

ALA-PDT Amplifies Intense Inflammatory Response in the Treatment of Acne Vulgaris via CXCL8

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Research

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Abstract

Background: Acne vulgaris is a chronic inflammatory cutaneous disease. 5-Aminolaevulinic acid photodynamic therapy (ALA-PDT) is a novel and effective therapy for severe acne vulgaris. However, the specific mechanism of ALA-PDT for acne still remain unclear. Here, we investigate the possible mechanism of intense inflammatory response of ALA-PDT for acne vulgaris.

Results: It appeared that ALA-PDT suppresses proliferation and lipid secretion of primary human sebocytes. And ALA-PDT could up-regulate the expression of CXCL8 in vivo and in vitro, amplifying inflammatory response by recruiting T cells, B cells, neutrophils and macrophages. We also found that ALA-PDT elevated the expression of CXCL8 via p38 pathway. SB203580, a p38 pathway inhibitor, decreased the expression of CXCL8 after ALA-PDT in sebocytes.

Conclusion: ALA-PDT amplifies intense inflammatory response in the treatment of acne vulgaris via CXCL8. Our data deciphers the mechanism of intense inflammatory response after ALA-PDT for acne vulgaris.

Introduction

Acne vulgaris is a chronic inflammatory cutaneous disease involving pilosebaceous follicles, and it affects almost 85% of the population.(1) Excessive sebum production and complex inflammatory reaction are two of the key factors of the pathogenesis of acne vulgaris. The dysregulation of innate immune signaling is a new sight for the physiopathology of acne.(2, 3) Topical or oral antibiotics and isotretinoin are routinely used to treat acne.(1, 4, 5) However, antibiotic resistance is increasing, more than 50% of *Propionibacterium acnes* strains are resistant to topical macrolides, affecting its effectiveness, as well as the system adverse effects of isotretinoin involving mucocutaneous, musculoskeletal, and ophthalmic systems.(1, 6) And long course of the traditional treatments also limits their application.(7)

5-Aminolaevulinic acid photodynamic therapy (ALA-PDT) is a relatively novel therapy for acne vulgaris. After applied to the lesion for a period of time, ALA preferentially accumulated in the pilosebaceous units, then light of an appropriate wavelength activates the process of reactive oxygen species (ROS) generating, promoting the photodynamic reaction.(8) ALA-PDT has been involved in many kinds of guidelines of acne vulgaris for the advantages of positive curative effects, tissue targeting, minimal invasion, repeatability, and no system side effects like oral agents.(1, 9) (10) However, the specific mechanism of the ALA-PDT for acne still remain undetermined.

Clinically, "intense inflammatory response", such as erythema and pustule, occurs in the early phase after ALA-PDT.(5) We also observed that there was a positive correlation between the degree of inflammatory reaction and the efficacy. (11, 12) However, the related research is rarely reported on the amplification of inflammation. The correlative mechanism is being unknown. In our study, we found that interleukin-6 (IL-6), tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β) and C-X-C motif chemokine (CXCL8) were increased significantly after ALA-PDT for acne vulgaris. We focused the role of CXCL8, which is a pro-

inflammatory chemokine, secreted by different cell types including blood monocytes, alveolar macrophages, fibroblasts, endothelial cells, and epithelial cells.(13) Various cytokines (IL-1, IL-6 and TNF α) and ROS could stimulate the up-regulation of CXCL8. (13, 14) However, the mechanism of CXCL8 in amplifying acute inflammatory response after ALA-PDT on acne vulgaris has not been fully elucidated.

Here, we found the optimum parameters of ALA-PDT on primary sebocytes and show that ALA-PDT could inhibit the proliferation and lipid secretion of sebocytes. Furthermore, we found that ALA-PDT regulates the expression of CXCL8 via p38 pathway, recruiting a variety of immune cells to amplify the inflammatory response. Our data deciphers the mechanism of intense inflammatory response after ALA-PDT for acne vulgaris.

