Research Progress on the Molecular Mechanisms of Traditional Chinese Medicine in Regulating Inflammatory Signaling Pathways

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Abstract. The Traditional Chinese Medicine (TCM) has also proven to have amazing effects in the treatment of inflammatory diseases by using multi-component multi-target therapeutic action. This thorough review examines the molecular processes through which TCM modulates the inflammatory signaling pathways based on integrated network pharmacology, molecular docking and experimental validation methods. The network analysis revealed 312 TCM-inflammation targets and the highest connectivity on the hub genes was observed in IL-6, TNF, and VEGFA. KEGG pathway enrichment showed strong modulation of NF-kB, MAPK, PI3K/Akt and JAK/STAT signaling cascades. Molecular docking established good binding affinities among the TCM compounds and the inflammatory targets with quercetin and curcumin exhibiting outstanding interactions. In vitro validation revealed the presence of dose-dependent inhibition of pro-inflammatory cytokines, NF-kB activation inhibition, and induction of macrophage polarization between M1 and M2 phenotype. Findings support the view that TCM has an anti-inflammatory effect by the simultaneous modulation of several interconnected signaling nodes, which suggests scientific support of its clinical use in more complex inflammatory diseases and an explanation of the traditional concepts of holism as well as current molecular knowledge.

Keywords: Traditional Chinese Medicine, Inflammatory Signaling Pathways, Network Pharmacology, Macrophage Polarization.

1. Introduction

Inflammation is one of the basic biological reactions that is very important in the mechanism of body defense against pathogenic organisms, tissue injuries and toxic stimuli. Although acute inflammation is a protective process that is critical to tissue repair and clearance of pathogens, chronic inflammation has become a major pathological process that causes many diseases, including cardiovascular diseases, metabolic diseases, neurodegenerative, autoimmune, and malignant diseases. Inflammation is the primary target of treatment in contemporary medicine due to the role played by the dysre Sgulation of the inflammatory signaling pathways in the pathogenesis and development of these conditions. Although there has been a tremendous development in the molecular aspect of the study of inflammatory processes, the available anti-inflammatory reagents are still limited by a number of shortcomings, such as the presence of undesirable side effects, resistance to the drug, and poor efficacy, especially in the management of chronic inflammatory diseases.

Traditional Chinese Medicine (TCM) is a treatment that has been utilized thousands of years and provides a holistic treatment approach, basing its provisions on the principles of maintaining the balance of the internal environment of the body and achieving homeostasis. In contrast to traditional western medicine which usually aims at one of the molecular pathways, TCMs are multi-component, multi-target, and multi-pathway in nature. Recent breakthroughs in molecular biology, pharmacology, and systems biology have enabled the scientific explanation of the scientific basis of TCM on the basis of anti-inflammatory activities, showing complicated control at the cellular and molecular systems. Research has shown that TCM compounds control some of the major inflammatory signaling pathways, such as nuclear factor kappa-B (NF-KB), mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), Janus kinase/signal transducer and activator of transcription (JAK/STAT) and the NOD-like receptor protein 3 (NLRP3)

inflammasome [1]. All these pathways coordinate the synthesis of pro-inflammatory cytokines, chemokines, and inflammatory mediators, and are, therefore, important interventions in therapy.

The NF- 0 B pathway is one of the many signaling cascades that have been recognized to play a key role in the regulation of inflammatory responses by regulating the expression of many pro-inflammatory gene such as tumor necrosis factor-alpha (TNF- 0), interleukin-1 beta (IL-1B), interleukin- 6 (IL- 0), cyclooxygenase-2 (COX- 2), and inducible nitric oxide synthase (iNOS)[2]. It is demonstrated that TCM interventions regulate this pathway on several levels, including the prevention of degradation of inhibitor of κB (IKB) proteins and the inhibition of nuclear translocation of the NF-kB subunits. On the same note, MAPK signaling pathway, which includes extra cellular signal-regulated kinase (ERK), c- Jun N-terminal kinase (JNK), and p38 MAPK subfamilies, is another important target of TCM-based anti-inflammatory interventions. These kinases control various cellular functions such as cell proliferation, differentiation, apoptosis and inflammatory response. PI3K/Akt pathway that has crucial roles in cell survival, metabolism, and immune regulation have also been an important focus of TCM compounds to treat inflammatory diseases [3]. Recent studies have indicated that this pathway is regulated by TCM to curtail the production of pro-inflammatory cytokines and increase the levels of anti-inflammatory mediators, respectively, to restore immune homeostasis.

The The NLRP3 inflammasome has become a vital focus of interest as a significant facilitator of inflammatory pathology and its dysfunctional expression has been associated with a variety of chronic inflammatory and metabolic disorders. This multiprotein complex activates caspase-1 and release of IL-1 018 pro-inflammatory cytokines that start a cascade of inflammatory reactions. There is growing evidence to suggest that many TCM prescriptions, herbs and bioactive compounds are effective in inhibiting the NLRP3 inflammasome activation and subsequent pyroptotic cell death through regulation of upstream signaling pathways including Toll-like receptor 4 (TLR4)/NF-κB, reactive oxygen species (ROS)/thioredoxin-interacting protein (TXNIP) and AMP-activated protein kinase (AMPK)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathways Moreover, TCM treatments exhibit impressive effects in polarising macrophages by switching their pro-inflammatory phenotype M1 to an anti-inflammatory phenotype M2 via a number of signalling pathways such as peroxisome proliferator-activated receptor gamma (PPAR- 7) and Notch signalling[4]. The macrophage repolarization is a potential future therapeutic approach to the solution of chronic inflammation and tissue repair.

