Molecular Medicine

Supplemental Data

GPR84 and TREM-1 Signaling Contribute to the Pathogenesis of Reflux Esophagitis

Heba Abdel-Aziz,[1] Mathias Schneider,[2] Winfried Neuhuber,[3] Abdel Meguid Kassem,[4] Saleem Khailah,[4] Jürgen Müller,[5] Hadeel Gamal Eldeen,[4] Ahmed Khairy,[4] Mohamed T Khayyal,[6] Anastasiia Shcherbakova,[7] Thomas Efferth,[2] and Gudrun Ulrich-Merzenich[7]

Online address: http://www.molmed.org

The Feinstein Institute for Medical Research
North Shore LIJ
Empowering Imagination. Pioneering Discovery.®

MATERIALS AND METHODS

Drugs

STW 5 (Iberogast®), is a multi-component herbal preparation consisting of well-characterized and standardized (1,2) hydroethanolic extracts of *Iberis amara L. (15%)*, *Melissa officinalis L. (10%)*, *Matricaria recutita L. (20%)*, *Carum carvi L. (10%)*, *Mentha piperita L. (5%)*, *Angelica archangelica L. (10%)*, *Silybum marianum L. Gaertner (10%)*, *Chelidonium majus L. (10%)* and *Glycyrrhiza glabra L. (10%)*. It is widely used to treat functional gastro-intestinal diseases based on strong clinical evidence (1A for functional dyspepsia) (3–10) and recommended by the guidelines of the German Society of Gastroenterology (DGVS) for the treatment of functional dyspepsia (11).

Antibodies

For the Western Blots the following antibodies (Ab) were used: phospho-p38 (Thr180/Tyr182) rabbit polyclonal Ab (1:1000), p38 rabbit polyclonal Ab (1:1000; Cell Signaling Technologies, Boston, USA), Anti-GPR84 (H300) rabbit polyclonal Ab (1:500; Santa Cruz Biotechnology, Heidelberg, Germany), anti-rabbit IgG and anti-mouse IgG (1:2000; Santa Cruz Biotechnology); rabbit anti-LOX-1 polyclonal Ab (1:1000), rabbit anti-β-actin polyclonal Ab (1:1000), and rabbit-Anti-ZO-1 polyclonal Ab (1:1000; Bioss, Hölzel Diagnostika Handels GmbH, Cologne, Germany); rabbit anti-p-NFκB p65 (Ser 468) polyclonal Ab (1:500; Santa Cruz Biotechnology); rabbit anti-IL17A polyclonal AB (1:150; Sigma, St. Louis). Human GPR84 recombinant protein purchased from Abnova GmbH (Heidelberg Germany) was used as reference protein.

For immunohistochemistry the rabbit anti-GPR84 polyclonal antibodies (Prestige antibodies, Sigma-Aldrich, Schnelldorf, Germany) was used.

Other Materials

PAXgene Blood RNA Tube (BD, Heidelberg, Germany); RNAlater® Tissue Protect Tube (Qiagen, Hilden, Germany); TransAM® complete lysis buffer (Active Motif, LaHulpe, Belgium); Leica Aristoplan microscope (Leica, Bensheim, Germany); charge-coupled device camera (Visitron Systems, Puchheim, Germany); rat Proteome-Profiler cytokine array kits (panel A, R&D Systems); GenoPlex system and GenoSoft software (VWR); TransAM NF-κB p65 immunoassay-based kit (Active Motif, LaHulpe, Belgium).

Animals

Adult male Wistar rats (8 wks, Charles River, Sulzfeld, Germany) were left to acclimatize for one week before starting experimentation. They were provided with standard pellet diet, given water ad libitum, and kept at a temperature of 22 ± 3°C and a constant relative humidity throughout the experimental period. The study was carried out according to The European Communities Council Directive of 1986 (86/609/EEC) and approved by the Ethical Committee for Animal Experimentation (Landesamt fuer Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany).

Experimental Design

Adult male Wistar rats were blindly allocated to five groups, namely sham operated (8 animals), esophagitis group (14 animals), STW5 and omeprazole treated groups (10 animals each). Two groups received water (sham and esophagitis groups), two received STW5 (0.5 or 2 mL/kg) and one received omeprazole (30 mg/kg) by oral gavage once daily starting 7 d prior to induction of esophagitis. One of the vehicle treated groups was sham-operated. Esophagitis was induced in all other groups as described above. Animals were treated for 10 d further then sacrificed under anesthesia. Experiments were carried out in four successive rounds, each round containing animals from all groups. The number of animals, which completed the study and were finally analyzed, was: 8 for sham operated, 9 for esophagitis group, 6 for the low dose and 7 for the higher dose STW5 treated groups, 6 for the omeprazole treated group (the remaining animals died during the study or had to be terminated early due to ethical considerations, mainly excessive weight loss).

Supplementary Table S1. Primer ID for the qRT-PCR

Gene Name	Gene Symbol	Gene ID	TAQ-Man Primer ID
Chemokine (C-C motif) ligand 4 (Mip1-b, Scya4)	*Ccl4*	116637	Rn00671924_m1
Claudin 3	*Cldn3*	65130	Rn00581751_s1
Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity α)	*Cxcl1*	81503	Rn00578225_m1
G-protein coupled receptor 84	*GPR84*	688730	Rn03037021_sH
Interleukin 6	*IL-6*	24498	Rn01410330_m1
Interleukin 17D	*IL17d*	691799	Rn01481920_m1
Mucin 16, cell surface associated	*Muc 16*	315451	Rn01749838_g1
Vascular adhesion molecule 1	*VCAM-1*	25361	Rn00563627_m1
Eukaryotic 18S rRNA, endogenous control (FAM™/MGB)	*18S*	Ref. seq.	4333760TX03205.1

During anesthesia, blood for RNA analysis was withdrawn from the posterior vena cava. Finally the lower 3 cm of the esophagus were excised, opened longitudinally, examined macroscopically and scored according to the severity of damage (0=no visible damage to 5=perforation).

Esophageal samples were weighed and cut into 3 parts: one fixed in 10% formalin and embedded in paraffin, one stored at -80 °C in an RNAlater® tube for RNA-array analysis and one used to prepare whole cell lysates and stored at -80 °C.

Induction of Reflux Esophagitis

Reflux esophagitis was induced as described by Omura *et al*. (12) with some modifications. Briefly, animals were deprived of food for 12 h prior to surgery. Under anesthesia (ketamine/xylazine, 50/6 mg/kg, i.p.), the abdomen was incised along the midline and the limiting ridge (transitional region between the fore-stomach and corpus) was ligated carefully with a 2-0 silk thread. The duodenum near the pylorus was covered with a 3 mm long piece of 18-Fr Nelaton catheter as a ring. A separate group of animals were only sham-operated, whereby they were anesthetized, their abdomens opened, and then sutured again without further surgical manipulation. Animals were sacrificed 10 d after induction of esophagitis or sham-operation.

