean expression level of *CTSS* under different experimental conditions.



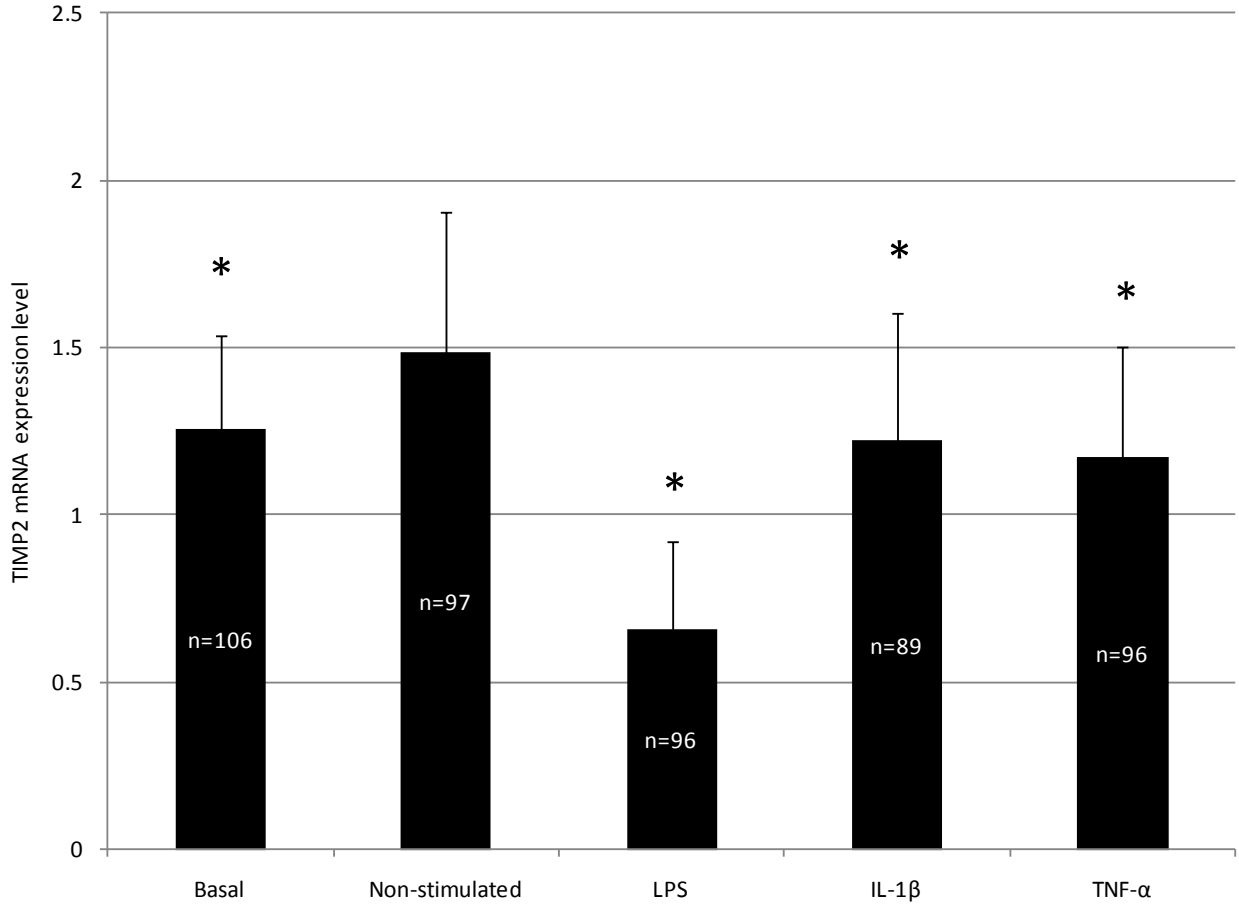
*p<0.0001 *vs.* non-stimulated
Error bars show standard deviation

Figure S6. Mean expression level of *TIMP1* under different experimental conditions.



