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Immunomodulatory effects of thymol and cinnamaldehyde in chicken cell lines

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Supplementary materials and methods

Cell lines and culture conditions

Human epithelial cell line Caco-2 (ACC 169) was purchased from DSMZ (Braunschweig, Germany). Cells were maintained at 37 °C in a 5% CO_2 atmosphere and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Foetal bovine serum (FBS). For the TEER assay, Caco-2 and LMH cells were differentiated on permeable filters to produce a polarized epithelial cell monolayer prior to measurements of TEER.

Quality control of TEER assay

The TEER of the cell monolayers in all replicate wells was measured on day 15 of differentiation, before addition of TCB, in order to assess whether epithelial integrity had been achieved. Only cell culture inserts producing resistance values of 450 Ω/cm^2 or above (indicative of epithelial integrity) were used in the subsequent assay.

Proteomic analysis

Cell culture and sample preparation

Chicken monocytes (HD-11) were cocultured with TCB (10 ng/ml) for 2 h at 37 °C with 5% CO_2 and then stimulated with/without LPS (100ng/ml) and further cultured for 6 h. Cells were then washed and the cell pellets collected for proteomic analysis. The protein areas used in the proteomic output graphs were the measured average chromatographic intensities of the three most intense and unique peptide ions, computed as the total area under the intensity curve (AUC) inside each peak region. A peak region is a square of a m/z interval and a retention time interval. For each protein the average area of the three most intense peptides are used. Each peptide can be represented by several charge states or detected variable modifications. A peptide signal is the sum of the detected isotope clusters determined by the peak areas. All experiments were performed 2 times (i.e. 2 biological replicates) with a total of 4 replicates for each experimental condition (i.e. 4 technical replicates).

Protein digestion

Cells were lysed in 5% SDS in 100 mM triethylammonium bicarbonate (TEAB), pH7.55. Lysates were heated to 95 °C for 10 min to denature the proteins. The DNA was sheered using a Bioruptor® Pico sonication device high energy water bath sonicator system (Diagenode Diagnostics, Belgium) with 10 cycles of sonication each of 10 seconds duration with a 30 seconds break in between. The temperature was set to 5 °C throughout the denaturation process. Protein concentrations were estimated in triplicate using a colorimetric BCA micro assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Cell protein was digested into peptides using the S-trap™ 96-well plate digestion protocol (Kall *et al.*, 2007). Based on the available protein concentrations, an amount equal to 50 µg was utilized from each sample. Protein was reduced in

20 mM DTT for 10 min at 95 °C, cooled to room temperature and alkylated with 40 mM iodoacetamide (IAA) for 30 min in the dark. Subsequently, 12% phosphoric acid was added in the volume ratio 1:10 to acidify samples. To each sample, 350 µl S-trap binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB, pH7.1) was added and samples was carefully mixed. Samples were loaded into a 96-well S-trap digestion plate and centrifugated at 1,500×*g* for 2 min. Each well was washed three times with 200 µl S-trap binding buffer followed by centrifugation at 1,500 × g for a further 2 min. Then, 125 µl digestion buffer (50 mM TEAB with 1.66 µg trypsin/125 µl buffer) was added to each well and wells incubated over night at 37 °C. Peptides were eluted from the S-trap wells using 80 µl 50 mM TEAB followed by centrifugation at 1,500×*g* for 2 min, then eluted again using 80 µl of 0.2% aqueous formic acid followed by centrifugation at 1,500×*g* for 2 min and finally eluted for a third time using 80 µl of 50% aqueous acetonitrile (can) containing 0.2% formic acid followed by centrifugation at 1,500×*g* for 2 min. Eluted peptides were dried down and resuspended in 50 µl 0.1% trifluoroacetic acid (TFA). Peptide concentrations were estimated using a colorimetric BCA micro assay kit (Thermo Scientific Pierce) and samples adjusted to equal concentrations. A QC sample (mixture of all samples) was prepared by taking 10 µl from all samples and mixing well prior to colorimetric analysis.

Data acquisition

Nano LC-MS/MS was performed using an UltiMate™ 3000 RSLCnano System (Thermo Scientific) interfaced to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific). Samples were dissolved in 0.1% TFA and loaded onto a 20 mm NanoViper Trap Column (Acclaim™ PepMap™ 100 C18, 3 µm particle size, i.d. 0.075 mm) connected to a 500 mm analytical column (Acclaim™ PepMap™ 100 C18, 2 µm particle size, i.d. 0.075 mm). Separation was performed at a flow rate of 250 nl/min using a 116-min gradient of 0-36% Solvent B (100% ACN, 0.1% FA) into the Nanospray Flex Ion Source (Thermo Scientific). The Orbitrap Fusion instrument was operated in data-dependent MS/MS mode using HCD fragmentation. The peptide masses were measured by the Orbitrap (MS scans were obtained with a resolution of 120,000 at 200 m/z). Ions exhibiting the greatest peak intensity determined within a 4 second cycle were selected and subjected to fragmentation. Ions were isolated by the quadrupole set at a frequency of 1.2 Da with an isolation window. Fragment spectra were recorded in the Orbitrap with a resolution 15,000. Dynamic exclusion was enabled with an exclusion duration of 60 s, and an exclusion mass tolerance width of ±10 mg/kg relative to masses on the list.

