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Molecular Pharmacology

Gedunin binds to MD-2 and impairs LPS-induced TLR4 signaling in macrophages

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Running Title: Gedunin impairs LPS-induced macrophage activation

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List of non-standard abbreviations: 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; CD, cluster of differentiation; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; FRAP, fluorescence recovery after photobleaching; HO-1, heme oxigenase-1; HSF-1, heat shock factor-1; Hsp, heat shock protein; IkB, I-kappa-B; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; MAL KO, MyD88-adapter-like knockout; MAL/MyD88, MyD88-adapter-like/Myeloid differentiation primary response 88; MAP, mitogen-activated protein; MD-2, myeloid differentiation protein 2; NFκB, nuclear factor κB; NO, nitric oxide; Pam3, Pam3Cys-Ser-(Lys)4 trihydrochloride;PGE2, prostaglandin E2; Poly I:C, Polyinosinic:polycytidylic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, PolyAcrylamide Gel Electrophoresis; SPR, Surface Plasmon Resonance; TAK-1, transforming growth factor β-activated kinase 1; TLR, toll like receptor; TRAM/TRIF, TRIF-related adaptor molecule/TIR-domain-containing adapter-inducing interferon-β; TRIF KO, TIR-domain-containing adapter-inducing interferon-β knockout; WT, wild type.

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Abstract

Recognition of bacterial lipopolysaccharide (LPS) by innate immune system is mediated by the CD14/TLR4/MD-2 complex. In the present study, we have investigated the modulatory effect of gedunin, a limonoid from species of the Meliaceae family described as heat shock protein (Hsp)90 inhibitor, on LPS-induced response in immortalized murine macrophages. The pretreatment of wild type (WT) macrophages with gedunin (0.01-100μM, non-cytotoxic concentrations) inhibited LPS (50ng/ml)-induced calcium influx, TNF-α and nitric oxide (NO) production, in a concentration-dependent manner. The selective effect of gedunin on MAL/MyD88- and TRAM/TRIF-dependent signaling pathways was further investigated. The pretreatment of WT, TRIF KO and MAL KO macrophages with gedunin (10μM) significantly inhibited LPS (50ng/ml)-induced TNF-α and IL-6 production, at 6h and 24h, suggesting that gedunin modulates a common event between both signaling pathways. Furthermore, gedunin (10μM) inhibited LPS-induced prostaglandin E_2 (PGE_2) production, cyclooxygenase-2 (COX-2) expression and nuclear factor κB (NFκB) translocation into the nucleus of WT macrophages, demonstrating a wide range effect of this chemical compound. In addition to the ability to inhibit LPS-induced pro-inflammatory mediators, gedunin also triggered anti-inflammatory factors IL-10, heme oxigenase-1 (HO-1) and Hsp70 in macrophages stimulated or not with LPS. *In silico* modeling studies revealed that gedunin efficiently docked into the MD-2 LPS binding site, a phenomenon further confirmed by surface plasmon resonance. Our results reveal that, in addition to Hsp90 modulation, gedunin acts as a competitive inhibitor of LPS, blocking the formation of TLR4/MD-2/LPS complex.

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Introduction

Recognition of lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, by the immune system, involves at least three receptor molecules - cluster of differentiation (CD)14, toll like receptor (TLR)4 and MD-2 -, which are mostly expressed by macrophages (Shimazu et al., 1999; Ulevitch and Tobias, 1995; Viriyakosol et al., 2000; Wright et al., 1990). LPS recognition by the TLR4 complex leads to the recruitment of the adaptor proteins MAL/MyD88 (MyD88-adapter-like/Myeloid differentiation primary response 88) and TRAM/TRIF (TRIF-related adaptor molecule/TIR-domain-containing adapter-inducing interferon-β), which in turn activate two distinct signaling pathways that present different kinetics. TLR4 initially recruits MAL/MyD88, leading to early phase activation and is further endocytosed and delivered to intracellular vesicles to only then form a complex with TRAM/TRIF, leading to the late phase activation (Hwang et al., 1997; Kagan et al., 2008; Kawai and Akira, 2011). These two pathways are required to drive robust NFkB and mitogen-activated protein (MAP) kinase activation and the subsequent induction of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β and nitric oxide (NO) (Byrd-Leifer et al., 2001; Fitzgerald et al., 2003; Gais et al., 2010; Li et al., 2005a; Sato et al., 2003). The overproduction of these mediators during inflammation can lead to tissue damage, multiple organ dysfunction and septic shock (Dos Santos and Slutsky, 2000; Marshall, 2001; Shen et al., 2004; Su, 2002).

It has been proposed that the heat shock protein (Hsp) chaperone machinery is implicated in TLR4/MD-2/CD14 signaling by maintaining the structural integrity of the multimeric LPS receptor complex and by regulating MAP kinase members (Echeverria et al., 2011; Triantafilou et al., 2001). In addition, it has been suggested that LPS is transferred from CD14 to Hsp70 and Hsp90, and then interacts with a large hydrophobic pocket of MD-2 (da Silva Correia et al., 2001; Park et al., 2009; Triantafilou et al., 2001). The Hsp90 is an abundantly and ubiquitously expressed chaperone that helps to maintain, at the expense of ATP, the structure of several membrane, cytoplasmic, and

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endoplasmic reticulum-associated client proteins (McClellan et al., 2007; Picard, 2002; Pratt and Toft, 1997; Zhao and Houry, 2005). Hsp90 chaperoning activity can be inhibited by geldanamycin, 17-allylamino-17-demethoxy-geldanamycin (17-AAG) and celastrol, which lead to increased degradation of Hsp90 client proteins (Matts et al., 2011). In experimental models, Hsp90 inhibitors have been shown to suppress different signaling pathways and display potent antiproliferative, cytoprotective and antiinflammatory activities (Ambade et al., 2012; Chow et al., 2013; Leung et al., 2015; Lewis et al., 2000; Poulaki et al., 2007). These effects result from nonfunctional conformational changes as well as from the induction of heat shock response via the activation of heat shock factor-1 (HSF-1), which leads to increased expression of Hsp90 and other Hsps, including Hsp70, Hsp40 and Hsp32 (heme oxygenase-1, HO-1) (Chow et al., 2013; Pritchard et al., 2001; Shamovsky and Nudler, 2008; Trott et al., 2008).

Gedunin and its analogues is an important bioactive limonoid-type tetranortriterpene isolated from the Meliaceae family and is reported to display a wide range of biological activities including antitumor, antimalarial, anti-allergic and antiinflammatory activity in different experimental models (Brandt et al., 2008; Conte et al., 2015; Ferraris et al., 2012; Ferraris et al., 2011; Henriques and Penido, 2014; Kamath et al., 2009; Miranda Junior et al., 2012). The molecular mechanism of action related to the biological effects of gedunin relies on the inhibition of Hsp90 activity via specific binding to the co-chaperone p23 and by disrupting the co-chaperone Cdc37-Hsp90 interaction (Patwardhan et al., 2013; Matts et al., 2011). However, here we demonstrate that gedunin mechanisms of action go beyond Hsp90 modulation. Using *in silico* and surface plasmon resonance analysis, we show that gedunin binds to MD-2, impairing TLR4/MD-2/CD14 signaling and decreasing LPS-induced inflammatory response in murine macrophages.

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Materials and Methods

Cell culture

Immortalized bone-marrow-derived macrophage cell lines generated from wild-type (WT), MAL KO, TRIF KO C57BL/6 mice (a kind gift from Douglas Golenbock; University of Massachusetts Medical School) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St. Louis, USA) supplemented with fetal bovine serum (FBS, 10%, Gibco/Life Technologies, Carlsbad, USA), sodium pyruvate (1mM, Sigma Aldrich, St. Louis, USA), and ciprofloxacin (10µg/ml, Fresoflox®, Fresenius Kabi, Barueri, Brazil).

Cytotoxicity assay

In order to perform *in vitro* assays with gedunin, we first examined the cytotoxic effects of this substance (and gedunin vehicle, dimethyl sulfoxide, DMSO) on WT immortalized macrophages. Viable cells were seeded in a flat bottom 96-well plate ($2x10^5$ cells/well, in quadruplicate) and cultured for 24h in the presence of different concentrations of gedunin (0.001 – 1000µM; 5% CO_2 at 37°C). The assay was assessed by resazurin reduction method. The absorbance was read at 555/585nm ($\lambda_{ex}/\lambda_{em}$) using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, USA) and results are expressed as percentages of viable cells (Table 1). The compound concentrations that induced ≥10% of cell death were considered cytotoxic and were not used in the biological assays. It is noteworthy that DMSO (≤0.1%, maximal concentration used) and LPS (≤1µg/ml) induced ≤10% of macrophage death.

Treatments and stimulation

WT, MAL KO and TRIF KO macrophages (10^6 cells/well) were plated in 6- or 24-well plates in DMEM (supplemented with 2% FSB, 1mM sodium pyruvate, and 10µg/ml ciprofloxacin) for 24h, and then treated with gedunin (0.01 – 100µM; Gaya Chemical Corporation, New Milford, USA). After 1h, macrophages were stimulated with LPS (50 – 1000 ng/ml, from *E. coli* O111:B4, Sigma

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Aldrich, St. Louis, USA), re-purified by a repeated phenolchloroform extraction (Hirschfeld et al., 2000) for 6 or 24h and the cell-free supernatants recovered for analysis. The Hsp90 inhibitor 17-AAG (1μM) and dexamethasone (100nM), used as reference inhibitors, were purchased from Sigma Aldrich (St. Louis, USA) and induced ≤10% of macrophage death at the concentrations used.