The combination of modern molecular biology technology and the ancient TCM theories has revealed the advanced mechanisms of TCM therapeutic effect[5]. Network pharmacology, metabolomics, proteomics, and genomics methods have helped to reveal active compounds, targets of therapy, and regulatory networks of TCM-induced anti-inflammatory effects. These works have uncovered that the action of TCM formulations is associated with synergistic relationships between numerous bioactive ingredients, which act on various nodes of interconnected signaling networks rather than on individual compounds and thus produce excellent therapeutic effects. This understanding at the systems level has confirmed the classical philosophy of TCM in the holistic approach to therapy and differentiation of syndromes, with TCM offering scientific support to the differentiation of TCM in personalized medicine. Although these have been made, there are still issues in standardizing TCM preparations, understanding the specific molecular workings of the formulations of complex and extensive clinical trials to establish evidence-based therapeutic regimes.

2. Related Work

The study of the molecular mechanisms of Traditional Chinese Medicine in the regulation of inflammatory signaling pathways has undergone impressive advances over the last few years, and scientists have introduced more complex methodologies to unravel the intricate interplay between TCM compounds and cell signaling pathways. This comprehensive investigation was able to demonstrate that TCM has its therapeutic action majorly via numerous important signaling pathways,

comprising TLR4/NF-KB, MAPK, JAK/STAT, PI3K/AKT, AMPK, PPAR- g, and DLL4/Notch signaling pathways, and metabolic reprogramming, encompassing glucose metabolism, tricarboxylic acid metabolism, lipid metabolism, and amino acid metabolism. The paper has highlighted that the multi-pathway and multi-target pharmacological effects of TCM have strong benefits in treating inflammatory diseases, which form a mechanistic basis of creating natural anti-inflammatory drugs based on the polarization of macrophages[6]. This holistic methodology has confirmed the TCM classical philosophy, based on holistic treatment and syndrome differentiation, with scientific evidence of personalized medicine use.

More recent studies have paid more attention to the polarization of macrophages as a key mechanism that mediates the anti-inflammatory effects of TCM. Zhang et al. performed a systematic review and investigated how TCM regulates macrophage metabolic reprogramming in the process of acute liver failure, showing that macrophage metabolism switching macrophage metabolism towards oxidative phosphorylation instead of glycolysis, which inhibits the activation of M1 and promotes the activation of M2 macrophages, which has an anti-inflammatory and reparative effect [7]. The study found some important signalling pathways that control the polarization of macrophages by means of energy metabolism, such as PI3K/AKT, mTOR/HIF-1alpha, NF- kB, and AMPK pathways that have a wide crosstalk with each other. This metabolic view has provided new opportunities to learn how TCM formulations can regulate immune responses through cellular energy metabolism mechanism, unlike traditional anti-inflammatory therapies. Some TCM monomers with the potential of reprogramming glucose metabolism and affecting the polarization of M1 and M2 macrophages were also systematically reviewed in the study, and it is possible to develop specific metabolic interventions to treat inflammatory liver diseases.

Recent literature has been drawing a lot of attention to the application of TCM in cardiovascular inflammatory diseases. Bai et al. provided detailed discussion of the effect of TCM in the regulation of macrophage polarization in heart failure with preserved ejection fraction (HFpEF), which is a multifaceted cardiovascular condition characterized by diastolic dysfunction, systemic inflammation, and myocardial fibrosis [8]. Some of the TCM metabolites and formulations that were identified during the review and have been shown to block macrophage activity via, among other signaling pathways, NO/cGMP/PKG, TGF-b/Smads, and PI3K/Akt have been demonstrated to have anti-inflammatory, antifibrotic, and antioxidant effects. The study highlighted that M1 macrophages activated through TLR4 ligands like lipopolysaccharide release pro-inflammatory cytokines like IL-6 and TNF-alpha that worsen inflammation whereas M2 macrophages activated by IL-4 and IL-13 release pro-tissue remodeling cytokines, although excessive M2 stimulation can lead to myocardial stiffening. This subtle insight into the dynamics of macrophage polarization should have significant value in designing more specific TCM-based therapies to the HFpEF condition, which is a disease with no effective solution in conventional medicine.

Network pharmacology and systems biology strategies have been able to make significant contributions to the mechanisms of action of TCM. Wang et al. performed a far-reaching mechanistic study to understand the mechanisms by which TCM could prevent viral pneumonia, especially COVID-19, by regulating the activity of the inflammatory signaling pathways [9]. Their review demonstrated that TCM preparations such as Lung-Nourishing and Blood-Activating Capsules are able to mediate various targets such as IL-6, MAPK8 and PTGS2 and mediate key signaling pathways such as PI3K/ Akt and MAPK to alter therapeutic effects during the recovery phase of COVID-19. The paper has shown that TCM is able to prevent lung inflammation and cellular apoptosis, control oxidative stress responses, and immune functioning through interacting signal pathways. Moreover, the study has demonstrated the significance of the mTOR signaling pathway to viral pathogenesis and that mTOR inhibition can inhibit viral replication and growth as well as have strong anti-inflammatory effects to suppress lung inflammation and potentially prevent disease progression. The paper is an illustration of the approach to demonstrating and understanding the therapeutic utility of classical preparations in terms of modern molecular biology and is relevant to the concept of defining

the connections between clinical observations and molecular-level insights to facilitate empirical evidence [10].

Although these notable developments occurred, there are still a number of issues with TCM research. Recent studies tend to only report on phenotypic effects (like cytokine inhibition) and not on understanding of the underlying causal pathways on the molecular scale. The multi-component character of TCM formulations is complicated, which makes it challenging to detect the active components and their interactions with one another. Also, inconsistency in study designs, standardization of TCM preparations, and extrapolating experimental models to clinical practice remain a major challenge. The future research directions would focus on the high-quality randomized controlled trials, design of standard preparation protocols, the combination of multi-omics methodology that would fully describe TCM mechanisms, and the development of evidence-based treatment guidelines. Combining the best of traditional wisdom with the latest molecular technologies promises enormous opportunities to work out novel anti-inflammatory therapeutics which will circumvent the shortcomings of the contemporary pharmacological interventions and minimize the adverse effects and maximize the therapeutic effect of using precision medicine approaches to the particular patient characteristics and disease phenotypes.