Protein database search and quantitative analysis

The LC-MS/MS data was processed (including data smoothing, background subtraction, and centroiding) using a Proteome Discoverer (Version 2.0, Thermo Scientific). The processed data was then used to identify sequence similarity with known proteins in the Chicken Uniprot database which contains 29,476 sequences, using an in-house Mascot server. Trypsin was chosen as the peptide with a maximum of 2 missed cleavages permitted. S-Carbamidomethyl cysteine was defined as a fixed modification and oxidation of methionine as a variable modification. The MS/MS results were searched with a peptide ion mass tolerance of ±8 mg/kg and a fragment ion mass tolerance of ±0.02 Da. Percolator (Silva *et al.*, 2006) was used for calculating false discovery rates (FDR). Only peptides identified as rank 1 peptides with a confidence value of 1% ($q<0.01$) were considered for further analysis.

All raw files were imported into Expressionist v11.0.5 (Genedata) for data analysis. Data were retention time (RT) aligned using a pairwise alignment, filtered and smoothed before peak detection based on volumes. Detected peaks were isotope-clustered and singletons were filtered out. Peak clusters were matched with Proteome Discoverer identifications and peptides were grouped based on protein identifications. Proteins were quantified based on the three most intense tryptic peptides according to the 'Hi-3' approach (Zougman *et al.*, 2014). Quantitative results were exported into Analyst v11.0.5 (Genedata) for normalization, statistical filtering and testing.

Quality control (QC)
To monitor the quality of the LC-MS/MS based proteomics analysis, QC samples were analysed alongside the experimental samples. The QC samples were prepared by taking equal volumes of all digested protein samples. The first 8 samples analysed were QC samples and thereafter a QC sample was analysed after every five samples throughout the sample sequence. The QC results were used to monitor the extent of variation in the data acquisition system and for data normalization.

Statistical analysis
A multivariate latent class analysis (LCA) was performed on the proteomics manifestations, followed by multiple pairwise treatment comparisons by associated t-tests controlling the FDR at a level 5%. All output values are presented as means ± SEM; $P<0.05$ was considered as statistically significant. Statistical analyses were conducted in SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

RT-PCR
RNA extraction, sample QC and data normalization
RNA was extracted from cells using the Qiasymphony RNA Kit (Qiagen, Hilden, Germany) and the QIAsymphony robot, according to the RNA_CT_400 protocol. The extracted RNA was eluted in 50 µl of H_2O and quantitated on a NanoDrop ND-8000. The RNA integrity numbers (RIN) were determined on an Agilent Bioanalyzer using the Agilent Nano chip. The RNA was then normalized to 10 ng/µl by dilution with H_2O, before cDNA synthesis.

cDNA synthesis
For cDNA synthesis, 100 ng of RNA was used as the input in a 20 µl cDNA reaction. cDNA was synthesized using the Applied Biosystems™ High Capacity cDNA Kit (ThermoFisher, #4368813)). For the preparation of cDNA standard curves, RNA from selected experimental samples was subsequently concentrated to 100 ng/µl.
A no template control (NTC) using H_2O instead of RNA was prepared in a standard cDNA synthesis reaction and a non-enzyme control (NEC) was prepared using 100 ng of pooled sample RNA without addition of reverse transcriptase. The following kit is used in the assay.

Specific target amplification (STA)
A pre-amplification reaction was run in accordance to the Fluidigm Specific Target Amplification Quick Reference (PN 69000133 RevB) and AROS SOP 28: 'Fluidigm gene expression analysis'. In short, 1.25 µl cDNA was mixed with 2.5 µl TaqMan PreAmp Master Mix (2×) (Applied Biosystems, PN 4391128) and 1.25 µl Pooled TaqMan assay mix (0.2×). The

5 µl reaction mix was amplified in a thermal cycler over a 14-cycle run. Briefly, the mixture was first heated to 95 °C for 10 min, and then followed 14 cycles. Each cycle comprised of 15 seconds at 95 °C followed by 4 min at 60 °C. Samples were maintained at 4 °C until harvest. The pre-amplified product was then diluted 1:5 by addition of 20 µl 1x TE buffer.