Calcium mobilization assay

Intracellular calcium concentrations on preplated WT immortalized macrophage cells (2×10^5 per well) pretreated with gedunin (0.01-100μM) and exposed to LPS (1μg/ml) over 600s using the FLIPR Calcium Plus Assay Kit on FlexStation II fluorometric microplate reader (Molecular Devices, Sunnyvale, USA), with fluorescence intensity ratios at 485/525nm ($\lambda_{ex}/\lambda_{em}$) recorded up to 5min, and analyzed using SoftMax Pro (Molecular Devices, Sunnyvale, USA).

Nitrite determination

Cells were seeded on 24-well plates in a final concentration of 1×10^6 cells/well (DMEM supplemented with 2% FBS, in 5% CO_2 at 37 °C) and allowed to grow to confluence. Confluent cells were pretreated with gedunin (0.01-10μM) and exposed to LPS (50ng/ml) for 24h. The supernatant was collected and nitrite, a stable metabolite of NO in aqueous solutions, was measured by Griess reaction, after the addition of 100 μl modified Griess reagent (Sigma Aldrich, St. Louis, USA) to the wells for 15 min at room temperature. Absorbance was read at 562nm using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, USA). The concentration of nitrite was calculated from a sodium nitrite standard curve (range 1.5-100μM).

Cytokine analysis

TNF-α, IL-6, IL-10 levels were evaluated in the supernatants of stimulated immortalized macrophages by ELISA using matched antibody pairs from R&D Systems (Quantikine, R&D Systems, Minneapolis, USA), according to the manufacturer's instructions. Results are expressed as picograms per milliliter.

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PGE_2 quantification

Concentrations of prostaglandin E_2 (PGE_2) were measured in the supernatants of LPS (50ng/ml)-stimulated macrophages pretreated or not with gedunin (10μM), 17AAG (1μM) or dexamethasone (100nM) using an EIA kit (Cayman Chemical Company, Ann Arbor, USA), according to the instructions provided by the manufacturer.

Western blot

Total protein content in cytoplasmatic and nuclear extracts were determined by the Bradford reagent (Sigma Aldrich, St. Louis, USA). Cell lysates were denatured in Laemmli´s sample buffer (50mM Tris-HCl, pH 6.8; 1% sodium dodecyl sulfate (SDS); 5% 2-mercaptoethanol; 10% glycerol and 0.001% bromophenol blue) and heated at 95°C for 3min. Aliquots containing 30μg of protein were re-suspended in SDS-PAGE loading buffer, resolved on 12% SDS acrylamide gels and transferred onto PVDF Hybond™ membranes (Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% non-fat dry milk/Tris-buffered saline containing 0.1% Tween-20 for 2h at room temperature, the membranes were probed overnight at 4°C with specific primary antibodies followed by horseradish peroxidase-labeled secondary antibodies. Rabbit polyclonal anti-mouse HO-1 (1:5000) and horseradish peroxidase-labeled goat polyclonal anti-rabbit antibodies (1:2500) were obtained from Enzo Life Sciences (Farmingdale, USA). Mouse monoclonal anti-goat Hsp70 (1:5000), mouse monoclonal anti-goat COX_2 (1:5000) and mouse monoclonal anti-rabbit NFκB p65 (1:5000) were obtained from Santa Cruz Biotechnologies (Santa Cruz, USA). PVDF sheets were incubated with streptavidin-conjugated horseradish peroxidase (1:10000) for 1h and developed by an ECL®-plus reagent (Enhanced Chemiluminescence, Amersham Biosciences) and visualized on Hyperfilm (Amersham Biosciences, Buckinghamshire, UK). The bands were quantified by densitometry, using Image J software program (public domain).

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In silico docking simulations

The structures of the human MD-2 and the co-chaperone p23 were taken from the protein data bank (PDB) codes 2E56 (Ohto et al., 2007) and 1EJF (Weaver et al., 2000), respectively. Before the docking simulation, the protein structure was prepared adding hydrogens and the protonation of titrable residues were calculated with the PROPKA program (Li et al., 2005b), inside the pdb2pqr program (Dolinsky et al., 2007; Dolinsky et al., 2004), considering a pH of 7.

All molecular docking calculations were performed using the Autodock Vina program (Trott and Olson, 2010), in a two-step approach: *i*. the blind docking procedure and *ii.* the pocket search procedure. The blind docking procedure consisted of searching the entire protein surface in order to determine the potential binding pocket(s). This was achieved using the grid center as the center of each protein, using a grid size big enough to cover the entire protein surface. After finding the binding pockets, we centered the grid center within the discovered binding pocket and performed a more accurate search by the pocket search procedure, using the following parameters: energy_range = 10, num_modes = 20 and exhaustiveness = 800.

Physicochemical binding assays of gedunin towards MD-2

The Surface Plasmon Resonance (SPR) analysis was performed on carboxyl sensor chips coated with nickel-nitrilotriacetic acid (HisCap; ICx Nomadics Inc, Stillwater, USA) previously treated with an activation solution (500µM $NiCl_2$) in a constant flow (10µl/min) at 37°C for 10min, for each binding assay cycle. Prior the binding assay, the recombinant human (rh)MD-2 fusion protein (R&D Systems, Minneapolis, USA) was immobilized (0.1750µg/ml, flow rate of 5µl/min, for 10min) on the chip surface with reaction buffer (150mM NaCl, 100mM HEPES pH 7.4). It is important to note that due to the lack of a commercially available recombinant murine (rm)MD-2, the rhMD-2 was used in the present study. The rhMD-2 shares 56% of identity with rmMD-2 at the amino acid level, and their common residues are essential for TLR4 activation by LPS (Park et al., 2009; Zimmer et

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al., 2008). The differences in surface charge distribution (electrostatic potential) of human and murine MD-2 binding pockets do not alter the hydrophobic interaction between LPS and MD-2 inside the cavity. The binding of gedunin and of LPS to immobilized MD-2 was performed using different concentrations in reaction buffer (gedunin: 0.001–1000μM and 0.482-482.0μg/ml; LPS: 0.482-482.0μg/ml). At the end of each interaction step, the HisCap chip was treated with regenerating buffer (0.7M imidazole, pH 8.0) at 37°C for 3 min (50μl/min). All binding assays were registered in real time using a sensorgram, and changes in the SPR angle (θ spr) were expressed as arbitrary resonance units (RU). To avoid artifacts, RU values from the reference channel were substracted from the RU values of test samples. All SPR analysis were performed in a SensíQ Pioneer optical transduction biosensor (ICx Nomadics Inc, Stillwater, USA).

The association and dissociation rates of complex formation were calculated based on the analysis of sensorgram graphs. Kinetic values were obtained using Qdat software (ICx Nomadics Inc, Stillwater, USA). Data of affinity constant (K_{eq}) and of Gibbs free energy (θ G°) for the complexes formed were derived from the K_a and K_d data (Souza-Silva et al., 2014). The reliability of the data was confirmed by double reciprocal plots (1/response vs 1/free analyte), as previously described (Pastushok et al., 2005; Samoylov et al., 2005). Inhibitory modes of gedunin for LPS (9.64μg/ml) binding to immobilized MD-2 was performed using different concentrations of gedunin (0.482–482.0μg/ml) in reaction buffer. The binding of gedunin (14.5μg/ml) to MD-2 in the presence of LPS (0.482–482.0μg/ml) was also compared.

Statistical analysis

Data are reported as the mean ± standard error of the mean (SEM) and were analyzed by means of ANOVA, followed by Student Newman-Keuls test. Values of $p<0.05$ were regarded as significant.

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RESULTS

Concentration effect of gedunin on LPS-induced macrophage activation

The effect of different concentrations of gedunin was evaluated *in vitro* on LPS-induced calcium influx and on TNF-α and nitrite production by WT macrophages. As observed in Fig. 1A and B, the stimulation of macrophages with LPS (1μg/ml) induced a sustained increased in the levels of intracellular calcium, which was inhibited by the pretreatment with gedunin, from 0.01 to 100μM. Gedunin pretreatment also impaired LPS (50ng/ml)-induced TNF-α production by macrophages, in a concentration-dependent manner (R^2=0.96, $p<0.001$, Fig. 1C). In addition, as shown in Fig. 1D, LPS (50ng/ml) plus IFN-γ (200IU/ml) increased nitrite production by macrophages, which was also inhibited by gedunin pretreatment from 0.01 to 10μM. Since 10μM of gedunin resulted in 100% viability (Table 1) and significantly impaired different parameters of macrophage activation, this concentration was used in all subsequent experiments.

Gedunin impairs LPS-induced cytokine production of early and late phase responses

LPS recognition by the TLR4/MD-2/CD14 complex induces a signaling cascade that culminates in the production of cytokines, such as TNF-α and IL-6, via early (MAL/MyD88-dependent) and late (TRAM/TRIF-dependent) pathways (Akira and Takeda, 2004). To investigate the selective effect of gedunin on the early and late activation triggered by LPS, we evaluated the production of cytokines by TRIF KO and MAL KO macrophages 6 and 24h after stimulation, respectively. As shown in Fig. 2 (A-D), TNF-α production through early and late pathways was significantly inhibited by gedunin, as well as by the reference inhibitors 17-AAG and dexamethasone pretreatments. Similarly, as shown in Fig. 2 (E-H), the pretreatment with gedunin, 17-AAG and dexamethasone inhibited IL-6 production via early and late phases.

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Gedunin diminishes PGE_2 production and COX-2 expression induced by LPS

We further evaluated the effect of gedunin on LPS-induced PGE_2 production through early and late pathways. As shown in Fig. 3A and B, the pretreatment with gedunin, 17-AAG and dexamethasone inhibited LPS-induced PGE_2 production by WT macrophages 6 and 24h after stimulation. The expression of COX-2, the enzyme responsible for the production of prostanoids including PGE_2 (Alhouayek and Muccioli, 2014), is shown to be induced after stimulation with LPS (Hwang et al., 1997; Rhee and Hwang, 2000) and IL-1β (Arias-Negrete et al., 1995; Endo et al., 2014). Here we show that the pretreatment with gedunin decreased LPS (50ng/ml)-induced COX-2 protein expression in WT immortalized macrophages (Fig. 3C). Decreased COX-2 expression was also observed for 17AAG and dexamethasone.

