Induction of glucocorticoid-induced leucine zipper (GILZ) contributes to anti-inflammatory effects of the natural product curcumin in macrophages

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Running Title: GILZ-dependent anti-inflammatory effects of curcumin

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ABSTRACT
GILZ is inducible by glucocorticoids and plays a key role in their mode of action. GILZ attenuates inflammation mainly by inhibition of NF-κB and MAP kinase activation but does not seem to be involved in the severe side effects observed after glucocorticoid treatment. Therefore, GILZ might be a promising target for new therapeutic approaches. The present work focuses on the natural product curcumin, which has previously been reported to inhibit NF-κB.

GILZ was inducible by curcumin in macrophage cell lines, primary human monocyte-derived macrophages, and murine bone marrow-derived macrophages (BMMs). The upregulation of GILZ was neither associated with glucocorticoid receptor activation nor with transcriptional induction, mRNA or protein stabilization, but was a result of enhanced translation. Since the GILZ 3′-UTR contains AU-rich elements (AREs), we analyzed the role of the mRNA binding protein HuR, which has been shown to promote the translation of ARE-containing mRNAs. Our results suggest that curcumin treatment induces HuR expression. An RNA-immunoprecipitation assay confirmed that HuR can bind GILZ mRNA. In accordance, HuR overexpression led to increased GILZ protein levels but had no effect on GILZ mRNA expression. Our data employing siRNA in LPS-activated RAW264.7 macrophages show that curcumin facilitates this anti-inflammatory action by induction of GILZ in macrophages. Experiments with LPS-activated BMMs from wild-type and GILZ knockout mice demonstrated that curcumin inhibits the activity of inflammatory regulators, such as NF-κB or ERK and subsequent TNF-α production via GILZ. In summary, our data indicate that HuR-dependent GILZ induction contributes to the anti-inflammatory properties of curcumin.

The polyphenol curcumin (diferuloylmethane) is the principal bioactive compound of turmeric (Curcuma longa) preparations, which are commonly used as traditional remedies or spices. Recently, extensive research has shown that curcumin has a broad range of therapeutic effects, including anti-inflammatory, anti-oxidant, anti-proliferative, hypoglycemic, lipid-lowering, anti-thrombotic, and anti-coagulant activity. At the same time, no dose-limiting toxicity was observed in clinical trials, indicating pharmacological safety. Thus, curcumin administration has been suggested as a potential therapeutic approach for the treatment of several pathologic conditions, especially inflammatory diseases such as cardiovascular pathologies, arthritis, asthma, ulcerative colitis, inflammatory bowel disease, and type II diabetes (1-5). Several in vivo studies suggested a profound...

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The effect of curcumin on cells of the mononuclear phagocyte system. In a mouse model of abdominal aortic aneurysms, oral administration of curcumin efficiently suppressed mononuclear inflammation in the aortic walls, i.e. pro-inflammatory cytokine expression and adverse connective tissue remodeling (6). In addition, curcumin decreased the number of pro-inflammatory M1 macrophages but increased the number of anti-inflammatory M2 macrophages in the myocardium in a rat model of experimental autoimmune myocarditis (7). In diabetic nephropathy, curcumin administration reduced the infiltration of macrophages into the glomeruli and impaired the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α (8). Likewise, oral administration of nano-emulsion curcumin diminished macrophage recruitment in a mouse model of peritonitis (9). Curcumin has been shown to interact with various molecular targets, including transcription factors, enzymes, and receptors (1,5,10). The anti-inflammatory effects of curcumin in macrophages and other mononuclear cells are associated with its ability to suppress nuclear factor (NF)-κB signaling (8-13).

Over the last years, the endogenous anti-inflammatory mediator glucocorticoid-induced leucine zipper (GILZ) has attracted increasing attention. GILZ mediates its anti-inflammatory activity mainly by direct binding to the pro-inflammatory transcription factors NF-κB and activator protein (AP)-1, thereby inhibiting their translocation (14-17). Additionally, GILZ has also been reported to be a negative regulator of mitogen-activated protein kinase (MAPK) signaling (18-20). Due to its strong induction by glucocorticoids (GCs) in the thymus, early studies on GILZ concentrated on its effects on thymocytes and T-lymphocytes (21). Several reports indicated that the effects of GILZ overexpression are similar to GC treatment regarding the induction of thymocyte and T-lymphocyte apoptosis (14,17,21-23). Moreover, lack of GILZ impairs GC-induced apoptosis in B-lymphocytes (24).

In monocytes and macrophages, GILZ can be induced by anti-inflammatory agents, such as GCs, interleukin (IL)-4 or IL-10 (15,25-27). GILZ overexpression in macrophage–like THP-1 cells resulted in GC-like effects, i.e. reduced expression of macrophage activation markers, chemokine expression, and NF-κB activity upon treatment with the Toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS) (15).

In the present study, we aimed to examine the influence of curcumin on GILZ expression in macrophages and to elucidate whether GILZ plays a role in the anti-inflammatory properties of curcumin in this cell type.

RESULTS
In the first series of experiments, we examined whether curcumin influences the expression of GILZ in the macrophage-like cell lines RAW264.7 and U937. After incubation with curcumin at non-toxic concentrations, increased GILZ protein levels were observed in murine RAW264.7 and human U937 cells as well as primary human monocyte-derived macrophages (HMDMs), as assessed by Western blot (Figure 1 A-D) and/or flow cytometric analysis (Figure 1 H and I). The specificity of the GILZ-PE antibody used for flow cytometry was evaluated using untreated and dexamethasone-treated WT and GILZ KO MPI macrophages (Figure 1 E-G).

To analyze the molecular mechanism underlying curcumin-mediated GILZ induction, we first examined whether curcumin was able to activate the glucocorticoid receptor (GR). HEK 293T cells were transfected with a vector encoding a GR-GFP fusion protein, followed by treatment with either dexamethasone or curcumin for up to 40 min. As expected, live microscopy revealed translocation of the GR into the nucleus upon dexamethasone treatment. In contrast, GR translocation was absent in curcumin-treated cells (Figure 2 A and Supplemental Figure 1), indicating that the upregulation of GILZ by curcumin was not associated with GR activation.

Next, we examined GILZ mRNA levels in RAW264.7 and U937 cells after curcumin exposure for up to 3 h. No difference in GILZ mRNA expression was detected in either cell line (Figure 2 B and C) and primary HMDMs (data not shown), suggesting that neither the level nor the stability of the GILZ transcript was significantly altered in response to curcumin. Analysis of GILZ mRNA levels after addition of the transcription inhibitor actinomycin D in the absence or presence of curcumin confirmed that curcumin did not affect GILZ mRNA stability (Figure 2 D).

The influence of curcumin on GILZ protein stability was assessed by incubating cells with cycloheximide, a protein synthesis inhibitor, either...
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alone or in combination with curcumin, for different time points. Cycloheximide treatment resulted in a significant decrease of GILZ protein levels after 4 h. However, this effect was not influenced by curcumin co-treatment (Figure 2 E), indicating that curcumin did not stabilize GILZ protein. Thus, we determined the impact of curcumin on the translation of GILZ. To this end we treated U937 cells with curcumin and subjected the resulting lysates to polysomal fractionation. The absorbance at 254 nm, reflecting the RNA content, decreased in the later fractions of the polysome profile, whereas the 60S and 80S peaks increased in response to curcumin, thus indicating a suppression of global translation upon curcumin treatment (Figure 3A). This is in line with a previous report showing that curcumin inhibited cap-dependent translation by suppressing the activation of translation initiation factors (28). Interestingly, while global translation appeared to be inhibited, the distribution of GILZ mRNA shifted from the subpolysomal fractions (fractions 1–3) into the late polysomal fractions (fractions 6–9) (Figure 3 B). This indicates that curcumin enhances GILZ translation.