Immunohistochemistry

Paraffin embedded tissues were cut into 4-μm-thick sections and mounted on object slides with charged surface (VWR, Darmstadt, Germany). After deparaffinization in xylol and rehydration in graded alcohol solutions, heat induced antigen retrieval was performed by incubating slides in citrate buffer (pH=6) for 25 min using a vegetable boiler. Afterwards endogenous peroxidase was blocked in 3% H_2O_2. After blocking with

Supplementary Table S2. Dependent variable (sumf1).

Group		Statistics	Bootstrap[a]			
					95%-CI	
			Distortion	Standard error	Lower value	Upper value
Esophagitis	Mean	80.3053	-0.1494[b]	4.5169[b]	72.2137[b]	89.1455[b]
	St.deviation	8.49069	-2.49555[c]	3.18314[c]	0.00000[c]	11.97259[c]
	N	3	0[b]	1[b]	1[b]	6[b]
Omeprazole	Mean	48.4049	0.1914[d]	23.7805[d]	12.6137[d]	98.5139[d]
	St.deviation	44.7041	-13.09117[e]	18.04118[e]	0.00000[e]	60.74060[e]
	N	3	0[d]	1[d]	1[d]	6[d]
Sham	Mean	5.6117	-0.0121[f]	0.8372[f]	3.9068[f]	7.0938[f]
	St.deviation	1.60514	-0.42665[g]	0.60533[g]	0.00000[g]	2.25354[g]
	N	3	0[f]	1[f]	1[f]	6[f]
STW5 0.5 mL/kg	Mean	28.6615	-0.1715[h]	6.1568[h]	16.7767[h]	40.0751[h]
	St.deviation	11.6563	-3.22885[i]	4.44537[i]	0.00000[i]	16.47449[i]
	N	3	0[h]	1[h]	1[h]	7[h]
STW5 2 mL/kg	Mean	11.7215	-0.0658[j]	4.9752[j]	1.2223[j]	19.0831[j]
	St deviation	9.33463	-2.73118[k]	3.78588[k]	0.00000[k]	12.62949[k]
	N	3	0[j]	1[j]	1[j]	6[j]
Total	Mean	34.9410	-0.3570	8.3982	19.2000	51.4773
	St deviation	33.4089	-1.73847	5.41529	18.64502	39.48919
	N	15	0	0	15	15

Supplementary Table S3. Sources of variance.

Source	SSQ Type III	df	MSQ	F	Sig.	Partial Eta-SQ
Corrected model	11033.933[a]	4	2758.483	6.007	0.010	0.706
Constant Term	18313.068	1	18313.068	39.878	0.000	0.800
Group	11033.933	4	2758.483	6.007	0.010	0.706
Failure	4592.267	10	459.227			
Total	33939.268	15				
Corrected total variation	15626.200	14				

Supplementary Table S4. Multiple comparisons (sumf1; Scheffé).

(I)Group	(J)Group	Mean difference (I-J)	St error	Sig.	95%-CI Lower value	95%-CI Upper value
Esophagitis	Omeprazole	31.9003	17.49718	0.535	-33.3625	97.1632
	Sham	74.6935*	17.49718	0.024	9.4307	139.9563
	STW5 0.5 mL/kg	51.6438	17.49718	0.145	-13.6190	116.9066
	STW5 2 mL/kg	68.5838*	17.49718	0.038	3.3210	133.8466
Omeprazole	Control	-31.9003	17.49718	0.535	-97.1632	33.3625
	Sham	42.7932	17.49718	0.275	-22.4696	108.0560
	STW5 0.5 mL/kg	19.7435	17.49718	0.859	-45.5194	85.0063
	STW5 2 mL/kg	36.6834	17.49718	0.409	-28.5794	101.9463
Sham	Control	-74.6935*	17.49718	0.024	-139.9563	-9.4307
	Omeprazole	-42.7932	17.49718	0.275	-108.0560	22.4696
	STW5 0.5 mL/kg	-23.0497	17.49718	0.781	-88.3125	42.2131
	STW5 2 mL/kg	-6.1097	17.49718	0.998	-71.3726	59.1531
STW5 0.5 mL/kg	Control	-51.6438	17.49718	0.145	-116.9066	13.6190
	Omeprazole	-19.7435	17.49718	0.859	-85.0063	45.5194
	Sham	23.0497	17.49718	0.781	-42.2131	88.3125
	STW5 2 mL/kg	16.9400	1749718	0.913	-48.3228	82.2028
STW5 2 mL/kg	Control	-68.5838*	17.49718	0.038	-133.8466	-3.3210
	Omeprazole	-36.6834	17.49718	0.409	-101.9463	28.5794
	Sham	6.1097	17.49718	0.998	-59.1531	71.3726
	STW5 0.5 mL/kg	-16.9400	17.49718	0.913	-82.2028	48.3228

*: The mean difference is significant (α =0.05).

Supplementary Table S5. Rotated component matrix for cytokine array.

	Factor 1	Factor 2	Factor 3	Factor 4
CINC1	**0.952**			
MIP-1a	**0.949**			
CINC2ab	**0.941**			
CINC3	**0.941**			
IL-10	**0.841**			
LIX	**0.810**		0.317	0.335
RANTES	**0.776**			0.471
MIP-3a	**0.774**	0.430		
IL-1ß	**0.755**			
MIG	**0.700**	0.447		0.405
CNTF		**0.897**		
Fractalkine		**0.866**		
ThymChemok	0.424	**0.700**		
IL-1ra		0.668	0.612	
VEGEF	0.608	0.649		
IL-1a			**0.949**	
L-Selectin			**0.814**	-0.435
TIMP-1	0.510		**0.753**	
sICAM-1				**-0.923**
IP-10	0.333			**0.726**

10% goat serum, slides were incubated overnight with rabbit anti GPR84 antibody. For determination of protein expression the UltraVision polymer detection method (kit from Thermo Fisher Scientific GmbH, Dreieich, Germany) was used as previously described in detail (13).

NF-κB p65 Measurement

NF-κB p65 DNA binding was measured in tissue whole cell lysates using a TransAM NF-κB p65 immunoassay-based kit (Active Motif, LaHulpe, Belgium). In brief, tissue lysates or HeLa whole cell extracts (for positive control, 2.5 μg/well) were incubated in duplicate for 1 h in a 96-well plate to which a double-stranded NF-κB consensus oligonucleotide sequence had been conjugated. Activated NF-κB p65 was detected by 1 h incubation with an anti-p65 antibody that recognizes an epitope as accessible only when NF-κB is bound to DNA. This was followed by 1 h incubation with a horseradish peroxidase-conjugated secondary antibody and finally by exposure to a 3,3′,5,5′-tetramethylbenzidine substrate solution. Reactions were measured colorimetrically at 460 nm.

