Exploring the modulatory effects of phytochemicals on human dendritic cell mediated immune responses
PD 135193
Final Report

Introduction:

In this project, we focused on dendritic cells (DCs), the most potent antigen presenting cells, which coordinate both innate and adaptive immune responses.[1] DCs are present in almost every tissue of the body, where they continuously scan their local microenvironment for invading pathogens. Via expressing a wide range of pattern recognition receptors such as Toll-like receptors (TLRs) DCs can rapidly sense the presence of pathogen-, and danger-associated molecular patterns that eventually leads to the activation of DCs. Once activated, DCs upregulate the expression of antigen presenting and costimulatory molecules, enhance the production of various cytokines and migrate to the draining lymph nodes to elicit antigen specific T cell responses. In the absence of activation signals DCs transport self-antigens from various tissues and facilitate the initiation of tolerance. Thus, DCs act as a double-edged sword: on the one hand, immunogenic DCs trigger active host defense, while, on the other hand tolerogenic DCs promote tolerance in the steady state. Nevertheless, accumulating evidence indicates that DCs are also implicated in the pathogenesis and pathomechanism of various autoimmune disorders and thus, modulating their functionality might provide a potential therapeutic approach to treat these conditions.[2] The principal aim of the proposed work was to study whether DC activities could be manipulated by plant-derived phytochemicals, specifically by the major bioactive compounds of ginger.

Background and aims:

Ginger has been used for thousands of years for the treatment of different illnesses such as cold, fever and migraine.[3] Lately, a myriad of studies have indicated that ginger also possesses various biological activities such as anti-inflammatory, antioxidant, anti-microbial, anti-cancer and neuroprotective effects. It has also been revealed that the pharmacological benefits of ginger can be mainly credited to gingerols and shogaols, the major bioactive compounds of its rhizome. Among those, 6-gingerol is the most abundant constituent of fresh ginger root, whereas, 6-shogaol is the predominant bioactive principle in the dried rhizome.[4] Several recent data indicate that bioactive compounds of ginger are able to influence the effector functions of different immune cell types such as macrophages, neutrophils and T cells. Although, shogaols

[1] Amodio G, Gregori S. Dendritic cells a double-edge sword in autoimmune responses. Frontiers in immunology. 2012;3:233.

[2] Coutant F, Miossec P. Altered dendritic cell functions in autoimmune diseases: distinct and overlapping profiles. Nat Rev Rheumatol. 2016 Dec;12(12):703-15.

[3] Valnet J. The Practice of Aromatherapy: A Classic Compendium of Plant Medicines and Their Healing Properties: Healing Arts Press 1990.

[4] Unuofin JO, Masuku NP, Paimo OK, Lebelo SL. Ginger from Farmyard to Town: Nutritional and Pharmacological Applications. Front Pharmacol. 2021;12:779352.

seem to exhibit more potent biological activities then gingerols, most of the reports have focused only on gingerols, when studying the immunomodulatory potential of ginger compounds. It is also important to note that the effects and mechanisms of action of these phytochemicals in human DCs have not been explored yet.

Therefore, in the present study we aimed to investigate the effects of both 6-gingerol and 6-shogaol on the TLR-mediated functions of human DCs as well as to study the molecular mechanisms underlying their actions. Due to the low availability of DCs in the peripheral blood we performed our experiments on human monocytes-derived DCs (moDCs), which can be found in different tissues both under physiological and inflammatory conditions.[5] Besides, moDCs serve as a potent source for DC-based immunotherapies, thus represent a suitable model to study human DC biology.[6]

Results:

We showed for the first time that 6-gingerol and 6-shogaol are able to attenuate the immunogenicity of human DCs through various mechanisms. First, we treated moDCs with increasing doses of 6-gingerol and 6-shogaol to find an effective dose that could modulate the phenotypical and functional properties of DCs without affecting their viability. Our results show that 6-gingerol and 6-shogaol applied in the concentration range of 10-50 μM did not affect the viability of either resting or TLR-stimulated moDCs. In parallel experiments, our dose-response analysis also revealed that 6-gingerol and 6-shogaol dose-dependently modulated the phenotypical and functional properties of moDCs. In particular, both compounds decreased the TLR-mediated expression of various cell surface markers including the activation marker CD83, the costimulatory molecules CD80 and CD86 and the MHC class II molecule HLA-DQ in a dose dependent manner. Similarly, both components reduced the production of the pro-inflammatory cytokines TNF and IL-6, and the anti-inflammatory cytokine IL-10 in response to various TLR stimuli. 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.

Since the expression levels of MHC and co-stimulatory molecules have a fundamental effect on the ability of DCs to activate T cells, we also investigated the impact of 6-gingerol and 6-shogaol on the T cell-stimulatory capacity of moDCs. In these experiments, moDCs were exposed to live Escherichia coli (E. coli), to better represent an in vivo situation. E.coli elicited a strong cytokine response by moDCs that was greatly decreased upon pre-treatment with 6-gingerol and 6-shogaol. The bioactive compounds of ginger also significantly reduced IL-12 production and, consequently, the T helper 1 (Th1)-polarizing ability of moDCs as it was show

[5] Backer RA, Probst HC, Clausen BE. Classical DC2 subsets and monocyte-derived DC: Delineating the developmental and functional relationship. Eur J Immunol. 2023 Mar;53(3):e2149548.

[6] Hopewell EL, Cox C. Manufacturing Dendritic Cells for Immunotherapy: Monocyte Enrichment. Mol Ther Methods Clin Dev. 2020 Mar 13;16:155-60.

by their decreased capacity to induce IFN-γ secreting CD4+ T cells. Nevertheless, neither of the treatment combinations were able to induce the generation of IL-17 producing CD4+ Th17 cells.

Our mechanistic studies further revealed that the ginger-derived bioactive compounds interfere with the TLR-mediated activation of different signaling pathways including the nuclear factor-kappa B (NF-κB), mitogen activated protein kinases (MAPK) and mammalian target of rapamycin (mTOR) cascades. While the TLR-mediated degradation of inhibitor of NF-kB (IkBα) was not affected by either 6-gingerol or 6-shogaol pre-treatment, both compounds significantly suppressed the translocation of NF-κB p65 subunit to the nucleus. Although, 6-gingerol and 6-shogaol mostly inhibited the activity of the MAPK signaling pathway, we observed TLR ligand-dependent differences. Further, we found that 6-gingerol decreased the mTOR complex 2 (mTORC2)-mediated phosphorylation of Akt, whereas had no effect on the mTORC1-mediated phosphorylation of p70S6K in TLR-stimulated moDCs. On the contrary, 6-shogaol could greatly suppress the TLR-mediated activation of both mTOR complexes. Furthermore, we also investigated the expression of key glycolysis-related genes. Although some differences could be observed between the effects of 6-gingerol and 6-shogaol, collectively our data imply that the investigated ginger phenolics interfere with both the TLR-triggered mTOR activity and metabolic reprogramming of moDCs.

Previous studies have indicated that several plant-derived components are able to induce heme oxygenase-1 (HO-1), which acts as an immunomodulator in DCs through inhibiting their inflammatory functions and maintaining them in a tolerogenic state.[7] Therefore, we sought to reveal whether 6-gingerol or 6-shogaol exert their anti-inflammatory effects through the upregulation and activation of HO-1 in human moDCs. Interestingly, 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. Nevertheless, our results demonstrate that the siRNA-mediated depletion of HO-1 did not restore the cytokine production of moDCs treated with 6-shogaol.

Since our results show that ginger phenolics do not regulate DC functionality through HO-1 upregulation we decided to further study the mechanism of action of ginger compounds instead of performing ex vivo experiments in the last year of the project. Most importantly, our new data demonstrate that 6-shogaol upregulates the activity of AMP-activated protein kinase (AMPK), a known inhibitor of mTORC1, whereas 6-gingerol does not affect that. Moreover, we found that 6-shogaol is also able to enhance both the cytosolic and nuclear expression of nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that plays a pivotal role in the regulation of anti-oxidant and anti-inflammatory responses.[8] 6-shogaol also greatly

[7] Chauveau C, Remy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert FX, et al. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. Blood. 2005 Sep 1;106(5):1694-702.

[8] Saha S, Buttari B, Panieri E, Profumo E, Saso L. An Overview of Nrf2 Signaling Pathway and Its Role in Inflammation. Molecules. 2020 Nov 23;25(22).

enhanced the nuclear translocation of NRF2, which has plenty of known target genes including HO-1. Consequently, the previously reported increased HO-1 levels are the consequence of enhanced NRF2 activity upon 6-shogaol treatment. In addition, we also investigated whether 6-gingerol and 6-shogaol could promote the tolerogenic potential of DCs. Our new data show that neither of the components were able to upregulate the expression of tolerogenic markers such as programmed death-ligand 1 (PD-L1), PD-L2, immunoglobulin-like transcript (ILT) 3, ILT4 and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) on the surface of moDCs. Besides, neither 6-gingerol nor 6-shogaol could induce IL-10 producing foxp3+ regulatory T cell polarization by moDCs. These data imply that the bioactive compounds of ginger decrease the immunogenic potential of DCs without increasing their tolerogenic capacity.

Conclusion:

Our data demonstrate for the first time that the ginger-derived compounds, 6-gingerol and 6-shogaol, are powerful modulators of human DC functionality. We found 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 provide evidence that the ginger-derived compounds modulate DC functionality via interfering with the NF-kB, MAPK and mTOR signaling pathways. Our results also indicate that 6-shogaol exerts a more potent anti-inflammatory capacity on moDCs compared to 6-gingerol. Finally, our data suggest that 6-shogaol attenuates human DC functionality via activating AMPK and the NRF2/HO-1 system.

Presentation of results:

The result were introduced in the form of 4 oral presentations and 1 poster presentation at scientific conferences. The abstracts of presentations were published in conference booklets or on the website of the conferences. Furthermore, 2 diploma theses were prepared by students working on this project. During the Covid-19 pandemic, I was also involved in the writing of 3 review articles on the regulatory mechanisms of DCs, the topic of which is not closely related to the recent research project; nevertheless, the grant number was indicated in the funding section. 2 manuscripts were prepared based on the results of the recent project. One manuscript was submitted to a high-ranking journal and is under revision now. In addition, a review was also prepared that is going to be submitted once the research manuscript is accepted.

Oral and poster presentations:

1. Beatrix Ágics, Tünde Fekete, Kitti Pázmándi: Modulation of human dendritic cell mediated inflammatory processes by phytochemicals, 14th Molecular, Cell and Immune Biology Winter Symposium (Online Conference) Debrecen, 7-8 January 2021
2. Beatrix Ágics, Tünde Fekete, Kitti Pázmándi: Modulation of human dendritic cell mediated inflammatory processes by phytochemicals, XXIV. Spring Wind Conference, 28-30 May 2021

3. Beatrix Ágics, Kitti Pázmándi, Tünde Fekete: Effects of phytochemicals on human dendritic cell functions, PhD Scientific Days 2021 Budapest, 7-8 July 2021 *The abstract was published in the abstract book of the conference (ISBN 978-615-5586-99-6).*
4. Kitti Pázmándi, Dóra Bence, Tünde Fekete: Investigating the immunomodulatory effects of phytochemicals on human monocyte-derived dendritic cells. 50th Congress of the Hungarian Society for Immunology, 20-22 October 2021, Kecskemét, Hungary. *The abstract was also published in Immunology Quarterly, Volume 13, Issue 3, page 30, October 2021 (ISSN 2061-0203)*
5. Benyhe-Kis Bernadett: The effects of bioactive compounds of ginger on the human dendritic cell mediated inflammatory responses. Scientific Student Council Conference (Session: Experimental Immunology, Microbiology,) 23-25 February, 2022 Debrecen, Hungary

Thesis works:

1. Bernadett Benyhe-Kis: The effects of bioactive compounds of ginger on the human dendritic cell mediated inflammatory responses. 2022, supervisor: Tünde Fekete
2. Zoltán Hegyi: Investigating the mechanism of action of ginger-derived compounds on the functionality of dendritic cells. 2023, supervisor: Tünde Fekete

Published works:

1. Dóra Bencze, Tünde Fekete, Kitti Pázmándi: Type I Interferon production of plasmacytoid dendritic cells under control, Int J Mol Sci. 22: (8) p. 4190., 2021. doi: 10.3390/ijms22084190.
2. Tünde Fekete, Dóra Bencze, Eduárd Bíró, Szilvia Benkő, Kitti Pázmándi: Focusing on the cell type specific regulatory actions of NLRX1, Int J Mol Sci. 2021 Jan 28;22(3):1316. doi: 10.3390/ijms22031316.
3. Dóra Bencze, Tünde Fekete, Kitti Pázmándi Correlation between Type I Interferon Associated Factors and COVID-19 Severity. Int J Mol Sci. 2022 Sep 19;23(18):10968. doi: 10.3390/ijms231810968.

Unpublished works:

1. Kitti Pázmándi, Beatrix Ágics, Attila Gábor Szöllősi, Attila Bácsi, Tünde Fekete: Ginger-derived bioactive compounds attenuate inflammatory responses of human dendritic cells. *Under revision at Frontiers in Immunology. Manuscript ID: 1244299 (submitted on 22th of June 2023).* Please find the latest submitted version of the manuscript below.
2. Kitti Pázmándi, Attila Bácsi, Tünde Fekete: Effects of ginger constituents on immune cells and their proposed mechanism of action (*prepared for submission at Frontiers in Immunology*). Please find the draft of the manuscript below.

Ginger-derived bioactive compounds attenuate inflammatory responses of human dendritic cells

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

TF and KP designed the research, performed experiments, analyzed and interpreted data, and wrote the manuscript. BA performed experiments and participated in data analysis. AGS and KP provided conceptual insight and revised the manuscript. KP and AB contributed with essential reagents. All authors reviewed and approved the manuscript.

Keywords

Dendritic cell1, TLR signaling2, Anti-inflammatory3, cytokine4, ginger5, Ginger constituents modulate DC functions

Abstract

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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 and anti-oxidative effects. Although, the precise effect 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 exert anti-inflammatory properties on 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 maturation and 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. Further, we 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 pathways. Particularly, both 6-gingerol and 6-shogaol significantly decreased the TLR-induced nuclear translocation of NF-κB p65 and the phosphorylation of various MAPK pathway compounds. In addition, the bioactive compounds of ginger substantially reduced the TLR-mediated phosphorylation of p70S6 kinase and Akt, the downstream targets of mTOR complex (mTORC) 1 and mTORC2, respectively. In line with that, 6-gingerol and 6-shogaol also decreased the expression of key glycolysis-related genes. Altogether these data suggest that the bioactive constituents of ginger might interfere with the TLR-induced metabolic changes of moDCs as well. Of note, our results also indicate that 6-shogaol exerts a more potent anti-inflammatory capacity on moDCs compared to 6-gingerol. These results expand our knowledge on the mechanism of action of ginger constituents in human immune cells and support their potential application as novel anti-inflammatory agent in the treatment of autoimmune and inflammatory conditions.

Contribution to the field

Dendritic cells (DCs) serve as sentinels of the immune system that can drive immune responses towards either immunity or tolerance depending on the stimuli they receive from their microenvironment. Nevertheless, DCs are also implicated in the pathogenesis and pathomechanism of various autoimmune diseases. Thus, modulating DC functionality might provide a potential therapeutic approach to treat these conditions. Recent data indicate that various phytochemicals including the main bioactive compounds of ginger are able to influence the effector functions of different immune cell types; however, there are no available data on the impact of these compounds on human DCs yet. Here we report for the first time that the major constituents of ginger are powerful modulators of DC functions. Our data demonstrate that 6-gingerol and 6-shogaol exert anti-inflammatory properties on human DCs by inhibiting their maturation, cytokine production and T cell stimulatory capacity upon Toll-like receptor (TLR) stimulation. Further, we provide evidence that the ginger-derived compounds modulate TLR-mediated DC functionality via interfering with the nuclear factor-kB, mitogen-activated protein kinase and mammalian target of rapamycin signaling pathways. Our data imply that the due to their strong immunosuppressive capacity ginger constituents might serve as a new tool to dampen DC-driven inflammatory responses.

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Data availability statement

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Ginger-derived bioactive compounds attenuate inflammatory responses of human dendritic cells

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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 and anti-oxidative effects. Although, the precise effect 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 exert anti-inflammatory properties on 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 maturation and 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. Further, we 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 pathways. Particularly, both 6-gingerol and 6-shogaol significantly decreased the TLR-induced nuclear translocation of NF-κB p65 and the phosphorylation of various MAPK pathway compounds. In addition, the bioactive compounds of ginger substantially reduced the TLR-mediated phosphorylation of p70S6 kinase and Akt, the downstream targets of mTOR complex (mTORC) 1 and mTORC2, respectively. In line with that, 6-gingerol and 6-shogaol also decreased the expression of key glycolysis-related genes. Altogether these data suggest that the bioactive constituents of ginger might interfere with the TLR-induced metabolic changes of moDCs as well. Of note, our results also indicate that 6-shogaol exerts a more potent anti-inflammatory capacity on moDCs compared to 6-gingerol. These results expand our knowledge on the mechanism of action of ginger constituents in human immune cells and support their potential application as novel anti-inflammatory agent in the treatment of autoimmune and inflammatory conditions.

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 (1). By contrast, in the absence of infection, tissue damage, or any other 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 (2). Nevertheless, a growing body of evidence implicates DCs in the pathogenesis and pathomechanism of various autoimmune conditions (3-5). 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.

To accomplish their tasks DCs are equipped with a wide array of Toll-like receptors (TLRs), which are specialized to sense a broad range of pathogen-associated molecular patterns (PAMPs) and self-derived damage-associated molecular patterns (DAMPs) (6). Among them, cell surface TLRs such as TLR2, TLR4 and TLR5 mainly recognize microbial cell wall and membrane components, whereas endosomal TLRs such as TLR7, TLR8 and TLR9 can detect both microbial and self-derived nucleic acids. TLR ligation activates common signal transduction cascades such as the nuclear factor-kappa B (NF-κB), mitogen activated protein kinases (MAPK), or interferon regulatory factor (IRF) pathways leading to the production of various inflammatory cytokines, chemokines, and type I interferons (IFNs) that are key elements of host defense against microbial infection (6). TLR signaling also activates the phosphoinositide 3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) signaling cascade that drives a metabolic switch from oxidative phosphorylation to glycolysis. Glycolytic reprogramming is a hallmark of activated DCs and is critical to meet the energetic demands of TLR-driven DC activation (7).