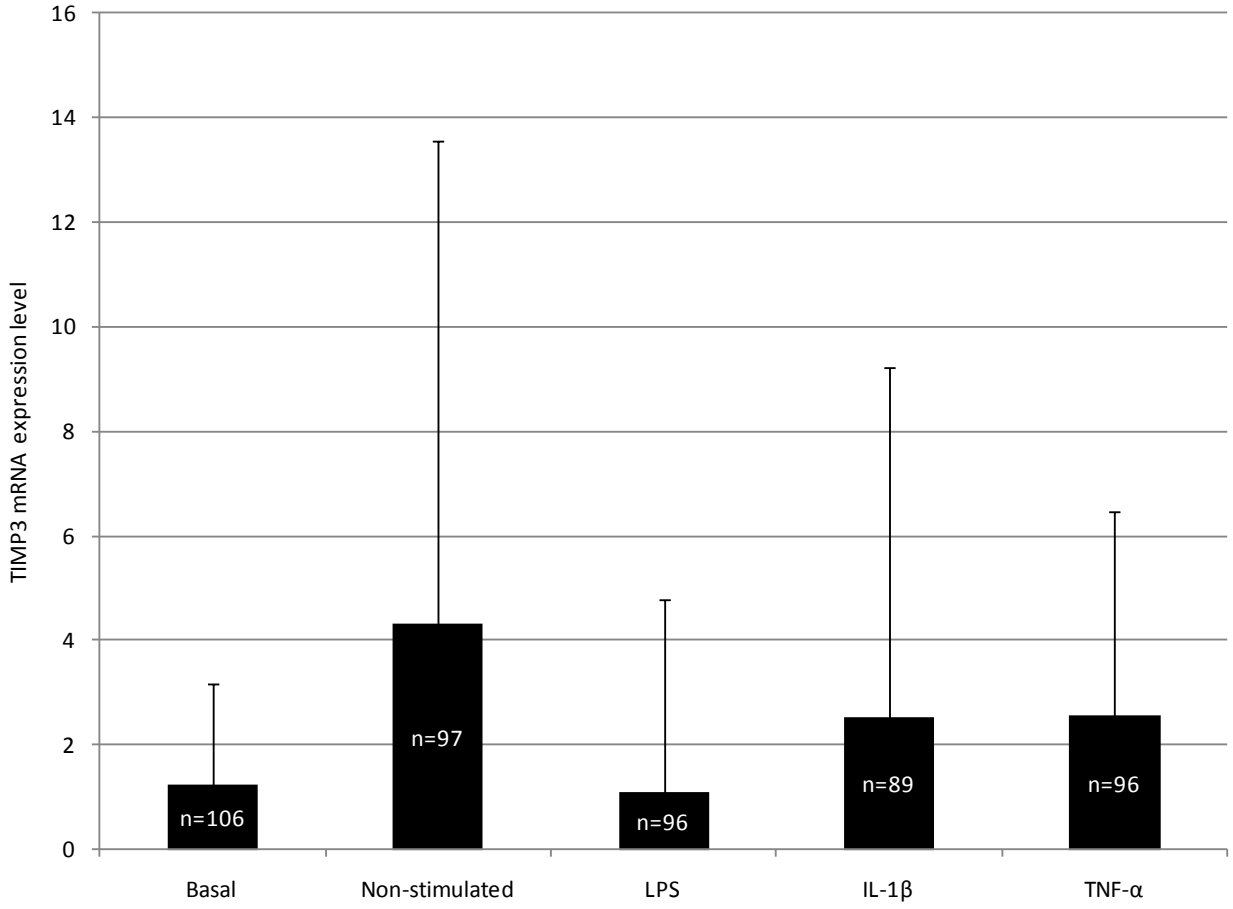
*p<0.0001 *vs.* non-stimulated
Error bars show standard deviation

Figure S7. Mean expression level of *TIMP2* under different experimental conditions.



