Bis-demethoxycurcumin and its cyclized pyrazole analogue differentially disrupt lipopolysaccharide signaling in human monocyte-derived macrophages

Supplemental material - Methods

Cell viability assay

At the end of differentiation, macrophages seeded in 48-well plates were washed and treated with increasing concentrations (2.5-10 µM) of curcumin or the analogues GG6 or GG9 in RPMI 1640 + 10% FBS (Zusso et al., 2017). After 24 h incubation, 10 µL (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/mL) was added to each well for 4 h. At the end of incubation the formazan crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO). MTT reduction was quantifed on a microplate reader (Victor2 Multilabel Counter, Wallac, Cambridge, MA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Results were expressed as raw optical density (O.D.) values.

Gene expression analysis

Macrophages were lysed immediately after the 24-hour polarization step using RNeasy Plus Mini Kit (Qiagen, Switzerland). cDNA was generated using RevertAid Reverse Transcriptase and random primers according to the manufacturer's instructions. qRT-PCR reactions were then run with Max SYBR Green PCR Master Mix on a CFX96 Real-Time PCR Detection System

thermocycler (Biorad, Milan, Italy). Primer pairs for human NLRP3 were designed ex novo using NCBI Primer-BLAST: Forward-AAGAGCCTTGCCCAGACATC; Reverse- AGCTTCAAACGACTCCCTGG. Results were normalized using the housekeeping gene GAPDH (Forward-CACCATCTTCCAGGAGCGAG; Reverse- CCTTCTCCATGGTGGTGAAGAC) and the ΔΔ cycle threshold method, and are expressed as relative fold of stimulated over control group, used as calibrator.

Legend to Supplemental Figures

Supplemental Figure 1: Human blood-derived monocytes were grown in RPMI + 10% FBS and differentiated into macrophages in the presence of 20 nM CSF-1 for 7 days at 37°C and 5% CO_2. At the end of differentiation, macrophage viability was measured by MTT assay following treatment with curcumin, GG6 and GG9 (2.5-10 µM) for 24 h. Data are expressed as mean raw absorbance values (±SEM) of 3 independent experiments performed in triplicate.

Supplemental Figure 2: Effects of curcumin, GG6, GG9 and CLI-095 on IL-1β accumulation in LPS-stimulated human macrophages. After differentiation, macrophages were pre-treated with curcumin, GG6, GG9 and CLI-095 as indicated for 30 min followed by stimulation with 1 µg/ml LPS for 24 h in FBS-free RPMI in the presence of brefeldin (10 µM) over the last 4 h to disrupt cytokine secretion pathways. Cell lysates were collected and analyzed for IL-1β release by ELISA. Bar graph represents the mean (±SEM) of 6 independent experiments. *$p<0.01$ *vs* resting, °$p<0.05$ *vs* LPS.

Supplemental Figure 3: Human NLRP3 mRNA levels in macrophages. cDNA from resting, LPS-stimulated M1 and test agent-pretreated M1 macrophages were assayed by qPCR after 90-min LPS incubation. GADPH served as loading control. Bars represent the mean (± SEM) of 3 independent experiments.

Supplemental Figure 4: Effects of GG9 and CLI-095 on cell-bound TNF-α expression in LPS-stimulated macrophages. After differentiation, macrophages were pre-treated with either GG9 or CLI-095 for 30 min, and then incubated with LPS for 6 h at 37°C in RPMI + 10% FBS in the presence of brefeldin (10 µM) over the last 4 h to disrupt cytokine secretion pathways. Prior to flow cytometry analysis fixed/permeabilized cells were stained with anti-TNF-α antibodies (eBioscience). Fluorescence plots are from representative experiments (panel **A**), and bar graphs represent the mean (± SEM) of 5 independent experiments (panel **B**). * $p < 0.05$ vs. resting; # $p < 0.05$ vs. LPS.

