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5,2′-Dibromo-2,4,5-trihydroxydiphenylmethanone, a novel immunomodulator of T lymphocytes by regulating the $CD4^+$ T cell subset balance via activating the mitogen-activated protein kinase pathway

Fan Yang[a], Hong-Hong Cai[a], Xiu-E Feng[a], Yuan-Lin Zhang[b], Rui Ge[a], Bao-Guo Xiao[b], Qing-Shan Li[a,b,*]

[a] School of Pharmaceutical Science, Shanxi Medical University, Taiyuan, Shanxi 030001, PR China
[b] Shanxi Key Laboratory of Chronic Inflammatory Targeted Drugs, School of Traditional Chinese Materia Medical, Shanxi University of Chinese Medicine, Taiyuan 030619, PR China

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ABSTRACT

5,2′-Dibromo-2,4′,5′-trihydroxydiphenylmethanone (LM49) exerted therapeutic effects against rat acute pyelonephritis by regulating immune responses, especially affecting T lymphocytes. However, its underlying action mechanism remains unclear. T lymphocytes play an irreplaceable role in immune responses. Therefore, we sought to understand whether LM49 is an immunomodulator of T lymphocytes. The results showed that LM49 promoted T lymphocyte proliferation, increased the number of $CD4^+$ T cells, and increased the $CD4^+/CD8^+$ T cell ratio. LM49 regulated the $CD4^+$ T cell subset balance by increasing the production of $CD4^+IL\text{-}2^+$, $CD4^+IL\text{-}4^+$, and $CD4^+IL\text{-}10^+$, and reducing the production of $CD4^+IL\text{-}17^+$, without changing the production of interferon-γ. LM49 had a significant effect on the mRNA expression of the transcription factors T-bet, GATA3, Foxp3, and RORγt. Furthermore, LM49 raised the phospho (p)-extracellular signal-regulated protein kinase 1/2, p-p38, and p-c-Jun N-terminal kinase expression levels. T cell proliferation, and the production of $CD4^+IL\text{-}2^+$, $CD4^+IL\text{-}4^+$, and $CD4^+IL\text{-}10^+$ induced by LM49, were decreased by inhibitors of mitogen-activated protein kinases (MAPKs). These results revealed that LM49 possesses immunomodulatory activity on T lymphocytes, in which the MAPK pathway plays an essential role.

1. Introduction

T lymphocytes are adaptive immune cells including T helper (Th) and T cytotoxic lymphocytes (Tc) [1–5]. They release many pro-inflammatory factors that participate in the resulting inflammation [6–8]. After recognition of an antigen, activated Th cells are classified into Th1 and Th2 subtypes [9–12]. Th1 cells secrete interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor-α that mainly mediate cellular immune responses, while Th2 cells secrete IL-4, IL-6, and IL-10 that mainly mediate humoral immune responses [11]. In normal circumstances, Th1 and Th2 cells are in a state of dynamic balance to maintain normal cellular and humoral immune functions. These immunomodulatory cytokines participate in T cell proliferation, differentiation, activation, and immunomodulation [12–15].

Mitogen-activated protein kinases (MAPKs) are part of a classical signal transduction pathway, directly related to T cell survival, differentiation, and function [16,17]. There are three MAPK subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. ERKs are activated by mitogens and growth factors, and are required for regulating cell proliferation and Th2 cell differentiation [18,19]. JNKs are essential for $CD4^+$ T cell differentiation and cytokine production [20]. In particular, p38 is activated specifically by a pathway that results in IL-2 secretion by T cells, and is necessary for IL-12 and IFN-γ production [21,22]. p38 MAPKs are involved in regulating the synthesis of inflammatory mediators at both transcriptional and translation levels, making them potential targets for anti-inflammatory therapy [12]. Furthermore, MAPKs activate the immune response in T cells [23–25].

LM49 is a novel marine bromophenol (2,4′,5′-trihydroxyl-5,2′-dibromo diphenylmethanone) derivative that displayed strong anti-inflammatory effects in our previous research in an animal model of acute pyelonephritis [26–28]. That in vivo study confirmed that LM49 treatment increased $CD4^+$ T cells compared with the acute pyelonephritis model group [27]. The current study was designed to explore

* Corresponding author at: School of Pharmaceutical Science, Shanxi Medical University, 56 Xinjian South Road, Taiyuan, Shanxi 030001, PR China.
E-mail address: sxlqs2012@163.com (Q.-S. Li).

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Table 1
Primers used for the RT-PCR study.

	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
FoxP3	AGGCACTTCTCCAGGACAGACC	GGCGTTGGCTCCTCTTCTTGC
GATA3	CAAGGCACGATCCAGCACAGAAG	AGTACCATCTCGCCGCCACAG
IL-2	CTGCGGCATGTTCTGGATT	CATCATCGAATTGGCACTCAA
IL-4	GAACGAGGTCACAGGAGAAGG	AATATGCGAAGCACCTTGGAA
IL-6	GATGGATGCTACCAAACTGGA	CCAGGTAGCTATGGTACTCCAGAA
IL-10	TAGAGCTGCGGACTGCCTTC	TTCCGATAAGGCTTGGCAAC
IL-17	CCTCAAGTTCTTCGTCCGCATCC	CATTGGACTTCGGCAGAGGTCTTC
INF-γ	CAGGCCATCAGCAACAACATAAGC	AGCTGGTGGACCACTCGGATG
T-bet	AAGTTCAACCAGCACCAGACAGAG	GCCACGGTGAAGGACAGGAATG
TNF-α	CCTCTAGCCCACGTCGTAGC	AGCAATGACTCCAAAGTAGACC
RoRγt	GTCCAGACAGCCACTGCATTCC	TGCCGTAGAAGGTCCTCCAGTC
GAPDH	GGCAAGTTCAACGGCACAGT	ATGACATACTCAGCACCGGG