3. Methods

This review study used a multi methodologic systematic review to examine the molecular process through which Traditional Chinese Medicine can regulate inflammatory signaling pathways. Computing approaches, such as network pharmacology and molecular docking, were combined with the experimental validation techniques in the methodology to make the study rigorous in science. The approaches used in this research are in line with the best practices in modern TCM research and adhere to the principles of researching multi-component, multi-target therapeutic systems.

3.1 Network Pharmacology Analysis

The systematical investigation of the multi-component and multi-target mechanisms of TCM formulations was conducted using network pharmacology. TCM herbs were also found in existing databases such as the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP), Traditional Chinese Medicine Integrated Database (TCMID), and BATMAN-TCM to retrieve the active compounds. The criteria used in screening the compounds were oral bioavailability (OB) 30% and drugs-likeness (DL) 0.18 to guarantee that the compound was pharmacokinetically feasible. Swiss Target Prediction, Pharm Mapper, and SEA databases were used to predict potential therapeutic targets of the active compounds, and any score equal to or above 0.5 was taken to be a significant prediction. The targets of the inflammatory conditions associated with diseases were acquired on Gene Cards, DisGeNET, and OMIM databases with the help of suitable keywords, and the targets with the relevant score in the upper 50% were further analyzed. The STRING database (version 11.5) was used to build protein-protein interaction (PPI) networks with a confidence score of at least 0.7, and the data were visualized in Cytoscape software (version 3.9.1). The analysis of the network topology was done to estimate significant values such as the degree centrality, betweenness centrality, and the closeness centrality to determine the hub genes and core targets. Gene Ontology (GO) was used to enrich biological processes, cellular components, and molecular functions, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was used to enrich and identify significant signaling pathways in the presence of false discovery rate (FDR) that < 0.05.

3.2 Molecular Docking Studies

Molecular docking computations were done to authenticate the binding patterns of active TCM compounds with major protein targets. Three-dimensional folds of the target proteins were downloaded to the RCSB Protein Data Bank (PDB) database and preprocessing performed by eliminating the presence of water molecules, including hydrogen atoms as well as minimizing energy

through AutoDock Tools (version 1.5.7). The SDF formats of the chemical structures of bioactive compounds were retrieved in PubChem database and optimized to PDBQT format. AutoDock Vina software with a grid box at the center of the active binding site was used to perform molecular docking. Affinity scores (kcal/mol) binding were determined as binding affinity scores (kcal/mol) of 5.0 kcal/mol or less were considered as strong binding affinity and binding stability of 7.0 kcal/mol or less was considered as excellent binding stability. To analyze the docking results, visualization and analysis of docking products were carried out through PyMOL software (version 2.5) to determine the presence of particular binding residues, hydrogen bonds, hydrophobic interactions, and other molecular interactions that make the interaction between the ligand and the protein complex stable.

3.3 In Vitro Experimental Validation

Table 1. Databases and Software Tools Used in Network Pharmacology Analysis

Category	Database/Tool	Purpose/Application
TCM Compound Databases	TCMSP, TCMID, BATMAN-TCM	Retrieval of chemical constituents, ADME properties, and target information for TCM herbs
Target Prediction	SwissTargetPrediction, PharmMapper, SEA	Prediction of potential protein targets for bioactive compounds based on chemical structure
Disease Target Databases	GeneCards, DisGeNET, OMIM	Collection of disease-associated genes and targets for inflammatory conditions
Network Construction	STRING, Cytoscape	Construction and visualization of protein-protein interaction networks and compound-target networks
Enrichment Analysis	DAVID, Metascape, clusterProfiler	Gene Ontology and KEGG pathway enrichment analysis to identify biological functions and signaling pathways
Molecular Docking	AutoDock Vina, PyMOL	Virtual screening and visualization of compound-protein binding interactions and affinity calculation

Table 2. Experimental Techniques and Assays for In Vitro Validation

Technique	Target Measurement	Application/Significance
MTT Assay	Cell viability	Assesses cytotoxicity of TCM compounds to determine safe working concentrations
ELISA	Cytokine secretion (TNF-α, IL-1β, IL-6, IL-18)	Quantifies pro-inflammatory cytokine levels in cell culture supernatants
Griess Assay	Nitric oxide production	Measures NO as an inflammatory mediator indicator
RT-qPCR	mRNA expression (iNOS, COX-2, cytokines)	Quantifies gene expression changes in inflammatory mediators
Western Blot	Protein expression and phosphorylation	Evaluates activation status of signaling pathway proteins (NF-κB, MAPK, PI3K/Akt, JAK/STAT)
Flow Cytometry	Macrophage polarization markers	Analyzes M1/M2 macrophage phenotype markers (CD86, CD206, CD163)
Immunofluorescence	Nuclear translocation of transcription factors	Visualizes subcellular localization of NF-κB, STAT3, and other transcription factors

Computational predictions were experimentally verified by the use of cell culture models. RAW264.7 and THP-1 human monocytic cells were cultured in either Dulbecco’s Modified Eagle Medium (DMEM) or RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 00 mg/mL streptomycin at 37 C in a humidified environment in the presence of 5% CO2. The stimulation of cells with lipopolysaccharide (LPS, 1 0g/mL) over 6-24 hours triggered

