Mechanism underlying the role of Miaoyao Fanggan sachet in up-regulating respiratory tract immunity through TLR-MyD88-NFκB signaling pathway

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Abstract

Background To elucidate the mechanism underlying the role of Miaoyao Fanggan sachet (MFS) in improving respiratory immunity through the TLR-MyD88-NFκB signaling pathway in MyD88 knockout mouse models and human normal lung epithelial cell line (BEAS-2B).

Methods Sixteen male C57BL/6J mice (SPF grade) were randomly divided into the control (n = 8) and MFS groups (n = 8) and 16 male MyD88 knockout mice were randomly assigned into the MyD88 knockout (MyD88-KO group, n = 8) and MyD88 KO + MFS groups (n = 8). Mice in the MFS intervention group were continuously treated with MFS inhalation from 8:00 am to 8:00 pm for 30 d and the sachets were replaced every 6 d. Hematoxylin-eosin (H.E.) staining was used to observe the pathological changes of lung tissues. The expression levels of TAK1, NFκB p65, IκB and IL6 in mouse lung tissues were detected by immunohistochemical staining, immunofluorescent staining, Western blot and qRT-PCR, respectively.

Results Mikawa's lung injury score did not significantly differ between the control and MFS groups (both $P > 0.05$). The parameters related to liver and kidney function did not significantly differ among four groups (all $P > 0.05$). The TAK1, NFκB p65, IκB and IL6 mRNA and protein in the MFS group were significantly up-regulated than those in the other groups (all $P < 0.05$). Compared with the MyD88-KO group, they were significantly up-regulated in the MyD88-KO + MFS group ($P < 0.05$).

Conclusion HFS can improve the respiratory immunity probably by up-regulating the expression level of MyD88, activating the TLR-MyD88-NFκB signaling pathway and stimulating the release of cytokines in the downstream pathway.

Introduction

Inherited from traditional Chinese medicine, Sachet therapy is filled with Chinese herbal medicine to prevent and treat pandemic influenza. It can be worn on the chest and is fully packed with volatile Chinese herbal medicine, which is capable of preventing influenza and expelling worms and filth. The therapeutic effect of treating diseases can be achieved by inhaling the smell and gas released from the medicine in the sachet [1, 2]. At the beginning of the pandemic spreading of influenza A (H1N1) throughout China, Liu *et al.* from the Second Military Medical University have observed that sachet therapy can yield clinical efficacy in lowering the prevalence rate of H1N1 in the local infants and children who wear the sachets. In addition, those who wear the sachets to experience a shorter course of H1N1 infection and less clinical symptoms compared with their counterparts who do not wear the sachets. In animal experiments, sachet therapy can significantly increase the content of secretory immunoglobulin A (SIgA) in the respiratory tract, and elevate the levels of multiple immune biomarkers, such as CD3+, CD4+, CD4+/CD8 + and interferon-gamma (INF-γ), *etc.* [3]. Wang *et al.* have established mouse models with influenza A and B viruses and subsequent experiments found that influenza-preventive sachet can reduce the mortality rate and lung lesion index in the established mouse models [4]. All these investigations have

proven that sachet-wear therapy plays an effective role in reducing the incidence rate and alleviating the symptoms in the prevention and treatment of influenza.

MFS is derived from the sachet therapy in traditional Chinese medicine. MFS is an ancient and unique treatment method to prevent and treat influenza, invented and passed down from generation to generation by the Miao ethnicity in the southeast Guizhou Province. To explore the mechanism underlying the role of MFS in preventing respiratory tract infection, our research team has conducted preliminary exploration and demonstrate that MFS can effectively up-regulate the expression levels of Toll-like receptor-2/4 (TLR2/4), natural killer (NK) cells, interleukin-2 (IL-2), interleukin-18 (IL-18) and other inflammatory cytokines [5]. Relevant clinical trials have shown that MFS is capable of effectively preventing the infection of influenza A virus (H1N1), prompting that MFS may play a role in preventing and treating influenza by improving the host immunity. Based on previous investigations, a mouse model of upper respiratory tract mucosal immunity dysfunction was established by cold stimulation [6], which were treated with continuous sachet inhalation and compared with those administered with pidotimod, a classical immunomodulatory drug. The experimental results have demonstrated that MFS and/or pidotimod can up-regulate the expression levels of MyD88, NF κ B p65 and Traf6 in the mouse lung tissues. Besides, under cold stimulation conditions, the expression levels of proteins also tend to be up-regulated and the release of the inflammatory cytokines, such as IL2, IL18, in the downstream signaling pathway can be facilitated [7], hinting that MFS possesses similar effect upon regulating the immunity compared with pidotimod. In addition, MFS can regulate the respiratory tract immune function of the mice probably via the TLR-MyD88-NF- κ B signaling pathway, thereby playing a key role in the prevention and treatment of the upper tract infectious diseases.