Gene Microarrays

RNA was isolated from tissue and blood samples by PaxGene tissue RNA kit and PaxGene blood RNA kit (Qiagen, Hilden, Germany). The gene modulation was determined by gene microarrays as described earlier (14) in four animals from each group. The RNA-Integrity numbers (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) of the isolated RNAs were between 5.8 and 8.6. For analysis single color hybridization of the rat RNA on the Rat Agilent Whole Genome Oligo Micorarrays (41013 genes) after T7 RNA amplification was performed (Miltenyi Biotec, Bergisch Gladbach, Germany). The Agilent Feature Extraction software (FES) was used to read out and process the microarray image files. For the determination of the differential gene expression FES derived output data files were further analysed using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware). The background corrected intensity data were used for the calculation of the ratios**:** experimental sample / control. The ratios were computed using a common "artificial reference" (4 control samples combined). This common reference was used as baseline for all samples. A global correlation analysis of all ratio data was performed. Data sets were filtered in order to remove genes which were not differentially regulated in any comparison. Computed ratios (sample/control) were further analysed by Ingenuity systems Inc., Qiagen, Hilden, Germany as described (14). Data were considered as up- or down-regulated if the expression ratio sample/reference control was ≥ 2 with a p-value <0.01. The gene bank was used as gene identifier and the Ingenuity® knowledge base genes were used as gene reference set. Filters were set for mammals for all analyses. With this general setting commonly or differentially regulated genes in each group were determined and the so-called canonical pathways (well-studied signalling and metabolic pathways within Ingenuity Pathways Analysis, IPA) were identified.

Benjamini–Hochberg multiple testing correction was used as statistical test to

Supplementary Table S6. Regulation of genes of interest in blood.

Genes	Inflammation	STW5 0.5 mL/kg	STW5 2 mL/kg	Omeprazole (30 mg/kg)
CCl2	1.4 (ns)	1 (ns)	-12.5***	-9.4***
CCl7	1.98 (ns)	-5.2***	-2.65*	-3.7*
CXCL1 (*CINC1*)	-1.0 (ns)	-25.4***	-22.5***	-22.5***
CXCL2 (*CINC3*)	22.92 *	-13.9***	-24.8***	-25.9***
CXCL9 (*MIG*)	Nd	-3.5*	-2.8 (ns)	-2.1 (ns)
GPR84	2.6**	-2.5*	-1.3 (ns)	-1.2 (ns)
	4.36*	-3.2*	-1.3 (ns)	-1.2 (ns)
	(+4.65)	(-1.51)	(-1.82)	(+1.39)
VCAM-1	19.87***	-10.1***	-19.3***	-21.7***
	(7.12)	(+16.1)	(-5.72)	(-44.6)
CD163	56.3 ***	-8.3*	-54.0***	-58.5***
IL-1α	1.43 (ns)	-2.16**	-1.78 (ns)	-1.66 (ns)
	2.74*	-2.49 (ns)	1.01 (ns)	-2.11*
IL-1β	1.26 (ns)	2.31**	1.44 (ns)	1.97*
IL-1R1	5.41**	-6.22**	-5.25**	-5.73**
IL-1RAP	-1.06 (ns)	3.21*	1.04 (ns)	1.08 (ns)
	1.02 (ns)	-1.09 (ns)	1.08 (ns)	-1.46 (ns)
IL-1RL1	3.09*	-3.97*	-3.37*	-3.98*
	3.54*	-2.33 (ns)	-3.10*	-3.21*
IL-1RL2	-1.61 (ns)	1.35 (ns)	1.87*	1.45 (ns)
IL-2	-1.49 (ns)	1.36 (ns)	1.01 (ns)	1.71*
IL-3RA	1.11 (ns)	1.05 (ns)	1.134 (ns)	1.31*
	1.76**	-1.20 (ns)	-1.12 (ns)	-1.01 (ns)
IL-4	-1.38*	1.11 (ns)	1.30 (ns)	1.08 (ns)
IL-4R	-1.36*	1.09 (ns)	-1.02 (ns)	1.46**
	1.39*	-1.50*	-1.19 (ns)	-1.18 (ns)
IL-6R	1.55*	1.16 (ns)	-1.26 (ns)	1.33 (ns)
IL-10RA	-1.07 (ns)	2.24**	-1.16 (ns)	2.01**
IL-10RB	1.32*	1.22 (ns)	-1.20 (ns)	1.16 (ns)
IL-11	-1.32*	1.04 (ns)	2.01***	1.25 (ns)
IL-11RA	4.24**	-6.44***	-4.45**	-3.66*
IL-12RB1	1.93*	1.08 (ns)	-2.01**	-1.02 (ns)
IL-13	-1.55 (ns)	1.31 (ns)	2.02**	1.66*
IL-13RA1	6.35***	-1.47 (ns)	-1.60 (ns)	-1.59 (ns)
	2.55*	1.15 (ns)	-1.76 (ns)	1.19 (ns)
IL-13 RA2	-1.17 (ns)	1.87*	1.07 (ns)	-1.02 (ns)
IL-17B	-1.15 (ns)	1.33**	1.43**	1.49***
IL-17C	-1.25 (ns)	1.48*	2.11***	1.28 (ns)
IL-17D	1.64 (ns)	-2.27*	1.01 (ns)	-1.05 (ns)
IL-17RB	1.25 (ns)	-1.06 (ns)	1.77**	1.18 (ns)
IL-17RE	15.29**	-9.13*	-3.0 (ns)	-6.33*
IL-18R1	-1.48**	1.09 (ns)	1.33 (ns)	1.20 (ns)
IL-18rap	3.65***	-1.36 (ns)	-1.55 (ns)	-1.28 (ns)
	-1.46 (ns)	1.00 (ns)	1.08 (ns)	1.09 (ns)
IL-19	1.59 (ns)	-3.59*	-2.86 (ns)	-3.03 (ns)
IL-22RA2	1.78*	1.96*	1.26 (ns)	2.02**
IL-23A	1.28 (ns)	2.02**	1.31 (ns)	1.32 (ns)
IL-27	4.72***	-1.55 (ns)	-1.45 (ns)	-1.22 (ns)
IL-34	3.83*	-2.89 (ns)	-3.63*	-4.54*
IL-36B	1.32 (ns)	2.21***	1.05 (ns)	2.20**
IL-36RN	-1.00 (ns)	1.33*	1.73***	1.02 (ns)
	-1.15 (ns)	-1.03 (ns)	3.31 (ns)	1.05 (ns)

Continued on the next page

calculate false discovery rate in IPA functions and pathways. Common as well as unique gene regulations in the different observational groups were determined by the "Compare Analysis Tool".