Adenylate- and uridylate-rich elements (AREs), commonly found in the 3′-untranslated region of mRNAs, are central elements of gene regulation and have been reported to affect the translation process. The mRNA binding protein HuR binds to mature mRNAs through ARE recognition motifs in their 3′UTRs, typically via U-rich sites, thereby stabilizing the respective mRNAs and modulating their translation (29-32). As the 3′-UTR of GILZ mRNA contains a U-rich region (33) and might, therefore, be regulated by HuR, we examined whether curcumin affects HuR expression. Western blot analysis showed that HuR levels were indeed increased in curcumin-treated cells (Figure 4 A). Similar results were obtained with primary HMDMs (Figure 4 B).

We then investigated whether HuR can bind to GILZ mRNA. Cell lysates were subjected to HuR immunoprecipitation, followed by quantification of HuR-bound mRNAs by real-time RT-PCR. In contrast to the negative control GAPDH, GILZ mRNA was enriched to a similar degree as the positive control CCNB1 (Figure 4 C) (34), demonstrating that HuR can bind to GILZ mRNA. To characterize the influence of enhanced HuR expression on GILZ, we overexpressed HuR in HEK 293T cells. GILZ protein levels were indeed increased in HuR-overexpressing cells, whereas GILZ mRNA was not (Figure 4 D-F). These observations strengthen our notion that curcumin increases GILZ mRNA translation via HuR induction.

To examine the functional consequences of increased GILZ levels after curcumin treatment, cells were treated with curcumin, followed by LPS exposure. Subsequently, NF-κB activity was measured by luciferase reporter gene assay. As shown in Figure 5 A, curcumin strongly inhibited LPS-induced NF-κB activation. NF-κB activity was restored upon siRNA-mediated GILZ knockdown, which supports the assumption that GILZ induction contributes to the anti-inflammatory actions of curcumin. In accordance, the inhibitory effect of curcumin on LPS-induced NF-κB-dependent iNOS activity was abrogated upon GILZ knockdown (Figure 5 C and D).

However, GILZ knockdown did not fully rescue either luciferase activity or nitrite accumulation. To evaluate whether the reasons underlying this observation involve residual GILZ expression or GILZ-independent mechanisms, primary murine bone marrow-derived macrophages (BMMs) obtained from wild type (WT) and GILZ knockout (KO) mice were employed. Curcumin was able to induce GILZ in BMMs, as indicated by flow cytometric analysis (Figure 6 A).

The effect of curcumin on NF-κB activity in GILZ-depleted BMMs was comparable to that seen in siGILZ-treated RAW264.7 cells (Figure 6 B), suggesting that GILZ, although considerably contributing to the anti-inflammatory actions of curcumin, is not the only factor involved.

Loss of GILZ has also been shown to enhance extracellular-signal regulated kinase (ERK) signaling in murine bone marrow-derived macrophages (20). Therefore, we examined LPS-induced ERK activation in curcumin-treated WT and GILZ KO BMMs. LPS-mediated ERK activation was strongly inhibited by curcumin in WT macrophages at concentrations that upregulated GILZ expression, whereas ERK signaling was preserved in equally treated GILZ KO BMMs (Figure 6 C-F). Both ERK and NF-κB are involved in LPS-induced TNF-α production in murine BMMs (20). Thus, we also examined the inhibitory effects of curcumin on TNF-α secretion in WT and GILZ KO cells. Pre-incubation of WT BMMs with curcumin decreased the induction of TNF-α by LPS in a dose-dependent manner. In contrast, curcumin...
failed to show any effect on GILZ KO cells under most of the tested conditions (Figure 6 G), thereby supporting the notion that curcumin exerts its anti-inflammatory actions at least in part via GILZ.

**DISCUSSION**

For centuries, the natural compound curcumin has been used for the treatment of inflammatory conditions in Eastern medicine, and various reports have confirmed its ethnopharmacological relevance in recent years (1,3). Several in vitro and in vivo studies suggested that curcumin may be used to treat distinct pathological conditions, including chronic inflammatory processes and neoplastic diseases (10,35).

The mechanisms underlying the anti-inflammatory properties of curcumin involve the regulation of different molecular targets, including transcription factors, growth factors, inflammatory cytokines, and protein kinases (10). The ability of curcumin to interfere with NF-kB signaling is central to its anti-inflammatory action. In general, the effect has been attributed to an attenuated nuclear translocation of NF-kB, which has previously been explained by an induction of its inhibitor IxB or downregulation of molecules required for TLR signaling (8-13,36). In the present study, we demonstrated for the first time that curcumin upregulates the anti-inflammatory mediator GILZ. GILZ can directly interact with NF-kB, thereby inhibiting its translocation. Our data suggest that GILZ induction considerably contributes to the inhibition of NF-kB by curcumin in macrophages.

In addition, GILZ has been reported to attenuate MAPK signaling, e.g. by binding to Ras/Raf, which then leads to the inhibition of downstream MAP kinases, such as ERK (18-20). It was recently shown that curcumin not only diminishes NF-kB activity, but also interferes with other pro-inflammatory signaling pathways in macrophages, including LPS-induced ERK signaling (36). Our experiments with LPS-activated BMMs from wild-type and GILZ knockout mice demonstrated that curcumin inhibits the activity of ERK, at least in part, via GILZ. In line with these findings, the production of proinflammatory effectors, such as NO and TNF, was decreased by curcumin in a GILZ-dependent manner.

We previously observed high constitutive GILZ expression levels in primary human in vitro differentiated and pulmonary macrophages (33). Interestingly, GILZ levels were significantly reduced in human alveolar macrophages as well as in vivo in mouse lungs upon TLR activation (20,33). Both siRNA-mediated GILZ knockdown in human macrophages and GILZ knockout in murine BMMs increased the responsiveness towards LPS, suggesting that repression of endogenous GILZ expression is a regulatory mechanism that enhances the pro-inflammatory response.

In addition to its upregulation by curcumin demonstrated in the present study, GILZ can be induced in monocytes and macrophages by GCs and anti-inflammatory cytokines, such as IL-10 (15,25). The potent inhibitory action of GCs on cytokine and chemokine production in human monocytes has been shown to be abrogated by siRNA-mediated GILZ silencing (25), indicating that GILZ is essential for anti-inflammatory GC actions. In accordance, GILZ overexpression in the macrophage cell line THP-1 resulted in GC-like effects upon LPS challenge (15). Macrophages from the inbred LPS-resistant mouse strain SPRET/Ei have been reported to highly express GILZ. SPRET/Ei macrophages, therefore, showed reduced IL-6 and IL-12 levels after LPS treatment, whereas GILZ knockdown by siRNA compensated the effect. In summary, these reports indicate that GILZ upregulation efficiently prevents inflammatory responses in myeloid cells. Of note, specific upregulation of GILZ might avoid the metabolic side effects of GC therapy, as GILZ and GCs have opposing consequences for the differentiation of mesenchymal stem cells (MSCs): whereas GCs favor adipocyte differentiation and suppress osteoblast formation, GILZ overexpression induces osteogenic differentiation (37,38).