On the basis of our previous findings, this study was designed to investigate and analyze multiple loci of the TLR-MYD 88-NF- κ B p65 signaling pathway in the C57BL/6J mouse models, aiming to explore the molecular mechanism underlying the effect of MFS upon enhancing the respiratory tract immunity and resist the respiratory infection.

Materials And Methods

Experimental animals and feeding conditions

Sixteen SPF grade male C57BL/6J mice and 16 male MyD88 knockout mice (MyD88-KO), 6-7 weeks old, weighing 25-35 g, were purchased from the Experimental Animal Center of Nanjing University. Standard feed was purchased from the Animal Center of Guizhou Medical University. All mice were kept in animal room (SPF grade) of Clinical Research Center of Affiliated Hospital of Guizhou Medical University. All 32 mice were maintained in PVC cage, 4 mice/cage, at a temperature of 22 $\pm$ 0.5 $^{\circ}$ C, a humidity of 40%-60%, illumination time from 7: 00 am to 7: 00 pm, free access to diet and water.

Animal grouping and model establishment

Sixteen male C57BL/6J mice with SPF grade were randomly divided into the blank control group (control group, n=8) and Miaoyao Fangan sachet group (MFS group, n=8). Sixteen male SPF grade C57BL/6J MyD88 knockout mice were randomly assigned into the MyD88 knockout group (MyD88-KO group, n=8) and MyD88 knockout+Miaoyao Fangan sachet group (MyD88-KO+MFS group, n=8). Mice in the MFS and MyD88-KO+MFS groups were given with sachet intervention: 10 g sachet was placed at the inlet end of static pressure window of PVC cage, continuous inhalation for 12 h every day (8:00 am-8:00 pm), and the sachet was replaced every 6 d. Mice in the control and MyD88-KO groups received no other treatment, were maintained under the same conditions and had free access to diet and water. All mice were sacrificed under anesthesia after intraperitoneal injection of chloral hydrate at the 31st d.

Serum sample collection

At 30 d after the sachet intervention, all mice were sacrificed by chloral hydrate injection, the skin around the eyes was disinfected, and blood sample was taken from the single eyeball. The mice were lifted, the eyeballs were enucleated by tweezers, letting the blood dripping into the anti-coagulation tube from the orbit and mixed evenly. When the blood was almost exhausted, the heart of the mice was pressed. Approximately 0.8-1 ml of blood was collected from each mouse. The collected blood was centrifuged at 3500 *r*/min for 5 min, and the supernatant and precipitate were separated and immediately sent to Clinical Laboratory of Affiliated Hospital of Guizhou Medical University for routine blood examination and liver and kidney function evaluation.

Tissue specimen collection

The thoracic cavity of the mice was dissected immediately after the mice were sacrificed. The lung tissue was collected, repeatedly irrigated with PBS, placed on dry filter paper to observe the morphology of the lung tissues. Partial left lung tissue was soaked in formalin solution and embedded in paraffin for H.E. staining, immunohistochemical and immunofluorescent analyses. The remaining lung tissue was immediately placed in a sterile frozen tube and quickly transferred to a refrigerator at -80°C for subsequent Western blot and qRT-PCR.

H.E staining

The lung tissue was soaked in 10% formalin solution, fixed for 24 h, dehydrated with gradient alcohol, xylene, embedded with paraffin and cut into slice thickness of 3 μ m. The slices were placed in an oven at 60°C for 40 min, placed in xylene I-II for 10 min each, 100% ethanol, 100% ethanol, 95% ethanol, 85% ethanol and 75% ethanol for 5 min each. Then, the slices were rinsed with distilled water for 3 times, stained with hematoxylin dye solution, placed in xylene I-II for 15 min each, sealed with neutral gum, and the slices were imaged with Leica DM2500 microscope. Mikawa's lung injury score was calculated to evaluate the severity of lung tissue injury, as illustrated in Table 1.

Table 1
Mikawa's lung injury scoring system.