Reverse Transcription and Quantitative Real-time Polymerase Chain Reaction

To validate the microarray data, genes that were highly affected by the treatment and/or belonged to different biological pathways were selected for qRT-PCR as follows: inflammatory mediators: *Interleukin 6* (*IL-6*), *IL-17D*, *CCL4*, *CXCL1*; barrier function: *Claudin3* (*Cldn3*); Receptor signalling: *G-protein coupled receptor 84* (*GPR84*), *vascular adhesion molecule 1* (*VCAM-1*); 18sRNA served as endogenous control. An average of 5 samples were analysed per group.

In brief, cDNA was synthesized from 1000 ng (blood) or 500 ng (tissue) of RNA with the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations for hexamer priming as described previously (14). The qRT-PCR was carried out using predesigned TaqMan® primers (FAM™ dye–labeled; Supplementary Table S1; Life Technologies GmbH, Darmstadt, Germany). The TaqMan® gene expression arrays were performed in 96-well plates on a TaqMan® Thermal Cycler 7300 System in a reaction volume of 20 µL with the ready to use TaqMan gene expression master mix. qRT-PCR conditions were as follows: 50°:5 min (1x); 95°:10 min (1x); 95°:15 s (40x); 60°: 1 min (40x).

The mean of the 18sRNA for each replicate was subtracted from the corresponding gene value to normalize the data (ΔCt). Results were calculated as $2^{-\Delta\Delta Ct}$ (15). Treatment data express the fold changes of gene expression compared to the mean of the esophagitis group. Data of the esophagitis group express the fold changes compared to the sham-control.

Western Blot (WB) Analysis

WB analysis was performed in rat tissue whole cell lysates. Protein concentra-

Supplementary Table S6. ***Continued.***

ILDR1	2.59*	-1.97 (ns)	-1.21 (ns)	-1.74 (ns)
	-1.62 (ns)	1.18 (ns)	1.59 (ns)	1.27 (ns)
ILDR2	-1.62*	1.25 (ns)	1.76*	1.38 (ns)
	2.05 (ns)	-2.83 (ns)	-2.41 (ns)	-2.40 (ns)
ILVBL	1.57**	-1.47*	-1.70**	-1.59**
MAPK1	1.29 (ns)	-1.09 (ns)	-1.36*	-1.44*
MAPK1IP1L	-1.26*	1.21 (ns)	-1.27 (ns)	1.62***
MAPK3	-1.20 (ns)	-1.06 (ns)	1.12 (ns)	-1.51***
MAPK4	1.50 (ns)	1.68*	-1.64 (ns)	-1.12 (ns)
	-1.49 (ns)	1.02 (ns)	1.14 (ns)	1.69 (ns)
MAPK8	-1.03 (ns)	-1.07 (ns)	-1.84*	-1.20 (ns)
	2.16*	1.62 (ns)	1.02 (ns)	-1.19 (ns)
MAPK8IP1	4.91**	-3.03*	-3.04*	-3.83**
MAPK8IP2	-1.24 (ns)	-1.29 (ns)	1.37 (ns)	1.28 (ns)
MAPK8IP3	-3.01**	2.88***	1.27 (ns)	4.17***
MAPK9	2.60**	-1.59 (ns)	-1.91 (ns)	-1.78*
MAPK11	-1.29 (ns)	-1.11 (ns)	1.96*	1.14 (ns)
	-1.28 (ns)	-1.14 (ns)	-1.01 (ns)	1.74 (ns)
MAPK13	-1.35 (ns)	1.16 (ns)	1.82***	1.07 (ns)
MAPKAP1	1.03 (ns)	-1.12 (ns)	-1.03 (ns)	1.18**
MAPKAPK2	1.48**	-1.00 (ns)	-1.44*	1.10 (ns)
	1.20*	1.14 (ns)	-1.25 (ns)	1.44**
MAPKAPK3	1.30 (ns)	1.20 (ns)	-1.62*	1.04 (ns)
MAPKAPK5	-1.22**	1.24*	-1.28*	1.60***
CP	63.5***	-16.5***	-63.1***	-62.6***
PTGIS	45.1***	-11.5***	-62.1***	-51.6***
CD163	56:3***	-8.3*	-54.0***	-58.5***
CD 14	2.1 (ns)	-1.4 (ns)	-2.1 (ns)	-1.2 (ns)
Muc 16	19.0*	-21.5***	-4.7*	-18.9*

Column 2 shows fold changes for the esophagitis group versus sham. Column 3-5 show fold changes for the treatment groups as compared to the esophagitis group. *p≤0.05, **p≤0.01, ***p≤0.001. Values between brackets were obtained by qRT-PCR. For abbreviations please see list of abbreviations.

tions were determined by BCA-assay (Pierce). Equal amounts of protein (70 μg; for p-NFkB p65: 500 μg) were diluted with sample buffer, boiled for 5 min, size separated using a 10% SDS-Page as previously described (16), and transferred to nitrocellulose (Transblotter, BioRad Laboratories GmbH, Munich, Germany). Membranes were blocked in 20 mM Tris (pH 7.6), 137 mM NaCL, 0.1% Tween-20 (TBST) supplemented with 2.5% BSA for 3 h at room temperature. Membranes were incubated overnight at 4°C or for 2 h at room temperature with the primary Ab dissolved in TBST containing 1% BSA. The primary Abs were detected using the respective secondary Abs and visualized by Western lightening chemiluminescense (Perkin Elmer, Waltham, Massachusetts, USA) after 6 washings of the membranes. Intensity of the digitally detected bands (PeqLab Biotechnologies, Erlangen, Germany) were evaluated densitometrically using the ImageJ software. A minimum of 5 independent samples per group were evaluated.

Cell Culture

The normal human esophageal squamous cell line HET-1A (ATCC, LGC Standards GmbH, Wesel, Germany) was originally derived from human normal esophageal autopsy tissue by transfection with plasmid pRSV-T. It has been shown to retain epithelial morphology, stains positively for cytokeratins and has remained non-tumorigenic.

Cells were cultured in the bronchial epithelial cell medium BEGM (BulletKit; Lonza, Cologne, Germany) in cell culture flasks precoated with a mixture of 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin (Biochrom, Berlin, Germany) at 37°C in a 5% CO_2-humidified atmosphere.

Cells of passage numbers 50 – 65 were used.

Western Blots Using Capsaicin-Stimulated HET-1A Cells

For experiments 35 mm dishes were pre-coated with 150 μL of the fibronectin/collagen I/BSA mixture (see above). Het-1A cells were trypsinized and seeded in a density of 1x 10^5 cells per dish and kept one night for attachment.

Cell culture medium was removed and replaced by fresh medium. Cells were then treated for 18 h (suitable time period was previously determined by HAA) with 50 μM capsaicin (Sigma-Aldrich) and/or STW5 (60 μL/mL) or omeprazole (10 μg/mL). In case of a combined treatment STW5 or omeprazole were applied directly before capsaicin.