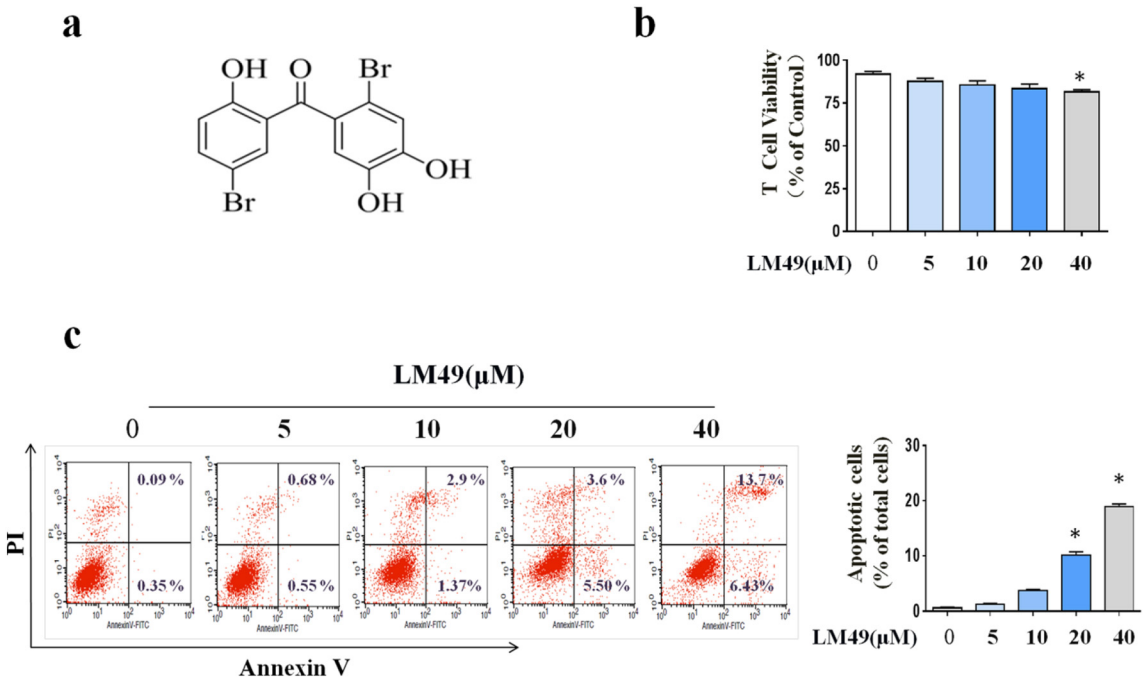

Fig. 1. Cytotoxicity of LM49 toward purified T lymphocytes.
T cells were treated with LM49 (0, 5, 10, 20, and 40 μM) for 24 h. (a) The chemical structure of LM49. (b) T cell viability was evaluated by the MTT assay. (c) T cells were stained with Annexin V and propidium iodide, and apoptosis was analyzed by flow cytometry. Quantitative data are presented as means ± SD (n = 5) from five independent experiments. *P < 0.05 for LM49 (5, 10, 20, and 40 μM) group vs. LM49 (0 μM) group.

possible mechanisms of this effect. We first purified T lymphocytes, then studied changes in their proliferation, the numbers of $CD4^+$ T cells, the $CD4^+/CD8^+$ T cell ratio, and the production of intracellular factors by $CD4^+$ T cells after LM49 treatment. We also investigated the mechanism underlying the immunomodulatory activity of LM49 on purified T lymphocytes. Overall, we aimed to demonstrate that LM49 could be an immunomodulator of T lymphocytes.

2. Materials and methods

2.1. Reagents

Anti-CD4-PE-Cyanine5 (15-0042-81), anti-mCD3-PE (12-0031-81), anti-mCD8-FITC (11-0081-81), anti-mIFN-γ-FITC (11-7311-41), anti-mIL-2-FITC (11-7021-41), anti-mIL-4-PE (12-7041-81), anti-mIL-10-PE (12-7101-82) and anti-mIL-17-FITC (11-7177-81) were purchased from eBioscience Biotechnology (San Diego, CA, US). Antibodies against ERK1/2 (8544), p-ERK1/2 (4370), p38 (8690), p-p38 (4511), JNK (9255), p-JNK (9251) were purchased from Cell Signaling Technology (Beverly, MA, USA), anti-βactin (sc-47778) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Concanavalin A (ConA) were provided from Sigma (St. Louis, MO, USA). Pan T cell isolation kit II (130-095-130) was provided from MACS (Miltenyi Biotec, Germany). All other chemicals and reagents were of analytical grade from China National Pharmaceutical Group Co. LM49 (2,4′,5′-trihydroxyl-5,2′-dibromo diphenylmethanone) was prepared by our research group. The purity of the compound was > 99.5% by high-performance liquid chromatography. Diluted to test concentrations with culture medium immediately prior to the experiment.

2.2. T lymphocytes purification

Male BALB/C mice (18–20 g) were provided by the Experimental Animal Center of Shanxi Medical University. The care and use of laboratory animal were in accordance with the guidelines of International Council for Laboratory Animal Science. All experimental protocols were approved by the Ethics Committee of Shanxi Medical University, Taiyuan, China. The spleens were removed aseptically and cut into pieces and then suspended with RPMI 1640 medium. Single cell suspensions were prepared by filtering the suspension through a sterile

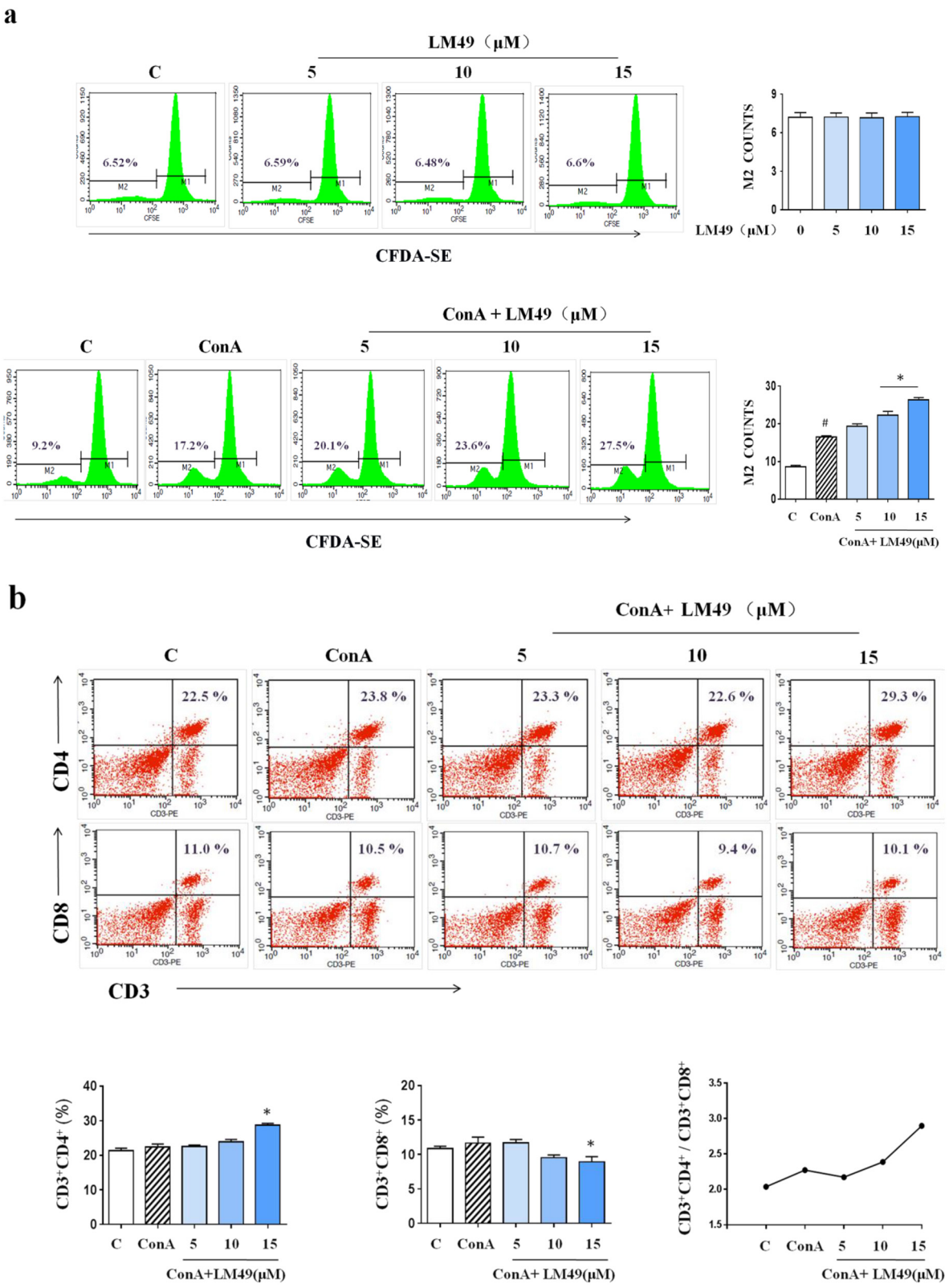

Fig. 2. Effect of LM49 on the proliferation and subsets of T lymphocytes.
(a) T cells were treated with LM49 (5, 10, 15 μM) and/or concanavalin A (ConA) (5 μg/mL) for 48 h. Cells were assessed by flow cytometry (b) LM49 regulated the $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets of T cells, and the $CD4^+/CD8^+$ ratio was calculated. Quantitative data are presented as means ± SD (n = 5) from five independent experiments. $^{\#}P < 0.05$ for concanavalin A (ConA) vs. control; $^{*}P < 0.05$ for LM49 (5, 10, 15 μM) + ConA vs. ConA.

