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Artemisinin, a potential option to inhibit inflammation and angiogenesis in rosacea

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ABSTRACT

Background: Rosacea is a facial chronic inflammatory skin disease with dysfunction of immune and vascular system. Artemisinin (ART), an anti-malaria drug, was reported to have several effects including anti-inflammation and anti-angiogenesis activities. However, the role of ART on rosacea remains unclear.
Objectives: To investigate the effects and molecular mechanism of ART on rosacea.
Method: In rosacea-like mouse model, the phenotype of rosacea lesions was evaluated by redness score, the inflammatory biomarkers were analyzed by qPCR, and the infiltration of inflammatory cells were assessed by IHC analysis and immunofluorescence. In *vitro*, LL37-induced expression of inflammatory factors in HaCaT cells was detected by qPCR, potential signaling pathways were detected by Western blotting or immunofluorescence. Migration ability of human umbilical vein endothelial cells (HUVECs) was evaluated by cell scratch and transwell assays.
Result: The skin erythema and histopathological alteration, as well as the elevated pro-inflammatory factors (IL-1β, IL6, TNFα) and TLR2 were significantly ameliorated by ART treatment in LL37-induced rosacea-like mice. In addition, ART reduced the infiltration of $CD4^+$ T cells, macrophages and neutrophils, and repressed the expression of immune cells related chemokines (CXCL10, CCL20, CCL2 and CXCL2) in mouse lesions. In HaCaT cells, ART significantly decreased the LL37-induced expression of inflammatory biomarkers. Moreover, we found that ART inhibited rosacea-like inflammation via NF-kB signaling pathways in HaCaT cells. Finally, for vascular dysregulation, ART repressed the angiogenesis in mouse model and inhibited the LL37-induced HUVECs migration *in vitro*.
Conclusion: ART ameliorated rosacea-like dermatitis by regulating immune response and angiogenesis, indicating that it could represent an effective therapeutic option for patients with rosacea.

1. Introduction

As a chronic facial inflammatory diseases, rosacea is characterized primarily by telangiectasia, flushing, erythema, papules, pustules, fibrosis or a combination of these symptoms [1,2]. According to clinical features, rosacea is classified into four subtypes including erythematotelangiectatic rosacea (ETR), papulopustular rosacea (PPR), phymatous rosacea (PhR) as well as ocular rosacea (OR) [1]. As a common disease in the world, previous investigations demonstrated the prevalence of rosacea ranged from 2 to 22% in different populations [3]. Although it is not a lethal disease, due to facial involvement, patients with rosacea have a diminished quality of life compared with the general population [4].

The pathophysiology of rosacea is not fully clear, and current evidence suggests that dysfunctional immune and vascular system are attributed to the occurrence and development of rosacea [5,6]. Excessively active innate immunity, especially the over-activation of Toll-like receptor 2 (TLR2) in keratinocytes, is an important pathological mechanism of rosacea [7]. Pro-inflammatory cytokines/chemokines and pro-angiogenic factors are released upon the activation of TLR2 in keratinocytes, which have been reported to be associated with rosacea symptoms such as erythema, telangiectasia, and inflammation [8–10]. Apart from innate immunity, adaptive immune cells including $CD4^+$T cells were found to be well involved in rosacea [11]. Additionally,

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dilated blood vessels in all subtypes of rosacea and evident angiogenesis in PhR could be found in histopathological features [12].

Due to complex pathophysiology, treatments for rosacea are full of challenges [13]. Doxycycline, tetracycline and brimonidine, which exert anti-inflammatory or anti-vascular effects, are commonly used in clinic. However, Oral administration of antibiotics for long-term treatment may lead to body dysbacteriosis [14], and such antibiotics treatment also tend to result in gastrointestinal side effects [15,16]. In addition, although reduced persistent erythema have been shown in two randomized controlled trials [17], immediate side effects associated with the use of brimonidine including erythema, pruritus, burning sensation, flushing and rebound erythema can also occur frequently [18]. Moreover, these treatments are usually administrated in combination, so increasing adverse reactions and reducing compliance could occur. Therefore, it is necessary to explore simpler, more effective and safer treatments.

Artemisinin (ART) is an antimalarial drug isolated from Artemisia annua L. As a drug used in more than one million malarial patients, ART is demonstrated without noticeable side effects and adverse reaction [19]. In addition to antimalarial effects, clinical and experimental studies also demonstrated that ART possesses functions of anti-inflammation [20] and anti-angiogenesis [21]. Since rosacea is a skin disease with immune and vascular system dysfunction, we speculate that ART can also be used to treat rosacea.

In the present study, we explored potential therapy role and mechanism of ART on rosacea *in vivo* and *in vitro*. We conducted a rosacea-like mouse model and observed the effects of ART intervention on rosacea phenotype, inflammatory biomarkers, inflammatory cells and angiogenesis. In order to explore the potential pharmacology mechanism, the roles of ART on immunity including rosacea-related inflammation factors and NF-kB signal pathway were studied in HaCaT cells. Finally, we also explored the action of ART on migration of human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Reagents

LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTE) was commercially synthesized by Sangon Biological Technology (Shanghai, China), it was identified by HPLC and purity was more than 95%. ART was obtained from Selleck Chemicals (California, USA). PDTC (Ammonium pyrrolidinedithiocarbamate, an inhibition of NF-kB signaling pathway) was purchased from Sigma (Sigma-Aldrich LLC, Shanghai). Cell Counting Kit-8 (CCK8) solution was purchased from Boster Biological Technology (Wuhan, China). Anti-mouse-CD4, -CD31, -F4/80 and–Ly6G antibodies were obtained from Invitrogen (Thermo Fisher Scientific, Shanghai), anti-p65, anti-phospho-p65 and β-Tublin were purchased from CST (Cell Signaling Technology, Shanghai). Alexa Fluor488 green fluorescently and Alexa Fluor546 red fluorescently labeled secondary antibodies were purchased from Life Technologies (Thermo Fisher Scientific,Shanghai).

