Gua Sha attenuates the pulmonary inflammation in mice infected with PR8 virus by balancing the ratio of Treg/Th17

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Abstract
Background: Gua Sha, an ancient Chinese treatment which produces the pressure on the skin, is used to prevent and treat cold for thousands of years. There’re evidences to approve that it can activate immune response and reduce the inflammation. However, how it has the effect on T helper 17 cells (Th17) and regulatory T cells (Treg) is poorly understood. Here, this study aims at the relationship between the pressure-stoke in the skin and pulmonary Th17 as well as Treg in PR8-infected mice.

Methods: ICR mice were randomly divided into five groups. The body weight and survival rates of all groups were monitored through the experiment. At the end of experiment, lung inflammation was detected by HE staining and the expression of Matrix metalloproteinase-9 (MMP-9) was measured by immunohistochemistry. Th17 and Treg from lung tissues was analyzed by flow cytometry.

Results: Our results indicated that the survival rates of prophylactic and therapeutic group respectively showed 20% and 10% though Gua Sha treatment didn’t restore the weight-loss of PR8-infected mice. What’s more important, Gua Sha remarkably inhibited inflammatory infiltration and the expression of MMP-9 of lung tissues in infected mice (p <0.05). Finally, the ratio of Treg/Th17 from lung tissues in PR8-infected mice was significantly increased as compared with control mice while Gua Sha treatment remarkably inhibited this enhancement. All these results indicated that Gua Sha has the efficacy on reducing the pulmonary inflammation in PR8-infected mice possibly via restoring the Treg/Th17 balance.

Conclusions: Our findings for the first time suggest that Gua Sha exhibits a significant inhibition of inflammatory infiltration with down-regulation of MMP-9 in lung tissues from RR8-infected mice, which might be associated with the differentiation of Th17 and Treg. Further research will be carried toward how Gua Sha functions on maintaining the homeostasis of Th17 and Treg in the lungs.

Background
Influenza is an infectious respiratory disease which usually manifests as a series of clinical symptoms such as cough, fever, headache and weakness [1,2]. The Influenza virus that is a common cause of influenza belongs to the orthomyxoviridae family, and the viral genome is a single-stranded (-) RNA in seven or eight fragments. There are three viral serotypes, including A, B, and C [3]. The influenza A
virus (IAV), including PR8, infects a wide variety of hosts, particularly mammals and poultry [4]. According to past research, multifarious inflammatory cells in lung, as well as bronchial epithelial cells, fibroblasts, smooth muscle cells, could increase the expression of MMP-9 after infection with influenza virus [5]. MMP-9 degrades components of the alveolar basement membrane which contributes to the destruction of structure in the lung [6]. During the influenza virus infection, both innate and adaptive responses are involved in host defense. Particularly, the ratio of Treg/Th17 changes a lot in infected models [7], suggesting the interplay of Treg/Th17 is essential to shape immune response after virus infection.

Gua Sha is an effective therapy for many diseases in traditional Chinese medicine. 'Gua' means 'to scrape or scratch', thus Gua Sha is a method to bring 'Sha' to the body's surface by means of scratching or scraping [8]. Gua Sha therapy is a therapeutic modality that involves using a smooth-edged instrument for skin frictioning to intentionally create transient red or purple petechiae and ecchymosis, which normally fades in a few days [9]. This therapy is generally well tolerated, with little or no discomfort. It is widely used and spread because of its simple operation and avoiding oral side effects of medications [10]. And research evidence reported Gua Sha has the effect on treating respiratory diseases [11–13].

Research on Gua Sha therapy is mainly focus on clinical trial reports and only a few studies discussed its physiological effects and potential therapeutic mechanisms [14–16]. Some studies showed that scraping can improve the immune function of the body. After administration of Gua Sha, the number of neutrophils and macrophages increased, and then keratinocytes release a large number of inflammatory cytokines which could activate the migration and accumulation of immune cells [17].

However, these findings lack the experiment of applying Gua Sha therapy alone and the mechanism of Gua Sha therapy in controlling respiratory inflammation remains unclear. In the present study, we demonstrate viral pneumonia model causes severe damage to lung tissue in infected mice. And Gua
Sha therapy could attenuate the pulmonary inflammation through decreasing the expression of MMP-9 and reversing the imbalance of Treg/Th17 in lung tissues.

