Int J Clin Exp Med 2017;10(12):15991-16003
www.ijcem.com /ISSN:1940-5901/IJCEM0057303

Original Article

Regulation of the phenotype and function of peritoneal macrophages by moxibustion in ulcerative colitis rats, and role of temperature

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Received May 12, 2017; Accepted October 31, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Macrophage (Mφ) phenotype and function play an important role in colonic inflammation, and Mφ regulation contributes to healing colonic damage. To elucidate the role of macrophage and related mechanism in moxibustion action in ulcerative colitis (UC). UC model was established by supplying rats with drinking water containing 3.5% dextran sulfate sodium salt. High-temperature moxibustion (HM) at 43±1°C was applied. Firstly, the colonic lesion was observed. Secondly, the functional phenotype and phagocytosis of peritoneal Mφ (pMφ) were detected and differentiation of Mφ due to the expression of specific cytokines were observed. UC model rats had a significantly larger number of $CD11b^{+}CD86^{+}$ Mφ in the abdominal cavity, with markedly more active phagocytosis and a significantly higher expression of inducible nitric oxide synthase (iNOs) mRNA compared to normal rats (all $p < 0.05$). They also showed apparent colonic damages. The abnormal expressions of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and migration inhibitory factor (MIF) were found. HM increased the number of $CD11b^{+}CD163^{+}$ Mφ in the peritoneal cavity and inhibited the phagocytic ability of pMφ, accompanied by high expressions of arginase I (Arg I) mRNA in pMφ and IL-4 and IL-13 in the abdominal cavity (all $p < 0.05$). HM encouraged the healing of colonic ulcers and alleviation of inflammation. Moxibustion promotes the healing of colonic injury and inflammation in UC rats, which is plausibly related to the thermal effect in modulating the activation of pMφ.

Keywords: Ulcerative colitis, moxibustion, thermal effect, peritoneal cavity, macrophage

Introduction

Ulcerative colitis (UC) is one of the major and common types of inflammatory bowel disease (IBD). The incidence of UC in the Asian region has increased annually along with the change of environment and lifestyle [1]. An epidemiologic survey of IBD in Northern China (Daqing) revealed that the incidence of UC was 1.64 per 100,000 people, with a mean diagnosis age of 48.9 years, and a morbidity rate for IBD of 1.77 per 100,000 people [2]. Recurrent abdominal pain, diarrhea, pus, and blood stool caused by UC, following long-term medical consequences, and financial burdens have a serious impact on the physical and mental health of the patients [3]. Therefore, the gradual recovery of intestinal function and improvement of the quality of life (QOL) are the major concerns for UC patients. Thus, the major anti-inflammation and immune suppression treatment protocols for UC, no longer satisfy patient requirements.

The unusual aggregation of immune cells and secretion of numerous cytokines in colon are the main histopathological features of UC. Abnormal immune response is considered a crucial pathological factor of UC, and either excessive immune reactions or immune deficiency may contribute to the development of chronic non-specific inflammation in the colon. Phagocytosis, antigen presentation, and cytokines-producing effect make macrophage (Mφ) a critical population among the cells of colonic innate immune system, and play a dual role in both normal immune defense and inflammatory injury. In the normal population, M2 Mφ represents the majority in colonic tissues. Compared to M1 Mφ, M2 is weak in reacting to bacteria but produces anti-inflammatory cytokines to maintain intestinal tolerance. On the contrary, M1, residing in colonic tissues of IBD patients, is the predominant type of Mφ, generates many pro-inflammatory factors, and is highly sensitive to bacterial stimulations [4-6]. With the

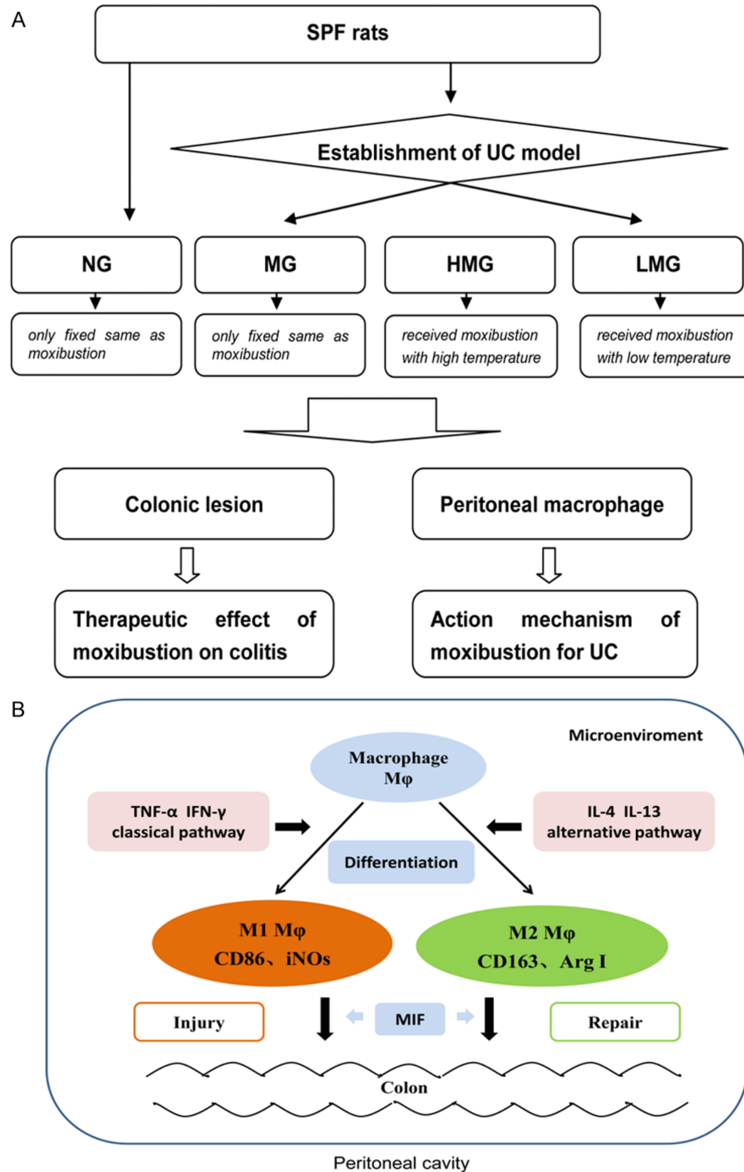

Figure 1. A. Study design. B. Macrophage (Mφ) can be polarized into M1 or M2 types through two metabolic pathways based on the co-factors in the peritoneal microenvironment. With the major presence of TNF-α and IFN-γ, the classical pathway is favored, thus producing M1 Mφ with increased expressions of CD86 and iNOs. And the alternative pathway is favored due to the prodominant presence of IL-4 and IL-13, producing M2 Mφ with increased expressions of CD163 and Arg I. MIF is the important cytokine inducing the migration of differentiated Mφ. 43°C temperature caused by moxibustion will probably regulate the Mφ differentiation and migration contributed to the recovery of mucosal damage.