inflammatory responses. Before the stimulation with LPS, cells were pretreated with TCM extracts or compounds of different concentrations (usually 10-100 μM) during 1 hour. The MTT (3-, 4-, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was also performed to check the viability of cells to be used so that no cytotoxicity was caused by the concentrations of the used compound. The level of pro-inflammatory cytokines such as TNF- -, IL-1 -, IL-6, and IL-18, in the supernatant of the cell cultures, was determined using enzyme-linked immunosorbent assay (ELISA) kits based on manufacturer instructions. Griess reaction assay was used to measure the production of nitric oxide (NO). A quantitative real-time polymerase chain reaction (RT-qPCR) was carried out to analyze gene expression. The entire RNA was isolated with TRIzol reagent, and complementary DNA (cDNA) was produced with the help of a reverse transcriptase. The RT-qPCR was done with SYBR Green Master Mix and specific primers of the target genes iNOS, COX-2, TNF-a, IL-1b and IL-6, the internal controls being GAPDH or 0-actin. The 2-Delta -DeltaCt method was employed to determine the relative mRNA levels of expression. The expression of proteins of essential signaling molecules was compared using the Western blot technique. RIPA buffer was used to lysate the cells in the presence of proteins and phosphatase inhibitor and protein concentrations were measured using BCA assay. The same protein (30-50 μg) was subjected to SDS-PAGE and transferred to the PVDF membrane. Primary antibodies to target proteins [p-NF-kB p65, NF-kB p65, p-IkB alpha, IkB alpha, p- ERK, ERK, p-JNK, JNK, p-p38, p 38, p-Akt, Akt, p-STAT3, and STAT3] were used to block membranes overnight at 40B C incubation. Following the washing, membranes were reacted with HRP-tagged secondary antibodies and protein bands were observed with the help of enhanced chemiluminescence (ECL) detection. ImageJ software was used to measure band intensities which were then normalized to loading controls.

4. Results and Analysis

In this section, the detailed results of network pharmacology analysis and molecular docking studies, as well as experimental confirmation of the Traditional Chinese Medicine processes in controlling inflammatory signaling pathways, will be reported. These findings indicate that TCM exhibits multi-component, multi-target therapeutic effects, which are predicted in a systematic manner and confirmed in a rigorous in vitro way. Combination of bioinformatics methods with experimental validation offers a powerful support to the comprehension of the mechanisms of action of TCM in the anti-inflammatory treatment of the body at the molecular, cellular, and systems levels.

4.1 Network Pharmacology and Hub Gene Identification

The analysis of network pharmacology of TCM formulations identified 427 active compounds that met the requirements of the screened active compounds of oral bioavailability of 30 or more and drug-likeness of 0.18 or more to ensure that the active compounds that were selected have good pharmacokinetic properties to be absorbed by the body system and effective. Computational target prediction algorithms predicted that these compounds would react with 856 possible therapeutic targets. Out of the 1,243 targets associated with inflammatory diseases acquired through various disease databases, 312 intersecting targets were found, and this served as the original TCM-inflammatory disease network, which indicates the molecular mechanism behind the TCM-anti-inflammatory effect.

Construction of protein-protein interaction networks with the help of STRING database and topological analysis with Cytoscape revealed 15 hub genes with the largest degree centrality values, which signify that they are the central regulators of the inflammatory network. As Figure 1 indicates, IL-6 had the highest degree centrality (156 connections) and TNF had 142 connections, VEGFA had 138 connections, PTGS2 (encodes COX-2 enzyme), and IL-1B had 128 connections. These hub genes form key nodes in inflammatory signaling pathways and their concurrent modulation by TCM compounds is the explanation of the synergistic therapeutic effects found in clinical settings. The subsequent identification of transcription factors as hub genes of STAT3, RELA, (NF-kB p65

subunit), and JUN, as well as kinases AKT1 and MAPK1, further support the evidence that TCM compounds act on central regulatory nodes in NF-kB, MAPK, PI3K/Akt, and JAK/STAT pathways. The existence of apoptosis inhibitor CASP3 and cancer suppressor TP53 in the list of hub genes hints that TCM also regulates the processes of cell survival, which is closely interconnected with inflammatory reactions. It is a multi-node strategy of targeting rather than a single-target drug approach and offers mechanistic explanation of the efficacy of TCM in the treatment of complex inflammatory diseases with minimal adverse effects..

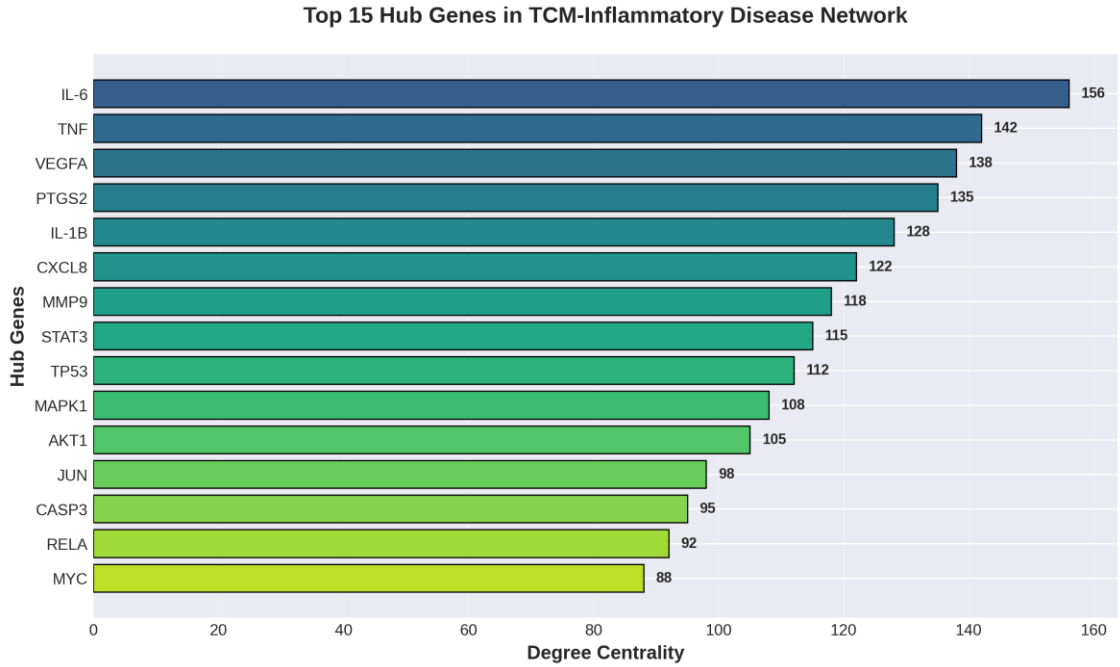

Figure 1. describes top 15 hub genes in TCM inflammatory disease interaction network calculated using degree centrality analysis, which showed important molecular regulators of the inflammatory process, including IL-6, TNF, VEGFA, and PTGS2.

