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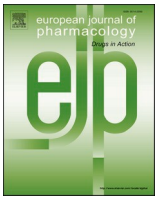

Ginger-derived bioactive compounds attenuate the Toll-like receptor mediated responses of human dendritic cells

Kitti Pázmándi [a], Beatrix Ágics [a,b], Attila Gábor Szöllősi [a], Attila Bácsi [a], Tünde Fekete [a,*]

[a] Department of Immunology, Faculty of Medicine, University of Debrecen, 1 Egyetem Square, H-4032, Debrecen, Hungary
[b] Doctoral School of Molecular Cell and Immune Biology, University of Debrecen, Debrecen, 1 Egyetem Square, H-4032, Hungary

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ABSTRACT

Ginger has been used for thousands of years for the treatment of many illnesses, from nausea to migraines. Recently, an interest has grown in ginger compounds in the context of autoimmune and inflammatory diseases due to their significant anti-inflammatory effects. Nevertheless, the effects and mechanism of action of these phytochemicals in human immune cells, particularly in dendritic cells (DCs) are unclear. In the present study, we investigated the effects of 6-gingerol and 6-shogaol, the major compounds found in ginger rhizome, on the functionality of primary human monocyte-derived DCs (moDCs). Here we report for the first time that 6-gingerol and 6-shogaol dampen the immunogenicity of human DCs by inhibiting their activation, cytokine production and T cell stimulatory ability. In particular, the bioactive compounds of ginger dose-dependently inhibited the upregulation of activation markers, and the production of different cytokines in response to synthetic Toll-like receptor (TLR) ligands. Moreover, both compounds could significantly reduce the *Escherichia coli*-triggered cytokine production and T cell stimulatory capacity of moDCs. We also provide evidence that the ginger-derived compounds attenuate DC functionality via inhibiting the nuclear factor-κB (NF-kB), mitogen activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR) signaling cascades. Further, 6-shogaol but not 6-gingerol activates the AMP-activated protein kinase (AMPK) and nuclear factor erythroid 2-related factor 2 (NRF2) pathways that might contribute to its anti-inflammatory action. Altogether, our results indicate that ginger-derived phytochemicals exert their anti-inflammatory activities via multiple mechanisms and suggest that 6-shogaol is more potent in its ability to suppress DC functionality than 6-gingerol.

1. Introduction

Dendritic cells (DCs) are considered to be the most potent antigen presenting cells, which play a central role in linking innate and adaptive immunity. By continuously scanning their local microenvironment, DCs can readily detect the presence of potentially harmful molecular structures and danger signals in peripheral tissues that results in the activation and migration of DCs to the draining lymph nodes, where they elicit antigen-specific T cell responses (Lanzavecchia and Sallusto, 2002). By contrast, in the absence of infection or inflammatory stimuli, DCs induce and maintain peripheral T cell tolerance. Therefore, DC acts as a double-edged sword, since on the one hand immunogenic DCs trigger active host defense, while, on the other hand tolerogenic DCs promote tolerance and protect against autoimmunity in the steady state (Amodio and Gregori, 2012). Nevertheless, a growing body of evidence implicates DCs in the pathogenesis and pathomechanism of various autoimmune conditions (Ganguly et al., 2013; Coutant and Miossec, 2016; Khan et al., 2022). Therefore, fine-tuning the effector function of DCs is essential to prevent self-reactive immune responses and maintain an optimal balance between inflammation and tolerance.

Several plant-derived compounds show promising anti-inflammatory properties (Furst and Zundorf, 2014); however, to date there are no studies investigating the effects of the bioactive compounds of ginger on human DC functionality. Ginger rhizome (*Zingiber officinale*) is a widely used spice that exhibits multiple beneficial biological activities, including anti-oxidant, anti-inflammatory, anti-microbial, and anti-tumor properties. More than 400 different chemical compound have been identified in ginger. Among those, 6-gingerol and 6-shogaol account for the major pharmacological activities of ginger (Li et al., 2019; Ma et al., 2021). So far a myriad of reports has proven the pharmacological potential of gingerols and shogaols. Nevertheless, only a few studies compared their anti-oxidant (Dugasani et al., 2010) and

* Corresponding author.
E-mail address: fekete.tunde@med.unideb.hu (T. Fekete).

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anti-inflammatory activities (Pan et al., 2008; Ho and Chang, 2018) in immune cells.

The anti-inflammatory effects of 6-gingerol have been first explored in a murine macrophage cell line. It was demonstrated that 6-gingerol inhibited the nitrogen oxide (NO) production and inducible NO synthase (iNOS) expression in lipopolysaccharide (LPS)-activated J774.1 macrophages (Ippoushi et al., 2003). Later it was published that 6-, 8- and 10-gingerols inhibited the activation, proliferation of mouse spleen-derived $CD3^+$ T cells and their production of interferon-gamma (IFN-γ) in response to anti-CD2/CD3/CD28-coated beads. Another study showed that 6-gingerol attenuated neutrophil extracellular trap release in response to LPS and various lupus-relevant stimuli (Ali et al., 2021). So far, only one study showed that 6-gingerol was able to modulate DC functionality in a mouse model of experimental autoimmune encephalomyelitis (Han et al., 2019). Much less is known about the role of 6-shogaol in modulating immune cell functions. A study reported that 6-shogaol inhibited the lipopolysaccharide (LPS)-induced activation of nuclear factor-kappa B (NF-κB), mitogen activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways in RAW 264.7 macrophages (Pan et al., 2008).

Since it has never been investigated how the bioactive compounds of ginger might affect the Toll-like receptor (TLR)-mediated responses of human DCs, in the present study we aimed to explore the effects of 6-gingerol and 6-shogaol on human DC functionality and to study the molecular mechanisms underlying their actions.

2. Materials and methods

2.1. Isolation and culturing of primary human cells

The collection of human heparinized leukocyte-enriched buffy coat samples complied with the guidelines of the Helsinki Declaration and was approved by the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Faculty of Medicine (OVSzK 3572-2/2015/5200, Hungary). Human buffy coats were obtained from healthy blood donors and peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll-Paque Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK, Cat. No. 17-1440-03) gradient centrifugation.

Monocytes were separated from PBMCs by positive selection using CD14 microbeads (Miltenyi Biotech, Bergish Gladbach, Germany, Cat. No. 130-050-201). For DC differentiation, freshly isolated monocytes were plated at a density of 10^6 cells/ml in 24-well cell culture plates in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. R8758) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies Corporation, Carlsbad, CA, USA, Cat. No. 10270-106), 2 mM L-glutamine (Biosera, Nuaille, France, Cat. No. XC-T1755/100), 100 U/ml penicillin, 100 μg/ml streptomycin (both from Biosera, Cat. No. XC-A4122/100), 80 ng/ml granulocyte-macrophage colony-stimulating factor (Gentaur Molecular Products, London, UK, Cat. No. 04-RHUGM-CSF) and 50 ng/ml interleukin (IL)-4 (PeproTech, Brussels, Belgium, Cat. No. 200-04) for 5 days.

Allogenic, naive $CD4^+$ T cells were isolated from PBMCs using the human naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec, Cat. No. 130-094-131), and were subsequently co-cultured with DCs as described below. Cells were incubated at 37 °C in 5% CO_2 humidified atmosphere.

2.2. Cell stimulation

5-day monocyte-derived DCs (moDCs) were pre-treated with different doses (0-10-25-50 μM) of 6-gingerol (Cayman Chemical, Ann Arbor, MI, Cat. No. 11707) or 6-shogaol (Cayman Chemical, Ann Arbor, MI, Cat. No. 11901) for 2 h as indicated in the figure legends. Both reagents were dissolved in ethanol, thus medium containing ethanol was used as control treatment (vehicle control) at a final concentration of 0.0014%, corresponding to the highest concentration used when testing the compounds. Cell were then stimulated with 500 ng/ml ultrapure LPS from *E. coli* 0111:B4 (Invivogen, San Diego, CA, USA, tlrl-3pelps), CL075 (Invivogen, tlrl-c75) or PAM3CSK4 (Invivogen, tlrl-pms) for different time periods. In separate experiments, cells were primed with Escherichia coli (E. coli) ATCC11775 at a MOI of 1. The *E. coli* strain was a kind gift from Dr. Walter Pfliegler (Department of Molecular Biotechnology and Microbiology, Faculty of Science and Technology, University of Debrecen, Debrecen, Hungary).

2.3. DC- T cell co-culture and intracellular cytokine staining

Following pretreatment with 6-gingerol or 6-shogaol moDCs were stimulated with *E. coli* as described above. Thereafter cells were washed two times in cell culture medium and then were co-cultivated with allogeneic naïve $CD4^+$ T cells in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Life Technologies Corporation), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma-Aldrich) and 1 μg/ml anti-human CD3 monoclonal antibody (BD Biosciences, Franklin, Lakes, NJ, USA, Cat. No. 555329). For intracellular cytokine staining naïve $CD4^+$ T cells were seeded on 48-well cell culture plates at a ratio of 1:10 (1×10^5 DCs; 1×10^6 T cells) in 500 μl RPMI 1640 medium. After 6 days of co-culture T cells were stimulated with 0.1 μg/ml phorbol myristate acetate (PMA, Invivogen, tlr-pma) and 1 μg/ml ionomycin (Sigma-Aldrich, Cat. No. 10634) for 2 h alone then in the presence of the protein transport inhibitor monensin (BD Biosciences, Cat. No. 554724) for an additional 5 h. Thereafter, the cells were stained with anti-CD4-FITC (BioLegend, clone OKT4, Cat. No. 317408), anti-CD25-FITC (BioLegend, clone BC96, Cat. No. 302604) and isotype matched control antibody (both from BioLegend, San Diego, CA, USA), fixed and permeabilized by the BD Cytofix/Cytoperm solution (BD Biosciences, Cat. No. 554714). Cells were then labeled with APC-conjugated anti–IFN–γ (BioLegend, clone 4 S.B3, Cat. No. 502512), Foxp3-PE (BioLegend, clone 206D, Cat. No. 320108) and fluorescence intensities were measured with FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