progress of UC, Mφ of different phenotypes and functions appear. Drugs that aim to regulate the function of colonic Mφ and induce immunosuppression promote mucosal healing and mitigate the symptoms in UC patients, producing similar effects as interleukin-4 (IL-4) and IL-13 [7, 8]. This suggests that the abnormality in phenotype and activity of Mφ is involved in the pathogenesis of colonic injuries in UC, and effective regulation on the differentiation and function of Mφ is probably a crucial way to recover the immune homeostasis and colonic tissues.

Acupuncture and moxibustion are effective in treating intestinal diseases characterized by diarrhea and abdominal pain. For example, they effectively improve the clinical symptoms and promote the recovery of intestinal function and colonic tissues in IBD [9-12]. However, there are no definite conclusions about the mechanism of moxibustion. The thermal effect has always been considered an important mechanism of moxibustion. Moxibustion has a certain regulatory effect on immune response and the activity of Mφ [13-15]. Therefore, our research used a UC rat model to comparatively observe the influence of mild moxibustion at different temperatures on the phenotype and activity of peritoneal macrophage (pMφ), and to determine the role of thermal stimulation during moxibustion treatment (**Figure 1**). The general purpose of our experiment is to explore the action mechanism of moxibustion in treating UC.

Materials and methods

Animals

Specific pathogen-free (SPF) grade rats weighing 150±10 g were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (SCXK2012-0002). Feeding conditions were as follows: indoor temperature at 23°C, relative humidity between 50 and 70%, 12/12 h light/dark cycle,

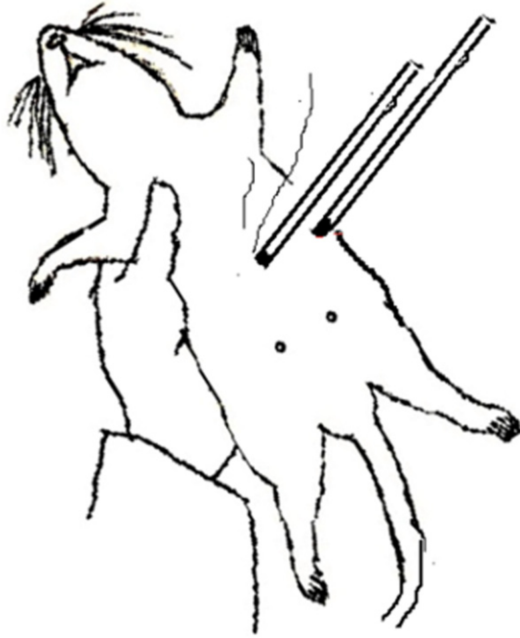

Figure 2. Illustration of moxibustion operation. (1) Tianshu (ST25) acupoints are located 5 mm lateral to the middle line of rats abdomen, at the intersection of upper 2/3 and lower 1/3 between xiphoid and pubic symphysis. (2) Moxibustion in the high-temperature moxibustion group (HMG) was operated at bilateral Tianshu (ST25) acupoints by lighting fine moxa, and the burning tip was suspended and kept away from the surface of acupoints by 1.5-2 cm for maintaining the skin temperature around 43±1°C. (3) Moxibustion in the low-temperature moxibustion group (LMG) was performed as in the HMG, but the burning end was kept away by 3-3.5 cm to maintain the temperature around 36±1°C.

and free access to food and drinking water. The experiment officially began after a 3-day adaptive feeding. All operations were strictly in accordance with the guidelines and approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine.

Establishment of UC rat model and grouping

The rats were randomly divided into four groups: a normal group (NG), a model group (MG), a high-temperature moxibustion group (HMG) (43±1°C), and a low-temperature moxibustion group (LMG) (36±1°C), ten rats each. Rats in the MG, HMG, and LMG groups were the UC models by being provided drinking water containing 3.5% dextran sulfate sodium salt (DSS, molecular weight: 36,000-50,000; MP Biomedicals Co., Ltd., USA) for 7 days, 40 mL total solution per day for each rat to develop UC [16, 17]. Rats in the NG group were provided plain drinking water. During the treatment, each model rat was continuously fed drinking water containing 1% DSS for 8 days, 40 mL total solution per day. For rats in the NG, normal drinking water was supplied for 15 days, 40 mL per day for each rat. The identification of UC model was mainly based on the pathological observation of the colon, such as the absence of a mucosal epithelium, formation of ulcers, and edematous lymphocyte infiltration into the lamina propria and submucosal connective tissues.

Treatment

After successful modeling, moxibustion intervention was performed (**Figure 2**). The rats in the NG and MG were only fixed same as moxibustion treatment. The rats in the HMG received mild moxibustion treatment at bilateral Tianshu (ST25) points [18] by using fine moxa sticks (Nanyanghanyi Moxa Co., Ltd., China) specifically for experimental animals. The burning end was kept away from the treated area by 1.5-2 cm, and the ash was flicked off every 5 s to maintaining the temperature at 43±1°C. The moxibustion treatment in the LMG was the same as that in the HMG, but the burning end was kept away from the surface of acupoints by 3-3.5 cm, and the ash was flicked off every 5 s for maintaining the temperature at 36±1°C. The moxibustion treatment was within the rat tolerance, considering whether the rat was struggling or not. The treatment was given once a day, 10 min each session, for a total of 8 sessions. The temperature on the surface of acupoints was measured by using an infrared thermometer (Optris Co., Ltd., Germany).