Although TLR-initiated responses are critical for host defense against pathogens and tissue repair, excessive TLR activation has been linked to the pathogenesis of various inflammatory and autoimmune diseases such as sepsis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and psoriasis (8). In SLE, abnormal stimulation of plasmacytoid DCs (pDCs) by self-nucleic acids through TLR7 and TLR9 leads to aberrant release of type I IFNs, which in turn stimulates autoantibody production by B cells (9). The antimicrobial peptide LL37 is continuously overexpressed in psoriatic skin lesions, where it forms complexes with self-DNA or -RNA and triggers TLR7, TLR8, and TLR9 signaling in DCs, which initiate the expansion of autoimmune T cells (10). Furthermore, TLR-stimulated conventional DCs (cDCs) have been proposed to be central to the initiation and perpetuation of RA by secreting pro-inflammatory cytokines and presenting antigens to autoreactive T cells (11). Therefore, targeting the TLR-mediated functionality of DCs might provide a powerful tool to dampen excessive inflammation, and thus to treat autoimmune and inflammatory disorders (12).

Several plant-derived compounds show promising anti-inflammatory properties (13); 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 compounds such as lipids, terpens, phenolics, and carbohydrates have been identified in ginger. Among those, the phenolic compounds, mainly gingerols and shogaols, account for the pharmacological activities of ginger (14). Gingerols are the main pungent components of fresh ginger that can be differentiated based on their unbranched alkyl side chain length. Among them, 6-gingerol is the most abundant constituent that is followed by 8-gingerol and 10-gingerol (15). Upon drying or heating the thermally labile gingerols undergo dehydration reactions to form the corresponding shogaols, which are twice as pungent as gingerols (16). Although shogaols are scarcely present in fresh ginger root,

6-shogaol is the predominant bioactive compound in the dried rhizome. Gingerols and shogaols are structurally similar; however, the double bound in the side chain of shogaols suggest a higher biological activity.

So far a myriad of reports has proven the pharmacological potential of gingerols and shogaols. Nevertheless, only a few studies compared their anti-oxidant (17) and anti-inflammatory activities (18, 19) 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 (20). In mouse peritoneal macrophages, 6-gingerol abrogated the production of pro-inflammatory cytokines but had no effect on the LPS-induced expression of costimulatory molecules and major histocompatibility (MHC) class II protein (21). Another study showed that 6-gingerol attenuates neutrophil extracellular trap (NET) release in response to LPS and various lupus-relevant stimuli (22). So far, only one study showed that 6-gingerol is able to modulate DC functionality in a mouse model of experimental autoimmune encephalomyelitis (EAE) (23). Much less is known about the role of 6-shogaol in modulating immune cell functions. A study reported that 6-shogaol inhibited the LPS-induced phosphorylation and nuclear translocation of NF-κB as well as the activation of PI3K/Akt and MAPK signaling pathways in RAW 264.7 macrophages (18). Another report presented that 6-shogaol decreased cancer development and progression by inhibiting the cancer-induced up-regulation of CC-chemokine ligand 2 (CCL2) in tumor-associated DCs (24).

Nevertheless, the effects of 6-gingerol and 6-shogaol on the TLR-mediated responses of human DCs have not been elucidated yet and the mechanism of action of these compounds remained largely unknown. Thus, in the present study we aimed to explore the effects of 6-gingerol and 6-shogaol on human DC functionality as well as 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 (clone OKT4, Cat. No. 317408) 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 4S.B3) 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 (Cat. No. 334306, Clone: 5C3), anti-HLA-DQ-PE (Cat. No.318106, Clone:HLADQ1), anti-CD83-PE-Cy5 (Cat. No. 305310, Clone: HB15e), anti-CD86-PE (Cat. No. 305406, Clone IT2.2) 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 Laemmli sample buffer 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).

208 **2.7 Subcellular fractionation**

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

217 **2.8 ELISA**

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

224 **2.9 SiRNA-mediated gene silencing**

225 5-day moDCs were transfected with transfection reagent alone (mock), Silencer Select
226 Negative Control siRNA (scr; Thermo Fisher Scientific, Cat. No. 4390844) or HO-1 specific
227 Silencer Select Validated siRNA (Thermo Fisher Scientific, Assay ID: s194530, Cat. No.4390824)
228 in Opti-MEM medium (Thermo Fisher Scientific, Cat. No. 11058021) in 4-mm cuvettes (Bio-Rad,
229 Cat. No. 1652088) using GenePulser Xcell instrument (Bio-Rad). Following transfection, cells
230 were plated at a density of 10^6 cells/ml in 24-well cell culture plates in RPMI 1640 medium. After
231 24 h of transfection cells were treated as indicated.

232 **2.10 Statistical analysis**

233 Data are expressed as Mean ± SD and statistical significance was analyzed using one-way
234 ANOVA, followed by Bonferroni post hoc test. Data analysis was performed with GraphPad Prism
235 v.6. Software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be
236 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 (cDC2) 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 (25, 26). 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 (27); 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 Figures 1A, B and 2A, B**). Meanwhile, we also analyzed the expression of various plasma membrane-bound proteins including the activation marker CD83, the 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 (**Figure 1A-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. 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 (**Figure 2A-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 (**Figure 3A**). 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 (**Figure 3B-C**). In parallel, we also tried to measure the Th17 polarizing capacity of *E. coli*-triggered moDCs; however, moDCs failed to induce detectable levels of IL-17 in CD4+ T cells (data not shown).

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 T cell 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 (6). 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 minutes. 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 (**Figure 4A-B**). In parallel, we have also measured the expression of the inhibitor of NF-κB (IkBα) in the cytosolic fraction. Interestingly, treatment with either 6-gingerol or 6-shogaol does not influence the TLR-mediated degradation of IkBα (**Figure 4A-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 (**Figure 5A-B**). Our results demonstrate that all three TLR ligands significantly induced the phosphorylation of MAPKs, which generally reached a peak after 30 minutes 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.

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 mTOR complex (mTORC) 1–mTORC2 network in innate immune cells, thereby controlling a wide range of basic cellular processes (28). 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 (29, 30). 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 (**Figure 6A-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 (**Figure 6A**). Pre-treatment with 6-shogaol could significantly decrease the TLR-driven phosphorylation of p70S6K, whereas 6-gingerol did not affect it (**Figure 6A-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 minutes of stimulation (**Figure 6A**). 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 (**Figure 6A-B**). These data suggest that the bioactive constituents of ginger can inhibit the TLR-induced activation of both mTOR complexes.

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 (31). Therefore, we also investigated how TLR-mediated metabolic switch is affected by the bioactive compounds of ginger. In 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 (32). 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 Figures 3A-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 Figures 3A**). 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 Figures 3B**). 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 Figures 3B**). 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 The 6-shogaol-mediated inhibition of cytokine production is independent of HO-1 upregulation in moDCs

Previous studies have indicated that several plant-derived components are able to induce HO-1 (33), which acts as an immunomodulator in DCs through inhibiting their inflammatory functions and maintaining them in a tolerogenic state (34). Therefore, we sought to reveal whether 6-gingerol or 6-shogaol exert their anti-inflammatory effects through the upregulation and activation of HO-1 in human moDCs. To do so, 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 (**Figure 7A-B**). Consistent with previous reports (35) immature moDCs constitutively express HO-1, which could be downregulated by TLR stimulation. Interestingly, 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 (**Figure 7A-B**).

Since 6-shogaol is able to upregulate HO-1 protein levels, next we investigated whether its anti-inflammatory capacity is dependent on HO-1 activity. To this end, we performed siRNA-mediated gene silencing to deplete HO-1 in 5-day moDCs. At 24 h post transfection cells were pre-treated with 6-shogaol, then were stimulated with different TLR agonists. After 24 h of activation

cell culture supernatants were analyzed for TNF, IL-6 and IL-10 release by ELISA (**Figure 7C**). In parallel, the efficacy of gene silencing was evaluated by western blot analysis (**Supplementary Figure 4A-C**). Our data show that the siRNA-mediated gene silencing significantly reduced the level of HO-1 in resting moDCs that was retained in activated moDCs as well. Nevertheless, our results demonstrate that HO-1 depletion did not restore the cytokine production of moDCs treated with 6-shogaol.

Though, 6-shogaol treatment could induce a strong and significant upregulation of HO-1 expression in moDCs, our data suggest that in our *in vitro* experimental setting 6-shogaol exerts its anti-inflammatory effect via a different mechanism.

4. Discussion

Depending on the environmental signals DCs can induce either tolerogenic or immunogenic responses, and thus are central regulators of immunological processes. Under physiological conditions DCs maintain tolerance, whereas in the presence of invading microorganisms they induce pathogen-specific immune responses (2). Emerging evidence also indicates that DCs play a critical role in the initiation and perpetuation of various autoimmune diseases, such as multiple sclerosis (MS), SLE or RA (3, 4). Thus, modulating DC functionality might provide a potential therapeutic approach to treat autoimmune conditions. Several recent data 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 SLE and MS demonstrated that 6-gingerol, the major pungent constituent of ginger exerts potent anti-inflammatory effects. In particular, 6-gingerol inhibited the infiltration of inflammatory cells to the central nervous system and thus ameliorated neuroinflammation in EAE mice (23). Moreover, 6-gingerol was able to reduce NET release in different animal models of SLE (22). Further, in a mouse model of intracerebral hemorrhage, 6-shogaol but not 6-gingerol exerted neuroprotective effects possibly through inhibiting the activity of microglia cells (36).

So far, only one study investigated the *in vitro* effects of 6-gingerol on the functionality of DCs using mouse bone marrow-derived DCs (BM-DCs). Nevertheless, little is known about the effect of ginger-derived compounds on primary human immune cells, and to our knowledge, there are no available data on the impact of these compounds on human DCs yet. Although there is an overlap between mice and humans in terms of DC development and functions, results must be interpreted carefully as the molecular mechanisms regulating DC physiology/biology are not fully conserved across mice and humans (37, 38). Due to these species differences, results obtained from mouse studies are not always predictive of human cell responses, and more importantly animal studies might not accurately translate to human reactions (39). This has already been proven to be the case with other natural compounds (such as capsaicin), where not only were the data generated in murine models not applicable to human cells, but they actually had the opposite effect (40). Thus, we aimed to study how the main bioactive compounds of ginger, 6-gingerol and 6-shogaol, are able to influence the *in vitro* functionality of human DCs in response to various TLR stimuli. To do this we used moDCs, a widely accepted model of human DCs (27). Immunogenic moDCs generated under laboratory conditions might be used to initiate antigen-specific T cell responses to kill cancer cells, whereas tolerogenic moDCs might be applied for the treatment of autoimmune diseases (41). To generate moDCs with tolerogenic properties several synthetic and natural agents have already been tested; however, whether ginger-derived phenolics are able to induce the anti-inflammatory properties of moDCs has not yet been investigated.

First, we treated moDCs with increasing doses of 6-gingerol and 6-shogaol to find an effective dose that could modulate the phenotypical and functional properties of DCs without affecting their viability. Based on *in vitro* experiments using other cell types including mouse macrophages (18), mouse DCs (23), neutrophils (22), and T cells (42) the bioactive compounds of ginger can be applied in the concentration range of 1-100 μM without inducing any cytotoxic effects. In line with literature data, our results show that 6-gingerol and 6-shogaol applied in the concentration range of 10-50 μM did not affect the viability of either resting or TLR-stimulated moDCs.

In parallel experiments, our dose-response analysis also 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 the pro-inflammatory cytokines TNF and IL-6 in response to various TLR stimuli. In line with our data, a study demonstrated that 100 μM of 6-gingerol could significantly decrease the production of IL-1β, IL-6, TNF and IL-23 as well as to inhibit the expression of CD80, CD86 and MHC II in LPS-stimulated mouse BM-DCs (23). Interestingly, another study demonstrated that 6-gingerol significantly reduced the production of TNF, IL-12 and CCL5, while

having no effect on the expression of CD80, CD86 and MHC II of LPS-challenged mouse peritoneal macrophages (21). Nevertheless, in that particular study the authors applied lower concentrations of 6-gingerol (1 μg/ml) compared to ours and other studies. Consistent with our data, 30 μM of 6-shogaol significantly decreased the LPS-triggered upregulation of IL-6 and IL-8 in human epithelial cells (43). Moreover, 20 μM of 6-shogaol greatly reduced the NLRP3 inflammasome-mediated IL-1β secretion in LPS-primed and ATP-activated THP-1 macrophages (19). Interestingly, 6-gingerol and 6-shogaol treatment also decreased the production of the tolerogenic cytokine IL-10. In addition, recent reports (44, 45) demonstrated that 6-gingerol downregulates the expression of programmed death-ligand 1 (PD-L1) as well. Generally, PD-L1 is a major tolerogenic marker for DCs (46), the level of which correlates with the expression level of IL-10 (47). Based on these data we could also hypothesize that the ginger-derived bioactive compounds might keep DCs in an inactivated state rather than inducing a tolerogenic phenotype; however, further investigation is warranted to support this assumption.

As DCs play a key role in T cell activation and polarization, next we investigated the impact of 6-gingerol and 6-shogaol on the T cell stimulatory capacity of moDCs. In those experiments, moDCs were exposed to live bacteria, which are more potent stimulators of DC responses than synthetic TLR agonists due to the presence of multiple PAMPs, and thus better represent *in vivo* situations. As expected, *E. coli* elicited a strong cytokine response by moDCs that was significantly decreased upon pre-treatment with 6-gingerol and 6-shogaol. The bioactive compounds of ginger also significantly reduced the Th1 polarizing ability of DCs as it was shown by their decreased capacity to induce IFN-γ production by CD4+ naïve T cells. By contrast, mouse BM-DCs pre-treated with 6-gingerol retained their ability to mount a Th1 response but could not prime Th17 cell differentiation (23). We also investigated the Th17 polarizing ability of *E. coli*-triggered moDCs but were unable to detect IL-17 in CD4+ T cells. This might be explained by the fact that moDCs favor Th1 response through IL-12 production and support Th17 immunity to a lesser extent (26).

Previous studies have shown that the NF-kB and MAPK signaling pathways play an important role in regulating the TLR-triggered inflammatory response of DCs (48). Our study demonstrated that both 6-gingerol and 6-shogaol inhibited the TLR-stimulation mediated activation of NF-κB by suppressing the translocation of NF-κB p65 to the nucleus. In line with that, Han et al. reported that 6-gingerol prevented NF-κB p65 phosphorylation evoked by LPS stimulation of BM-DCs (23). Further, 6-shogaol reduced the LPS induced phosphorylation and nuclear translocation of NF-κB p65 in RAW 264.7 macrophages (18). Moreover, pre-treatment of primary microglia cells with 6-shogaol decreased the phosphorylation and degradation IkB in response to LPS (49). In contrast to the results obtained in LPS-stimulated mouse macrophages and microglia, we found that the TLR-mediated degradation of IkBα 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.

Investigating 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 activity of ERK, it could effectively inhibit its phosphorylation upon CL075 and PAM3CSK4 challenge. Further, we found that 6-gingerol did not affect p38 and JNK phosphorylation in response to any of the applied stimuli. In line with our results, 6-gingerol significantly impaired the phosphorylation of ERK, while not affecting p38 and JNK activity in LPS- and ATP-primed bone marrow-derived macrophages (50). Similarly, in mouse BM-DCs 6-gingerol suppressed the MAPK pathway by inhibiting the LPS-mediated phosphorylation of ERK1/2 and JNK (23). Similar to our results, p38 phosphorylation was not affected by 6-gingerol treatment in these cells either. 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. Similarly, in RAW 264.7 macrophages 6-shogaol attenuated LPS-stimulated ERK phosphorylation, but did not affect p38 activation. In primary microglia cells 6-shogaol reduced the phosphorylation of p38 and JNK in response to LPS, but had no effect on ERK activity. In primary human

endothelial cells LPS-induced JNK phosphorylation was markedly reduced upon 6-shogaol treatment, while the LPS-triggered p38 activation was not impaired (43). 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 (28-30). Emerging evidence highlights the importance of mTOR activity in DC development and function, including their TLR-driven responses. So far, only one study demonstrated that 6-shogaol is able to attenuate the PI3K/Akt signaling in LPS-activated RAW264.7 macrophages (18). A few studies have also suggested that 6-gingerol and 6-shogaol might exert their anti-cancer effects by suppressing the Akt/mTOR signaling cascade (51, 52); however, to date no study has investigated the effect of these compounds on the activity of mTOR in DCs. Here we demonstrated that 6-gingerol decreased the mTORC2-mediated phosphorylation of Akt, whereas had no effect on the mTORC1-mediated phosphorylation of p70S6K in TLR-stimulated moDCs. Moreover, we found that 6-shogaol could greatly suppress the TLR-mediated activation of both mTOR complexes. It is also known that TLR stimulation of DCs is accompanied by a metabolic transition, in which cells become committed to aerobic glycolysis. Since sustained glycolytic reprogramming in moDCs is mTOR-dependent (31) we also investigated the expression of key glycolysis-related genes including *HIF1A*, *LDHA* and *HK2*. Interestingly, only LPS could induce a substantial upregulation of these genes, which was significantly suppressed by 6-shogaol treatment. Furthermore, 6-shogaol was also able to reduce the CL075-triggered upregulation of *HIF1A* and *LDHA*. On the contrary, 6-gingerol could only marginally decrease the LPS-induced expression of HK2, while not exerting any effects on *HIF1A* and *LDHA*. Although some differences could be observed between the effects of 6-gingerol and 6-shogaol, 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.