Definition of lung injury	Severity of lung injury (score)			
	None or extremely slight injury	Slight injury	Moderate injury	Extremely severe injury
Alveolar congestion	0	1	2	3
Hemorrhage	0	1	2	3
Neutrophil infiltration or aggregation in space or vascular wall	0	1	2	3
Alveolar septum thickening or hyaline membrane formation	0	1	2	3

Immunohistochemical staining

The slices were placed in an incubator at 60°C for 40 min, xylene I-II for 15min each, soaked in anhydrous ethanol, 90% ethanol, 80% ethanol and 70% ethanol for 5min each, immersed in 2 L 0.001 mmol/L citrate for 3min, natural cooling and soaked for with PBS 5 min × 3 times. The lung tissue was marked with a histochemical pen, and 3% hydrogen peroxide was supplemented to incubate for 15 min at room temperature, 5% goat serum blocking solution was added to the range of lung tissue enclosed by the pen and blocked for 30 min at room temperature. Primary antibody (TAK1, IL6 rabbit monoclonal antibody diluted at 1:100, NFκB p65 rabbit monoclonal antibody diluted at 1:500, NFκB p50 rabbit monoclonal antibody was diluted at 1:500, IκB rabbit monoclonal antibody was diluted at 1:500, PBS-diluted antibody) was incubated in a refrigerator at 4°C overnight, goat anti-rabbit IgG antibody (diluted ratio: 1:500) was incubated at 37°C water bath for 45 min, 50 ml DAB working solution, hematoxylin staining for 30 s, soaked in 70% ethanol, 80% ethanol, 95% ethanol, anhydrous ethanol soaked slices for 5 min each, xylene I-II for 15 min each. The slices were observed under Leica DM2500 light microscope for 8 fields at random (400×). The imaging data were collected and semi-quantitatively analyzed by Image-Pro Plus 6.0 software, and the integral optical density (IOD) of positive particles in each image was measured and the mean value was calculated.

Immunofluorescent staining

The experimental procedures are the same as described in immunohistochemical staining. The fluorescent secondary antibody was supplemented, and the slices were soaked in PBST for 3 min × 3 times. After absorbing the excess liquid on slices with absorbent paper, the diluted fluorescent secondary antibody was added, incubated at 37°C for 1 h in dark room, soaked in PBST for 5 min × 3 times, supplemented and incubated with DAPI in dark room for 30 s-1 min. The obtained samples were stained

with nuclear dye, washed in PBST for 5 min× 4 times to remove the excess DAPI, added with anti-fluorescence quencher, and then observed under fluorescent microscope.

Western blot

A portion of 60 mg mouse lung tissues were collected in 1.5 ml EP tube, the suspension was centrifuged at 12000 *rpm* for 15 min at 4°C, the supernatant was obtained and washed with PBS, centrifuged for 1 min at 3000 *rpm*, the supernatant was discarded, supplemented with cytoplasmic extraction buffer, homogenized, and centrifuged at 12000 *rpm* for 15 min to obtain the nucleoprotein. Each sample was added with the loading buffer at a ratio of 1:4, stirred and mixed well, boiled at 100°C for 10 min, and then the sample was collected. The 12% SDS-PAGE gel kit was purchased from BIO-RAD (U.S.). Resolver A 2.5 ml, Resolver B 2.5 ml, 10% APS 25 ul, TEMED 2.5 ul were mixed into the glass plate, and supplemented with Stacker A 1 ml, Stacker B 1 ml, 10% APS 10 ul and TEMED 2 ul. The prepared 12% SDS-PAGE gel was placed into the electrophoresis tank, added with electrophoresis liquid, pre-dyed Marker and protein samples of each group for constant pressure electrophoresis. Then, the PVDF membrane was placed into TBST and washed by a low-speed shaking table at room temperature for 5 min×3 times, and 5% defatted milk was sealed for 1 h and then the PVDF membrane was washed by TBST for 10 min×3 times. The TAK1 and IL6 rabbit monoclonal antibody was diluted at a ratio of 1:500, 1:1000 for NFκB p65 rabbit monoclonal antibody and 1:500 for IκB rabbit monoclonal antibody. The primary antibody was diluted and added with PVDF membrane overnight at 4°C, washed with TBST for 10 min × 3 times, added with goat anti-rabbit IgG antibody (dilution ratio: 1:2500), incubated for 1 h at room temperature, then washed with TBST for 10 min × 3 times. The prepared chemiluminescent solution was evenly dropped on PVDF membrane and incubated in dark for 1 min. The results were statistically analyzed and calculated by Image J software.