Results

1. ALA accumulates in sebaceous gland specifically.

We observed that ALA-PDT had a direct effect on sebaceous gland in acne vulgaris in clinical. To verify this, we detected if ALA accumulated in targeting cells and tissues. As shown in Fig. 1A, red fluorescence of PpIX in sebocytes induced by ALA was observed by fluorescence microscope. As the fluorescence intensity of PpIX was related to the effect of ALA-PDT, we investigated the optimum parameters of ALA. Our results showed that fluorescence intensity of PpIX was strongest in sebocytes when incubated with 0.5 mM ALA for 6 h (Fig. 1B, 1C). But if incubated for 6 h, the proliferation of sebocytes would be decreased significantly. No significant differences were detected when incubated for 4 h (Fig. 1D). So the optimum parameter of ALA was 0.5 mM for 4 h on sebocytes.

To further confirm that ALA was accumulated in sebaceous gland directly, 8% ALA was applied on the right sebaceous gland on back of golden hamster for 3 h. As shown in Fig. 1E, red fluorescence of PpIX was observed around both right and left sebaceous gland. Freezing section of the tissue also showed that PpIX was accumulated in follicle sebaceous gland units directly (Fig. 1F). Taken-together, ALA could specifically accumulate in sebaceous gland.

2. ALA-PDT suppresses proliferation and lipid secretion of sebocytes

To substantiate the inhibitory effect we evaluated the proliferation, apoptosis and lipid secretion of sebocytes after ALA-PDT. After 0.5 mM ALA-PDT (10 J/cm²), ROS accumulates in sebocytes (Fig. 2A). According to the previous study, the accumulation of ROS was related to the effect of ALA-PDT.

Next we used CCK-8 assay to evaluate the cell viability. As the energy intensity of 0.5 mM ALA-PDT increased from 0 to 40 J/cm², cell viability of sebocytes was reduced from 100 \pm 8.99% to 20.18 \pm 8.09% and IC50 was at 10 J/cm² (Fig. 2B). The apoptotic population in sebocytes was evaluated by flow cytometry. As expected, 0.5 mM ALA-PDT (10 J/cm²) significantly decreased the relative number of live

sebocytes and concurrently increased the relative number of apoptotic sebocytes (Fig. 2C, 2D). Fluorescence microscope was used to detect the secretion of lipids in sebocytes treated by ALA, Red light and ALA-PDT respectively. (Fig. 2E). Lipid in sebocytes was found to be significantly reduced 24 h after ALA-PDT. The above results indicated that ALA-PDT inhibited the ability of lipid secretion and proliferation of sebocytes.

3. ALA-PDT induces up-regulation of CXCL8 in sebocytes and sebaceous gland.

Clinically, intense inflammatory reaction occurs in the early phase after ALA-PDT. Therefore, we tested whether inflammatory cytokines changed after ALA-PDT. Sebocytes treated by ALA, Red light and ALA-PDT were collected 24 h later. The results showed that the expression of CXCL8, IL-6, TNF- α and IL-1 β was significantly increased after ALA-PDT (Fig. 3A). In view of the effect of the recruitment of CXCL8 on immune cells, our research focused on the regulatory effect of ALA-PDT on CXCL8.

Further experiment substantiated that the expression of CXCL8 was elevated by ALA-PDT in sebaceous gland of golden hamster. After treated by ALA-PDT, tissues were taken from treated sites at 1 h, 3 h, 6 h, 12 h, 24 h after ALA-PDT and untreated tissues were used for comparisons. We found that positive staining for CXCL8 started to increase at 3 h after ALA-PDT around sebaceous gland. These findings illustrated ALA-PDT upregulated the expression of CXCL8 on sebocytes and sebaceous gland.

4. CXCL8 amplifies intense inflammatory response after ALA-PDT in sebaceous gland.

CXCL8 is a kind of chemokines, recruiting inflammatory cells. To further investigate the role of CXCL8, we detected the expression of CD3, CD11b, CD19 and CD68 in sebaceous gland after ALA-PDT by immunohistochemistry. After treated by ALA-PDT, tissues were taken from treated sites at 1 h, 3 h, 6 h, 12 h, 24 h after ALA-PDT and untreated tissues were used for comparisons. In HE staining, inflammatory cells started to collect towards sebaceous gland at 1 h after ALA-PDT. The results showed that positive staining for CD3(+) T cells and CD11b (+) neutrophils started to increase gradually at 1 h after ALA-PDT around sebaceous gland. Expression of CD19(+) B cells and CD68(+) macrophages slightly increased from 3 h after ALA-PDT (Fig. 4). Collectively, the results implied that CXCL8 could recruited several inflammatory cytokines, which were mainly T cells with a few neutrophils, B cells and macrophages.