2.4. Phenotypical analysis of moDCs by flow cytometry

Phenotypical analysis of moDCs was performed by flow cytometry using anti-CD40-FITC (clone 5C3, Cat. No. 334306), anti-HLA-DQ-PE (clone HLADQ1, Cat. No.318106), anti-CD83-PE-Cy5 (clone HB15e, Cat. No. 305310), anti-CD86-PE (clone IT2.2, Cat. No. 305406), anti-programmed death-ligand 1 (PD-L1)-PE (clone 29E2A3, Cat. No. 329705), anti-PD-L2-APC (clone 24 F.10C12, Cat. No. 329608), anti-immunoglobulin-like transcript 3 (ILT3)-PE (clone ZM4.1, Cat. No. 333007), anti-ILT4-APC (clone 42D1, Cat. No. 338707) and their isotype-matched control antibodies (all from BioLegend). The viability of moDCs was assessed by 7-aminoactinomycin-D (7-AAD; 10 μg/ml; Sigma-Aldrich, Cat. No. A9400) staining. Fluorescence intensities were measured with FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

2.5. Quantitative real time PCR

Total RNA was isolated using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA, Cat. No. TR118) then was treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. AM2222) to exclude amplification of genomic DNA. Reverse transcription was performed using the High Capacity cDNA RT Kit (Thermo Fisher Scientific, Cat. No. 4368813). Gene expression assays were purchased from Thermo Fisher Scientific for hexokinase 2 (HK2, Assay ID: Hs00606086_m1, Cat. No: 4331182), lactate dehydrogenase A (LDHA, Assay ID: Hs00855332_g1, Cat. No: 4331182), hypoxia-inducible factor 1-alpha (HIF1A, Assay ID: Hs00153153_m1, Cat. No: 4331182), and from Integrated DNA Technologies (Coralville, IA, USA) for PPIA

(cyclophilin A; Assay ID: Hs. PT.58v.38887593. g). Quantitative PCR was performed using the ABI StepOne Real-Time PCR System (Thermo Fisher Scientific). Cycle threshold values were determined using the StepOne v2.1 Software (Thermo Fisher Scientific) and were normalized to the housekeeping gene PPIA.

2.6. Western blotting

Protein extraction was performed by lysing the cells in 2x Laemmli sample buffer (62.5 mM TRIS-HCL, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) and then heated at 100 °C for 10 min. Proteins samples were separated by SDS-PAGE using 10% polyacrylamide gels then transferred to nitrocellulose membranes (Bio-Rad Laboratories GmbH, Munich, Germany, Cat. No. 162-0115). Non-specific binding sites were blocked with 5% non-fat dry milk diluted in TBS Tween buffer (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4). The following antibodies were ordered from Cell Signaling (Danvers, MA, USA): anti-phospho-Akt (Ser473; Cat. No. 4060), anti-Akt (pan; Cat. No. 4685), anti-phospho-p70S6 kinase (p70S6K, Thr389; Cat. No. 97596) anti-p70S6K (Cat. No.2708), anti-phospho-p38 MAPK (Cat. No. 9216), anti-p38 MAPK (Cat. No. 9212), anti-phospho-extracellular-regulated kinase (ERK) 1/2 (Thr202/Tyr204; Cat. No. 9106); anti-ERK 1/2 (Cat. No. 9102), anti-phospho-Jun amino-terminal kinases (JNK) (Thr183/Tyr185; Cat. No. 4668), anti-JNK (Cat. No. 9252), anti-histone H3 (Cat. No. 9715), anti-heme oxygenase-1 (HO-1), (Cat. No. 5843). Anti-β-actin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA; Cat. No. sc-47778). The bound antibodies were labeled with anti-mouse (Bio-Rad, Cat. No. 1721011) or anti-rabbit (GE Healthcare, Cat. No. NA934) horseradish peroxidase-conjugated secondary antibodies and were visualized by the ECL system using SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific, Cat. No. 34580) or Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095) and X-ray film exposure. Densitometric analysis of immunoreactive bands was performed using Image Studio Lite Software version 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

2.7. Subcellular fractionation

Cytosolic and nuclear extracts were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Cat. No. 78833) according to the manufacturer's protocol. Protein concentrations for each extract were determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23227) and then extracts were subjected to Western blot analysis. 5 μg of nuclear extracts and 2 μg of cytosolic extracts were loaded on 12.5 % SDS-polyacrylamide gel then all subsequent steps were performed as described above. Beside anti-β-actin the following antibodies were used: anti- NF-κB p65 (Cell Signaling, Cat. No. 8242), anti-NRF2 (Thermo Fisher Scientific, Cat. No. PA5-27882), anti-phospho-AMPK (Thr172; Cell Signaling, Cat. No. 2535), anti-AMPK (Cell Signaling, Cat. No. 5831), anti-histone H3 (Cell Signaling, Cat. No. 9715).

2.8. ELISA

Cell culture supernatants were collected after a 24-h stimulation and then tumor necrosis factor (TNF) (Cat. No. 555212), IL-6 (Cat. No. 555220), IL-10 (Cat. No. 555157) and IL-12 (Cat. No. 555183) levels were determined by the BD OptEIA human ELISA kits (all from BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Absorbance was measured by a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 450 nm.

2.9. Statistical analysis

Data are expressed as mean ± SD and statistical significance was analyzed using one-way ANOVA, followed by Bonferroni post hoc test. Data analysis was performed with GraphPad Prism v.6. Software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. The active constituents of ginger dose-dependently inhibit the TLR-mediated activation of human moDCs

Due to the low availability of cDCs in the peripheral blood we performed our experiments on human moDCs, which highly resemble the type 2 subset of cDCs (cDC 2) both in their phenotypical and functional properties. Although moDC was initially identified as an inflammatory DC subtype recent findings show that moDCs can be found in different tissues under physiological conditions as well (Segura, 2022; Backer et al., 2023). These findings also suggest that monocytes contribute to the maintenance of the peripheral steady-state DC network. Besides, moDCs also serve as a potent source for DC-based immunotherapy (Hopewell and Cox, 2020); therefore, moDCs represent a suitable model to study human DC biology.

First, we sought to determine an optimal dose of 6-gingerol and 6-shogaol that could efficiently modulate DC functionality. Based on literature data the bioactive compounds of ginger are usually used at concentrations of 1–100 μM under *in vitro* conditions. Therefore, we pre-treated 5-day moDCs with increasing doses of 6-gingerol and 6-shogaol in the range of 10–50 μM for 2 h. Thereafter the cells were activated with different TLR ligands including the TLR4 agonist ultrapure LPS, the synthetic TLR7/8 ligand CL075, and the synthetic TLR2/1 agonist PAM3CSK4. After 24 h cells were subjected to flow cytometry analysis to assess cell viability and the expression pattern of various cell surface markers. In parallel experiments, cell culture supernatants were collected and analyzed for various secreted cytokines by ELISA. Our results demonstrate that none of the applied doses of 6-gingerol or 6-shogaol influence the viability of resting and activated moDCs(Supplementary Fig. 1A and B and 2A, B). Meanwhile, we also analyzed the expression of various plasma membrane-bound proteins including the activation marker CD83, the major histocompatibility (MHC) class II molecule HLA-DQ, and the co-stimulatory molecules CD86 and CD40. We found that both bioactive compounds exhibited a dose-dependent inhibition on the TLR-mediated upregulation of the cell surface molecules, while having no effect on their expression when applied alone (Fig. 1A–C). Representative histograms showing the effects of the highest doses of 6-gingerol and 6-shogaol are shown in Supplementary Figs. 3A–C.

In particular, the 50 μM dose of 6-gingerol or 6-shogaol could significantly decrease the expression of CD40, CD83, CD86 and HLA-DQ in DCs activated through cell-surface TLRs. Nevertheless, we could see a weaker or no effect of the applied compounds when moDCs were stimulated through the endosomal TLR7/8 receptor. In separate experiments, we also measured the surface expression of various inhibitor molecules (Fig. 2A–C). Our data show that both 6-gingerol and 6-shogaol significantly decrease the TLR-triggered expression of PD-L1 and PD-L2. Interestingly, both bioactive compounds of ginger could slightly upregulate the expression of ILT-3 on the surface of immature moDCs; however, this effect was counteracted in the presence of different TLR stimuli. When moDCs were challenged with LPS, 6-gingerol and 6-shogaol even induced a significant downregulation of ILT-3. In addition, exposure to LPS and CL075 but no to PAM3CSK4 increased the surface expression of ILT-4, which was downregulated upon pre-treatment with 6-gingerol or 6-shogaol. Altogether, these data suggest some TLR agonist-dependent differences in the effects of ginger constituents on the cell surface marker expression of moDCs. Similar to the cell surface markers we could see a dose-dependent reduction in the TLR-triggered cytokine production of moDCs as well (Fig. 3A–C). Representative histograms showing the effects of the highest doses of 6-gingerol and 6-