Western blots were performed as previously described (16). In brief, cells were lysed in RIPA-buffer containing 1 mM sodium orthovanadate and protein inhibitor cocktail (10 μg/mL) for 20 min. The cell lysate was removed by scrapping and centrifuged for 10 min at 14000 rpm (4°C). The protein content of the supernatants was determined by Bradford (1949) or by BCA-protein assay. Equal amounts of protein (60-70 μg) were mixed 1:4 with 5x Laemlibuffer, boiled for 5 min, size separated by 10% SDS-page and transferred to nitrocellulose. Membranes were blocked in 20 mM Tris (ph 7.6), 137 mM NaCl, 0.1% Tween-20 (TBST) for 2 to 3 h at RT. Membranes were incubated overnight at 4°C or for 2 h at RT with the primary antibody dissolved in TBST containing 1% BSA. The primary Ab was detected using the respective Ab and visualized by Western lightning chemiluminescense (ECL, Perkin Elmer Life Science) after extensive washing of the membranes. Bands were evaluated by a Peqlab Gel-documentation station and quantified with the ImageJ software (NIH).

Supplementary Table S7. Regulation of genes of interest in esophageal tissue.

Gene	Esophagitis	STW5 0.5 mL/kg	STW5 2 mL/kg	Omeprazole 30 mg/kg
IL-1α	1.44 (ns)	-1.89*	-1.02 (ns)	-1.21 (ns)
	2.27*	-2.06*	-1.63*	-1.80**
IL-1β	13.91***	-2.21 (ns)	-22.28***	-5.87**
IL-1F10	-3.61**	2.59*	2.15 (ns)	1.98 (ns)
IL-1R1	3.65***	-1.94**	-3.33***	-3.82***
IL-1R2	2.02 (ns)	-1.90(ns)	-1.61 (ns)	-2.29*
IL-1RAP	1.26 (ns)	-1.57 (ns)	-1.35 (ns)	2.33 (ns)
	1.67 (ns)	-1.66 (ns)	-2.02*	1.60 (ns)
IL-1RL1	1.85 (ns)	-1.48 (ns)	-3.95***	-1.79 (ns)
	19.72***	-2.95**	-7.91**	-7.38**
IL-1RN	2.39***	-2.36 ***	-1.99***	-2.51***
IL-2RB	3.54*	-1.57 (ns)	-5.16**	-2.15 (ns)
IL-2RG	3.50***	-1.47 (ns)	-4.60***	-2.53***
	-1.07 (ns)	-1.07 (ns)	1.18 (ns)	1.19 (ns)
IL-3RA	1.81**	-2.01**	-1.83**	-1.94**
	2.53***	-2.23***	-2.80***	-2.90***
IL-4	1.86*	-1.18 (ns)	2.65 (ns)	-1.56 (ns)
IL-4R	3.33***	-3.02**	-3.19***	-2.80**
	3.79***	-3.07**	-3.65***	-4.01***
IL-6	11.44***	-4.95*	-9.75***	-9.38***
IL-6R	2.61***	-1.54**	-2.48***	-2.33***
IL-6ST	1.74**	-1.34*	-3.02***	-1.72***
	1.09 (ns)	-1.08 (ns)	-1.09 (ns)	-1.05 (ns)
IL-7R	12.69***	-2.59*	-19.20***	-6.63***
	7.68***	-2.65**	-9.31***	-5.32***
IL-10	10.56***	-5.20**	-9.75***	-9.29***
IL-10RA	1.74*	-1.83 (ns)	-1.70*	-1.49 (ns)
IL-10RB	2.04***	-1.32**	-1.88***	-2.05***
IL-12A	-1.24 (ns)	2.11*	-1.09 (ns)	1.27 (ns)
IL-12RB2	1.07 (ns)	-1.34 (ns)	-1.02 (ns)	-1.72**
IL-13RA1	-1.20 (ns)	1.18 (ns)	1.63***	1.08 (ns)
	-1.17 (ns)	1.00 (ns)	1.28*	1.09 (ns)
IL-13RA2	1.61 (ns)	-1.10 (ns)	-6.62***	-4.24***
IL-16	1.80***	1.00 (ns)	-2.77***	-1.87***
IL-17A	-1.31 (ns)	-2.02 (ns)	-8.44***	2.55 (ns)
IL-17D	-2.30***	1.82***	1.92**	1.71**
IL-17RA	4.04***	-1.85**	-4.10***	-2.97***
IL18	2.84***	-1.38 (ns)	-3.44***	-2.03***
IL18BP	2.26**	-1.70 (ns)	-2.00**	-1.68*
IL18R1	1.54 (ns)	-1.47 (ns)	-1.08 (ns)	1.07 (ns)
IL18RAP	5.68**	-6.40**	-2.70 (ns)	-6.55**
	1.02 (ns)	-1.01 (ns)	-1.00 (ns)	1.00 (ns)
IL19	3.51*	-3.97*	-4.57**	-3.42*
IL20	1.09 (ns)	-1.02 (ns)	1.14 (ns)	1.88 (ns)
IL20RA	-3.11***	1.70**	3.15***	2.61***
IL20RB	-1.97*	1.09	2.83***	1.71***
IL21R	9.65***	-2.12*	-7.77***	-5.96***
IL23A	2.13**	-1.88*	-2.78**	-2.51***
IL24	2.20 (ns)	-1.16 (ns)	-10.82**	-1.12 (ns)
IL27	1.15 (ns)	-2.38*	-1.27 (ns)	-1.67 (ns)
IL34	1.44*	1.51***	-2.44***	-1.58**
IL36A	1.11 (ns)	-2.02**	-1.00 (ns)	-1.17 (ns)
IL36B	-2.26*	1.19 (ns)	3.40***	2.13**

Continued on the next page

GPR84 Expression in Human Esophageal Samples

Tissue preparations were obtained from 34 patients who underwent routine upper gastrointestinal endoscopy (UGE) and presented either with endoscopic normally appearing mucosa or reflux esophagitis. Patients with Barrett's esophagus, esophageal polyps or moniliasis were not included. An informed consent was obtained from all patients and all procedures were approved by the local ethics committee and were conducted according to the ethical guidelines of the declaration of Helsinki (as revised in 2000).

A full medical history was taken from all patients with special emphasis on indications for upper gastrointestinal endoscopy, symptoms and signs of upper and lower gastrointestinal tract (see Table 1 in the article). Endoscopic biopsies were obtained from the distal esophagus above the Z-line. Esophagogastroduodenoscopy (EGD) was done using video chip OLYMPUS CV-240 (Tokyo, Japan) or PENTAX EPM-3500 (Tokyo, Japan) endoscope under conscious sedation (intravenous midazolam). According to endoscopic findings patients were graded according to the Los Angeles classification into the following: endoscopically normal esophagus, Grade A esophagitis, Grade B esophagitis, Grade C esophagitis. Biopsies were taken using biopsy forceps and immediately preserved in formalin (10% solution). They were embedded in paraffin within 24 h to form a tissue block which was kept at room temperature. GPR84 was assessed by immunohistochemical staining.