sieve mesh. Red blood cells were lysed with red blood cell Lysis Buffer. The cells were washed twice with cold phosphate-buffered saline (PBS, pH 7.2), and adjusted to the concentration of 5×10^6 cells/mL in RPMI 1640 medium with 15% fetal bovine serum, following by incubating in cell culture dishes for 2 h. The suspended cells were the spleen lymphocytes. Then T lymphocytes were isolated by using Pan T cell

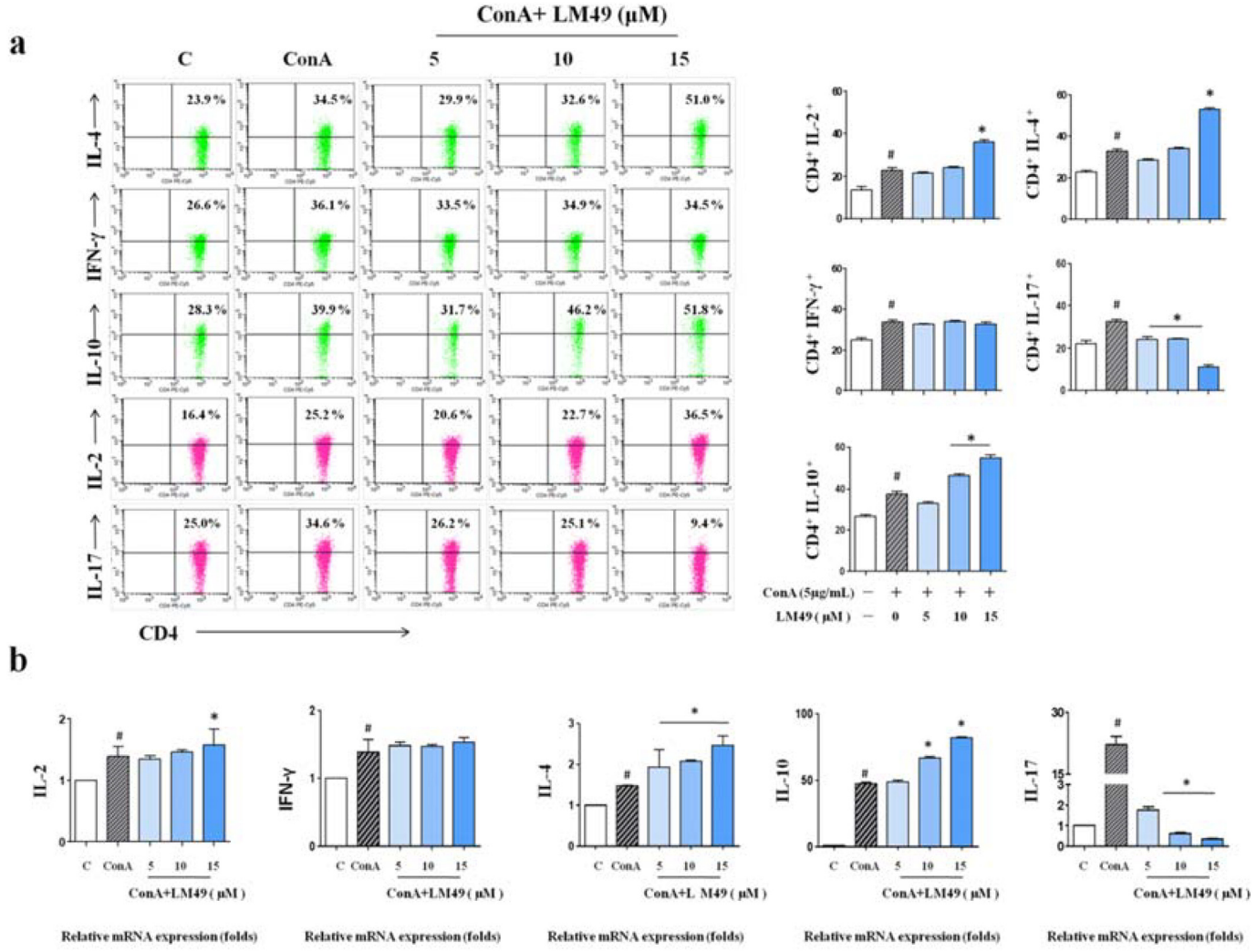

Fig. 3. Effect of LM49 on the production of cytokines by T lymphocytes.
(a) The $CD4^+$interleukin (IL)-2^+, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}10^+$, $CD4^+$interferon (IFN)-γ^+, and $CD4^+IL\text{-}17^+$ populations of cells were assessed by flow cytometry. (b) The expression of IL-2, IL-4, IL-10, IFN-γ, and IL-17 mRNAs in T lymphocytes was measured by the real-time polymerase chain reaction. Quantitative data are presented as means ± SD (n = 5) from five independent experiments. $^{\#}P < 0.05$ for concanavalin A (ConA) vs. control; $^{*}P < 0.05$ for ConA + LM49 (5, 10, 15 μM) vs. ConA.

isolation kit II according to the manufacturer's instruction. According to the flow cytometry, the purity of T cells was higher than 90%. The survival rate was higher than 95% as detected by trypan blue.

2.3. Cell culture and LM49 exposure

T cells were cultured in RPMI 1640 medium supplemented with 15% FBS, penicillin (100 U/mL) and streptomycin (100 U/mL), maintained in a humidified incubator with 5% CO_2 at 37 °C. When cells were at logarithmic phase, cells in LM49 groups were treated with different concentrations of LM-49 (5, 10, 15 μM). Besides, cells in LM49 groups were treated with ConA (5 μg/ml). The non-treated cells were used as control. Each group was cultured for 24–48 h prior to the following experiments.

2.4. Proliferation assay

T lymphocytes (5×10^6 cells/well) were incubated with medium alone as the control group. When cells are at logarithmic phase, they were stained with 5 μM CFDA-SE (carboxyfluorescein diacetate, succinimidyl ester), then were treated with ConA (5 μg/ml) and different concentrations of LM-49 (5, 10, 15 μM) in 96-well plate. After 48 h, T lymphocyte proliferation was stained with fluorescently labeled anti-CD4 antibodies and analyzed via flow cytometry.

2.5. Flow cytometry analysis

The T cells were harvested and washed twice with ice-cold PBS (supplemented with 2% FBS and 0.1% sodium azide). After filtration and washing, cells were stained with PE-conjugated-CD3, PE-Cy5conjugated-CD4.To analyze the expression of cell surface and intracellular proteins, we used double staining for cell surface antigens and intracellular cytokines for flow cytometry analysis. Lymphocytes from spleen were incubated with anti-mouse CD4- PE-Cy5 antibodies for 30 min at 4 °C. After two washes with PBS, the cells were fixed by adding Intracellular Fixation Buffer (eBiosciences) for 20 min, then fixed by Permeabilizatio Fixation Buffer for 40 min at room temperature. For intracellular cytokine staining, the cells were stained for anti-mIFN-γ-FITC, anti-mIL-2-FITC, anti-mIL-4-PE, anti-mIL-10-PE and anti-mIL-17-PE at 4 °C for 45 min in dark. The results were acquired using a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using Flow Jo software.