5. ALA-PDT regulates the expression of CXCL8 via p38 signaling pathway.

The activation of CXCL8 gene promoter, trans-activation by JNK pathways and stabilization by p38 pathway are involved in the up-regulation of CXCL8 expression. To further study the mechanism of ALA-PDT's regulation of CXCL8 in sebocytes, we analyzed the expression of p38, Erk1/2, and JNK pathways by western blot. Sebocytes were collected for western blotting after ALA-PDT immediately. The results showed that ALA-PDT upregulated p-p38 and decreased expression of p-Erk1/2 and p-JNK significantly (Fig. 5A, 5B).

Next, to verify whether suppression of p38 pathway could block the effect of ALA-PDT in sebocytes, p38 inhibitor SB203580 was employed. Cells were collected for further assays at 24 h post-treated with ALA-

PDT and SB203580 (10 μ M). The results showed that pretreated with SB203580 attenuated the upregulated CXCL8 mRNA expression by ALA-PDT in sebocytes (Fig. 5C). The results indicated that ALA-PDT induce CXCL8 production through p38 pathway.

Discussion

ALA-PDT is a common and effective treatment for severe acne vulgaris.(9) Key pathogenic factors involve hyperkeratinisation, disturbance of microbiota, sebum production and complex inflammatory mechanisms.(15) There are many research developments on the effect of ALA-PDT on keratinocytes and *Propionibacterium acnes* (*P. acnes*). But the effect of ALA-PDT on sebocytes received limited attention. In clinic, there is a specific absorption of ALA on sebaceous gland, and after ALA-PDT, a temporary and serious inflammatory reaction happens around the target lesions to achieve the expected therapeutic efficacy.(9, 16)

A systematic review of randomized controlled trials to evaluate the efficacy and safety of PDT on acne showed that the ALA-PDT could significantly decrease the sebum secretion. Xiang et al have demonstrated that sebaceous glands atrophy and reduction of sebum secretion after ALA-PDT may be caused by the suppression of lipogenesis and cell growth in SZ95 cells by mTOR signaling pathway.(17) However, there are few studies about the effect of ALA-PDT on primary sebocytes in vivo. In this study, we used primary human sebocytes and animal models to verify the effect of ALA at different points in time and to further investigate the effect of ALA-PDT on sebaceous gland.

We provided the optimum parameters of ALA-PDT in vitro and in vivo. After incubated with 0.5 mM ALA for 4 h, red fluorescence was observed in sebocytes, then after ALA-PDT (10J/cm²), the lipid secretion was inhibited and sebocytes were induced to apoptosis (Fig. 1, 2). Sebaceous gland on the back of gold hamster could absorb ALA specifically. When ALA was applied on one side, the other side of sebaceous gland could also absorb and transform to PpIX (Fig. 1).

Clinically, the obvious inflammatory response aggravated temporarily after ALA-PDT. And there is a positive relationship between inflammatory reaction and effectiveness of ALA-PDT.(9) However, the mechanism of complex inflammatory reaction after ALA-PDT is not clear. Multiple data have demonstrated ALA-PDT can enhance immunogenicity around target lesion, providing a promising strategy for inducing a systemic immune response.(18) The induction of CXCL8 by ALA-PDT was further supported by our findings in vitro and in vivo. As shown in Fig. 3, expression levels of CXCL8 in sebocytes and sebaceous gland of golden hamster were increased after ALA-PDT. According to immunohistochemical results, we observed positive expression of T cells, neutrophils, B cells and macrophages at different points in time, finding a recruitment path of them (Fig. 4). This process changed the microenvironment in pilosebaceous unit, accelerating inflammatory process in order to eliminate the lesions. So up-regulation of CXCL8 after ALA-PDT to recruit more inflammatory factors is beneficial in treating acne vulgaris. Then, we found that p38 pathway might be involved in up-regulation of CXCL8 after ALA-PDT (Fig. 5). When treated with p38 inhibitor SB203580, the expression of CXCL8

showed decreased after ALA-PDT in sebocytes. Thus, CXCL8 was up-regulated after ALA-PDT via p38 MAPK pathway in sebocytes. However, there are still other inflammatory-related factors or pathways could regulate the microenvironment after ALA-PDT. Further studies are necessary to determine the mechanisms of complex inflammatory reactions induced by ALA-PDT for severe acne vulgaris.