RT-PCR

Total RNA was extracted from 60 mg lung tissues by Trizol method. A portion of 2 ul of RNA sample was taken and added into a de-RNA enzyme EP tube filled with 48 ul of DEPC. The absorbance value (OD) of RNA by ND2000C ultramicro ultraviolet spectrophotometer at a wavelength of 260 nm and 280 nm was measured. RNA purity was calculated by the ratio of OD_{260 nm} to OD_{280 nm}. DEPC was supplemented as the blank control before the sample was measured. Reverse transcription cDNA was operated by using kits (Thermo). The total reaction system was 20 μl. According to the RNA concentration results, 1 μl RNA template was added to each reaction system, primer oligo (dT) 18 μl adjusted to the total volume of 12 μl with enzyme-free water. After mixing, the quantity of total RNA template was calculated as 1 μl. After incubation for 5 min at 65°C, the total RNA template was transferred for ice cooling. The reaction system in the previous step was added with 5×reaction solution, RNA enzyme inhibitor, 10dNTP Mix, reverse transcriptase Rim in sequence to 20 μl. The reaction system (10 μl) included 2×SYBR Green 5 μl, reverse transcription product 2 μl, primer 1 μl and RNase-free H₂O 3 μl. Fluorescent quantitative PCR conditions

were set as follows: 40 cycles of pre-denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing/extension at 60°C for 60 s. β -actin was used as the internal reference. The obtained results were calculated by $2^{-\Delta\Delta CT}$ method. The primer design was illustrated in **Table 2**.

Table 2 Primer sequences.

Gene	Forward	Reverse
Mouse β -actin	TGACAGGATGCAGAAGGAGA	GTACTTGCGCTCAGGAGGAG
Mouse TAK1	GCCTTGTTAGACCGTGAGGA	GGGACACTGCTTTCCACTCT
Mouse NF κ Bp65	GCAGTTTGATGCTGATGAAGAC	ACCAGGCGAGTTATAGCTTCAG
Mouse I κ B	GATGTCAACAGGGTAACCTACCA	CCTCCAAACACACAGTCATCATA
Mouse IL 6	CCTCTCTGCAAGAGACTTCCAT	AATTAAGCCTCCGACTTGTGAA

Statistical analysis

All data processing was performed by using SPSS 17.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 6.0 statistical software. The measurement data of the experimental results are expressed as mean $\pm$ standard deviation (SD). Multiple group comparison was conducted by using one-way ANOVA. Two-group comparison was performed by using Dunnett’s *t*-test. A *P* value of less than 0.05 was considered as statistical significance.

Results

General conditions

No mouse died throughout the experiment in the control, MFS, MyD88-KO and MyD88-KO + MFS groups with a mortality rate of 0%. All mice in four groups were in good mental state, had free access to diet and water, they were sensitive in reaction, black and bright in hair color, and obtained stable weight growth. The general conditions of all mice in four groups did not significantly differ which were observed by naked eyes.

Routine Blood Test, Liver And Kidney Function Detection

Routine blood test demonstrated that the white blood cell count of the mice in the MFS group was significantly higher compared with that in the control group (*P* < 0.05), whereas the results of other parameters including neutrophil, hemoglobin, platelet levels did not significantly differ between the MFS and control groups (all *P* > 0.05). In addition, the parameters related to liver and kidney function including

ALT, AST, BUN, SCr levels did not remarkably differ among four groups (all $P>0.05$), as illustrated in Tables 3 and 4.

Table 3
Detection results of routine blood routine test.

Group	WBC (× 10 ⁹ /L)	RBC (× 10 ¹² /L)	NEU (× 10 ⁹ /L)	Hg (g/L)	PLT (× 10 ⁹ /L)
Control group	4.586 ± 1.056	9.606 ± 0.599	0.660 ± 0.362	153.333 ± 11.719	396.000 ± 74.101
MFS group	6.304 ± 1.581 [□]	9.296 ± 0.373	0.460 ± 0.050	147.667 ± 5.033	449.333 ± 15.044
MyD88-KO group	4.386 ± 0.592	9.606 ± 0.396	0.417 ± 0.257	147.667 ± 2.082	491.000 ± 64.133
MyD88-KO + MFS group	4.368 ± 0.575	9.376 ± 0.315	0.493 ± 0.221	149.667 ± 4.726	450.666 ± 16.653
Note: * denotes $P<0.05$ compared with the control group.					

Table 4
Detection results of blood biochemical test.