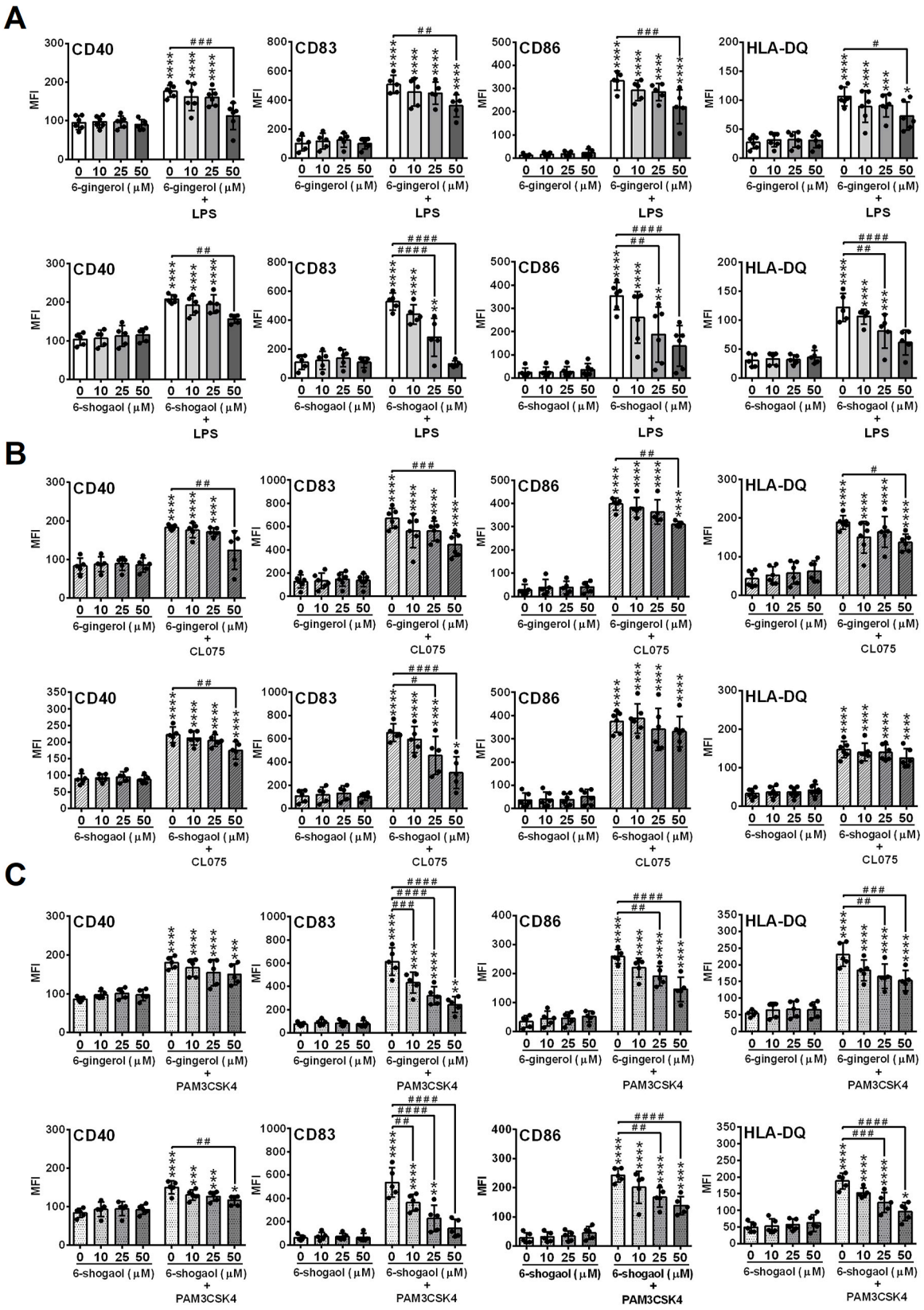

Fig. 1. 6-gingerol and 6-shogaol dose-dependently reduce the expression of activation and co-stimulatory molecules on the surface of moDCs. Immature moDCs were pre-treated with vehicle control, increasing doses of 6-gingerol or 6-shogaol for 2 h and then stimulated with 0.5 μg/ml LPS (A), CL075 (B) or PAM3CSK4 (C) for 24 h. The changes in the expression level of cell surface molecules were assessed by flow cytometry. Bar graphs represent the mean ± SD of 5–6 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$, ****$p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

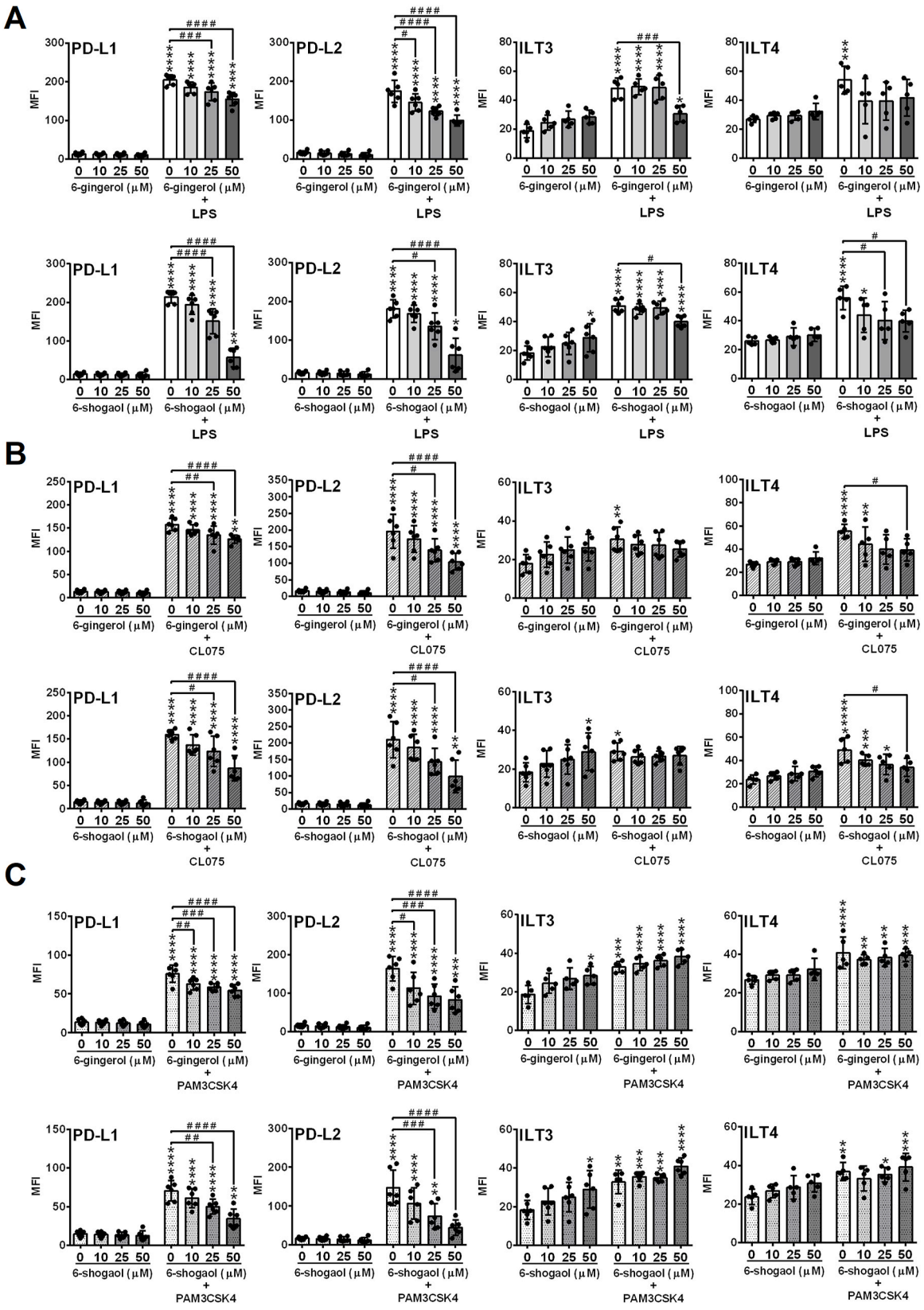

Fig. 2. 6-gingerol and 6-shogaol dose-dependently decrease the surface expression of inhibitory molecules on moDCs. Immature moDCs were pre-treated with vehicle control, increasing doses of 6-gingerol or 6-shogaol for 2 h and then stimulated with 0.5 μg/ml LPS (A), CL075 (B) or PAM3CSK4 (C) for 24 h. The changes in the expression level of inhibitory cell surface markers were assessed by flow cytometry. Bar graphs represent the mean ± SD of 5–6 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$, ****$p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

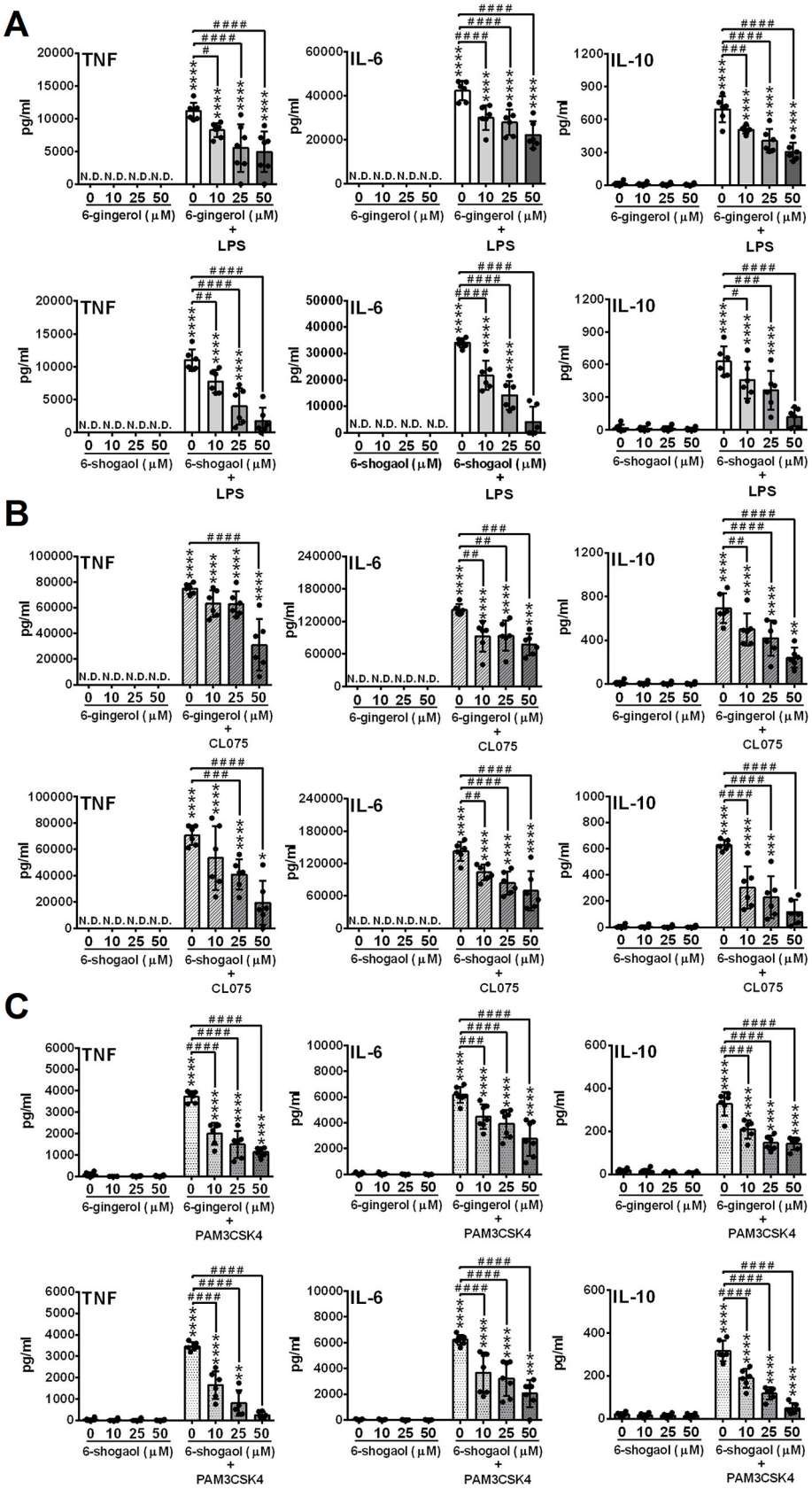

Fig. 3. 6-gingerol and 6-shogaol decrease the cytokine production of TLR-stimulated moDCs in a dose-dependent manner. Immature moDCs were pre-treated with vehicle control, increasing doses of 6-gingerol or 6-shogaol for 2 h and then stimulated with 0.5 μg/ml LPS (A), CL075 (B) or PAM3CSK4 (C). TNF, IL-6 and IL-10 protein levels were measured by ELISA 24 h after stimulation. Bar graphs represent the mean ± SD of 6–9 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$, ****$p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$. N.D.: Not determined.

shogaol are shown in Supplementary Figs. 4A–C. Both 6-gingerol and 6-shogaol could significantly reduce the secretion of the pro-inflammatory cytokines TNF and IL-6, as well as the anti-inflammatory cytokine IL-10 by TLR-stimulated moDC. As with the phenotypic markers, 6-gingerol or 6-shogaol applied alone had no effect on the cytokine production of resting moDCs.

