Phosphatidylserine Topically Attenuates Imiquimod-induced Psoriasis Through Inflammation Inhibition in Mice

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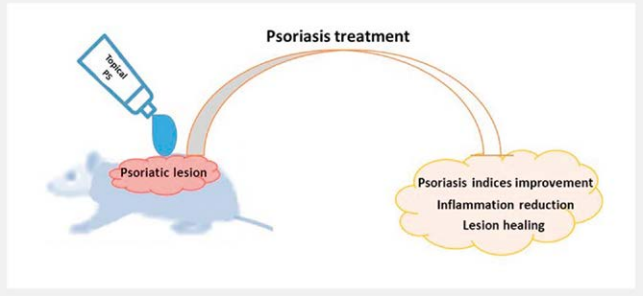
ABSTRACT

Background Psoriasis is a chronic skin condition that is associated with persistent inflammation and skin lesions. Topical therapy has been a promising approach to the alleviation of psoriasis through the application of anti-inflammatory agents. Phosphatidylserine (PS) administration has shown anti-inflammatory effects in the trials. Consequently, the objective of this study was to evaluate the effects of topical PS on the potential improvement of an imiquimod (IMQ)-induced psoriasis model. Additionally, cyclosporine A was utilized as a comparative anti-psoriatic agent in our study.

Methods The psoriasis model was established by topically applying IMQ to the dorsal skin of mice once daily for five consecutive days. The efficacy of topical PS was assessed using the Psoriasis Area and Severity Index (PASI) score to evaluate skin lesions. Subsequently, the skin samples were analyzed using Baker's scoring system, Masson's trichrome staining, immunohistochemistry, and real-time PCR analysis.

Results IMQ-induced plaque-type psoriasis resulted in a significant increase *(P < 0.05)* in dermal thickness, hyperkeratosis, PASI score, and inflammatory cytokines at the lesion site. The topical PS and cyclosporine A significantly *(P < 0.05)* reduced PASI score and dermal thickness, while also alleviating erythema and scaling when compared to untreated mice. Furthermore, biomolecular assessments revealed that PS significantly *(P < 0.05)* inhibited the gene expression of IL-17, IL-23, and TNF-α cytokines in the IMQ-induced lesions.

Conclusion Topical PS may pointedly alleviate psoriasis through the inhibition of inflammation. The beneficial effects of the PS recommend further investigation in both experimental and clinical studies in the control of skin psoriasis.



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Introduction

Psoriasis is a chronic and immune-mediated skin disease, which can be caused by hereditary, environmental, and lifestyle factors or a combination of them [1]. The disease is characterized by the presence of erythematous plaques covered with white or silvery scales, which may be as localized or extensive on the skin [2]. It is established that abnormalities in keratinocyte proliferation and differentiation contribute to the development of acanthosis, hypogranulosis, parakeratinization, and proliferation of cutaneous blood vessels, all of which are hallmarks of psoriatic plaques [3]. Furthermore, in psoriatic skin lesions, there is an elevation in the levels of inflammatory cytokines, which exacerbates disease severity and promotes neovascularization within the skin [4]. Based on the critical role of the IL-23/Th17 axis in the pathogenesis of psoriasis, several IL-23 and IL-17 antagonists have been approved or are under investigation [5]. However, it is noteworthy that not all patients exhibit a favorable response to IL-17 and IL-23. Moreover, TNF-α and IL-17 collaborate in a sustained inflammation within psoriatic lesions, hence the simultaneous inhibition of both cytokines can represent a promising therapeutic strategy [5, 6].

Despite considerable research that has been conducted on the mechanisms underlying the attenuation of psoriasis [7, 8], no effective treatment has been established that can completely eradicate the disease so far. Even the mildest form of the disease, characterized by localized inflammatory lesions, has the potential to rapidly progress into a more severe manifestation that affects over 10 % of the body surface area [9, 10]. Typically, treatment options for patients with psoriasis include local therapy, phototherapy, and systemic treatments. However, these approaches have demonstrated certain limitations in clinical practice [11–13], indicating a continued necessity for the exploration of new therapeutic choices for psoriasis.

PS is a dietary supplement that has received approval from the Food and Drug Administration (FDA) and is recommended for the prevention of memory impairment and dysfunction in older adults [14]. A clinical study involving patients with AD demonstrated that those receiving oral PS exhibited significantly higher scores in semantic memory tests, vocabulary assessments, and picture-matching tasks compared to the control group [15]. Moreover, experimental studies have reported the anti-inflammatory and immunomodulatory effects of PS phospholipids [16–19].