Group	ALT (U/L)	AST (U/L)	BUN (umol/L)	SCR(umol/L)
Control group	33.723 ± 7.915	155.640 ± 51.892	10.965 ± 1.547	10.660 ± 1.010
MFS group	38.590 ± 7.345	180.205 ± 78.243	11.257 ± 2.078	10.435 ± 1.554
MyD88-KO group	36.65 ± 14.794	143.703 ± 57.539	11.460 ± 1.433	10.952 ± 2.260
MyD88-KO + MFS group	31.490 ± 7.645	200.268 ± 62.888	10.663 ± 1.242	11.748 ± 1.986

Pathological changes of lung tissues

The lung tissue structure of the mice was in normal in contour, smooth cross-sectional plane, the surface of the lung tissue was in uniform color, soft in texture and free of lung nodule, as shown in **Figure 1**. H.E. staining demonstrated that the lung tissue contour was complete with clear structures of organs, bronchi and capillaries and no obvious exudation of alveolar wall was observed in all four groups, as illustrated in **Figure 2**. A small amount of mononuclear cells and neutrophil infiltration were seen in the alveolar cavity of mice in the MFS group, and Mikawa’s lung injury score was 0 point in the control, MFS, MyD88-KO and MyD88-KO+MFS groups with no statistical significance (all $P>0.05$).

Immunohistochemical staining

Immunohistochemical staining showed that TAK1, NFκB p65, IκB and IL6 were mainly expressed in the alveolar epithelial cells, bronchial epithelial cells and vascular endothelial cells, *etc.* The positive expression was seen in brownish yellow particles. Semi-quantitative analysis of Image-Pro Plus demonstrated that compared with the control group, the expression levels of TAK1, NFκB p65, IκB and IL6 in lung tissue of mice in the MFS group were significantly up-regulated (all $P<0.05$), whereas the expression levels of TAK1, NFκB p65, IκB and IL6 in lung tissues of mice in the MyD88-KO and MyD88-KO+MFS groups were considerably down-regulated (all $P<0.05$). The expression levels of TAK1, NFκB p65, IκB and IL6 in the MyD88-KO+MFS group were higher compared with those in the MyD88-KO group (all $P<0.05$), as demonstrated in **Figures 3-4**.

Immunohistochemical fluorescent staining

Immunohistochemical fluorescent staining revealed that red fluorescence in **Figure 5A** represents the expression level at the NFκB p65 site, green fluorescence in **Figure 5B** denoted the expression level at the NFκB p50 site, blue fluorescence in **Figure 5C** represented the nucleus, and **Figure 5D** was the overlapping image of the above three images. Compared with the control group, the expression level of NFκB in the nucleus of lung tissues in the MFS group was significantly up-regulated, whereas those in the MyD88-KO and MyD88-KO+MFS groups were considerably down-regulated. Compared with the MyD88-KO group, the expression level of NFκB in the nucleus in the MyD88-KO+MFS group was slightly up-regulated in **Figure 5**.

Western blot

β-actin was used for semi-quantitative analysis. Compared with the control group, the expression levels of TAK1, IκB and IL6 proteins were significantly up-regulated in the lung tissues of mice in the MFS group (all $P<0.05$), whereas those in the MyD88-KO and MyD88-KO+MFS groups were remarkably down-regulated (all $P<0.05$). Compared with the MyD88-KO group, the expression levels of TAK1, IκB and IL6 proteins in the MyD88-KO+MFS group were considerably higher (all $P<0.05$). Compared with the control group, the expression of NFκB p65 protein in the lung tissue nucleus of mice in the MFS group was up-regulated, while those in the MyD88-KO and MyD88-KO+MFS groups were down-regulated. Compared with the MyD88-KO group, the expression level of NFκB p65 protein in the nucleus was slightly higher in the MyD88-KO+MFS group. PCNA as the nuclear reference was used for semi-quantitative analysis, as demonstrated in **Figure 6**.

qRT-PCR

The gene amplification and dissolution curves were shown in **Figure 7**. Compared with the control group, the expression levels of TAK1, NFκB p65, IκB and IL6 mRNA in lung tissues of mice were significantly up-regulated in the MFS group (all $P<0.05$), whereas the expression levels of TAK1, NFκB p65, IκB and IL6

mRNA in lung tissues of mice were remarkably down-regulated in the MyD88-KO and MyD88-KO+MFS groups (all $P < 0.05$). Compared with the MyD88-KO group, the expression levels of TAK1, NF κ B p65, I κ B and IL6 mRNA in lung tissues of mice in the MyD88-KO+MFS group were considerably up-regulated (all $P < 0.05$), as illustrated in **Figure 8**.