Gedunin inhibits nuclear translocation of NFκB

NFκB activation is critical for the synthesis of inflammatory mediators, including cytokines (D'Acquisto et al., 2002). Thus, we investigated the ability of gedunin to inhibit NFκB activation *in vitro* by analyzing its translocation into the nucleus by western blot. NFκB/p65 protein levels were determined in nuclear extracts of WT immortalized macrophages stimulated with LPS (50ng/ml) for 24h. LPS stimulation enhanced the presence of the p65 subunit in the nucleus, when compared to non-stimulated WT immortalized macrophages (Fig. 3D). NFκB translocation was inhibited by gedunin, 17-AAG and dexamethasone pre-treatments.

Gedunin triggers antiinflammatory mechanisms in macrophages

The induction of antiinflammatory and pro-resolving mechanisms may be an additional means by which antiinflammatory substances exert their effects. Moreover, it has been demonstrated that some Hsp90 modulators can induce the expression of antiinflammatory factors (Chow et al., 2013; Der Sarkissian et al., 2014; Shamovsky and Nudler, 2008; Trott et al., 2008). Here we show that gedunin pretreatment enhanced HO-1 expression on LPS (50ng/ml)-stimulated WT

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immortalized macrophages, whereas 17-AAG and dexamethasone pretreatment did not enhanced the expression of HO-1 (Fig. 4A). The fact that LPS failed to increase HO-1 expression 6h after stimulation is in accordance with previous reports (Rushworth et al., 2005; Song et al, 2003). Incubation of unstimulated macrophages with gedunin induced more prominent expression of HO-1 when compared to cells incubated with gedunin and LPS ($p \leq 0.05$). In addition, gedunin and 17-AAG pretreatments were able to induce Hsp70 expression on WT immortalized macrophages that were stimulated with LPS (Fig. 4B). Similarly, the increase in Hsp70 expression observed in macrophages that were pretreated with dexamethasone was not statistically significant when compared to LPS-stimulated macrophages. Furthermore, LPS-induced IL-10 production was enhanced in WT immortalized macrophages by pretreatments with gedunin, 17-AAG and dexamethasone (Fig. 4C). Interestingly, the incubation of unstimulated macrophages with gedunin was able to induce increased HO-1 and IL-10 expression. Thus, our results show that gedunin is also capable to induce antiinflammatory factors in macrophages.

Gedunin docks to the MD-2 protein surface at the LPS binding site

The complexity of the TLR4 signaling pathway (e.g. numerous proteins acting at different levels) hinders the identification of molecular targets of gedunin. We first analyzed the structure and interaction of known targets of gedunin. It has been recently demonstrated that gedunin is able to bind and inactivate p23, avoiding the formation of its complex with the heat shock protein, Hsp90 (Patwardhan et al., 2013). Structurally, p23 presents an antiparallel beta-sandwich fold and the gedunin binding site is located within the region that forms the complex to Hsp90. Using a binding docking scheme, we were able to find the same previously reported binding site that allowed us to obtain an equivalent binding mode of gedunin to p23 (Supplemental Figure 1).

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Among the potential candidates of proteins of the TLR4 pathway, we considered proteins that directly bind and recognize LPS, including the TLR4 accessory protein MD-2 (Meng et al., 2010). Considering that gedunin is a very hydrophobic molecule, we tested whether it would bind to MD-2.

Comparison between the sequences and crystal structures of MD-2 and p23 revealed that these two proteins possess a similar fold, as represented by the structural alignments of their crystal structures (Fig. 5). Thus, both MD-2 and p23 present an antiparallel beta-sandwich fold with a well-defined hydrophobic pocket (p23 being more compact).

Mapping gedunin-binding site in MD-2

We then used an *in silico* docking approach to identify putative gedunin binding sites in the MD-2 structure. Our hypothesis is based on two assumptions: *i*) p23 and MD-2 share some structural similarity; *ii*) since gedunin binds to p23, it probably binds to MD-2.

Our results show that gedunin docked to the large hydrophobic site that overlaps to the LPS binding site (Fig. 6). As illustrated in figure 6A, all the 16 high-score poses were docked at the most hydrophobic region of the MD-2 protein surface, within the LPS binding pocket (the hydrophobic pocket). Moreover, the MD-2 residues interacting with the higher-score docked conformation of gedunin are all hydrophobic Val24, Ile32, Ile46, Val48, Ile52, Leu61, Leu78, Phe119, Phe121, Cys133, Val135, Phe151 and Ile153 (with the exception of the polar Ser120) (Fig. 6B-C). Thus, according to our predictions, the binding of gedunin to MD-2 surface would impair LPS binding, avoiding the formation of the complex between MD-2 and LPS.

Physicochemical binding assays to MD-2 protein

The biosensor analysis performed here was useful to prove the binding property of MD-2 with its ligand, gedunin. The kinetics of the interaction was evaluated after activation of the HisCap sensor chip upon immobilization of the complexes, which was activated to recognize and bind to the

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histidine region of the MD-2. The binding of MD-2 protein to the HisCap chip exhibited a significant binding rate of 452±18 RU/s for the interaction with nickel-nitrilotriacetic acid. Therefore, the binding site of MD-2 was accessible to interact with gedunin in solution. The time RU/s variation in 890s was used to evaluate the interactions between MD-2 and gedunin (Fig. 7A).

The dissociation values of MD-2 binding (RU/s) were: 1.3 ± 0.1 RU/s, 1.6 ± 0.2 RU/s, 3.4 ± 0.4 RU/s, 4.0 ± 0.2 RU/s, 5.7 ± 0.6 RU/s, 13 ± 0.1 RU/s and 33.4 ± 0.3 RU/s for gedunin concentrations of 0.001 µM, 0.01 µM, 0.10 µM, 1.0 µM, 10.0 µM, 100.0 µM and 1000.0 µM, respectively (Fig. 7A,B). The kinetic values of gedunin interaction with MD-2 was assessed the affinity constant, as KD 280±29µM. Gedunin was further analyzed using a series of concentrations, which demonstrated an increase in the SPR signal (RU/s), indicating that this limonoid directly binds to immobilized MD-2 in a concentration-dependent fashion (R^2=0.925; Fig. 7B). Double-reciprocal plot linearization shows that gedunin binding to MD-2 occurs in a concentration-dependent fashion (Fig. 7C). Figure 7D shows the dose-response curves of gedunin and LPS binding to MD-2, demonstrating RU_{max} = 38.653 (R^2 = 0.952) for gedunin and RU_{max} = 69.953 (R^2 = 0.9846) for LPS. The double-reciprocal plot was performed to calculate 50% of saturation of binding of gedunin and LPS to immobilized MD-2 and revealed the values of 14.5µg/ml and 9.64µg/ml, respectively. The addition of gedunin (14.5µg/ml) to LPS previously added to immobilized MD-2 at different concentrations diminished RU values of LPS binding (R^2=0.9883), evaluated 20 seconds after dissociation time (Figure 7E). A similar binding inhibition curve was observed when LPS (9.64µg/ml) was added to gedunin at different concentrations (R^2=0.8511), suggesting that gedunin might compete with LPS for the same binding region of MD-2. Figure 7F shows the percentage of inhibition calculated using the values demonstrated in figure 7E, considering 100% as the resonance signal of 1/RUmax of gedunin (RU 19.33) and LPS (RU 35.00).

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Discussion

In this study, we demonstrate that gedunin has a remarkable suppressive effect on macrophage activation induced by LPS. We also provide evidence that gedunin binds to the MD-2 component of the TLR4 receptor complex and, by impairing the upstream activation of LPS signaling cascade, gedunin inhibits both early (MAL/MyD88 dependent) and late (TRAM/TRIF dependent) pathways.

Our group has previously demonstrated that gedunin presents important antiinflammatory and immunomodulatory effects in different experimental models *in vivo*, including experimental arthritis, allergic pleurisy and allergic lung inflammation, in which macrophages play a pivotal role (Conte et al., 2015; Henriques and Penido, 2014; Penido et al., 2006a; Penido et al., 2006b; Penido et al., 2005). Here we demonstrate that gedunin directly modulates macrophages *in vitro*, in a concentration-dependent manner, inhibiting early and classical parameters of macrophage activation after PAMP (pathogen-associated molecular pattern) recognition. The fact that different pathways are involved in calcium influx and TNF-α and NO production reinforces that gedunin blocks an upstream and common mechanism involved in these three responses, most likely due to MD-2 binding. However, the modulation of Hsp90 by gedunin (Brandt et al., 2008; Patwardhan et al., 2013) also explains the impairment of LPS-induced NO production, since NO synthesis has been shown to be modulated by Hsp90, through Hsp90-iNOS interaction (Luo et al., 2011; Yoshida and Xia, 2003). Indeed, it has been previously reported that Hsp90 inhibition by geldanamycin also impaired NO production by macrophages stimulated with LPS (Luo et al., 2011).