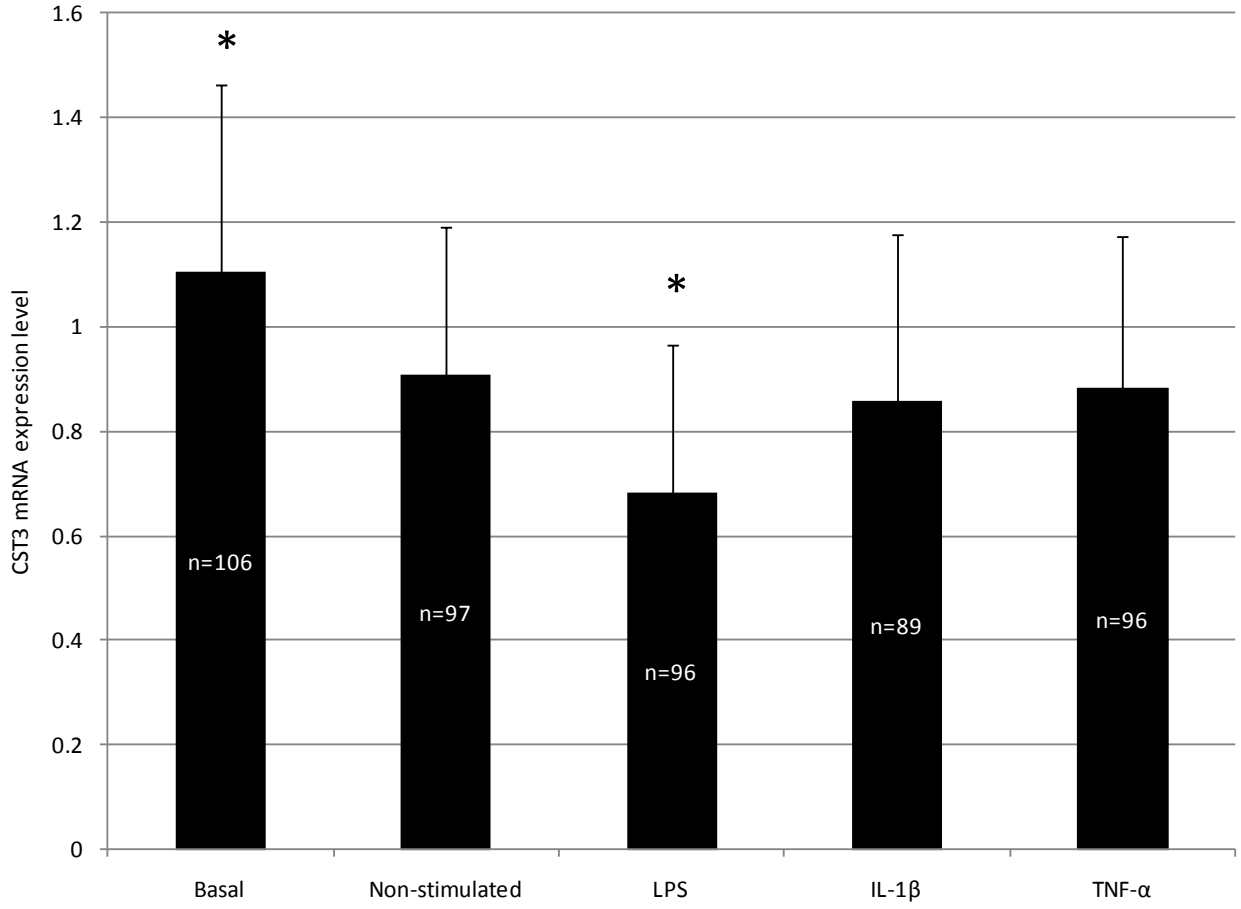
*p<0.0001 *vs.* non-stimulated
Error bars show standard deviation

Figure S8. Mean expression level of *TIMP3* under different experimental conditions.



All p>0.0001 *vs*. non-stimulated
Error bars show standard deviation

Figure S9. Mean expression level of *CST3* under different experimental conditions.