Finally, we hypothesized that the ginger-derived compounds exert their anti-inflammatory effects on DC functionality through HO-1 upregulation. Recent data indicate that several natural compounds, including 6-shogaol are able to effectively induce HO-1 expression without cytotoxic effects (33). HO-1 is an inducible enzyme, which is involved in heme metabolism and has potent anti-oxidant and anti-inflammatory capacity. The beneficial effects of HO-1 induction in inflammation are related to the degradation of toxic free heme and generation of anti-inflammatory products, such as carbon monoxide and biliverdin, the latter of which is subsequently converted into bilirubin (35). Recently, substantial studies have suggested that beyond its enzymatic activity, HO-1 is able to affect cellular processes through its non-canonical functions. In particular, HO-1 is able to interact with several intracellular signaling molecules that not only determines its cellular location but also implicates HO-1 in a variety of signal transduction pathways (53). Thus, we were curious to see whether 6-gingerol and 6-shogaol are able to influence the expression of HO-1. Interestingly, only 6-shogaol was able to strongly upregulate HO-1 levels, whereas 6-gingerol had no effect on it. Interestingly, 6-gingerol had no effect on HO-1 expression, whereas 6-shogaol strongly upregulated its levels both in resting and TLR-stimulated moDCs. These results are in line with a previous study showing that 6-shogaol but not 6-gingerol increases the protein levels of HO-1 in primary cultured microglia (36). The authors also demonstrated that 6-shogaol had a neuroprotective effect via the upregulation of HO-1 in an *in vivo* mouse model of hemorrhagic brain injury. Furthermore, 6-shogaol markedly increased HO-1 protein levels in LPS-stimulated human umbilical vein endothelial cells as well (43). Similarly, curcumin, another plant-derived phenolic compound has been reported to be a strong inducer of HO-1 in human moDCs as well (54). Campbell et al. demonstrated that tin-protoporphyrin IX, a competitive HO-1 enzymatic inhibitor attenuated the ability of curcumin to reduce the production of TNF in human LPS-stimulated moDCs. While the authors found that the inhibitory effects of curcumin are, at least, partially mediated by HO-1 activity, our results demonstrated that HO-1 depletion was not able to reverse the inhibitory effects of 6-shogaol on the cytokine production of TLR-stimulated moDCs.

Nevertheless, we suppose that the strong HO-1 upregulation by 6-shogaol might influence other functional or phenotypical properties of human DCs. It is noteworthy that 6-shogaol induced high HO-1 expression regardless of the activation state of DCs. Based on literature data HO-1 is essential for the suppression of antigen-specific T-cell responses by immature DCs. In a murine model of allergic asthma intratracheal administration of HO-1-expressing DCs decreased the severity of lung inflammatory responses probably through increasing the differentiation of antigen-specific regulatory T (Treg) cells (55). Another study reported that adoptively transferred HO-1 expressing immature DCs more efficiently suppressed the alloantigen-specific responses of recipient T cells and the alloantibody production of recipient B cells than conventional immature DCs in a murine cardiac allotransplant model (56). Furthermore, both studies demonstrated that the induction of high HO-1 levels by cobalt protoporphyrin (CoPP) inhibited LPS-induced maturation of mouse BM-DCs and reduced their T cell stimulatory capacity in vitro as well (55, 56). These observations suggest that the induction of high HO-1 levels might improve the tolerogenic capacity of resting DCs to subsequent stimuli. CoPP treatment also significantly increased the production of the anti-inflammatory cytokine IL-10 in BM-DCs, thereby promoting their ability to induce Treg polarization (56). In contrast to CoPP, 6-shogaol greatly reduced the secretion of IL-10 in human TLR-stimulated DCs, thus it is plausible that 6-shogaol is not able to support Treg polarization to such a high extent by human DCs. Nevertheless, further investigation is warranted to reveal the exact role of HO-1 induction by 6-shogaol in human DCs.

Numerous *in vitro* experiments and *in vivo* animal studies have already shown the beneficial anti-inflammatory capacity of ginger-derived compounds, especially that of 6-gingerol on different immune cell types. 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. Moreover, we provide evidence that the ginger-derived compounds modulate DC functionality via interfering with the NF-kB, MAPK and mTOR signaling pathways. Although, the exact mechanism of action of these compounds are unknown, some studies suggested that ginger constituent might affect these signaling pathways by targeting various upstream regulatory molecules such as AMP-activated protein kinase (AMPK), nuclear factor erythroid 2-related factor 2 (Nrf2), or peroxisome proliferator-activated receptor gamma (PPARγ). Multiple reports indicate that AMPK indirectly blocks mTORC1 activation by phosphorylating two of its regulatory proteins and thus eventually inhibits anabolic processes and inflammation (57). A study demonstrated that 6-gingerol inhibited oral cancer cell growth and migration by activating AMPK and suppressing the AKT/mTOR signaling pathway (51). Other studies reported that 6-gingerol ameliorated inflammation through activating AMPK in the liver of high fat diet mice (58) and rats (59). We also made attempts to measure AMPK activity in moDCs but we could not detect consequent changes in its phosphorylation level (data not shown). Recent studies suggest that 6-gingerol might exert its anti-oxidant and anti-inflammatory effects through activating the NRF2 transcription factor. As a response to oxidative stress and inflammation, NRF2 is translocated to the nucleus to trans-activate the expression of various cytoprotective genes, such as HO-1 (60). Via activating the Nrf2/HO-1 axis 6-gingerol effectively limited sepsis-induced liver injury in mice (61), while 6-shogaol repressed UVB-induced inflammation in human epidermal keratinocytes (62). In our studies, only 6-shogaol was able to upregulate HO-1 in moDCs, the possible implication of which in the anti-inflammatory responses of moDCs is discussed above. Some studies proposed that ginger phenolics exert their anti-inflammatory effect through peroxisome proliferator-activated receptor gamma (PPARγ), which can suppress inflammatory responses by regulating NF-κB activation. 6-Shogaol was found to inhibit LPS-induced inflammation in BV2 microglia by activating PPARγ (63). It was also reported that the anti-inflammatory activity of 6-gingerol on ventilation-induced lung injury in mice are partially mediated by the PPARγ/NF-κB pathway (64). The above-mentioned studies give a deeper mechanistic insight into the anti-inflammatory actions of ginger-derived compounds and suggest that ginger phytochemicals most likely exert their pleiotropic effects via targeting multiple

regulatory molecules and signaling pathways. Nevertheless, further investigations are needed to reveal their exact mechanism of action.

It must be noted that many of our results suggest that 6-shogaol exerts a more potent anti-inflammatory capacity on moDCs compared to 6-gingerol. Although comparative studies are rare, our results are in line with previous reports showing that 6-shogaol exerts a stronger anti-inflammatory and anti-oxidant capacity then 6-gingerol (17, 18). Up to now, the reason for these differences and the exact mechanism of action of these compounds are unknown. Still, it was proposed that the loss of hydroxyl group in the side chain of shogaols makes them more lipophilic that potentially increases their bioavailability compared to gingerols (16). This hypothesis was supported by another study suggesting that the α,β-unsaturated ketone moiety in the chemical structure of 6-shogaol explains its higher efficacy in different bioactivities compared to 6-gingerol (65), further studies are needed to support this hypothesis.

Several reports proposed ginger compounds as candidates for the treatment of autoimmune diseases due to their potent immunomodulatory capacity. Ginger has been used traditionally as an herbal medicine for the treatment of many maladies, including chronic diseases such as arthritis, yet clinical studies are lacking due to the poor water solubility and adsorption of ginger phenolics. To overcome these problems, novel technological approaches such as nanodrug delivery systems are under development to increase the bioavailability of ginger compounds (66). Therefore, 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.

5. Ethics Statement

The collection of human heparinized leukocyte-enriched buffy coat samples complied with the guidelines of the Helsinki Declaration and was approved by 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).

6. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions

TF and KP designed the research, performed experiments, analyzed and interpreted data, and wrote the manuscript. BA performed experiments and participated in data analysis. AGS and KP provided conceptual insight and revised the manuscript. KP and AB contributed with essential reagents. All authors reviewed and approved the manuscript.

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9. Figure legends

Figure 1. 6-gingerol and 6-shogaol dose-dependently reduce the expression of cell surface 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 cell surface molecules were assessed by flow cytometry. Bar graphs represent the mean ± SD of at least 3 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

Figure 2. 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 at least 3 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

Figure 3. 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-C**) The moDCs pre-treated with the indicated reagents were co-cultured with allogeneic naïve CD4+ T cells. After 6 days of co-cultivation 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. IFN-γ production of CD4+ T cells was measured by intracellular cytokine staining using flow cytometry. (**B**) Representative dot plots are shown. Numbers indicate the percentage of cells in each quadrant. (**C**) Bar graphs represent the mean ± SD of 4 independent experiments. *p<0.05, **p<0.01, ***p<0.01, ****p < 0.0001 vs. control (**A**) or T cell control (**C**); $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.01, $^{\#\#\#\#}$p < 0.0001.

Figure 4. 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 IkBα, 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 at least 4 independent experiments. **p < 0.01, ***p<0.01, ****p < 0.0001 vs. control; $^{\#\#}$p<0.01, $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001.

Figure 5. 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 at least 3 independent experiments. *p < 0.05, ***p < 0.01, ****p < 0.0001 vs. control; $^{\#}$p < 0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001.

Figure 6. 6-gingerol and 6-shogaol decrease the mTOR-mediated signaling and metabolic reprogramming 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. Kinetics of p70S6K and Akt 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. Bar graphs represent the mean ± SD of at least 4

694 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.01, ****p < 0.0001 vs. control; $^{\#}$p <
695 0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001.
696
697 **Figure 7.** HO-1 depletion does not influence the 6-shogaol-mediated inhibition of cytokine
698 production in moDCs. Immature moDCs were pre-treated with vehicle control, 50 μM 6-gingerol
699 or 6-shogaol for 2 h then stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4 for 24 h. The
700 protein levels of HO-1 were measured by western blotting. (**A**) Representative blots are shown. (**B**)
701 Bar graphs represent the mean ± SD of at least 3 independent experiments. (**C**) 5-day moDCs were
702 transfected with tranfection reagent alone (mock), scrambled siRNA (scr) or HO-1 specific siRNA.
703 After 24 h moDCs were pre-treated with vehicle control or 50 μM 6-shogaol for 2 h then stimulated
704 with 0.5 μg/ml LPS, CL075 or PAM3CSK4. After 24 h of stimulation TNF, IL-6 and IL-10 protein
705 levels were measured by ELISA. Bar graphs represent the mean ± SD of 4 independent experiments.
706 ****p < 0.0001 vs. control; $^{\#\#\#\#}$p < 0.0001

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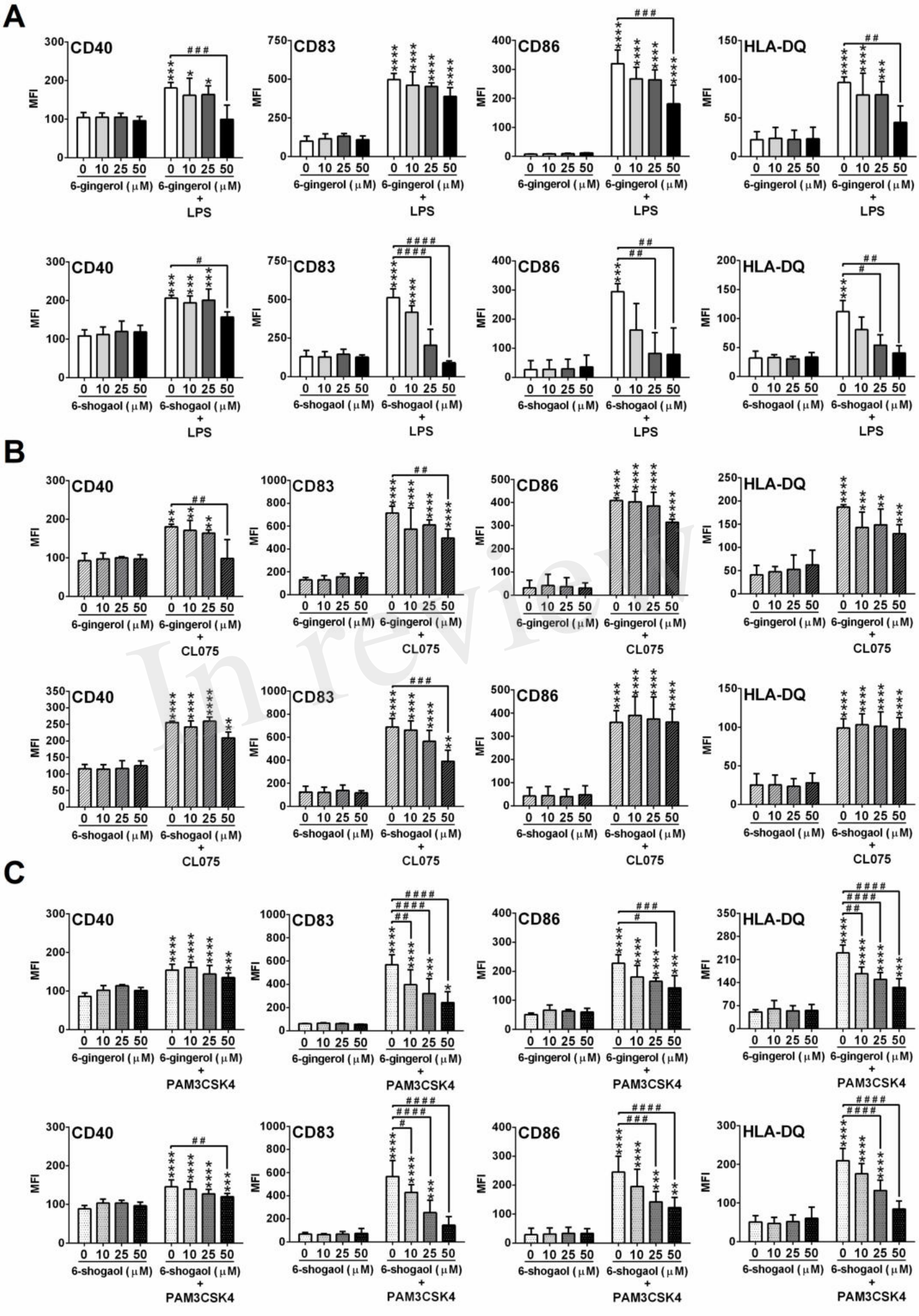
Figure 1.TIF



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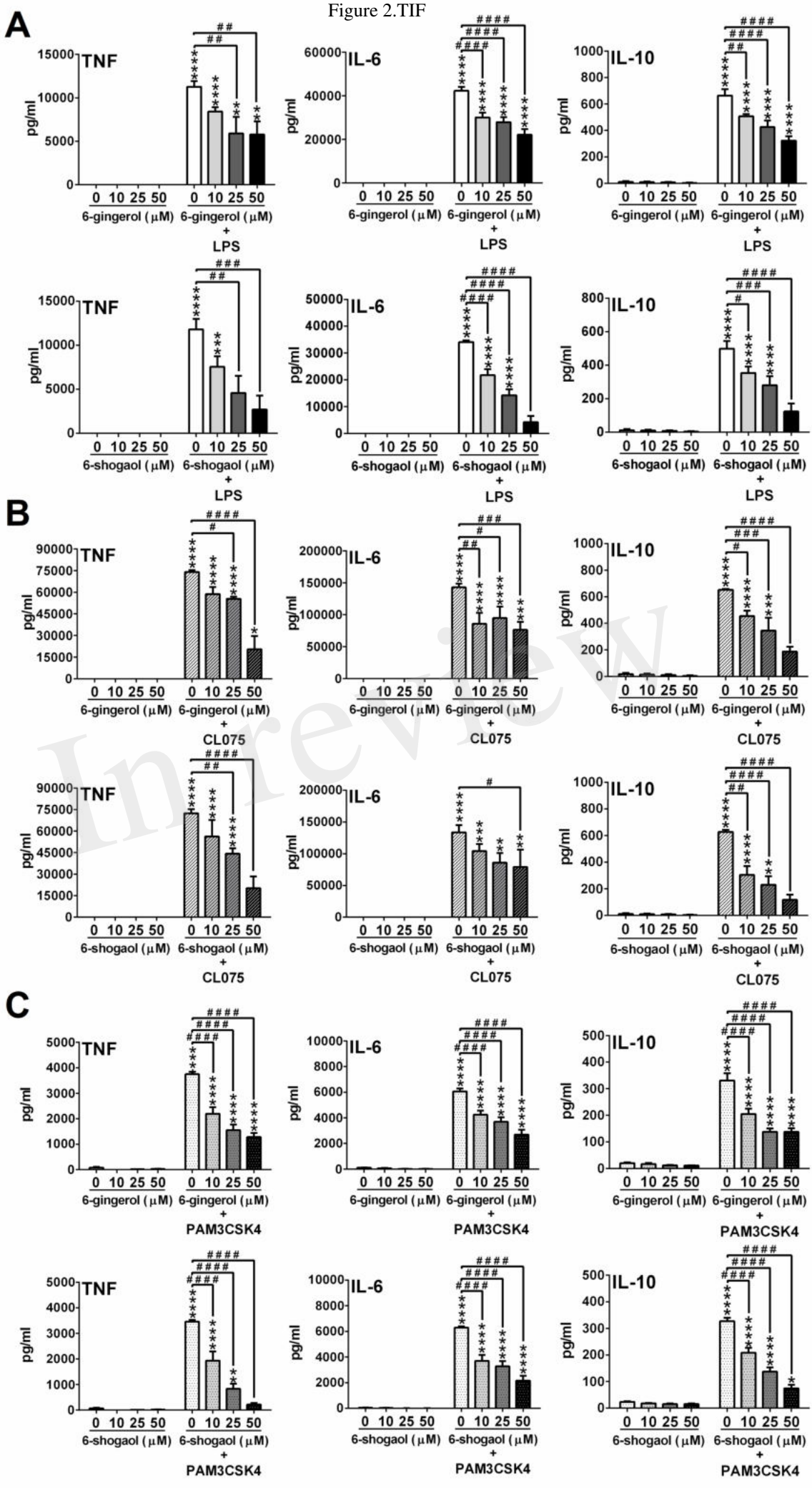