The recognition of LPS by TLR4/MD-2/CD14 complex culminates in the production of TNF-α and IL-6 via both MAL/MyD88 and TRAM/TRIF dependent pathways (Akira and Takeda, 2004; Wax et al., 2003). Hsp90 (in association with Hsp70 and other chaperones) maintains the conformation and activity of MAL/MyD88 and TRAM/TRIF-dependent kinases involved in TNF-α

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and IL-6 production, such as p38, ERK1/2and JNK (Beutler et al., 2003; Bode et al., 2003; Davis and Carbott, 1999; Echeverria et al., 2011; Richter and Buchner, 2001; Yamamoto et al., 2003). Indeed, geldanamycin has been shown to inhibit TNF-α and IL-6 mRNA as well as protein levels in LPS-stimulated macrophages (Vega and De Maio, 2003; Wax et al., 2003). Whether gedunin distinctly modulates MAL/MyD88 and TRAM/TRIF pathways was investigated here by means of TRIF and MAL KO macrophages. The fact that this limonoid impaired cytokine production via both signaling cascades supports our assumption that gedunin acts upstream in LPS-induced macrophage activation. This effect is likely to occur either via MD-2 blockade (as revealed by our *in silico* and SPR experiments) or via modulation of TLR4-associated Hsp90, as previously proposed by Triantofilou and coworkers (Latz et al., 2002; Triantafilou et al., 2004; Triantafilou et al., 2008; Triantafilou and Triantafilou, 2004). These authors have suggested, by means of FRAP (fluorescence recovery after photobleaching) analysis, that Hsp90 is part of the multimeric LPS receptor complex (Triantafilou et al., 2001). It is noteworthy that the ability of gedunin to modulate both MAL/MyD88 and TRAM/TRIF pathways independently of TLR4/MD-2 was also observed, since gedunin treatment impaired TNF-α production by macrophages stimulated with Pam3 (TLR2 selective agonist) and Poly I:C (TLR3 selective agonist) (Supplemental Figure 2). These data reinforce the role of Hsp90 in this phenomenon, since several kinases involved in MAL/MyD88 and TRAM/TRIF pathways are regulated by Hsp90 chaperoning activity (Hinz et al., 2007; Shi et al., 2009; Yang et al., 2006; Yun et al., 2011).

In addition to cytokines, LPS *in vitro* stimulation triggers increased COX-2 expression and PGE_2 production by macrophages via the TRIF signaling pathway (Endo et al., 2014). In accordance, we demonstrate here that WT macrophages produced higher amounts of PGE_2 24h, rather than 6h, after LPS stimulation, a time point in which the TRIF signaling pathway is most prominent. Nonetheless, gedunin pretreatment diminished PGE_2 production to basal levels during early and late responses. As mentioned above, Hsp90 plays an important role in the regulation of mitogen-activated

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protein kinase (MAPK) family members and, by stabilizing TAK-1 (transforming growth factor β-activated kinase 1), triggers MAPKs and NFkB activation, involved in COX-2 expression (Bode et al., 2012; Echeverria et al., 2011; Eliopoulos et al., 2002; Shi et al., 2009). There are different mechanisms proposed for Hsp90 modulation of NFkB. It has been shown that geldanamycin reduces LPS-mediated NFkB nuclear translocation in murine macrophages (Byrd et al., 1999). Malhotra and coworkers (2001) demonstrated that modulation of Hsp90 activity by geldanamycin did not impair IkB degradation or NFkB translocation into the nucleus; however, it reduced the formation of the NFkB/DNA complex and, therefore, inhibited activation of cytokine promoter. Other reports have demonstrated that the inhibition of Hsp90 activity by geldanamycin reduced the stability of LPS-induced TNF-α and IL-6 transcripts, a phenomenon partially dependent of p38 (Wax et al., 2003). Our results demonstrate that, in our model, gedunin impaired LPS-induced NFkB translocation in macrophages, reinforcing the additional role of this limonoid in modulating Hsp90 in LPS-triggered response, i.e., blocking the upstream signaling of TLR4/MD-2.

In addition to the ability to inhibit LPS-induced pro-inflammatory mediators, gedunin also triggered antiinflammatory factors, namely HO-1 (Hsp32), Hsp70 and IL-10. Supporting our data, the induction of heat shock proteins, including HO-1, by the Hsp90 inhibitor celastrol has been previously demonstrated *in vitro* and *in vivo* (Chow et al., 2013; Der Sarkissian et al., 2014; Trott et al., 2008). HO-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation, which is widely expressed during cellular stress and inflammation, triggered by diverse stimuli, including LPS (Camhi et al., 1995; Camhi et al., 1998). During inflammation, HO-1 plays antiinflammatory and immunomodulatory responses, mediated by the degradation of pro-inflammatory free heme, as well as via the production of bilirubin and carbon monoxide (CO), which present antiinflammatory properties (Gozzelino et al., 2010; Naito et al., 2014). As described in the literature, LPS *in vitro* stimulation triggers increased HO-1 expression, induced by IL-10, via a p38 MAPK-dependent pathway (Lee and Chau, 2002). In addition, it has been demonstrated that CO

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attenuates Hsp90 activity and promotes dissociation of its client proteins (Lee et al., 2014). Here we demonstrate that this stress-inducible protein was increased by gedunin incubation in the presence or in the absence of LPS, suggesting that, in our experimental model, gedunin effects are likely a result of both MD-2 binding and Hsp90 modulation. The fact that HO-1 expression was higher in gedunin pretreated macrophages compared to medium- or LPS-stimulated cells reinforces that gedunin can modulate macrophage function independently of TLR4/MD-2 activation, likely via Hsp90.

The inhibition of Hsp90 activity has been shown to induce increased expression of Hsp70 and Hsp90 via HSF-1 activation, and is related to cell protection during stress (Leung et al., 2015; Paul and Mahanta, 2014; Sharp et al., 2013). Accordingly, we have observed the induction of Hsp90 expression in LPS-stimulated macrophages pretreated with gedunin, 17-AAG and dexamethasone (that also modulates HSF-1 activity [(Knowlton and Sun, 2001)]) (Supplemental Figure 3). Overexpression or induction of intracellular Hsp70 (by stress or by Hsp70-inducing compounds) have been shown to decrease nuclear NFkB translocation, production of TNF-α, IL-6, IL-1β, NO, as well as iNOS expression (Dokladny et al., 2010; Kim et al., 2012; Muralidharan et al., 2014; Shi et al., 2006). Accordingly, it has been demonstrated that extracellular Hsp70 diminishes LPS-induced TNF-α by bone marrow-derived dendritic cells *in vitro* and induces IL-10 production by synovial cells from arthritis patients (Detanico et al., 2004). The antiinflammatory cytokine IL-10 suppresses LPS-induced macrophage activation by inhibiting the expression of specific TLR-induced pro-inflammatory genes referred to as “IL-10 counter-regulated genes” (Cardwall and Weaver, 2014; Moore et al., 1993). It is noteworthy that IL-10-mediated anti-inflammatory response is also mediated by HO-1 expression (Lee and Chau, 2002). Interestingly, our study revealed that gedunin acts as an Hsp70-inducer and also increases the production of IL-10, supporting its antiinflammatory and immunomodulatory effects.

Based on the assumptions that p23 and MD-2 share some structural similarity and that gedunin binds to p23, we hypothesized that gedunin might bind to MD-2. Supporting the biological

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effects observed with LPS-activated macrophages pretreated with gedunin, our *in silico* results showed that gedunin docked to the MD-2 large hydrophobic site that overlaps to the LPS binding site, avoiding the formation of the complex between CD14/MD-2/TLR4 and LPS. Furthermore, the binding of gedunin to MD-2 proposed by our docking experiments was proved by the biosensing surface assays performed by us. Even though the physicochemical conditions in the SPR assays might not exactly reflect that of the cellular microenvironment, the affinity of gedunin to MD-2 corroborates our *in vitro* data. The measurement of kinetic values strongly indicates that gedunin is capable to form complexes with MD-2 and, therefore, acts as antiinflammatory in LPS-stimulated macrophages. Based on our SPR data, the Gibbs free energy of the formed complexes suggest that gedunin spontaneously binds to MD-2. This finding is a physicochemical prove that gedunin interferes with the binding of LPS to MD-2 as a competitive inhibitor and, therefore, impairs the formation of TLR4/MD-2/LPS complex. Overall, our data suggest that - in addition to Hsp90 modulation - gedunin impairs TLR4 signaling pathway by inhibiting the binding of LPS to MD-2 in macrophages.

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Authorship Contributions

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Performed data analysis: Borges, P.V., Moret, K.H., Souza-Silva, F., Batista, P.R., Monteiro, C.M.M., Penido, C.

Wrote or contributed to the writing of the manuscript: Penido, C., Borges, P.V., Moret, K.H., Batista, P.R., Alves, C.R.

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Footnotes section

Co-author information: P.V.B and K.H.M. are considered coauthors for this publication

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Legends for Figures

Figure 1. Concentration-dependent inhibition of LPS-induced macrophage activation. *In vitro* pretreatment of WT immortalized macrophages (10^6 cells/well) with gedunin (Ged; 0.1-100μM) for 1h impaired (A-B) intracellular calcium influx, (C) TNF-α and (D) NO production by macrophages stimulated with LPS. (A) Kinetics of calcium influx of LPS (1μg/ml)-stimulated macrophages over 600s measured by FLIPR Calcium Plus Assay Kit and (B) means of values obtained within 600s for each group. (C) TNF-α levels were determined by ELISA in the supernatants of macrophages 6h after LPS (50ng/ml) stimulation. (D) NO was determined in supernatants of macrophages stimulated with LPS (50ng/ml) plus IFN-γ (200IU/ml) at 24h by the colorimetric Griess. (C-D) Results are expressed as the mean ± SEM for triplicate wells per group from three independent experiments, and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by * (p<0.05), *** (p<0.001). Whereas $^{++}$ (p<0.01), $^{+++}$ (p<0.001) represent differences between treated and stimulated groups.

Figure 2. Gedunin impairs LPS-induced cytokine production during early and late phase activation. Pretreatment of WT (A, B, E, F), TRIF KO (C, G) and MAL KO (D, H) macrophages (10^6 cells/well) with gedunin (Ged, 10μM), 17-AAG (1μM) or dexamethasone (Dexa, 100nM) for 1h impaired LPS (50ng/ml)-induced TNF-α (A-D) and IL-6 (E-H) at 6h (left column graphs) and 24h (right column graphs), as determined by ELISA in cell free supernatants. Results are expressed as the mean ± SEM for quadruplicate wells per group from three independent experiments, and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by *** (p<0.001). Whereas $^{++}$ (p<0.01), $^{+++}$ (p<0.001) represent differences between treated and stimulated groups.