Conclusions

In conclusion, our study demonstrated that ALA-PDT up-regulates the expression of CXCL8 in sebocytes and sebaceous gland via p38 pathway, then CXCL8 amplifies intense inflammatory reaction, suggesting a potential basis for the clinical application of ALA-PDT in the treatment of severe acne.

Methods

Reagents

ALA hydrochloride powder was from Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Co, Ltd. (Shanghai, China). Keratinocyte-SFM Medium was from Gibco (Rockville, MD, USA). Fetal bovine serum (FBS) was supplied by Hyclone (Logan, UT, USA). Annexin-V-FITC Apoptosis Detection kit was obtained from BD Biosciences (San Diego, CA, USA). The following reagents and commercially available assay kits were used: CXCL8 polyclonal antibody (27095-1-AP, Proteintech, Rosemont, USA), anti-CD3 antibody (ab16669, Abcam, Cambridge, MA, USA), anti-CD11b antibody (ab133357, Abcam, Cambridge, MA, USA), anti-CD19 antibody (ab245235, Abcam, Cambridge, MA, USA), anti-CD68 antibody (ab54333, Abcam, Cambridge, MA, USA), human CXCL8 ELISA kit (Biotech Well, Shanghai, China). Complementary DNA (cDNA) Reverse Transcription Kit was from Applied Biosystems (ThermoFisher, Loughborough, UK). Primer CXCL8 was from Shanghai Personal Gene Technology Co. Ltd. (Shanghai, China). The primary antibody p-Erk1/2, Erk1/2, p-JKN, JNK, p-p38 and p38 were purchased from CST (Danvers, MA, USA). p38 inhibitor SB203580 were from Sigma (St. Louis, MO, USA). Other chemicals were purchased from local commercial sources and were of analytical grade.

Cells and animals

Primary human sebocytes were isolated from pilosebaceous unit (face) and used at passages 4–8. The human sebocytes were maintained in Keratinocyte-SFM supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in 5% CO₂ air atmosphere. Male golden hamster (42 ~ 48 days old) were obtained from Vital River Laboratories (Beijing, China).

Sebaceous gland

Sebaceous glands were obtained from the back of golden hamster after incubated with 8% ALA for 4 h in the dark. The samples were frozen at -20 °C for about 30 min. The frozen tissue together with the vise were quickly observed under a stereomicroscope. The vertical plane of the skin sample was cut by a sterile scalpel to obtain slices that contained intact pilosebaceous units. The sebaceous glands in the

hand-made slices were observed under white and UV light, and the spectrum of sebaceous glands was documented by the microfluorospectrometer.(19)

Protoporphyrin IX (PpIX) absorption

Sebocytes were treated with ALA (0.5 mM) for 4 h. After 24 h, the cells were observed by fluorescence microscope (FM-YG100; Soochow FZM Optical Tech, China) with blue light (peak at 365 nm). After 10 min in dimethyl sulfoxide, the supernatant was collected, then detected by fluorescence spectrophotometer with excitation at 405 nm wavelength.

ALA-PDT treatment

Sebocytes were incubated with ALA (0.5 mM) in serum-free medium for 4 h at 37 °C, then washed twice with phosphate buffered saline (PBS) and were irradiated by 10 J/cm² red LED light (635 nm) at 16.67 mW/cm². In vivo study, 8% ALA cream was topically applied on the sebaceous gland on the back of golden hamster. After incubated for 3 h in the dark, excess ALA cream was removed, and the sebaceous gland was irradiated by a red LED light (635 nm) at a power density of 80 mW/cm² and energy density of 38.4 J/cm².

Cell viability and apoptosis assay

Cell viability was measured using a CCK-8 Kit (Beyotime Biotechnology, Shanghai, China). Sebocytes (5 × 10³ per well) were seeded into 96-well plates, then treated with ALA (0.5 mM), red light and ALA-PDT respectively. 24 h after the treatments, 10 µL of CCK-8 was added to each well for 1 h. The absorbance of cell culture at 450 nm was detected with a Thermo Scientific Microplate Reader. Cell apoptosis was monitored by flow cytometry. Sebocytes had been treated with ALA (0.5 mM), red light or ALA-PDT. 24 h later, these cells were harvested and quantitatively analyzed with a FACScan flow cytometer (BD, Franklin Lakes, NJ, USA).