PS phospholipids are integral components of the cell membrane, with those located on the outer membrane being recognized by the PS receptors of phagocytes [20]. Research also indicates that the interaction between phagocytes and PS liposomes simulates the recognition of apoptotic cells during the process of efferocytosis [20]. This interaction leads to anti-inflammatory and immunosuppressive effects within the organism [16, 17, 19]. PS liposomes have been shown to reduce the production of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interferon-gamma IL-1, interleukin-1 (IL-1), IL-6 interleukin-6 (IL-6), while also suppressing humoral and cellular immune systems responses by antigen antigens in *in vitro* and *in vivo* studies [17, 21–23]. The study's results of Xuemei et al. [16] determined that therapeutic stem cells have immunomodulatory effects through the release of PS due to apoptotic stem cells. The data indicate PS regulate the immune system by the increase of the IL-10-production, decrease of neutrophil infiltration, inhibition of NK cell activation, and induction of macrophage polarization towards M2 [16].

In this study, we aimed to investigate the anti-psoriatic properties of topical PS using a murine model. To evaluate the severity of skin inflammation, we employed the Psoriasis Area and Severity Index (PASI) score, with mice subjected to IMQ-induced psoriasis. Moreover, the effect of topical PS on the other hallmarks of the psoriasis model was evaluated through Baker's scoring system, histopathological parameters, real-time polymerase chain reaction (PCR), and immunohistochemistry analysis.

Results

Body and spleen weights

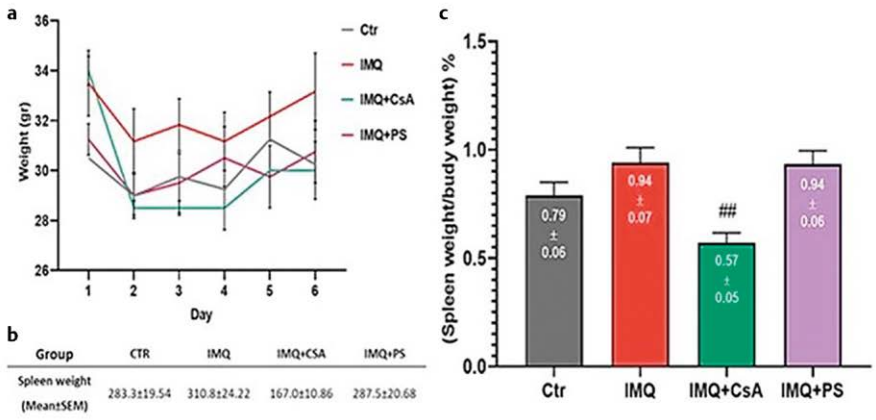
In this study, the alterations in the spleen weight of the mice were assessed independently of changes in the animals' body weight. Consequently, the ratio of spleen weight to body weight was calculated to facilitate comparisons between the groups. According to our data (▶ **Fig. 1a**), there is no statistical difference ($P > 0.05$) in body weight changes among the various groups over time. Furthermore, the spleen weight in the IMQ group exhibited an increase, whereas a decrease was observed in the treated CsA group (▶ **Fig. 1b**). The percentage of spleen weight/body weight reduced significantly ($P < 0.05$) in the IMQ + CsA group compared to the IMQ group (▶ **Fig. 1c**). Furthermore, the percentage of spleen weight to body weight did not demonstrate any significant difference *(P > 0.05)* in the IMQ + PS group compared to the IMQ group (▶ **Fig. 1c**).