Samples were examined by an experienced pathologist blinded to the group assignment. GPR84 immuno-reactivity was scored for each sample in six representative high-power fields. The staining intensity (SI) was semi-quantitatively assessed using the following score: 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong).

Descriptive Statistics

Factor analysis is a statistical method for explaining the variation of a large number of observed, correlated variables

Supplementary Table S7. ***Continued.***

IL36G	-2.08 (ns)	1.18 (ns)	2.05*	2.00*
IL36RN	1.29 (ns)	-1.35 (ns)	-1.13 (ns)	1.30 (ns)
	1.98 (ns)	1.12 (ns)	3.30***	2.08**
ILDR1	-1.06 (ns)	1.16 (ns)	-1.26 (ns)	-1.31 (ns)
	-1.19 (ns)	1.47 (ns)	2.84*	1.32 (ns)
ILF3	1.22*	-1.33***	-1.42**	-1.55***
ILK	1.82***	-1.42*	-2.27***	-1.94***
ILVBL	-1.44**	1.18 (ns)	1.58***	1.30*
MAPK1	-1.25 (ns)	1.18*	1.56***	1.58***
MAPK1IP1	-1.06 (ns)	1.06 (ns)	1.17***	-1.05 (ns)
MAPK1IP1L	1.49***	-1.68***	-1.21 (ns)	-1.59***
MAPK3	-1.09 (ns)	1.26*	1.30*	1.21 (ns)
MAPK8IP1	-1.43**	1.29*	1.46**	1.11 (ns)
MAPK8IP2	-1.16 (ns)	-1.11 (ns)	1.45**	-1.11 (ns)
MAPK9	-1.10 (ns)	-1.05 (ns)	-1.04 (ns)	-1.36*
MAPK10	1.29 (ns)	-1.45 (ns)	-2.10*	-1.52 (ns)
MAPK11	-2.33*	1.21 (ns)	3.69***	1.59**
	-1.57**	1.04 (ns)	1.95***	1.12 (ns)
MAPK12	-1.75*	1.17 (ns)	1.49*	1.18 (ns)
	1.02 (ns)	1.03 (ns)	-1.49 (ns)	1.10 (ns)
MAPK13	-1.42**	1.09 (ns)	1.76***	1.20***
MAPK14	-1.10 (ns)	1.04 (ns)	1.11*	1.11*
MAPKAP1	-1.07 (ns)	-1.00 (ns)	1.22***	1.01 (ns)
MAPKAPK2	1.41*	-1.48**	-1.52***	-1.53**
	1.69***	-1.65***	-1.74***	-1.68***
MAPKAPK3	-1.20 (ns)	1.05 (ns)	1.24*	1.00 (ns)
MAPKAPK5	-1.14 (ns)	-1.26 (ns)	1.29*	1.28 (ns)
CP	5.1***	-1.2 (ns)	-8.4***	-3.4***
PTGIS	1.8*	1 (ns)	-1.4	-1.4
CD163	4.6*	-1.26 (ns)	-6.99***	-3.4**
CD 14	10.6***	-4.6***	-12.6***	-8.1***
Muc 16	-13.6**	1.8 (ns)	9.9**	2.0 (ns)
	(-2.4)	(3228)	(9808)	(13453)
PAR1 (*F2R*)	2.18***	-1.1 (ns)	-2.83***	-2.48***
PAR3 (*F2RL2*)	4.15 (ns)	-2.08 (ns)	-14.70***	-5.98*
JAM 2	1.42 (ns)	1.55***	-2.64***	-1.19*
MarvelD1	1.31*	-1.14 (ns)	-1.66**	-1.04 (ns)
MarvelD2	-2.20***	1.16 (ns)	2.67*	1.73***
Dsg1b	-2.58**	1.46 (ns)	3.17**	2.37**
	-1.79 (ns)	1.15 (ns)	2.36*	2.33**
Dsc3	-2.04*	1.20 (ns)	1.94*	1.91*
TJp1 (*ZO1*)	-1.54**	1.18 (ns)	1.92***	1.57***
	-2.41**	2.44***	3.92 (ns)	3.64 (ns)
TJp2 (*ZO2*)	-1.48**	-1.43**	1.90 (ns)	1.39**
Tjp3 (*ZO3*)	-2.25***	1.24 (ns)	3.11 (ns)	1.70**
OCLN	-1.73***	1.85**	3.27***	1.97***
CLDN3	-2.94**	2.01 (ns)	1.7 (ns)	2.80**
	(0.59)	(3.93)	(3.83)	(3.52)
CLDN23	-2.28***	0.32 (ns)	2.83***	2.33***

Column 2 shows fold changes for the esophagitis group versus sham. Column 3-5 show fold changes for the treatment groups as compared to the oesophagitis group. *p≤0.05, **p≤0.01, ***p≤0.001. Values between brackets were obtained by qRT-PCR.
For abbreviations please see list of abbreviations.

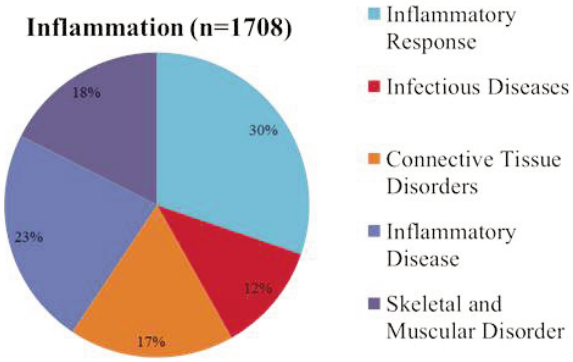
using a smaller number of unobserved, latent variables (factors) (17). The original variables (mediators) with the highest loadings (correlations) on these factors are then used to interpret the meaning of the factors. A linear combination of these variables is used to define the dependent variable for an ANOVA.