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Figure 3. Gedunin diminishes LPS-induced PGE_2 production, COX-2 protein expression and NFκB nuclear translocation. *In vitro* pretreatment of WT immortalized macrophages (10^6 cells/well) with gedunin (Ged, 10μM), 17-AAG (1μM) or dexamethasone (Dexa, 100nM) for 1h impaired LPS (50 ng/ml)-induced (A-B) PGE_2 levels in cell free supernatants at 6 and 24h, (C) COX-2 expression in whole cell extracts at 24h and (D) NFκB expression in nuclear fractions 24h after stimulation. (C-D) Representative western blots are shown in top, whereas densitometric analyses are shown in the graphs. Results are expressed as the mean ± SEM for quadruplicate wells per group from at least three independent experiments and statistically analyzed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by ** (p<0.01), *** (p<0.001). Whereas $^{++}$ (p<0.01), $^{+++}$ (p<0.001) represent differences between treated and stimulated groups.

Figure 4. Gedunin triggers antiinflammatory mechanisms in macrophages. Expression of HO-1 (A) and Hsp70 (B) in whole WT immortalized macrophage extracts (10^6 cells/well), 6 and 24h after LPS (50ng/ml) stimulation, respectively, evaluated by western blot. Cell were pretreated for 1h with gedunin (Ged, 10μM), 17-AAG (1μM) or dexamethasone (Dexa, 100nM) before stimulation. Representative western blots from three independent experiments are shown (top panels). (C) Effect of gedunin pretreatment on LPS (50 ng/ml)-induced IL-10 production by WT macrophages, evaluated in 24h-cell-free supernatants by ELISA. Results are represented as the mean ± SEM for at least triplicate samples per group from three independent experiments. Statistical analysis was performed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between treated and stimulated groups are indicated by $^{+}$ (p<0.05), $^{++}$ (p<0.01). Whereas $^{###}$ (p<0.001) represents differences between treated and non-stimulated groups.

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Figure 5. Structural alignment of the crystal structures of the human MD-2 and the co-chaperone p23. (A) Representation of human MD-2 (PDB ID 2E56) in red on the left, p23 (PDB ID 1EJF) in blue in the middle, and the superposition of both structures on the right. (B) Secondary structure-based alignment of the sequences from MD-2 and p23 structures using the Protein Structure Alignment module of Maestro (2012).

Figure 6. *In silico* molecular docking reveals the binding mode of gedunin on the MD-2 structure (PDB ID 2E56). (A) 16-top high score conformations of gedunin within the hydrophobic pocket. Representation plus molecular surface of human MD-2. (B) Best-score predicted pose of gedunin on the molecular surface of MD-2 (colored accordingly the Eisenberg normalized consensus hydrophobicity scale (Eisenberg et al., 1984)). (C) 2D-Ligand Interactions Diagram of gedunin within the hydrophobic pocket, generated using Maestro software (2012). Polar and hydrophobic aminoacids are illustrated in blue and green circles.

Fig. 7. Biosensing surface assays to assess the binding of gedunin and LPS to immobilized MD-2. After covering the HisCap chip with MD-2, the binding assay of gedunin (0.001-1000μM) was followed by the variation of response throughout 890s. (A, B) Resonance signals are represented by sensorgram in arbitrary resonance units (RU) analyzed after subtraction of a reference line using Qdat software. (C) Double-reciprocal plot linearization of gedunin binding to MD-2. (D) Concentration curves of binding of gedunin and LPS (0.482-482μg/ml) to immobilized MD-2. (E) Binding inhibition curves of gedunin by LPS (R2=0.9883) and of LPS by gedunin (R2=0.8511), analyzed using 14.5 μg/ml of gedunin and 9.64 μg/ml of LPS, after 20 seconds of dissociation time. (F) Percentage of binding inhibition. The degree of inhibition was measured considering 100% as the resonance signal of 1/RUmax of gedunin (RU 19.33) and LPS (RU 35.00). Data are representative of 5 experiments.

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Table 1. Macrophage viability after exposure to gedunin

Medium	Tween 20 (3%)	Gedunin (μM)						
		0.001	**0.01**	**0.1**	**1**	**10**	**100**	**1000**
100 ± 2.90	2.7 ± 0.05	100 ± 1.23	100 ± 3.46	100 ± 0.22	100 ± 1.97	100 ± 4.74	91.5 ± 3.96	86.9 ± 0.39

Results are expressed as percentage of cell viability (%) from quadruplicate wells (2 x 10^5 cell/well), after incubation of macrophages with gedunin from 24h (37°C, 5% CO_2). Cell viability was assessed by resazurin reduction method, as described in Material and methods.