Macroscopic observation and PASI score

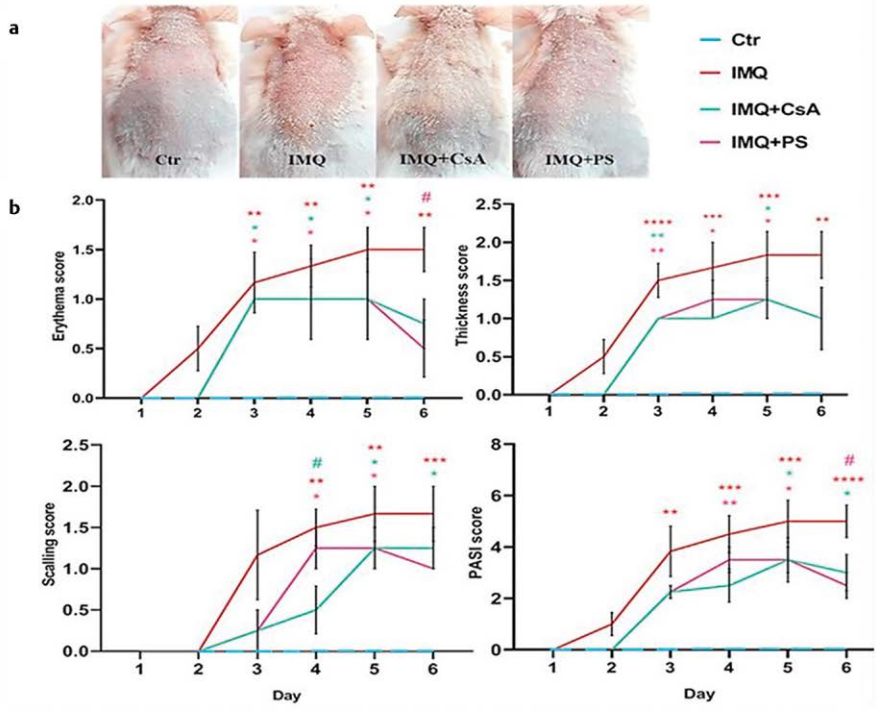
As given in ▶ **Fig. 2a, b**, the inflammatory signs including erythema, scaling, thickness, and cumulative PASI score, exhibited a significant reduction in the IMQ + PS and IMQ + CsA groups when compared to the IMQ group. Moreover, the comparison between the Ctr group and the IMQ group revealed a significant *(P < 0.05)* in inflammatory signs during the application of IMQ on the depilated dorsal skin of the mice. The IMQ + PS group demonstrated a significant *(P < 0.05)* in the PASI score on the final day of treatment. No significant differences *(P > 0.05)* were observed between the IMQ + PS and IMQ + CsA groups regarding the inflammatory indicators (see ▶ **Fig. 2b**). Additional data detailing the results at various time points are presented in ▶ **Fig. 2b**.

Histopathology

As illustrated in ▶ **Fig. 3**, hematoxylin and eosin (H&E) staining, along with Baker's score, were employed to assess psoriatic-like reactions in murine models. The histopathological findings indicated that IMQ induces psoriatic-like reactions characterized by hyperkeratosis, parakeratosis, acanthosis, the presence of Munro micro-abscesses, and a significant lymphocytic infiltrate (see ▶ **Fig. 3a**). Therefore, the IMQ group showed a significant *(P < 0.01)* increase in Baker's score in comparison with the Ctr group (▶ **Fig. 3c**). The data presented in ▶ **Fig. 3** indicate that the inflammatory response was significantly reduced in the IMQ + PS and IMQ + CsA groups, with p-values of < 0.05 and < 0.01, respectively, compared to the IMQ group. IMQ + CsA improved Baker's score rather than the IMQ + PS group but we did not



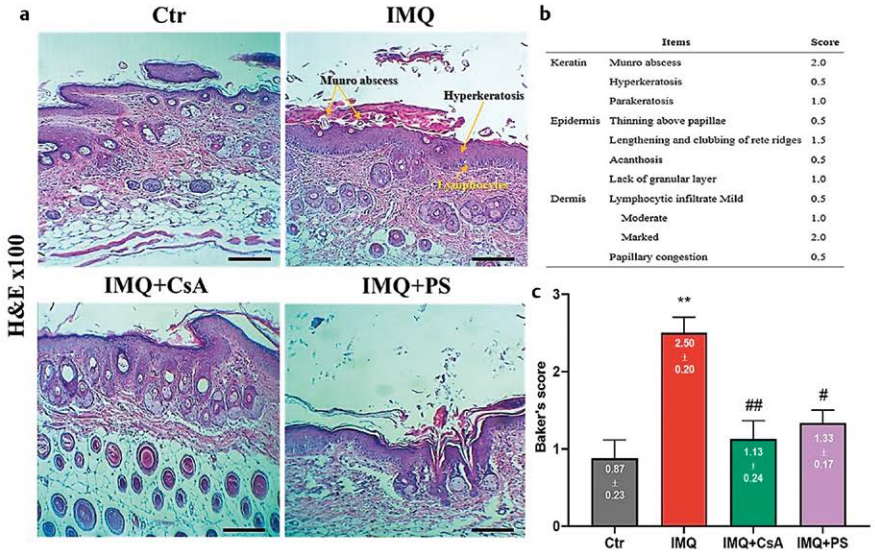
▶ **Fig. 1** Diagrams of **a**) the changes in body weight over six consecutive days, **b**) the average spleen weight of the experimental mice, and **c)** the percentage of spleen weight/ body weight on the sixth day of the trial showed that significantly decreased in the IMQ + CsA group. Data are presented with n = 6 per group and mean ± SEM. *##P < 0.01* is compared to the IMQ group.