Intracellular lipids determination

Sebocytes were treated with ALA (0.5 mM), red light or ALA-PDT. After 24 h, the cells were washed with PBS and neutral lipids were labeled with the Nile red (10 µg/ml in DMSO). After 5 min in dimethyl sulfoxide, the cells were observed by fluorescence microscope (FM-YG100; Soochow FZM Optical Tech, China) with excitation at 485 nm and emission at 565 nm to detect red fluorescence.(20)

Elisa analysis

Sebocytes were cultured in six-well tissue culture plates and treated with ALA (0.5 mM), red light or ALA-PDT. After centrifugation, the supernatants were collected at 24 h post-treatment and analyzed using an ELISA-based IL-6, TNF-α, IL-1β and CXCL8 detection kit.

Quantitive real-time PCR analysis

Sebocytes were treated with ALA (0.5 mM), red light or ALA-PDT. Then, the cells were harvested using TRIzol according to the manufacturer's instruction and reverse transcribed to cDNA. Following reverse

transcription, the samples were subjected to Taqman qPCR analysis on a 7900 H T Fast Real-Time PCR System (Life Technologies, ThermoFisher, Loughborough, UK). CXCL8 mRNA levels were detected by SYBR Green qPCR (Life Technologies) using the following primers: 5'- TTG CCA AGG AGT GCT AAA G-3' (human CXCL8 forward primer), 5'- CACTCTCAATCACTCTCAGTTC-3' (human CXCL8 reverse primer). GAPDH mRNA level was used as control.

Immunohistochemical studies

Freshly isolated tissue from sebaceous gland on the back of golden hamster was obtained in 1 h, 3 h, 6 h, 12 h and 24 h after ALA-PDT. The tissue was stored in formalin and 5 μ m sections were dewaxed (30 min 56 °C, 2 \times 10 min xylene), followed by rehydration, antigen unmasking, and blocking. Then, the samples were stained with anti-CD3, anti-CD19, anti-CD68, and anti-CXCL8 primary antibodies at 1 μ g/mL in blocking solution for 30 min at 37 °C. The slides were rinsed in PBS and incubated with a goat anti-rabbit IgG secondary antibody (Boster, China) diluted in blocking solution for 30 min. The slides were inculcated with streptavidin-biotin complex (Boster, China) for 30 min, rinsed in PBS, stained using DAB chromogen and hematoxylin counterstain, and observed under a light microscope. Exposure to PBS was used for negative control sections.

Western blot analysis

Sebocytes were cultured in six-well tissue culture plates and treated with ALA (0.5 mM), red light and ALA-PDT respectively. Total protein was extracted from the cells with RIPA lysis buffer. Afterwards, Western blotting was performed as described previously.

Statistical analysis

All quantified data were expressed as mean values \pm standard error (mean \pm SE). The Student's t test for nonpaired replicates was used to identify statistically significant differences between treatment means. Significance was accepted at $P < 0.05$.

Abbreviations

ALA-PDT, 5-Aminolaevulinic acid photodynamic therapy; ROS, reactive oxygen species; PpIX, protoporphyrin IX; IL-6, interleukin-6; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin-1 β ; CXCL8, C-X-C motif chemokine 8.

Declarations

Ethics approval

The study protocol of golden hamsters was approved by the Ethics Committee of Shanghai Skin Disease Hospital (NO.11400700312027). The license of golden hamsters was SCXK2016-0011.

Consent for publication

Not applicable.

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 no conflict of interest.

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Authors' contributions

LZ and JY conceived and designed the analysis. JY, LS and XL collected the data. DX, JL and XW contributed data or analysis tools. LZ, XL and XW performed the analysis. JY and QZ wrote the paper. All authors read and approved the final manuscript.