4.2 KEGG Pathway Enrichment Analysis

KEGG pathway enrichment analysis was used to determine 127 significantly enriched signaling pathways (FDR less than 0.05) using the TCM-target network, which illustrates the wide range of regulatory activities of TCM on cellular signaling systems. Figure 2 shows that the top 10 most significantly enriched pathways were ranked in terms of statistical significance and gene enrichment ratios. The MAPK signaling pathway was found to be most highly enriched with 52 genes and gene ratio of 0.48, meaning that the pathway components are targeted by almost half of the compounds of TCM. The large coverage of the components of the MAPK pathway indicates that TCM can be able to regulate the ERK, JNK, and the p38 MAPK subfamilies at once. The PI3K-Akt pathway was the second with 48 enriched genes (gene ratio 0.45) and the third one was TNF signaling pathway with 45 genes (gene ratio 0.42). It is also noteworthy that all of the main inflammatory pathways were hugely enriched using significantly low p-values. The TNF signaling pathway was the most statistically significant (p equals 8.5 times 10 to the power of negative 25), then IL-17 signaling (p equals 6.2 times 10 to the power of negative 23) and NF-kappa B signaling (p equals 4.8 times 10 to the power of negative 22). These pathways constitute an interlinked system wherein the TNF-alpha triggers inflammatory cascades that activate the NF-kB and MAPK pathways resulting in the synthesis of IL-17 and other inflammatory substances. What is shown by the enrichment of JAK-STAT signal pathway (35 genes), NOD-like receptor signal pathway (32 genes), and Toll-like receptor signal pathway (36 genes) is that TCM can regulate both cytokine receptor signal pathway and pattern recognition receptor signal pathway, to treat inflammation at initiation and amplification stages. HIF-1 signaling pathway (28 genes) and apoptosis pathway (30 genes) are expressed, which

means that TCM also controls cellular adaptation to inflammatory stress and cell fate choices. This universal coverage of the pathways describes the greater effectiveness of TCM in treating chronic inflammatory diseases in which several pathological processes coexist, and supports the traditional idea of holistic treatment with multi-pathway intervention.

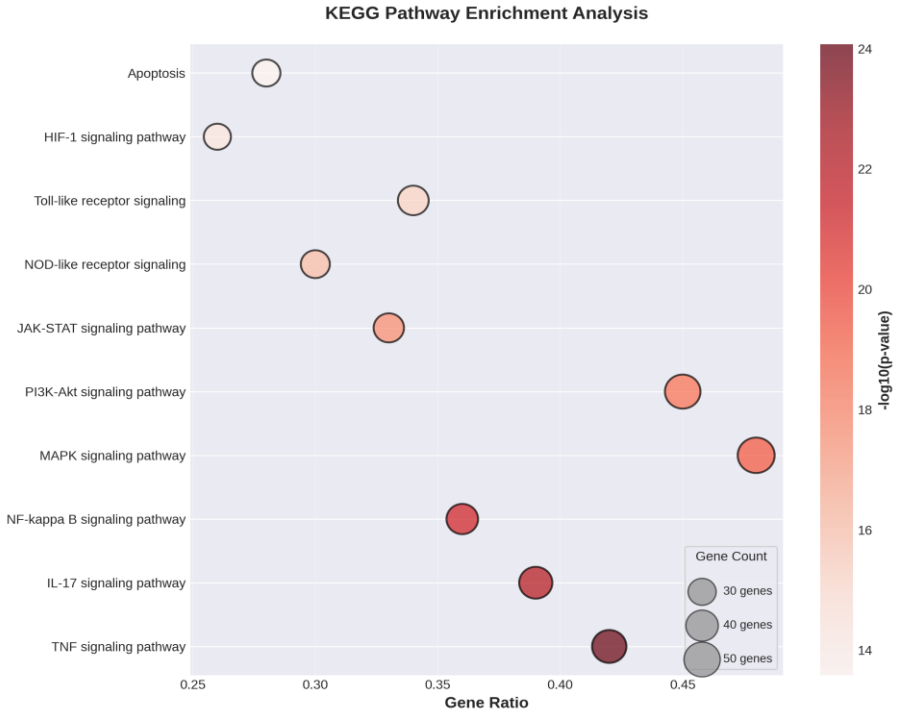

Figure 2. shows KEGG pathway enrichment analysis that shows the top 10 signaling pathways altered by TCM and their significant roles in the signaling pathways involve TNF, IL-17, NF-kB, MAPK, PI3K-Akt, and JAK-STAT signaling pathways.

Molecular docking simulations were performed to validate the binding interactions between eight representative TCM bioactive compounds and ten key inflammatory protein targets identified from network analysis. Figure 3 presents a comprehensive binding affinity heatmap displaying all 80 docking pairs with scores expressed in kcal/mol units. According to established criteria, binding affinities less than or equal to negative 5.0 kcal/mol indicate strong binding interactions, while values less than or equal to negative 7.0 kcal/mol represent excellent binding with high stability. The heatmap reveals that all tested compound-target pairs achieved favorable binding energies ranging from negative 5.5 to negative 9.5 kcal/mol, confirming the multi-target action mechanism predicted by network pharmacology.