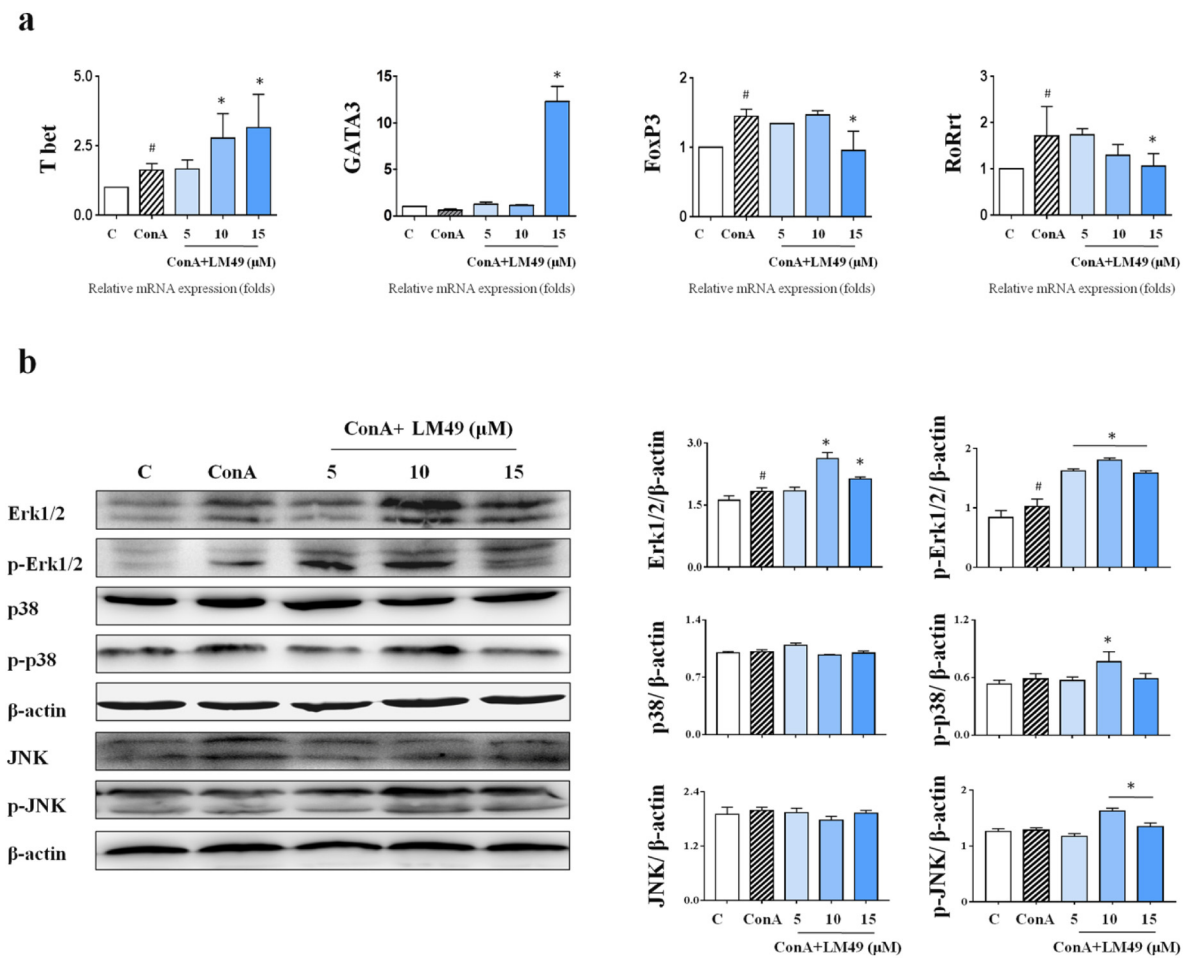

Fig. 4. Potential mechanisms for the action of LM49 in T lymphocytes.
(a) Expression of T-bet, GATA3, Foxp3, and RORγt mRNAs in T lymphocytes was measured by the real-time polymerase chain reaction. (b) Expression of phospho (p)-extracellular signal-regulated kinase (ERK)1/2, ERK1/2, p-c-Jun-N-terminal kinase (JNK), p-p38, and p38 proteins was assessed by western blot. Quantitative data are presented as means ± SD (n = 5) from five independent experiments. $^{\#}P < 0.05$ for concanavalin A (ConA) vs. control; $^{*}P < 0.05$ for ConA + LM49 (5, 10, 15 μM) vs. ConA.

2.6. Quantitative real-time PCR (Real-time PCR)

Total RNA was isolated from cells or tissues by subjecting them to Trizol reagent (Takara Bio) and reverse-transcribed to cDNA using iScript cDNA synthesis kit (Takara Bio). The cDNA was amplified by the multiple kit (SYBR Premix Ex TaqTM, DRR041A, Takara Bio) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Primers are indicated in Table 1. GAPDH served as an internal control. Gene expression was calculated as previously described by the following equation = $2^{-\Delta\Delta Ct}$ [21].

2.7. Western blot

Total protein was isolated from RAW264.7 cells and the frozen liver using RIPA (Boster Bio, Wuhan, China) according to the manufacturer's instruction. Protein concentration was quantified using the BCA Protein Assay Kit (Boster Bio, Wuhan, China). Equal amounts of proteins (about 30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred electrophoretically to nitrocellulose membrane (Millipore). After blocking with 5% skimmed milk at RT for 1 h, the membranes were incubated with anti-ERK1/2 (1:2000), anti-p-ERK1/2 (1:2000), anti-p38 (1:1000), anti-p-p38 (1:1000), anti-JNK (1:1000), anti-p-JNK (1:1000), anti-βactin (1:2000) at 4 °C for overnight, followed by HRP-conjugated secondary antibodies at RT for 1 h. Immunoblots were developed with an enhanced chemiluminescence system and determined using ImageJ (version 1.48) software.

2.8. Statistical analysis

All results were represented as means ± SD. Statistically significant differences were determined by one-way ANOVA with SPSS17.0 software. For the significance of mRNA or protein levels, mRNA (relative expression) and protein (densitometry values) levels were measured, comparing to respective housekeeping controls. In all cases, statistical significance was considered at values of $P < 0.05$.

3. Results

3.1. Cell viability

Prior to investigating the immune-regulatory activities of LM49, its cytotoxic effects at concentrations of 0, 5, 10, 20, and 40 μM were examined on T cells using the MTT reduction assay. LM49 had no obvious effects on cell viability at concentrations between 5 and 20 μM for 24 h (Fig. 1b). Cell viability was about 81% at 40 μM LM49. The induction of T cell apoptosis by LM49 was detected by flow cytometric analysis (Fig. 1c). Apoptotic cells increased from 0.6% in the control group to 19.2% in the LM49 (40 μM) treated group. Accordingly, we used concentrations of LM49 up to 15 μM in all additional experiments. In