*p<0.0001 *vs.* non-stimulated
Error bars show standard deviation

Figure S10. The relationship between basal mRNA expression of *MMP1* in alveolar macrophages and genotype of the *MMP1* rs1799750 polymorphism (p=0.0071). Means and standard deviations are shown for each genotypic group.

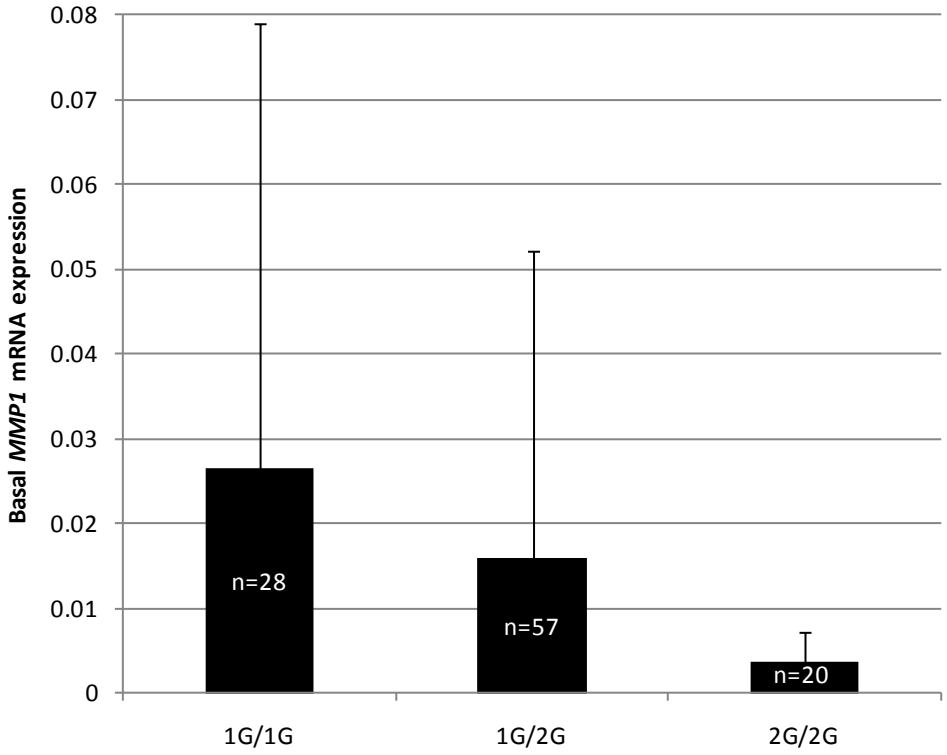


Figure S11. The relationship between expression of *MMP1* protein in alveolar macrophages stimulated with LPS and genotype of the *MMP1* rs1799750 polymorphism (p=0.0033). Means and standard deviations are shown for each genotypic group.