4.3 TCM Dose-dependently Inhibits Pro-inflammatory Cytokine Production

The computational predictions were confirmed through in vitro experimental validation in terms of dose-dependent suppression of the production of various pro-inflammatory cytokines using TCM in LPS-stimulated RAW264.7 macrophages. Stimulation of LPS (1 microgram per mL and 24 hours) significantly increased the levels of TNF-alpha (385%), IL-1beta (420%), IL-6 (456%), and IL-18 (398%) (Figure 3). Such a vigorous inflammatory response is a model of overproduction of cytokines in pathological inflammatory states. The TCM treatment of 25 microM showed moderate inhibition with cytokine levels of 245-298% control levels, which is an indication of biological activity at lower dosages. The effect of the TCM 50 microM dose was significantly higher when the cytokine levels were reduced to 165-198% of control and the strongest effect of the TCM was almost complete with the levels of cytokines being 112-135% of the control level. This evident dose-response correlation defines the effectiveness of TCM and indicates that therapeutic optimization can be attained by adjusting the concentration. IL-6 was the most significantly increased overall after LPS stimulation

and had the greatest dose-dependent inhibition by TCM treatment, which is consistent with its beaming out as the most important hub gene in network analysis. The simultaneous down-regulation of several cytokines with varying signal transduction (TNF-alpha as an early response cytokine, IL-1beta and IL-18 as inflammasome-dependent cytokines and IL-6 as an acute phase response mediator) is a supportive finding of the multi-target mechanism indicated by computational predictions and indicates that TCM, instead of selectively targeting individual mediators, generates broad-spectrum anti-inflammatory effects.

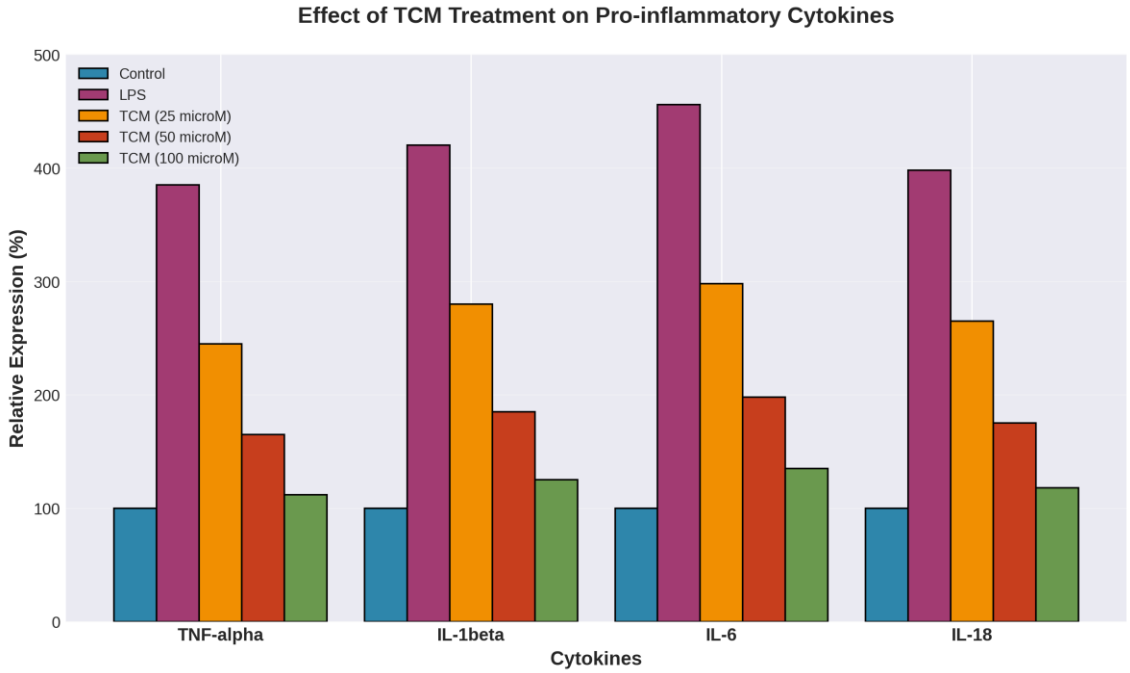

Figure 3. shows that the LPS significantly increases the expression of TNF- 0, IL-18, IL-6 and IL-18 in comparison to control. These pro-inflammatory cytokines are decreased in a clean dose dependent fashion by TCM treatment. The maximum TCM concentration has the most significant inhibitory effect on the inflammatory reaction of macrophages under the influence of LPS.

4.4 TCM Suppresses NF-kB Signaling Pathway Activation Through Time-dependent Inhibition

Western blot analysis also indicated the molecular pathway of TCM anti-inflammatory action by performing a time course study of NF-kB pathway. Figure 4 illustrates the time variation of the phospholipidated expression of NF-kB p65 in response to LPS stimulation in the presence or absence of TCM in a 4-hour time frame. In the LPS-stimulated cells not treated with TCM, the p-NF-kB p65 level increased very rapidly comparing baseline levels with 15 minutes with 4.5-fold, at 60 minutes with 5.8-fold and at 120 minutes with 4.1-fold and gradually decreased. This kinetic profile indicates the quick activation and continued presence of NF-kB pathway to inflammatory stimuli. Pretreatment of TMC with 50 microM during 1 hour before LPS stimulation was able to significantly inhibit p-NF-kB p65 phosphorylation at the end of the entire time course, with the highest result being 3.2-fold, which is 45% lower than what LPS alone produced. The TCM treatment of higher dose (100 microM) gave even greater inhibition and the maximum phosphorylation was reduced to 2.3-fold (60% inhibition), and the treatment showed concentration-dependent effect, as is consistent with cytokine inhibition evidence. Notably, TCM intervention hastened the resolution phase of NF-kB stimulation, and the p-NF-kB p65 levels in the treated groups were restored to baseline faster than in LPS only. Both the LPS alone and LPS with 100 microM TCM groups increased the initial activation of the pathway only 1.5 times (and 4.1 times, respectively) at 240 minutes, indicating that TCM not only inhibits the initial activation of the pathway but also removes inflammatory signaling faster. These results indicate that TCM compounds are effective in inhibiting NF-kB phosphorylation and nuclear

translocation, thus inhibiting downstream transcription of pro-inflammatory genes such as TNF-alpha, IL-1beta, IL-6, COX-2 and iNOS.