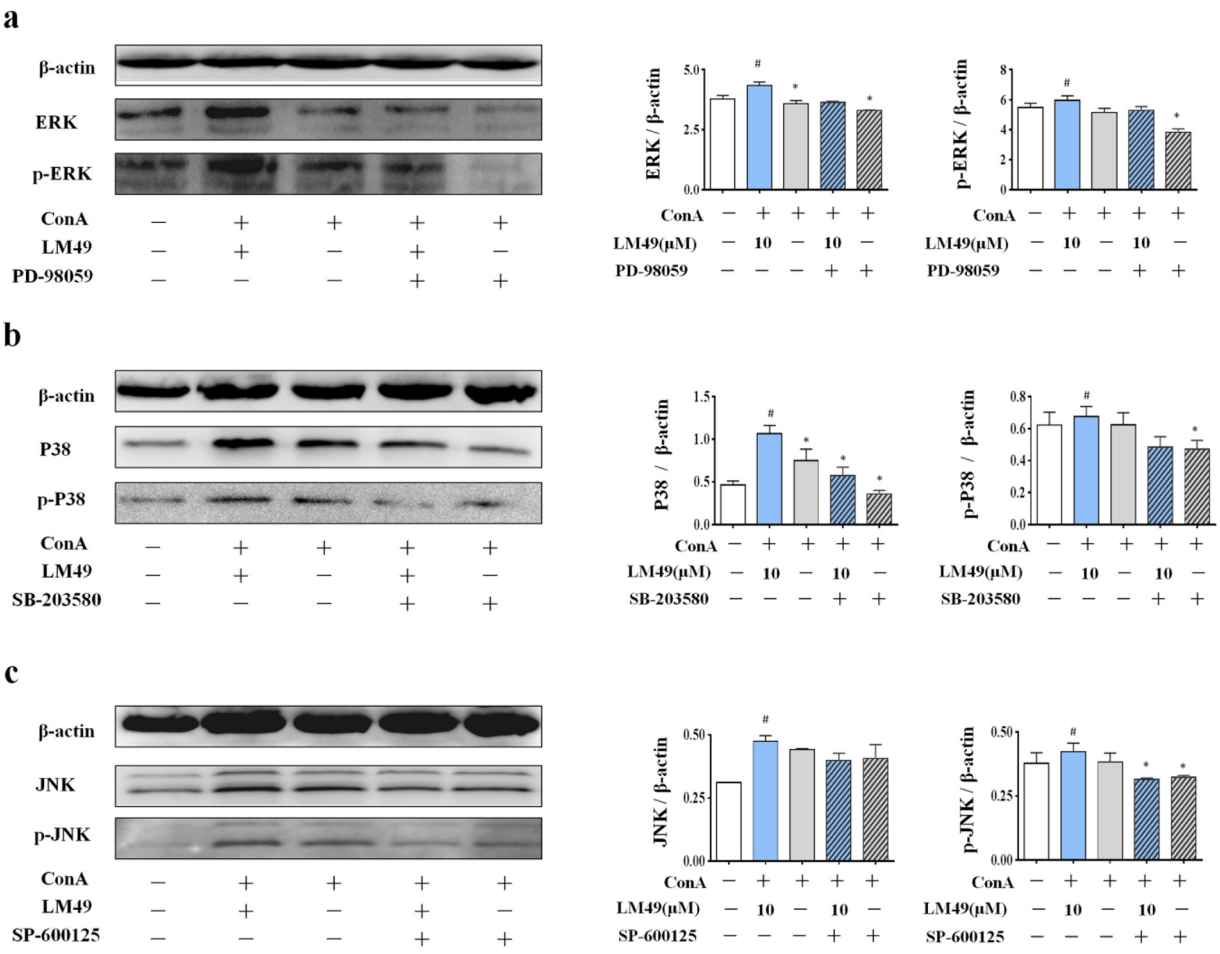

Fig. 5. Effect of mitogen-activated protein kinase inhibitors on T lymphocytes.
T lymphocytes were pretreated with PD98059, SP600125, or SB203580 (40 μM) for 30 min. The cells were then treated with LM49 (10 μM) and/or concanavalin A (ConA) (5 μg/mL) for 24 h. Protein expression was assessed by western blot. (a) Expression of phospho (p)-extracellular signal-regulated kinase (ERK)1/2 and ERK1/2 proteins. (b) Expression of p-c-Jun-N-terminal kinase (JNK) and JNK proteins. (c) Expression p-p38 and p38 proteins. Quantitative data are presented as means ± SD (n = 5) from five independent experiments. $^{\#}P < 0.05$ for concanavalin A (ConA) + LM49 vs. control; $^{*}P < 0.05$ for LM49 or LM49 + PD98059/SP600125/SB203580 vs. ConA + LM49.

general, these results demonstrate that LM49 exhibits little toxicity to T cells and may possess good biocompatibility.

3.2. LM49 promotes the proliferation of T lymphocytes

The effect of LM49 on the proliferation of T lymphocytes was measured by CFDA-SE assays. As shown in Fig. 2a, using LM49 alone to stimulate T lymphocytes did not directly induce T cell proliferation. When LM49 was used in combination with ConA, CFDA-SE staining indicated that the proliferation of T lymphocytes was increased significantly. This suggested that LM49 synergized with ConA to promote the proliferation of T lymphocytes.

3.3. LM49 regulates subsets of T lymphocytes

Flow cytometry was used to investigate the effect of LM49 on the $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ lymphocyte subsets [29]. The results showed that LM49-15 μM treatment significantly increased the proportion of $CD3^+CD4^+$ T cells while LM49-(10, 15 μM) treatment decreased the proportion of $CD3^+CD8^+$ T cells. Compared with the control group, the $CD4^+/CD8^+$ T cell ratio following LM49-15 μM treatment increased significantly (Fig. 2b).

3.4. LM49 modulates the production of intracellular factors by T lymphocytes

To determine whether Th1 and/or Th2 effector cell subsets developed in response to LM49 treatment, we assessed cells that were doubly stained for surface CD4, and intracellular IL-2, IL-4, IL-10, and IL-17. As shown in Fig. 3a, the LM49-(10, 15 μM) group exhibited a significant increase in the number of $CD4^+$ T cells expressing IL-2, IL-4, and IL-10 ($CD4^+IL\text{-}2^+$, $CD4^+IL\text{-}4^+$, and $CD4^+IL\text{-}10^+$ cells, respectively), and a significant decrease in the number of $CD4^+$ T cells expressing IL-17 ($CD4^+IL\text{-}17^+$), without changing the production of IFN-γ. We also analyzed the expression of IL-2, IL-4, IL-10, IL-17, and IFN-γ mRNAs in $CD4^+$ T cells by RT-PCR (Fig. 3b). These results were consistent with direct measurements of these factors.

3.5. LM49 affects the expression of transcription factors

The differentiation direction of $CD4^+$ T cells is mainly determined by several transcription factors [29–35]. Specifically, T-bet controls T cell differentiation into Th1 cells, GATA-3 controls T cell differentiation into Th2 cells, RORγt determines T cell differentiation into Th17 cells, and the Treg cell transcription factor is FoxP3 [35,36,38–40]. To investigate the effects of LM49 on expression of these transcription factors, Th cells were exposed to LM49 (5, 10, 15 μM) for 24 h and