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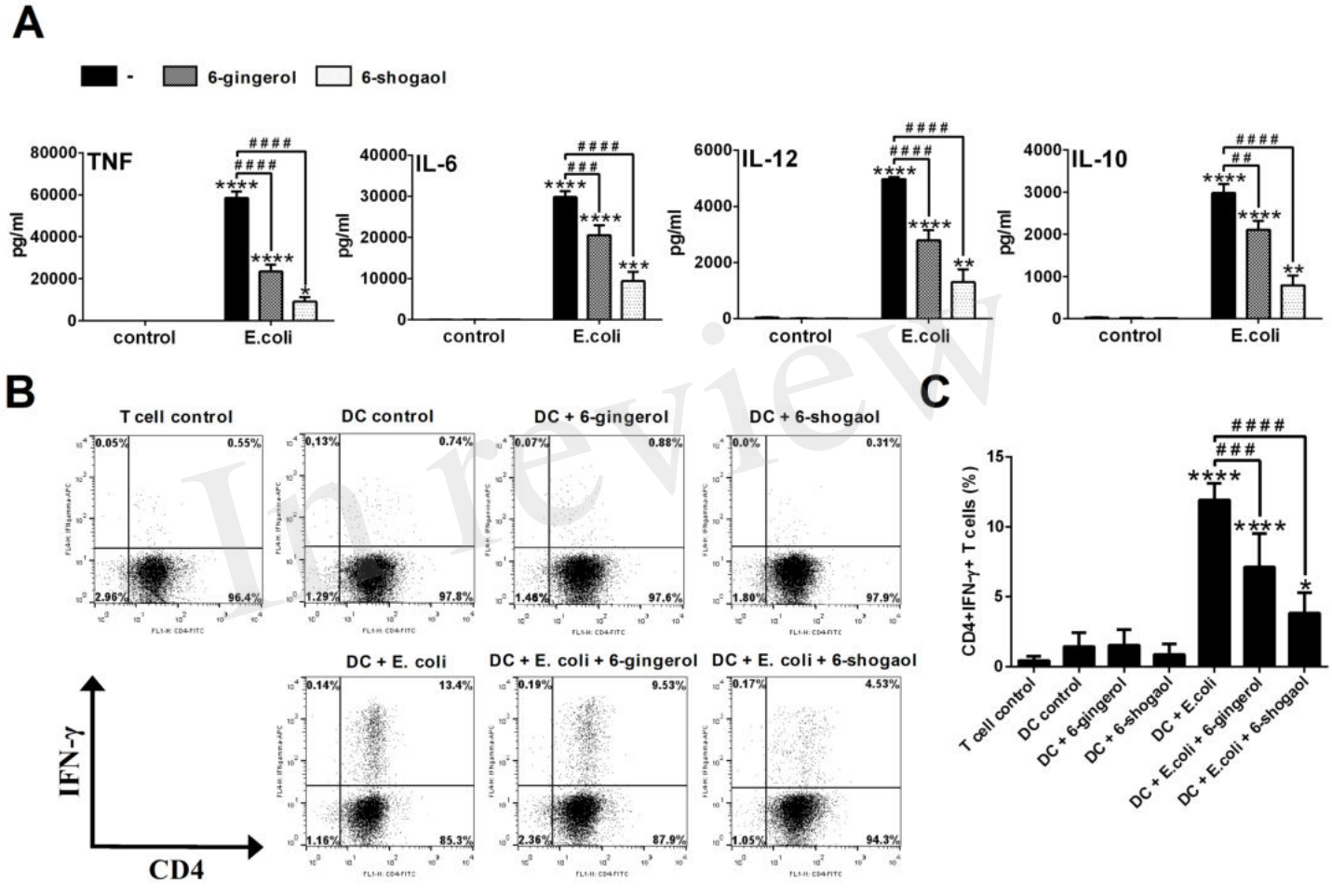

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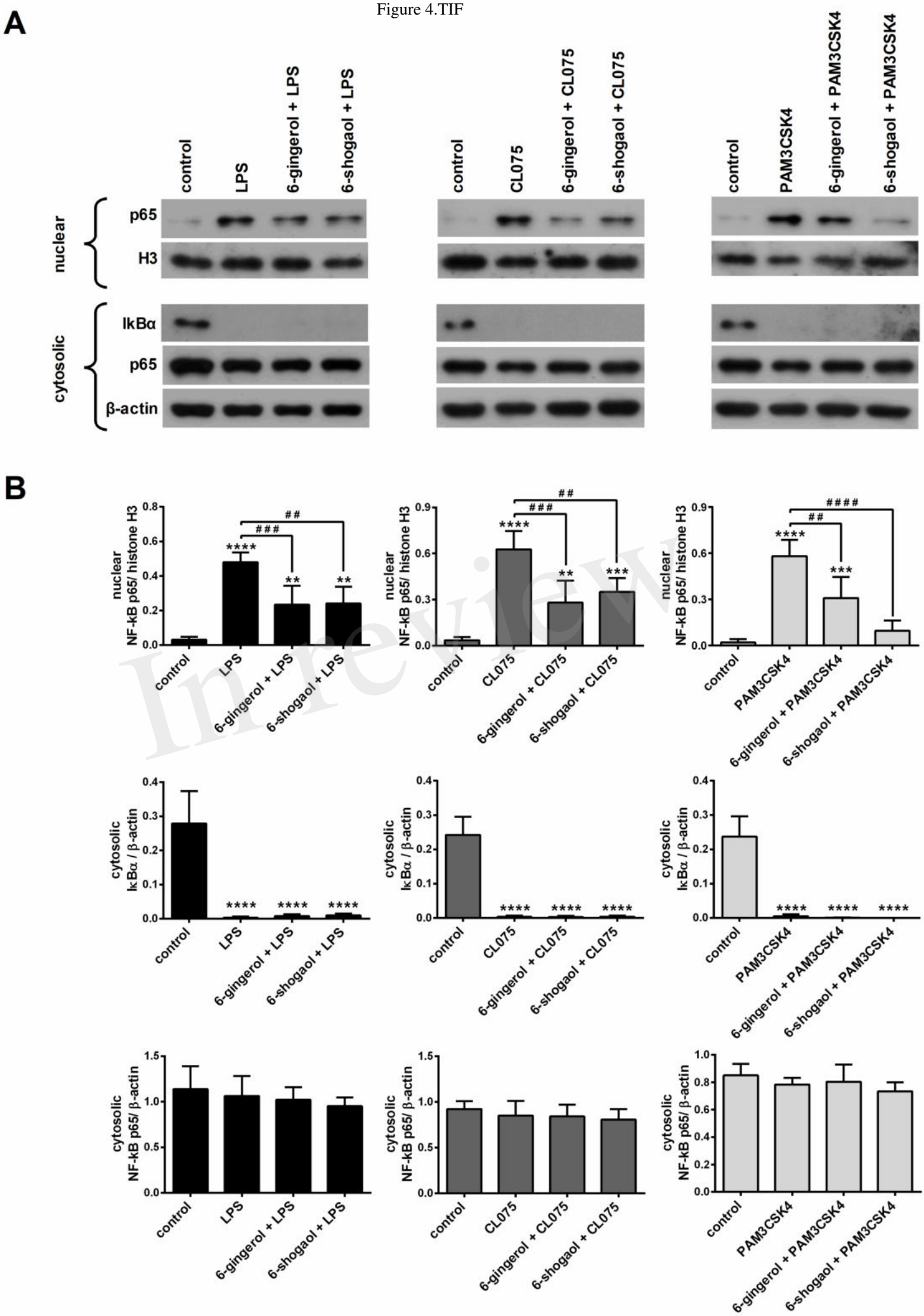

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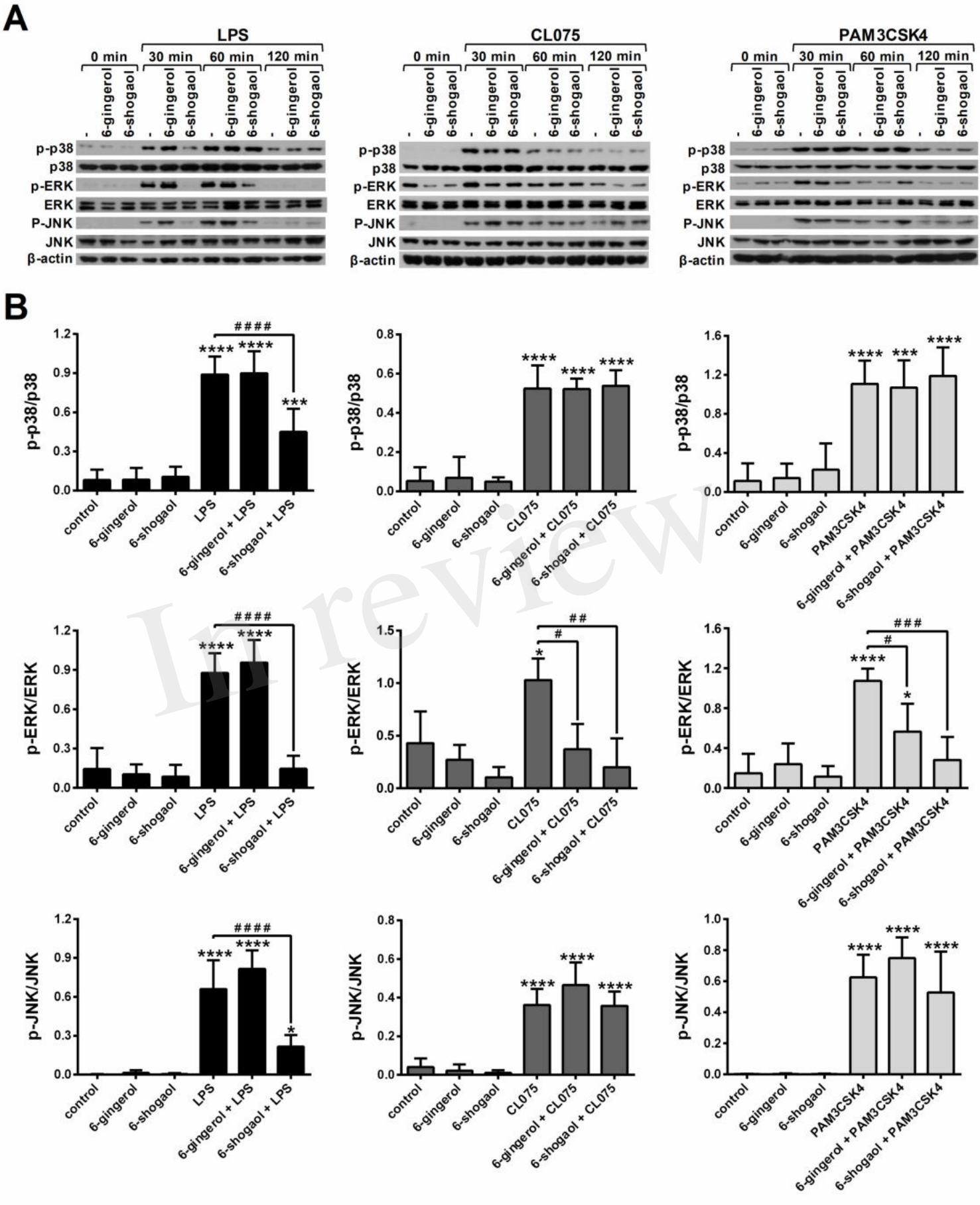

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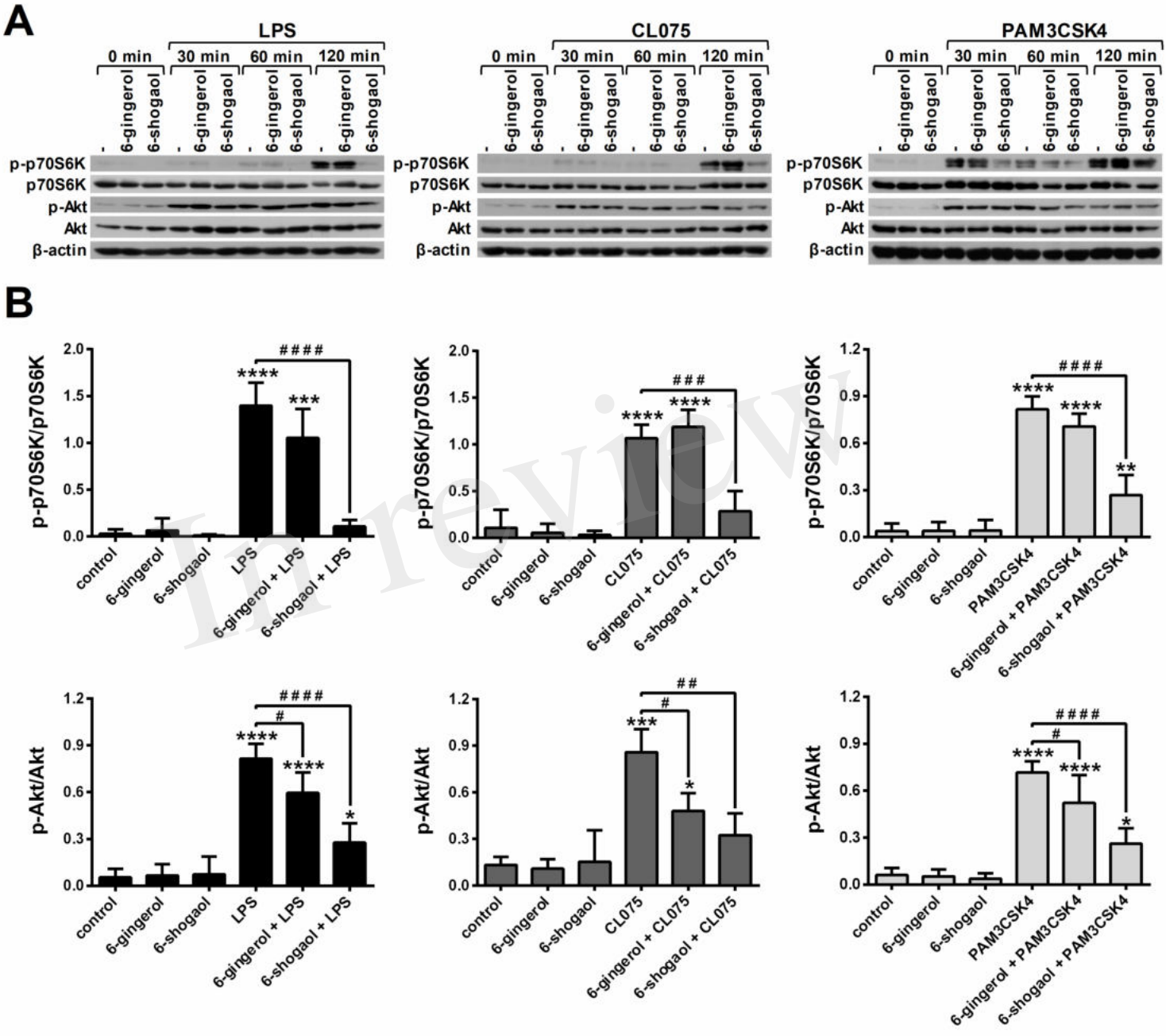

