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Artesunate alleviates imiquimod-induced psoriasis-like dermatitis in BALB/c mice

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ABSTRACT

Artesunate (ART), a derivative of artemisinin, is a medication to treat malaria. Beyond that, the anti-inflammatory and immunoregulatory activities of ART have been identified in autoimmune diseases. However, whether ART functions in psoriasis-like dermatitis induced by imiquimod (IMQ, a TLR7/8 agonist) is currently unkown. There, we found that the cumulative score, epidermal thickening and expression of Ki-67 of ART-treated BALB/c mice were significantly lower than those in the IMQ psoriatic model group. In addition, ART treatment ameliorated mice from systemic inflammation. Mechanistically, ART reduced γδ T cells in draining lymph nodes, which might be benefit the improvement of dermatitis. These findings suggested that ART could be a promising drug of psoriasis in clinic.

1. Introduction

Psoriasis is a common chronic inflammatory skin disease in humans, which affects approximately 1% to 3% of world's population and nearly 0.5% in China [1]. Psoriasis vulgaris, also known as chronic plaque psoriasis, the most common disease variant, is observed in about 90% psoriasis cases, which manifests as erythematous plaques covered by white scales. In spite the lesions and pruritus, the psychosocial and economic burden is even heavy [2]. Physiologically, psoriasis vulgaris lesions exhibit hyperkeratosis and parakeratosis in epidermis, contorted blood vessels and inflammatory infiltration in dermis. Although the pathogenesis of psoriasis remained unclear, the immune system especially the T cells, such as Th1, Th17 and γδ T cells played a crucial role in process [3]. Of note, systemic treatments, which included methotrexate (MTX), cyclosporin A, acitretin, biologic agents, and sometimes steroids are necessary for moderate to severe patients [4]. However, substantial side effects such as myelosuppression, abnormal hepatic function and metabolic disorder are noticeable in above-mentioned drugs, leading to limitation of the application in clinic. Therefore, it is urgent to explore new psoriasis medicine.

Artemisia annua L., a herb from the Compositae family, has been used for treatment of fever and inflammatory diseases for nearly two thousand years in China [5]. Artemisinin, the active component purified from that herb, has been exploited as anti-malaria medicine for decades. Recently, several non-malarial indications of artemisinin have been explored, including anti-biotic, anti-proliferation, anti-inflammation and immunoregulation [6,7]. However, due to the poor solubility and short *in-vivo* half-lives of artemisinin, researchers have developed its derivatives such as dihydroartemisinin and artesunate, which shared a endoperoxide linkage in their basic chemical structures. Artesunate (ART) is the semi-synthetic hemisuccinate ester of dihydroartemisinin (Fig. 1). Comparing to its predecessors, ART possess great water-solubility, which broaden its clinical application [8]. Based on the immunoregulatory function of ART, it is reasonable to assume that ART can be conducted as a promising medicine for immunological or papulosquamous diseases, which has been confirmed in systemic lupus erythematosus and rosacea [9,10].

Imiquimod (IMQ), a toll-like receptors (TLRs) 7/8 agonist, is used as a topical anti-wart medicine in human. Histopathologically, tropical IMQ application on mice could induce skin inflammation, erythematous lesions, skin thickening, scaling, epidermal hyperkeratosis, parakeratosis, proinflammatory cells infiltration and neoangiogenesis [11]. Generally, the IMQ-induced psoriasis closely resembles human plaque psoriasis lesions in terms of phenotypic and histological characteristic

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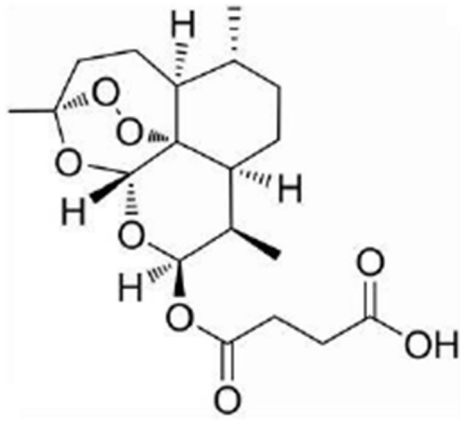

Fig. 1. The chemical structure of ART.

and is therefore used for the study of the pathogenesis of inflammatory psoriasis, plus the development of novel therapeutic agents [13,14]. In this study, by the used of IMQ-psoriasis mouse model, we make effort to determinethepotential therapeutic effect of ART on IMQ-inducedpsoriasis like dermatitisin mice.