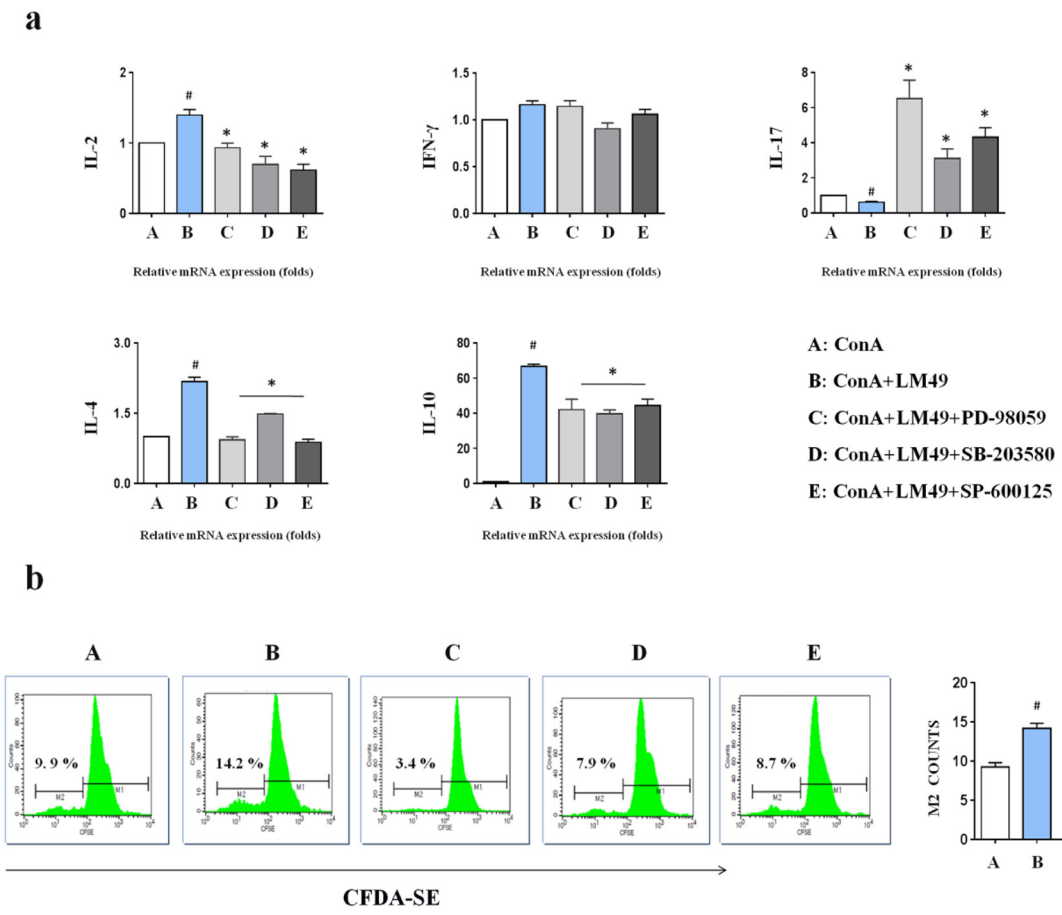

Fig. 6. Effect of mitogen-activated protein kinase inhibitors on cell proliferation and cytokine production.
T lymphocytes were pretreated with PD98059, SP600125, or SB203580 (40 μM) for 30 min. The cells were then treated with LM49 (10 μM) and/or ConA (5 μg/mL) for 24 h. (a) Expression of interleukin (IL)-2, IL-4, IL-10, interferon (IFN)-γ, and IL-17 mRNAs in T lymphocytes was measured by the real-time polymerase chain reaction. (b) Inhibitors of extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 decrease LM49-induced T-cell proliferation. Quantitative data are presented as means ± SD (n = 5) from five independent experiments. $^{\#}P < 0.05$ for concanavalin A (ConA) + LM49 vs. ConA; $^{*}P < 0.05$ for LM49 + ConA + PD-98059/SP-600125/SB-203580 vs. ConA + LM49.

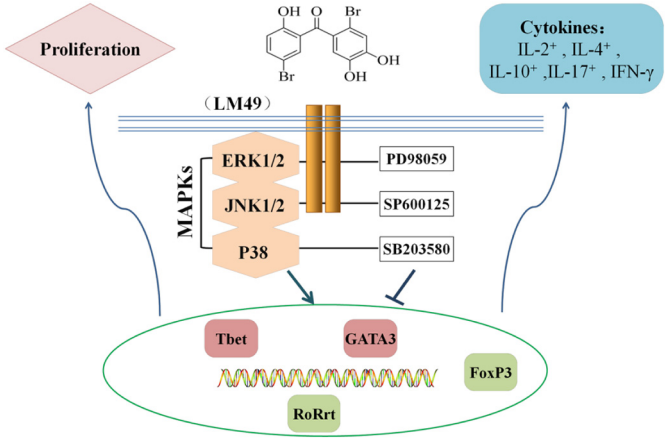

Fig. 7. Schematic diagram of the immunomodulatory activity of LM49 on purified T lymphocytes.
LM49 conferred immunomodulatory effects by promoting T lymphocyte proliferation and modulating $CD4^+$T cell subset balance through MAPK pathway.

transcription factor mRNA levels were measured by RT-PCR. The results indicated that LM49-(10, 15 μM) increased the mRNA levels of T-bet and GATA3, and decreased the levels of Foxp3 and RORγt, compared with the ConA group (Fig. 4a). These findings indicated that LM49 augmented $CD4^+IL\text{-}10^+$ Th2 cells, but inhibited Th17 and Treg cells.

3.6. LM49 mediates $CD4^+$ T cell signaling via the MAPK pathway

Based on the RT-PCR results, the MAPK signaling pathway may be closely related to the effect of LM49 on immune cell functions. Thus, we used western blots to examine the expression of ERK1/2, p-ERK1/2, JNK, p-JNK, p38, and p-p38 in T cells treated with LM49 to explore its immunomodulating effect. The data showed that LM49 (5, 10, 15 μM) (especially LM49-15 μM), compared with the ConA group, increased the levels of ERK1/2, p-ERK1/2, p-p38 and p-JNK proteins. Meanwhile, LM49 treatment had no obvious effect on JNK and p38 proteins (Fig. 4b).

3.7. Effect of MAPK inhibitors on T lymphocytes

T lymphocytes were exposed to inhibitors of ERK, JNK, and p38 MAPK for 30 min. The data showed that expression of all proteins was decreased by these inhibitors compared with control cells (Fig. 5a–c). To determine the role of MAPKs in regulating IL-2, IL-4, IL-10, IL-17, and IFN-γ secretion in LM49-treated T lymphocytes, cells were pretreated with PD98059, SP600125, or SB203580 for 30 min, followed by exposure to LM49 (10 μM) and ConA for 48 h. All inhibitors markedly reduced the production of IL-2, IL-4, and IL-10, and increased the production of IL-17, without changing the production of IFN-γ (Fig. 6a). The inhibitors also significantly suppressed T lymphocyte proliferation; the ERK inhibitor (PD98059) suppressed T lymphocyte proliferation to the greatest extent (Fig. 6b).

4. Discussion

In our previous study, LM49, a novel halophenol, did not directly inhibit *Escherichia coli* in vitro, but did show a good therapeutic effect when treating acute pyelonephritis in rats [27]. This protective effect can be attributed, at least in part, to the anti-inflammatory and immunomodulatory actions of LM49. The present study provides initial evidence for the immunoregulatory activity of LM49 by promoting T lymphocyte proliferation and altering the balance of $CD4^+$ T cell subsets.

Spleen is the largest peripheral lymphoid organ. T lymphocytes account for about 35% of lymphocytes and play an important role in immune diseases [41,42]. Through an exploration of T lymphocyte functions, it is possible to more fully understand corresponding links with the immune response, which has great significance for researching the immune response of the body [43–46]. To demonstrate the immunoregulatory effect of LM49 on T lymphocytes, we first measured proliferation of these cells by CFDA-SE assays. The results showed that LM49 could synergize with ConA to promote the proliferation of T lymphocytes. Secondly, we measured T lymphocyte subsets and found that LM49-(10,15 μM) could significantly increase the proportion of $CD3^+CD4^+$ T cells and decrease the proportion of $CD3^+CD8^+$ T cells. Compared with the control group, the $CD4^+/CD8^+$ ratio in lymphocytes treated with LM49 increased significantly.