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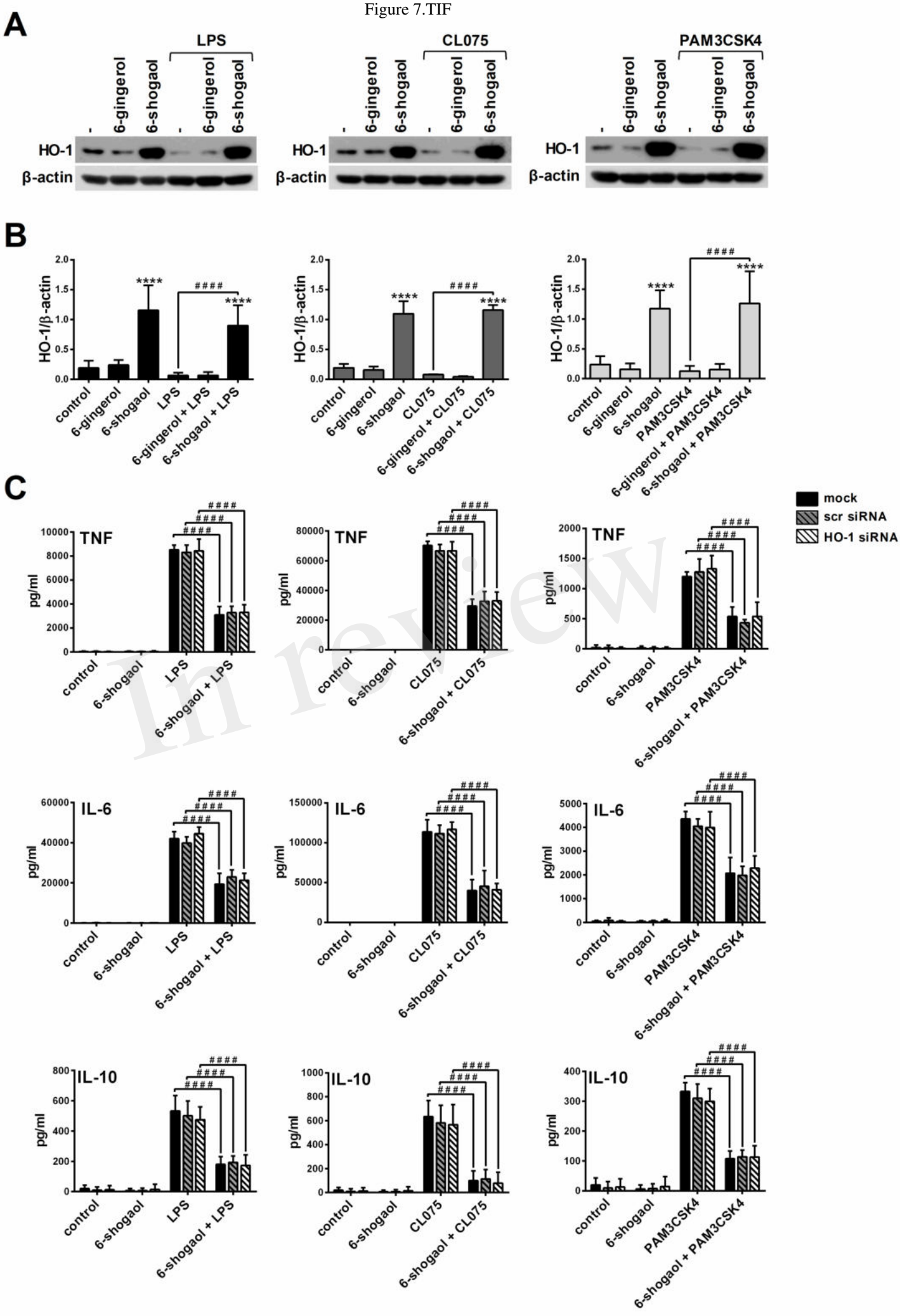
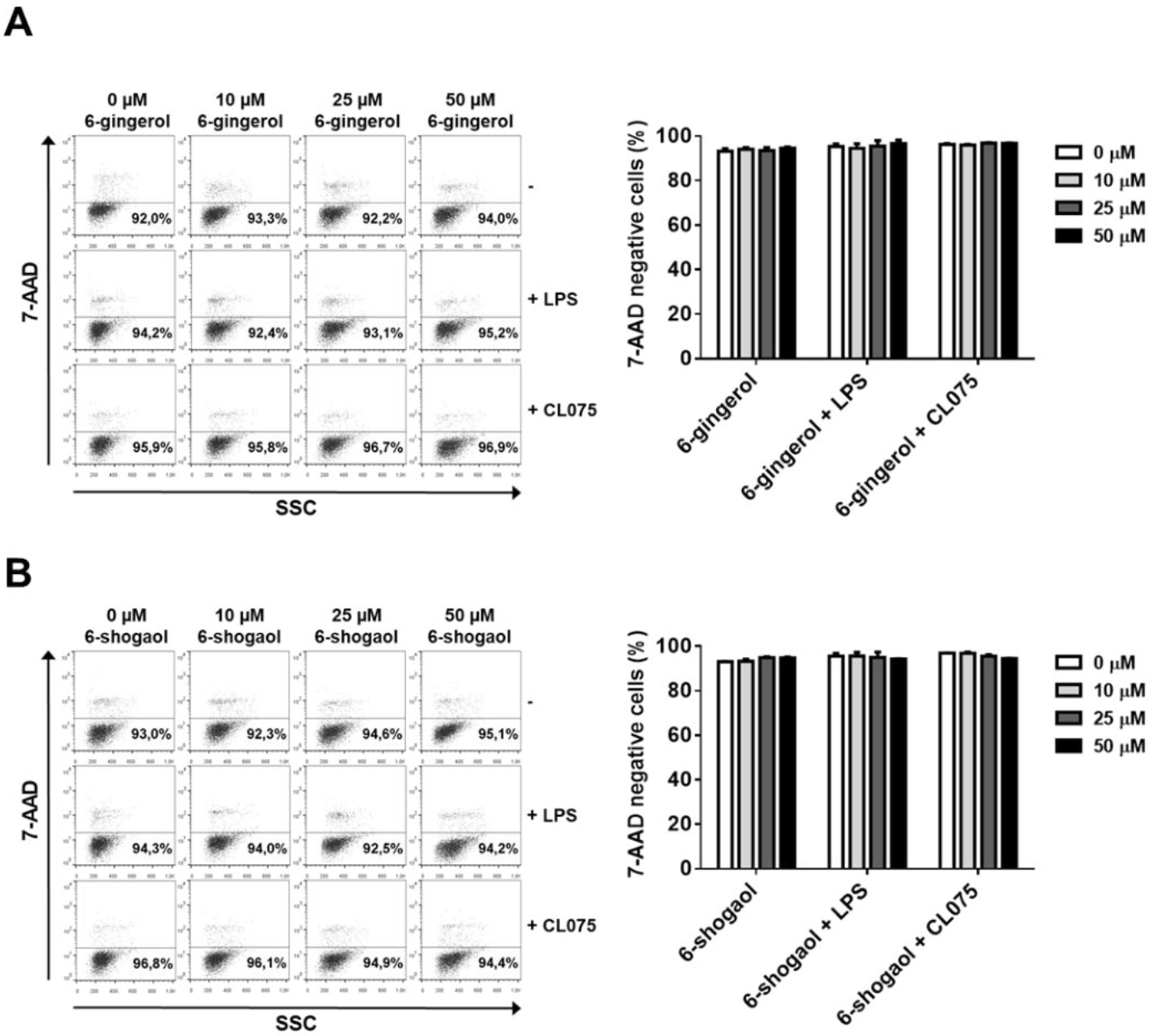
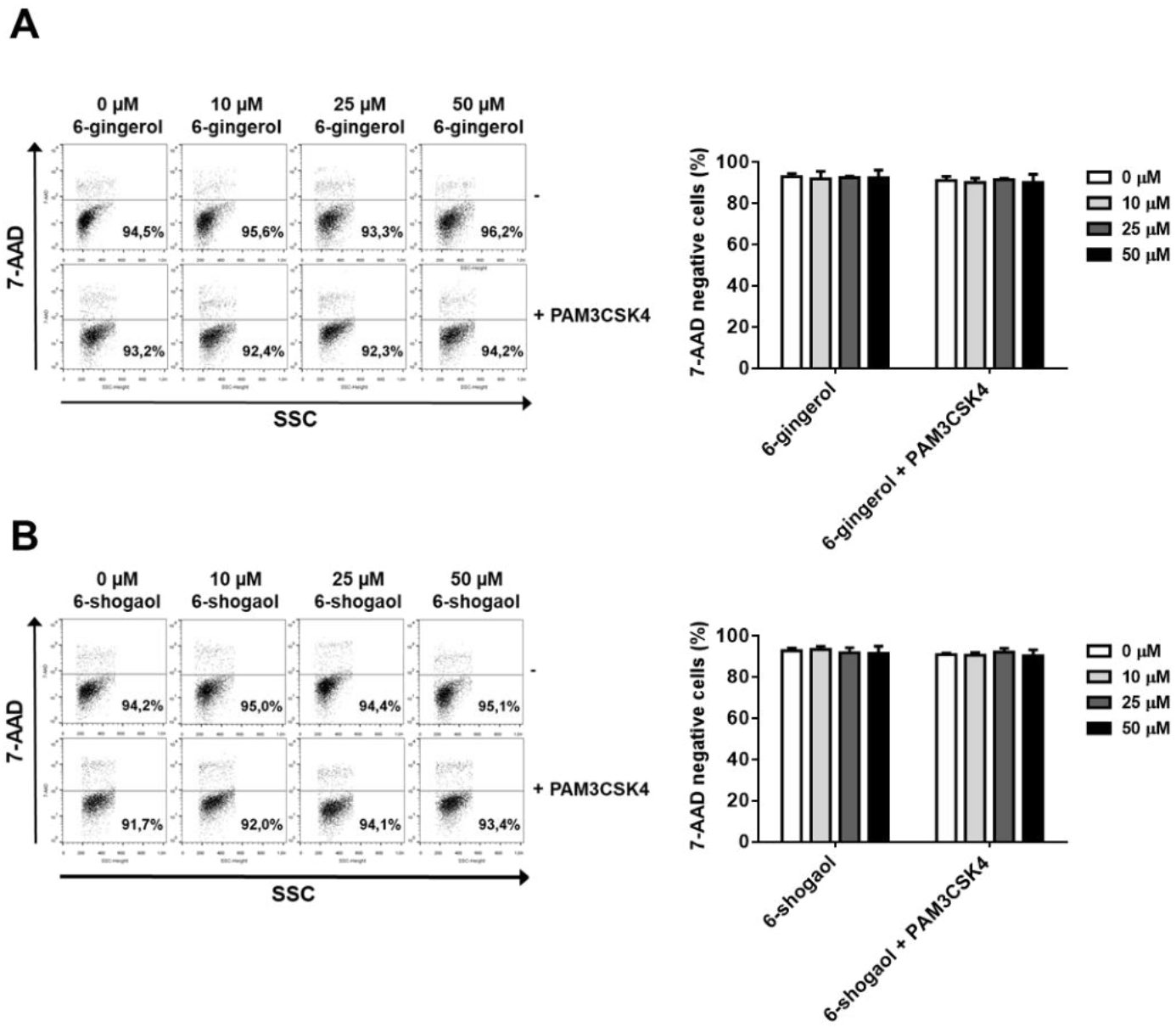


Figure 7.

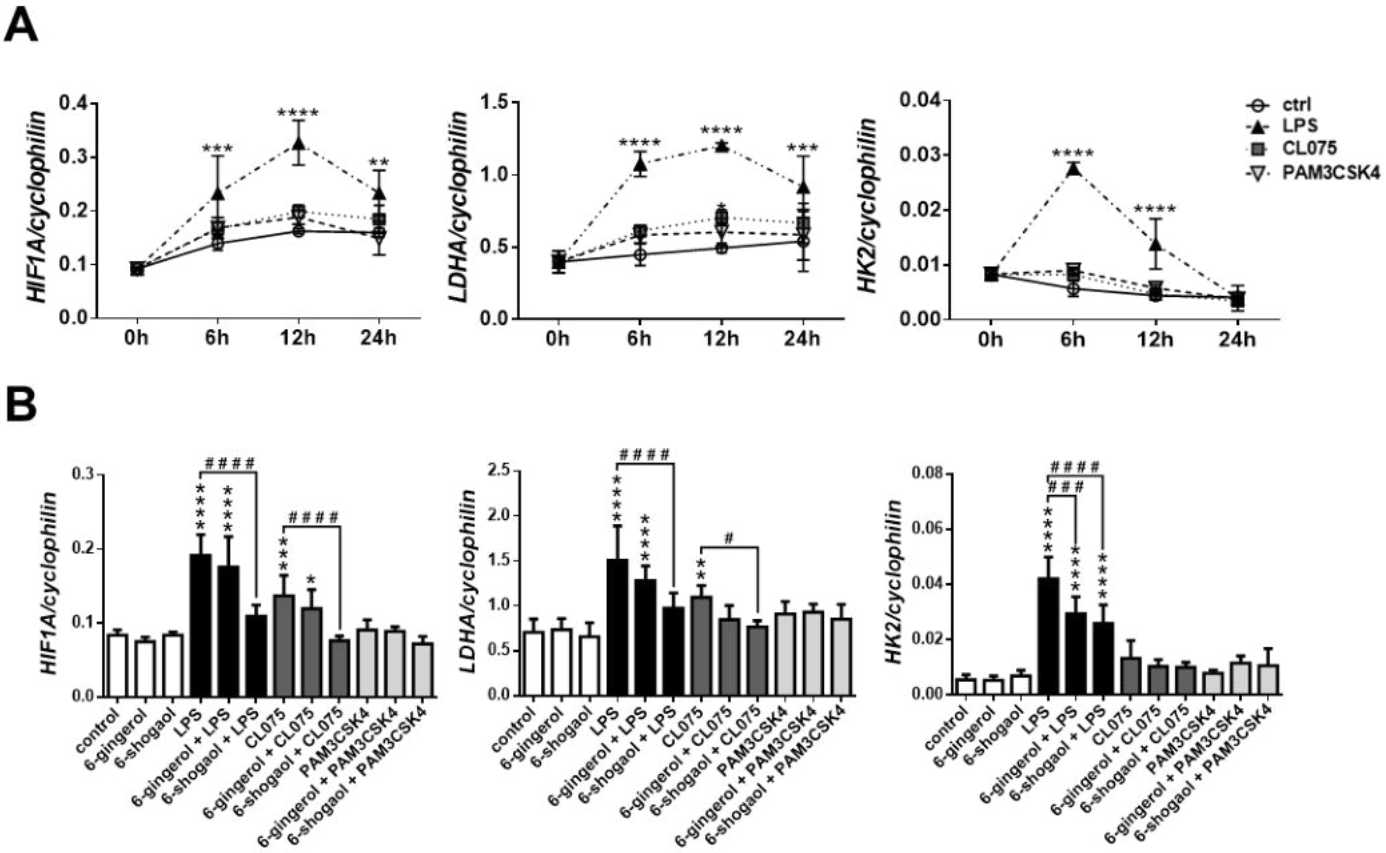
Supplementary Material



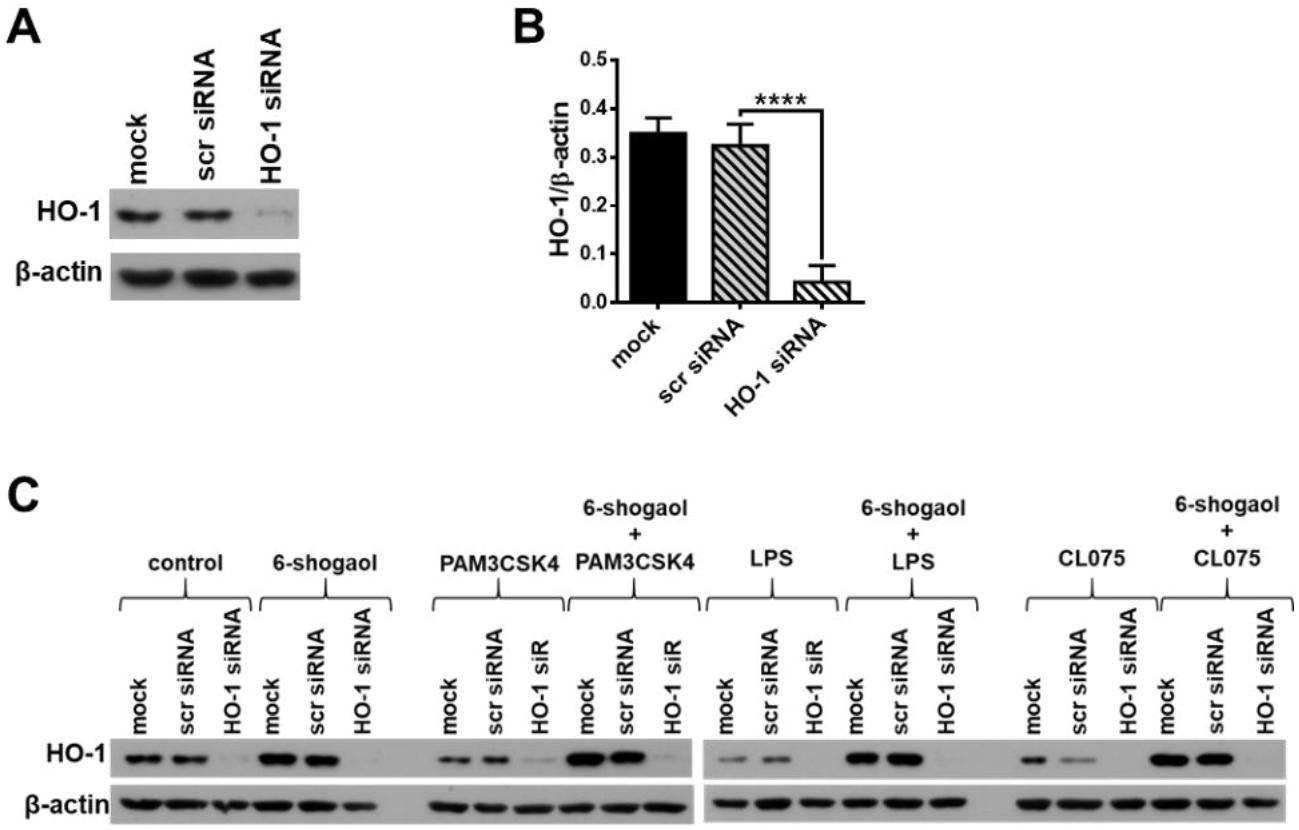
Supplementary figure 1. 6-gingerol or 6-shogaol treatment does not influence the viability of TLR4-, and TLR7/8-stimulated moDCs. 5-day moDCs were pre-treated with vehicle control, or with increasing concentrations of 6-gingerol (**A**) or 6-shogaol (**B**) for 2 h then stimulated with 0.5 µg/ml LPS or CL075. After 24 h cell viability was measured by 7-aminoactinomycin D (7-AAD) staining using flow cytometry. Numbers in representative dot plots indicate the percentage of 7-AAD negative cells. Bar graphs represent the mean ± SD of 3 individual experiments.



Supplementary figure 2. 6-gingerol or 6-shogaol treatment does not influence the viability of TLR2/1-stimulated moDCs. 5-day moDCs were pre-treated with vehicle control, or with increasing concentrations of 6-gingerol (**A**) or 6-shogaol (**B**) for 2 h then stimulated with 0.5 µg/ml PAM3CSK4. After 24 h cell viability was measured by 7-aminoactinomycin D (7-AAD) staining using flow cytometry. Numbers in representative dot plots indicate the percentage of 7-AAD negative cells. Bar graphs represent the mean ± SD of 3 individual experiments.



Supplementary figure 3. Analysis of glycolysis-related genes in moDCs. (**A**) Immature moDCs were stimulated with 0.5 µg/ml LPS, CL075 or PAM3CSK4 then the expression of *HIF1A*, *LDHA* and *HK2* was measured in a time-dependent manner at the mRNA level by Q-PCR. Figures represent the mean ± SD of 4 independent experiments. (**B**) 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. The expression of *HIF1A* and *LDHA* was assessed at 12 h of stimulation, whereas the expression of *HK2* was measured at 6 h of activation at the mRNA level by real-time PCR. Bar graphs represent the mean ± SD of 6 independent experiments. Data were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.



Supplementary figure 4. The efficacy of HO-1 siRNA gene silencing in moDCs. 5-day moDCs were transfected with HO-1 specific siRNA (HO-1 siRNA), scrambled siRNA (scr siRNA) or without siRNAs (mock). (**A-B**) After 48 h the efficacy of gene silencing was assessed by western blot analysis. (**A**) Representative blots are shown. (**B**) Bar graphs represent the silencing efficiency of HO-1 in control moDCs. Data are shown as means ± SD of 4 independent experiments and statistical analysis was performed by Student's t-test. ****$p<0.0001$ (**C**) After 24 h of transfection cells were pre-treated with vehicle control or 50 μM 6-shogaol for 2 h and then stimulated with 0.5 μg/ml LPS, CL075 or PAM3CSK4. After 24 h of stimulation the expression of HO-1 was assessed by western blot analysis. Representative blots of 3 independent measurements are shown.

Effects of ginger constituents on immune cells and their proposed mechanism of action

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Abstract

Ginger (Zingiber officinale) is a worldwide known spice and medicinal plant that has been used since ancient times to treat a plethora of diseases including cold, gastrointestinal complaints, nausea or migraine. Beyond that, a growing body of literature demonstrates that ginger exhibits anti-inflammatory, antioxidant, anti-cancer and neuroprotective actions as well. The beneficial effects of ginger can be attributed to the biologically active compounds of its rhizome such as gingerols, shogaols, zingerone and paradols. Of these bioactive compounds, gingerols are the most abundant in fresh roots, whereas shogaols are the major phenolic compounds of dried ginger. Over the last two decades numerous *in vitro* and *in vivo* studies demonstrated that the major ginger phenolics are able to influence the function of various immune cells including macrophages, dendritic cells, T cells and neutrophils. Although the mechanism of action of these compounds is not fully elucidated yet, some studies provide a mechanistic insight into their anti-inflammatory effects by showing that ginger constituents are able to target multiple signaling pathways. In this review, we aimed to summarize our current knowledge about the immunomodulatory actions of ginger compounds and to provide a deeper understanding about the mechanism of action underlying their anti-inflammatory effects.