2.2. Mouse experiments

BALB/c (7-weeks old) mice were obtained from Slac Laboratory Animal Co. Ltd (Shanghai, China). Briefly, for ART administration, ART was diluted in filtered DMSO and 200 mg/kg feed by means of gavage daily for consecutive 7 days. To induce rosacea-like mouse model, mouse back was shaved 24 h before treatments, and 40 μl of LL37 (320 μM) was administered by means of intradermal injection (twice a day) at the last 2 days as previous described [8]. 12 h after the final injection of LL37, skin inflammation was assessed by the severity of erythema and edema as previously described [22]. Skin was then biopsied for RNA extraction, hematoxylin-eosin staining and immunofluorescence.

2.3. Cells culture

Calcium-free DMEM (Gibco, ThermoFisher Scientific, USA) was used as medium for HaCaT cells (Human keratinocytes), and cMedium containing 10% fetal bovine serum (FBS), streptomycin (50U/ml) and penicillin (50U/ml). For further experiments, HaCaT cells (2×10^5) were seeded into 35 mm cell culture dishes and cultured in DMEM (containing 1.8 M calcium) medium (Gibco, ThermoFisher Scientific, USA), and when confluence reached 90%, cells were stimulated with ART (50 μM) or PDTC (10 mM) for 1 h before LL37 (4 μM) administration. HUEVCs were cultured in 1640 Medium (Gibco, ThermoFisher Scientific, USA) containing 10% FBS, streptomycin (50U/ml) and penicillin (50U/ml). All cells were cultured in an incubator at 37 °C and 5% CO_2.

2.4. Cell viability assay

HaCaT cells (2×10^3) and HUVEC (2×10^3) were cultured in a 96-well plate and treated with various concentrations (25 μm, 50 μm, 75 μm and 100 μm) of ART for 24 h. After removing the supernatant of each well, 10 μl CCK8 solution and 100 μl media were introduced. After incubation for 1 h in 5% CO_2 at 37 °C for 1 h, the cell viability was evaluated by measuring the absorbance intensity of the cell suspension at 450 nm by a microplate reader(PerkinElmer EnSight).

2.5. Histologic analysis

4-mm biopsy specimens were fixed overnight in 4% buffered formalin, embedded in paraffin, sectioned, then stained with hematoxylin-eosin, finally the sections were observed by an optical microscope (OLYMPUS, Japan) [22].

2.6. Immunofluorescence

Briefly, the frozen mouse lesion sections or HaCaT cells grown on round glass coverslips in 24-well plates were fixed with 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline (PBS), then blocked with 5% BSA (Shenggong, Shanghai, China) which containing 0.3% TritonX-100 for 1 h at room temperature. Consequently lesion sections or HaCaT cells were treated with anti-CD31 (1:100), -CD4 (1:100), -F4/80 (1:100), -Ly6G (1:100), -IL17 (1:100) or -p65 (1:400) antibodies at 4 °C overnight. The following day Alexa Fluor 488 or 546 antibodies were used as secondary antibody for 1 h. Subsequently, lesion sections or HaCaT cells were washed and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min. Finally, we captured images by Zeiss Axio Scope A1 (Zeiss, Germany).

2.7. Western blotting assay

Briefly, HaCaT Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Proteins were quantified with bicinchoninic acid assay, then 35 μg protein were separated by 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (PVDF). After blocked with 5% non-fat milk for 1 h, PVDF was incubated with the primary antibody (rabbit anti-phospho-p65, 1:1000, and mouse anti-β-Tubulin, 1:2000) at 4 °C overnight. Then, the membranes were washed with TBST (0.1% Tween) and immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibodies (1:5000) and detected with HRP substrate (Luminata; Millipore) by the ChemiDocTM XRS + system (Bio-Rad). The level of β-Tublin was used as controls.

2.8. Real-time quantitative PCR analysis

According to the manufacturer's protocol, 1 μg RNA isolated from skin lesion or HaCaT cells was transcribed to cDNA using PrimeScript

RT reagent Kit (Takara, Shiga, Japan), and RT-PCR was performed with SYBR GREEN (Bio-Rad, California, USA) in Real-Time PCR System® (Applied Biosystems, Life Technologies) under the following conditions: a cycle of 95 °C for 3 min, 40 cycles of 95 °C for 5 s, and a cycle of 60 °C for 30 s. All samples were analyzed in triplicate, and the relative expression was obtained according to the formula 2 -(ΔΔCt). The primer sequences were described in supplement table 1.

2.9. HUVEC scratch assay

HUVECs (5×10^5 per well) were seeded into 6-well plates. When cells grown to 100% confluence, the 'scratch wound' was made by a razor blade, then each well was rinsed with PBS and cultured in cell medium containing ART (50 μM) or LL37 (4 μM). HUVECs were incubated in an incubator at 37 °C, 5% CO2 for 24 h, then the migration ability of cells was evaluated by the distance between scratches of 3 randomly chosen fields.

2.10. HUVEC transwell assay

8-mm pore size transwell chambers (Corning Incorporated, State of New York, USA) were used to measure HUVECs migration. In a 24-well culture plates, 500 μl 1640 Medium with 10% FBS, LL37 (4 μM) or equal amount of PBS was added into each lower chamber. HUVECs (1×10^4) with 200 μl 1640 Medium without FBS co-incubated with 50 μM ART were seeded into each upper chamber. After 24 h, HUVECs adherent to the lower surface were stained with 1% crystal violet and counted under an optical microscope (OLYMPUS, Japan) in three random visual fields.

2.11. Statistical analyses

All data were presented as means ± SEM. The Student's *t*-test analyzed with GraphPad Prism 5 was used for compare differences between two groups. *, $P < 0.05$, **, $P < 0.01$ and ***, $p < 0.001$ are considered significant.