Morphological observation of the fixed colon samples

The distal colon (6-8 cm) was collected from 2 cm above the anus and rinsed. After gross scoring, the colon samples were fixed in 10% neutral formalin, followed by dehydration, embedding, slicing, and roasting by a pathological analysis system (Leica Co., Ltd., Germany). After being dewaxed and dehydrated by xylene and gradient alcohol, the samples were stained with hematoxylin solution (Nanjing Jiancheng Co., Ltd., China) for 10 min, differentiated by 1% hydrochloric acid alcohol, blued in 1% ammonia, stained by 0.5% eosin solution (Nanjing

Table 1. General morphological scoring

General morphological manifestation		Score
Colon adhesion	No adhesion	0
	Mild adhesion	1
	Severe adhesion	2
Ulcer and inflammation	No ulcer and no inflammation	0
	Local congestion, no ulcer	1
	1 ulcer without congestion or bowel wall thickening	2
	1 ulcer with inflammation	3
	> 2 ulcer and inflammation	4
	> 2 ulcer and/or inflammation area > 1 cm	5
	> 2 ulcer and/or inflammation area > 2 cm	6-8
	One more damage, plus 1	

Table 2. Histomorphological scoring

Histomorphological manifestation		Score
Ulcer	No ulcer	0
	Ulcer area < 3 cm	1
	Ulcer area < 3 cm	2
Inflammation	No inflammation	0
	Mild inflammation	1
	Severe inflammation	2
Granuloma	No granuloma	0
	Granuloma	1
Lesion depth	No lesion	0
	Sublesion	1
	Muscular layer	2
	Serosa layer	3
Fibrosis	No fibrosis	0
	Mild fibrosis	1
	Severe fibrosis	2

Jiancheng Co., Ltd.), and dehydrated again by gradient alcohol solution. Finally, the samples were hyalinized in xylene, and neutral gum was used for sealing. The morphological observation was accomplished by optical microscopy (Olympus Co., Ltd., Japan). The gross and histological scoring criteria are shown in **Tables 1** and **2** [19, 20].

Separation of pMφs [21]

The rats were anesthetized with 3% pentobarbital sodium (Merck Millipore Co., Ltd., USA), and Dulbecco's modified Eagle's medium (DMEM)/F12 medium was injected into the abdominal cavity. After being massaged, the abdomen was dissected along the midline, and the peritoneal irrigation fluid was collected. The peritoneal irrigation fluid was centrifuged at 2,000 rpm, and the supernatant was separated. Phosphate buffer solution (PBS) was added to resuspend the cells. The above operation was repeated twice. The lymphocyte separation solution (Cedarlane Co., Ltd., USA) was added without mixing and centrifuged at 3,000 rpm, and the middle layer of the solution was kept. The cell solution was prepared by adding 1 mL PBS, and the remaining cells were counted for further application.

Detection of $CD86^+$ and $CD163^+$

The number of cells was counted, and the concentration was adjusted to 1 × 10^6 cells/mL. Seven detection tubes were prepared for each sample: a negative control, a homotypic control, a single tube (CD86, PE-labeled), a single tube (CD163, PE-labeled), a single tube (CD11b, Alex488-labeled), a double tube (CD86 and CD11b), and another double tube (CD163 and CD11b). All antibodies involved were purchased from Bio-Rad Co., Ltd. (USA). Cells and corresponding antibodies were added into the tubes and incubated for 30 min at room temperature (25°C) in the dark, and all the tubes were centrifuged at 3,000 rpm per min for 10 min. The supernatant was discarded, and the remaining cells were resuspended by adding 200 μL PBS. After two-color fluorescence compensation, double-marked tubes were detected by using Attune flow cytometer (Thermo Fisher Scientific Co., Ltd., USA).

Phagocytosis of pMφs

The cells separated from the peritoneal cavity were resuspended into 1640 medium (Thermo Fisher Scientific Co., Ltd.) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Co., Ltd.), and the number of cells was counted. The peritoneal cells were inoculated into a 6-well plate (1 × 10^6 cells per well) and incubated at 37°C for 5 h in 5% CO_2. The supernatant was removed, and the remaining cells were cultured in 1 mL 1640 medium containing 10

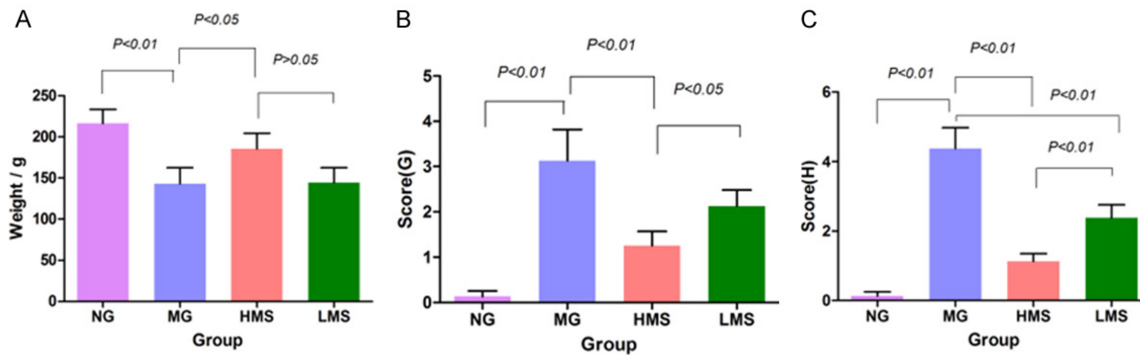

Figure 3. Increased weight, gross and histological score of colon. The ulcerative colitis (UC) model rats experienced diarrhea partially with blood, poor spirit, and significantly slower increase of body weight compared to normal rats [*n* = 8, (150.10±19.54) vs. (108.81±20.20)]. Their gross score and histological score simultaneously increased [*n* = 8, 0 (0,0) vs. 3 (1.25,4); 0 (0, 0) vs. 4 (3, 5)]. Moxibustion at 43±1°C notably improved the colonic lesion of UC rats, and their general and histological scores were both reduced compared to those in the model group (MG) and low-temperature moxibustion group (LMG) [*n* = 8, 1 (1, 1.75) vs. 3 (1.25, 4), 2 (1.25, 2.75); 1 (1, 1.75) vs. 4 (3, 5), 2.5 (1.25, 3)]; rat stool in the high-temperature moxibustion group (HMG) was well formed without any blood, and the body weight increased steadily [*n* = 8, (129.06±15.99) vs. (108.81±20.20); (129.06±15.99) vs. (113.38±19.29)].