1. Introduction

The rhizome of ginger (Zingiber officinale) has been used as a dietary food supplement (ingredient) across China and Southeast Asia since ancient times. As early as 3000 BC China's first written documentation of medicinal plants mentioned ginger as a herbal medicine and recommended it as a remedy for cold, fever, leprosy and tetanus (1). Other medicinal uses of ginger include the treatment of nausea, upset stomach and to aid digestion (2). The U.S. Food and Drug Administration classified ginger root as a safe herbal supplement that can be used in complementary and alternative medicine preparations (3). Due to its proven beneficial effects, in 2012 the European Medicines agency listed ginger rhizome in the prevention of nausea and vomiting associated with motion sickness (4). In the last few years a myriad of studies indicated that ginger possesses various biological activities such as anti-inflammatory, antioxidant, anti-microbial, anti-cancer and neuroprotective effects as well. It has also been revealed that the pharmacological benefits of ginger can be credited to the bioactive compounds of its rhizome.

To date, more than 400 chemical compounds such as lipids, terpens, phenolics, and carbohydrates have been identified in ginger rhizome (5, 6). The phenolic compounds, which are usually referred to as the nonvolatile components of ginger, are mainly responsible for its pharmacological activities and consist of gingerols, shogaols, paradols and zingerone (7). Gingerols are the main pungent components of fresh ginger that can be differentiated based on their unbranched alkyl side chain length. Among them, 6-gingerol is the most abundant constituent that is followed by 8-gingerol and 10-gingerol (8). Upon drying or heating of ginger root the thermally labile gingerols undergo dehydration reactions to form the corresponding shogaols, which are twice as much pungent as gingerols (9). Although shogaols are scarcely found in fresh ginger root, 6-shogaol is the predominant bioactive principle in the dried rhizome. At high temperatures or by microbial metabolism shogaols might be partly transformed to paradols, which represent a minor but important bioactive constituent of ginger (10). Besides, cooking or drying also converts gingerol into zingerone through a retro-aldol reaction (11). In addition, a number of other gingerol derivatives such as gingerdion, gingerdiol, dehydrogingerdione and other minor components have been isolated from ginger rhizome (12). A recent study using synthetic strategy to prepare 6-gingerol derivatives indicates that several of those show promising anti-platelet and anti-oxidant activities; however, their biological effects needs to be further elucidated (13).

In the last two decades, especially since the outbreak of Covid-19 pandemic, an interest has grown in plants with medicinal properties (14, 15). Due to its long-known health benefits and high bioactive agent content ginger is one of the most researched medicinal plant. In the last few years a number of studies have proven the pharmacological potential/importance of ginger-derived phytochemicals; however, the cellular and molecular mechanism behind its activities are not fully revealed yet. It was shown that, to a certain extent, ginger phytochemicals exert their anti-inflammatory effects by modulating the function of various immune cells such as macrophages, T cells or dendritic cells (DC), the latter of which are central in the coordination of immune responses (5). It was also demonstrated that ginger constituents have the potential to regulate key signaling pathways such as the nuclear factor-kappa B (NF-kB), mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling cascades in these cell types. Some studies also provided a deeper insight into the mechanism of action of ginger constituent by showing that those are able to target several signaling molecules such as the AMP-activated protein kinase (AMPK), nuclear factor erythroid 2-related factor 2 (NRFT2), heme oxygenase-1 (HO-1), and peroxisome proliferator-activated receptor gamma (PPARγ).

Although different bioactive compounds are present in ginger rhizome, the *in vitro* and *in vivo* studies are mainly focusing on gingerols and shogaols, which possess the most significant pharmacological effects. Some reports also suggest that shogaols exhibit more potent biological activities then gingerols due to the presence α, β-unsaturated ketone moiety (16). Besides, it was proposed that the loss of hydroxyl group in their sidechains increases their lipophilicity and thus their bioavailability compared to gingerols (9). Reviews published in the last few years focused on

the general biological effects (5), anti-cancer (17, 18) or neuroprotective (19) activities of ginger constituents. A review from 2021 discusses the anti-inflammatory actions of ginger extract and 6-shogaol (20), while another one from 2022 reviews that of gingerols in general (21). Nevertheless, a comprehensive review on the immunomodulatory effects of ginger phytochemicals /phenolic substances at the cellular and molecular level is still lacking. Most importantly, an update is needed to discuss the scientific merit of the most recently published research articles as well. Therefore, in this recent review we aimed to collate all the available evidence on the effects of the major ginger constituents on different immune cell types and to highlight the latest advances in our understanding of their mechanisms of action.

2. Effects of ginger compounds on the cells of the immune system

Ginger has been used for centuries for its pain-relieving properties. Recent clinical trials even suggest that the analgesic effects of ginger constituents are similar to that exerted by the non-steroidal anti-inflammatory drugs such as ibuprofen (22, 23). Research data also indicates that the pain reducing effect of ginger is linked to its high anti-inflammatory capacity (reviewed in (24)). Indeed, a number of *in vitro* and *in vivo* studies demonstrated that the bioactive compounds of ginger are able to dampen the inflammatory responses of various immune cells. Most of these studies focused on the innate immune machinery; however, there are also a few reports available on the effects of ginger compounds on T cells as well.

2.1. Macrophages

Macrophages serve as the first line of defense against invading pathogens and represent a highly plastic cell population with divergent phenotypes and functions. Generally, macrophages play an essential role in clearing out microbial pathogens by producing antimicrobial molecules such as reactive oxygen species (ROS) and nitric oxide (NO). In response to pathogenic stimuli macrophages also secrete various types of cytokines and chemokines and thus contribute to the initiation inflammatory responses. Besides, owing to their high functional plasticity, macrophages are involved in the resolution of inflammation and restoration of homeostasis as well (25). Nevertheless, abnormal activation and polarization of macrophages has been suggested to contribute to the pathogenesis of different autoimmune diseases (26).

The very first data investigating the effects of ginger components on immune cells was published in 2003. Ippoushi et al. demonstrated that 6-gingerol inhibits nitric oxide (NO) production of lipopolysaccharide (LPS)-stimulated J774.1 murin macrophages (27). Moreover, the authors found that 6-gingerol markedly decreased the protein levels of inducible NO synthase (iNOS) that might contribute to the reduction of NO production. Further, it was shown that 6-gingerol protects against DNA and protein damage by suppressing the peroxynitrite-induced single strand breaks in supercoiled plasmid DNA as well as the formation of nytrotyrosine in J744.1 cells. Similarly, another study on LPS-stimulated mouse peritoneal macrophages demonstrated that 6-gingerol decreased the LPS-triggered production of the inflammatory cytokines interleukin 1 beta (IL-1β), tumor necrosis factor (TNF), IL-12 and the chemokine CCL5 (RANTES), whereas had no effect on the upregulation of major histocompatibility complex (MHC) class II and the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2). The authors also gave some mechanistic insight by demonstrating that 6-gingerol was able to suppress the LPS-induced activation of NF-kB (28). In the next publication the anti-inflammatory activity of ginger compounds was tested on the monocytic U937 cell line (29). Both the organic ginger extracts and the standard compounds including 6-, 8-, 10-gingerols and 6-shogaol were able to inhibit the LPS-induced production of prostaglandin E_2 (PGE_2). Interestingly, the gingerols were more potent inhibitors of PGE2 production compared to 6-shogaol. Further, 6-, 8-, and 10-gingerols also efficiently reduced the mRNA level of cyclooxygenase-2 (COX-2), a key enzyme responsible for PGE2 production, while 6-shogaol did not affect that. On the contrary, a subsequent study showed that 6-shogaol inhibited

more effectively the production of PGE2 than 6-gingerol. Besides, 6-shogaol significantly blocked the protein and mRNA expression of COX-2 and iNOS in LPS-stimulated murine RAW 264.7 cells (30), while 6-gingerol only slightly reduced iNOS but not affected COX-2 expression. Thereafter, the authors examined the inhibitory effects of only 6-shogaol in details. It was demonstrated that 6-shogaol blocked the phosphorylation and degradation of inhibitor kBα (IkBα) that suppressed the subsequent phosphorylation and nuclear translocation of NF-kB p65 in LPS-stimulated mouse macrophages. In parallel, it was demonstrated that 6-shogaol inhibits the transcriptional activity of NF-kB as well. Furthermore, 6-shogaol also reduced the activation of PI3K and Akt and interfered with the MAPK signaling pathway by attenuating the phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2) but not that of p38 in LPS-stimulated RAW264.7 macrophages. Based on the results the authors concluded that 6-shogaol was able to inhibit iNOS and COX-2 expression by affecting different signaling pathways. The next study also used LPS-stimulated mouse macrophages as a model of inflammation to further explore the anti-inflammatory activity of 6-gingerol (31). Similar to the previous publications, 6-gingerol was found to inhibit iNOS and TNF through the suppression of IkBα phosphorylation and NF-kB activation. Further, it was demonstrated that 6-gingerol inhibits the LPS-triggered intracellular Ca^{2+} mobilization and ROS generation probably via blocking the cytosol-to-membrane translocation of protein kinase C-α (PKC-α).

Similar results were obtained to those reported by Pan et al. when the effects of 6-shogaol were investigated in BV2 and primary microglia cell cultures (32). In particular, 6-shogaol significantly suppressed PGE2 by downregulating COX-2 expression and reduced the LPS-elicited production of IL-1β and TNF by inhibiting the phosphorylation and degradation of IkBα, thereby the activation of NF-kB. In contrast to the results obtained by RAW264.7 macrophages, 6-shogaol inhibited the LPS-stimulated activation of p38 and JNK but not that of ERK1/2 in primary microglia cells. A few years later similar results were published showing that 6-shogaol suppressed LPS-induced IL-1β, IL-6, TNF and PGE2 production by inhibiting the phosphorylation and nuclear translocation of NF-kB in BV2 cells (33). As a new concept the authors suggested that 6-shogaol blocks the LPS-induced inflammatory mediator production through activating PPARγ, which is a known inhibitor of NF-kB activation. Similar to 6-shogaol, 6-gingerol also effectively inhibited the LPS-stimulated expression of iNOS and production of NO, IL-1β and IL-6 in primary mouse microglia (34). Furthermore, 6-gingerol was able to suppress the phosphorylation of Akt, mTOR and signal transcducer and activator of transcription 3 (STAT3), the latter of which is a crucial signaling intermediate for TLR4-induced inflammatory responses in macrophages (35).

In primary mouse calvarial osteoblasts 6-shogaol also inhibited the IL-1-induced expression of PGE2 by suppressing the enzymatic activity of COX-2 and PGE synthase that resulted in decreased receptor activator of NF-kB (RANKL) production and thus reduction of osteoclast differentiation (36).

Finally, two reports investigated the effect of ginger compounds on the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome-mediated responses. The first study compared the inhibitory effects of 6-, 8-, 10-gingerols/ shogaols in THP-1 macrophages stimulated with LPS and adenosine 5'-triphosphate (ATP), (37). It was demonstrated that 10-gingerol and all the shogaols effectively reduced the secretion of TNF and IL-1β as well as the protein levels of NLRP3 and caspase-1. In general, shogaols show a higher anti-inflammatory capacity than the corresponding gingerols that could be attributed to the α, β-unsaturated carbonyl group in the structures of shogaols. Among the investigated phytochemicals 6-shogaol was the most potent inhibitor of NLRP3-mediated inflammasome activation. Interestingly, the results also indicate that the increase in alkyl side chain length weakens the anti-inflammatory potential of shogaols, whereas enhances that of gingerols. The other study was conducted on RAW264.7 cells and mouse bone marrow-derived macrophages (BM-DMs), in which 6-gingerol greatly reduced the release of caspase-1 p20 as well as the production of IL-1β and IL-18 in response to ATP and LPS (38). 6-gingerol significantly suppressed the phosphorylation of p38, JNK and ERK1/2 in RAW264.7 cells, while inhibited only ERK1/2 activation in BM-DMs.

Treatment of RAW264.7 cells with an ERK agonist reversed the inhibitory effects of 6-gingerol on caspase-1 p20 release suggesting that 6-gingerol exerts its effect through blocking MAPK activation (Table 1).

2.2. Dendritic cells

DCs represent a heterogeneous population, which as the most potent professional antigen presenting cells (APCs) bridge the innate and adaptive immune systems. Expressing a wide repertoire of innate receptors DCs can recognize a broad range of pathogen-, and danger-associated molecular patterns, and then migrate to secondary lymphoid organs to present antigens to T cells. Activated DCs upregulate costimulatory molecules and produce polarizing cytokines to drive T cell differentiation, and initiate different types of antigen-specific adaptive immune responses. In the steady-state, in the absence of activation signals, antigen presentation by DCs leads to T cell unresponsiveness and tolerance (39). Besides their well-known functions of controlling innate and adaptive immunity, accumulating evidence indicates that DCs are implicated in the pathogenesis and pathomechanism of various autoimmune disorders (40).

Although, DCs play a central role in the coordination of immune responses, only two studies investigated how ginger-derived phytochemicals might affect their functionality. Han et al. studied the effects of 6-gingerol on mouse bone marrow-derived DCs (BM-DCs) and found that 6-gingerol was able to remarkably reduce the production of TNF, IL-1β, IL-6 and IL-23 as well as the expression of CD80, CD86 and MHC II in response to LPS exposure (41). Besides, 6-gingerol treated BM-DCs had an inferior capacity to prime T helper 17 (Th17) cell polarization upon co-culture with CD4+ naïve T cells. Further, it was demonstrated that 6-gingerol prevented the LPS-induced phosphorylation of NF-kB, JNK and ERK1/2 suggesting that 6-gingerol suppressed the activation of BM-DCs by interfering with the NF-kB and MAPK signaling cascades.

We have recently reported that ginger phenolics are also able to modulate the phenotypical and functional properties of human DCs as well (Pazmandi et al., 2023). So far, the studies have investigated the effect of 6-gingerol or 6-shogaol on LPS-stimulated immune cells. In our experiments, monocyte-derived DCs (moDCs) were first treated with 6-gingerol and 6-shogaol then were exposed to various TLR ligands including agonists of TLR4, TLR2/1 and TLR7/8. We found that 6-gingerol and 6-shogaol could significantly decrease the expression of CD40, CD83, CD86 and HLA-DQ in moDCs triggered through cell-surface TLRs, while we observed a weaker or no inhibition when moDCs were stimulated through the endosomal TLR7/8 receptor. Furthermore, 6-gingerol and 6-shogaol significantly reduced the secretion of TNF, IL-6 and IL-10 by TLR-stimulated moDCs. Similarly, both ginger compounds significantly reduced the *Escherichia coli*-triggered secretion of inflammatory cytokines, and thus their capacity to promote a Th1 phenotype in CD4+ T cells. Investigating the mechanism underlying the actions of these components, we found that both 6-gingerol and 6-shogaol could significantly decrease the TLR-induced nuclear translocation of NF-κB p65. Interestingly, 6-gingerol did not affect p38 and JNK activity, while 6-shogaol could significantly reduce their phosphorylation in response to LPS, but not to other TLR agonists. 6-shogaol was also able to suppress ERK phosphorylation in response to all three TLR lingands, while 6-gingerol did not affect that upon LPS triggering. We also demonstrated that 6-gingerol decreased mTOR complex 2 (mTORC2)-mediated Akt phosphorylation, whereas had no effect on mTORC1-mediated p70S6K phosphorylation in TLR-stimulated moDCs. In contrast, 6-shogaol could greatly suppress the TLR-mediated activation of both mTOR complexes. Further, we found that 6-shogaol could increase the activity of AMPK, the nuclear translocation of NRF2 and the expression of HO-1. Altogether, our data imply that the ginger-derived compounds attenuate human DC functionality via suppressing the NF-kB, MAPK and mTOR signaling pathways and by inducing the activity of AMPK and NRF2/HO-1 system (Table 1).

2.3. Neutrophil granulocytes

As phagocytic cells of the innate immune machinery, neutrophil granulocytes take an important part in clearing infectious agents from the human body. Besides their phagocytic capacity, activated neutrophils extrude neutrophil extracellular trap (NET) composed of DNA, histones, and antimicrobial enzymes, in which pathogens are immobilized and exposed to a lethal dose of effector proteins (42). Nevertheless, a large body of evidence indicates that through NET release neutrophils are implicated in the pathogenesis of autoimmune diseases such as lupus (43).

First it was published in 2010 that 6-,8-,10-gingerols and 6-shogaol are able to effectively inhibit ROS generation by human neutrophils in response to formylmethionine-leucyl-phenylalanine (fMLP), a strong inducer of neutrophil activation (16). Among the tested ginger phenolics, 6-shogaol showed the highest potential to suppress fMLP-induced ROS release, while 6-gingerol had the weakest inhibitory capacity. More than 10 years later, a study from 2021 investigated the anti-inflammatory properties of gingerols in neutrophils as well. Ali et al. demonstrated that 6-, 8-, and 10-gingerol suppressed netosis of human neutrophils in response to LPS, phorbol 12-myristate 13-acetate (PMA) and various lupus-relevant stimuli as well (44). All three gingerols suppressed the formation of H_2O_2 in neutrophils indicating that gingerols attenuate netosis by inhibiting the generation of ROS. Further, 6-gingerol also decreased the activity of phosphodiesterase 4 (PDE4), which by regulating cAMP levels plays a prominent anti-inflammatory effect in basically any cells involved in inflammation (45). Concomitantly, 6-gingerol increased the intracellular levels of cAMP and thus that of cAMP-dependent protein kinase A, which exerts anti-inflammatory activity. Mechanistically, the authors proposed that the antineutrophil effects of 6-gingerol depend, at least partially, on its ability to inhibit PDE activity.