3. Result

3.1. Mouse rosacea-like phenotype could be inhibited by ART

In the present study, we used a mouse rosacea-like dermatitis model to explore the pharmacologic mechanisms of ART. We found that ART significantly attenuated LL37-induced rosacea-like erythema (Fig. 1 a and b). The histological analysis demonstrated the inflammatory infiltration in rosacea-like dermatitis [8] was ameliorated by ART treatment (Fig. 1c). A number of studies demonstrated that TLR2 and pro-inflammatory cytokines were significantly up-regulated in rosacea [7,10]. Therefore, we detected the mRNA expression of TNFα, IL-1β, IL6 as well as TLR2 by qPCR. As shown in Fig. 1d, these inflammatory factors were repressed by ART treatment in mouse lesions. Thus, these results indicated that ART ameliorated the rosacea-like phenotype in LL37-induced mouse model.

3.2. ART suppressed the infiltration of $CD4^+$ T cells, neutrophils and macrophages in LL37-induced rosacea-like mouse

Importantly, previous studies demonstrated that both of $CD4^+$ T cells infiltrate and activation of Th1/Th17 pathways were involved in lesions of rosacea patients [11], indicating that adaptive immunity is an important pathogenesis for rosacea. Therefore, we detected $CD4^+$T cells in rosacea-like mouse lesion via immunofluorescence. Here, a greatly increasing number of $CD4^+$ T cells were observed in mouse lesions (Fig. 2a and b). Moreover, the administration of ART significantly repressed the LL37-induced $CD4^+$ T cell infiltration (Fig. 2a and b) in rosacea-like mouse lesion. Consistent with the decreased infiltration of $CD4^+$ T cells, the up-regulation of Th1 (IFN-γ, CXCL10) and Th17 (IL17A, CCL20)-associated cytokines and chemokines were repressed by ART in rosacea-like mouse skin (Fig. 2c). In addition to $CD4^+$ T cells, innate immune cells including neutrophils and macrophages have been reported to accumulate during rosacea inflammation. We found that ART also inhibited the innate immunity in rosacea, which based on LL37-induced neutrophils and macrophages invasion were repressed (Fig. 2d, e and g, h). Correspondingly, the expression of CXCL2 (mouse homolog of human neutrophil-recruiting chemokines CXCL8/IL8) and CCL2 (monocyte chemoattractant protein-1, MCP-1) were repressed by ART (Fig. 2f and i). Thus, these results supported that ART repressed adaptive and innate immunity in rosacea treatment.

3.3. ART repressed LL37-induced rosacea associated inflammatory factors in HaCaT cells

As epidermal keratinocytes play a similar role as innate immune cells, over-expression of pro-inflammatory factors in keratinocytes exerts an initial role on the pathogenesis of rosacea [8]. Thus, we explored the actions of ART on these pro-inflammatory factors in LL37-treated human keratinocyte (HaCaT) model. We found that different concentrations of ART (25 μM, 50 μM, 75 μM and 100 μM) presented slightly promoting effects on the viability of HaCaT cells (Fig. 3a) and we chose a drug concentration of 50 μm in cell experiment. Recent study showed a derivative of ART could inhibit the inflammation induced by LL37 in HaCaT cells [23]. In the present study, consistent with the anti-inflammation effects *in vivo*, we found ART suppressed LL37-induced up-regulation of IL-1β, IL6, IL8, TNFα, CCL2, CXCL10, CCL20 and TLR2 in HaCaT cells. (Fig. 3b). Collectively, these results indicated that ART repressed LL37-induced up-regulation of TLR2, cytokines and chemokines in keratinocytes.

3.4. ART inhibited LL-37 induced activation of NF-kB signaling pathway in HaCaT cells

It is well known that NF-kB nuclear translocation regulates pro-inflammatory gene expression and cytokine release [24,25]. To further analyze whether NF-kB signaling pathways were involved in inflammatory inhibition of ART on keratinocytes, HaCaT cells were pretreated with ART (50 μM) for 1 h and sequentially treated with LL37 (4 μM) for 30 min or 5 h, and the levels of phosphorylated-p65 were detected by immunoblot analysis. We found that LL-37 could promote the phosphorylation of p65, while ART could inhibite this process in HaCaT cells (Fig. 4a). Moreover, ART treatment significantly inhibited LL37-induced NF-kB p65 nuclear translocation in HaCaT cells, which was determined by immunofluorescence (Fig. 4b). Finally, in order to evaluate the role of ART on inflammatory signal pathway, a NF-kB signal pathway inhibitor PDTC (10 mM) was used in HaCaT cells. Similar with the inflammatory inhibition role of ART, PDTC also suppressed LL37-induced IL-1β, IL6, IL8 and TNFα in HaCaT cells (Fig. 4c). Together, these data supported that ART may attenuate inflammation in HaCaT cells partly through NF-kB signal pathways.

3.5. ART reduced angiogenesis and inhibited LL37-induced migration of HUVECs

To explore the effect of ART on vascular dysregulation in rosacea-like mouse model, we conducted mRNA expression of VEGF (vascular endothelial growth factor), Angpt (angiopoietin) and immunofluorescence of CD31 (a marker of blood vessels) in mouse lesions. LL37 significantly induced angiogenesis and pro-angiogenic factors expression in mouse model (Fig. 5a and b). Treatment with ART abrogated the angiogenesis with the decreased number of micro-vessels in dermis (Fig. 5a). Although ART failed to play a repression role on VEGFa, VEGFb and Angpt2, it showed significant inhibitory effects on LL37-induced VEGFc and Angpt2 in mouse lesions (Fig. 5b). *In vitro*, different