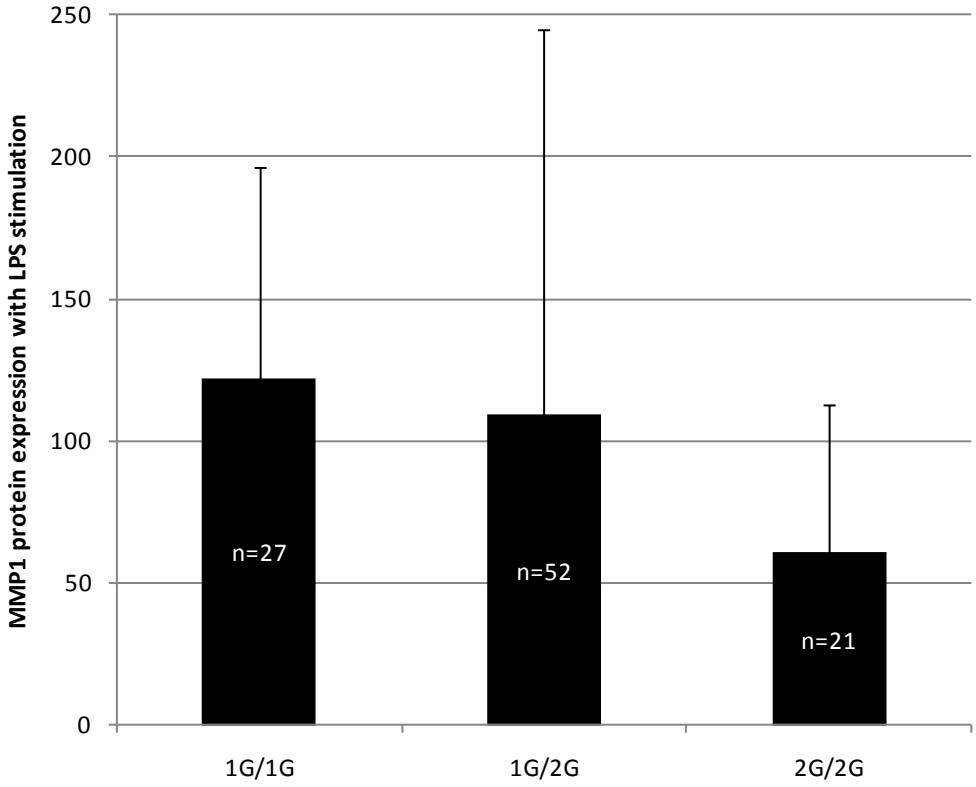


Figure S12. The relationship between expression of *CTSL* protein in alveolar macrophages cultured without stimulation and genotype of the *CTSL* rs2274611 polymorphism (p=0.0007). Means and standard deviations are shown for each genotypic group.