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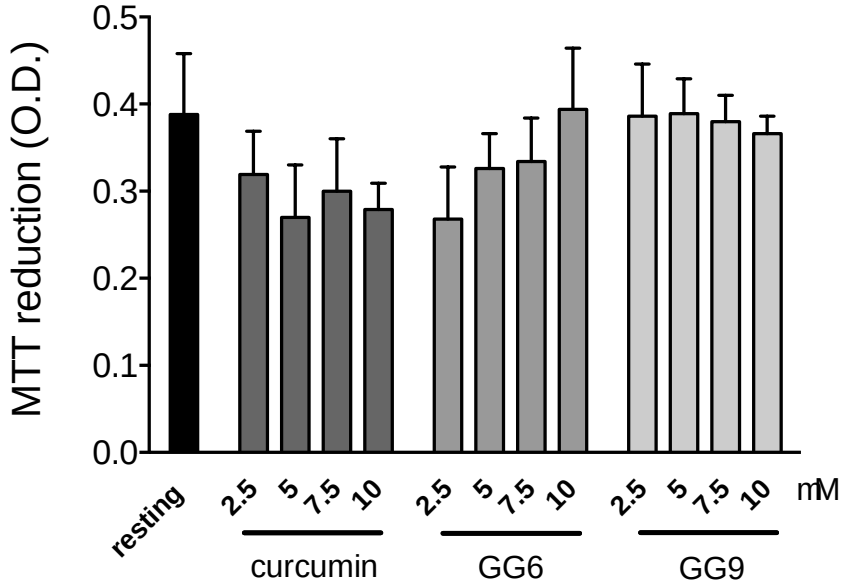
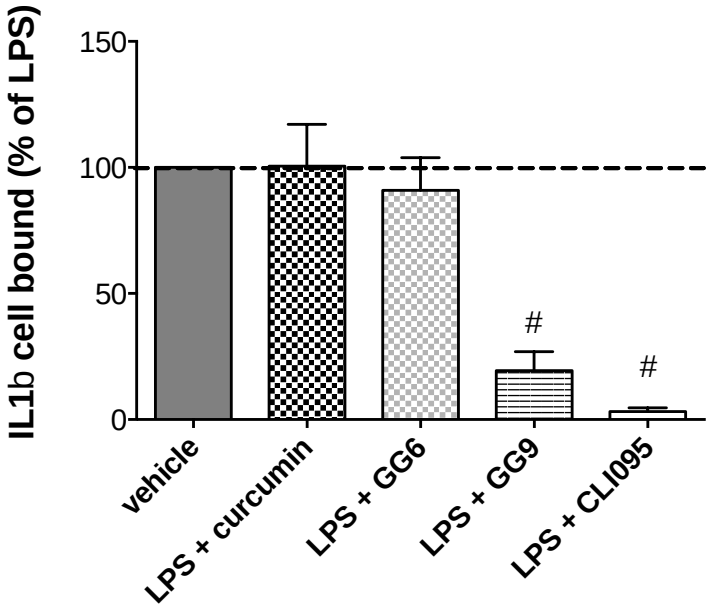
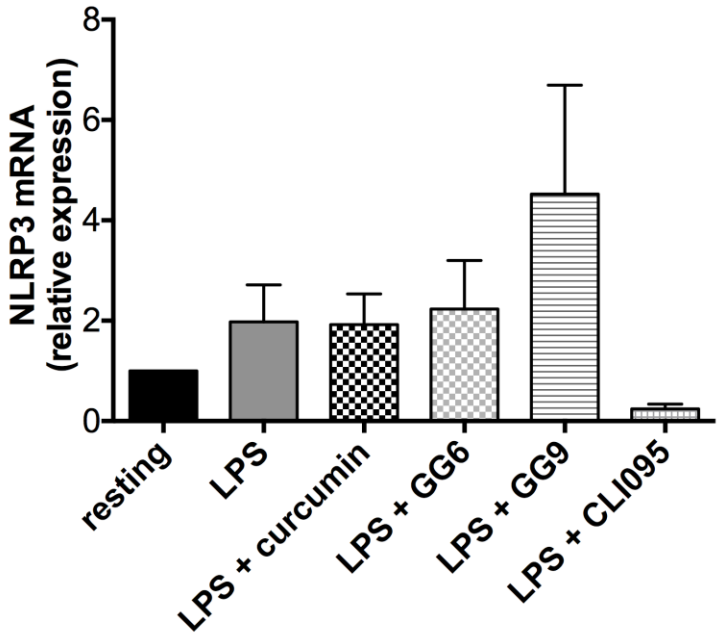
Supplemental Fig. 1



Supplemental Fig. 2



Supplemental Fig. 3



Supplemental Fig. 4