Discussion

MFS is invented and produced in southeast region of Guizhou Province, China, where endemic diseases and epidemics constantly emerge due to its remote location, humid climate and underdeveloped economy. Miao medicine inherited from the traditional Chinese medicine has provided valuable experience for local residents' life and health, disease prevention and control with its unique culture. The design of this experiment coincided with the rapid global spread of the 2009 H1N1 influenza epidemics, and the infected cases showed a trend of aggregation and increased along with the seasonal changes in China. At the beginning of the epidemics, researchers have applied the preventive deodorant sachets in more than 100 local children. Animal experiments have proven that the sachet plays a role in lowering the incidence of influenza, relieving the symptoms of patients, shortening the clinical course, and improving the immunity of respiratory mucosa. On this basis, this study was designed to explore the mechanism underlying the prevention of respiratory tract infection by MFS from the perspective of molecular immunology and taking advantage of the safe and cheap Miao medicine resources in Guizhou Province, China.

Inherent immune response is the first line of defense against the invasion of pathogens [8]. Inherent immune recognition and regulation are mainly realized by a series of embryo-coded pattern recognition receptor (PRR) to recognize those pathogens associated molecular pattern (PAMP) expressed on the pathogenic microorganisms. Toll-like receptors (TLRs) are important pattern recognition receptor families in natural and acquired immunity, and act as pivotal priming proteins for mammals to transmit extracellular antigen signal information into cells [9]. TLR can initiate a series of signal transduction pathways, activate the effect factors in the pathway, amplify the cascade responses of downstream signal pathway, cause the aggregation of granulocytes, macrophages and natural killer cells, increase vascular permeability, stimulate the release of downstream inflammatory mediators, cause slight inflammatory reactions in the body, and finally activate the immune regulation function of the body to achieve the effect of controlling and eliminating pathogens. Currently, 9 TLRs have been reported. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell membrane, which mainly recognize the bacteria, whereas TLR3, TLR7, TLR8 and TLR9 are distributed within cells to primarily recognize viral nucleic acids. TLRs family plays an important role in the discovery and elimination of bacteria and viruses [10].

Previous researches [11] have demonstrated that TLRs initiates the downstream inflammatory pathway mainly through two patterns. The first is the myeloid differentiation factor 88 (MyD88) pattern, which is a key adaptor protein in TLRs-mediated intracellular signaling pathway. Under the synergistic effect of myeloid differentiation protein-2 (MD-2), the activated Toll receptor unites with MyD88, and then interleukin-1 receptor-associated kinase (IRAK) is recruited through the domain of MyD88. Recruitment-

binding IRAK phosphorylation binds with tumor necrosis factor receptor associated factor 6 (TRAF6), which can activate transforming growth factor β activated kinase 1 (TGF- β activated kinase 1, TAK1), thereby regulating the NF κ B signaling pathway, the activation, expression and immune activation of downstream genes, *etc.* MyD88 is the first adaptor protein in the inflammatory signaling pathway. Once it receives the cell membrane signal, it can be immediately recruited and activated, and transmit the signal to the downstream pathway. Some scholars have compared MyD88 as the "bottleneck" of the entire signaling pathway. The expression level of MyD88 will significantly affect the signal transmission. The second pattern is the MyD88-independent signaling pathway. After TLR is activated, TRIF-related adaptomol-eeule (TRAM) binds with TIR-domain-containing adaptor inducing interferon B (TRIF), which can induce the expression of interferon and bind with TRAF6 to activate the downstream signaling pathway and secrete cytokines.

TRAF6, as an important conduction protein of inflammatory signals, is able to mediate signal transduction of MyD88-dependent and -independent signaling pathways and activate the nuclear factor kappa B (NF κ B) and mitogen-activated protein kinase (MAPK) signaling pathways [12]. Self-activation and degradation can be regulated according to the intensity of inflammatory signals in mediated signal transduction. TRAF6 can activate TAK1 and inhibitor of NF κ B (IKK) by binding with IRAK. The degradation of TRAF6 can undergo its own ubiquitination degradation through the monomer polymerization, thereby affecting the regulation of NF κ B signaling pathway [13].