▶ **Fig. 2** The representative data about various experimental groups of IMQ-induced psoriasis and their corresponding treatments include **a**) The dorsal skin of the mice on the sixth day. **b**) Diagrams of inflammatory signs score (numbered from 0 to 4) and cumulative PASI score (numbered from 0 to 12) in 6 consecutive days. Data are presented with n = 6 and mean ± SEM. The sign * is compared to the Ctr group and the sign # is compared to the IMQ group: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ #$P < 0.05$.

observe any significant *(P > 0.05)* difference in the mean of Baker's score between them, as shown in ▶ **Fig. 3c**.

Moreover, while there were no significant *(P > 0.05)* changes in dermal volume between the IMQ + CsA and IMQ + PS groups, our findings indicated that 5 % CsA reduced the number of infiltrating lymphocytes in the dermal layer more effectively than 10 % PS. Additionally, pathological changes, including Munro's microabscesses in the keratin layer and elongation of the epidermis, observed in the PS-treated group demonstrated an improvement in psoriatic signs similar to that seen in mice receiving CsA (▶ **Fig. 3a**).



▶ **Fig. 3** **a**) The representative H&E staining of the dorsal skin of mice indicated that IMQ group has Munro's microabscesses in the keratin layer, lengthening and clubbing of rete ridges, and moderate-severe dermal lymphocytic infiltration on the sixth day, even though PS similar to CsA drug reversed the IMQ-induces pathologic changes. **b**) The parameters of Baker's scoring system [37] for pathological changes evaluation. **c**) Diagram of Baker's pathology scores reflected in the method section, was used for histopathology evaluation. Data are presented with n = 6 per group and mean ± SEM. The sign * is compared to the Ctr group, ** *P < 0.01*, and the sign # is compared to IMQ group, *#P < 0.05*, *##P < 0.01*.

Epidermal thickness

Histological examination of the epidermal thickness of the back skin in mice was conducted using Masson's trichrome staining on the sixth day of the experiment. As illustrated in ▶ **Fig. 4a, b**, the volume of hyperkeratosis and epidermal thickness was significantly reduced in the IMQ + PS group *(P < 0.01)* and the IMQ + CsA group *(P < 0.0001)* compared to the IMQ group In contrast, the IMQ group exhibited a significant increase *(P < 0.0001)* in epidermal thickness and hyperkeratosis when compared to the Ctr group. Although CsA treatment resulted in a more reduction of hyperkeratosis compared to PS, there was no significant difference *(P > 0.05)* between the two treatments in IMQ-induced psoriasis, as shown in ▶ **Fig. 4a, b**.

Immunohistochemistry

As shown in ▶ **Fig. 5**, TNF-α marker in IHC analysis of the IMQ + PS and IMQ + CsA groups were in lower levels (mild expression) with a moderate cytokine expression compared to the IMQ group. IMQ-induced psoriasis resulted in an increase of the TNF-α inflammatory cytokine in the back skin of mice compared to the control group on the sixth day (▶ **Fig. 5**).