**Methods**

1.  *Mice, virus and drugs*

Male ICR mice at 6-8 weeks of age, were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). The mice were randomly divided into 6 groups: control group, model group, prophylactic group, therapeutic group, sham group and ribavirin group. Mice were monitored for survival, weight loss, and clinical signs of illness (e.g., inactivity, ruffled fur, hunched posture, poor appetite, rapid shallow breathing and audible crackling) for 14 days. The influenza A/PR/8/H1N1 virus was kindly gifted by professor Yu Hao from the Department of Immunology and Microbiology, Beijing University of Chinese Medicine (Beijing, China). The LD$_{50}$ was determined in mice after serial dilution of the stock. We challenged ICR mice with 5 LD$_{50}$ A/PR8 (25 mL). Infection was established by intranasal inoculation in mice after anesthetized by isoflurane. Ribavirin was purchased from Biokin Pharmaceutical (Sichuan, China). The ribavirin group was given ribavirin (100mg/kg), once a day for 7 days. All experimental procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care Committee of Beijing University of Chinese Medicine.

2.  *Gua Sha treatment on experimental mice*

Mice were anesthetized by 5% isoflurane and maintained at 1.5-2%. In the prophylactic and therapeutic groups, Gua Sha was performed on the side of the mouse's back by using a buffalo-horn Gua Sha plate, after the hair was shaved with a clipper a day prior to the experiment. The shaved skin area was wiped with 70% ethanol and left to dry. Then, scrape the back 100-150 times/min from neck to tail in a unidirectional manner, with an angle of about 90° between the Gua Sha plate and the mouse's back. The force of scrape was based on the appearance of red spots or freckles. Meanwhile, the sham group was scraping at the same frequency and force in the left thigh of the mouse.
3. **Pathological analysis**

At the end of experiment, the whole lungs of the mice were removed, washed in phosphate buffer and fixed in 10% formaldehyde at room temperature. Then lung tissues were dehydrated in graded concentration of ethanol, embedded in paraffin and sliced. Tissue sections of 4 μm thickness were stained with hematoxylin-eosin (HE). Then two experienced pathologists blinded with mice group observed sections of lung tissue under a light microscope and scored the lung injury through the method as described by Mikawa [18]: (a) alveolar congestion, (b) hemorrhage, (c) neutrophil infiltration in the alveolar and vascular wall, and (d) alveolar wall thickening/the formation of the hyaline membrane. Each of the above items was graded into five levels: 0=no damage, 1=slight damage, 2=moderate damage, 3= severe damage, and 4=extremely severe damage. The sum of the four items was the final score with a maximum of 16.

4. **Immunohistochemistry**

Immunohistochemistry (IHC) method was adopted to detect the expression of MMP-9 in the paraffin sections of mouse lung tissue. After being sliced, dewaxed, and hydrated, sections were placed in 3% H$_2$O$_2$ to incubate for 10 min, then rinsed 5 min three times with phosphate-buffered saline (PBS). Then they were incubated with 0.01 M citrate buffer for 15 min in 95°C water and flushed 5 min three times with PBS. For primary antibody incubation, anti-mouse MMP-9 antibody (from BioLegend, Inc., San Diego, CA) was diluted at 1:1000 with PBS and added into sections at 4°C overnight. Horseradish Peroxidase (HPR)-labeled secondary antibody (from Zsbio Commerce Store, Beijing, China) was incubated at 37°C for 20 min. Peroxidase activity was detected by using 3,3-diaminobenzidine tetrachloride (DAB; Beijing solarbio science & technology co., ltd., Beijing, China). Sections were counterstained by using hematoxylin and then observed under microscope. Ten visual fields were randomly selected for each group, and their integral optical density (OD) was measured by use of Image Pro software, and then semiquantitative analysis was conducted by means of statistical software.
5. **Cell isolation from lung tissues**

After sacrificing on day 6, lung tissues from mice were aseptically collected. To isolate single cell suspension from lung tissues, lungs were minced and digested with 1 mg/ml type IV collagenase (Worthington) and 50 μg/ml DNase I (Roche) for 45min at 37°C on a rotator. Then digested tissues liquid were filtered through 70-μm cell strainers and enriched with 40% Percoll gradient after red blood cells were lysed. Single-cell suspensions from lungs were used for subsequent flow cytometry staining.

6. **Flow cytometry analysis**

For intracellular cytokine staining, cells were stimulated with Cell Stimulation Cocktail (eBioscience) and incubated for 5 hours at 37°C. Cells were preincubated with anti-mouse CD16/32 (BioLegend) to block Fc receptors and washed before further staining. Then cells were stained with FITC conjugated anti-CD4 (BioLegend) and PercPCy5.5 conjugated anti-CD25 (BioLegend), following by fixing and permeabilizing with BD Cytofix/Cytoperm buffer. At last, cells were stained with PE conjugated IL-17A (BioLegend). For measurement of transcription factors, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions and stained with antibodies APC-FoxP3 (BioLegend). Cells were detected by Cantoll (BD, Biosciences) and analyzed by FlowJo software.