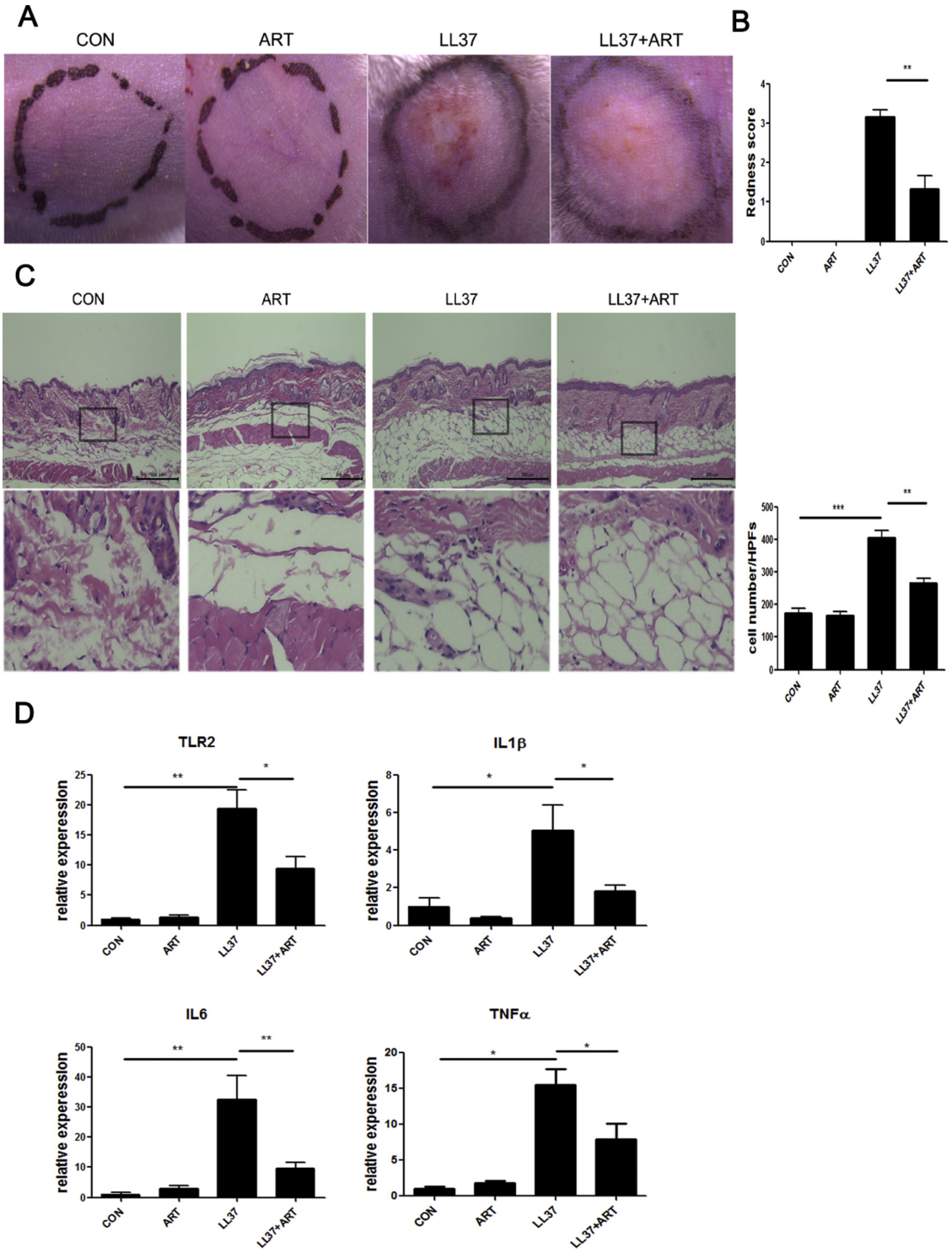

Fig. 1. Treatment with ART ameliorated rosacea-like dermatitis. (a) After the removal of hair, LL37 was injected intradermally into the dorsal skin to induce rosacea-like phenotype, ART was pretreated by gavage. The severity of the rosacea-like phenotype was assessed based on the redness score (b). (c) H&E for histological analysis of rosacea-like skin.Scale bars, 300 μm. (d) The expression levels of TLR-2,TNFα, IL-1βand IL-6 in mouse lesions were detected by qPCR analysis. Results are representative of three independent experiments. Data represent the means ± SEM, *P < 0.05, **P < 0.01 and ***P < 0.001.

concentrations of ART (25 μM, 50 μM, 75 μM and 100 μM) exerted no effects on the viability of HUVECs (Fig. 5c). However, 50 μM ART evidently repressed LL37-induced migration of HUVECs in the scratch test (Fig. 5d and e) and transwell assay (Fig. 5f and g). Thus, these results indicated that ART could attenuate angiogenesis in rosacea.

4. Discussion

The dysfunction of skin immune and vascular system is well considered as major pathogenesis of rosacea for a long term. ART is an effective treatment of various diseases with the anti-inflammation and anti-angiogenesis activities, however the therapeutic effect of ART on rosacea remains unclear. In the present study, we found that ART