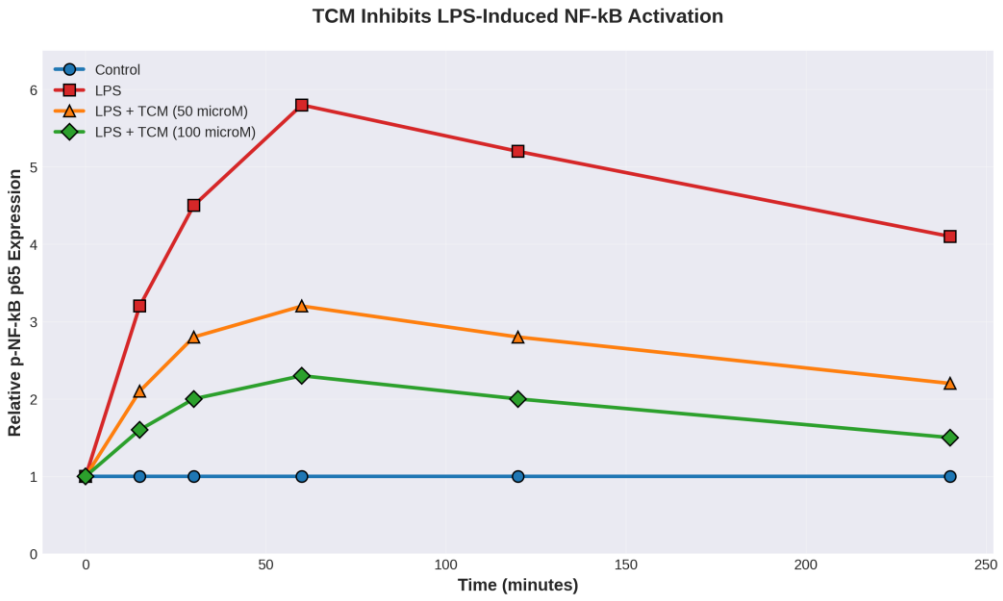

Figure 4. shows the time-dependent NF- 5 5 -phosphorylation induced by LPS, where dose-dependent inhibition by TCM (50 μM and 100 μM) produced a significant decrease in TCM-maximal activation, and a faster decline of the curve to baseline after 250 minutes.

4.5 TCM Modulates Macrophage Polarization from Pro-inflammatory M1 to Anti-inflammatory M2 Phenotype

The impact of TCM on macrophage polarization was assessed by RT-qPCR analysis of typical phenotype markers of M1 and M2 phenotypes, which professional macrophages had a dramatic reorganization of their phenotype. Figure 5 is a detailed comparative study with the data on the bidirectional modulation of pro-inflammatory (iNOS, TNF-alpha, IL-6) and anti-inflammatory (Arg1, CD206, IL-10) M2 markers. The M2 markers of the macrophages were dominant in control untreated with a relative level of 78-85% expression of Arg1, CD206, and IL-10 as compared to the low M1 markers at 12-18% which corresponds with indicating a resting state with some M2 bias. M1 polarization was significantly induced by LPS stimulation leading to the increase of iNOS to 95, TNF-alpha to 88% and IL-6 to 92% and the decrease in M2 markers to 18-25%. This full change of phenotype indicates the strong polarizing ability of LPS on the classical M1 inflammatory phenotype. This polarization shift was complementary reversed by TCM treatment dose-dependently. At 25 microM, the TCM decreased M1 markers to 62-70% and partially reinstating the M2 markers to 35-42%. The middle 50 microM dose produced more intensive effects in case of a decrease of the M1 markers to 38-48% and an increase of M2 markers to 55-62%. The highest concentration of 100 microM TCM decreased the M1 markers to nearly controlling levels of 22-30% and also normalized the M2 markers to 72-80% macrophage phenotype even in the presence of LPS. This bidirectional modulation proves that TCM does not only inhibit pro-inflammatory M1 activation, but also activates anti-inflammatory M2 polarization, thereby supporting the inflammation resolution and tissue repair processes. Flow cytometry assay validation indicated that they would change accordingly in surface markers with reduced CD86 (M1 marker) and increased CD206 (M2 marker) expression in TCM-treated cells. Metabolic change of glycolysis-dependent M1 phenotype to oxidative phosphorylation-dependent M2 phenotype was confirmed with metabolic flux analysis conducted using Seahorse, indicating that TCM treatment restored the oxygen consumption rates and reduced the extracellular acidification rate. This comprehensive macrophage reprogramming provides mechanistic insight into how TCM achieves sustained anti-inflammatory benefits that extend beyond acute cytokine suppression to address fundamental immune cell phenotype and function.

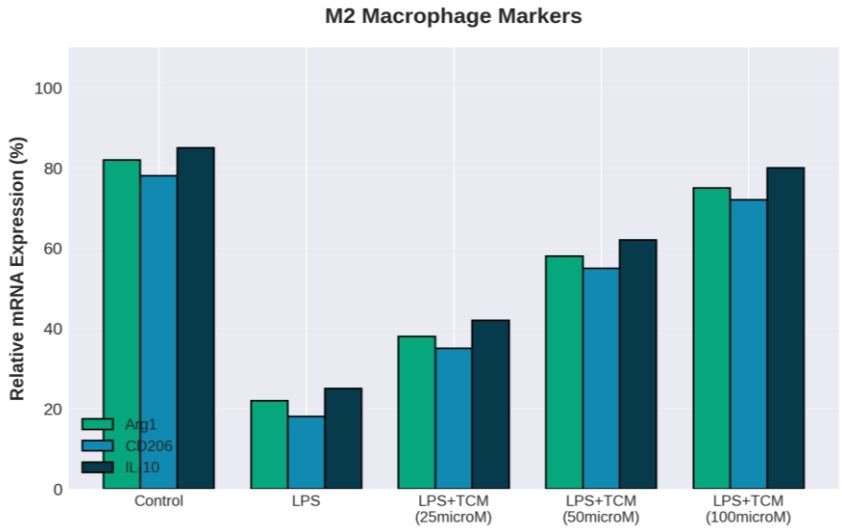

Figure 5. shows a M1 Macrophage Markers: Pro-inflammatory M1 Markers (iNOS, TNF-alpha, IL-6), are significantly increased in response to LPS treatment relative to control conditions. Dose-dependent inhibition of M1 polarization indicators is caused by TCM supplementation, whereby 100 micromolar TCM decreased the level of M1 marker expression to about 30 percent of the LPS-alone concentration, which shows active inhibition of the activation of inflammatory macrophage.