As intercellular signaling proteins, cytokines play vital roles in immunomodulation. The results of the current study demonstrated that LM49 treatments significantly increased the numbers of $CD4^+IL\text{-}2^+$, $CD4^+IL\text{-}4^+$, and $CD4^+IL\text{-}10^+$ cells, and reduced the number of $CD4^+IL\text{-}17^+$ cells, without changing the production of $CD4^+IFN\text{-}\gamma^+$.

Most cytokines regulate the expression of characteristic genes by specifically activating transcription factors [47]. T-bet and GATA-3 are the characteristic transcription factors of Th1 and Th2 cells, respectively [48]. They both play decisive roles in cell differentiation [49]. This study demonstrated that LM49 could restore the Th1/Th2/Th17 balance by regulating immune function at the levels of T-bet, GATA3, and RORγt. If the expression of transcription factors can be induced or regulated selectively by drugs, intervention upstream of cell differentiation to promote the cell dynamic balance may be a more specific and less toxic treatment strategy.

MAPKs are evolutionarily conserved enzymes that affect many physiological and cellular processes in mammalian species, including immune responses [50]. Our results showed that LM49 elevated the expression levels of MAPKs. When exposed to inhibitors, the phosphorylation of MAPKs was decreased to relatively low levels. To explore whether LM49 stimulated T lymphocyte proliferation and cytokine production through the MAPK pathway [51,52], we used inhibitors of ERK, JNK, and p38, and found that the proliferation of T lymphocytes was inhibited to varying degrees, with PD98059 having the strongest inhibitory effect [52,53]. Furthermore, all inhibitors markedly reduced the secretion of IL-2, IL-4, and IL-10. These results suggested that the MAPK pathway was involved in the immunomodulatory activity of LM49 on purified T lymphocytes.

In conclusion, the results of the current study indicate that LM49 can promote T lymphocyte proliferation and alter the $CD4^+$ T cell subset balance through the MAPK pathway. This study further suggests that the MAPK pathway plays an important mechanistic role in the immunomodulatory activity of LM49 on purified T lymphocytes (Fig. 7). LM49, a polyphenol that has been used rarely in the treatment of bacterial infections, is an attractive candidate for treating acute pyelonephritis. This study reveals the potential of LM49 to be developed as a novel immunomodulator, suggests that LM49 might have a role in future treatments for acute pyelonephritis, and provides a new perspective for research of bioactive components from marine plants. However, because all experiments were done in vitro, and the immunomodulatory mechanism of T lymphocytes in vivo is complex, more detailed investigations are required before this potential treatment can be verified.

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Conflict of interest

The authors declared that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Author contributions

Qing Shan Li and Fan Yang conceived and designed the experiments. Fan Yang wrote the manuscript. Hong-Hong Cai and Fan Yang performed the experiments. Xiu-E Feng,Yuan-Lin Zhang and Rui Ge helped in the preparation of experiments. Bao-Guo Xiao helped draft the final manuscript. All authors reviewed and approved the final manuscript.

References

[1] R.L. Coffman, Origins of the Th1-Th2 model: a personal perspective, Nat. Immunol. 7 (2006) 539–541.
[2] S. Guan, B. Fang, B. Song, Y. Xiong, J. Lu, Immunosuppressive activity of alpinetin on activation and cytokines secretion of murine T lymphocytes [J], Immunopharmacol. Immunotoxicol. 36 (2014) 290–296.
[3] F. Sallusto, D. Lenig, R. Förster, et al., Two subsets of memory T lymphocytes with distinct homing potentials and effector functions [J], Nature 401 (6754) (1999) 708–712.
[4] S. Saito, A. Nakashima, T. Shima, M. Ito, Review article: Th1/Th2/Th17 and regulatory T cell paradigm in pregnancy, Am. J. Reprod. Immunol. Microbiol. 63 (2010) 601–610.
[5] A. O'Garra, Cytokines induce the development of functionally heterogeneous T helper cell subsets, Immunity 8 (1998) 275–283.
[6] N. Marx, B. Kehrle, K. Kohlhammer, et al., PPAR activators as antiinflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis [J], Circ. Res. 90 (6) (2002) 703–710.
[7] R.M. Zwacka, Y. Zhang, J. Halldorson, et al., CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver [J], J. Clin. Investig. 100 (2) (1997) 279–289.
[8] X.Y. Lu, M. Chen, D.H. Chen, et al., Remifentanil on T lymphocytes, cognitive function and inflammatory cytokines of patients undergoing radical surgery for cervical cancer [J], Eur. Rev. Med. Pharmacol. Sci. (2018) 2854–2859.
[9] S. Romagnani, The Th1/Th2 paradigm [J], Immunol. Today 18 (6) (1997) 263–266.
[10] M.F. Neurath, S. Finotto, L.H. Glimcher, The role of Th1/Th2 polarization in mucosal immunity [J], Nat. Med. 8 (6) (2002) 567–573.
[11] C.D. Mills, K. Kincaid, J.M. Alt, et al., Pillars article: M-1/M-2 macrophages and the Th1/Th2 paradigm. J. Immunol. 2000. 164: 6166-6173 [J], J. Immunol. 199 (7) (2017) 2194.
[12] S. Zhang, X. Liu, C. Sun, J. Yang, L. Wang, J. Liu, L. Gong, Y. Jing, Apigenin attenuates experimental autoimmune myocarditis by modulating Th1/Th2 cytokine balance in mice [J], Inflammation 39 (2016) 678–686.
[13] M. Diao, J. Min, F. Guo, et al., Effects of salbutamol aerosol combined with magnesium sulfate on T-lymphocyte subgroup and Th1/Th2 cytokines of pediatric asthma [J], Exp. Ther. Med. 13 (1) (2017) 117–120.
[14] Y.Y. Shao, Y.M. Zhou, M. Hu, et al., The anti-allergic rhinitis effect of traditional Chinese medicine of Shenqi by regulating mast cell degranulation and Th1/Th2 cytokine balance [J], Molecules 22 (3) (2017) 504.
[15] B.C. Della, M. Benagiano, G.M. De, et al., T cell clones in human trichinellosis: evidence for a mixed Th1/Th2 response [J], Parasite Immunol. 39 (3) (2017) e12412.
[16] W. Zhang, H.T. Liu, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res. 12 (2002) 9–18.
[17] A. VanderBorght, P. Geusens, J. Raus, P. Stinissen, The autoimmune pathogenesis of rheumatoid arthritis: role of autoreactive T cells and new immunotherapies, Semin. Arthritis Rheum. 31 (2001) 160–175.
[18] R.J.D. Chen Dong, Richard A. Flavell, MAP kinases in the immune response, Annu.