2. Materials and methods

2.1. Experimental animals

38 male specific pathogen-free (SPF)BALB/c mice (weight, 22–27 g; age, 8–10 weeks) were obtained from the Animal Experiment Center at Sun Yat-Sen University (Guangzhou, China). The mice were maintained in a SPF colony at Forevergen experimental animal center (Permit No: SYXK Guangdong 2018–0186). The present study was performed in accordance with the National Institutes of Health Guidelines on Laboratory Research and approved by the Animal Care Committee at Sun Yat-Sen University and Forevergen.corporation.

2.2. Establishment of psoriasis animal model and drug treatment

The mice were randomly divided into 6 groups, which were IMQ + ART^{high}(60 mg/kg), IMQ + ART^{low}(30 mg/kg), IMQ + MTX (1 mg/kg), IMQ model, ART(60 mg/kg) and mockgroup. All the mice were shaved for a 3 × 4 cm area on the back three days before the modeling. The model establishment followed the previous study [15]. The mice were administered a daily topical dose of 62.5 mg of 5% IMQ (Aldara; 3 M Pharmaceuticals, UK) to the shaved area on their backs for 7 consecutive days. Vaseline was used instead of IMQ in ART(60 mg/kg) and mock groups. ART(Macklin Inc., Shanghai, China) or MTX (Pudepharma Inc., Datong, China) was prepared at 10 mg/ml and 0.1 mg/ml respectively in normal saline. Later two hours after topical application, based on the body weight, ART or MTX was intraperitoneally injected respectively on day 0, continuing through day 6.All the mice were sacrificed 24 h after the final administration.

2.3. Judgement of skin inflammatory severity

For the assessment of the swelling of ear lesions, a vernier caliper (resolution to 0.01 mm) were used to measure the thickness of ear daily. The severity of dorsal skin lesions was evaluated according to Psoriasis Area and Severity Index (PASI) score system [11], which consists of measures for skin erythema, scaling and thickness. Each parameter was scored independently on a scale from 0 to 4, where 0 = no clinical signs; 1 = slight clinical signs; 2 = moderate clinical signs; 3 = marked clinical signs; and 4 = very marked clinical signs. The cumulative score denotes the severity of inflammation.

2.4. Histopathology and measurement of epidermal thickness

The back skin and left ear samples were isolated and fixed in 10% formalin and embedded in paraffin. For the histopathological examination, 4-μm paraffin sections were cut and stained with hematoxylin and eosin (HE) and observed under a light microscope (Olympus, Tokyo, Japan). The HE sections were imaged under 100× view by a digital camera (Canon, Tokyo, Japan) and the data were analyzed by Image Pro Plus 6.0 software (Media Cybernetics, Rockville, USA.).

2.5. Immunohistochemical (IHC) examinations

IHC examination was performedper the instructions for the IHC kits (Zhongshan Jinqiao Inc., Beijing, China).Briefly,4-μm sections were deparaffinized and rehydrated followed by endogenous peroxidase quenching, antigen retrieval (saline sodium citrate, microwaving) and non-specific binding site blockade. Then rabbit polyclonal anti-mouse Ki-67 antibody (Abcam, Cambridge, UK) was used at 1:200 dilution.

2.6. Evaluation of spleen and draining lymph nodes

Followed by sacrificing, the spleen and draining lymph nodes (DLNs, including bilateral axillary, brachial and inguinal lymph nodes) were harvested and ground into single-cell suspension using nylon net bags (200 mesh). Cell counting was performed by Countstar Biotech Cell-counter (ALIT Life Science, Shanghai, China).

2.7. Flow cytometry of γδT cells subset

For detection of γδ T cells in the DLNs, single-cell suspensions were stained with Viability Dye eFluor 780 (eBioscience, Waltham, USA), PerCP-conjugated CD3e antibody and FITC-conjugated γδ TCR antibody for 15 mins at 4 degree (CD3e and γδ TCR antibody were from BD Biosciences, San Jose, USA). The cells were acquired on BD FACSVerse cytometer and analyzed by Flowjo VX software (BD Biosciences, San Jose, USA).