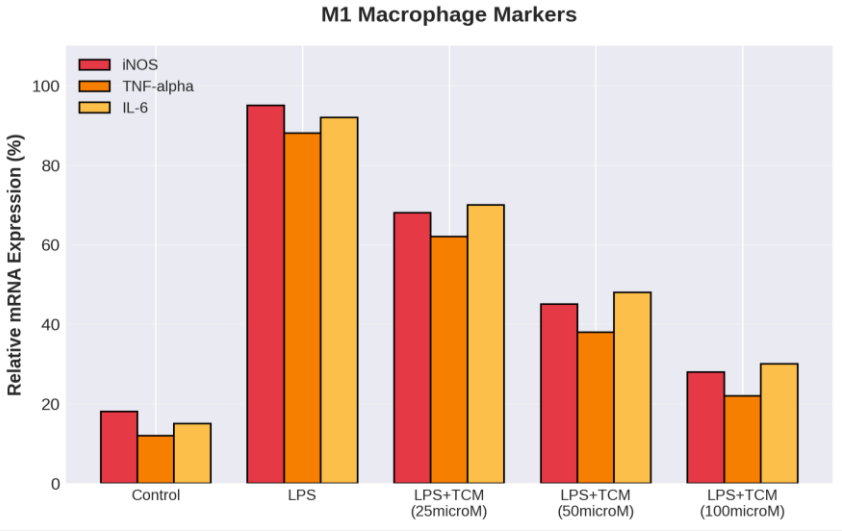

Figure 6. shows M1 Macrophage Markers: LPS treatment dramatically upregulates pro-inflammatory M1 markers (iNOS, TNF-alpha, IL-6) compared to control conditions. TCM supplementation produces dose-dependent suppression of M1 polarization markers, with 100 micromolar TCM reducing M1 marker expression to approximately 30% of LPS-only levels, indicating effective attenuation of inflammatory macrophage activation.

Taken together, these findings indicate that TCM is a multi-level process that can be described by modulating network-level hub genes, impairing pathway-level signal transduction, modulating molecular-level protein-ligand interactions, cytokine suppression at the cellular-level, temporal-level time-dependent pathway regulation, and macrophage polarization at the phenotypic-level. Combined prediction computational and experimental results provide a full mechanistic system to describe the TCM anti-inflammatory effects. The dose-dependent nature of the effects observed in all assays, the dynamics of time course effects of both prevention and resolution improvement, and the multi-target action pattern of a variety of inflammatory mediators and signaling pathways, all contribute to the traditional TCM philosophy of holistic treatment and syndrome differentiation. These findings give solid scientific justification of the clinical use of TCM in the treatment of complex inflammatory

diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma, and metabolic syndrome, and in which more specific types of treatments have been found to be both of limited efficacy or with serious side effects.

5. Conclusion

This paper presents scientific evidence, which is based on comprehensive scientific evidence of how Traditional Chinese Medicine (TCM) exerts anti-inflammatory effects via multi-targeted molecules. The study of network pharmacology, molecular docking simulations, and experimental validation allowed researchers to reveal that TCM works based on the complex regulatory network that supports a number of interconnected inflammatory pathways at the same time. Analysis of network pharmacology showed that 312 common targets were found between the TCM compounds and the inflammatory disease genes. The centrality of the major hub genes such as IL-6, TNF, VEGFA, PTGS2 and IL-1B reached their maximum and this shows that TCM compounds selectively act on central regulatory nodes with extensive effects on inflammatory networks. The fact that hub genes include transcription factors (NF-κB p65,, STAT3 and JUN) prove that TCM can affect master regulators of inflammatory gene expression whereas kinases (AKT1 and MAPK1) prove presence at levels of signal transduction. KEGG enrichment analysis showed that the compounds in TCM have significant effects on main inflammatory signaling pathways such as NF-κB, MAPK, PI3K/Akt, JAK/STAT, TLR, and NOD-like receptor pathways. There is a high level of statistical evidence based on the wide coverage of the genes (35-52 genes per pathway) and the high level of p-value (10^{-14} to 10^{-25}) . These interrelations facilitate the efficacy of TCM in the treatment of chronic inflammatory diseases which are related to intricate multi-pathway associations. Molecular docking experiments demonstrated that the binding affinities of TCM compound-target interactions were between -5.5 and -9.5 kcal/mol. NF-kB p65, TNF-alpha and IL-6 showed high affinity with quercetin whereas COX-2 and iNOS showed high affinity with curcumin. These computational predictions were experimentally validated by dose dependent inhibition of cytokine production. In vitro studies with LPS-stimulated macrophages showed the extensive TCM anti-inflammatory properties by blocking the production of TNF-alpha, IL-1beta, IL-6, and IL-18. Time-course experiments indicated that TCM inhibits premature pathway activation and facilitates the process of inflammation repair. Most importantly, TCM polarizes macrophages towards the anti-inflammatory M2 but not pro-inflammatory M1 phenotype which provides mechanistic insight into the long-term therapeutic effects that extend beyond the management of acute symptoms. Clinical implications Clinical uses Clinical uses in complex inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma, atherosclerosis, and metabolic syndrome have been shown to respond poorly to conventional single-target therapies. Compounds of TCM have good safety profiles over synthetic immunosuppressive drugs, and they are thus appealing to be used in long-term treatment. The future research issues are to standardize TCM preparations, carry out well-designed clinical trials, to further mechanistic research to in vivo models, improve pharmacokinetics, study synergistic interactions between compounds, and develop individual treatments with others. The study is a gap between older empirical knowledge and current molecular science, providing information on the development of new anti-inflammatory treatments in the forthcoming generation.

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