TAK1 is a member of the MAPK family and is functionally located in the inhibitory protein kinase of MAPK and I κ B. As an important kinase in the upstream cellular signal transduction in inflammatory response, TAK1 can be activated by the stimulation signals by UV, proinflammatory factors and hormones [14]. Previous studies [15] have demonstrated that the activated TAK1 can phosphorylate and activate NF- κ B induced kinase (NIK), thereby enabling NF- κ B to enter the nucleus to participate in gene transcription, play an important role in cell growth, differentiation, apoptosis and regulate the expression of multiple genes, such as inflammatory cytokines, cell adhesion molecules and growth factors.

NF κ B is an important transcription factor in the downstream inflammatory pathway, which regulates the transcription of various inflammatory mediators and cytokine genes. The activation of its transduction pathway is the molecular biological mechanism for amplification and persistence of inflammatory response, and ultimately affects the transcription and expression of different pro-inflammatory cytokines and body defense proteins [16]. Under normal circumstances, NF κ B binds to the inhibitory protein I κ B in the cytoplasm in the form of heterodimer and does not show activity. When stimulated, TLR/MyD88 undergoes a cascade of amplification reactions in the MyD 88-dependent signaling pathway, activates the downstream transcription factor NF κ B-induced kinase, provokes the I κ B phosphorylation and induces the separation between NF κ B and I κ B. The activated NF κ B can be transferred into nucleus to bind to corresponding target gene sites, which can regulate the over-expression of inflammatory mediators and cytokine genes, leading to the release of a large quantity of inflammatory mediators, infiltration of inflammatory cells and exertion of early immune response [17]. Meantime, the activation of NF κ B is explicitly regulated by both positive and negative feedbacks, mainly the negative feedback, which can

suppress the activity of NF κ B through two intra- and extra-cellular mechanisms, thereby maintaining the balance of cytokine network and preventing excessive inflammatory reaction in the host body [18].

In terms of the anti-infection immunity, pathogens bind with the IL-1 homologous domain (Toll/IL-1 receptor, TIR). Through the interaction between MyD88 and TIR adaptor protein (Toll/IL-1 receptor domain-containing adaptor protein, TIRAP), it can activate the interleukin-1 (IL-1) receptor associated kinase (IRAK), Traf6, TAK1 in sequence, activate MAP kinase (MAP3K or MKKK)) and NF- κ B, stimulate the expression of downstream inflammatory cytokines, such as IFN α / β , IL-1 β /6/8/12, thereby regulating the host immunity and exerting anti-infection effect [19].

Our research team has conducted drug toxicity experiment in the previous study to compare and verify the effect of sachets of different concentrations and durations upon improving the respiratory tract immunity in mouse models. Experimental results have demonstrated that continuous inhalation of 10 g sachets for 12 months fails to increase the mortality rate of mice. The expression levels of TLR2 and SIgA in the mice which continuously inhaled the sachet odor are significantly up-regulated compared with those in the mice that inhaled sachets intermittently. Nevertheless, the results of mice inhaled sachets continuously for 4 weeks are similar to those of mice inhaled for 6 weeks [20]. Based on these preliminary experimental results, our research team optimized and modified the previous experimental conditions by establishing a hyp immunity model under cold stimulation. In addition, the immunity-enhancing effect between MFS and pidotimod was also statistically compared. Pathological examination revealed that the hyp immunity model was successfully established. Under normal and cold stimulation conditions, both MFS and pidotimod could up-regulate the expression levels of MyD88, TRAF6, NF- κ B (P65) proteins and mRNA in the lung tissues, prompting that MFS probably play an effective role in preventing respiratory tract infection by regulating the TLR-MyD88-NF κ B signaling pathway and regulating the inherent immune function of respiratory tract.

Therefore, the previous method for model establishment was adopted and modified in this experiment. The mice were kept into PVC cages with a quantitative air flow rate to ensure the accuracy of drug concentration. Prior to the experiment, the MyD88 knockout mice were bred by Animal Institute of Nanjing University, and the successfully-bred mouse models subject to gene sequencing to ensure the success of model establishment, aiming to verify whether MyD88 is a key factor for the immunity-enhancing effect of MFS and whether MFS can prevent and treat respiratory tract infection via the TLR-MyD88-NF κ B signaling pathway mediated by MyD88.