RESULTS

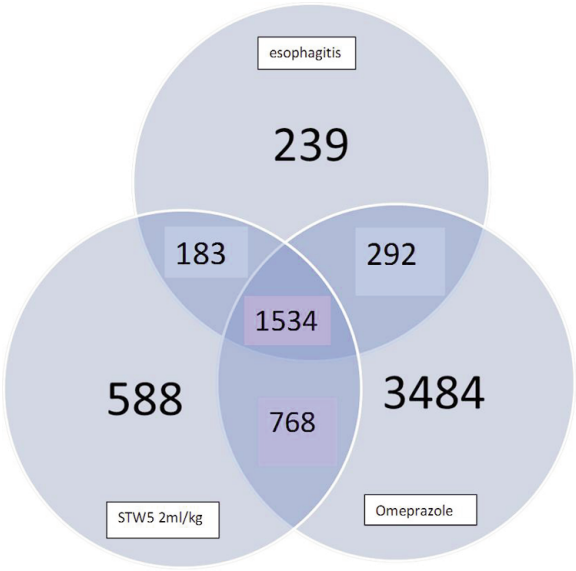
Factor Analysis Identifies CINC1-3, MIP-1/3α, MIG, RANTES and IL-1β as Highly Relevant Mediators

Factor analysis showed a 4 component outcome which interprets 85% of the total variance and therefore the internal structure of the mediators in a statistical connotation: factor1 41%, factor2 18%, factor3 15%, factor4 11%. The rotated component matrix (Supplementary Table S5) supports the determination of what the components (1 to 4) represent. Only the mediators with loadings (correlations) of ≥ 0.7 (bold) for each factor were used. The first component is most highly correlated with CINC1 to 3 and with MIP-1α, whereas IL-10 and IL-1β show a high correlation with component 1 without being related to any other component. Component 2 is highly correlated with CNTF and fractalkine with the two factors not being correlated to any other component. Component 3 is correlated to IL-1α which is not correlated to any other factor. Component 4 is correlated in this way to sICAM-1. Data suggest that at least components 3 and 4 are represented by IL-1α and sICAM-1 respectively and one can narrow down the measurements for component 3 and 4 to IL-1α and sICAM-1 respectively. Component 1 is obviously not represented by a single molecule but requires the measurement of several components.

After determining the internal structure of the mediators, the factors were analysed with respect to a functional influence in terms of differences between the treatment groups. A linear combination of the highlighted mediators of each factor was used as a new dependent variable of the calculated analysis of variance



Supplementary Figure S1. Grouping of modulated genes in the esophagitis group according to disorders.

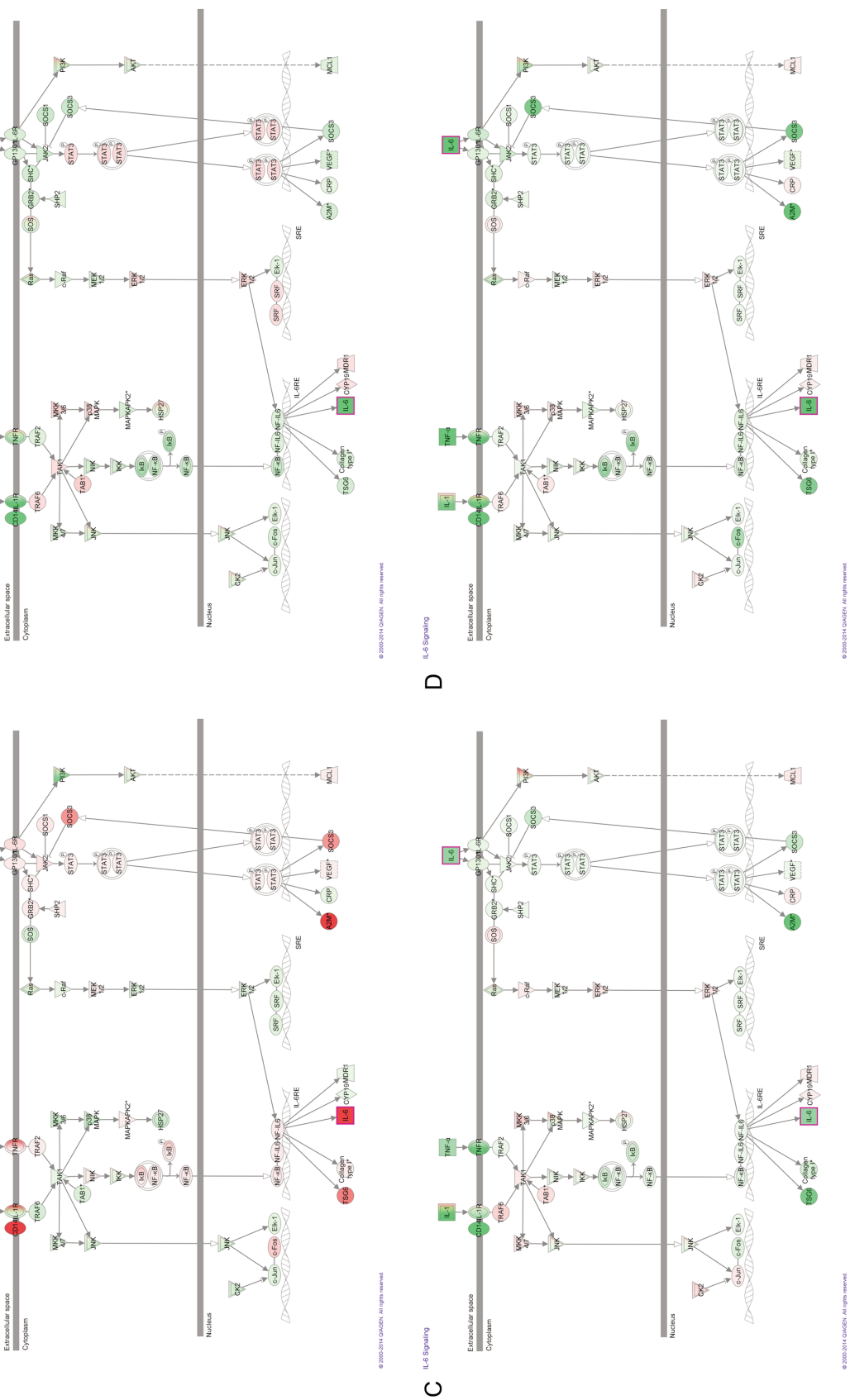


Supplementary Figure S2. Comparison of the gene modulation of STW5 and omeprazole vs. the esophagitis group. STW5 and omeprazole while combatting inflammation modulate 2302 (1534+768) genes in common, of which 1534 genes are modulated in the esophagitis group. The lower and higher doses of STW5 show a common modulation of 72.3% (766) (not shown). Data related to esophageal tissue.