2.8. Statistical analysis

SPSS V20.0 software (SPSS Science, Chicago, USA) was used to conduct statistical analysis. One-way ANOVA was performed to analyze among-group differences and determined by LSD test; a p-values less than 0.05 were considered as significant($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

3. Results

3.1. ART suppressed IMQ-induced dermatitis in mice

Firstly, to assess how ART acted on IMQ-induced dermatitis or not, the IMQ model was eastblished in BALB/c. A typical example is shown in Fig. 2A.In addition, as shown in Fig. 2B, the dorsal skin of IMQ model mice started to exhibit erythema and white scaling on day 2 and got thickened on day 3 after model induction. The three parameter scores and cumulative score increased up to the end of experiment, indicating the success of modeling. Compared with the IMQ model group, the initiation and progression of dermatitis were delayed about 1 day in ART and MTX treated diseased mice. Furthermore, administration of ART notably ameliorated the severity of IMQ-psoriasis compared to the IMQ model group according to the scores of erythema, thickness and

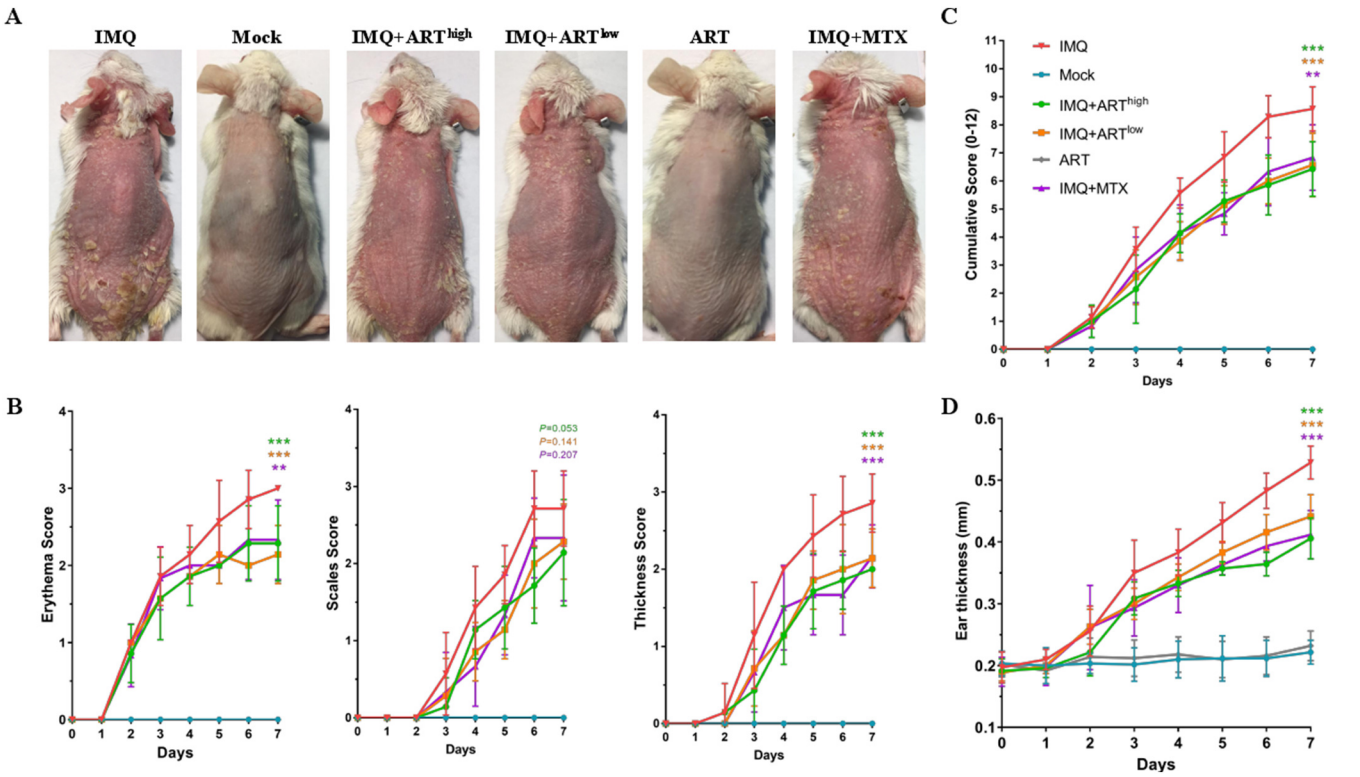

Fig. 2. ART treatment ameliorated IMQ-induced dermatitis. (A) Phenotypical presentation of mice back skin after 7 days application. (B) daily scoring of erythema, scales and thickness; (C) daily cumulative score (PASI); (D) ear thickness changes of mice. The data are presented as the means ± SDs (n = 6–7 per group). IMQ + ARThigh(60 mg/kg) , IMQ + ARTlow(30 mg/kg) , IMQ + MTX(1 mg/kg) group *versus* normal saline-injected mice following IMQ application ($P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