A year later it was published that zingerone, a less-studied component of ginger, also displays antineutrophil potentials (46). Zingerone significantly suppressed ROS production and NET release in PMA-stimulated mouse bone marrow-derived neutrophils. Interestingly, the bactericidal activity of neutrophils was not affected by zingerone treatment neither in vitro or in vivo. In particular, zingerone did not alter the phagocytic capacity of neutrophil in vitro and had no effect on bacteria dissemination in vivo. Furthemore, zingerone treatment significantly increased the levels of the nuclear Nrf2 and HO-1in PMA-stimulated neutrophil. In vivo experiments further showed that applying a specific inhibitor of Nrf2 reversed the protective effect of zingerone in septic mice.

In contrast with the previous findings, a recent study published that 6-gingerol increased the fMLP-stimulated CXCL8 chemokine secretion and ROS production in primary human neutrophils (47). Furthermore, 6-gingerol also increased the expression of neutrophil surface markers such as CD11b and CD66 as well as the expression of formyl peptide receptor 1, which may lead to increased responsiveness to its ligand, fMLP. In this particular study, the authors used very low concentration of 6-gingerol (50nM), which is generally hundred- and thousand-fold lower compared to any other previous publication on neutrophils or other immune cells. The report also suggested that 6-gingerol applied in a low dose facilitates neutrophil functions through binding to the transient receptor potential cation channel subfamily V member 1 (TRPV1), the potential of which as a mechanism of action we discuss in chapter *4.4* in details (Table 2).

2.4. T cells

As specialized cells of the adaptive immunity T cells play a central role in directing immune responses against pathogens, allergens, or tumors. While CD4+ T helper cells modulate immune responses by facilitating the activity of other immune cells, regulatory T cells (Treg) contribute to the maintainance of immune homeostasis and CD8+ cytotoxic T cells are essential to the elimination of cancer cells and virally infected cells. Nevertheless, it must be noted that specific populations of T cells such as Th17 cells might contribute to the pathogenesis of various chronic inflammatory disorders.

The first study regarding the effects of gingerols on the activation and effector function of T cells was published in 2015. Bernard et al. found that 6-, 8- and 10-gingerols inhibited the

proliferation of mouse spleen-derived CD3+ T cell in response to anti-CD2/CD3/CD28-coated beads (Dynabeads) or syngeneic DCs (48). 8- and 10-gingerols also significantly decreased the expression of the activation markers CD25 and CD69. All three gingerols reduced the Dynabead-stimulated production of interferon-gamma (IFN-γ), while not affected IL-4 synthesis. Moreover, 8- and 10- gingerols decreased IL-2 secretion as well. In general, the data suggest that 8- and 10-gingerols are more potent inhibitors of T cell activation and proliferation than 6-gingerol.

In accordance with that, another study demonstrated that 6-gingerol is able to suppress the production of IFN-γ and IL-4 by mouse spleen-derived CD4+ T cells cultured under Th1 or Th2 polarizing conditions, respectively (49). 6-gingerol also significantly suppressed the proliferation of pan T cells isolated from ovalbumin (OVA)-sensitized mice. In addition, 6-gingerol abrogated the staphylococcal enterotoxin B (SEB)-induced proliferation of unprimed T cells and the proliferation of Jurkat T cells in response to anti-CD3/CD28 and IL-2. Pre-treatment with 6-gingerol also inhibited the activation of p38, ERK1/2 and JNK as well as the nuclear translocation of NF-kB and c-fos transcription factors in Jurkat cells stimulated with PMA and ionomycin. Thus, the results suggest that 6-gingerol suppresses T-cell activation and proliferation through inhibiting NF-kB and activating protein 1 (AP1) activation (Table 2).

The effects of 6-gingerol on CD8+ T cells were studied only in the context of cancer so far. Two publications reported that 6-gingerol is able to increase the number of tumor-infiltrating CD8+ T cell in mice that might play an important role in the anti-tumor effect of ginger (50, 51). The anti-cancer activity of ginger compounds is not in the scope of the recent review; however; it has been discussed in details elsewhere (17, 18).

3. Protective effects of ginger compounds in animal models of autoinflammatory and autoimmune diseases

Abnormal activation of the immune system can lead to the generation of autoinflammatory or autoimmune disorders. The pathogenesis of autoimmune diseases is characterized by loss of tolerance against self-tissues, appearance of autoreactive T and B cells, and production of auto-antibodies. On the contrary, autoinflammatory diseases are mainly caused by altered innate immunity and are characterized by the activation of inflammasomes, and lack of self-reactive antibodies and T cells (52). Although there is a wide range of treatment options to manage symptoms, currently there is no cure for these disorders. As we introduced above several reports suggest that gingerols and shogaols might also alleviate disease symptoms due to their strong anti-oxidant and anti-inflammatory activities. These assumptions were later also supported by data from animal models of inflammation such as sepsis (38, 53, 54) or neuroinflammation (32, 34). Here we summarize the most recent in vivo animal data on the efficacy of gingerols and shogaols in the treatment of autoinflammatory and autoimmune diseases (Table 3).

Ulcerative colitis (UC) is one of the main forms of inflammatory bowel disease, which is characterized by chronic recurrent inflammation of the large intestine. The etiology and pathogenesis of these diseases is not yet fully understood, although it appears to involve both autoinflammatory and autoimmune traits (55). A study investigated the therapeutic efficacy of intraperitoneally-injected 6-, 8-, and 10-gingerols in a dextran sulfate sodium (DSS)-induced rat colitis model (56). Results show that all three gingerols attenuated DSS-induced symptoms of colitis and accelerated mucosal damage healing. Gingerols elevated the activity of the anti-inflammatory enzyme superoxide dismutase, while reduced the activity of myeloperoxidase, a marker of neutrophil infiltration, in the colon tissue. Besides, all three gingerols reduced the DSS-induced serum levels of the pro-inflammatory cytokines TNF and IL-1β.

A subsequent study further explored the mechanism behind the anti-inflammatory effects of 6-gingerol in the DSS-induced mouse colitis model (57). Orally-administered 6-gingerol significantly decreased the DSS-induced weight loss of mice. It was demonstrated that 6-gingerol decreased IL-17 levels, while increased IL-10 levels both in the serum and bowel tissues of DSS-treated mice.

Moreover, 6-gingerol inhibited the DSS-induced phosphorylation of IkB and the phosphorylation and nuclear translocation of p65 in the bowel tissue.

Another study investigated the efficacy of orally-administered 6-shogaol-loaded nanoparticles in the mouse model of DSS-induced colitis (58). Similar to gingerols, 6-shogaol also alleviated colitis symptoms and accelerated wound repair. In addition, 6-shogaol significantly decreased the levels of TNF, IL-6, IL-1β and iNOS, while increased the expression of the anti-inflammatory genes HO-1 and NRF2 in colon tissues of DSS-treated mice.

A recent study compared the anti-colitis efficacy of 6-, 8-, 10-gingerols and shogaols in the DSS-induced colitis mouse model (59). All components were able to prevent DSS-induced weight loss, colon length reduction and IL-1β, IL-6 and IFN-γ serum levels in DSS-treated mice. Among the ginger compounds, 8-, and 10- shogaols, which were also able to significantly decrease DSS-induced serum levels of TNF, showed the greatest efficacy compared to the other compounds. 6-, 8-, and 10-gingerols and 6-, 8-, and 10-shogaols also downregulated iNOS and COX-2 protein levels and significantly reduced phosphorylation of NF-kB in colonic tissue. Again, 10-shogaol was found to be the most potent in its ability to block iNOS and COX-2 expression and NF-kB activity. Besides it was the most effective in increasing the expression of tight junction proteins, thus the intestinal integrity of DSS-treated mice. The study concluded that 8-, and 10-shogaols are the most potent in their ability to suppress colitis symptoms and inflammation, thus may serve as better candidates for the treatment of colitis than the corresponding gingerols.

Multiple sclerosis (MS) is a T cell-mediated autoimmune disease of the central nervous system that is characterized by immune-mediated demyelination in the spinal cord and cerebral cortex (60). The neuroprotective effects of ginger compounds were extensively studied in animal models of neurotoxicity and brain damage (reviewed in (19)); however, so far only two studies investigated the immunomodulatory activity of gingerols and shogaols in experimental autoimmune encephalomyelitis (EAE), the mouse model of MS. The first study showed that 6-gingerol decreased the inflammatory infiltration and demyelination in the white matter of spinal cord in EAE mice. 6-gingerol-treated mice had lower number of inflammatory cells including DCs and T cells in the spleen. Further, splenocytes from 6-gingerol treated mice produced significantly lower levels of IL-17 and GM-CSF indicating that 6-gingerol inhibited Th17 polarization *in vivo* (38). In addition, 6-gingerol lowered the percentage of leukocytes such as CD11c+MHC II+ DCs, CD45+CD11b+ monocytes and Th17 cells in the central nervous system (CNS) of EAE mice indicating that 6-gingerol inhibits inflammatory cell infiltration in the CNS. The next study investigated whether 6-shogaol or its metabolite, 6-paradol could ameliorate EAE symptoms and inflammation (61). Administration of 6-shogaol or 6-paradol significantly reduced the clinical signs of the disease. Histological analysis show that both components decreased demyelination, cell accumulation and TNF expression in spinal cord of EAE mice. In addition, 6-shogaol and 6-paradol markedly reduced astrogliosis and microglial activation of EAE mice. These findings suggest that the neuroprotective effects of ginger phenolics in EAE might be associated to the dampened inflammatory responses in the CNS.

The protective role of ginger phenolics were also investigated in the context of systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). SLE affects almost every organ in the body and is characterized by the appearance of anti-nuclear autoantibodies and circulating immune complexes. It was demonstrated that 6-gingerol administration significantly reduced serum levels of anti-dsDNA, cell-free DNA and also myeloperoxidase-DNA complexes, the latter of which serves as a marker of NET formation (44). 6-gingerol also greatly reduced the serum levels of the pro-inflammatory cytokines TNF and IFN-γ. In line with that, 6-gingerol suppressed netosis and thus large-vein thrombosis in a mouse model of antiphospholipid syndrome as well. Pharmacokinetic studies further revealed that 6-gingerol accumulated in neutrophils, while its plasma level was dropping. These results are in line with pervious reports showing that ginger phenolics are converted into glucuronide conjugates and thus are rapidly cleared from the plasma (62, 63). In tissues, these conjugated forms are then reconverted into their free active form by specific enzymes such as β-glucuronidase.

RA is also a systemic chronic immune-mediated disorder, which is characterized by synovial inflammation, joint damage, loss of function and deformities. IL-17 is one of the key cytokine in promoting inflammation and thus cartilage damage in RA (64). Therefore, in a recent study, 315 natural extracts were tested in their ability to inhibit IL-17-induced IL-6 production by synovial cells (65). Among these extracts, dried ginger and in particular its specific component 8-shogaol showed the highest inhibitory activity against IL-17-mediated IL-6 secretion by synovial cells and macrophages. Thereafter, the anti-arthritic potency of 8-shogaol was investigated in adjuvant induced arthritis (AIA), a rat model of RA. Treatment with 6-shogaol reduced paw thickness and weight loss of AIA rats and improved their walking performance as well. Furthermore, 8-shogaol treatment decreased bone erosion and cellular infiltration to the joints of AIA rats. Upon 8-shogaol treatment the levels of TNF, IL-6 and IL-8 were markedly downregulated both in the serum and synovial tissues of AIA rats. These data suggest that 8-shogaol has the potential to ameliorate disease severity and inflammation in RA.

4. Proposed mechanism of action of gingerols and shogaols

As we have introduced above ginger constituents are able to interfere with common inflammatory signaling pathways including the NF-kB, MAPK and PI3K/Akt/mTOR cascades. It is still a question, though, what it the exact mechanism of action behind their inhibitory activity. Lately, mounting evidence indicates that ginger constituent might affect these signaling pathways by targeting different upstream regulatory molecules such as AMPK, NRF2, or PPARγ. Recent studies also suggest that these compounds exert their anti-inflammatory actions through binding to the TRPV1 ion channel. In this chapter we summarize our current knowledge about the possible mechanisms of immunomodulation by ginger phenolics.

4.1. AMPK

AMPK is a central regulator of diverse physiological and metabolic processes, which can be dysregulated in pathological conditions, such as cancer, obesity or chronic inflammatory diseases (66). Generally, AMPK blocks mTORC1 activation by phosphorylating two of its regulatory proteins. On the one hand AMPK phosphorylates and thereby increases the activity of TSC2, an upstream inhibitor of mTORc1. On the other hand, it phosphorylates raptor, a scaffold protein of mTORc1 that also leads to mTORc1 inactivation and consequently to the suppression of anabolic processes and inflammation (67). Accumulating evidence suggest a negative correlation between AMPK activity and inflammation (66). Chronic inflammation is associated with reduced AMPK activity and thereby aberrant mTOR activity. Thus, targeting AMPK might serve as a potential therapeutic strategy for treating inflammatory-related diseases (68). Many phytochemicals such as resveratrol, quercetin, curcumin act as natural activators of AMPK (69). Recent studies also suggest that ginger phytochemicals might also exert their anti-inflammatory effects on immune cells via suppressing mTOR and increasing AMPK activity.

Initially, studies reported that ginger phenolics inhibit inflammation by inhibiting the phosphorylation of key mTOR pathway components. First it was demonstrated that 6-shogaol inhibited the LPS-induced phosphorylation and activation of NF-κB by interfering with the PI3K/Akt and MAPK signaling pathways in murine macrophages (30). Thereafter, it was shown that 6-shogaol inhibited TNF-induced disassembly of tight junctions via inhibition of NF-κB and PI3K/Akt in the human colonic epithelial cell line HT-29/B6 (70). Another study further demonstrated that 6-gingerol suppressed LPS-induced microglia activation by down-regulating the Akt/mTOR pathway activity (34).

Later it was also demonstrated that ginger phenolics indirectly suppress mTOR activity by promoting the activation of AMPK. First it was demonstrated that ginger extract restored the high fat diet (HFD)-induced downregulation of AMPK activity in the liver of rats (71). Another study presented that 6-gingerol attenuates hepatic steatosis, inflammation and oxidative stress in HFD-

fed mice via activating AMPK (72). In particular, intragastrical administration of 6-gingerol decreased the concentration of TNF, IL-6 and ROS, while increased the phosphorylation level of liver kinase B1 (LKB1) and AMPK in the liver of HFD-fed mice. LKB1 is one of the master upstream kinases of AMPK, which forms a trimeric complex with two other proteins and then phosphorylates and activates AMPK. The authors suggest that 6-gingerol enhanced the stability of the LKB1 complex that consequently increased LKB1/AMPK pathway activity in the liver of mice. These results were also strengthened by in vitro experiments using palmitic acid-treated HepG2 cells, in which 6-gingerol reduced lipid accumulation and oxidative stress by increasing LKB1 complex stability and thus LKB1/AMPK pathway activity.

Recent observations further suggest that the anti-diabetic and anti-cancer efficacy of ginger compounds are also associated with their ability to induce AMPK activation. It was reported that 6-shogaol and 6-paradol promote glucose utilization in mouse adipocytes that could be attributed to the increased activity of AMPK (73). Furthermore, 6-gingerol was found to suppress oral cancer cell growth by inducing the activation of AMPK and blocking mTOR pathway activity (74), while 10-gingerol was shown to inhibit the proliferation and migration of vascular smooth muscle cells by AMPK activation (75). The latter study also demonstrated a stable binding between 10-gingerol and AMPK by molecular docking studies and surface plasmon resonance imaging analysis. This was the first study showing a direct interaction between 10-gingerol and AMPK that proposed 10-gingerol as a natural agonist of AMPK. Nevertheless, there are no available data on the binding affinity of other ginger phytochemicals to AMPK.

Although, some reports demonstrated that ginger phenolics decrease PI3K/Akt/mTOR signaling pathway activity in immune cells, the regulatory role of AMPK activity was not investigated in these studies. We have recently reported that 6-shogaol was able to inhibit mTORC1 signaling through upregulating AMPK activity in moDCs, while 6-gingerol was not able to do so (Pazmandi, 2023). However, further studies are needed to reveal, whether the anti-inflammatory activities of gingerols and shogaols could be generally linked to increased AMPK activity.

4.2. NRF2

NRF2 is a transcription factor that plays a pivotal role in the regulation of anti-oxidant and anti-inflammatory responses (76). Besides inducing the expression of an array of enzymes involved in antioxidant defense and detoxification it is able to negatively regulate NF-κB activity by multiple mechanisms. For instance, by inducing anti-oxidant enzymes NRF2 decreases ROS levels that consequently inhibits oxidative stress-mediated NF-κB activation. NRF2 is also able to prevent IκBα proteasomal degradation and thus the nuclear translocation of NF-κB. In addition, a series of in vitro and in vivo studies indicated that NRF2-mediated HO-1 expression substantially contributes to the anti-inflammatory activity of NRF2. As an important antioxidant enzyme HO-1 catalyzes the degradation of toxic free heme that leads to the release of anti-inflammatory products, such as carbon monoxide and biliverdin (77). Beyond its enzymatic functions, HO-1 exerts non-canonical, signaling functions, through which HO-1 is able to control essential cellular signaling processes such as gene expression, protein translation, and DNA repair mechanisms (78).