Our findings implicate the curcumin-inducible RNA-binding protein (RBP) HuR as a novel positive regulator of GILZ expression. RBPs can bind to coding as well as noncoding regions of mRNAs. Upon binding, HuR is able to modulate the translation of its target transcripts. HuR has been shown to promote the translation of many target mRNAs, such as HIF1A, TP53, DUSP1, CYCS, and HMOX1 (32,39-43). The molecular mechanisms by which HuR modulates translation are poorly understood, but may involve competition or cooperation with microRNAs. Alternatively, HuR might interfere with internal ribosome entry sites (IRESs) of target transcripts (32).
Unlike most other RBPs that induce mRNA destabilization, ELAV/Hu proteins preferentially stabilize U-rich transcripts. HuR has been reported to increase the stability of several mRNAs, including VEGFA, DUSP1, and cyclins CCNA2, CCNB1, CCNE and CCND1 (32,34,44-47). The underlying mechanisms are not entirely characterized, but it has been suggested that HuR competes with other RBPs that facilitate mRNA recruitment to sites of mRNA degradation, such as the exosome and P-bodies (32). However, the interaction of HuR with GILZ mRNA did not affect its stability in our hands. The influence of other HuR targets on the anti-inflammatory effects of curcumin presently remains elusive and might represent an interesting subject for future studies.

In summary, our data demonstrate that curcumin induces GILZ in macrophages, which contributes to its anti-inflammatory actions. These new insights into the effects of curcumin in the suppression of inflammatory responses suggest that curcumin treatment may be beneficial in various inflammatory diseases associated with excessive macrophage activation. The clinical use of curcumin is restricted by its low solubility and poor systemic bioavailability. Therefore, orally administered curcumin has mainly been used for the treatment of gastrointestinal disorders, including inflammatory bowel disease and colon cancer (48-50). New therapeutic approaches aiming to overcome these limitations, e.g. nano-emulsion or silica nanoparticle formulations, have recently been described (9,51) and might expand the number of pathologic conditions that may benefit from curcumin treatment in the near future. Alternatively, curcumin might serve as a lead for GILZ-inducing derivates with a better bioavailability.

**EXPERIMENTAL PROCEDURES**

**Materials** - Cell media and fetal calf serum (FCS) were from Sigma-Aldrich. Anti-GILZ antibodies were either obtained from Santa Cruz Biotechnology (polyclonal goat anti-GILZ Ab, sc-26518) or Sigma-Aldrich (polyclonal rabbit anti-GILZ Ab, SAB1101125). PE-labelled anti-GILZ antibodies and matching isotype controls were purchased from eBioscience (12-4033). PE-anti-F4/80 (130-102-943) and REA Control-PE were from Miltenyi Biotec. The anti-HuR antibody was obtained from Santa Cruz Biotechnology (sc-5261). Anti-tubulin (T9026) and anti-IgG (I5381) antibodies were from Sigma-Aldrich. Anti-rabbit IRDye 680- and anti-mouse IRDye 800- conjugated secondary antibodies were from LI-COR Biosciences. Anti-p44/42 (ERK1/2) (L34F12) mouse antibody and anti-phospho-p44/42 MAPK (Thr202/Tyr204) (20G11) rabbit mAbs were obtained from Cell Signaling Technology. The pRL-TK renilla vector was obtained from Promega. The pEGFP-GR vector was a gift from Alice Wong (Addgene plasmid # 47504). The pZeoSV2(-) vector containing the coding sequence of HuR and the matching control vector (52) were kind gifts from Prof. Dr. Hartmut Kleinert (Johannes Gutenberg University, Mainz, Germany). GILZ-targeting siGENOME SMARTpool siRNA and the non-targeting control siRNA were purchased from Dharmacon. Ultrapure LPS from Escherichia coli K12 was purchased from Invivogen. Curcumin was obtained from Enzo. Other chemicals were purchased either from Sigma-Aldrich or Carl Roth unless stated otherwise.

**Cell lines** - RAW264.7, U937, and L929 cells were cultivated in standard medium (RPMI 1640, 10% FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM glutamine). HEK 293T cells were grown in high glucose DMEM medium with supplements (10% FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM glutamine).

**Human monocyte-derived macrophages (HMDMs)** – Buffy coats were obtained from healthy adult blood donors (Blood Donation Center, Saarbrücken, Germany). The use of human material for the isolation of primary cells was approved by the local ethics committee (permission no. 130/08). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphocyte Separation Medium (PAA) and Leucosep tubes (Greiner). After washing with PBS, monocytes were purified from PBMC by plastic adherence for 1 h. Monocytes were differentiated into macrophages in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM glutamine, and 20 ng/ml GM-CSF at 37°C and 5% CO2 for 8 d. Medium was changed every other day.

**Murine bone marrow-derived macrophages (BMMs)** - BMMs were obtained from 8-12 week old male wildtype (WT) or GILZ knockout (KO) mice as described previously (20). Briefly, femurs and
tibias were flushed with standard medium. Erythrocytes contained in the cell pellet after centrifugation were lysed with hypotonic buffer. Cells were resuspended in standard medium supplemented with M-CSF (50 ng/ml, Biomol), transferred into a culture flask and allowed to adhere overnight. Non-adherent cells were collected and cultivated for another 5 d in M-CSF-containing medium. Subsequently, cells were detached with accutase (Sigma-Aldrich), suspended in standard medium with 10 ng/ml M-CSF and seeded in 96 well plates (7.5 x 10^4 cells/well in 150 µl medium) for MTT assays and TNF-α quantification or 12 well plates (0.5 x 10^6 cells/well in 1 ml medium) for Western blot analyses. BMMs were found to be > 95% pure as indicated by flow cytometric analysis using an antibody against F4/80 (data not shown).

MPI cells - MPI cells, i.e. non-transformed self-renewing primary murine macrophages, were obtained from C57BL/6 mice based on a method described previously (20,53). Briefly, MPI cells were prepared from fetal livers of male 15-d-old mouse embryos and grown in standard medium supplemented with 30 ng/ml murine GM-CSF (Biomol). Proliferating cells were subcultured by splitting them 1:5 after 6-8 d. The purity of the cell preparations was ≥ 95%, as assessed by flow cytometric analysis of F4/80 expression (data not shown).

Determination of cell viability - In order to ensure that non-toxic concentrations of curcumin were used, the MTT colorimetric assay was performed as described previously (20,54). Absorbance measurements were carried out at 550 nm with 630 nm as the reference wavelength using a microplate reader (Tecan Sunrise). The cell viability obtained from at least three independent experiments was calculated relative to untreated and solvent controls (data not shown).

HuR overexpression - HEK 293T cells were seeded at a density of 2 x 10^5 cells per well into a 12 well plate and were grown for 24 hours. Cells were either transfected with the pZeoSV2(−)HuR or the empty pZeoSV2(−) control vector using PolyFect (Qiagen) according to the manufacturer’s recommendations. Cells were harvested for RNA or protein isolation 48 h after transfection.

Western blotting - Cells were harvested with 100 - 200 µl lysis buffer (50 mM Tris-HCl, 1% (m/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.004% (m/v) bromphenol blue) supplemented with a protease inhibitor mix (Complete; Roche Diagnostics) as described previously (20,33,55). Blots were incubated with primary antibody dilutions at 4°C overnight and subsequently with secondary antibody dilutions at room temperature for 1.5 h. Blots were scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences), and relative protein amounts were determined using either Odyssey or ImageJ software.