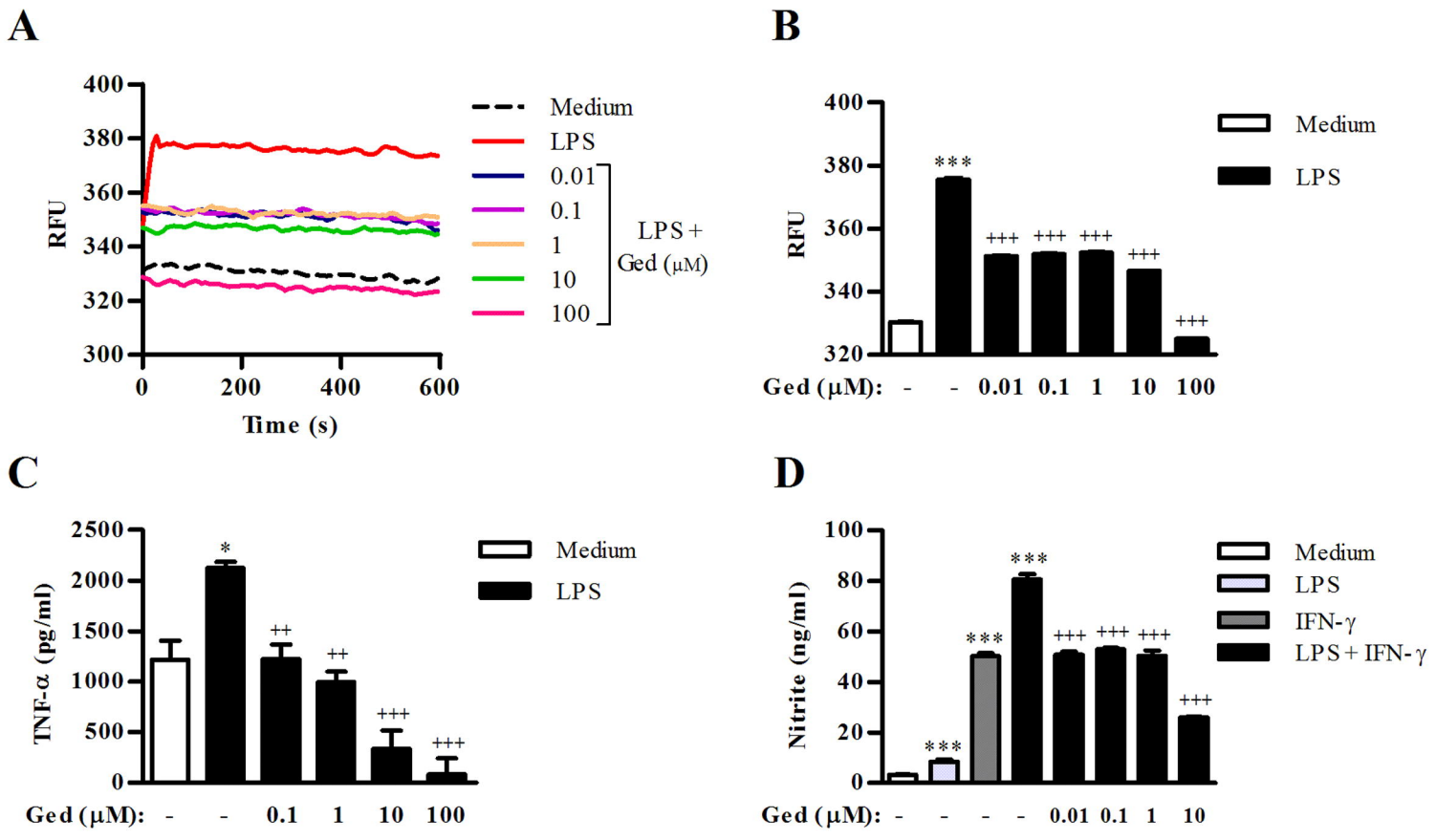


Figure 1

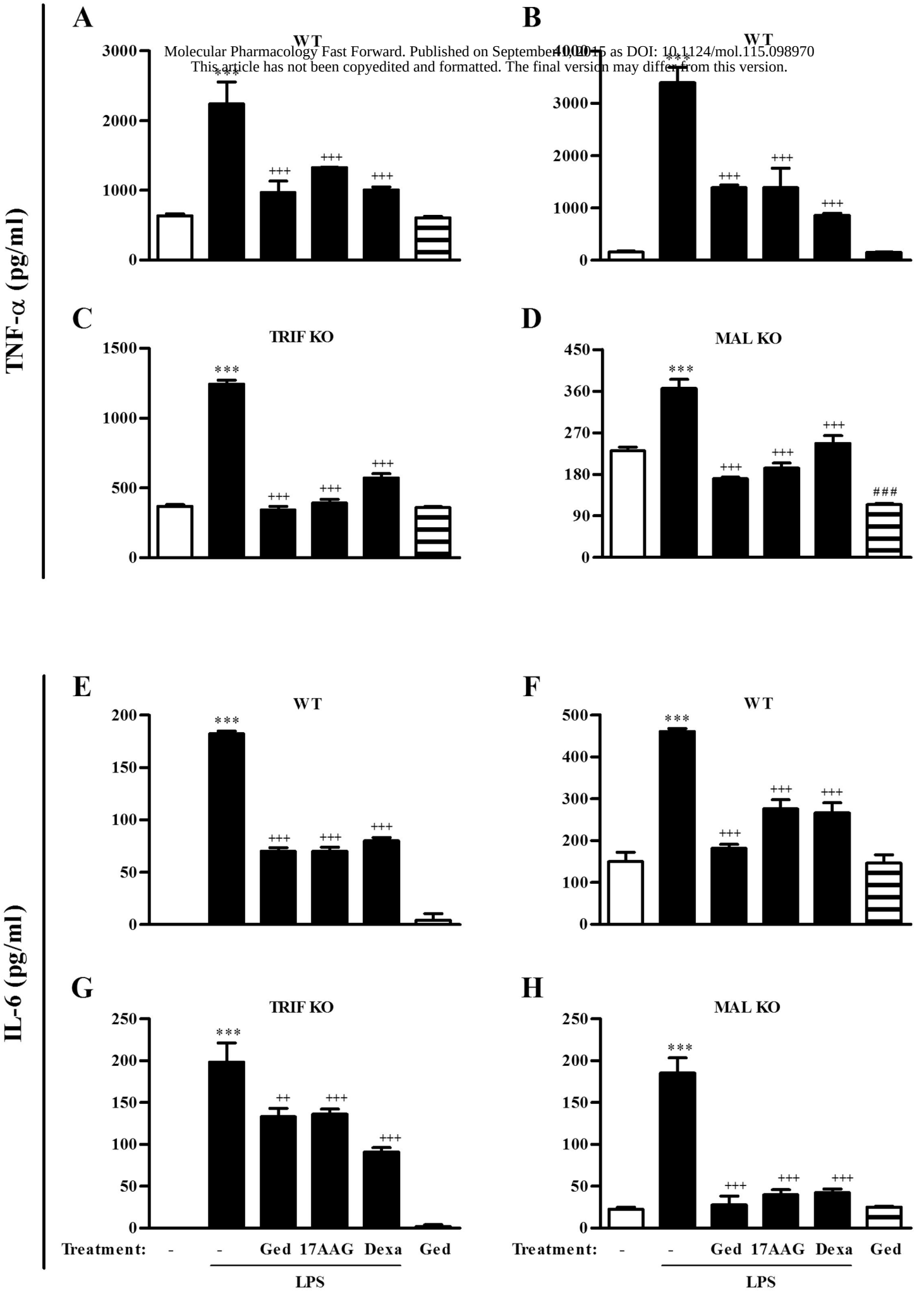


Figure 2

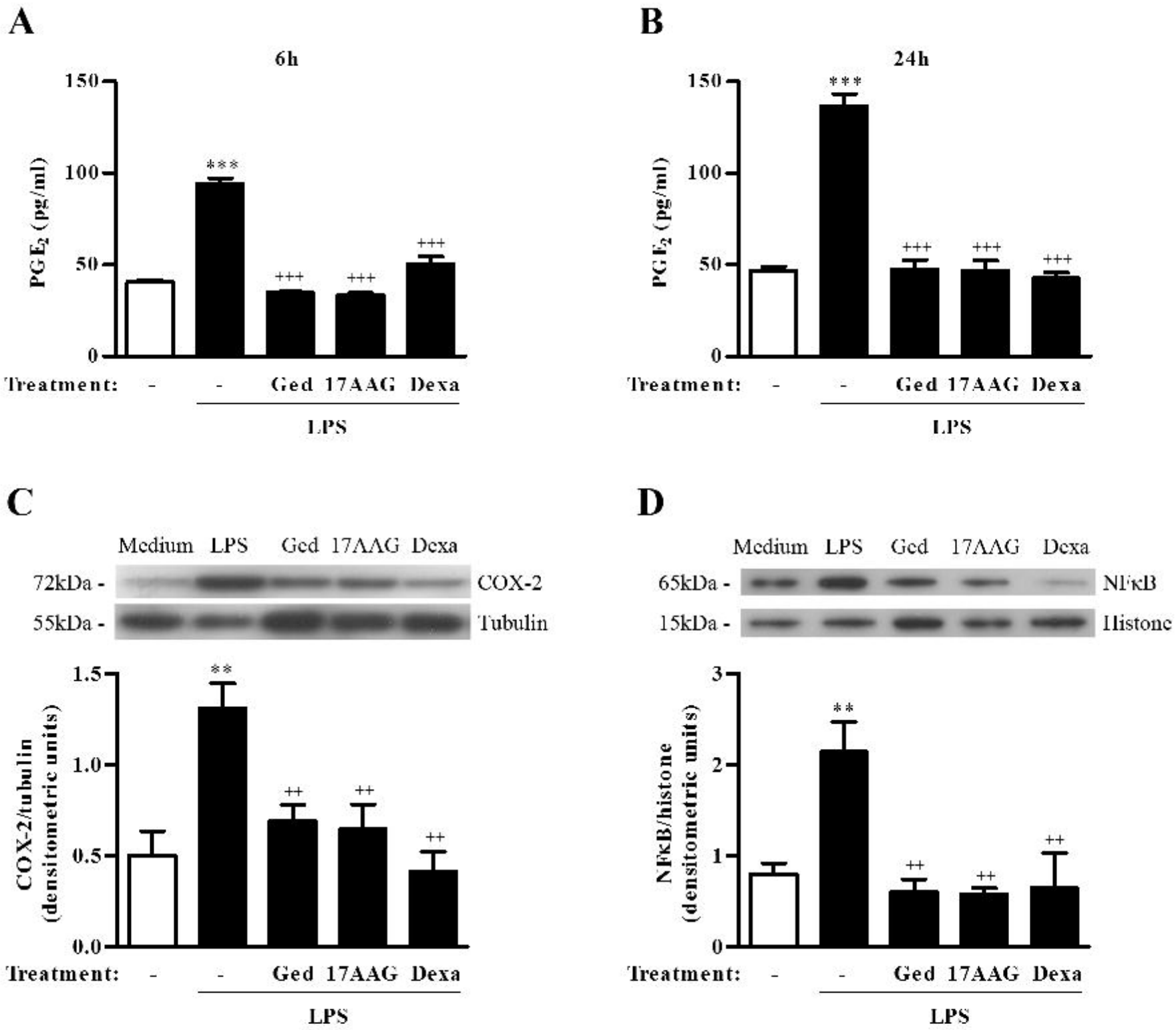
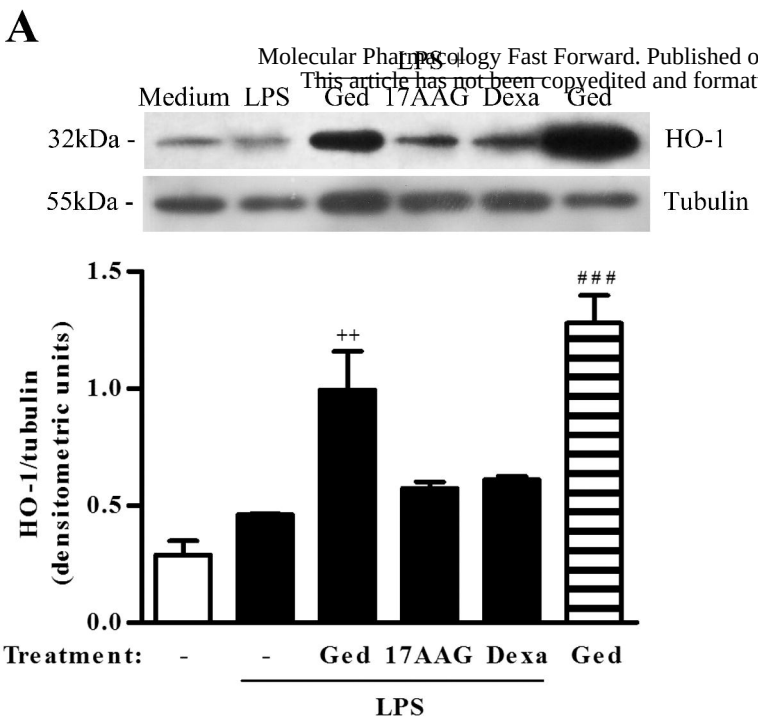
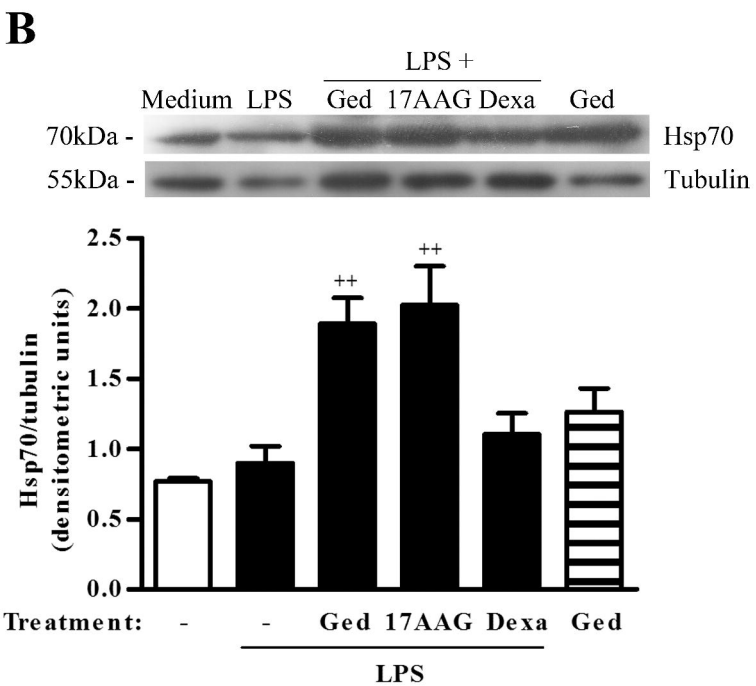