Gene expression

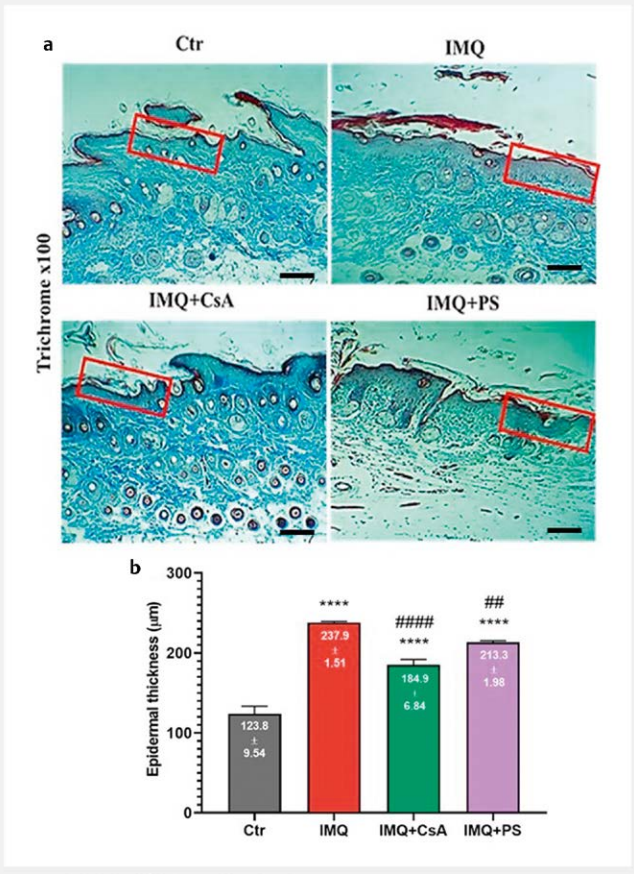
In this study, the gene expression of key inflammatory mediators including IL-17, IL-23, and TNF-α cytokines was analyzed, which are involved in the pathogenesis of psoriasis-like inflammation. The levels of IL-17, IL-23, and TNF-α cytokines were reduced significantly (*P < 0.01*) in the IMQ + CsA treated group compared to the IMQ group. Moreover, the IMQ + PS treatment significantly decreased the levels of IL-17 *(P < 0.01)*, IL-23 *(P < 0.05)*, and TNF-α *(P < 0.05)* cytokines compared to the IMQ group, as illustrated in ▶ **Fig. 6a–c**.

Additionally, topical CsA treatment reduced the levels of inflammatory mediators to a greater extent than the PS drug in psoriatic mice, with no significant difference *(P > 0.05)* observed between the two groups (▶ **Fig. 6a–c**).

Discussion

The present study aimed to investigate the potential therapeutic effects of topical PS in the improvement of IMQ-induced psoriasis in mice. Our data indicated that PS improved tissue parameters, including erythematous plaques, skin thickness, and hyperkeratosis, which were observed in IMQ- induced psoriasis in mice. The histopathological analysis also revealed a reduction in skin inflammation, as indicated by Baker's score, in the PS treatment group. Furthermore, PS significantly suppressed the gene expression of inflammatory cytokines IL-17, IL-23, and TNF-α in psoriatic lesions. The anti-psoriatic effects of PS application align with Al-Harbis' study, which demonstrated that lymphocyte-specific protein tyrosine kinase has an inhibitory effect on IMQ-induced psoriatic inflammation in a mouse model [24]. The investigation revealed that psoriatic inflammation is directly influenced by cytokine expression such as NFkB and STAT3, TNF-α, IL-17A, etc. and clinical features like skin thickness, acanthosis and leukocytic infiltration [24].

PS phospholipid plays a crucial role in cell signaling and exhibits immunomodulatory and anti-inflammatory effects [17, 21–23]. It is an FDA-approved dietary supplement that is currently on the market and has not been associated with any significant side effects to date [25]. Our recent study demonstrated that topical PS enhances wound healing and reduces necrosis by inhibiting inflam-

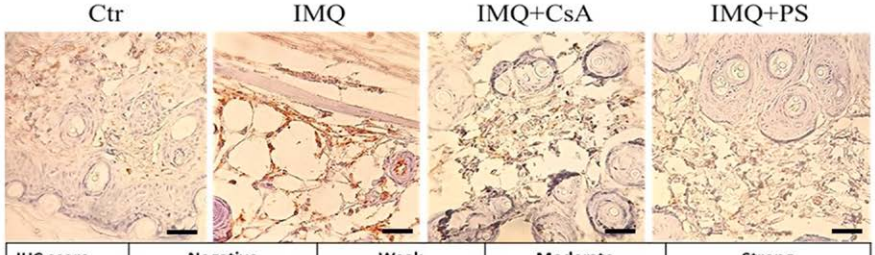


▶ **Fig. 4** **a**) The histological examination utilizing Masson's trichrome staining was conducted on the dorsal skin of mice from the respective groups on the sixth day. The application of topical PS resulted in a reduction of epidermal thickness, which was evaluated for each experiment based on four smooth regions delineated by red rectangles. **b**) Diagram of epidermal thickness measurement (by ImageJ software) on the sixth day. Data are presented with n = 6 and mean ± SEM. The sign * is compared to Ctr group, $^{****}P<0.0001$, and the sign # is compared to IMQ group, $\#\#P<0.01$, $\#\#\#\#P<0.0001$.

matory cell infiltration and activating growth factors in animal models [26].