Flow cytometry - Staining was essentially performed as described previously (20,56,57). Briefly, cells were detached from the plates using accutase (Sigma-Aldrich). For extracellular staining of F4/80, cells were washed with PBS, resuspended in FCB (flow cytometry buffer, PBS containing 2.5% (v/v) bovine calf serum and 0.05% (w/v) NaN₃). 5 x 10^5 cells were incubated with Mouse BD Fc Block (BD Biosciences) as recommended by the supplier, followed by addition of F4/80 or isotype control antibody (0.3 µg in 100 µl FCB) for 15 min on ice. The cells were washed in FCB and resuspended in 1% (w/v) cold paraformaldehyde in PBS, pH 7.6. To detect intracellular GILZ, cells were incubated with the Fc Blocking reagent, fixed for 10 min in 1% (w/v) paraformaldehyde in PBS, pH 7.6, followed by permeabilization in SAP (FCB with 0.2% (w/v) saponin and blocking for 30 min in 20% FCS (w/v, diluted in SAP). Cells were stained with the GILZ-specific antibody or the matching isotype control (0.5 µg in 100 µl SAP) for 30 min on ice. The stained cells were examined on a BD LSRFortessa™ cell analyzer, and results were analyzed using the FACS Diva software (BD Biosciences). Curcumin itself did not interfere with flow cytometric analysis (data not shown).

Determination of NF-κB activity - For reporter gene assays, a vector containing NF-κB responsive elements (RE) driving firefly luciferase expression (pGL4.32[luc2P/NF-κB-RE/Hygro], Promega) was used. The pRL-TK vector provided constitutive expression of renilla luciferase and served as an internal control value, to which expression of the firefly luciferase reporter gene was normalized. To assess the influence of GILZ on curcumin-induced
NF-κB-inhibition, RAW 264.7 macrophages were co-transfected with the firefly / renilla luciferase vectors (3.33 and 0.67 µg/ml, respectively) and either GILZ-targeting (siGILZ) or control siRNA (siCo, 10 nM) using the JetPrime reagent (Polyplus transfections) as recommended by the supplier. Knockdown efficiency was assessed by flow cytometry after 24 h (siGILZ: 50.5 ± 10.7% GILZ expression when compared with siCo (= 100%), p < 0.05).

For luciferase assays, RAW 264.7 cells were seeded at 10^4 cells per well into a 96 well plate, treated as indicated and harvested by addition of 1 x passive lysis buffer (Promega). BMMs were transfected and processed as reported previously (20). Luciferase activity was determined by addition of firefly luciferase substrate (470 µM D-luciferin, 530 µM ATP, 270 µM CoA, 33 mM DTT, 20 mM tricine, 2.67 mM MgSO_4_, and 0.1 mM EDTA, pH 7.8) or renilla substrate solution (0.1 M NaCl, 25 mM Tris-HCl pH 7.5, 1 mM CaCl_2, 1 µM coelenterazine), followed by luminescence measurement using a POLARstar OPTIMA Luminometer (BMG Labtech) or the Glomax Discover multiplate reader (Promega) as described in (20,33,54,55).

**RNA immunoprecipitation -** Immunoprecipitation of HuR-associated RNA was performed using the magnetic SureBeads system (Bio-Rad) according to the manufacturer’s specifications. For four samples, 4.5 x 10^5 U937 cells were centrifuged for 5 min at 500 x g and washed with cold PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4_, 1.8 mM KH_2PO_4_, pH 7.4). The cell pellet was lysed in 2 ml RIPA buffer (50 mM Tris-HCl (pH 7.5), 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.05 % sodium dodecyl sulfate, 1 mM EDTA, 150 mM NaCl) and centrifuged for 10 min at 16,000 x g and 4°C. All buffers were supplemented with RNaseOut (Invitrogen) and a protease inhibitor cocktail (Complete; Roche Diagnostics) as suggested by the suppliers. The beads were pretreated according to the manufacturer’s recommendations and incubated in antibody dilutions. The concentration of the HuR antibody or the IgG control was 5 µg in 200 µl 150 mM NaCl solution per sample. 500 µl of the samples were added to the pretreated beads, incubated on a rotator for 1 h at room temperature and washed according to the manufacturer’s protocol. RNA extraction was performed using the Qiazol reagent (Qiagen). 500 µl Qiazol were added to the beads. After adding 100 µl chloroform, samples were thoroughly vortexed and centrifuged at 16,000 x g and 4°C for 10 min. The aqueous phase was transferred to a tube containing 6 µl linear acrylamide (5 mg/ml, Ambion), 60 µl 5 M ammonium acetate and 600 µl isopropanol, followed by vortexing and precipitation at -80°C overnight. Tubes were thawed on ice and centrifuged at 16,000 x g and 4°C for 10 min. The pellet was washed once with 0.5 ml 70% ethanol, dried and dissolved in 20 µl RNase-free water.

**RNA isolation, reverse transcription, and real-time RT-PCR -** Total RNA from cultured cells was extracted using either Qiazol (Qiagen) or the High Pure RNA Isolation Kit (Roche) as recommended by the manufacturers. After removing residual DNA (DNA-free kit, Applied Biosystems), 1 µg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). DNase treatment was skipped for IP samples. Quantitative real-time RT-PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad), gene-specific primers (Eurofins MWG Operon, table 1), and 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus. Standards from 60 to 0.0006 attomoles of the PCR product cloned into pGEMTeasy (Promega), were run alongside the samples to generate a standard curve. Alternatively, a cDNA dilution series were used. All samples and standards were analyzed in triplicate, except for IP samples, which were measured in duplicate. The PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of 20 s at 95°C, 20 s at the primer-specific annealing temperature and 20 s at 72°C.

**Griess assay -** RAW 264.7 cells were cultured in 96 well plates and treated with LPS (0.1 µg/ml) in the presence or absence of curcumin (3.1 and 6.3 µM) or DMSO as a solvent control. After 20 h, the concentration of nitrite, a stable metabolite of NO, was measured in the supernatants by Griess assay as described previously (58). Briefly, 90 µl 1% sulfanilamide in 5% H_3PO_4_ and 90 µl 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O were added to 100 µl of the cell culture supernatant, followed by absorbance measurement at 550 nm and the reference wavelength 630 nm using a Tecan Sunrise microplate reader. A standard curve of sodium nitrite dissolved in medium was used to determine nitrite concentrations. GILZ knockdown was determined by transfecting RAW264.7 cells
with control siRNA or siGILZ (40 nM) using the jetPrime reagent (PolyPlus transfections) as recommended by the supplier. Knockdown efficiency was assessed by flow cytometry 24 h after transfection (siGILZ: 29.7 ± 7.8% GILZ expression when compared with siCo (= 100%), p < 0.001).

**TNF bioassay** - The TNF bioassay was performed as previously described (20, 59). In brief, L929 cells were seeded at a density of 3×10^4 cells per well into a 96 well microplate. After 24 h, medium was replaced by actinomycin D solution (1 µg/ml in standard medium). After 1 h of pre-incubation with actinomycin D, BMM supernatants were added. Dilution series of recombinant murine TNF-α (100-2,500 pg/ml, Biomol) in standard medium were run alongside the samples to generate a standard curve. The plates were incubated for an additional 24 h at 37°C. The MTT assay was used to assess cell viability.