μL fluorescent microspheres (1 μm; Thermo Fisher Scientific Co., Ltd.) at 37°C for 1 h. The medium was removed, and the wells were washed with PBS once or twice. The adherent cells were dissociated with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd., China) to prepare a cell suspension, then centrifuged at 3,000 rpm and resuspended into 1 mL PBS for detection.

Content of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-4, IL-13, and migration inhibitory factor (MIF) in peritoneal fluid

According to the instructions of the enzyme-linked immunosorbent assay (ELISA) kit (ColorfulGene Co., Ltd., China), the samples, standards, and avidin were added into each well, mixed, and incubated at 37°C for 30 min. The supernatant was discarded, the plates were washed five times with the washing liquid, the appropriate enzyme-labeled reagent added and incubated at 37°C for 30 min. The supernatant was discarded and washed five times with the washing solution. The reagents A and B were added, mixed, and incubated at 37°C for 15 min. The plates were analyzed by a microplate reader (Bio-Tek Co., Ltd., USA) at a wavelength of 450 nm. The concentration of each cytokine in the sample was determined by plotting a standard curve.

Expression of inducible nitric oxide synthase (iNOs) and arginase I (Arg I) mRNA in pMφs

The sequences of the primers for iNOs were 5'-ATGTCTGGTTTGGAGAGAGCA-3' and 5'-GAGGAGCAGGGACTTCTTGAG-3', and the polymerase chain reaction (PCR) fragment size was 156 bp. The sequences of the primers for Arg I were 5'-TGTGGTAGCAGAGACCCAGAAG-3' and 5'-GTCAGCGGAGTGTTGATGTCAG-3', and the PCR fragment size was 138 bp. The sequences of the primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5'-CCGAGGGCCCACTAAAGG-3' and 5'-GCTGTTGAGTCACAGGAGCAA-3', and the PCR fragment size was 116 bp. The extraction of RNA from peritoneal cells was performed by successively adding TRIzol (Thermo Fisher Scientific Co., Ltd.), chloroform, isopropanol, 75% ethanol, absolute ethanol, and diethylpyrocarbonate (DEPC) water. Internal amplification was performed by the ABI PCR instrument (Thermo Fisher Scientific Co., Ltd.) at 95°C for 10 min and 40 cycles (95°C for 15 s; 60°C for 45 s). The data were analyzed by ABI Prism 7300 SDS software. The relative expression of iNOs and Arg I mRNA was expressed as $2^{\Delta CT}$ × 100% (Δ threshold cycle (CT) = CT value of target gene-CT value of GAPDH) as the statistical value.

Statistical analysis

The data were statistically analyzed using SPSS (version 18.0) software. Normally distributed data (eg. weight, number of different functional phenotypes of phagocytes in peritoneal cavity, phagocytic activity, mRNA expression of iNOS/Arg I, genetic content of various cells from colon sample and expression of MIF in peritoneal cavity) were expressed as mean ± standard

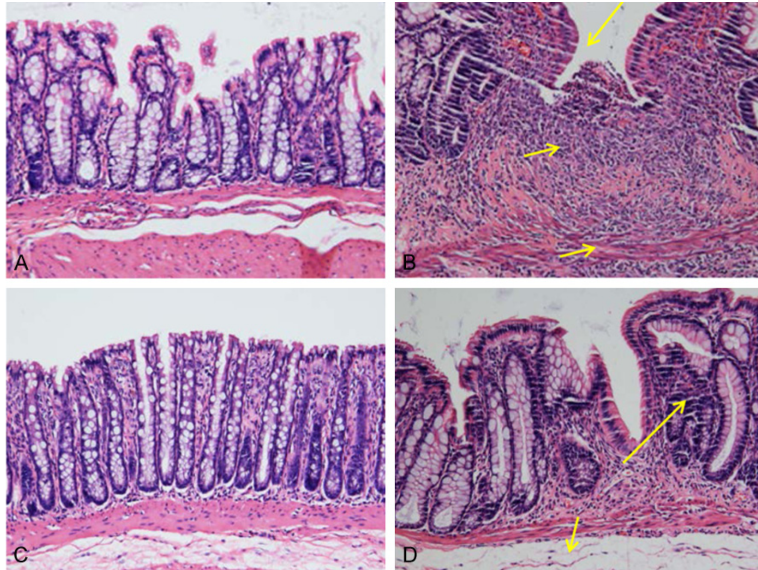

Figure 4. Morphological observation of colon. The colon of ulcerative colitis (UC) model rats showed absence of mucosal epithelium, formation of ulcers, and edematous lymphocyte infiltration into the lamina propria and submucosal connective tissues, as visualized under the microscope. More macrophages were detected in the submucosa of UC model rats than in that of the normal rats. After moxibustion treatment, the colonic damage of rats was notably alleviated in the high-temperature moxibustion group (HMG) and low-temperature moxibustion group (LMG), indicated by complete coverage of mucosal epithelium, hyperplasia of colon glands, and regular glandular cavity. Although the HMG had some scattered macrophages in colonic submucosa, it showed a more significant improvement of colon structure than the model group (MG) and LMG. (Magnification × 200) A. Normal group (NG), B. MG, C. HMG; D. LMG.

deviation and analyzed with one-way analysis of variance (ANOVA) if the homogeneity of variance was confirmed. Fisher's least significant difference (LSD) test was used for comparison between two treatment groups. The data with heterogeneity of variance were analyzed by Dunnett's T3 test. Non-normally distributed data (eg. gross and histological scores) were expressed as median (quartile) and analyzed by a non-parametric test. $P < 0.05$ indicated statistical significance.