cumulative score on day 7 (erythema: IMQ = 3.00 ± 0, IMQ + ART^{high} = 2.29 ± 0.49***, IMQ + ART^{low} = 2.14 ± 0.38***; thickness: IMQ = 2.86 ± 0.38, IMQ + ART^{high} = 2.00 ± 0***, IMQ + ART^{low} = 2.14 ± 0.38***; cumulative score: IMQ = 8.57 ± 0.78, IMQ + ART^{high} = 6.43 ± 0.98***, IMQ + ART^{low} = 6.57 ± 1.13***). The average scales scores were slightly decreased (IMQ = 2.71 ± 0.49, IMQ + ART^{high} = 2.14 ± 0.69, IMQ + ART^{low} = 2.29 ± 0.49). In addition, ART exhibited similar therapeutic effect in controlling psoriatic symptoms as compared to MTX (Fig. 2B,C).

At the meantime, the severity of ear lesion and skin inflammation assessed by measurement of ear thickness. As shown in Fig. 2D, the left ear got swelling after twice IMQ cream challenge. Compared to the IMQ model group, ART treatment delayed swelling for 1 to 2 days. By day 7, the ear thickenings of high and low dosage ofART-treated groups were greatly decreased (IMQ: 0.52 ± 0.03 mm, IMQ + ART^{high}: 0.41 ± 0.03 mm***, IMQ + ART^{low}: 0.44 ± 0.04 mm***). Taking together, our finding indicated that ART efficiently mitigate IMQ-induced dermatitis in mice.

3.2. ART relieved IMQ-induced histopathological changes

IMQ application often results in influx of various cells as well as hyperplasia of epidermis. In addition, scaling of the skin is often an indication of parakeratosis which is a typical phenomenon of psoriasis skin lesions. Therefore, we further investigated the effect of ART on dermatitis histologically. The pathologic changes converts induced by imiquimod, such as epidermis hyperplasia and parakeratosis, were shown in Fig. 3A. Acanthosis was also obviously observed due to the abnormal proliferation of keratinocytes. Analysis of H&E sections showed that the number of keratinocyte layers increased from 1 to 3 in mock group mice to more than 10 in IMQ model. In contrast, ART could noticeably reduce the above-mentioned pathological symptoms, especially epidermis hyperplasia (Fig. 3B,C). The thickness of epidermis was decreased in both ART-treated groups similarly with MTX-treated group. In conclusion, the histological analysis revealed that ART alleviate IMQ induced histopathological changes.

3.3. Decreased expression of Ki-67 in ART-treated mice

To further demonstrate the anti-hyperplasia effect of ART on keratinocytes, we performed IHC examination of Ki-67 of dorsal skin samples. In the epidermis of mock group, only some basal layer cells were Ki-67 positive visualized by immunohistochemical staining, whereas in IMQ group, nearly all the basal layer cells even some superbasal ones exhibited Ki-67 expressed (Fig. 4). Beside of epidermis thickening, the number of Ki-67 expression were reduced in ART treated mice. Detailed, the average number of Ki-67 positive basal layer cells in IMQ group was 19.1 ± 2.3, while the number decreased to 12.5 ± 1.6 in IMQ + ART^{high} group. Moreover, the number In IMQ + ART^{low} and IMQ + MTX groups were 13.9 ± 3.1 and 12.6 ± 3.4, respectively. Taking together, our data suggested that treatment of ART could efficiently reduce epidermal proliferation during psoriasis lesion development.

3.4. IMQ induced splenomegaly is decreased upon ART treatment

To investigate the effect on systematic immune responses, we dissected the spleens and draining lymph nodes (DLNs). We weighted the spleens and calculated the cell number in spleen and DLNs. At end point of IMQ treatment, we consistently noted a significant spleen enlargement as shown in Fig. 5,IMQapplication led to increased proliferation of lymphocytes in spleen and DLNs. Additionally, ART at high dose significantly decreased IMQ-induced splenomegaly (Fig. 5A). Both high and low dose of ART treatment successfully inhibited the proliferation