7. **Statistical analysis**

The SPSS16.0 Software was used to complete the statistical analysis. Student’s t-test was used to compare continuous variables between two groups, and ANOVA was used to compare continuous variables across multiple groups. Mantel-Cox test was used for Survival data. A p-value less than 0.05 was considered statistically significant.

Results
1. **The effect of skin scraping on survival and body weight of PR8-infected mice.**

Protocol for experimental influenza infection and skin scraping treatment were showed in Figure 1a. PR8-infected mice showed the signs of dehydration, greasy fur, and inactive condition from 3 days after infection. Ribavirin alleviated the condition of dehydration and recovered the activity in PR8-infected mice. No obvious recovery in the behavioral appearance were observed in Gua Sha prophylactic and therapeutic groups. Mice in model group showed significant weight loss and survival decrease during the influenza infection. Although the body weight in ribavirin group began to lower from day 3, ribavirin quickly recovered their loss in PR8-infected mice from day 4. In addition, ribavirin protected infected mice from dying (Fig. 1b and c). It’s worth noted that 20% and 10% survival rate existed respectively in Gua Sha prophylactic and therapeutic groups though scraping didn’t prevent the loss of body weight in PR8-infected mice.

2. **Skin scraping attenuated pulmonary hyperemia and lung inflammation in PR8-infected mice.**

To detect the pulmonary damage caused by PR8 virus infection in mice, we measured lung histopathology from anatomical observation and H&E staining of lung sections. The control group showed a normal lung appearance with pink color and normal intact alveoli structures (Fig.2a and b). PR8-infected mice possessed a dark red lung with severe congestive edema. H&E staining of the lung sections revealed that a large amount of inflammatory exudate was both around bronchus and in the alveoli interstitium in PR8-infected mice, which caused the interstitial thickening of the lung and broke the integrity of alveoli structure. Both Gua Sha prophylactic and therapeutic groups indicated red-pale lungs, significant decreasing of inflammation infiltration and amendment of damage for alveoli structure. Meanwhile, Gua Sha administration ameliorated the lung injury obviously in PR8-infected mice according to analysis of pathological scores (Fig.2c). These results indicate skin scraping has preventive and curative effect on alleviating pulmonary inflammation in PR8-infected mice.
3. **Skin scraping suppressed the expression of MMP-9 in lung tissue in PR8-infected mice**

The main function of MMP-9 is to degrade IV, V collagen and gelatin, which are the most important components in the extracellular matrix [19]. MMP-9 is secreted by bronchial epithelial cells, neutrophils, eosinophils, mast cells and alveolar macrophages suggesting that MMP-9 can be expressed both in normal lung and the lung tissue infiltrated by inflammatory exudate[20,21]. After the infection of influenza virus, remarkably increased expression of MMP-9 was from both various inflammatory cells and parenchymal cells, which was detected by immunohistochemistry staining (Fig.3a) while only shown in epithelial cells of the control group ($p<0.01$). Gua Sha administration significantly reduced the expression of MMP-9 in lung tissue from PR8-infected mice. What’s more, this expression of MMP-9 was restricted in lung parenchyma cells in Gua Sha prophylactic and therapeutic groups. (Fig. 3b, c). These results suggest that skin scraping significantly inhibits the expression of MMP-9 in lung tissue cells from PR8-infected mice.

4. **Skin scraping rectified the ratio of Treg/Th17 in PR8-infected mice.**

Th17 cells are a subset of pro-inflammatory T helper cells defined by their production of interleukin 17 (IL-17) and their main effector cytokines are IL-17A, IL-17F, IL-21, and IL-22. They play the role of a double-edged sword during influenza infection [22,23]. Treg cells are another unique T lymphocyte subset in the body that secretes IL-10, and TGF-β, which is regulated by the transcriptional factor foxhead box P3 (FoxP3) [24,25]. Treg cells suppress activity of a variety of immune cells and therefore inhibit immune responses, which are closely related to influenza [26]. The numbers of Tregs and Th17 cells from lung tissues were detected by flow cytometry so as to analyze whether and how skin scraping influenced the differentiation of T helper cell during PR8 infection in mice. On day 6 after infection, single cell suspensions from lung tissues of every group were obtained. Then intracellular cytokines staining was used to detect CD3$^+$CD4$^+$IL-17A$^+$ T cells as Th17 after stimulation of PMA plus ionomycin while Treg was defined as CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ by flow cytometry. From
Figure 4, the model group showed a significantly higher proportion of Treg and lower Th17 compared with control group. However, skin scraping remarkably inhibited the enhancement of Treg and promoted the differentiation of Th17. It’s interesting that ribavirin induced the highest proportion of Th17 in PR8-infected mice. The ratio of Treg/Th17 in PR8-infected mice was 2.16:1, while this ratio in prophylactic and therapeutic group was significantly lower than that in model group. All these results indicated that Gua Sha has the efficacy on reducing the pulmonary inflammation in PR8-infected mice possibly via restoring the Treg/Th17 balance.