Results

Improvement of general condition and colonic lesion

Our results demonstrated that moxibustion produced some notable effects in improving the general condition of UC rats and promoted the repairation of colonic damages. Compared to the normal rats, the UC rat models had loose stool, partially with blood, slower increase in body weight, and severe colonic damage with ulcers and inflammatory infiltration, with significantly higher gross scores and histological scores (all $p < 0.05$, **Figures 3** and **4**). Compared to the MG, the colonic tissue lesion in the HMG was notably improved, with integration of colonic mucosa and alleviation of inflammation, and the gross and histological scores were significantly lower (all $p < 0.05$, **Figures 3** and **4**). In the HMG, rat stool was without blood, and the body weight increased steadily. Compared to the LMG, the gross and histological scores of colon in the HMG were both significantly lower (all $p < 0.05$, **Figures 3** and **4**). No significant differences in the weight gain and the general score between the LMG and the MG were detected (all $p > 0.05$).

Influence on the number of different functional phenotypes of pMφ in peritoneal cavity

Furthermore, moxibustion adjusted the number of pMφs with different functional phenotypes in the peritoneal cavity by increasing $CD11b^+CD163^+$ Mφ and decreasing $CD11b^+CD86^+$ Mφ. CD11b antibody was used to identify Mφs, and CD86 or CD163 antibody was co-incubated to distinguish their functional status inducing injury or recovery respectively. Compared to that in normal rats, the number of $CD11b^+CD86^+$ Mφ significantly increased, while the number of $CD11b^+CD163^+$ Mφ decreased simultaneously in the peritoneal cavity of UC rat models (all $p < 0.05$, **Figure 5**). Compared to that in the MG and LMG, the number of $CD11b^+CD86^+$ Mφ was down-regulated and the number of $CD11b^+CD163^+$ Mφ was up-regulated in the peritoneal cavity of rats in the HMG (all $p < 0.05$, **Figure 5**). No significant difference in the number of $CD11b^+CD163^+$ Mφ in the peritoneal cavity between the LMG and the MG was detected ($p > 0.05$).

Inhibition of the phagocytosis of pMφ

Moreover, moxibustion suppressed the phagocytic activity of pMφ to some extent. Mφ was

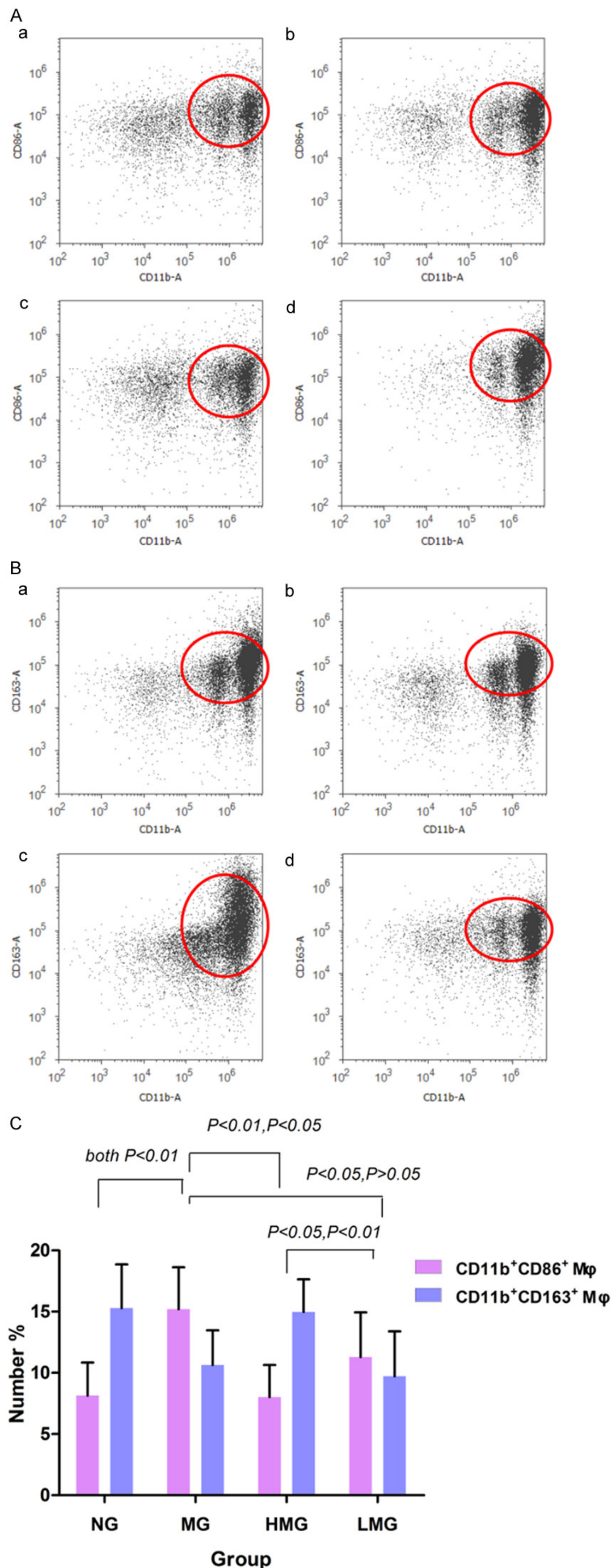

Figure 5. Number of CD11b$^+$CD86$^+$ Mφ and CD11b$^+$CD163$^+$ Mφ in the peritoneal cavity. The number of CD11b$^+$ CD86$^+$ Mφ significantly increased, and the number of CD11b$^+$CD163$^+$ Mφ decreased in the model rats compared to that in the normal rats [n = 8, (8.14±2.70) vs. (15.21±3.42); (15.29±3.57) vs. (10.64±2.83)]. The number of CD11b$^+$CD86$^+$ Mφ was significantly lower, and the number of CD11b$^+$CD163$^+$ Mφ was significantly higher in the high-temperature moxibustion group (HMG) compared to that in the model group (MG) and low-temperature moxibustion group (LMG) [n = 8, (8.02±2.62) vs. (15.21±3.42), (11.29±3.66); (14.97±2.68) vs. (10.64±2.83), (9.73±3.66)]. A. Distribution of peritoneal CD11b$^+$ CD86$^+$ Mφ. B. Distribution of peritoneal CD11b$^+$CD163$^+$ Mφ. C. Number of CD11b$^+$CD86$^+$ Mφ and CD11b$^+$CD163$^+$ Mφ.