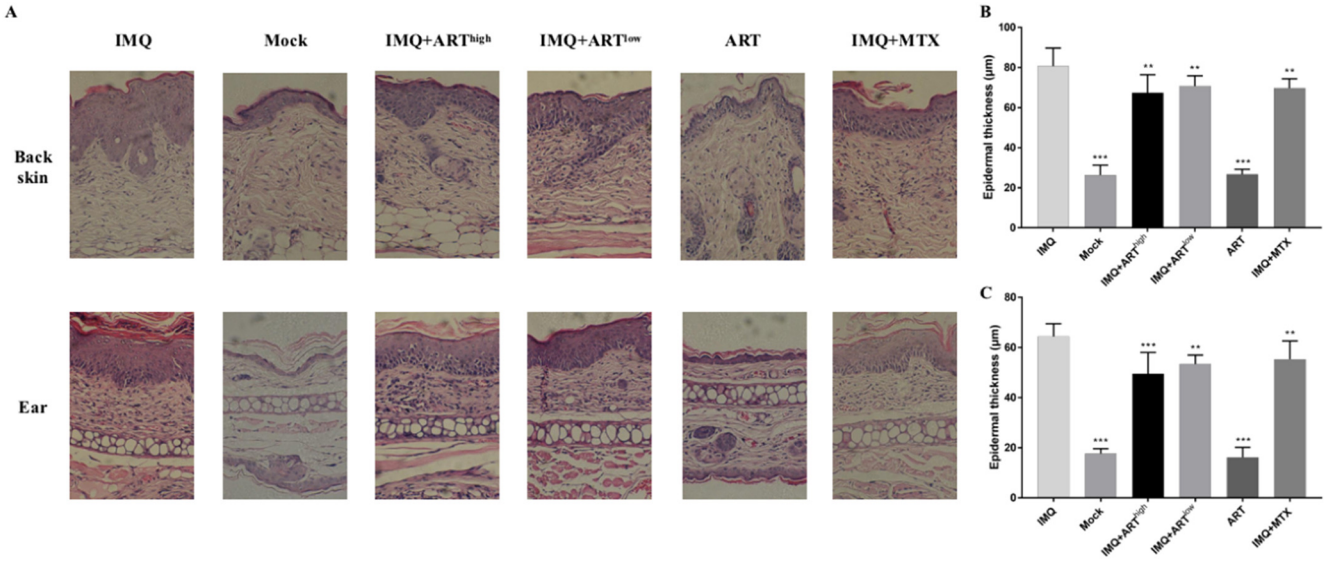

Fig. 3. ART attenuated IMQ-induced histopathological changes.(A) H&E histological sections of back skin and ear (20×); epidermal thickness of back skin (B) and ear (C). Statistical analysis was carried out by ANOVA followed by LSD to compare all groups to IMQ group (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

of spleen and DLNs cells. Furthermore, high dose of ART displayed a stronger inhibition ability than that of low dose one ($P = 0.04$). Of note, MTX also revealed immunosuppression function in IMQ model. In sum, our findings clearly showed that ART treatment resulted in rescue in splenomegaly.

3.5. ART inhibited γδ T cells subset

Th17 cells were classic IL-17A producer. Our previous results suggested that ART had no effects on the proportions of Th17 subsets in the DLNs of IMQ-treated mice (Fig. 6), indicating ART might selectively function in other immune cells in psoriasis.

A subset of γδ T cells which can resident in dermis and express IL-17A has been identified in the differentiation of keratinocytes and the pathogenesis. Therefore γδ T cell role in ART ameliorated psoriasis disease is of our interest. Indeed, as it showed in Fig. 7, compared to mock group, the proportion of γδ T cells in IMQ model group was significantly increased: IMQ (1.88 ± 0.78)% *vs.* Mock (0.89 ± 0.51)% ($P = 0.004$). After the treatment of 60 mg/kg ART, the proportion was partially decreased: IMQ-ART^{high} (1.29 ± 0.33)%*vs.* IMQ (1.88 ± 0.78)% ($P = 0.074$). Although ART had no significant effect on percentage of γδ T cells, the total number of γδ T cells was repressed in either high or low dose of ART or MTX treated mice: IMQ: (0.57 ± 0.22) × 10^6; IMQ + MTX: (0.30 ± 0.12) × 10^6 **; IMQ-ART^{high}: (0.24 ± 0.05) × 10^6 ***; IMQ-ART^{low}: (0.41 ± 0.08) × 10^6**. In addition, the inhibition of ART on γδ T cells was in a dose dependent manner: IMQ + ART^{high}: (1.29 ± 0.33)%*vs.* IMQ + ART^{low} (1.65 ± 0.35)% ($P = 0.048$). Therefore, the aforementioned finding indicated that reduced γδ T cells population in DLNs might contribute to the therapeutic effects of ART on skin disease.