Discussion
Gua Sha is a traditional Chinese physical therapy. Under the guidance of the theory of meridians and acupoints of traditional Chinese medicine, red splotchy marks appear on the skin that look like scrapes or light bruising, but in actuality it is a vascular response called transitory therapeutic petechiae after Gua Sha treatment [27]. We selected the erector spinae muscle on both sides of the back spine of the mouse in prophylactic and therapeutic group for scraping, which is named “Urinary Bladder Meridian of Foot-Taiyang” by traditional Chinese medicine (TCM), because it was always used to prevent and treat pulmonary diseases in clinic [28].

In our study, we first observed the effect of scraping on the survival rate and body weight of influenza mice. It is well known that the influenza virus attaches airway epithelial cells through hemagglutinin (HA) protein on its surface, causing disease by a large number of replications in the airways and alveolar epithelium [22]. In our experiments, though Gua Sha as a physical therapy which induced the moderate inflammatory response and immune responses failed to prevent weight loss and the decreasing of survival rate in infected mice, it had the significantly protective effect against pulmonary inflammatory exudates. This discrepancy might be reasonably explained that Gua sha is performed on the skin which delivers its therapeutic effect on lung, according to “lung governing skin and hair”. However, PR8 viruses not only induced viral pneumonia by intranasal administration, but also entered the blood and triggered systemic failure.
In addition, Immunohistochemistry revealed that the secretion amount of MMP-9 in PR8-infected mice was higher than other groups. These results together showed that the expression of MMP-9 is associated with lung pathology induced by PR8 infection. MMP-9, referred to as a zinc-binding endopeptidase whose main components are type IV, V collagen and gelatin, which can degrade a variety of extracellular matrix molecules, modulates tissue remodeling upon acute lung injury and interstitial lung disease [6,19]. When the lungs were infected with influenza virus, MMP-9 could directly act on the basement membrane of the alveolar capillaries to increase its permeability, and at the same time destroyed the connection of cadherin to the vascular endothelial cells and increase the microvascular permeability [29]. Pharmacological inhibition of MMP-9 also has been reported to partially reduce lung pathology in mice caused by influenza virus, and MMP-9 deficiency protects mice from severe influenza A viral infection [30,31]. An in vitro study showed that influenza virus infection increases MMP-9 secretion and promoter activity in Vero cells [33]. Our data showed that an increase of MMP-9 in the lung immunohistochemical analysis of the model and the sham group on day 6 after infection, and a remarkable decrease in Gua Sha treatment. Recently, Joselyn et al. showed that the increasing pulmonary adaptive immune response to IAV, with higher CD4\(^+\) T lymphocytes and lower frequencies of anti-inflammatory Tregs, appeared in MMP-9 \(^{-/-}\) mice after IAV infection [31]. And MMP-9 might decrease IL-17 production in the lungs by selectively inhibiting IL-23 expression [32]. Coincidental with theirs, our data showed that MMP-9 production, which affected the balance of regulatory T cell and other effector CD4\(^+\)T lymphocytes, was critical to severe lung pathology caused by influenza A virus.

It is reported that virus-induced Tregs can become activated by a pathogen-derived peptide and downregulate antigen-specific effector CD4\(^+\) and CD8\(^+\) T cell accumulation and cytokine production correlated with their antigen specificity, so as to modulate an antiviral immune response in PR8-infected mice [26,34]. Some researches demonstrated that Treg cells limit Th17 cell inflammation by serving as principal amplifiers of negative regulatory circuits operating in immune effector cells.
Interleukin-10 (IL-10) signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation during PR8 infection [35,36]. In our study, the higher ratio of Treg/Th17 emerged in PR8-infected mice compared with the control group. Meanwhile, this dominant Treg was highly rectified and a significant proportion of Th17 was induced after Gua Sha or ribavirin treatment in PR8-infected mice. Th17 was a double-edged sword during influenza infection. Although some studies have suggested a pathological role for IL-17 secreted by Th17 cells in host immunity to influenza, other studies have suggested a protective function. For instance, it has been documented that IL-17 depletion resulted in increased weight loss as well as reduced survival in mouse model of influenza [23,37]. These higher Th17 cells might be necessary for clearing the infection and promoting tissue repair.