Gene expression analysis on the Fluidigm BioMark instrument

The gene expression analysis was performed using the Fluidigm Biomark system according to the manufacturers' protocol and run under standard conditions. The set-up of the PCR reaction was performed as described in Fluidigm Quick Reference Cards [PN 68000130, Rev.B] and AROS SOP 28: 'Fluidigm gene expression analysis'.

Loading of the Fluidigm Dynamic Arrays

The samples and assays were loaded on to the chip. The assays were each loaded into 1 to 3 (dependent on the specific experiment) separate inlets on the chip (format 96.96). Each sample inlet was mixed with each assay inlet on the chip. The Fluidigm PCR was performed using the cycling protocol (standard setting, see Supplementary Table S1).

PCR data processing and delivery

The real-time PCR data were analysed using Fluidigm Real-Time PCR Analysis software ver. 4.3.1 (Fluidigm Corporation, CA, USA). The initial data were analysed using the Linear (Derivative) for baseline correction Baseline and User (Detectors) Ct Threshold settings. Data analysis was performed as follows:

1. Control samples (NEC) were evaluated, in which there should have been no significant amplification. Amplification was considered generally acceptable if it occurred at least 5-10 cycles later than the amplification of the positive control ($\Delta Ct \geq 5\text{-}10$). The amplification of the positive control should have been similar to that of known reference sample values. If either the negative or the positive controls did not perform as expected, a closer assessment of the result and decisions for further troubleshooting were taken on a case-by-case basis.
2. Average Ct values for each target / sample were calculated.
3. The standard deviations of the Ct values for each target / sample were calculated. (High standard deviations are usually associated with high Ct values where the Ct value is close to the Limit of Quantification (LOQ) for the assay.).
4. The data collected during the processes were delivered in a report sheet with all raw as well as average Ct values and standard deviations associated with the Ct values for each sample.
5. Reference gene stability was calculated using Normfinder v0.953.

Taq Man ID and PCR probes sequences

Please see Supplementary Tables S2 and S3.

Supplementary references

Käll, L., Canterbury, J.D., Weston, J., Noble, W.S. and MacCoss, M.J., 2007. Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nature Methods. 4: 923-925. https://doi.org.10.1038/nmeth1113

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Zougman, A., Selby, P.J. and Banks, R.E., 2014. Suspension trapping (STrap) sample preparation method for bottom-up proteomics analysis. Proteomics 14: 1006-1010. https://doi.org.10.1002/pmic.201300553

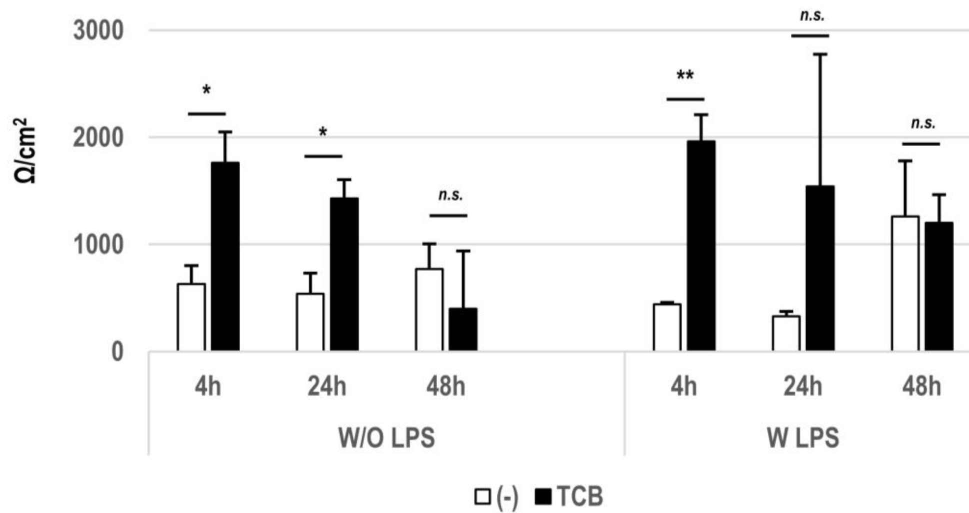

Figure S1. Thymol and cinnamaldehyde blend (TCB) positively modulated epithelial barrier integrity of human Caco-2 cells. Human epithelial cells (Caco-2) were cocultured for 2 h with TCB (10 ng/ml) prior to stimulation with/without LPS, as indicated. Cell cultures were incubated for a further 48 h prior to measurement of trans epithelial electrical resistance (TEER) at 4, 24 and 48 h respectively. Each column represents the mean (± SEM) of two independent experiments each with 4 technical replicates. W/O LPS: LPS was not added to cells; W LPS: LPS was added to cells. *: P<0.05, **: P<0.01, n.s.: not statistically significant.