**GR translocation assay** - HEK 293T cells were transfected with pEGFP GR vector (glucocorticoid receptor tagged with GFP) by electroporation using the Amaxa nucleofection device (Lonza) and were grown in µ-Slide VI0.4 (ibidi). After 24 h, cells were analyzed by live microscopy (60). The cells were analyzed with an Axio Observer Z1 epifluorescence microscope equipped with an AxioCam Mrm, Incubator Xlmulti S1, TempModul S1, and CO₂ Modul S1 (Zeiss). All cell images were obtained using a Fluar 40x/1.30 Oil M27 objective. The nuclei staining, Hoechst 33342 (2 µM) was added 5 min before the acquisition. Cells were either treated with 50 µM curcumin or 100 nM dexamethasone. Video recording was started directly after drug application.

**Polysomal fractionation** - 8 × 10⁶ U937 cells were seeded into 75 cm² flasks. After 1 d, the cells were treated with 6.25 µM curcumin or vehicle control for 3 h, followed by polysomal fractionation as described previously (61). Briefly, after incubation with 100 µg/ml cycloheximide (CHX) for 10 min at 37°C, cells were harvested in PBS/CHX (100 µg/ml) and lysed in 750 µl polysome buffer (140 mM KCl, 20 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 0.5% NP40, 0.5 mg/ml heparin, 1 mM DTT, 100 U/ml RNasin (Promega), 100 µg/ml CHX). After pelleting, the cytoplasmic lysates were layered onto 11 ml 10–50% continuous sucrose gradients. The gradients were centrifuged at 35,000 rpm for 2 h at 4°C without brake, using an SW40 rotor in a Beckman ultracentrifuge. Thereafter, the gradients were collected in 1 ml fractions using a Gradient Station (Biocomp). Absorbance was measured at 254 nm. RNA was precipitated by addition of sodium acetate (3 M) and isopropanol, and further purified using the Nucleospin RNA Kit (Macherey-Nagel) according to the manufacturer’s manual. mRNA derived from polysomal fractions or total RNA extracted from the gradients was reverse transcribed using the Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific). GILZ mRNA was subsequently quantified by real-time RT-PCR using the iQ SYBR Green Supermix (BioRad).

**Statistical analysis**

Data are expressed as means ± SEM. Statistical significances between multiple groups were determined either by one-way ANOVA with Bonferroni’s post hoc test for normally distributed values or Mann-Whitney test for data that were not normally distributed. Comparison of normally distributed data in data sets that only contained two groups was performed using Student’s t-test. Values with p < 0.05 were considered significant.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
JH, NH, JVVP, SL, MMK, KA, TS, and BD designed, performed, and analyzed the experiments. JH wrote the paper. AKK, NH, SL, TS, SB, and CR contributed to drafting the manuscript. SB and CR provided materials and discussed the data. AKK initiated the study and participated in data interpretation and manuscript preparation. All authors read and approved the final manuscript.
REFERENCES

1. Gupta, S. C., Kismali, G., and Aggarwal, B. B. (2013) Curcumin, a component of turmeric: from farm to pharmacy. *Biofactors* **39**, 2-13

2. Aggarwal, B. B., Gupta, S. C., and Sung, B. (2013) Curcumin: an orally bioavailable blocker of TNF and other pro-inflammatory biomarkers. *Br J Pharmacol* **169**, 1672-1692

3. Aggarwal, B. B., Sundaram, C., Malani, N., and Ichikawa, H. (2007) Curcumin: the Indian solid gold. *Adv Exp Med Biol* **595**, 1-75

4. Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., Ko, J. Y., Lin, J. T., Lin, B. R., Ming-Shiang, W., Yu, H. S., Lee, S. H., Chen, G. S., Chen, T. M., Chen, C. A., Lai, M. K., Pu, Y. S., Pan, M. H., Wang, Y. J., Tsai, C. C., and Hsieh, C. Y. (2001) Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* **21**, 2895-2900

5. Shishodia, S., Sethi, G., and Aggarwal, B. B. (2005) Curcumin: getting back to the roots. *Ann N Y Acad Sci* **1056**, 206-217

6. Parodi, F. E., Mao, D., Ennis, T. L., Pagano, M. B., and Thompson, R. W. (2006) Oral administration of diferuloylmethane (curcumin) suppresses proinflammatory cytokines and destructive connective tissue remodeling in experimental abdominal aortic aneurysms. *Ann Vasc Surg* **20**, 360-368

7. Gao, S., Zhou, J., Liu, N., Wang, L., Gao, Q., Wu, Y., Zhao, Q., Liu, P., Wang, S., Liu, Y., Guo, N., Shen, Y., Wu, Y., and Yuan, Z. (2015) Curcumin induces M2 macrophage polarization by secretion IL-4 and/or IL-13. *J Mol Cell Cardiol* **85**, 131-139

8. Soetikno, V., Sari, F. R., Veeraveedu, P. T., Thandavaranay, R. A., Harima, M., Sukumaran, V., Lakshmanan, A. P., Suzuki, K., Kawachi, H., and Watanabe, K. (2011) Curcumin ameliorates macrophage infiltration by inhibiting NF-kappaB activation and proinflammatory cytokines in streptozotocin induced-diabetic nephropathy. *Nutr Metab (Lond)* **8**, 35

9. Young, N. A., Bruss, M. S., Gardner, M., Willis, W. L., Mo, X., Valiente, G. R., Cao, Y., Liu, Z., Jarjour, W. N., and Wu, L. C. (2014) Oral administration of nano-emulsion curcumin in mice suppresses inflammatory-induced NFkappaB signaling and macrophage migration. *PLoS One* **9**, e111559

10. Zhou, H., Beevers, C. S., and Huang, S. (2011) The targets of curcumin. *Curr Drug Targets* **12**, 332-347

11. Ziaei, A., Hoppestädter, J., Kiemer, A. K., Ramezani, M., Amirghofran, Z., and Diesel, B. (2015) Inhibitory effects of teuclatriol, a sesquiterpene from salvia mirzayanii, on nuclear factor-kappaB activation and expression of inflammatory mediators. *J Ethnopharmacol* **160**, 94-100

12. Chen, F., Guo, N., Cao, G., Zhou, J., and Yuan, Z. (2014) Molecular analysis of curcumin-induced polarization of murine RAW264.7 macrophages. *J Cardiovasc Pharmacol* **63**, 544-552

13. Kim, G. Y., Kim, K. H., Lee, S. H., Yoon, M. S., Lee, H. J., Moon, D. O., Lee, C. M., Ahn, S. C., Park, Y. C., and Park, Y. M. (2005) Curcumin inhibits immunostimulatory function of dendritic cells: MAPKs and translocation of NF-kappaB as potential targets. *J Immunol* **174**, 8116-8124

14. Ayyroldi, E., Migliorati, G., Bruscoli, S., Marchetti, C., Zollo, O., Cannarile, L., D’Adamio, F., and Riccardi, C. (2001) Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood* **98**, 743-753

15. Berrebi, D., Bruscoli, S., Cohen, N., Foussat, A., Migliorati, G., Bouchet-Delbos, L., Maillot, M. C., Portier, A., Couderc, J., Galanaud, P., Peuchmair, M., Riccardi, C., and Emilie, D. (2003) Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10. *Blood* **101**, 729-738