separated by time-dependent adherent culture and incubated with 1 μm fluorescent microspheres. Compared to the normal rats, the number of CD11b$^+$ Mφ that had internalized 1 μm fluorescent microspheres in the peritoneal cavity of UC rat models has notably increased ($p < 0.05$, **Figure 6**). Compared to the MG and LMG, the number of CD11b$^+$ Mφ that had internalized 1 μm fluorescent microspheres in the peritoneal cavity of the HMG was significantly lowered ($p < 0.05$, **Figure 6**). No significant difference in the phagocytosis of Mφ in the peritoneal irrigation fluid between the MG and the LMG was detected ($p > 0.05$).

Decrease in the expressions of iNOs mRNA and increase in Arg I mRNA in pMφ

In addition, moxibustion notably down-regulated the expression of iNOS mRNA and up-regulate expression of Arg I mRNA in pMφ. Compared to normal rats, the expression of iNOs mRNA in the pMφ of the UC rats significantly increased, and the expression of Arg I mRNA in the pMφ significantly decreased (all $p < 0.05$, **Figure 7**). Compared to the MG, the expression of iNOs mRNA in the HMG significantly decreased, while

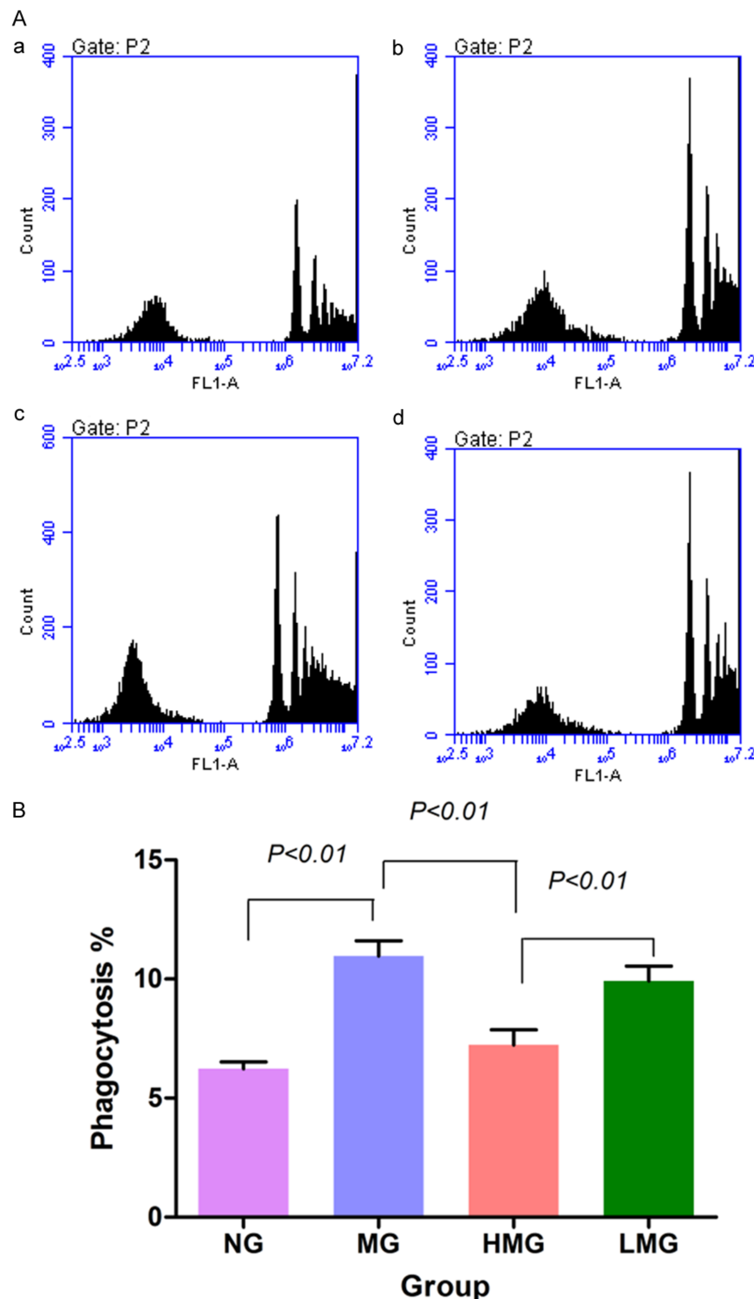

Figure 6. Phagocytic capacity of Mφ in the peritoneal cavity. Positive cells referred to those swallowed more than 1 microsphere and were entirely counted and analyzed. The phagocytic capacity of peritoneal macrophage (pMφ) in ulcerative colitis (UC) rats was significantly strengthened compared to that in the normal rats [n = 8, (6.23±0.79) vs. (10.97±1.80)]. The phagocytic ability in the high-temperature moxibustion group (HMG) was significantly weakened compared to that in the model group (MG) and low-temperature moxibustion group (LMG) [n = 8, (7.23±1.79) vs. (10.97±1.80), (9.92±1.79)]. A. Number of peritoneal macrophage (pMφ) that swallowed microspheres and fluorescent intensity. B. Phagocytic capacity of pMφ.

the expression of Arg I mRNA markedly increased ($p < 0.05$). The expression of Arg I mRNA in the pMφ in the HMG was significantly higher than that in the pMφ in the LMG (all $p < 0.05$, **Figure 7**). No significant difference in the expressions of iNOs and Arg I mRNAs between the MG and the LMG was detected ($p > 0.05$).