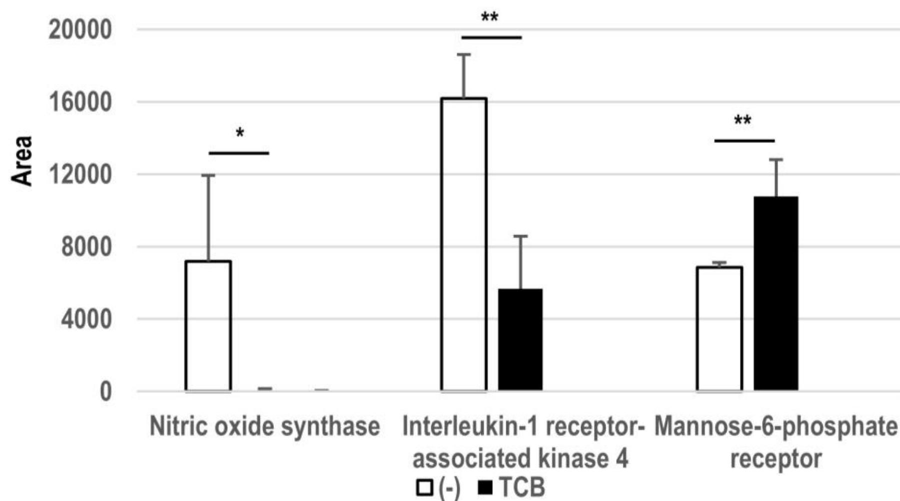

Figure S2. Thymol and cinnamaldehyde blend (TCB) alters relative expression of cell transcription factors by HD-11 monocytes/macrophages, as determined by proteomic analysis. Chicken monocytes (HD-11) were cocultured 2 h with TCB, and then stimulated with/without LPS and further cultured for 6 h. Data are shown as area (absolute values). Each column represents the mean from 2 independent experiments each with 4 technical replicates. *: P<0.05, **: P<0.01.

Table S1. PCR Cycling protocol.

Stage	Temperature (°C)	Time (S)	# of cycles
Thermal Mix	50	120	1
Thermal Mix	70	1800	1
Thermal Mix	25	600	1
UNG	50	120	1
Hot Start	95	600	1
Denaturation	95	15	40
Annealing	60	60	40

Table S2. Taq Man ID for PCR.

Markers chicken	TaqMan ID no.
TLR1 / TLR6	Gg03372556_s1
TLR2	Gg03357653_s1
TLR3	Gg03359006_m1
TLR4	Gg03354643_m1
TLR5	Gg03344019_s1
TLR7	Gg03812600_m1
IL-6	Gg03337980_m1
IL-10	Gg03358689_m1
TGF beta-3	Gg03371522_m1
TNF (alpha)	Gg03364359_m1
iNOS	Gg03347749_m1
MyD88	Gg03355571_m1
NFkappaB-1	Gg03366668_m1
NFkappaB-2	Gg03365205_m1
TRIF	Gg03813681_s1
IRF3	Gg03339759_m1
SMAD-2	Gg03347188_m1
BCL2	Gg03349196_m1
COX-2	Gg03320004_m1
GM-CSF	Gg03370156_m1
CCL4 (MIP-1 beta)	Gg03338617_m1
CCL20 (MIP-3 alpha)	Gg03365414_m1
pIgR	Gg03359883_m1
CD14	Gg03812062_s1
CD107a	Gg03367377_m1
CD28	Gg03349036_m1
CTLA-4	Gg03359551_m1
IL-1 beta	Gg03347154_g1
IL-8	Gg03348119_m1
IL-12B	Gg03349677_m1
MUC2	Gg03326003_m1
CCL5 (RANTES)	Gg03360168_m1
CXCL10 (IP-10)	Gg03312108_m1
Beta-Actin	Gg03815934_s1
TBP	Gg03366486_m1
POLR2B	Gg03337168_m1

Table S3. PCR probe sequences.

Markers	Genebank acc. no.	F-Primer sequence (5'-3')	R-Primer sequence (5'-3')	Probe seq (5'-3')
G-CSF	NM_205279	gcccaatcacacgacgttg	gctggatgttggaggagagg	tggagacgctgcagttggac
M-CSF	GQ249404	cgactctgtctgctacgtga	cgtcttcatcttgttggcgt	aagccgccttccctttgct
CCL3	EU999777	tcaacctgctgcttgtccta	tccctccctttcttggtcac	cctcattgcctccgcctaca
CLDN4	XM_003642382	gccaaagccaaggtgatgat	tagaagtcccggatgatggc	gcgtcctcatcctcatcccc
OCLN	NM_205128	tcctcatcgtcatcctgctc	ttcttcacccactcctccac	aacatctactgggaccgcgc