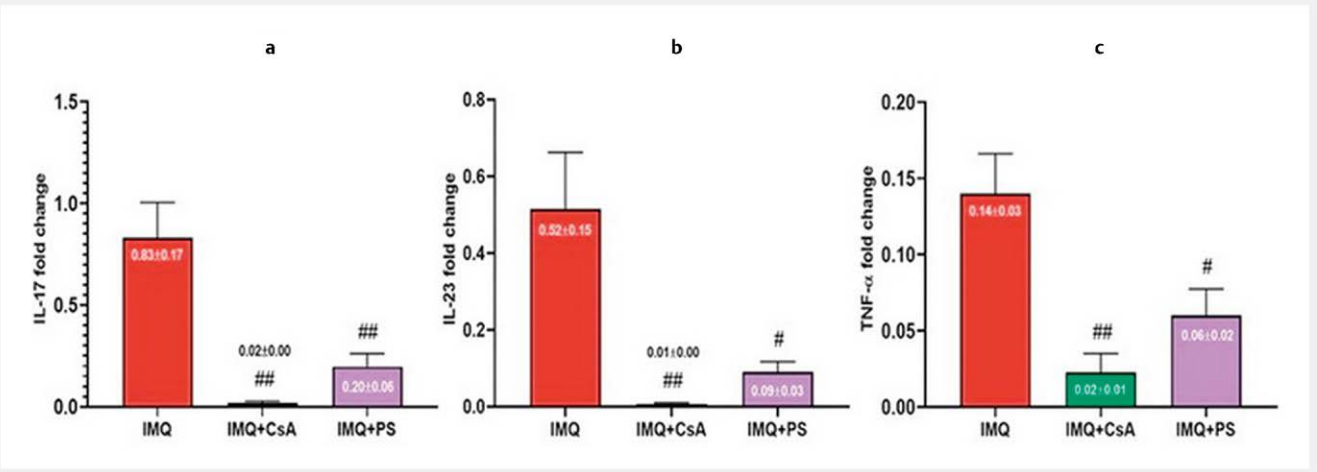
IMQ-induced psoriasis demonstrated significant alterations in the expression of inflammatory cytokine genes, characterized by erythematous plaques, overlying scales, skin thickening, and hyperkeratosis at the psoriatic site. In contrast, topical PS, similar to the drug CsA, exhibited comprehensive properties in alleviating skin complaints and exerting anti-inflammatory effects associated with cytokine gene expression in psoriatic mice. The CsA drug has been utilized in clinical settings for severe skin disorders with immuno-inflammatory origins, such as psoriasis and atopic dermatitis [27]. On the other hand, side effects and poor absorption of CsA present challenges for its short- and long-term topical use. [28].

In our study, a 5-day topical treatment with PS significantly improved the PASI score and reduced skin thickness and hyperkeratosis in psoriatic mice. The PASI score is utilized to assess the severity of psoriasis by calculating the values of thickness, redness, and scaling in experimental models. [29]. Moreover, histopathological examinations indicated that skin thickness and hyperkeratosis were significantly reduced following topical therapy with PS and CsA in psoriatic mice. Furthermore, the expression of inflammatory cytokines, including IL-17, IL-23, and TNF-α, was suppressed after a 5-day treatment with topical PS therapy on the dorsal skin of psoriatic mice. Therefore, these findings suggest that the anti-psoriatic properties of PS may be attributed to the suppression of IL-17, IL-23, and TNF-α expression, which are associated with skin inflammation [30]. It is known that the IL-23/IL-17 axis has a key role in inflammation promotion of psoriasis. Furthermore, the strategy of blocking TNF-α, in conjunction with monoclonal antibodies targeting IL-17 and IL-23 cytokines, has proven effective in the treatment of patients with psoriasis [31–33]. These pro-inflammatory cytokines contribute to the proliferation of epithelial cells and hyperkeratosis, as well as the recruitment of additional inflammatory immune cells in psoriatic lesions [33]. Skin-resident dendritic cells (DCs) and macrophages are one of the main primary sources of



IHC score	Negative (No expression)	Weak (Mild expression)	Moderate (Moderately expression)	Strong (Highly expression)
Ctr	+++	-	-	-
IMQ+CsA	+	++	-	-
IMQ	-	+	++	-
Imiq+PS	-	+++	-	-

▶ **Fig. 5** IHC analysis was conducted on the dorsal skin of the mice on the sixth day. The scores of the TNF-α marker were given qualitatively with n = 3 per each group as follows: negative (no expression), weak (mild expression), moderate (moderately expression), and severe (highly expression).