Regulation of key cytokines inducing the differentiation of pMφ

Furthermore, moxibustion simultaneously reduced the contents of TNF-α, IFN-γ and increased the contents of IL-4 and IL-13 in the abdominal microenvironment. Compared to the normal rats, the levels of TNF-α and IFN-γ in the peritoneal lavage fluid significantly increased in the UC rat models, while the levels of IL-4 and IL-13 significantly decreased (all $p < 0.05$, **Figure 8**). Compared to MG and LMG, the contents of TNF-α were lowered in the HMG, while the contents of IL-4 and IL-13 obviously increased (all $p < 0.05$, **Figure 8**). There was a slight difference in the content of IFN-γ between the LMG and the HMG but without statistical significance ($p > 0.05$). No significant differences in comparing the expressions of TNF-α, IFN-γ, IL-4, and IL-13 between the MG and the LMG were detected ($p > 0.05$).

Different effects on the expression of MIF in peritoneal cavity

Additionally, moxibustion inhibited the expression of MIF in peritoneal cavity. Compared to the normal rats, the expression of MIF in peritoneal cavity of the UC rat models significantly increased ($p < 0.05$, **Figure 9**). Compared to MG and LMG, the expression of MIF in peritoneal cavity was sig-

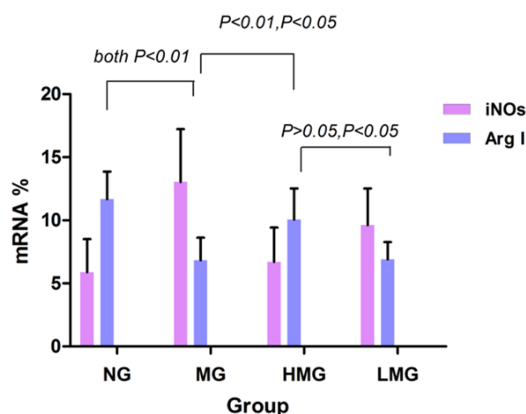

Figure 7. Number of different phenotypes of peritoneal macrophage (pMφ) and expression of inducible nitric oxide synthase (iNOs) and arginase I (Arg I) mRNAs in pMφ. The expression of iNOs mRNA significantly increased, and the expression of Arg I mRNA decreased in the UC model rats compared to that in the normal rats [n = 6, (5.89±2.61) vs. (13.04±4.19); (11.68±2.17) vs. (6.84±1.79)]. The expression of Arg I mRNA was significantly higher, and the expression of iNOs mRNA was lower in the high-temperature moxibustion group (HMG) compared to that in the model group (MG) and low-temperature moxibustion group (LMG) [n = 6, (10.66±2.46) vs. (6.84±1.79); (6.71±2.71) vs. (13.04±4.19)].

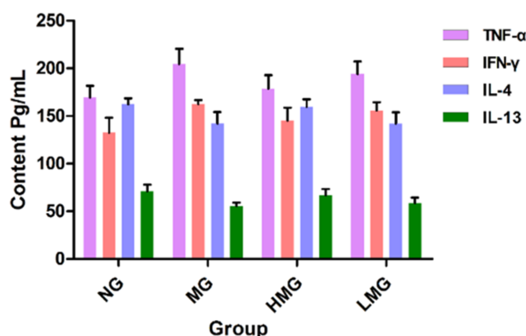

Figure 8. Contents of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-4 (IL-4), and IL-13 in the peritoneal cavity. The levels of TNF-α and IFN-γ significantly increased, and the levels of IL-4 and IL-13 decreased in the ulcerative colitis (UC) rats compared to those in the normal rats [n = 8, (169.48±12.28) vs. (204.68±15.92); (132.51±15.68) vs. (162.57±4.30); (162.39±6.27) vs. (142.10±12.15); (71.14±6.92) vs. (55.52±3.74); all p < 0.05]. The content of TNF-α was significantly lower, and the contents of IL-4 and IL-13 were higher in the high-temperature moxibustion group (HMG) compared to those in the model group (MG) and low-temperature moxibustion group (LMG) [n = 8, (178.79±13.96) vs. (204.68±15.92), (194.07±13.10); (159.68±7.90) vs. (142.10±12.15), (142.06±11.69); (67.05±6.32) vs. (55.52±3.74), (58.56±5.87); all p < 0.05)].

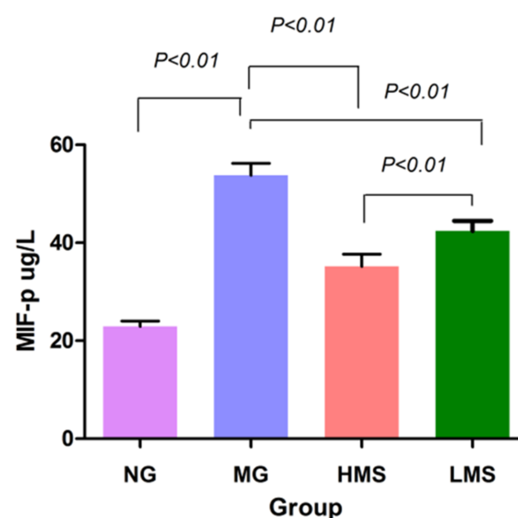

Figure 9. Expression of migration inhibitory factor (MIF) in the peritoneal cavity. The expression of MIF has significantly increased in the ulcerative colitis (UC) rats compared to that in the normal rats [n = 8, (22.94±2.64) vs. (53.78±5.98)]. After moxibustion treatment, the expression of MIF was significantly lower in the high-temperature moxibustion group (HMG) compared to that in the model group (MG) and low-temperature moxibustion group (LMG) [n = 8, (35.17±6.09) vs. (53.78±5.98), (42.41±5.07)].

nificantly lower in HMG (p < 0.05, **Figure 9**). There was significant difference in the expression of MIF in peritoneal cavity between the LMG and the MG (p < 0.05).