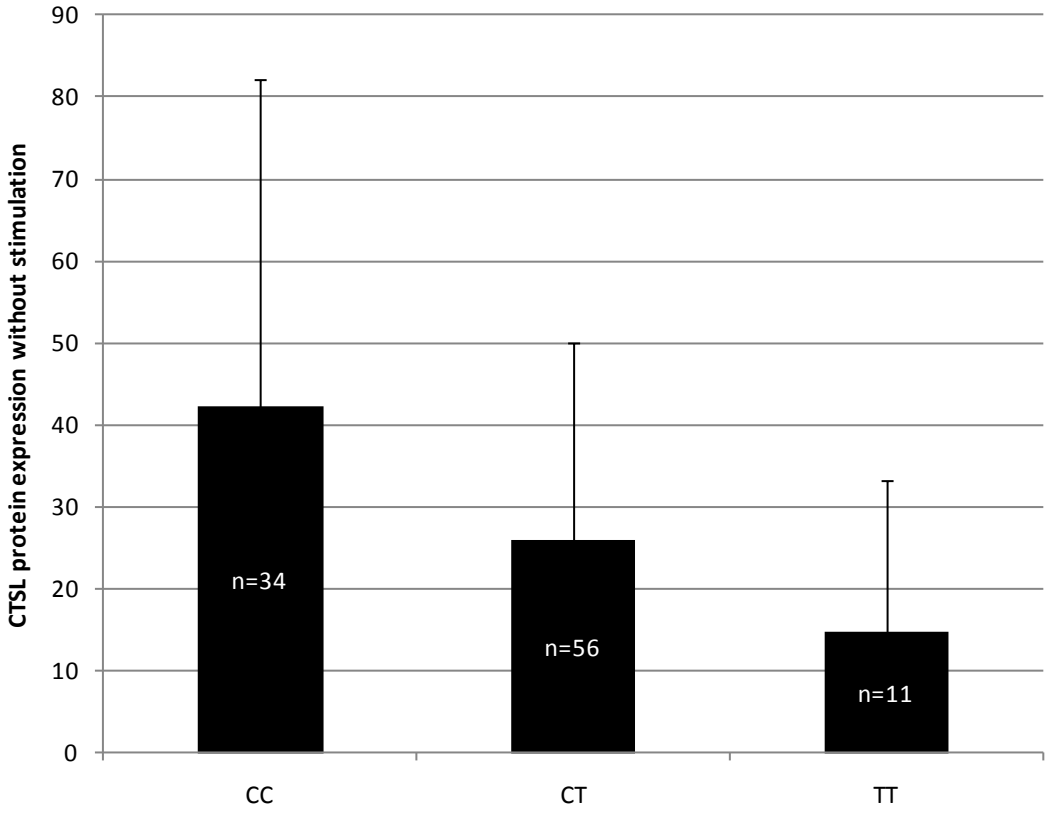
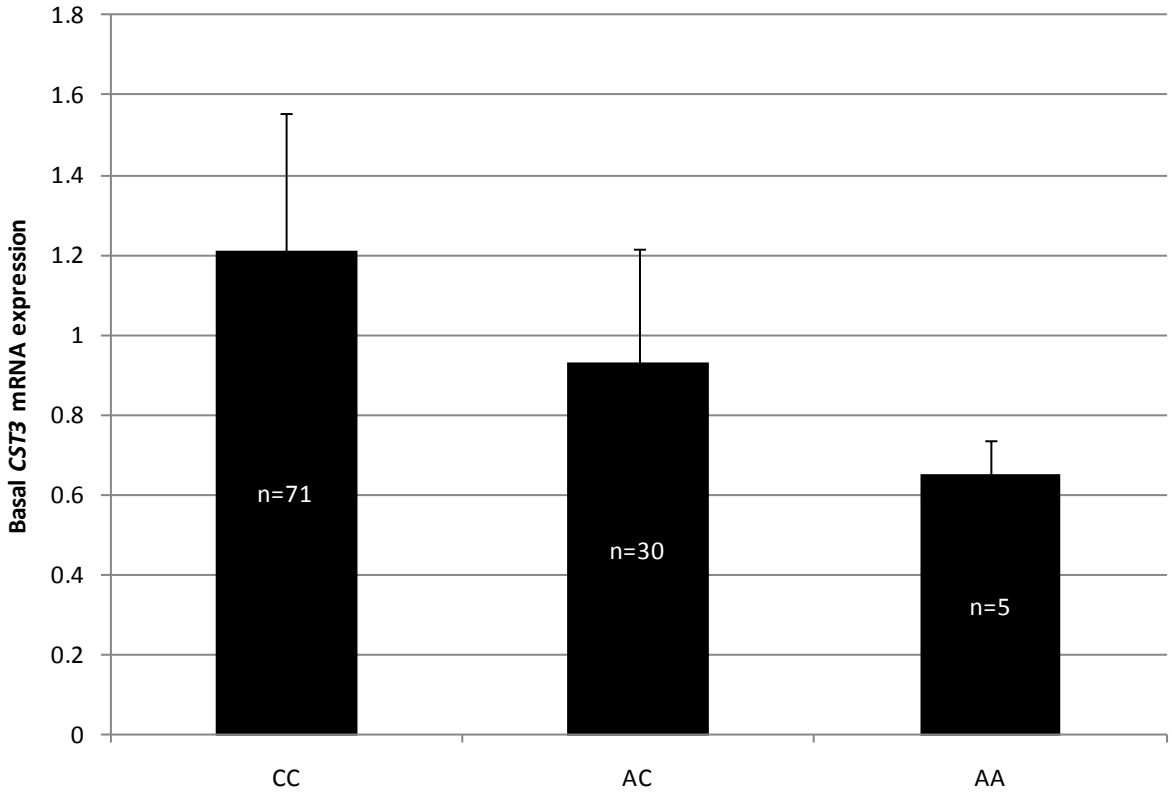


Figure S13. The relationship between basal mRNA expression of *CST3* in alveolar macrophages and genotype of the *CST3* rs6036478 polymorphism ($p=1\times10^{-7}$). Means and standard deviations are shown for each genotypic group.



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