4. Discussion

In this study, we demonstrated ART, an artemisinin derivative, could alleviate IMQ induced psoriasiform dermatitis in mice. These mice with ART treat revealed lower cumulative scores, epidermal thickening and expression of Ki-67. In addition, ART also reduced enlargement of spleen and DLNs. Moreover, ART inhibited γδ T cells population in DLNs. Compared to MTX, a classical remedy for psoriasis [1], ART showed a similar effect on improvement of dermatitis,

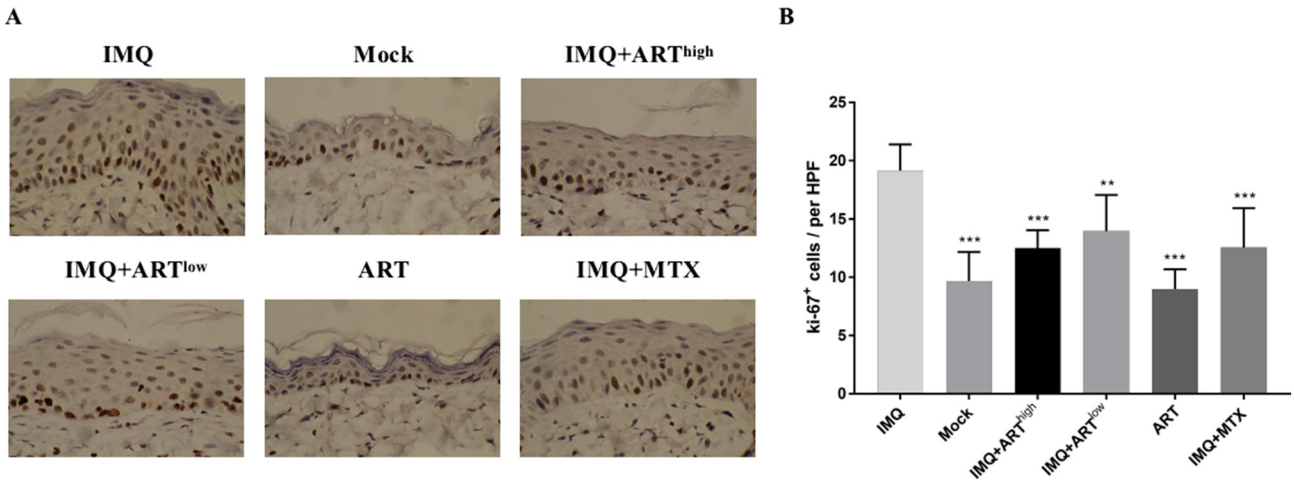

Fig. 4. ART decreased Ki-67 expression in IMQ-induced dermatitis. (A) IHC staining of Ki-67 in back skin samples (40×). (B) Numbers of ki-67 positive cells per one 40 × HPF, three HPF photos for each sample(* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

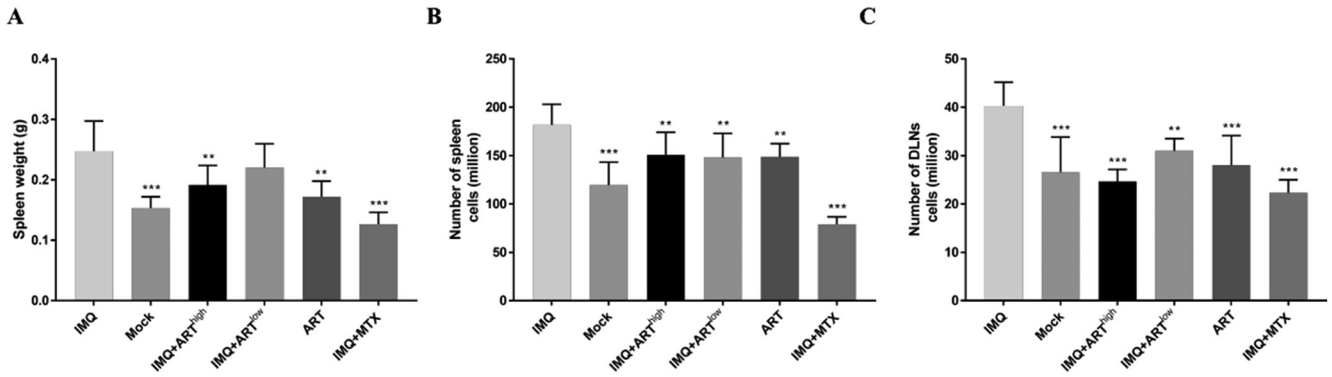

Fig. 5. ART inhibited IMQ-induced proliferation of lymphocytes. (A) dissected spleen weight of each group at day 7; (B) splenic cell-counting of each group; (C) cell-counting of draining lymph nodes (DLNs) of each group. Data were shown as the mean ± SD, compared with IMQ group (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