Discussion

UC is an unexplained chronic colonic inflammatory disease with diffuse, continuous, and superficial (localized mucosa) characteristics in lesions. Abnormalities of innate immunity in colon have been considered as the root cause of initiation and development of UC [22]. As an important part of the innate immune system, Mφ of different differentiation status shows a high heterogeneity in its function. The amount of inflammatory type Mφ with aberrant activation and migration is the core mechanism of colonic injuries in UC. Moxibustion is one of the effective alternative therapies for UC, characterized by gentle stimulation, non-drug property, and simple application. Moxibustion is also well accepted by patients [9, 23]. Clinical studies showed that moxibustion improved the QOL and promoted the recovery of intestinal function in UC. However, the action mechanism of moxibustion is still unclear, limiting its further

application in the clinic. Thermal effect is the main factor in the action of moxibustion [24-26]. Our study has observed the influence of moxibustion at two different temperatures on the colonic injury of UC rats and explored the action mechanism of moxibustion in treating UC from the perspective of pMφ. The results showed that moxibustion at 43±1°C increased the number of $CD163^+$ Mφ, which is responsible for repairing, inhibiting the phagocytosis ability of pMφ, up-regulating the amount of Arg I mRNA and the contents of IL-4 and IL-13, and down-regulating the expression of MIF in abdominal cavity. Moxibustion at 43±1°C displayed a more powerful regulation on the differentiation and migration of pMφ than moxibustion at 36±1°C. Moxibustion repairs the colonic damages and alleviates the inflammation of UC rats, through its thermal effect on regulating the balance of M1/M2 Mφs and their migration as well as the rebuilding of the homeostasis of the innate immune system. Our study proved that an appropriate temperature was the important basis of moxibustion therapeutic effects, and this is similar to the results of a previous study [27].

In this study, two temperatures were chosen: 36±1°C in the LMG, which is close to normal body temperature producing no or only low heat stimulation, and 43±1°C in the HMG, which is definitely higher than normal body temperature, producing a significant thermal stimulation. The results showed that moxibustion at 43±1°C improved the situation of diarrhea and hematochezia, increased the body weight, alleviating colonic injuries and significantly lowering the gross and histological colon scores in UC rat models. This indicates that an optimal temperature is an essential factor of moxibustion in ameliorating colonic damage and abdominal symptoms in treating UC. Some studies reported that a high temperature ranging from 38°C to 41°C modulated both innate and acquired immune systems, and this phenomenon is known as hyperthermia. The immune-regulatory effect of temperature at this range was different from that induced by exogenous heat stimulation [27]. Studies also showed that at 40°C, the phagocytosis ability of mononuclear macrophages in human peripheral blood was improved, while the activity of natural killer cells was inhibited at 42°C [28, 29]. We concluded that the therapeutic efficacy of moxibustion required the maintenance of an appropriate temperature to induce a bilateral regulation of the immune response.

Our study focused on the possible action mechanism of moxibustion in treatment of UC by looking into the change of pMφ. M1 and M2 are the two major cell types differentiated from Mφ in different microenvironments, and they have different cell phenotypes and functional activities [30, 31] (**Figure 1**). Activation and aberrant migration of M1 Mφ in colonic tissue are important mechanisms that cause destabilization of innate immunity and inflammatory injury in colon. Different phenotypes of Mφ indicate its functional heterogeneity, and its activity is involved in the injury and repair of colon in UC rats. Experiments on 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced IBD rat models demonstrated that the activities of colonic macrophage (cMφ) and pMφ were similar, the isolated pMφ highly reacted to lipopolysaccharide (LPS) stimulation *in vitro*, and the expression of TNF-α, IL-1β, and iNOs were strengthened [32, 33]. Thermal stimulation at 39.5°C increases the ability of pMφ in responding to LPS both *in vivo* and *in vitro*, indicated by higher expressions of TNF-α and 70-kDa heat-shock protein (HSP70) and activated nuclear factor-kappa B (NF-κB) signaling pathway, indicating that the variations of pMφ function are capable of reflecting the repair ability of the body triggered by high fever after infection [34]. Thus, we assume that moxibustion improves the symptoms of UC and promotes the healing of intestinal injury plausibly by regulating Mφ [35-38]. After verifying the role of temperature in the action of moxibustion, this study further observed the regulatory effect of moxibustion on the phenotype and function of pMφ in order to prove that moxibustion at Tianshu (ST25) acupoints eased colonic inflammation and healed the colonic damages via regulating Mφ. Our research showed that moxibustion at 43±1°C increases the number of $CD11b^+CD163^+$ Mφ, lowers the number of $CD11b^+CD86^+$ Mφ in the abdominal cavity, up-regulates the expression of Arg I mRNA, and down-regulates iNOs mRNA in pMφ, and the phagocytosis was weakened in the UC rats. We detected the level of key cytokines during Mφ differentiation and MIF, which determines pMφ migration in peritoneal lavage fluid, finding that moxibustion at 43±1°C promoted the expression of IL-4 and IL-13, inhibited the production of IFN-γ and TNF-α, and down-regulated the expression of MIF in peritoneal cavity. These

results suggest that moxibustion at 43±1°C regulate the differentiation and migration of Mφ, and improve the microenvironment to generate M2 Mφ to promote the recovery of colonic injury.

Peritoneal Mφ has the characteristic of migration, and previous study confirmed that the colonic mucosal lymph cells partially originated from pMφ migration [39]. However, the detailed relationships between intestinal lymph cells and pMφ require further studies. Although moxibustion at 43±1°C produced more significant effects, moxibustion at 36±1°C significantly lowered the colonic histological score and the number of $CD11b^+CD86^+$ Mφ and MIF in the abdominal cavity. Thus, we hypothesize that there are other factors in the therapeutic action of moxibustion such as moxa smoke. However, further studies are required in order to elucidate the mechanisms underlying moxibustion action.

Moxibustion at 43±1°C reduces the number of $CD86^+$ pMφ, increases the number of $CD163^+$ pMφ, regulates the expression of iNOs and Arg I mRNAs in pMφ, inhibits the phagocytosis ability of pMφ, down-regulates the content of TNF-α, up-regulates the expression of IL-4 and IL-13 in the abdominal cavity, and influences the transformation of pMφ in the UC rat models. Moxibustion alleviates the colonic damages in UC rats plausibly via affecting the phenotype, function, and chemotaxis of pMφ through thermal effect.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81202754, 81674073); National Key Basic Research Program of China (973 Program) (No. 2015CB554501); Project of Shanghai Municipal Commission of Health and Family Planning (No. 20144Y0153).

Disclosure of conflict of interest

None.

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