▶ **Fig. 6** **a**, **b** & **c**) A diagram illustrating the levels of IL-17, IL-23, and TNF-α in the dorsal skin of mice on the sixth day, as measured by real-time PCR. Data are presented with n = 6, mean ± SEM. The sign # is compared to IMQ group: #*P*<0.*05*, ##*P*<*0.01*.

IL-23 [34], and activated plasmacytoid dendritic cells (pDCs) in the dermis secrete type I interferon and TNF-α. These DCs will produce IL-12 and IL-23, and then lead immature T cells into T helper (Th)1, Th17, and Th22 cells [33].

The previous reports have shown that parenteral and oral administrations of PS reduce Ag-specific CD4 + T cells [22] and delayed-type hypersensitivity reaction (DTH) [17, 18] that are results of inhibiting productively activated T lymphocytes [22]. Furthermore, PS liposomes reduced the levels of inflammatory cytokines, including TNF-α, IFN-γ, IL-1β, IL-2, IL-6, and IL-8, in various experimental studies [16–19, 23] as well as the other biological activities [35]. PS phospholipids on apoptotic cells or within liposomal structures interact with PS receptors expressed on phagocytes. This interaction triggers the production of anti-inflammatory cytokines while reducing pro-inflammatory cytokines. [20, 21]. Furthermore, our results demonstrated a significant reduction in spleen mass percentage following a 5-day topical treatment with CsA in psoriatic mice. The reports indicated that an increase in spleen weight occurs as a result of the topical application of IMQ in the experiments. This application leads to the activation of T cells and systemic inflammation, which play a significant role in the pathogenic process of human psoriasis [36, 37]. Therefore, changes in spleen weight may serve as a marker in IMQ-induced psoriasis models.

Our data proposed that the topical application of PS interacts with individual leukocytes in skin tissues, such as lymphocytes and dendritic cells, thereby inhibiting inflammatory responses in psoriatic skin. Nevertheless, further studies are necessary to elucidate the role of inflammatory cytokines, such as IL-17A, by administering PS in IL-17a knockout mice or through pretreatment with an anti-IL-17A receptor to confirm its anti-inflammatory pathways. Given the multiple interactions between PS and the immune system, other signaling pathways, including NFATc1, NF-κB, and STAT3, should be considered for investigation in psoriatic conditions in future studies.

Conclusion

Our data indicate that PS hydrogel, similar to the CsA drug, has beneficial effects on psoriatic inflammation in a mouse model. PS therapy reduced plaque-type psoriasis indices, including skin thickness, hyperkeratosis, and scores associated with dermatitis and inflammation. The expression of the cytokines IL-17, IL-23, and TNF-α was suppressed during the 5-day treatment with PS in psoriatic mice. Furthermore, further investigation is recommended to explore other signaling pathways of PS in the future, which may be involved in the control of psoriasis.

Method

Drug and preparation

Carboxymethyl cellulose (CMC) and cyclosporine A (CsA) powder were purchased from Sigma (USA). Phosphatidylserine (PS) and Imiquimod 5 % cream were obtained from Pharmin USA, LLC (USA) and Pharmaceuticals Meda (UK), respectively. Phosphatidylserine 10 % (w/v) [19] as well as cyclosporine 5 % (w/v) were prepared with ethanol 10 % (v/v). The CMC (1 %) was added to the suspensions and stirred at warm conditions. To prepare the vehicle, 1 ml of pure ethanol was mixed with 9 ml of distilled water and CMC 1 % without the drug.

Animal

Male BALB/c mice with an average weight of 30 gr were obtained from the School of Medicine, Tehran University of Medical Sciences (Tehran, Iran). Their health was checked and they were randomly placed in separate cages with suitable numbers to adapt before the experiment. They were provided with unrestricted access to water and food and were subjected to a 12-hour light and 12-hour dark cycle, with a temperature maintained at 21 ± 2 °C. The cages were cleaned and maintained regularly to ensure hygiene and a suitable living environment for the mice. The experimental methods were deeply in line with the experiment and ethics followed the guidelines set out by the Canadian Council for Animal Care and

approved by the Institutional Animal Care and use of committee IR.TUMS.MEDICINE.REC. 1403.247.