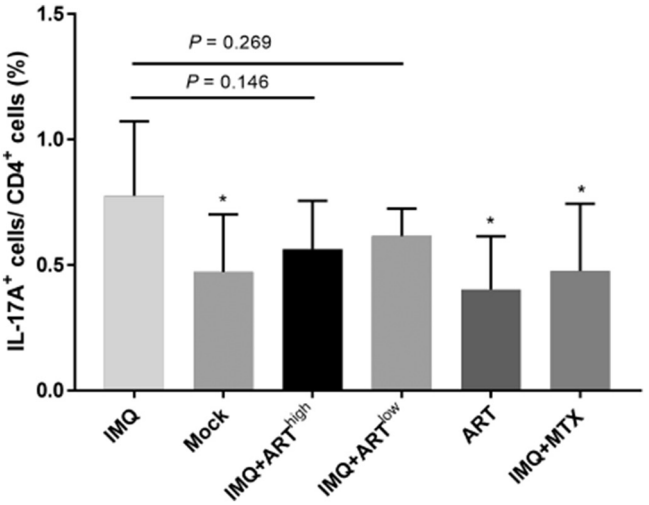

Fig. 6. The percentage of Th17 cells in draining lymph nodes (* $P < 0.05$).

epidermal thickening and inhibition of systemic inflammation without obvious side effects. Therefore, our results shed a light of ART in psoriasis and provide an alternative effective approach for anti-psoriasis therapy.

Although the roles of epidermal hyperplasia, neoangiogenesis, and dermal infiltration of inflammatory cells, were studied in psoriasis, the etiology of psoriasis including immune stimulation and keratinocytes was still poorly understood [16]. The interaction between immune system and keratinocytes was considered as the major pathogenesis [17]. The phenotype the immune-pathogenesis additionally involves the induction of interleukin 17 (IL-17) and IL-17 producing cells. All those features consistent in both human and murine IMQ-induced psoriatic inflammation [18]. Biologic agents which neutralizing IL-17 or blocking its receptor IL-17RA, such as secukinumab and brodalumab, exhibited great clinical remission of psoriasis [19]. In IMQ mouse model, IL-17 signaling plays a critical in the initiation of psoriatic dermatitis and neutrophil infiltration [20]. Our previous results suggested that ART had no effects on the proportions of Th17 subsets in the DLNs of IMQ-treated mice, indicating ART may selectively function in other IL-17 producing cells in psoriasis. Increasing evidences showed that γδ T cells and Th17 cells are key resource of IL-17 production in dermis [21]. γδ T cells, especially the IL-17-producing-γδ T cells, was highlighted in the development of skin disorders [22]. γδ T cells were γδ T-cell-receptor expressing subset without CD4 and CD8, only accounted about 1%–5% $CD3^+$ T cells [23]. As the primary and active effector T cells in IMQ-induced psoriasis, dermal γδ T cells rapidly produced IL-17A and IL-22, which initiated and amplified the inflammation [24]. Furthermore, dermal γδ T cells migrated from skin to the draining lymph nodes and underwent further expansion after IMQ treatment [25,26]. Actually, ART decreased slightly serum level of IFN-γ and IL-17, but the statistical difference was not significant (sFig. 1). In our study, ART exhibited immunoregulation and suppressive effect on γδ T cells, which might be responsible for its anti-psoriasis functions. We found ART could decreased IL-17A+ γδ T cells significantly (sFig. 2). Moreover, recent studies suggested that ART or dihydroartemisinin could enhance the proliferation and function of tumor infiltrating γδ T cells and contribute to anti-tumor effects [27,28]. Furthermore, as TLRs are elemental receptors of immune system, the intensive stimulation of TLRs would activate the innate immune system and the subsequent adapt immune system, which induces systemic inflammation including splenomegaly, lymph nodes enlargement [11,12]. It is therefore plausible that, ART might exhibita dual role in the regulation of γδ T cells in environment-dependent. Such a notion remains important and warrants further investigated.

Keratinocytes disorder (eg, epidermal hyperproliferation, hyperkeratosis) was the central in pathology and directly reflected to the symptoms. It was not only the result of interaction of keratinocytes to immune cells and cytokines, but also led to a pathologic cycle. For instance, LL-37, an antimicrobial peptide secreted by kerationocytes, could inhibit the apoptosis of neutrophils and served as an autoantigen to deteriorate skin inflammation [17,29]. In our study, ART performed anti-proliferation effect in psoriatic lesions, resulting in lower skin thickening in skin tissue. Ki-67, as a cell proliferative maker, can be detected in the keratinocytes from the basal layer of normal epidermis and act as an indicator of effective psoriasis therapies [30]. In the skin of mouse with psoriasiform dermatitis induced by IMQ, the spreading and immune-positive rate of Ki-67 are much higher [31]. Furthermore, we noted ART-treatment could reduce Ki-67 expression, which further demonstrated the anti-proliferation effect of ART.