A
6h
150
100
50
0
PGE2 (pg/ml)
***
+++
+++
+++
Treatment: - - Ged 17AAG Dexa
LPS
B
24h
150
100
50
0
PGE2 (pg/ml)
***
+++
+++
+++
Treatment: - - Ged 17AAG Dexa
LPS
C
Medium LPS Ged 17AAG Dexa
72kDa - COX-2
55kDa - Tubulin
1.5
1.0
0.5
0.0
COX-2/tubulin (densitometric units)
**
++
++
++
Treatment: - - Ged 17AAG Dexa
LPS
D
Medium LPS Ged 17AAG Dexa
65kDa - NFκB
15kDa - Histone
3
2
1
0
NFκB/histone (densitometric units)
**
++
++
++
Treatment: - - Ged 17AAG Dexa
LPS

Figure 3





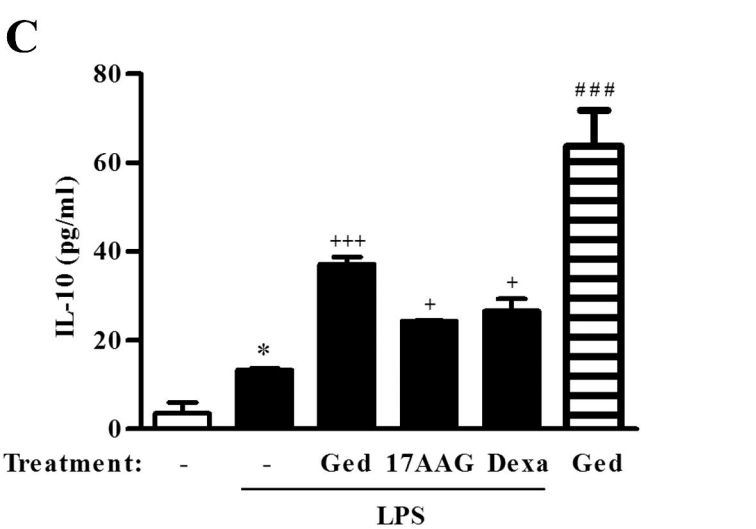
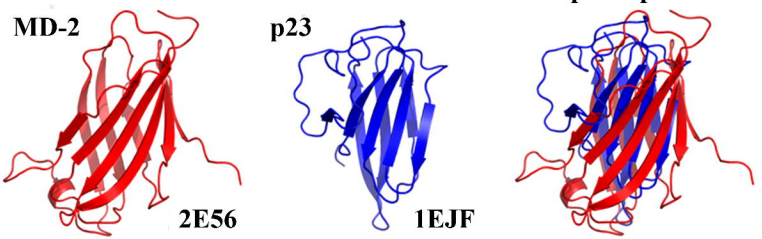


Figure 4

A



B

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                    .........+.........+.........+.........+.........+.....
2E56   SSA 1    17 --------------------CCCCCEEEEEECEEEEEEEECCCCCCC-EEEEEC
1EJF   SSA 2     1 CEEEEEEEEECEEEEEEEE----------------------CC-CECCEEE-
2E56       1    17 --------------------EAQKQYWVCNSSDASISYTYCDKMQYPI-SINVNP
1EJF       2     1 MQPASAKWYDRRDYVFIEFCV----------------------ED-SKDVNVN-

                    .........+.........+.........+.........+.........+.....
2E56   SSA 1    51 CCCCCCEEEEEEEEECCCCCCCCEEEEEEEEEECEEECCEEEECCCCCCCCCCC
1EJF   SSA 2    31 -EE-CEEE-EEEEEE-C-----------------------------------
2E56       1    51 CIELKGSKGLLHIFYIPRRDLKQLYFNLYITVNTMNLPKRKEVICRGSDDDYSFCR
1EJF       2    31 -FE-KSKL-TFSCLG-G-----------------------------------

                    .........+.........+.........+.........+.........+.....
2E56   SSA 1   107 CECCECEEEEEEEEECCCCCC--EEEEEEEEEEECCCCECCEEEEEEEECCCCC
1EJF   SSA 2    44 ---CCCEEEEEEEEC-CC-CCCCE-EEEEECE---------EEEEEEC----C
2E56       1   107 ALKGETVNTTISFSFKGIKFSK--GKYKCVVEAISGSPEEMLFCLEFVILHQPNSN
1EJF       2    44 ---SDNFKHLNEIDLF-HC-IDPND-SKHKRTDR---------SILCCLRK----G

                    .........+.........+.........+.........+.........+.....
2E56   SSA 1       ------------------------------
1EJF   SSA 2    81 CCCCCCCCCCCCCCCCCEEECCCCCCCCCC
2E56       1       ------------------------------
1EJF       2    81 ESGQSWPRLTKERAKLNWLSVDFNNWKDWE


Alignment Score:  0.808
RMSD:  4.495 Angstrom
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Figure 5