For further experiments, we decided to use the 50 μM dose of both compounds, which could remarkably modulate both the phenotypical and functional properties of moDCs without affecting their viability.

3.2. 6-Gingerol and 6-shogaol reduces the E. coli-triggered cytokine production and T cell stimulatory capacity of moDCs

Since DCs play a central role in coordinating T cell responses we were curious to see how the bioactive compounds of ginger could modulate their T cell stimulatory capacity. To this end, 5-day moDCs were pre-treated with 6-gingerol and 6-shogaol then were stimulated with live *E. coli* to provide appropriate signals for the efficient activation of T cells. After 24 h supernatants were collected to assess cytokine secretion by ELISA, while DCs were harvested for co-culture with allogeneic naïve $CD4^+$ T cells. After 6 days of co-culture the intracellular cytokine production of T cells was measured by flow cytometry. Our results show that 6-gingerol and 6-shogaol significantly reduced the *E. coli*-triggered secretion of the pro-inflammatory cytokines IL-6 and TNF, as well as the production of the T cell polarizing cytokines IL-12 and IL-10 by moDCs (Fig. 4A). As expected, moDCs pre-conditioned with 6-gingerol or 6-shogaol show a lower capacity to induce IFN-γ production, and thus to promote a Th1 phenotype in $CD4^+$ T cells (Fig. 4B and C). In parallel, we also tried to measure the Th17 and Treg polarizing capacity of *E. coli*-triggered moDCs; however, moDCs failed to induce detectable levels of IL-17 and IL-10 in $CD4^+$ T cells (data not shown). Further, neither of the treatment conditions resulted in Foxp3 upregulation (Fig. 4 D, E). Nevertheless, the ability of moDCs to upregulate CD25 expression on T cells was decreased upon pre-treatment with 6-gingerol or 6-shogaol that refers to their reduced capacity to initiate T cell activation (Fig. 4 D, E).

Taken together these data indicate that both 6-gingerol and 6-shogaol are able to effectively inhibit the TLR-mediated functionality of moDCs including their cytokine producing and Th1 polarizing capacity.

3.3. 6-Gingerol and 6-shogaol modulate the TLR-mediated activation of NF-κB and MAPK signaling pathways in moDCs

TLR-induced maturation and activation of DCs are promoted by the activation of NF-kB and MAPK signaling cascades (Kawasaki and Kawai, 2014). Therefore, we wanted to see how these pathways are affected when moDCs are treated with 6-gingerol or 6-shogaol prior to TLR stimulation.

First, we investigated the TLR-triggered nuclear translocation of the p65 subunit of NF-κB. Therefore, immature moDC were pre-conditioned with 6-gingerol or 6-shogaol for 2 h then were stimulated with different TLR ligands for 30 min. Thereafter the cells were lysed and fractionated into nuclear and cytosolic extracts. NF-κB p65 protein levels were tested in both fractions by Western blot. Histone H3 and β-actin were used as loading controls for the nuclear and cytosolic fractions, respectively. Our results demonstrate that both compounds could significantly decrease the TLR-induced nuclear translocation of NF-κB p65, whereas they did not exert any effect on its cytosolic levels (Fig. 5A–B). In parallel, we have also measured the expression of the inhibitor of NF-κB (IκBα) in the cytosolic fraction. Interestingly, treatment with either 6-gingerol or 6-shogaol did not influence the TLR-mediated degradation of IκBα (Fig. 5A–B).

Next, we studied the activity of the MAPK signaling cascade in TLR-stimulated moDCs. Therefore, immature moDCs were pre-treated with 6-gingerol or 6-shogaol and then stimulated with different TLR ligands in a time-dependent manner. As a readout we measured the phosphorylation of the three main MAPKs: p38, ERK and JNK (Fig. 6A–B). Our results demonstrate that all three TLR ligands significantly induced the phosphorylation of MAPKs, which generally reached a peak after 30 min of activation. Therefore, we analyzed phosphorylated protein levels by densitometry at this time point. Our results show that 6-shogaol could significantly decrease the phosphorylation of p38 in moDCs stimulated with LPS, but not in those triggered by CL075 or PAM3CSK4. Interestingly, 6-gingerol did not affect p38 phosphorylation induced by any of the applied stimuli. Further, we found that 6-shogaol significantly decreased ERK phosphorylation induced by all TLR ligands, whereas 6-gingerol reduced ERK phosphorylation only in moDCs triggered by CL075 and PAM3CSK4. Our results further demonstrate that 6-gingerol pre-treatment did not affect the TLR-triggered phosphorylation of JNK in moDCs. Interestingly, 6-shogaol significantly decreased the LPS-triggered JNK activity, while did not affect that upon stimulation with CL075 or PAM3CSK4 (Fig. 6A–B). All these data imply that the bioactive compounds of ginger suppress the TLR-mediated activation and effector functions of moDCs via modulating the NF-κB and MAPK signaling pathways.

3.4. The bioactive compounds of ginger decrease the TLR-mediated activation of mTOR signaling and metabolic changes in moDCs

Various extracellular signals including TLR ligands are able to activate the mammalian target of rapamycin (mTOR) complex (mTORC) 1–mTORC2 network in innate immune cells, thereby controlling a wide range of basic cellular processes (Weichhart et al., 2015). Many studies, including ours, have demonstrated that mTOR plays an essential role in regulating the effector function of DCs such as their cytokine production and T cell stimulatory ability (Fekete et al., 2014, 2020). In order to investigate whether 6-gingerol and 6-shogaol could modulate the TLR-driven activation of mTOR in moDCs, we analyzed the kinetics of phosphorylation of p70S6K (Thr389) and Akt (Ser473), which are the downstream targets of mTORC1 and mTORC2, respectively (Fig. 7A–B). First, immature moDCs were pre-treated with 6-gingerol or 6-shogaol, and then stimulated with different TLR ligands in a time-dependent manner. Our results demonstrate that the applied TLR stimuli significantly increased the phosphorylation of p70S6K after 2 h of activation (Fig. 7A). Pre-treatment with 6-shogaol could significantly decrease the TLR-driven phosphorylation of p70S6K, whereas 6-gingerol did not affect it (Fig. 7A–B). Further, we found that all TLR ligands could induce a strong increase in the phosphorylation of Akt as early as 30 min after stimulation that was either sustained or decreased by 120 min, depending on the donor and the activation stimulus. The effects of 6-gingerol and 6-shogaol on the TLR-induced Akt phosphorylation were most evident at 60 or 120 min of stimulation (Fig. 7A). At these time points, densitometric analysis shows that 6-gingerol slightly but significantly reduced the TLR-induced phosphorylation of Akt, whereas 6-shogaol exerted a more pronounced inhibitory effect (Fig. 7A–B). These results indicate that 6-shogaol is able to block the activity of both mTOR complexes, whereas 6-gingerol only inhibits mTORC2. These data also prompted us to investigate the activity of AMPK, which is a well-known upstream negative regulator of mTORC1 (Fig. 7C–D). To these experiments, we used the cytosolic fraction of cell lysates generated for nuclear translocation studies. Our results show that 6-gingerol or 6-shogaol alone were not able to trigger AMPK phosphorylation in resting DCs (Fig. 7C–D). Interestingly, after 30 min of stimulation with TLR ligands, 6-shogaol was able to induce AMPK phosphorylation in moDCs, whereas 6-gingerol was not able to do so (Fig. 7E–F). Altogether these data imply that 6-shogaol suppresses mTORC1 activity, most likely via activating AMPK.