In conclusion, artesunate acted against IMQ-induced dermatitis and systemic inflammation. Furthermore, the cutaneous therapeutic function of ART was comparable to that of methotrexate. Mechanistically, it selectively inhibited the expression of γδ T cells and expressed the proliferation of keratinocytes. Together with its effectiveness and safety, the current study offered the evidence to support the notion that ART could be a promising remedy for psoriasis.

Acknowledgements

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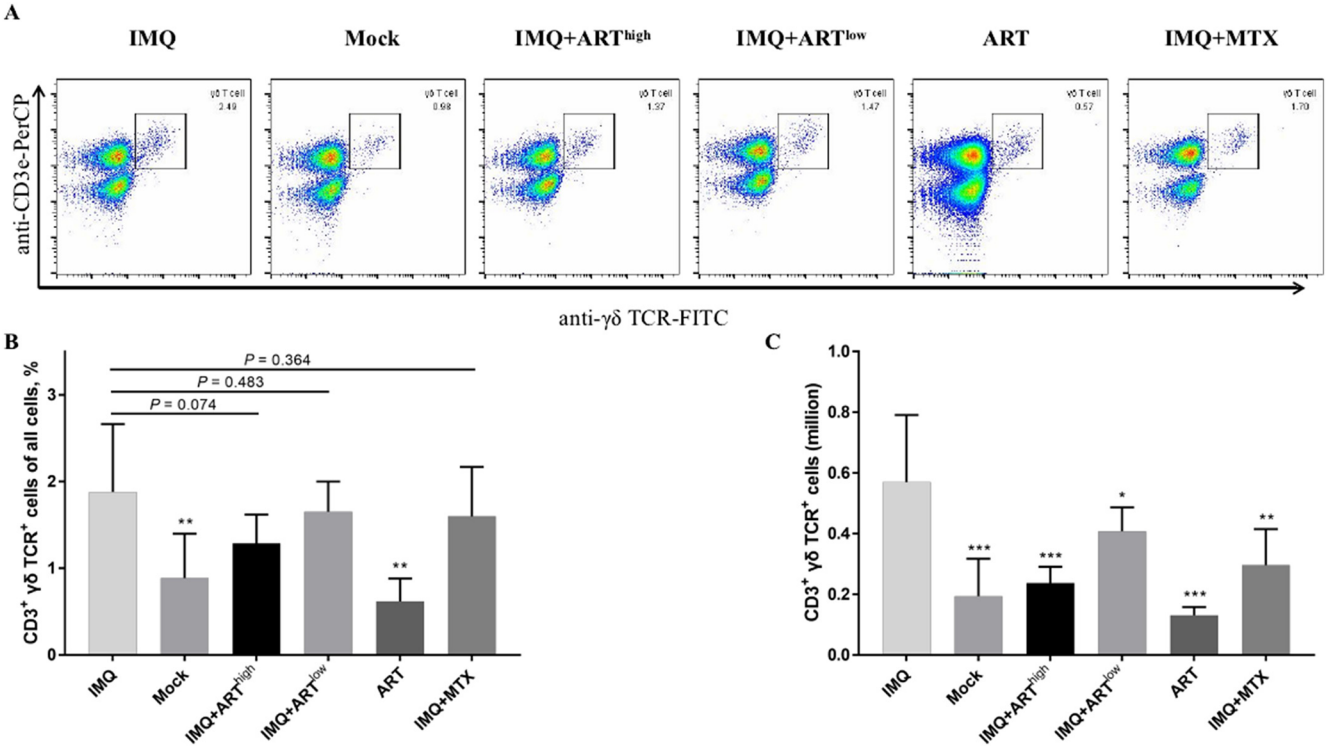

Fig. 7. Effect of ART on γδ T cells in draining lymph nodes (DLNs). (A) Results of flow cytometry. (B) The percentage of γδ T cells from all cells from DLNs. (C) The absolute numbers of γδ T cells in DLNs. Data were shown as the mean ± SD, compared with IMQ group (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

Declaration of competing interest

The authors have declared no conflicting interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105817.

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