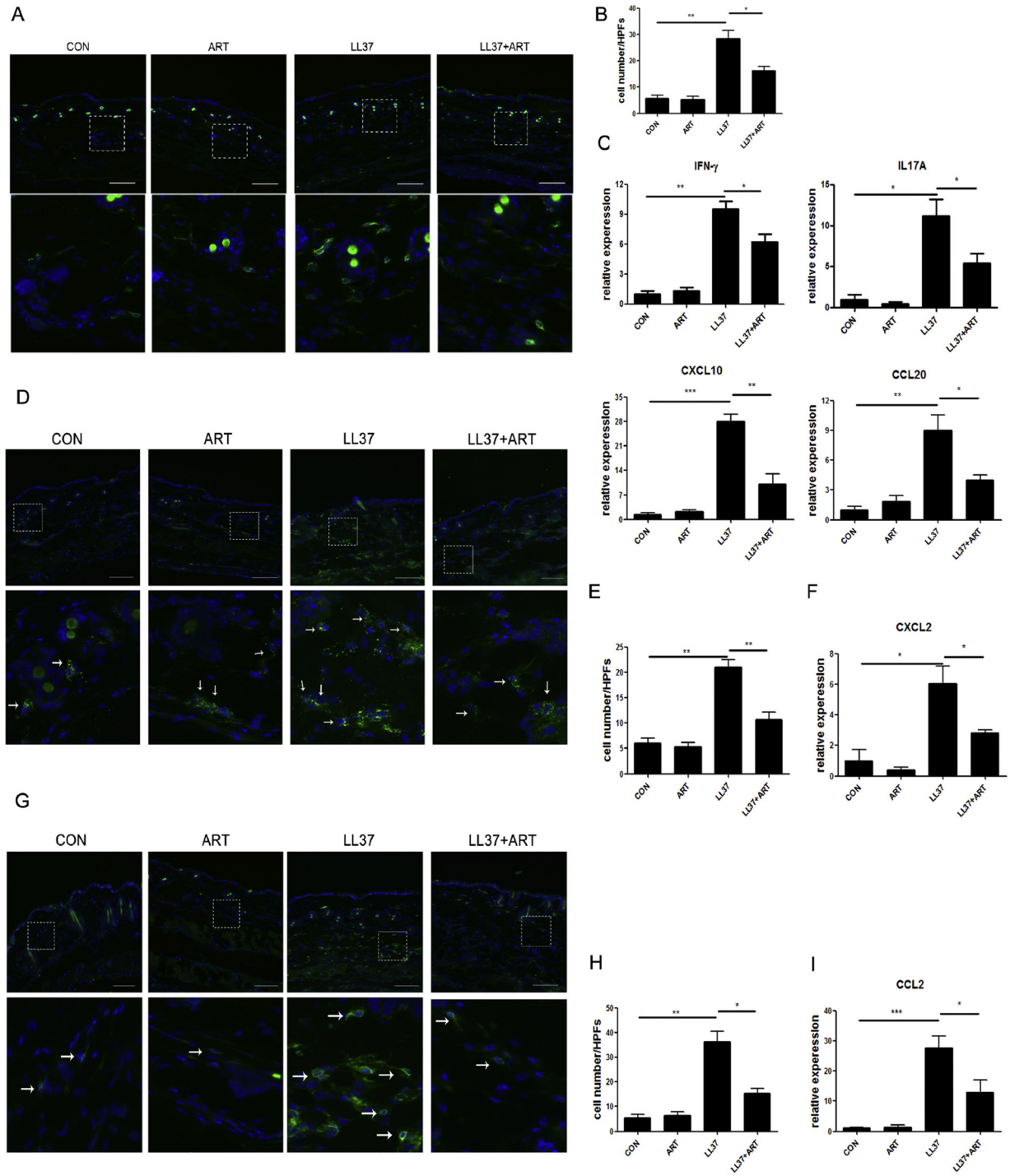

Fig. 2. ART reduced the infiltration of CD4 + T cells, neutrophils and macrophages in LL37-induced rosacea-like mouse. (a. b)Expression of CD4 in skin visualized by immunofluorescence. Green indicates $CD4^{+}$T cell. Blue indicates DAPI. Scale bars, 100 μm. (c)Th1 (CXCL10, IFN-γ) and Th17 (CCL20, IL17A) cell-related genes expression significantly modulated in mouse model. (d. e) Expression of Ly6G in skin visualized by immunofluorescence. Green indicates Ly6G (neutrophils), Blue indicates DAPI. Scale bars, 100 μm. (f) Neutrophils chemokine (CXCL2) significantly was modulated in mouse model. (g. h) Expression of F4/80 in skin visualized by immunofluorescence. Green indicates F4/80 (macrophages), Blue indicates DAPI. Scale bars, 100 μm. (i) Macrophages chemokine (CCL2) significantly was modulated in mouse model. Data represent the means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001.

attenuated the rosacea-like dermatitis partly by repressing inflammation and angiogenesis, which providing a valuable option for clinical treatment on rosacea.

The excessive activation of TLR2 could promote the release of inflammatory cytokines and chemokines, which are associated with erythema and inflammation in rosacea [26]. TLR2 is also able to activate NLRP3 inflammasome and mediate IL-1β release, thus inducing further inflammatory reactions [27]. In the present research, we found ART can inhibit LL37-induced up-regulation of TLR2 in rosacea-like mouse model. Consistent with the anti-inflammatory effects of ART in some reports [20,28], IL-1β, IL-6 and TNFα were also repressed by ART in rosacea-like mouse. Moreover, ART repressed the expression of CCL2 (monocyte chemokine), CXCL2 (neutrophil chemokine) in LL37-induced mouse model, which could exert chemotaxis on macrophages