It is well known that mTORC1 regulates the TLR-induced reprogramming from oxidative phosphorylation to aerobic glycolysis that is essential for the activation and immunogenic functions of DCs (Snyder and Amiel, 2018). Therefore, we also investigated how TLR-mediated metabolic switch is affected by the bioactive compounds of ginger. In

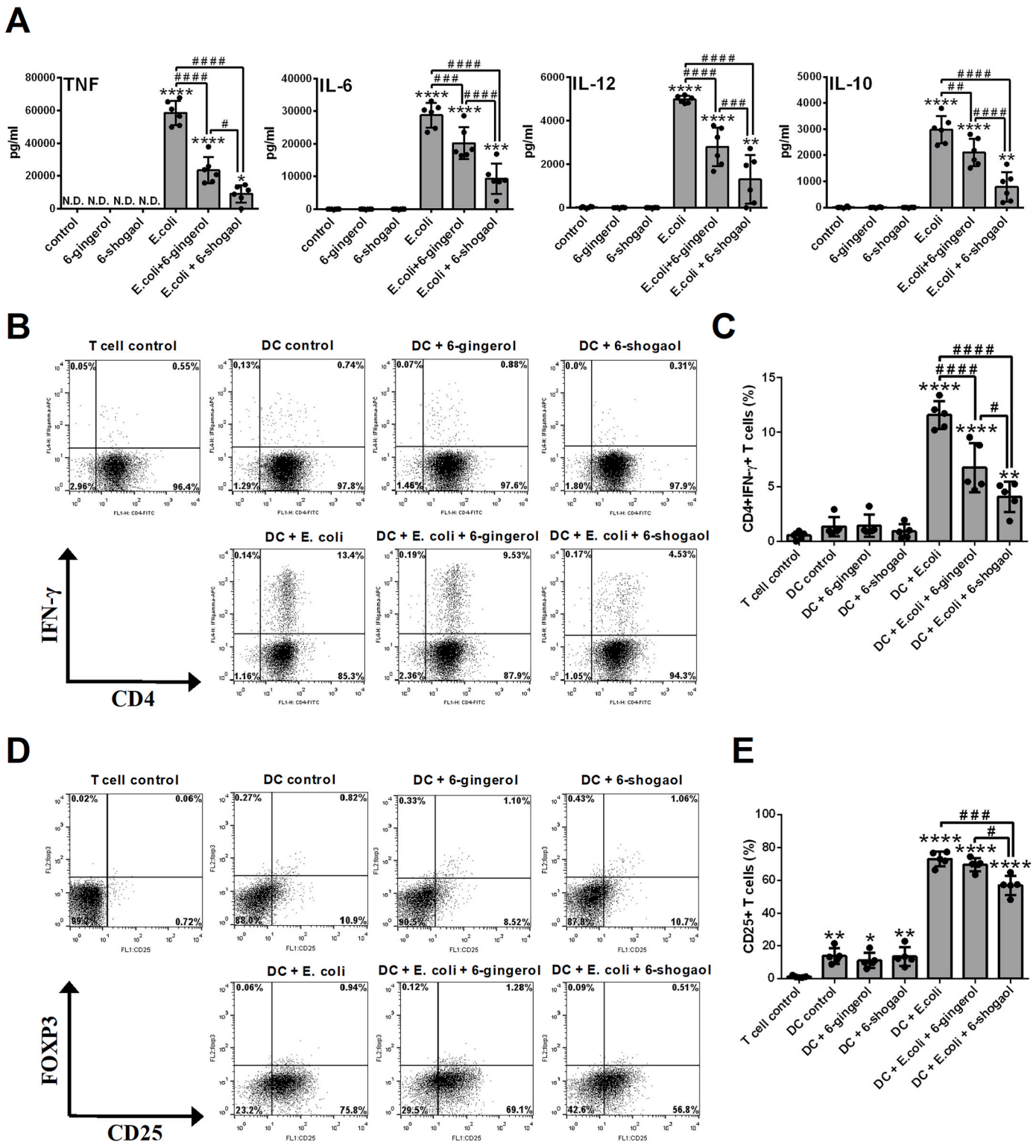

Fig. 4. The bioactive compounds of ginger decrease the E. coli-triggered cytokine production and T cell stimulatory capacity of moDCs. 5-Day moDCs were pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol then exposed to *E. coli* for 24 h. (A) TNF, IL-6, IL-12 and IL-10 levels in supernatants were measured by ELISA. Bar graphs represent the mean ± SD of 5–6 independent experiments. (B–E) The moDCs pre-treated with the indicated reagents were co-cultured with allogeneic naïve $CD4^+$ T cells. After 6 days of co-culture, T cells were stimulated with phorbol myristate acetate (0.1 μg/ml) and ionomycin (1 μg/ml) in the presence of monensin for 5 h. The percentage of IFN-y-producing (B, C) and CD25 and FOXP3 expressing $CD4^+$ T cells (D, E) was measured by flow cytometry. (B, D) Representative dot plots are shown. Numbers indicate the percentage of cells in each quadrant. (C) Bar graphs represent the mean ± SD of 5–6 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$, ****$p < 0.0001$ vs. control (A) or T cell control (C); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$. N.D.: Not determined.

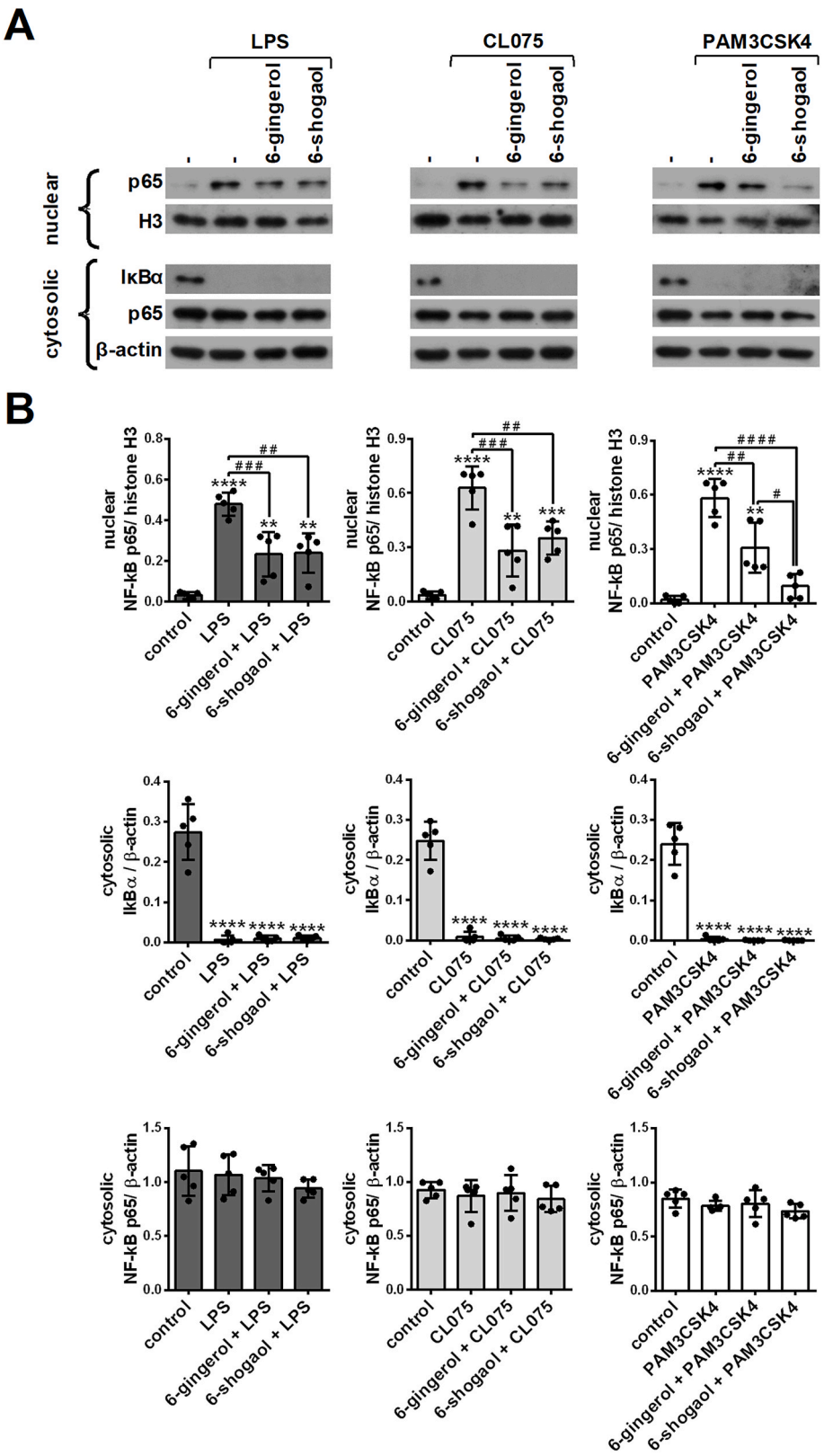

Fig. 5. Treatment with 6-gingerol or 6-shogaol inhibit the TLR-mediated nuclear translocation of NF-kB p65 in moDCs. Immature moDCs were pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol for 2 h and then stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4. After 30 min of stimulation cells were lysed and then fractionated into cytosolic and nuclear fractions. Expression levels of IκBα, NF-κB p65 and β-actin in the cytosolic fraction, and NF-κB p65 and histone H3 in the nuclear fraction were determined by Western blot. (A) Representative blots are shown. (B) Bar graphs represent the mean ± SD of 5 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.01$, $^{****}p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