Conclusions
In summary, our findings for the first time suggest that Gua Sha exhibits a significant inhibition of inflammatory infiltration with down-regulation of MMP-9 in lung tissues from RR8-infected mice, which might be associated with the differentiation of Th17 and Treg. Further research will be carried toward how Gua Sha functions on maintaining the homeostasis of Th17 and Treg in the lungs.

Abbreviations
Th17: T helper 17 cells; Treg: Regulatory T cells; MMP-9: Matrix metalloproteinase-9; IAV: Influenza A virus; PR8: A/PR/8/H1N1 virus; HE: Hematoxylin-eosin; IHC: Immunohistochemistry; PBS: Phosphate-buffered saline; HPR: Horseradish Peroxidase; OD: optical density; TCM: Traditional Chinese medicine; HA: Hemagglutinin.

Declarations

Ethics approval and consent to participate
All procedures were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Consent for publication
All authors agree to publish this paper.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding
author on reasonable request.

*Competing interests*

The authors declare that they have no competing interests.

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*Authors’ contributions*

Each author has contributed significantly to this study. GYP, YLL and JWK conceived and designed the study. YLL, YAW, JWK and ZRL performed the animal experiments and flow cytometry detection. DYG and RJD performed pathological and histochemical experiments. YLL and YAW performed the statistical analysis. GYP, YLL, YAW, JWK drafted and revised the manuscript. All authors read and approved the final manuscript.

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Figures
The effect of skin scraping on survival and body weight of PR8 infected mice. a The mice in the prophylactic group began to scrape 3 days before PR8 infection, once every other day for 2 times. The therapeutic group and the sham group began to scrape at 2 hours after infection, once every other day for 3 times. The control group and the ribavirin group were intragastrically administered at 2 hours after infection. The ribavirin group was given ribavirin (100 mg/kg) 0.2 ml/day for 7 days. Normal diet and drinking water in mice. b Body weight was monitored after scraping. c Survival was monitored after PR8 infection. n=10, Mean ± SD. ****p<0.0001 compared with the control group. ns: not significant.
Figure 1

The effect of skin scraping on survival and body weight of PR8 infected mice. a The mice in the prophylactic group began to scrape 3 days before PR8 infection, once every other day for 2 times. The therapeutic group and the sham group began to scrape at 2 hours after infection, once every other day for 3 times. The control group and the ribavirin group were intragastrically administered at 2 hours after infection. The ribavirin group was given ribavirin (100 mg/kg) 0.2 ml/day for 7 days. Normal diet and drinking water in mice. b Body weight was monitored after scraping. c Survival was monitored after PR8 infection. n=10, Mean ± SD. ****p<0.0001 compared with the control group. ns: not significant.
Skin scraping attenuates pulmonary hyperemia and lung inflammation in PR8 infected mice.

a The macroscopic pathology of lung among different groups were showed on day6 after PR8 infection. b Representative pathological micrographs of lung sections (HE staining, ×200). c Lung injury scores of the mice in different groups on day6 after PR8 infection. Mean ± SD. n=3. ***p<0.001 compared with the control group. #p<0.05, ##p<0.01 compared with the model group.
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Figure 3

Skin scraping can suppress the expression of MMP-9 in lung tissue. a Representative IHC results for MMP-9 in mice lung tissue of each group on day6 after infection (original magnification, ×200). b, c Semiquantitative analysis of IHC by the method of the average optical density (AOD). Mean ± SD. n=3. **p<0.01, compared with the control group. #p<0.05, ##p<0.01 compared with the model group.
 Skin scraping can suppress the expression of MMP-9 in lung tissue. a Representative IHC results for MMP-9 in mice lung tissue of each group on day 6 after infection (original magnification, ×200). b, c Semiquantitative analysis of IHC by the method of the average optical density (AOD). Mean ± SD. n=3. **p<0.01, compared with the control group. #p<0.05, ##p<0.01 compared with the model group.
Figure 4

Changes in Th17 cells and Tregs in influenza mice by scraping. Single cell suspensions from lung tissues of every group were obtained 6d after PR8 infection in mice. Representative flow cytometry charts illustrated percentage of Th17 cells and FoxP3+ regulatory T cells in lungs. These were pre-gated from CD3+CD4+ cells.
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