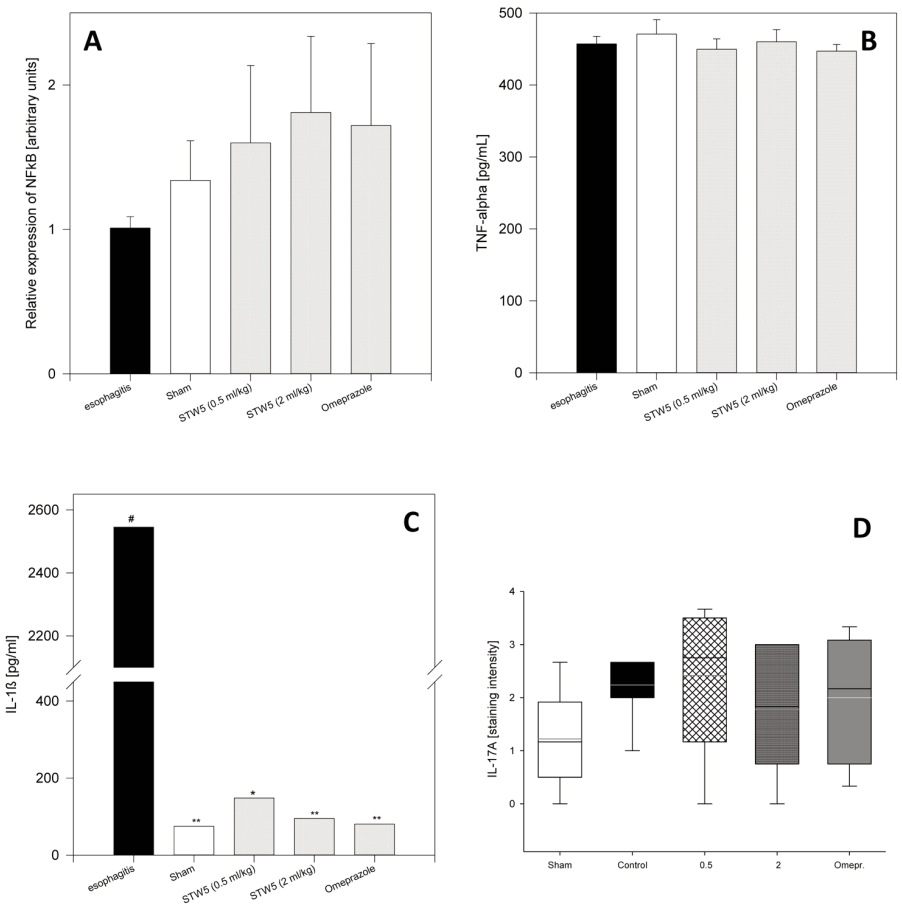
(ANOVA). Factor 1, which was the most relevant factor, showed a significant difference between the groups ($P \leq 0.01$). Multiple comparison testing (Scheffé) showed a significant difference between the control and sham group and between the control and the STW5 2 mL/kg group, but not between the control and omeprazole group. The variables derived from the other factors in the same way did not show any significant difference between the groups using ANOVA.

REFERENCES

1. Kroll U, Cordes C. (2006) Pharmaceutical prerequisites for a multi-target therapy. *Phytomedicine.* 13 Suppl 5:12–9.
2. Wegener T, Wagner H. (2006) The active components and the pharmacological multi-target principle of STW 5 (Iberogast). *Phytomedicine.* 13 Suppl 5: 20–35.
3. Rösch W, *et al.* (2006) Phytotherapy for functional dyspepsia: a review of the clinical evidence for the herbal preparation STW 5. *Phytomedicine.* 13:114–21.
4. Madisch A, *et al.* (2004) Treatment of functional dyspepsia with a herbal preparation. *Digestion.* 69:45–52.
5. Gundermann K, Godehardt E, Ulbrich M. (2003) Efficacy of a herbal preparation in patients with functional dyspepsia: a meta-analysis of double-blind, randomized, clinical trials. *Adv. Ther.* 20:43–9.
6. Melzer J, *et al.* (2004) Meta-analysis: phytotherapy of functional dyspepsia with the herbal drug preparation STW 5 (Iberogast). *Aliment. Pharmacol. Ther.* 20:1279–87.
7. von Arnim U, *et al.* (2007) STW 5, a phytopharmacon for patients with functional dyspepsia: results of a multicenter, placebo-controlled double-blind study. *Am. J. Gastroenterol.* 102:1268–75.
8. Rösch W, Vinson B, Sassin I. (2002) A randomised clinical trial comparing the efficacy of a herbal preparation STW 5 with the prokinetic drug cisapride in patients with dysmotility type of functional dyspepsia. *Z. Gastroenterol.* 40:401–8.
9. Holtmann G, Adam B, Vinson B. (2004) Evidence-based medicine and phytotherapy for functional dyspepsia and irritable bowel syndrome: a systematic analysis of evidence for the herbal preparation Iberogast [in German]. *Wien. Med. Wochenschr.* 154:528–34.
10. Holtmann G, *et al.* (2013) A placebo controlled randomised treatment trial for functional dyspepsia including post-treatment drug withdraw and placebo withdraw effects. *Gastroenterology.* 144:733.
11. Madisch A. (2013) Leitlinienbasierte Phytotherapie bei funktionellen Magen-Darm-Störungen. *Z. Phytotherapie.* 34:270–3.
12. Omura N, *et al.* (1999) Establishment of surgically induced chronic acid reflux esophagitis in rats. *Scand. J. Gastroenterol.* 34:948–53.
13. Krishna S, *et al.* (2015) A randomised, double blind, placebo-controlled pilot study of oral artesunate therapy for colorectal cancer. *EBioMedicine.* 2:82–90.
14. Ulrich-Merzenich G, *et al.* (2012) Novel neurological and immunological targets for salicylate-based phytopharmaceuticals and for the anti-depressant imipramine. *Phytomedicine.* 19:930–9.
15. Schierwagen R, *et al.* (2013) Serum markers of the extracellular matrix remodelling reflect antifibrotic therapy in bile-duct ligated rats. *Front. Physiol.* 4:195.
16. Ulrich-Merzenich G, *et al.* (2007) Vitamin C promotes human endothelial cell growth via the ERK-signaling pathway. *Eur. J. Nutr.* 46:87–94.
17. Do K-A, Qin ZS, Vannucci M. (2013) *Advances in Statistical Bioinformatics: Models and Integrative Inference for High-Throughput Data.* New York: Cambridge University Press.



Supplementary Figure S3. IL-6 Signaling - Comparing STW5 and omeprazole. The signaling pathways (Ingenuity pathway analysis) of IL-6 are shown. Genes which were modulated in the esophageal tissue are highlighted. Red indicates an up-regulation, green a down-regulation. A) regulated genes during esophagitis. B) regulated genes after treatment with STW5 (0.5 mL/kg). C) regulated genes after treatment with STW5 (2 mL/kg). D) regulated genes after omeprazole treatment.



Supplementary Figure S4. Modulation of NF-kB, TNF-α, IL-1ß and IL-17 in esophageal tissue of rats with RE. (A) Relative expression of NF-kB as determined by Western Blot analysis normalized with β-actin. (B and C) Tissue concentrations of TNF-α (B) and IL-1β (C) determined by ELISA. For TNF-α bars represent mean + SEM, for IL-1β bars represent median (since data were not normally distributed) (D) Immunohistochemical staining of IL-17A presented as box-plot. Data represent the analysis of a minimum of 5 tissue samples from 5 animals per group. *p<0.05; **p<0.01 vs esophagitis group, #p<0.05 vs Sham.