16. Di Marco, B., Massetti, M., Bruscoli, S., Macchiariulo, A., Di Virgilio, R., Velardi, E., Donato, V., Migliorati, G., and Riccardi, C. (2007) Glucocorticoid-induced leucine zipper (GILZ)/NF-kappaB interaction: role of GILZ homo-dimerization and C-terminal domain. *Nucleic Acids Res* **35**, 517-528
17. Mittelstadt, P. R., and Ashwell, J. D. (2001) Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J Biol Chem* **276**, 29603-29610
18. Ayroldi, E., Zollo, O., Macchiariulo, A., Di Marco, B., Marchetti, C., and Riccardi, C. (2002) Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Mol Cell Biol* **22**, 7929-7941
19. Ayroldi, E., Zollo, O., Bastianelli, A., Marchetti, C., Agostini, M., Di Virgilio, R., and Riccardi, C. (2007) GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling. *J Clin Invest* **117**, 1605-1615
20. Hoppstädter, J., Kessler, S. M., Bruscoli, S., Huwer, H., Riccardi, C., and Kiemer, A. K. (2015) Glucocorticoid-Induced Leucine Zipper: A Critical Factor in Macrophage Endotoxin Tolerance. *J Immunol* **194**, 6057-6067
21. D'Adamio, F., Zollo, O., Moraca, R., Ayroldi, E., Bruscoli, S., Bartoli, A., Cannarile, L., Migliorati, G., and Riccardi, C. (1997) A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* **7**, 803-812
22. Delfino, D. V., Agostini, M., Spinicelli, S., Vito, P., and Riccardi, C. (2004) Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice. *Blood* **104**, 4134-4141
23. Delfino, D. V., Agostini, M., Spinicelli, S., Vacca, C., and Riccardi, C. (2006) Inhibited cell death, NF-kappaB activity and increased IL-10 in TCR-triggered thymocytes of transgenic mice overexpressing the glucocorticoid-induced protein GILZ. *Int Immunopharmacol* **6**, 1126-1134
24. Bruscoli, S., Biagioli, M., Sorcini, D., Frammartino, T., Cinino, M., Sportoletti, P., Mazzon, E., Bereshchenko, O., and Riccardi, C. (2015) Lack of Glucocorticoid-induced leucine zipper (GILZ) deregulates B cell survival and results in B cell lymphocytosis in mice. *Blood* **126**, 1790-1801
25. Hamdi, H., Bigorgne, A., Naveau, S., Balian, A., Bouchet-Delbos, L., Cassard-Doulcier, A. M., Maillot, M. C., Durand-Gasselin, L., Prevot, S., Delaveauacoupet, J., Emilie, D., and Perlemuter, G. (2007) Glucocorticoid-induced leucine zipper: A key protein in the sensitization of monocytes to lipopolysaccharide in alcoholic hepatitis. *Hepatology* **46**, 1986-1992
26. Vago, J. P., Tavares, L. P., Garcia, C. C., Lima, K. M., Perucci, L. O., Vieira, E. L., Nogueira, C. R., Soriani, F. M., Martins, J. O., Silva, P. M., Gomes, K. B., Pinho, V., Bruscoli, S., Riccardi, C., Beaulieu, E., Morand, E. F., Teixeira, M. M., and Sousa, L. P. (2015) The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution. *J Immunol* **194**, 4940-4950
27. Hoppstädter, J., and Kiemer, A. K. (2015) Glucocorticoid-induced leucine zipper (GILZ) in immunosuppression: master regulator or bystander? *Oncotarget* **6**, 38446-38457
28. Chakravarti, N., Kadara, H., Yoon, D. J., Shay, J. W., Myers, J. N., Lotan, D., Sonenberg, N., and Lotan, R. (2010) Differential inhibition of protein translation machinery by curcumin in normal, immortalized, and malignant oral epithelial cells. *Cancer Prev Res (Phila)* **3**, 331-338
29. Zhou, Y., Chang, R., Ji, W., Wang, N., Qi, M., Xu, Y., Guo, J., and Zhan, L. (2015) Loss of Scribble Promotes Stem Translation through Translocation of HuR and Enhances Cancer Drug Resistance. *J Biol Chem* **291**, 301-302
30. Yu, T. X., Rao, J. N., Zou, T., Liu, L., Xiao, L., Ouyang, M., Cao, S., Gorospe, M., and Wang, J. Y. (2013) Competitive binding of CUGBP1 and HuR to occludin mRNA controls its translation and modulates epithelial barrier function. *Mol Biol Cell* **24**, 85-99
31. Yoon, J. H., Abdelmohsen, K., Srikantan, S., Guo, R., Yang, X., Martindale, J. L., and Gorospe, M. (2014) Tyrosine phosphorylation of HuR by JAK3 triggers dissociation and degradation of HuR target mRNAs. *Nucleic Acids Res* **42**, 1196-1208
32. Abdelmohsen, K., and Gorospe, M. (2010) Posttranscriptional regulation of cancer traits by HuR. *Wiley Interdiscip Rev RNA* **1**, 214-229
33. Hoppstädter, J., Diesel, B., Eifler, L. K., Schmid, T., Brune, B., and Kiemer, A. K. (2012) Glucocorticoid-induced leucine zipper is downregulated in human alveolar macrophages upon Toll-like receptor activation. *Eur J Immunol* **42**, 1282-1293
GILZ-dependent anti-inflammatory effects of curcumin

34. Wang, W., Caldwell, M. C., Lin, S., Furneaux, H., and Gorospe, M. (2000) HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. *EMBO J* **19**, 2340-2350

35. Gupta, S. C., Patchva, S., and Aggarwal, B. B. (2013) Therapeutic roles of curcumin: lessons learned from clinical trials. *AAPS J* **15**, 195-218

36. Zhou, Y., Zhang, T., Wang, X., Wei, X., Chen, Y., Guo, L., Zhang, J., and Wang, C. (2015) Curcumin Modulates Macrophage Polarization Through the Inhibition of the Toll-Like Receptor 4 Expression and its Signaling Pathways. *Cell Physiol Biochem* **36**, 631-641

37. Shi, X., Shi, W., Li, Q., Song, B., Wan, M., Bai, S., and Cao, X. (2003) A glucocorticoid-induced leucine-zipper protein, GILZ, inhibits adipogenesis of mesenchymal cells. *EMBO Rep* **4**, 374-380

38. Zhang, W., Yang, N., and Shi, X. M. (2008) Regulation of mesenchymal stem cell osteogenic differentiation by glucocorticoid-induced leucine zipper (GILZ). *J Biol Chem* **283**, 4723-4729

39. Kuwano, Y., Rabinovic, A., Srikantan, S., Gorospe, M., and Demple, B. (2009) Analysis of nitric oxide-stabilized mRNAs in human fibroblasts reveals HuR-dependent heme oxygenase 1 upregulation. *Mol Cell Biol* **29**, 2622-2635

40. Kuwano, Y., Kim, H. H., Abdelmohsen, K., Pullmann, R., Jr., Martindale, J. L., Yang, X., and Gorospe, M. (2008) MKP-1 mRNA stabilization and translational control by RNA-binding proteins HuR and NF90. *Mol Cell Biol* **28**, 4562-4575

41. Galban, S., Kuwano, Y., Pullmann, R., Jr., Martindale, J. L., Kim, H. H., Lal, A., Abdelmohsen, K., Yang, X., Dang, Y., Liu, J. O., Lewis, S. M., Holcik, M., and Gorospe, M. (2008) RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1alpha. *Mol Cell Biol* **28**, 93-107