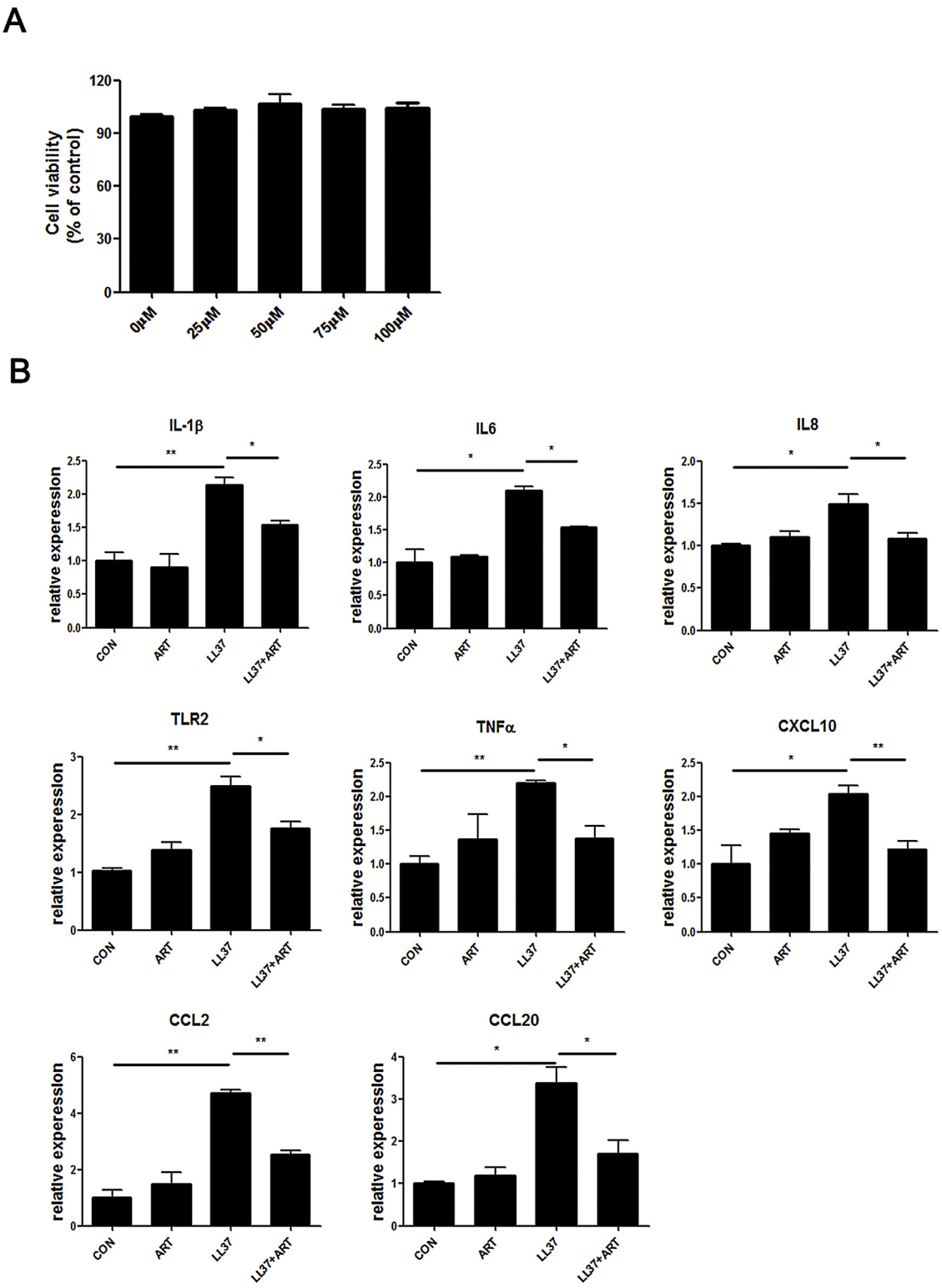

Fig. 3. ART reduced cytokines and chemokines expression in HaCaT cells. (a) The effect of different concentrations of ART on cell stability in CCK8 experiment. (b) ART repressed inflammatory cytokines and chemokines expression in HaCaT cells. HaCaT cells were pre-treated with ART (50 μM) for 1 h and stimulated by LL37(4 μM) for 12 h, the transcription levels of cytokines and chemokines were detected by RT-PCT. Data represent the means ± SEM. *P < 0.05 and **P < 0.01.

and neutrophils in rosacea [9]. Thus, as demonstrated in histologic analysis and Immunofluorescence in mouse lesions, rosacea-like dermatitis and subcutaneous inflammatory cell infiltration including macrophages and neutrophils were significantly suppressed by ART.

In addition to innate immunity, the adaptive immune system, including Th1/Th17 cells, is also significantly activated in rosacea [11]. ART derivative had been reported to reduce joint inflammation by suppressing the infiltration of Th17 cells [29]. In this study, ART evidently inhibited the up-regulation of IFN-γ and IL17A (Th1 and Th17 cells cytokines), CXCL10 and CCL20 (Th1 and Th17 cells chemokine) in rosacea-like lesions, correspondingly the invasion of subcutaneous $CD4^+$ T cells were also reduced. Thus, these evidences suggested that