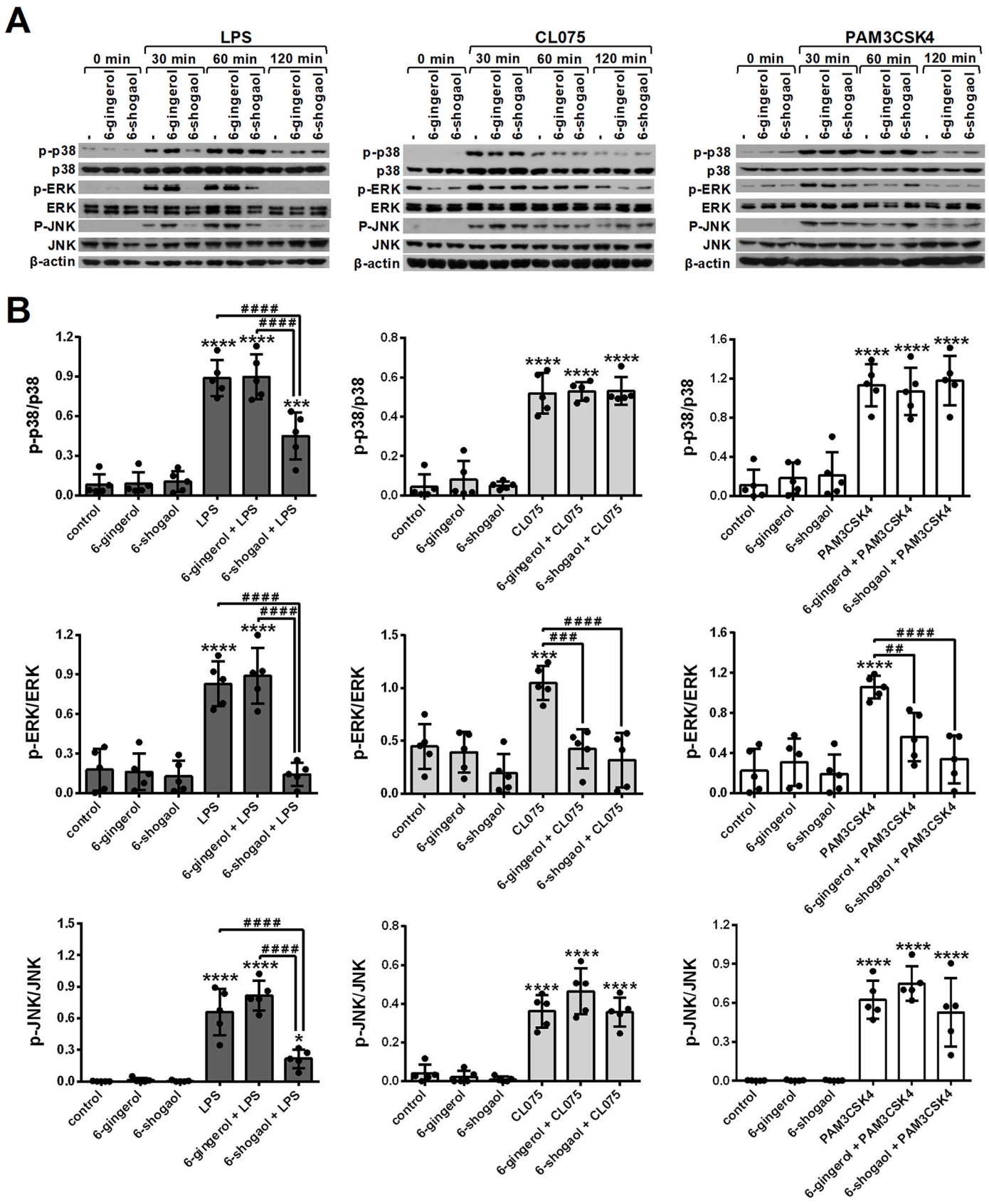

Fig. 6. The bioactive compounds of ginger modulate the TLR-induced activation of the MAPK pathway in moDCs. Immature moDCs were pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol for 2 h then stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4 for different time periods. Kinetics of p38, ERK and JNK were determined by western blotting. (A) Representative blots are shown. (B) Band intensities of samples collected at 0 and 30 min were analyzed by densitometry. Bar graphs represent the mean ± SD of 5 independent experiments. *$p < 0.05$, ***$p < 0.01$, ****$p < 0.0001$ vs. control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

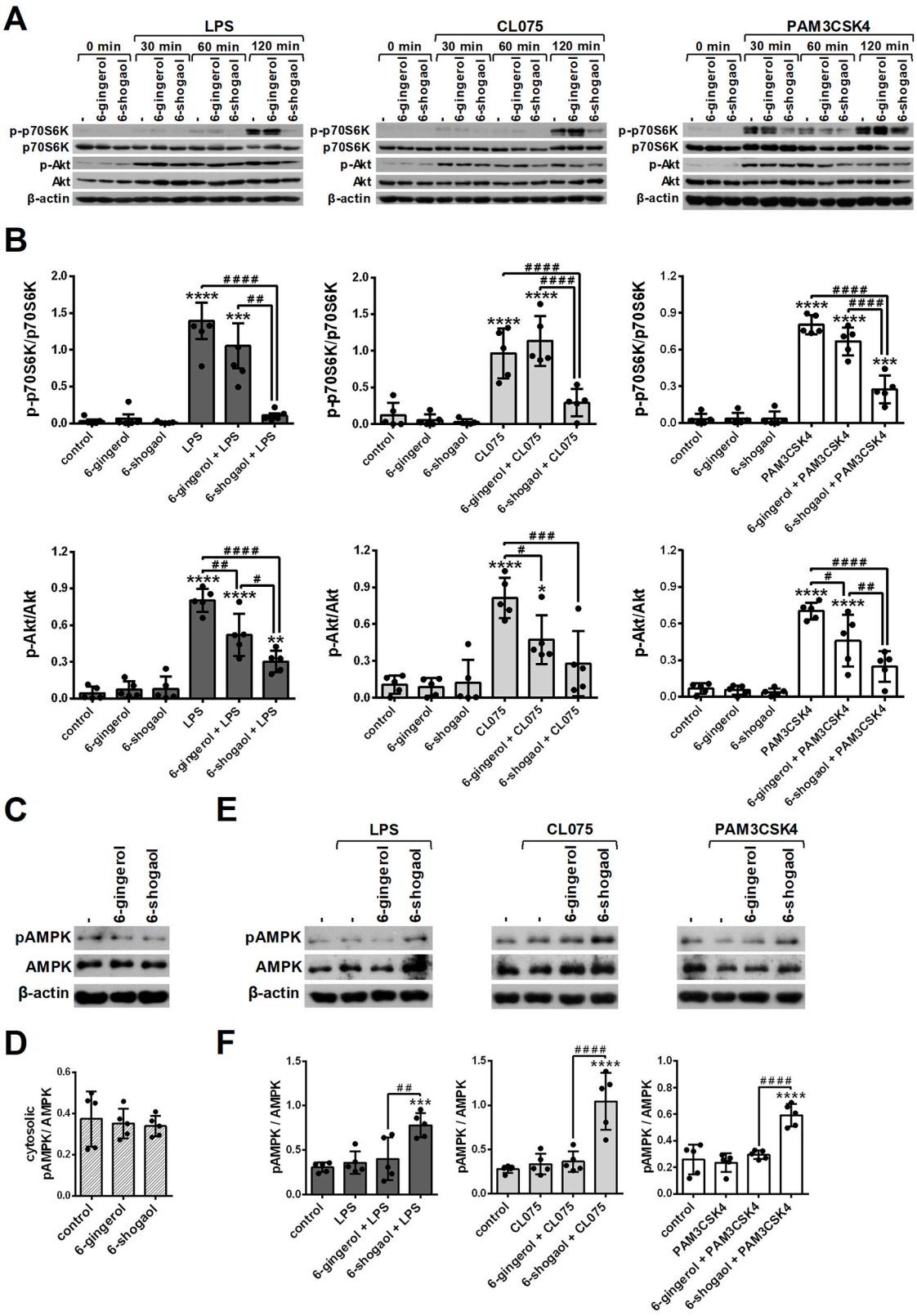

Fig. 7. 6-shogaol affects both the mTOR and AMPK signaling pathways, while 6-gingerol influences only mTORC2 activity in TLR-stimulated moDCs. Immature moDCs were pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol for 2 h then stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4 in a time-dependent manner. (A, B) Kinetics of p70S6K and Akt phosphorylation were determined by western blotting. (A) Representative blots are shown. (B) Band intensities of samples collected at 0 and 60 or 120 min were analyzed by densitometry. (C, D) Phosphorylation of AMPK was detected in the cytosol of immature moDCs pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol for 2 h. (E, F) In separate experiments, cells were further stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4 for 30 min then the protein levels were determined by western blotting. Representative blots (C, E) and densitometric bar graphs are shown (D, F). (B, D, F) Bar graphs represent the mean ± SD of 5 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$, ****$p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

activated immune cells including DCs HIF1-α is one of the main drivers of enhanced glycolysis, which promotes the transcription of all glycolytic enzymes, including HK2, LDHA (Taylor and Scholz, 2022). To this end, first we examined the mRNA expression of key glycolysis-related enzymes including *HIF1A*, *LDHA* and *HK2* in a time-dependent manner (Supplementary Figs. 4A–B). The time response curve shows that only LPS could substantially induce the upregulation of *HIF1A*, *LDHA* and *HK2*. Interestingly, CL075 only slightly increased the levels of *HIF1A* and *HK2*, whereas PAM3CSK4 did not seem to exert any effect on the expression of the glycolysis-related genes (Supplementary Fig. 4A). Since the LPS-triggered expression of *HIF1A* and *LDHA* reached a peak at 12 h of activation, and *HK2* peaked at 6 h, we investigated the effect of 6-gingerol and 6-shogaol pre-treatment at these time points (Supplementary Fig. 4B). Our results demonstrate that 6-gingerol significantly decreased the LPS-triggered expression of *HK2* but did not affect that of *HIF1A* and *LDHA*. On the contrary, 6-shogaol significantly reduced the LPS-mediated expression of all three genes. In addition, 6-shogaol could decrease the CL075-induced expression of *HIF1A* and *LDHA* as well (Supplementary Fig. 4B). These data indicate that at some extent the bioactive compounds of ginger could also interfere with the TLR-mediated glycolytic changes in moDCs.

3.5. 6-Shogaol, but not 6-gingerol induces the upregulation of the NRF2/HO-1 system

In vitro and *in vivo* studies indicated that several phytochemicals exert their beneficial effects via activating NRF2, a transcription factor that is able to induce various anti-inflammatory mechanisms. NRF2 can suppress inflammation through transcriptional induction of anti-inflammatory genes such as HO-1 (Funes et al., 2020), and it is also able to directly inhibit NF-κB activity by preventing IκBα proteasomal degradation or blocking NF-κB nuclear translocation (Saha et al., 2020). Therefore, we sought to reveal whether 6-gingerol or 6-shogaol exert their anti-inflammatory effects through activation of the NRF2 signaling pathway in human moDCs (Fig. 8A–D).

Under homeostatic conditions, Kelch-like ECH-associated protein 1 (Keap1) constitutively ubiquitinates Nrf2 and thus subjects it to proteasomal degradation (Saha et al., 2020). In line with that, we found that NRF2 is barely detectable in resting moDCs (Fig. 8A–C). Nevertheless, a 2-h treatment with 6-shogaol but not 6-gingerol increased both the cytosolic and nuclear levels of NRF2 indicating that 6-shogaol induced both the stability and the nuclear translocation of NRF2. 6-Shogaol triggered NRF2 expression and nuclear translocation was also increased when moDCs were subsequently stimulated with various TLR ligands (Fig. 8B–D).

As a readout of NRF2 activation, we also measured the protein levels of HO-1. Therefore, immature moDCs were pre-treated with 6-gingerol and 6-shogaol, and then were activated with different TLR stimuli. After 24 h of activation HO-1 expression was assessed by western blotting (Fig. 8E and F). Consistent with previous reports (Campbell et al., 2021) immature moDCs constitutively express HO-1, which could be downregulated by TLR stimulation. More importantly, we found that 6-gingerol did not influence the expression of HO-1, whereas 6-shogaol could greatly enhance it both alone and in the presence of TLR agonists (Fig. 8E and F).

Altogether, our data demonstrate that 6-shogaol but not 6-gingerol is able to target the NRF2 signaling pathway that might substantially contribute to its anti-inflammatory actions in moDCs.