42. Kawai, T., Lal, A., Yang, X., Galban, S., Mazan-Mamczarz, K., and Gorospe, M. (2006) Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR. *Mol Cell Biol* **26**, 3295-3307

43. Mazan-Mamczarz, K., Galban, S., Lopez de Silanes, I., Martindale, J. L., Atasoy, U., Keene, J. D., and Gorospe, M. (2003) RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. *Proc Natl Acad Sci U S A* **100**, 8354-8359

44. Fan, X. C., and Steitz, J. A. (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J* **17**, 3448-3460

45. Peng, S. S., Chen, C. Y., Xu, N., and Shyu, A. B. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J* **17**, 3461-3470

46. Simone, L. E., and Keene, J. D. (2013) Mechanisms coordinating ELAV/Hu mRNA regulons. *Curr Opin Genet Dev* **23**, 35-43

47. Levy, N. S., Chung, S., Furneaux, H., and Levy, A. P. (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J Biol Chem* **273**, 6417-6423

48. Holt, P. R., Katz, S., and Kirshoff, R. (2005) Curcumin therapy in inflammatory bowel disease: a pilot study. *Dig Dis Sci* **50**, 2191-2193

49. Vecchi Brumatti, L., Marcuzzi, A., Tricarico, P. M., Zanin, V., Girardelli, M., and Bianco, A. M. (2014) Curcumin and inflammatory bowel disease: potential and limits of innovative treatments. *Molecules* **19**, 21127-21153

50. Carroll, R. E., Benya, R. V., Turgeon, D. K., Vareed, S., Neuman, M., Rodriguez, L., Kakarala, M., Carpenter, P. M., McLaren, C., Meyskens, F. L., Jr., and Brenner, D. E. (2011) Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. *Cancer Prev Res (Phila)* **4**, 354-364

51. de Oliveira, L. F., Bouchmella, K., Goncalves Kde, A., Bettini, J., Kobarg, J., and Cardoso, M. B. (2016) Functionalized Silica Nanoparticles As an Alternative Platform for Targeted Drug-Delivery of Water Insoluble Drugs. *Langmuir* **32**, 3217-3225

52. Rodriguez-Pascual, F., Hausding, M., Ihrig-Biedert, I., Furneaux, H., Levy, A. P., Forstermann, U., and Kleinert, H. (2000) Complex contribution of the 3'-untranslated region to the expresional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem* **275**, 26040-26049

53. Fejer, G., Sharma, S., and Gyory, I. (2015) Self-renewing macrophages--a new line of enquiries in mononuclear phagocytes. *Immunobiology* **220**, 169-174
54. Hoppstädter, J., Seif, M., Dembek, A., Cavelius, C., Huwer, H., Kraegeloh, A., and Kiemer, A. K. (2015) M2 polarization enhances silica nanoparticle uptake by macrophages. *Front Pharmacol* **6**, 55

55. Hahn, R. T., Hoppstädter, J., Hirschfelder, K., Hachenthal, N., Diesel, B., Kessler, S. M., Huwer, H., and Kiemer, A. K. (2014) Downregulation of the glucocorticoid-induced leucine zipper (GILZ) promotes vascular inflammation. *Atherosclerosis* **234**, 391-400

56. Kiemer, A. K., Senaratne, R. H., Hoppstädter, J., Diesel, B., Riley, L. W., Tabeta, K., Bauer, S., Beutler, B., and Zuraw, B. L. (2009) Attenuated activation of macrophage TLR9 by DNA from virulent mycobacteria. *J Innate Immun* **1**, 29-45

57. Hoppstädter, J., Diesel, B., Zarbock, R., Breinig, T., Monz, D., Koch, M., Meyerhans, A., Gortner, L., Lehr, C. M., Huwer, H., and Kiemer, A. K. (2010) Differential cell reaction upon Toll-like receptor 4 and 9 activation in human alveolar and lung interstitial macrophages. *Respir Res* **11**, 124

58. Kiemer, A. K., and Vollmar, A. M. (2001) Elevation of intracellular calcium levels contributes to the inhibition of nitric oxide production by atrial natriuretic peptide. *Immunol Cell Biol* **79**, 11-17

59. Kiemer, A. K., Müller, C., and Vollmar, A. M. (2002) Inhibition of LPS-induced nitric oxide and TNF-alpha production by alpha-lipoic acid in rat Kupffer cells and in RAW 264.7 murine macrophages. *Immunol Cell Biol* **80**, 550-557

60. Astanina, K., Koch, M., Jüngst, C., Zumbusch, A., and Kiemer, A. K. (2015) Lipid droplets as a novel cargo of tunnelling nanotubes in endothelial cells. *Sci Rep* **5**, 11453

61. Kunze, M. M., Benz, F., Brauss, T. F., Lampe, S., Weigand, J. E., Braun, J., Richter, F. M., Wittig, I., Brüne, B., and Schmid, T. (2016) sST2 translation is regulated by FGF2 via an hnRNP A1-mediated IRES-dependent mechanism. *Biochim Biophys Acta* **1859**, 848-859
FIGURE LEGENDS

Figure 1 – Curcumin induces GILZ. GILZ protein expression in RAW264.7 (A, C) and U937 cells (B, D) after exposure to curcumin (6.25 µM) or the solvent control DMSO (0.2%, Co) was examined by Western blot analysis. Tubulin was used as a loading control. A, C: One representative blot out of 2 (A) or 5 (B) is shown. C, D: Signal intensities were quantified, normalized to tubulin and expressed as x-fold of solvent control (Co). * p < 0.05 compared with solvent control. E-G: Detection of GILZ by intracellular flow cytometry. MPI cells were isolated from fetal livers of WT and GILZ KO animals. E: GILZ KO was confirmed by real-time RT-PCR (n = 4, duplicates). F, G: MPI cells were treated with dexamethasone (Dex, 100 nM) or solvent (Co, 0.05% ethanol) for 24 h, followed by intracellular GILZ staining. Mean fluorescence intensities were measured by flow cytometry. F: Representative histograms. G: Data were expressed as x-fold of isotype control (n = 3, triplicates). * p < 0.05, *** p < 0.001 compared with WT cells or as indicated. H-I: RAW264.7, U937 and human monocyte-derived macrophages (HMDMs) were treated with curcumin (6.25 µM) or solvent control (0.025% DMSO, Co) for 3 h. Intracellular GILZ expression was measured by flow cytometry. (H) Representative histograms are shown. (I) Background-subtracted mean fluorescence intensities (MFI) obtained for solvent controls were set as 1. Data are expressed as means ± SEM (cell lines: n = 2, triplicates; HMDMs: 3 donors, duplicates). * p < 0.05, ** p < 0.01 compared with solvent control.