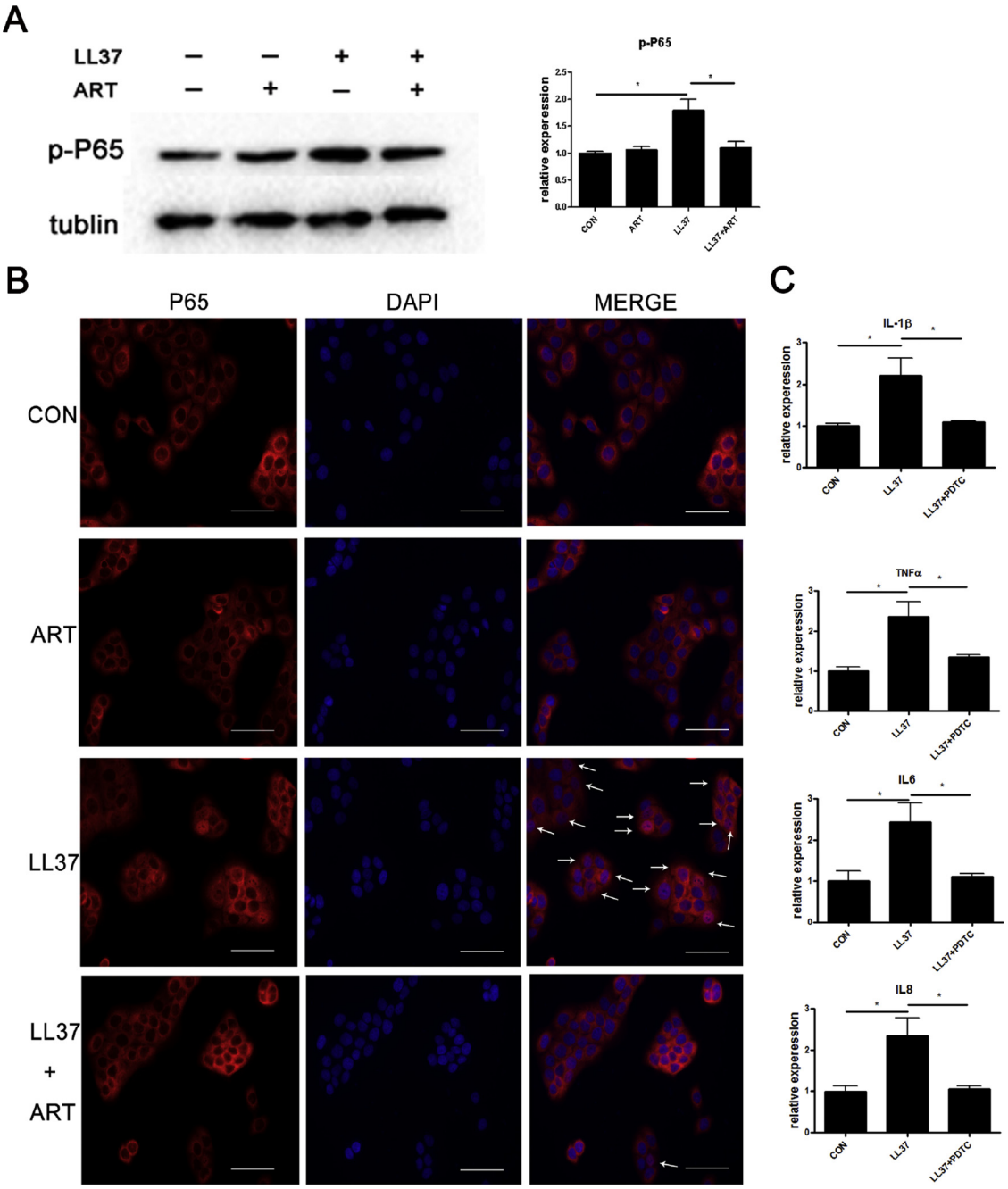

Fig. 4. ART inhibited LL-37 induced activation of NF-kB signaling pathways and the nuclear translocation of NF-κB p65 in HaCaT cells. (a) HaCaT cells were pre-treated with ART (50 μM) for 1 h and stimulated by LL37 (4 μM) for 30 min,the phosphorylation levels of p65 was analyzed by immunoblot. (b) Immunofluorescence staining for NF-kB p65. HaCaT cells were pre-treated with ART (50 μM) for 1 h and stimulated by LL37(4 μM) for 5 h, nuclear NF-kB p65 expression is increased in the LL37 group, but cytoplasmic expression is increased in the LL37 plus ART group. The nucleus is labeled with DAPI (blue). Scale bars, 40 μm. (c) HaCaT cells were pre-treated with PDTC (10 mM) for 1 h and stimulated by LL37(4 μM) for 12 h, the transcription levels of cytokines were detected by RT-PCT. Results are representative of three independent experiments.

ART exerting its therapeutic effects on rosacea may majorly via anti-inflammation and immunomodulatory properties.

Keratinocytes can perceive pathogens and tissue damage mediated by pattern recognition receptors (PRRs), and consequently release inflammatory factors such as antimicrobial peptides, cytokines as well as chemokines [30,31]. For rosacea, when TLR2 is activated by external stimuli or triggering factors, IL-1β, IL8 (CXCL2 in mouse) and TNFα could be released from keratinocytes [26]. In the present study, we found ART could inhibit LL37 induced TLR2 expression in HaCaT cells, and correspondingly, the expression of IL-1β, IL8 and TNFα were also repressed by ART. As in our previous study, LL37 could induce expression of cytokines and chemokines in keratinocytes [22], and the release of cytokines induces the occurrence of inflammation, while the chemokines exacerbates inflammation via recruiting immune cells in rosacea [9]. Here, LL37-induced upregulation of chemokines (CCL2, CXCL10 and CCL20) was also inhibited by ART in HaCaT cells. Thus, we considered ART attenuated rosacea-like inflammation mainly by inhibiting the expression of cytokines and chemokines in keratinocytes. Additionally, LL37 could decrease keratinocytes viability and increase apoptosis *in vitro* [23]. Inflammatory factors released from potential cell damage process may exacerbate inflammation in *vivo*. In our study, ART presented slightly promoting effects on the viability of HaCaT cells. This