4. Discussion

Emerging evidence indicate that various phytochemicals including the main bioactive compounds of ginger are able to influence the effector functions of different immune cell types. Studies using mouse models of systemic lupus erythematosus (SLE) and multiple sclerosis (MS) demonstrated that 6-gingerol, the major pungent constituent of ginger exert potent anti-inflammatory effects. In particular, 6-gingerol inhibited the infiltration of inflammatory cells to the central nervous system and thus ameliorated neuroinflammation in experimental autoimmune encephalomyelitis mice (Han et al., 2019). Moreover, 6-gingerol was able to reduce netosis in different animal models of SLE (Ali et al., 2021). In a mouse model of intracerebral hemorrhage, 6-shogaol but not 6-gingerol exerted neuroprotective effects possibly through inhibiting microglia activity (Ohnishi et al., 2019). In ischemic stroke 6-shogaol also exerted neuroprotective activity by attenuating inflammation (Na et al., 2016). Since little is known about the effect of ginger-derived compounds on primary human immune cells and to the best of our knowledge there are no available data on the impact of these compounds on human DCs yet, especially that of 6-shogaol, we aimed to study how the main bioactive compounds of ginger are able to influence the functionality of human DCs.

Our initial analysis revealed that 6-gingerol and 6-shogaol dose-dependently modulated the phenotypical and functional properties of moDCs. In particular, both compounds could decrease the expression of various cell surface activation markers and the production of various cytokines in response to TLR stimuli. In line with our data, a study demonstrated that 6-gingerol could significantly decrease the production of IL-1β, IL-6, TNF and IL-23 in LPS-stimulated mouse bone marrow-derived DCs (BM-DCs) (Han et al., 2019). Another study also demonstrated that 6-gingerol greatly reduced the production of TNF, IL-12 and CCL5 by LPS-challenged mouse peritoneal macrophages (Tripathi et al., 2007). Consistent with our data, 6-shogaol also significantly decreased the LPS-triggered upregulation of IL-6 and IL-8 in human epithelial cells (Bischoff-Kont et al., 2022). Further, we found that 6-gingerol and 6-shogaol also decreased the TLR-triggered expression of the CD83, MHC-II and different co-stimulatory molecules in DCs. Consistent with these results, in a previous study 6-gingerol significantly inhibited the expression of CD80, CD86 and MHC-II molecules in LPS-treated mouse BM-DCs (Han et al., 2019). We also investigated the surface expression of different inhibitory molecules, the upregulation of which might render DCs tolerogenic (Manavalan et al., 2003; Bakdash et al., 2013). Similar to the activation molecules, both ginger-derived compounds reduced the TLR-mediated upregulation of PD-L1 and PD-L2. Further, we found that the TLR-mediated upregulation of ILT3 and ILT4 was also reduced by 6-gingerol or 6-shogaol pre-treatment. These results suggest that the bioactive compounds of ginger dampen the immunogenicity of DCs without increasing their tolerogenic potential.

Next we studied the impact of 6-gingerol and 6-shogaol on the T cell-stimulatory capacity of moDCs. As expected, 6-gingerol and 6-shogaol significantly reduced the *E. coli* elicited cytokine response by moDCs. Consequently, the T cell activating and Th1-polarizing ability of moDCs was also decreased. By contrast, mouse BM-DCs pre-treated with 6-gingerol retained their ability to mount a Th1 response and could not prime Th17 cell differentiation (Han et al., 2019). We also investigated the Th17 priming ability of *E. coli*-triggered moDCs, but were unable to detect IL-17 that might be explained by the fact that moDCs favor Th1 response through IL-12 production and support Th17 immunity to a lesser extent (Backer et al., 2023). 6-Gingerol or 6-shogaol pre-treated moDCs were also unable to induce Treg differentiation that might be attributed to their reduced expression of surface inhibitory molecules and decreased capacity to produce IL-10. Our findings also suggest that, compared to 6-gingerol, 6-shogaol exerts a stronger inhibitory capacity on the cytokine production and T cell stimulatory ability of DCs.

Investigating the TLR-mediated signaling cascades we found that both 6-gingerol and 6-shogaol suppressed NF-κB signaling by blocking the nuclear translocation of NF-κB p65. In line with that, Han et al. reported that 6-gingerol prevented NF-κB p65 phosphorylation evoked by LPS stimulation of BM-DCs (Han et al., 2019). 6-Shogaol also reduced the LPS induced nuclear translocation of NF-κB p65 in RAW 264.7 macrophages (Pan et al., 2008). Moreover, 6-shogaol decreased the phosphorylation and degradation of IkB in primary microglia cells in response to LPS (Ha et al., 2012). In contrast to the results obtained in

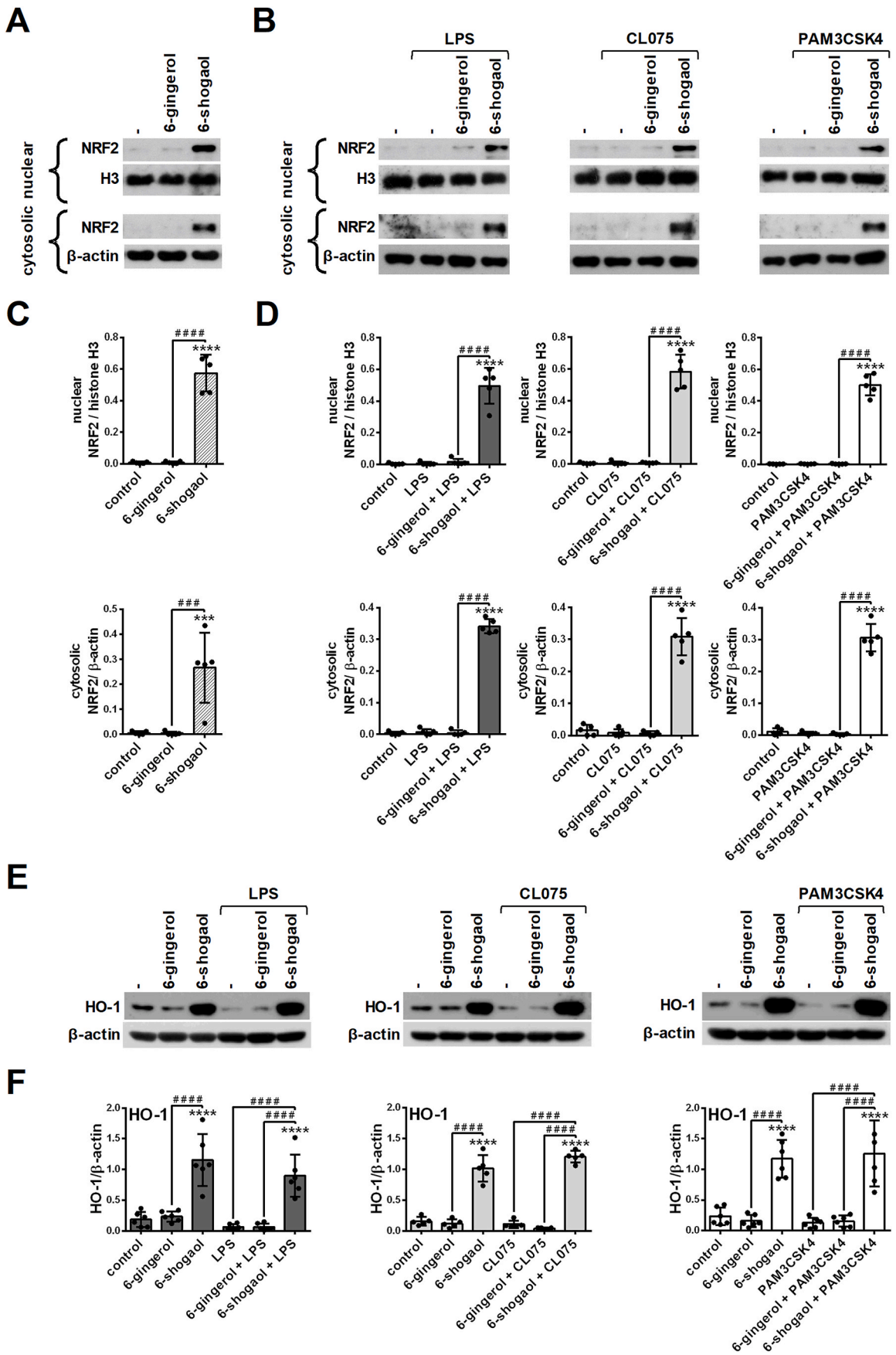

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Fig. 8. 6-shogaol, but not 6-gingerol induces the upregulation of the NRF2/HO-1 system. (A, C) Immature moDCs were pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol for 2 h then cells were lysed and fractionated into cytosolic and nuclear fractions. Expression levels of NRF2 and histone H3 in the nuclear fraction and NRF2 and β-actin in the cytosolic fraction were determined by Western blot. (B, D) In separate experiments, cells were also stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4 for 30 min and then the levels of NRF2, histone H3 and β-actin of fractionated samples were measured as described above. (E, F) Immature moDCs were pre-treated with vehicle control, 50 μM 6-gingerol or 6-shogaol for 2 h then stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4 for 24 h. HO-1 and β-actin protein levels of whole cell lysates were measured by western blotting. (A, B, E) Representative blots are shown. (C, D, F) Bar graphs represent the mean ± SD of 5–6 independent experiments. ***$p < 0.01$, ****$p < 0.0001$ vs. control; $^{###}p < 0.001$, $^{####}p < 0.0001$.

LPS-stimulated mouse macrophages and microglia, we found that the TLR-mediated degradation of IκBα is not affected either by 6-gingerol or 6-shogaol pre-treatment of human moDCs. These data imply that the mechanism of action of ginger phenolics might vary between different cell types and organisms.