Experimental design

In this study, the experimental groups were divided into 4 groups with 6 mice which were selected randomly as follows: Vehicle (Ctr): Vehicle administration with no intervention of imiquimod. Imiquimod (IMQ): Intervention of imiquimod and no administration (negative control); Imiquimod/cyclosporine (IMQ + CsA): Intervention of imiquimod and CsA administration (positive control); Imiquimod/phosphatidylserine (IMQ + PS): Intervention of imiquimod and PS administration.

On day 1, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), and then the hair of the fixed space between their shoulder blades was completely shaved. A quarter of each sachet of IMQ cream (62.5 mg) was applied with a spatula to the mentioned area (except Ctr group) and this action was repeated for 5 days. Two hours later, the three specified compounds were administered topically in a volume of 200 µl on the backs of the mice, with the treatment repeated for five consecutive days. Animals were weighed regularly once a day for 6 days. Photos were taken from the back of the animals for PASI score evaluation, and this action was repeated for 6 days (before the intervention of IMQ).

On day 6, the mice were euthanized using carbon dioxide (CO2). Half of the dorsal skin samples were preserved in 10 % formalin, while the other half was stored at −80 °C for molecular analysis. Additionally, the spleen tissues were rinsed in saline and subsequently weighed. Changes in spleen weight are regarded as a hallmark of T cell activation and systemic inflammation in the IMQ-induced psoriasis model [37].

Macroscopic observation and PASI score

For psoriasis area and severity index (PASI) in 6 days, evaluated inflammatory signs that including erythema, scaling, and thickness, with these scores :0) none; 1) slight; 2) moderate; 3) marked; 4) very marked, and this cumulative scores (numbered from 0 to 12). The evaluation was done independently by two researchers (n = 6 for each parameter) and the mean of values was then calculated [29].

Histopathology

For histological parameters characteristic of psoriasis, an expert pathologist evaluated hematoxylin-eosin-stained skin sections (3–4 µm) using the Baker scoring system. The scores included keratinosis, development of abscesses, acanthosis, and dermal lymphocyte infiltrations which were graded by the histopathological scores on a scale of 0 –11 [38]. To evaluate the precise histological characteristics of the tissues, the prepared samples were stained with Masson's trichrome dye, and the thickness of the epidermis was measured using the length measurement tool in ImageJ software. Briefly, the skin samples were deparaffinized and stained with a hematoxylin solution, followed by a mixture of Orange G, Ponceau S, and a light green staining agent.

Immunohistochemistry

This method was employed to assess TNF-α cytokine expression in the dorsal skin of mice. Consistent with our previous study [39], we utilized specific primary and secondary antibodies, and the analysis was conducted by an unbiased pathologist. The presence of brown-stained cells in the sections indicates relative cytokine expression in the tissue.

Gene expression

For each mouse, a proper skin sample was also harvested sterilely and immediately flash-frozen in liquid nitrogen and then stored at −80 °C. The mRNA expressions of IL-17, IL-23, and TNF-α factors were measured by real-time quantitative PCR analysis. Total RNA was isolated from all tissues using an RNA X-Plus kit (Cinna Gen, Tehran, Iran) and was quantified by NanoDrop spectrophotometer (Thermo Scientific Nanodrop 2000). In the next step, cDNA was synthesized (Bio fact, South Korea), and Real-time PCR was performed with the Cyber Green method (Yekta Tajhiz Co, Cat No: YT2551) with the following program; 40 cycles of denaturation (15 seconds at 95 °C), annealing (20 seconds at 60 °C) and elongation process (30 seconds at 72 °C). β-actin was used as the housekeeping gene and positive control. The relative expression of transcript level of each case was calculated according to $2^{-\Delta\Delta ct}$[40].

Statistical analysis

We used Prism version 8 software for statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) and presented as mean ± standard error of the mean (SEM). $P < 0.05$ was considered as significant.

Data availability

Research data are not shared.

Disclosure statement

All authors declare that they have no conflict of interest.

Authors' contributions

Bahareh Farasati Far: Writing and editing.
Partow Mirzaee Saffari: Animal study, Data analysis.
Razieh Mohammad Jafari: Methodology.
Ramin Goudarzi: Reviewing and editing.
Ahmad Reza Dehpour: Methodology.
Alireza Partoazar: Supervision, Conceptualization, Reviewing and editing.
All authors reviewed the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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