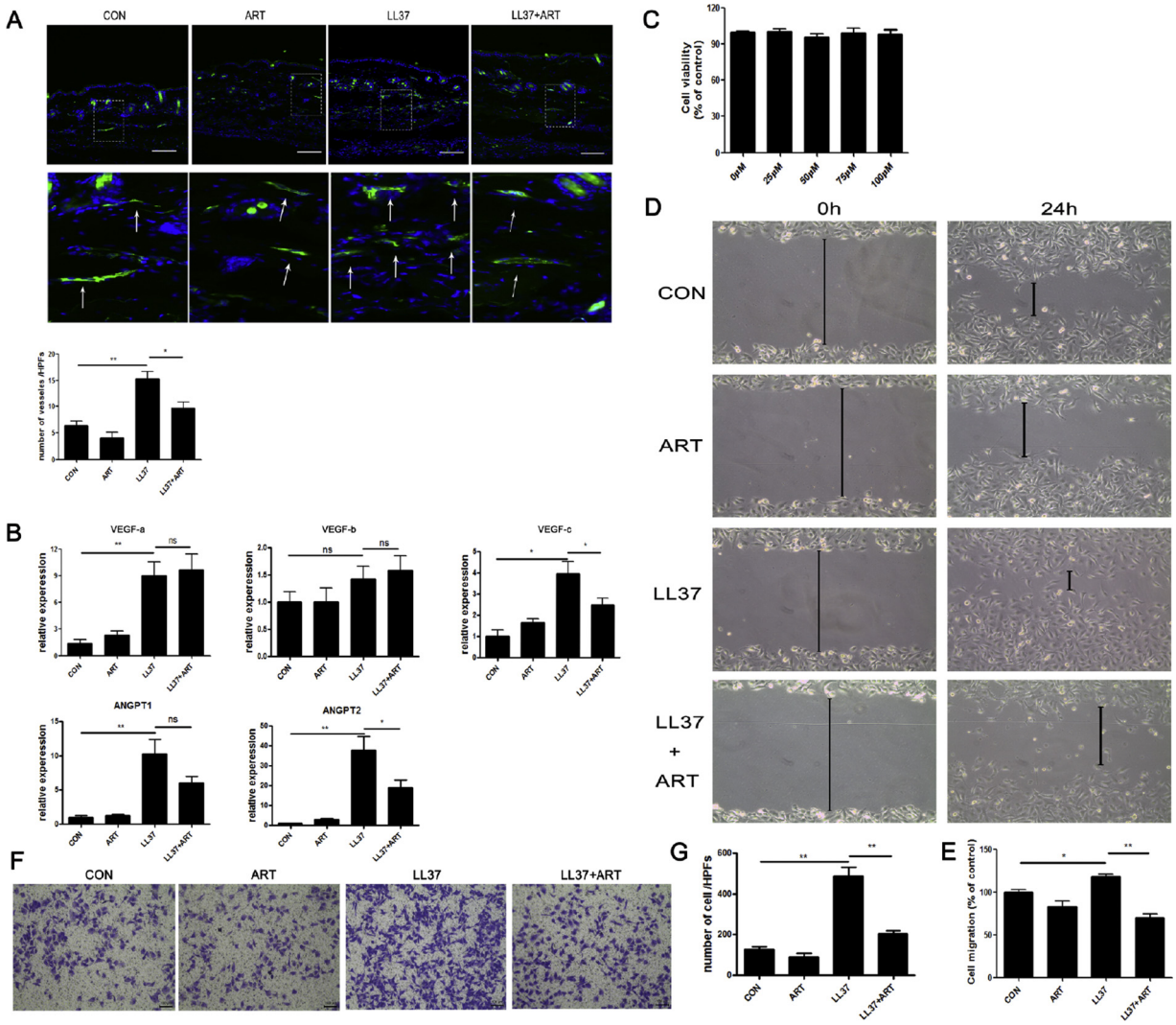

Fig. 5. ART reduced angiogenesis and inhibited LL37-induced migration of HUVECs. (a)Expression of CD31 in skin visualized by immunofluorescence. Green indicates CD31 cell. Blue indicates DAPI. Scale bars, 100 μm. (b) ART suppress the expression of pro-angiogenic factors in rosacea-like mice. (c)The effect of different concentrations of ART on HUVECs stability in CCK8 experiment. (d. e) ART inhibited LL37-induced migration of HUVECs in the cell scratch test. (f. g) ART inhibited LL37-induced migration of HUVECs in transwell assay. Results are representative of three independent experiments. Data represent the means ± SEM.*P < 0.05, and **P < 0.01.

may be another protective role on rosacea-like inflammation in *vivo*, however further research is needed.

For a long term, NF-kB signaling pathways have been considered to exert an important role on inflammation [25,27]. ART had been reported to inhibit the phosphorylation of p65 [32] in macrophages cells. For potential therapy mechanisms of ART on rosacea, interestingly, we found ART inhibited the phosphorylation and the nuclear translocation of NF-kB p65 induced by LL37 in HaCaT cells. Moreover, PDTC, the inhibitor of NF-kB, showed similar effect with ART on inflammation repression in LL37-induced HaCaT cells. Thus, the repression of NF-kB signaling pathways may contribute to the anti-inflammatory activity of ART in rosacea.

As an important pathogenesis for rosacea, vascular dysfunction characterized as dilated blood vessels and angiogenesis is well established for a long time [10]. A number of studies showed ART could exert therapeutic effect on tumor disease via anti-angiogenesis [33]. In this study, ART evidently reduced angiogenesis (CD31) in rosacea like mouse model. Although the level of VEGF-a and VEGF-b failed to be inhibited by ART, VEGF-c and angiopoietin 2 promoted by LL37 were reduced by ART administration in mouse lesions. Previous study showed angiopoietin 2 other than VEGF was significantly up-regulated in rosacea patients [12], therefore the inhibitory effects of ART on angiopoietin 2 would contribute to its therapy for rosacea in the future. In addition to the inhibition of VEGF-c and angiopoietin 2, the effects of ART on angiogenesis may also be attributed to its inhibition of TLR2, CCL2, CXCL2, IL-1β and TNFα *in vivo* and *vitro*, which have been reported to be associated with angiogenesis in rosacea and tumor [5,9,34]. Moreover, we found LL37 could promote the migration of human umbilical vein endothelial cells, and ART can inhibit this effect. Although LL37 only showed a slight migration-promoting effect in HUEVCs scratch test, it significantly increased the migration of the cells in the transwell assay. Those evidence *in vitro* further support the potential role of LL37 on vessel. Thus, as an anti-angiogenesis drug, ART is a potential option for vascular dysfunction of rosacea patients.

In conclusion, our study explored the role and potential mechanism of ART on rosacea, and finally found ART attenuated rosacea phenotype by inhibiting immune response and angiogenesis both *in vivo* and *in vitro*, indicating that it could represent an effective therapeutic option for patients with rosacea.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biopha.2019.109181.

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