After 30-d MFS intervention, the mice in each group were in good mental state, had free access to diet and water, sensitive in response, black and bright in hair color, and obtained stable weight gain. The blood routine test and liver and kidney function parameters did not significantly differ among four groups (all $P > 0.05$) except that the white blood cell count of mice in the MFS group was increased. H.E. staining of the lung tissues indicated that the alveolar tissue contour in the four groups was normal, and the structures of trachea, bronchi and blood vessels were intact, which did not significantly differ from those in the control group, suggesting that MFS exerts no evident toxic effect upon mice after the model

establishment conditions are modified. The increase of white blood cell count in the MFS group is probably correlated with the activation of relevant signaling pathways and the accumulation of inflammatory cells induces the immune response of the host body. The expression levels of TAK1, NFκB p65, and IκB were significantly up-regulated in the MFS group. With the up-regulation of TAK1 expression, NFκB p65 was trans-located into the nucleus, and the expression in the nucleus was up-regulated correspondingly, which led to the up-regulation of the expression level of IL6, indicating that TLR-MyD88-NFκB signaling pathway was activated during the prevention of respiratory tract infection by MFS and resulting in the release of inflammatory cytokines and cell aggregation, such as natural killer cells, and early immune response. However, the expression levels of TAK1, NFκB p65, IκB and IL6 in the MyD88 KO and MyD88 KO + MFS groups were lower than those in the control group, indicating that MFS mainly relies upon the TLR-MyD88-NFκB signaling pathway mediated by MyD88 to achieve the purpose of improving the animal immunity. However, compared with MyD88 KO group, the expression levels of TAK1, NFκB p65, IκB and IL6 in the MyD88 KO + MFS group were slightly higher, which does not the possibility that MFS can activate alternative signaling pathways to function by up-regulating these target genes.

Conclusion

The findings in this animal experiment demonstrate that MFS can up-regulate the expression levels of TAK1, NFκB p65, IκB and IL6. Besides, it is capable of effectively activating the TLR-MyD88-NFκB signal transduction pathway mediated by MyD88. the Miao medicine fragrant capsule may regulate the body immunity through the TLR-MyD88-NFκB signaling transduction pathway. These findings remain to be subsequently confirmed by subsequent *in vitro* experiment.

Abbreviations

MFS: Miaoyao Fanggan sachet; H.E.: Hematoxylin-eosin; SIgA: secretory immunoglobulin A; TLR2/4: Toll-like receptor-2/4; NK: natural killer; IL-2: interleukin-2; IL-18: interleukin-18; MyD88-KO: MyD88 knockout; IOD: integral optical density; SD: standard deviation; PRR: pattern recognition receptor; PAMP: pathogens associated molecular pattern; TLRs: Toll-like receptors; MD-2: myeloid differentiation protein-2; IRAK: interleukin-1 receptor-associated kinase; TRAF6: tumor necrosis factor receptor associated factor 6; TGF-β-activated kinase 1: transforming growth factor β activated kinase 1; TRAM: TRIF-related adaptomolecule; NFκB: nuclear factor kappa B; MAPK: mitogen-activated protein kinase; NIK: NF-κB induced kinase; IL-1: interleukin-1; IRAK: interleukin-1 receptor associated kinase.

Declarations

Ethics approval and consent to participate

All mice were kept in animal room (SPF grade) of Clinical Research Center of Affiliated Hospital of Guizhou Medical University.

Consent for publication:

Not applicable.

Availability of data and material:

Not applicable.

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Competing interests:

There are no potential conflicts of interest to disclose.

Author Contributions

Mingjie Zhu is responsible for the literature research, experimental studies, data acquisition, manuscript preparation & manuscript editing; Guoquan Jia is responsible for the literature research, experimental studies, data acquisition; Quan Zhang is responsible for the guarantor of integrity of the entire study, study concepts & design, definition of intellectual content, manuscript editing & review; Guojun Li, Xiaofen Zhang and Junli Li are responsible for the clinical studies, data acquisition; Hong Li and Mingliang Cheng are responsible for the data acquisition; Baofang Zhang is responsible for the data analysis; Changqin Xin is responsible for the statistical analysis. All authors read and approved the final manuscript.

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Figures

Figure 1

Anatomical structure of lung tissues in mouse models among four groups.

Figure 2

Hematoxylin and eosin staining of lung tissues in each group (×400).

Figure 3

Expression levels of TAK1, NFκB p65, IκB and IL6 in lung tissues of mice (×400).

Figure 4

Immunodeficiency was used to detect the relative expression of TAK1, NFκB p65, IκB and IL6 proteins in lung tissue of mice, $P < 0.05$.

Figure 5

Immunofluorescent staining of NF-kappa B in lung tissue of mice (x 400).

Figure 6

Figure 6 is not available in this version

Figure 7

Gene amplification and dissolution curve.

Figure 8

Relative amount of TAK1, NFκB p65, IκB and IL6 mRNA in lung tissues in each group of mice, $P < 0.05$.