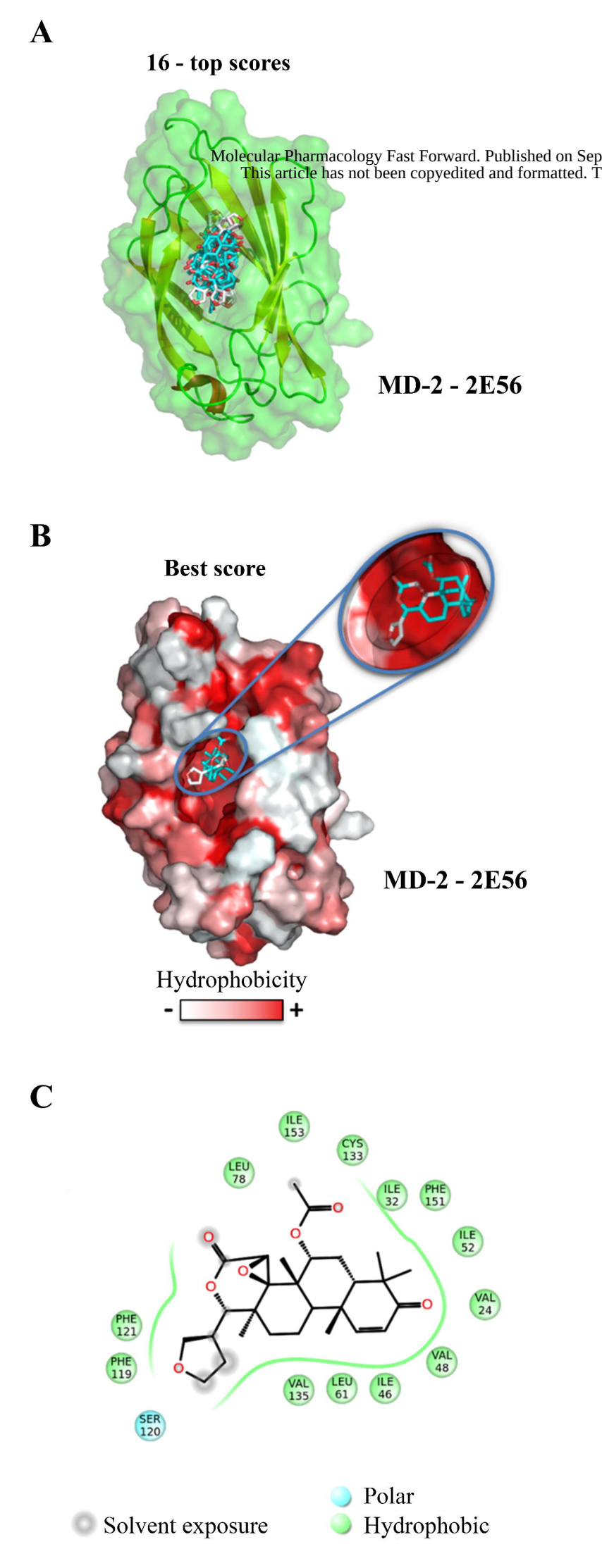

Figure 6

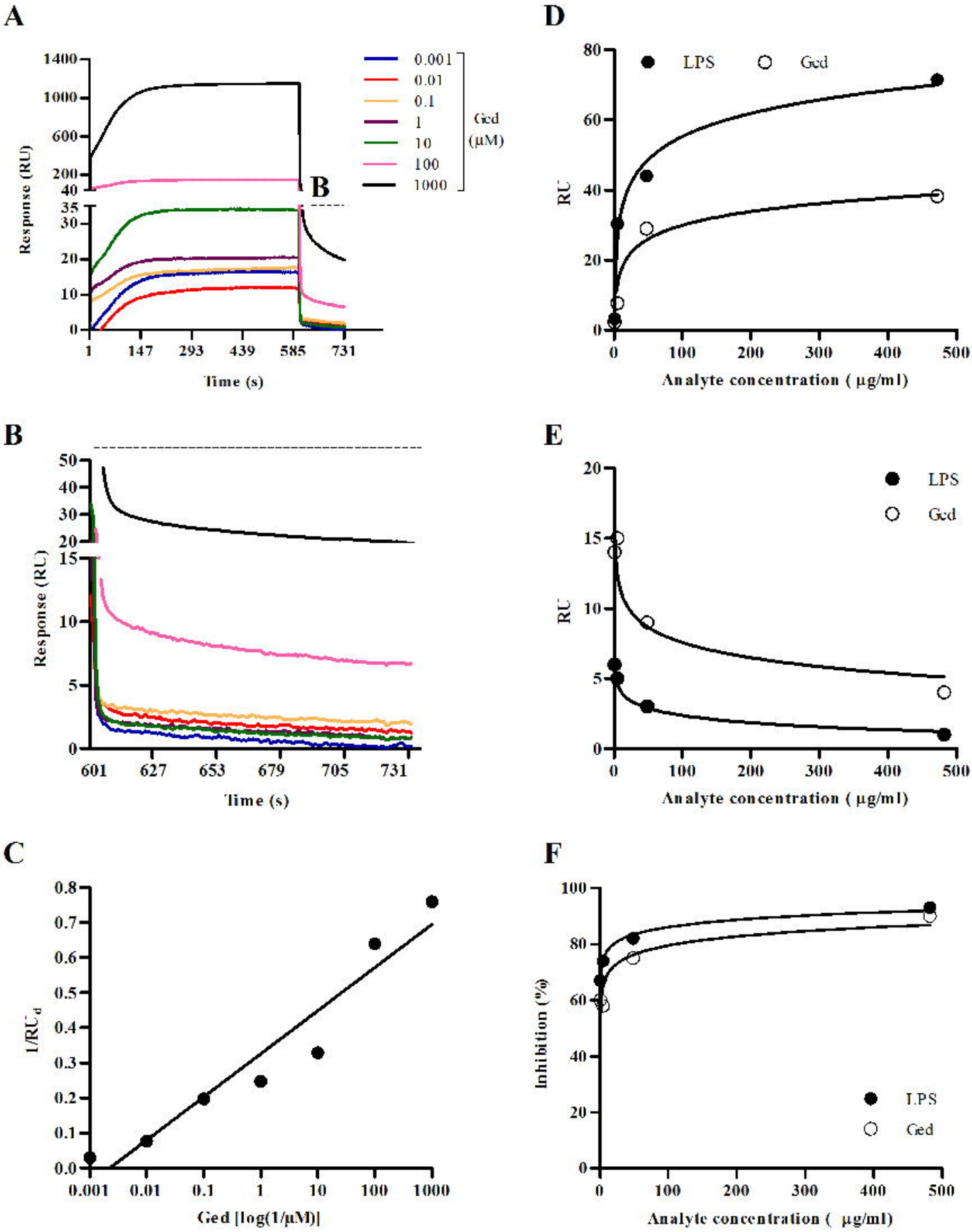
A
B
C
D
E
F
Response (RU)
Time (s)
0.001
0.01
0.1
1
10
100
1000
Ged (μM)
RU
LPS
Ged
Analyte concentration (μg/ml)
Ged [log(1/μM)]
1/RU_d
Inhibition (%)

Figure 7

Perla V. Borges, Katelim H. Moret, Clarissa M. Maya-Monteiro, Franklin Souza-Silva, Carlos R. Alves, Paulo R. Batista, Ernesto R. Caffarena, Patrícia Pacheco, Maria G. Henriques, Carmen Penido. **"**Gedunin binds to MD-2 and impairs LPS-induced TLR4 signaling in macrophages" *Molecular Pharmacology*

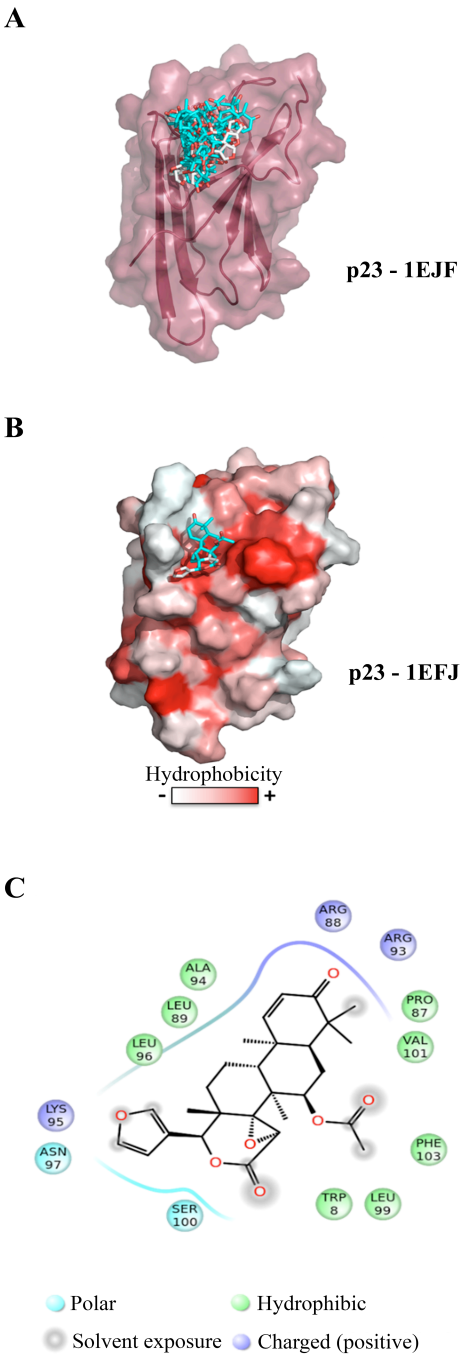

Figure S1. *In silico* molecular docking confirms the binding mode of gedunin on the crystal structure of the human co-chaperone p23 (PDB ID 1EJF). (A) 16-top high score conformations of gedunin within the hydrophobic pocket. Cartoon representation + molecular surface of human MD-2. (B) best-score predicted pose of gedunin on the molecular surface of the p23, colored accordingly the Eisenberg normalized consensus hydrophobicity scale (Eisenberg et al., 1984). (C) the 2D-Ligand Interactions Diagram, generated with Maestro program (2012). Polar, hydrophobic and positively charged aminoacids are illustrated in blue, green and purple circles.

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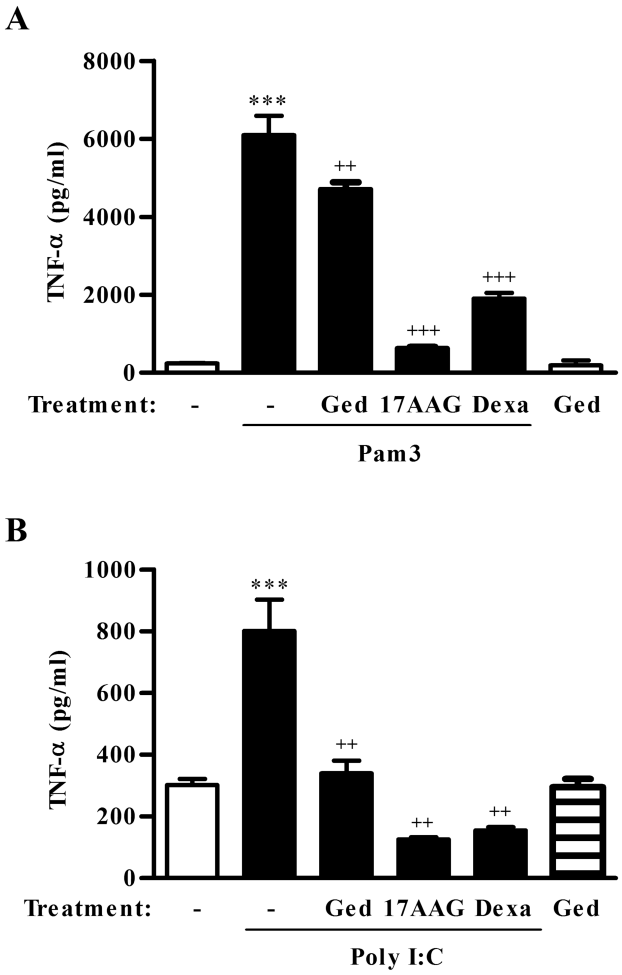

Figure S2. Gedunin impairs TLR2- and TLR3-dependent TNF-α production. WT immortalized macrophages (10^6 cells/well) were pretreated for 1h with gedunin (Ged, 10μM), 17-AAG (1μM) or dexamethasone (Dexa, 100nM) and stimulated with (A) Pam3 (palmitoyl-3-Cys-Ser-(Lys)4, Sigma Aldrich, 1μg/ml) or Poly I:C (Polyinosinic-polycytidylic acid, Sigma Aldrich, 10μg/ml,) for 24h. TNF-α levels were evaluated in the supernatants, as described in methods. Results are represented as the mean ± SEM for quadruplicate samples per group. Statistical analysis was performed by means of analysis of variance (ANOVA), followed by Newman-Keuls. Statistically significant differences between stimulated and non-stimulated groups are indicated by *** (p<0.001), whereas $^{++}$ (p<0.01) and $^{+++}$ (p<0.001) represent differences between treated and non-treated groups.

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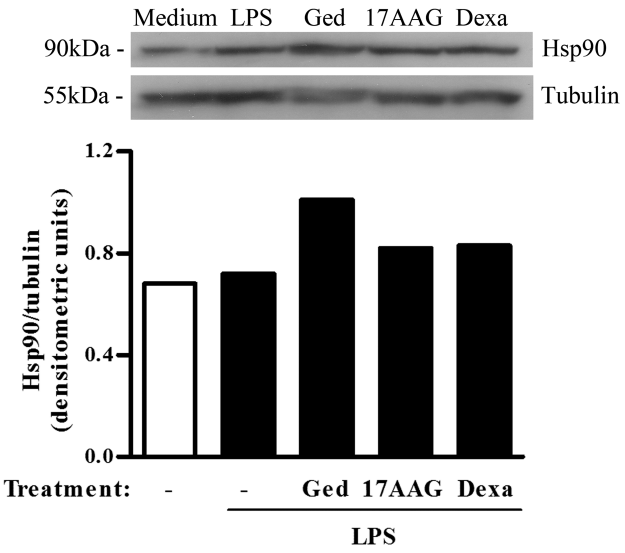

Supplemental Figure 3

Figure S3. Gedunin induces Hsp90 expression in macrophages. WT immortalized macrophages (10^6 cells/well) were pretreated for 1h with gedunin (Ged, 10μM), 17-AAG (1μM) or dexamethasone (Dexa, 100nM) and stimulated with LPS (50ng/ml) for 24h. Hsp90 expression in whole cell extracts evaluated by western blot, as described in methods. Mouse monoclonal anti-mouse/human/rat Hsp90 (1:5000) was obtained from Santa Cruz Biotechnologies (Santa Cruz, USA). Representative western blot of one experiment is shown in the top panel.