By studying the effects of 6-gingerol and 6-shogaol on the MAPK signaling pathway we observed TLR ligand-dependent differences. While 6-gingerol did not affect the LPS-induced MAPK activity, it could effectively inhibit ERK phosphorylation upon CL075 and PAM3CSK4 challenge. In line with our results, 6-gingerol significantly impaired the phosphorylation of ERK, while not affected p38 and JNK activity in LPS- and ATP-primed bone marrow-derived macrophages (Zhang et al., 2020). In mouse BM-DCs, 6-gingerol suppressed the LPS-triggered phosphorylation of ERK1/2 and JNK, while not affected p38 phosphorylation (Han et al., 2019). We also found that 6-shogaol could significantly reduce the LPS-induced phosphorylation of p38 and JNK but had no effect on their activity upon stimulation with CL075 and PAM3CSK4. Furthermore, 6-shogaol was also able to suppress ERK phosphorylation in response to all three TLR agonists. Here we must note that ERK activation is essential to the induction of IL-10 production by TLR-activated DCs (Saraiva and O'Garra, 2010). Thus, our data also indicate that 6-gingerol and 6-shogaol might suppress IL-10 secretion by downregulating ERK activity. Similar to our data, in RAW 264.7 macrophages 6-shogaol attenuated LPS-stimulated ERK phosphorylation, but did not affect p38 activation (Pan et al., 2008). In primary microglia 6-shogaol reduced the phosphorylation of p38 and JNK in response to LPS, but had no effect on ERK activity (Ha et al., 2012). In primary human endothelial cells 6-shogaol markedly reduced LPS-induced JNK phosphorylation, while did not alter p38 activation (Bischoff-Kont et al., 2022). All these data imply that both compounds of ginger are able to modulate the activity of different MAPKs; however, their exact effects on the MAPK signaling pathway vary depending on the cell type and stimuli applied.

In addition to activating the NF-κB and MAPK signaling pathways, many studies including ours have demonstrated that TLR ligands induce the PI3K/Akt/mTOR signaling pathway in DCs as well (Fekete et al., 2014, 2020; Weichhart et al., 2015). Emerging evidence highlights the importance of mTOR activity in DC development and function, including their TLR-driven responses. A study demonstrated that 6-gingerol attenuated microglia-mediated neuroinflammation and ischemic brain injury by suppressing the mTOR signaling pathway (Liu et al., 2020). Another study found that 6-shogaol was also able to attenuate the PI3K/Akt signaling in LPS-activated RAW264.7 macrophages (Pan et al., 2008). A few studies have also suggested that 6-gingerol and 6-shogaol might exert their anti-cancer effects by suppressing the Akt/mTOR pathway (Pei et al., 2021; Zhang et al., 2021). Nevertheless, to date no study has investigated the effect of these compounds on the activity of mTOR in DCs. Here we demonstrated that 6-shogaol greatly suppressed the TLR-mediated activation of both mTOR complexes, while 6-gingerol only decreased the activation of mTORC2. Altogether our data imply that the bioactive compounds of ginger can influence both the TLR-mediated activation of mTOR and TLR-triggered metabolic reprogramming of moDCs. Next, we investigated the effects of ginger phenolics on the activity of AMPK, a known mTORC1 inhibitor, in DCs. Interestingly, 6-gingerol did not affect AMPK activity, while 6-shogaol significantly increased AMPK phosphorylation in TLR-triggered moDCs. These observations explain why only 6-shogaol was able to inhibit mTORC1 activity in TLR stimulated DCs. In line with that, 6-shogaol and 6-paradol were also shown to stimulate glucose utilization in mouse adipocytes via increasing the activity of AMPK (Wei et al., 2017).

Recent studies suggest that the bioactive compounds of ginger might exert their anti-oxidant and anti-inflammatory effects through activating the NRF2 signaling pathway. It was reported that 6-gingerol effectively limits sepsis-induced liver injury in mice (Hong et al., 2020), while 6-shogaol represses UVB-induced inflammation in human epidermal keratinocytes (Chen et al., 2019) via activating the Nrf2/HO-1 axis. We found that 6-shogaol effectively increased the expression and nuclear translocation of NRF2 and enhanced the protein levels of HO-1, while 6-gingerol did not affect that in human moDCs. Our results are in line with a previous study showing that only 6-shogaol was able to increase the nuclear transport of NRF2 and upregulate the protein levels of HO-1 in rat microglia, while 6-gingerol was not able to do so (Ohnishi et al., 2019). Molecular modeling also revealed that 6-shogaol was able to increase NRF2 activity through a Michael reaction between its α, β-unsaturated carbonyl group and Keap1 that allowed the release of NRF2 from Keap1 and resulted in its nuclear translocation. It was further suggested that the α, β-unsaturated carbonyl group might be necessary for the interaction of bioactive compounds with Keap1 that would explain why 6-gingerol, which does not contain such a group in its side chain, was unable to activate the NRF2/HO-1 system in moDCs. Besides, it was proposed that the loss of hydroxyl group in the side chain of shogaols makes them more lipophilic that potentially increases their bioavailability and thus their efficacy compared to gingerols (Bhattarai et al., 2001). Here, it is worth noting that despite the *in vitro* immunomodulatory potential of ginger compounds, animal experiments are sparse and clinical studies are lacking due to the poor water solubility and adsorption of ginger phenolics. The current limitation of *in vitro* experiments is the use of relatively high doses of ginger compounds (Pan et al., 2008; Bernard et al., 2015; Han et al., 2019; Ali et al., 2021) that might be difficult to reach under *in vivo* conditions. To overcome these problems, novel technological approaches such as nanodrug delivery systems are under development to improve the specific delivery and bioaccessibility of ginger phytochemicals (Zhang et al., 2018; Yucel et al., 2022).

Consistent with that and other previous reports (Pan et al., 2008; Dugasani et al., 2010), we have seen at many instances that 6-shogaol exerted a more potent anti-inflammatory effect on moDCs compared to 6-gingerol. Altogether, our data suggest that ginger phytochemicals exert their anti-inflammatory effects via targeting multiple regulatory molecules and signaling pathways in DCs (Fig. 9).

5. Conclusion

Here we reported for the first time that the ginger-derived compounds, 6-gingerol and 6-shogaol, are powerful modulators of human DC functions. Our data demonstrated that 6-gingerol and 6-shogaol exert anti-inflammatory properties on human DCs by inhibiting their maturation, cytokine production and T cell stimulatory ability. We also provide evidence that both ginger-derived compounds modulate DC functionality via interfering with the NF-kB, MAPK and mTOR signaling pathways. Our findings further imply that, compared to 6-gingerol, 6-shogaol has a stronger anti-inflammatory potential that might be attributed to its ability to induce different signaling pathways including the AMPK and NRF2 cascades. Several natural compounds including berberine (Mo et al., 2014), resveratrol (Xu et al., 2022), and curcumin

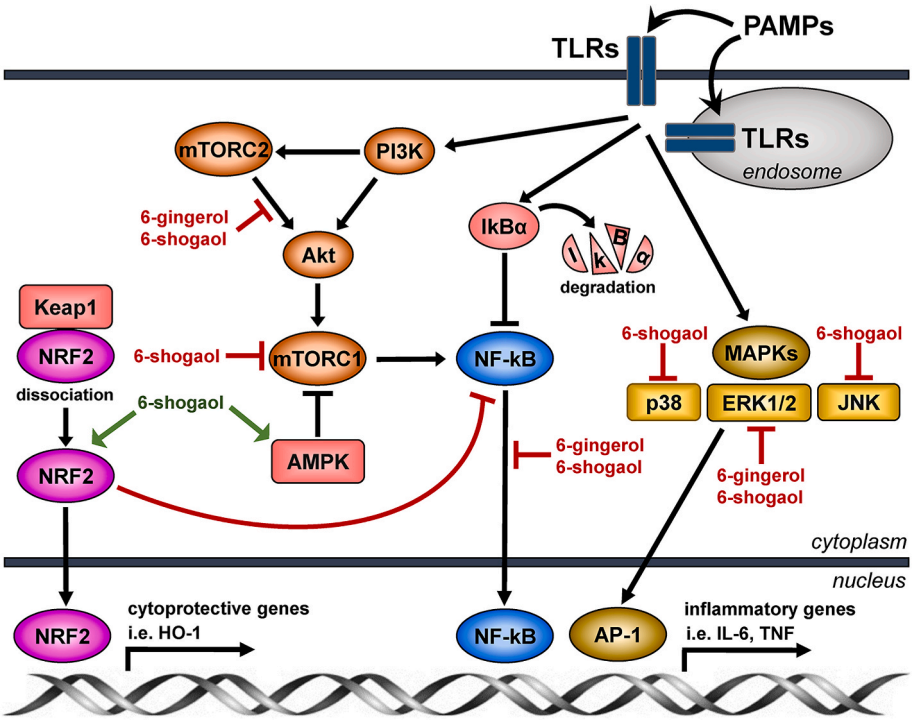

Fig. 9. Schematic illustration of the proposed mechanism of action underlying the anti-inflammatory activites of 6-gingerol and 6-shogaol in DCs. TLR stimulation triggers intracellular signaling cascades, including the NF-κB, MAPK and mTOR pathways, which ultimately lead to the expression of inflammatory genes. Ginger phenolics exert their anti-inflammatory effects by inhibiting the TLR-mediated nuclear translocation of NF-kB, the phosphorylation of different MAPKs and the activity of mTORC2. 6-Shogaol is also able to inhibit the TLR-triggered activation of mTORC1 probably through inducing AMPK activity. In addition, 6-shogaol increases the expression and nuclear translocation of NRF2, which might suppress inflammation by inhibiting NF-kB nuclear translocation and by inducing the expression of cytoprotective genes. The inhibitory actions of ginger compounds are indicated by red lines, whereas the activating effects are marked by green lines. AP-1: activator protein 1.

(Hu et al., 2023) were also found to exert their beneficial effects via activation of both the AMPK and NRF2 regulatory pathways. Altogether, our data suggest that ginger phytochemicals exert their anti-inflammatory effects via targeting multiple regulatory molecules and signaling pathways in DCs. Moreover, our results support the hypothesis that in the future ginger supplements might provide an alternative or more possibly a complementary therapy for individuals with autoimmune conditions. It is also plausible that the bioactive constituents of ginger might serve as a new tool to generate DCs with anti-inflammatory properties for DC-based therapies to treat autoimmune diseases. Although, 6-gingerol is the most studied bioactive compound of ginger, our findings indicate that 6-shogaol exhibits a greater anti-inflammatory activity, thus might possibly serve as a more potent therapeutic agent than 6-gingerol.

CRediT authorship contribution statement

Kitti Pázmándi: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Beatrix Ágics:** Validation, Methodology, Investigation. **Attila Gábor Szöllősi:** Writing – review & editing, Funding acquisition. **Attila Bácsi:** Writing – review & editing, Resources. **Tünde Fekete:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2024.176399.

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