In the last few years many studies proposed that ginger-derived bioactive compounds exert their anti-inflammatory effects by activating the NRF2/HO-1 axis. A study suggest that this is especially true for shogaols, which in contrast to gingerols, bear an α,β-unsaturated carbonyl group in their side chain (79). Molecular modeling revealed that 6-shogaol is able to increase HO-1 levels through a Michael reaction between its α, β-unsaturated carbonyl group and Kelch-like ECH-associated protein 1 (Keap1), which acts as a negative regulator of NRF2. This interaction initiates conformational changes in Keap1 that allows the release of NRF2 from Keap1 leading to its nuclear translocation. In the nucleus NRF2 binds to the antioxidant response element (ARE) and induces the expression of various antioxidant and anti-inflammatory genes. In that particular study, 6-shogaol was found to upregulate the protein levels of HO-1 and suppress the thrombin-induced NO release in rat microglia, while 6-gingerol was not able to do so. 6-shogaol also increased the nuclear

import of NRF2 in BV2 microglia and significantly enhanced the HO-1 mRNA level in primary-cultured microglia. Similarly, we have recently demonstrated that 6-shogaol increases the expression and nuclear translocation of NRF2 and enhances the protein levels of HO-1, while 6-gingerol does not affect that in human moDCs (ref). In an in vivo mouse ICH model, 6-shogaol increased striatal HO-1 protein levels and rescued neuron loss (79). Another study demonstrated that 6-shogaol protects against renal ischemia-reperfusion (I/R) injury as well. 6-shogaol pretreatment significantly decreased the expression of various pro-inflammatory cytokines and chemokines in mice subjected to renal I/R. Mechanistically, 6-shogaol reduced kidney inflammation by attenuating NF-κB activation and inducing HO-1 expression (80). The same year it was published that 6-shogaol prevented UVB-induced inflammation and oxidative stress through modulating NRF2 signaling in human epidermal keratinocytes (HaCaT cells), (81). 6-shogaol significantly decreased the UVB-triggered expression of IL-6, TNF and IL-10, while reduced the phosphorylation of ERK, JNK and p38 MAPKs in human epidermal keratinocytes. The authors further demonstrated that 6-shogaol prevented UVB-induced depletion of NRF2 and elevated HO-1 protein levels in HaCaT cells. In another in vitro model, 6-shogaol treatment increased the expression of HO-1 in LPS-stimulated human umbilical vein endothelial cells (HUVECs) as well (82). Similar to 6-shogaol, 6-gingerol was also found to activate the NRF2/Keap1 signaling pathway. It was demonstrated that 6-gingerol significantly reduced the expression of Keap1 and increased that of NRF2 in the nuclear fraction of buccal pouch tissues of 7,12-dimethylbenz(a)anthracened (DMBA)-treated hamsters (83). By doing so, 6-gingerol prevented buccal pouch carcinogenesis through inhibiting the expression of inflammatory and cell proliferation markers such as IL-6, TNF-α, IL-1β, iNOS, COX-2 and cyclin D, while inducing pro-apoptotic markers such as Bax in DMBA-induced hamsters. These findings were further strengthened by another study showing that 6-gingerol also alleviates sepsis-induced liver injury through activating the NRF2 pathway (54). Pre-treatment with 6-gingerol attenuated cecal ligation and puncture (CLP)-induced hepatic inflammation and injury by increasing the protein levels of NRF2 and HO-1 in liver homogenates of C57BL/6 mice. These results were further confirmed by an in vitro model. Here, 6-gingerol suppressed ATP-induced pyroptosis, IL-1β and caspase-1 secretion and ROS production by preventing the downregulation of NRF2 and HO-1 protein levels in LPS-primed RAW 264.7 cells. Furthermore, it was shown that 6-gingerol decreased cardiac injury via the NRF2/HO-1 pathway in both mouse and cell models of diabetic cardiomyopathy (DCM), (84). In the mouse heart 6-gingerol inhibited the expression of ferroptosis-related proteins, while enhanced the expression of anti-ferroptosis-related proteins. In addition, 6-gingerol treatment decreased the levels of IL-1β, IL-6, and TNF-α in serum and heart tissues of diabetic mice. In the H9c2 cardiac cells, 6-gingerol also inhibited ferroptosis and inflammation evoked by palmitic acid and high glucose administration. Most importantly, 6-gingerol enhanced the NRF2 and HO-1 protein levels both in the mouse and cell models of DCM. Hence, the authors suggest that 6-gingerol might protect against DCM by inhibiting ferroptosis and inflammation via activating the NRF2/HO-1 pathway. A novel study suggest that 6-gingerol attenuates sepsis-induced acute lung injury by suppressing NLRP3 inflammasome activation via NRF2 activation (85). In LPS-induced rats, 6-gingerol repressed the expression of various oxidative stress markers, inflammatory cytokines and NLRP3 inflammasome components in the lung tissues and inhibited the infiltration of inflammatory cells into the lungs. In addition, 6-gingerol prevented the LPS-mediated downregulation of NRF2 and HO-1 levels in the lung tissues of rats. Intraperitoneal injection of ML385, an NRF2 inhibitor, reversed the protective effect of 6-gingerol against LPS-induced oxidative stress and inflammation suggesting that 6-gingerol exerts its anti-inflammatory effects through activating the NRF2/HO-1 axis. Similar to 6-gingerol, zingerone, a less-studied natural compound of ginger, also reduced organ injury via activation of the NRF2 signaling pathway in a CLP-induced sepsis model (46). In particular, administration of zingerone alleviated ROS accumulation and systematic inflammation in septic mice and inhibited NET formation both in vivo and in vitro. The results further suggest that zingerone attenuates NET formation and inflammation via NRF2-dependent ROS inhibition.

As the above data indicate many studies have demonstrated that the bioactive compound of ginger are able to activate the NRF2/HO-1 axis that seems to greatly contribute to their anti-inflammatory effects. Lately, emerging evidence indicate a cooperation between AMPK and NRF2 signaling as well. In particular, AMPK might function as a positive upstream regulator of the NRF2/HO-1 system and thus can lead to the transactivation of specific target genes (86). Interestingly, the AMPK-mediated enhancement of the NRF2/HO-1 response does not seem to depend on mTOR inhibition (87). In our own studies, we found that 6-shogaol is able to increase the activity of both AMPK and NRF2 in human DCs (Pazmandi, 2023). Nevertheless, it needs to be further elucidated whether these components contribute separately to the ant-inflammatory potential of 6-shogaol or AMPK acts as an upstream regulator of NRF2.

4.3. PPARy

PPARy is a member of the nuclear receptor superfamily that is widely expressed in immune and endothelial cells (88). It is able to regulate various biological functions, such as inflammatory responses and lipid metabolism. Most importantly, PPARγ can suppress inflammation by promoting the inactivation of NF-κB through direct or indirect mechanisms. PPARγ can cause ubiquitination and proteolytic degradation of p65 NF-κB and it can also promote the expression of inhibitory proteins such as IκBα or HO-1. Many dietary phytochemicals target PPARγ (89), only a few studies investigated whether gingerols and shogaols act as PPARγ agonists.

In rats subjected to ventilator-induced lung injury GW9662, a selective PPARγ inhibitor abolished the protective effect of 6-gingerol (90). In particular, the 6-gingerol-mediated decrease in pro-inflammatory cytokine release, neutrophil accumulation and oxidative stress in lung tissues of rats challenged with mechanical ventilation was partially reversed by the inhibition of PPARγ. 6-shogaol was also suggested to exert its anti-inflammatory effects in LPS-activated BV2 microglia by activating PPARγ (33). In these cells 6-shogaol significantly reduced LPS-induced NF-κB activation, pro-inflammatory cytokine and PGE2 release that could be reversed by GW9662 treatment. In contrast, another study presented that GW9662 could not reverse the inhibitory effect of 6-shogaol on TNF-induced disassembly of tight junctions in human colonic HT-29/B6 cells (70).

Although, data on the relation of ginger phytochemicals to PPARγ activity are scares and contradictory, we cannot exclude the possibility that the anti-inflammatory effects of gingerols and shogaols are at least partially dependent on PPARγ activity.

4.4. TRPV1

TRPV1 is primarily expressed in sensory neurons and plays an essential role in heat sensation and nociception. Accumulating data suggest that TRPV1 is also expressed in various mammalian immune cells, especially in macrophages, DCs, T cells and neutrophils, where it modulates various functions such as cytokine release, migration, or phagocytic activity (91).

The major pungent compounds of ginger share certain structural characteristics with capsaicin. In particular, gingerol, shogaol and zingerone contain the same vanillyl head group as capsaicin; therefore, these ginger constituents can directly activate the TRPV1 channels, though with a much lower potency than capsaicin (92). A study showed that capsaicin show a strong potency, while 6-shogaol and 6-gingerol have a moderate potency against TRPV1 (93). Further it was demonstrated that 6-shogaol, 6-gingerol, and zingerone bind to the same ligand-binding pocket in TRPV1 channels as capsaicin; however, the distinct structural features in their tails cause large differences in their potency (94). 6-shogaol is the most similar to capsaicin regarding its molecular structure, thus it is represents the strongest agonist of TRPV1 among the ginger compounds. In comparison to 6-shogaol, 6-gingerol is slightly weaker in its potency to induce TRPV1 activity due to the presence of a hydroxyl group instead of a double bond in its tail. Finally, zingerone acts as a weak agonist for TRPV1 channels due to the lack of most of its aliphatic tail. Altogether, the length of the aliphatic tail, the additional hydroxyl group and the position of the C═C bond in the tail all

contribute to the stability of the ligand–channel interaction, and consequently to the ligand-induced channel activity.

In a rat model of I/R injury 6-gingerol was found to inhibit NLRP3-mediated inflammation and neuronal apoptosis while upregulated autophagy (95). The mechanistic findings indicate that 6-gingerol exerts anti-apoptotic and anti-inflammatory effects during cerebral I/R injury via dissociating TRPV1 from Fas-associated factor 1 (FAF1), which is able to modulate the sensitivity and thus the activity of TRPV1 to various stimuli.

The ligand-induced activation of TRPV1 by ginger compounds in non-neuronal cell types is poorly characterized. So far, only one study investigated whether the ligand-induced activation of TRPV1 by 6-gingerol is able to affect the functionality of neutrophils (47). The analyses showed that 6-gingerol increased intracellular Ca2+ concentrations, the expression of common surface markers such as CD11b and CD66b in neutrophils. Moreover, 6-gingerol enhanced fMLP-stimulated CXCL8 secretion and ROS production that could be reversed by pharmacological inhibition of TRPV1. Here the authors applied 6-gingerol at a low dose of 50 nM, which is a dietary relevant concentration that could be reached in blood plasma after consumption of 1 liter of ginger tea. In contrast, 50 μM of geranylacetone, a natural sesquiterpenoid, acting as a TRPV1 agonist inhibited fMLP-induced migration of neutrophils and CXCL8-induced intracellular Ca2+ mobilization (96). These observations suggest that lower concentrations of TRPV1 ligands might suppress immune cell functions, while higher TRPV1 ligand concentrations in the μM range rather inhibit immune responses.

5. Concluding remarks

In this review, our goal was to compile the most relevant findings on the immunomodulatory actions of ginger compounds. In the last decade, numerous *in vitro* and *in vivo* studies have reported that the major bioactive compounds of ginger, especially 6-gingerol and 6-shogaol, exert potent anti-inflammatory activity on different immune cell types. Though, comparative studies are spares, most of the findings indicate that 6-shogaol exhibit a stronger anti-inflammatory and anti-oxidant capacity than 6-gingerol that might be attributed to the presence of the α,β-unsaturated ketone moiety in its skeleton. Although the exact mechanism of action of ginger-derived bioactive compounds are not fully elucidated yet, data show that these compounds result in the successful inhibition of common signaling pathways such as the NF-κB and PI3K/Akt/mTOR signaling cascades that consequently leads to the suppression of inflammatory responses by immune cells. Interestingly, contradictory results were obtained regarding the action of gingerols and shogaols on the activity of MAPK signaling components; however, the reason for the varying results are unknown so far. Research data summarized above further suggest the involvement of AMPK, PPARγ, NRF2/HO-1 system and TRPV1 for the actions of ginger phenolics implying that ginger phytochemicals exert their pleiotropic effects via targeting multiple molecular signaling pathways. Ginger has been used traditionally as an herbal medicine for the treatment of many maladies, and due to their potent immunomodulatory capacity several reports proposed ginger compounds as candidates for the treatment of autoimmune diseases. Nevertheless, clinical studies are lacking probably due to the poor water solubility and adsorption of ginger phenolics. To improve the bioavailability and thus the therapeutic efficiency of ginger compounds more and more projects are focusing on the development of novel formulation techniques and drug-delivery systems (91, 97-100). Thus, it is feasible that in the future ginger supplements might provide an alternative or most probably a complementary therapeutic approach to treat autoimmune disorders.

6. Author Contributions

T.F. wrote the manuscript in consultation with K.P. A.B. and K.P provided conceptual insight, revised the manuscript and were responsible for final approval. All authors have read and agreed to the published version of the manuscript.

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8. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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874 **Table 1. Effect of ginger compounds on macrophages and dendritic cells**

Macrophages					
Compound	**Cell type**	**Challenge**	**Observed effects**	**Potential mechanism**	**Ref.**
6-gingerol	J774.1 macrophage cell line	LPS	• reduced NO, iNOS levels • protection against DNA and protein damage	-	(27)
6-gingerol	mouse peritoneal macrophage	LPS	• decreased IL-1β, IL-12, TNF, CCL5	• suppressed NF-kB activity	(28)
6-, 8-, 10-gingerols and 6-shogaol	monocytic U937 cell line	LPS	• reduced PGE2 production • reduced COX-2 expression	-	(29)
6-shogaol	RAW 264.7 macrophages cell line	LPS	• reduced PGE2 production • reduced COX-2, iNOS levels	• blocked IkBα phosphorylation and degradation • suppressed NF-kB activity • inhibited PI3K/Akt and ERK1/2 activation	(30)
6-gingerol	RAW 264.7 macrophages cell line	LPS	• inhibited iNOS and TNF expression • reduced intracellular Ca2+ mobilization • decreased ROS generation	• suppressed I-κBα phosphorylation, NF-κB nuclear translocation and PKC-α translocation	(31)
6-shogaol	BV2 murine microglial cell line and primary microglia	LPS	• reduced IL-1β, TNF and PGE2 production • reduced NO, iNOS levels • downregulated COX-2 expression	• decreased NF-kB activity • inhibited p38 and JNK activation	(32)
6-shogaol	BV2 murine microglial cell line	LPS	• suppressed IL-1β, IL-6, TNF and PGE2 production	• inhibited PPARγ activity	(33)
6-gingerol	primary mouse microglia cell	LPS	• reduced NO, iNOS levels • reduced IL-1β and IL-6 production	• reduced Akt, mTOR and STAT3 phosphorylation	(34)
6-shogaol	primary mouse calvarial osteoblast	IL-1α	• reduced PGE2 expression • decreased RANKL production and osteaoclast differentiation	• suppressed COX2 and PGE2 synthase activity	(36)
6-, 8-, 10-gingerols/ shogaols	THP1 human monocytic cell line	LPS+ATP	• reduced TNF and IL-1β secretion • reduced NLRP3 and caspase-1 levels	-	(37)
6-gingerol	RAW 264.7 macrophages cell line and mouse BM-DM	LPS+ATP	• reduced IL-18 and IL-1β secretion • reduced caspase-1p20 release	• suppressed ERK1/2 activation	(38)
Dendritic cells					
6-gingerol	mouse BM-DC	LPS	• reduced TNF, IL-1β, IL-6 and IL-23 secretion • reduced CD80, CD86 and MHC II expression • reduced Th17 priming capacity	• inhibited NF-kB, JNK and ERK1/2 activation	(41)
6-gingerol, 6-shogaol	human moDC	various TLR ligands	• reduced TNF, IL-6 and IL-10 production • decreased costimulatory molecule and MHC II expression • decreased capacity to prime Th1 T cells	• inhibited NF-κB nuclear translocation • reduced mTOR and MAPK activity • increased AMPK and NRF2 activity	Paz 2023

875 **Table 2. Effect of ginger compounds on neutrophil granulocytes and T cells**

Neutrophil granulocytes					
6-,8-,10-gingerols and 6-shogaol	human neutrophil	fMLP	• suppressed ROS release	-	(16)
6-, 8-, and 10-gingerol	human neutrophil	LPS, PMA	• suppressed netosis • reduced ROS generation	• decreased PDE4 activity • increased cAMP levels and PKA	(44)
zingerone	mouse BM-derived neutrophil	PMA	• suppressed ROS production • reduced netosis	• activated the NRF2 signaling pathway	(46)
6-gingerol	human neutrophil	fMLP	• increased ROS and CXCL8 production • increased CD11b, CD66, formyl peptide receptor 1 expression	• binding to TRPV1	(47)
T cells					
6-, 8- and 10-gingerols	mouse spleen-derived CD3+ T cell	Dynabead, syngeneic DCs	• decreased proliferation • reduced expression of CD25 and CD69 • reduced IFNγ, IL-2 and IL-12 production	-	(48)
6-gingerol	mouse spleen-derived CD4+ T cell	ovalbumin, Th1 or Th2 polarizing conditions	• decreased proliferation • reduced IFNγ and IL-4 production	-	(49)
6-gingerol	Jurkat human T cell line	PMA, ionomycin	• reduced proliferation • decreased T cell activation	• inhibited MAPK activation • suppressed NF-kB and AP-1 activation	(49)

876

877 **Table 3. In vivo effects of ginger phenolics in animal models of autoimmune diseases**

Compound	Model	Administration route	Observed effects	Potential mechanism	Ref.
6-, 8-, and 10-gingerols	DSS-induced rat colitis model	intraperitoneal	• attenuated DSS-induced symptoms of colitis • accelerated mucosal damage healing • decreased neutrophil infiltration in the colon • reduced TNF and IL-1β serum levels	• increased SOD activity	(56)
6-gingerol	DSS-induced mouse colitis model	oral	• decreased IL17 levels and increased IL-10 levels both in serum and bowel tissue	• inhibited IkB phosphorylation • suppressed NF-κB nuclear translocation in the bowel tissue	(57)
6-shogaol	DSS-induced mouse colitis model	oral	• alleviated colitis symptoms • accelerated wound repair • decreased the levels of TNF, IL-6, IL-1β and iNOS	• increased HO-1 and NRF2 expression in the colon tissues	(58)
6-, 8-, 10-gingerols and shogaols	DSS-induced mouse colitis model	oral	• prevent DSS-induced weight loss, colon length reduction • reduced IL-1β, IL-6 and IFN-γ serum levels • downregulated iNOS and COX-2 levels	• reduced NF-κB phosphorylation	(59)
6-gingerol	EAE mice	intraperitoneal	• decreased inflammatory cell infiltration and demyelination in spinal cord and CNS • lowered number of inflammatory cells in the spleen • inhibited Th17 polarization	-	(41)
6-shogaol, 6-paradol	EAE mice	oral	• decreased demyelination, cell accumulation and TNF expression in spinal cord • reduced astrogliosis and microglial activation	-	(61)
8-shogaol	AIA mice	intraperitoneal	• reduced paw thickness and weight loss • improved walking • decreased bone erosion and cellular infiltration to the joints • reduced, TNF, IL6 and IL-8 levels in serum and synovial tissues	-	(65)

878