Rev. Immunol. 20 (2002) 55–72.
[19] K.L. Jeffrey, M. Camps, C. Rommel, C.R. Mackay, Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses [J], Nat. Rev. Drug Discov. 6 (2007) 391–403.
[20] L. Huang, M. Wang, Y. Yan, et al., OX40L induces helper T cell differentiation during cell immunity of asthma through PI3K/AKT and P38 MAPK signaling pathway [J], J. Transl. Med. 16 (1) (2018) 74.
[21] Y. Zhang, C. Dong, MAP kinases in immune responses [J], Cell. Mol. Immunol. 2 (2005) 20–27.
[22] C.W. Chen, S. Hu, K.H. Tsui, et al., Anti-inflammatory effects of gossypol on human lymphocytic Jurkat cells via regulation of MAPK signaling and cell cycle [J], Inflammation 41 (6) (2018) 2265–2274.
[23] S.R. Paccani, M. Boncristiano, C. Ulivieri, et al., Nonsteroidal anti-inflammatory drugs suppress T-cell activation by inhibiting p38 MAPK induction [J], J. Biol. Chem. 277 (2) (2002) 1509–1513.
[24] Q.Y. Yi, Z.S. Bai, B. Cai, et al., HSV-TK/GCV can induce cytotoxicity of retinoblastoma cells through autophagy inhibition by activating MAPK/ERK [J], Oncol. Rep. 40 (2) (2018).
[25] L.L. Sharp, D.A. Schwarz, C.M. Bott, C.J. Marshall, S.M. Hedrick, The influence of the mapk pathway on t cell lineage commitment [J], Immunity 7 (5) (1997) 609.
[26] F. Zheng, S. Ban, X. Feng, C. Zhao, G. Du, Q. Li, Synthesis and biological activities of new halophenols [J], Med. Chem. 9 (2013) (-).
[27] Y. Zhang, X. Feng, R. Chang, et al., Therapeutic effects of 5,2′-dibromo-2,4′,5′-trihydroxydiphenylmethanone (LM49) in an experimental rat model of acute pyelonephritis by immunomodulation and anti-inflammation [J], Int. Immunopharmacol. 62 (2018) 155–164.
[28] X. Feng, T. Liang, J. Gao, D. Kong, R. Ge, Q. Li, Heme Oxygenase-1, a key enzyme for the Cytoprotective actions of Halophenols by upregulating Nrf2 expression via activating Erk1/2 and PI3K/Akt in EA.hy926 cells, Oxidative Med. Cell. Longev. 2017 (2017) 1–13.
[29] L. Huang, X.D. Xiang, W.J. Wang, et al., [Effect of rosiglitazone on the expression of T-bet/GATA-3 in T lymphocytes in patients with acute asthma] [J], Chin. J. Tuberc. Respir. Dis. 30 (2) (2007) 116.
[30] A.S. Abuelsaad, G. Allam, A.A. Al-Solumani, Hesperidin inhibits inflammatory response induced by Aeromonas hydrophila infection and alters $CD4^+/CD8^+$ T cell ratio [J], Mediat. Inflamm. 2014 (2014) 393217.
[31] I. Gutcher, B. Becher, APC-derived cytokines and T cell polarization in autoimmune inflammation, J. Clin. Invest. 117 (2007) 1119–1127.
[32] S.F. Ahmad, K.M.A. Zoheir, H.E. Abdel-Hamied, A.E. Ashour, S.A. Bakheet, S.M. Attia, A.R.A. Abd-Allah, Amelioration of autoimmune arthritis by naringin through modulation of T regulatory cells and Th1/Th2 cytokines [J], Cell. Immunol. 287 (2014) 112–120.
[33] Q. Du, X. Gu, J. Cai, M. Huang, M. Su, Chrysin attenuates allergic airway inflammation by modulating the transcription factors T-bet and GATA-3 in mice [J], Mol. Med. Rep. 6 (2012) 100–104.
[34] F. Gao, D. Wei, T. Bian, P. Xie, J. Zou, H. Mu, B. Zhang, X. Zhou, Genistein attenuated allergic airway inflammation by modulating the transcription factors T-bet, GATA-3 and STAT-6 in a murine model of asthma, Pharmacology 89 (2012) 229–236.
[35] K. Zhu, J. Ye, M. Wu, et al., Expression of Th1 and Th2 cytokine-associated transcription factors, T-bet and GATA-3, in peripheral blood mononuclear cells and skin lesions of patients with psoriasis vulgaris [J], Arch. Dermatol. Res. 302 (7) (2010) 517–523.
[36] Q. Wang, S.H. Ren, W. Dong, et al., [Effects of transcription factor T-bet, GATA-3, FoxP3 and CD4(+)CD25(+)regulatory T cells in pathogenesis of child Hench-Schonlein purpura] [J], J. Exp. Hematol. 20 (1) (2012) 133–136.
[38] Z.W. Lin, L.X. Wu, Y. Xie, et al., The expression levels of transcription factors T-bet, GATA-3, RORγt and FOXP3 in peripheral blood lymphocyte (PBL) of patients with liver cancer and their significance [J], Int. J. Med. Sci. 12 (1) (2015) 7–16.
[39] P. Chen, Z. Zhang, Q. Chen, et al., Expression of Th1 and Th2 cytokine-associated transcription factors, T-bet and GATA-3, in the eutopic endometrium of women with endometriosis [J], Acta Histochem. 114 (8) (2012) 779–784.
[40] C. Yang, C. Li, G. Luo, et al., [Expression of transcription factors T-bet and GATA-3 and relationship to the numbers of eosinophils in rat model of allergic rhinitis] [J], Lin Chuang Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 25 (23) (2011) 1090.
[41] A.K. Abbas, Basic Immunology: Functions and Disorders of the Immune System, 2nd ed., vol. 32, (2004), pp. 1030–1045.
[42] R. Houben, H. Voigt, C. Noelke, et al., MAPK-independent impairment of T-cell responses by the multikinase inhibitor sorafenib [J], Mol. Cancer Ther. 8 (2) (2009) 433.
[43] O.D. Jr, D.H. Katz, The allogeneic effect in inbred mice. IV. Regulatory influences of graft-vs.-host reactions on host T lymphocyte functions [J], J. Exp. Med. 138 (4) (1973) 825.
[44] Y. Matzner, A.M. Konijn, Z. Shlomai, et al., Differential effect of isolated placental isoferritins on in vitro T-lymphocyte function [J], Br. J. Haematol. 59 (3) (2010) 443–448.
[45] R. Dantzer, J.C. O'Connor, G.G. Freund, et al., From inflammation to sickness and depression: when the immune system subjugates the brain [J], Nat. Rev. Neurosci. 9 (1) (2008) 46–56.
[46] B. Song, S. Guan, J. Lu, Z. Chen, G. Huang, G. Li, Y. Xiong, S. Zhang, Z. Yue, X. Deng, Suppressive effects of fisetin on mice T lymphocytes in vitro and in vivo, J. Surg. Res. 185 (2013) 399–409.
[47] H.R.C. Biefer, T. Heinbokel, H. Uehara, et al., Mast cells regulate CD4+ T cell differentiation in absence of antigen presentation [J], J. Allergy Clin. Immunol. 142 (6) (2018) 1894–1908.
[48] C.J. Wang, M. Chen, Z.J. Lei, Functional state of T cells and role of transcription factors T-bet and GATA3 in children with Kawasaki disease [J], Chin. J. Pathophysiol. 30 (4) (2014) 733–737.
[49] Y.W. Shi, D.Y. Chen, J. Liu, et al., Effect of transcription factors T-bet and GATA-3 on esophageal carcinoma patients' immunity [J], J. Jiangsu Univ. (02) (2008) 156–158.
[50] W. Kolch, Coordinating ERK/MAPK signalling through scaffolds and inhibitors [J], Nat. Rev. Mol. Cell Biol. 6 (11) (2005) 827–837.
[51] J.M. English, M.H. Cobb, Pharmacological inhibitors of MAPK pathways [J], Trends Pharmacol. Sci. 23 (1) (2002) 40–45.
[52] J.Y. Yap, R.C. Wirasinha, A. Chan, et al., Indirect presentation in the thymus limits naïve and regulatory T cell differentiation by promoting deletion of self-reactive thymocytes [J], Immunology 154 (3) (2018).
[53] M.M. Pillat, B.L. Correa, R.C. Da, et al., Changes in T cell phenotype and activated MAPKs are correlated to impaired cellular responses to antigens and glucocorticoids during HTLV-I infection [J], J. Neuroimmunol. 216 (1) (2009) 76–84.