Figure 2 – GILZ induction by curcumin is not associated with enhanced GR activity, transcription, mRNA or protein stability. A: HEK 293T cells were transfected with the pEGFP GR vector and treated with either 50 µM curcumin or 100 nM dexamethasone. Video recording was started immediately after drug application. The GFP-tagged GR is shown in white. Arrows indicate cells in which nuclear translocation occurred. Scale bar, 50 µm. B, C: RAW264.7 (B) and U937 cells (C) were treated with curcumin (6.25 µM) for the indicated time points, and GILZ mRNA levels were determined by real-time RT-PCR and normalized to Ppia (B) or ACTB (C). The mean of the values obtained for untreated cells (0 h) were set as 100% and data are presented as means ± SEM (n = 3, duplicates). D: RAW264.7 cells were treated with actinomycin D (5 µg/ml, ActD) in the absence or presence of curcumin (6.25 µM). RNA was isolated at the indicated time points, and real-time RT-PCR analysis for Gilz was performed. Data are normalized to Ppia and represent means ± SEM (n = 3, duplicates). E: RAW264.7 cells were treated with cycloheximide (CHX, 5 µg/ml) and solvent control DMSO (0.07%) or CHX and curcumin (6.25 µM) for the indicated time points. Protein expression was determined by Western blot, and GILZ signal intensities were expressed as a percentage of 0 h values ± SEM (n = 4, triplicates).

Figure 3 – GILZ is translationally upregulated by Curcumin. U937 cells were treated with curcumin (6.25 µM) or vehicle control (Co) for 3 h and analyzed by polysomal fractionation. (A) Representative absorbance profiles measured at 254 nm during polysomal fractionation. (B) RNA was isolated from single fractions and the mRNA abundance of GILZ mRNA was analyzed by real-time RT-PCR. The distribution of GILZ mRNA across the gradient was determined relative to the total RNA extracted from the gradients. Changes of GILZ mRNA distribution induced by curcumin were normalized to Co. Data are presented as means ± SEM (n = 4, * p < 0.05, ** p < 0.01).

Figure 4 – HuR is involved in GILZ upregulation by curcumin. A, B: U937 cells were treated with solvent control DMSO (0.07%) for 4 h or curcumin (6.25 µM) for the indicated time points (A), and HMDMs were treated accordingly for 3 h (B). HuR expression was determined by Western blot. One representative blot is shown. Signal intensities (SI) were normalized to tubulin and expressed as x-fold of solvent control DMSO (A: n = 5, triplicates, B: n = 3, duplicates). C: U937 cells were lysed, followed by IP with IgG or HuR antibodies. Co-precipitated mRNAs were quantified by real-time RT-PCR. CCNB1: Cyclin B1, positive control for HuR binding mRNAs; GAPDH: negative control (n = 4, duplicates). D: HEK 293T cells were either transfected with control or HuR expression vector, followed by Western Blot analysis. D: One representative experiment out of three is shown. E: Signal intensities were normalized to tubulin as a loading control and are expressed as x-fold of control vector-transfected cells ± SEM (n = 3, duplicates).
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duplicates). F: ELAVL1 (HuR) and GILZ mRNA levels in control vector- or HuR vector-transfected HEK 293T cells were determined by Real Time RT-PCR. Values were normalized to ACTB and are presented as x-fold of control vector-transfected cells (n = 3, triplicates). * p < 0.05, ** p < 0.01, *** p < 0.001 compared with solvent control or as indicated.

Figure 5 – GILZ contributes to curcumin-induced inhibition of NF-κB and iNOS activity. A-D: RAW264.7 cells were left untreated (Co) or pre-treated with either solvent control (0.07% DMSO) or curcumin at the indicated concentrations for 30 min, followed by LPS stimulation (A, B: 1 µg/ml, 4 h; C, D: 0.1 µg/ml, 20 h). B, D: Cells were transfected with control (siCo) or Gilz-targeting (siGilz) siRNA prior to treatment. NF-κB activity (A, B) and nitrite accumulation (C, D) were measured by reporter gene assay or Griess assay, respectively. Values for LPS-induced activation in DMSO-pretreated cells were set as 100%, and data are presented as means ± SEM (n = 3-6, quintuplicates). * p < 0.05, ** p < 0.01, ***p < 0.001.

Figure 6 – GILZ is involved in curcumin-mediated inhibition of ERK activity and TNF secretion in BMMs. A, B: WT BMMs were treated with curcumin at the indicated concentrations or solvent control (0.025% DMSO, Co) for 3 h. Intracellular GILZ expression was measured by flow cytometry. Representative histograms are shown. Background-subtracted mean fluorescence intensities (MFI) are indicated within the graph. Values obtained for solvent controls were set as 1. Data are expressed as means + SEM (n = 4, duplicates). **p < 0.01 compared with solvent control. B: NF-κB activity in LPS-stimulated BMMs was measured by luciferase reporter gene assay. Cells were left untreated (Co) or pre-treated either with solvent control (0.05% DMSO) or with curcumin at the indicated concentrations for 60 min, followed by LPS stimulation (1 µg/ml) for 4 h. Values for LPS-induced activation in DMSO-pretreated cells were set as 100%, and data are presented as means + SEM (n = 3, triplicates). B, E-G: *p < 0.05, **p < 0.001 vs. equally treated cells of the same genotype without curcumin pretreatment; *p < 0.05, **p < 0.01, ***p < 0.001 vs. equally treated cells of the same genotype with 12.5 µM curcumin pretreatment; *p < 0.05, **p < 0.01, ***p < 0.001 vs. equally treated WT cells.
TABLES

Table 1 – Primers as used for Real Time RT-PCR.

| Gene                | Primer 5’-3’                       |
|---------------------|-----------------------------------|
| Human ACTB          | For.: TGC GTG ACA TTA AGG AGA AG  |
|                     | Rev.: GTC AGG CAG CTC GTA GCT CT   |
| Human ACTB (IP)     | For.: CCC CGC GAG CAC AGA G        |
|                     | Rev.: TAT CAT CAT CCA TGG TGA GCT GG |
| Human GILZ          | For.: TCC TGT CTG AGC CCT GAA GAG  |
|                     | Rev.: AGC CAC TTA CAC CGC AGA AC   |
| Human GILZ (IP, polysomal fractionation) | For.: GTT AAG CTG GAC AAC AGT GCC T |
|                     | Rev.: TTC TCC ACC AGC TCT CGG AT    |
| Human GAPDH         | For.: TTC GAC AGT CAG CCG CAT CT    |
|                     | Rev.: GCC CAA TAC GAC CAA ATC CGT T |
| Human CCNB1         | For.: ATG GTG AAT GGA CAC CAA CTC T |
|                     | Rev.: CAT TCT TAG CCA GGT GCT GC    |
| Murine Gilz         | For.: GGG ATG TGG TTT CCG TTA AAC TGG A |
|                     | Rev.: TGC TCA ATC TTG TTG TCT AGG GCC A |
| Murine Ppia         | For.: GGC CGA TGA CGA GCC C         |
|                     | Rev.: TGT CTT TGG AAC TTT GTC TGC   |
| Murine Rn18s        | For.: AGG TCT GTG ATG CCC TTA GA    |
|                     | Rev.: GAA TGG GGT TCA ACG GGT TA    |
Figure 3

A

![Graph showing polysome profiles under Co and curcumin treatments.](image)

B

![Bar graph showing GILZ mRNA distribution in curcumin treated samples.](image)
Figure 5

A

Luciferase activity [%] vs. curcumin concentration and LPS treatment.

B

Luciferase activity [%] vs. LPS treatment and siGILZ silencing.

C

Nitrite accumulation [%] vs. curcumin concentration and LPS treatment.

D

Nitrite accumulation [%] vs. LPS treatment and siGILZ silencing.
Induction of glucocorticoid-induced leucine zipper (GILZ) contributes to anti-inflammatory effects of the natural product curcumin in macrophages

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