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Figures

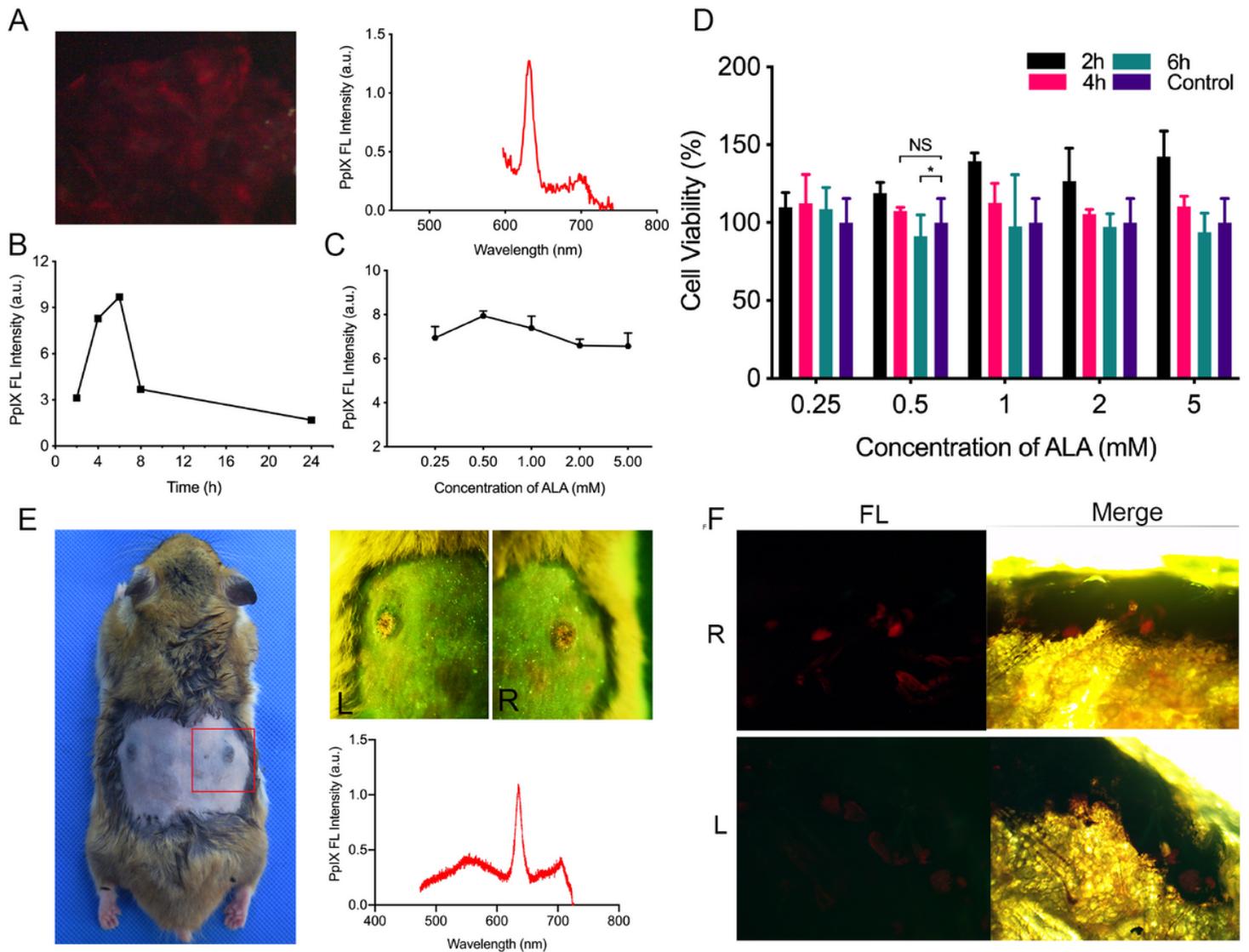


Figure 1

Absorption of ALA on sebocytes and sebaceous gland of golden hamster. (A) Red fluorescence of PpIX in sebocytes were observed by fluorescence microscope after incubated with 0.5mM ALA for 4h. (B) Fluorescence intensity of 0.5mM ALA incubated for different times on sebocytes was detected by fluorescence spectrophotometer. (C) Fluorescence intensity of different concentration of ALA incubated for 4h on sebocytes was detected by fluorescence spectrophotometer. (D) CCK8 was used to detect the proliferation of sebocytes incubated with different concentration of ALA for different time. (E) 10% ALA for 3h was applied on the right sebaceous gland on back of golden hamster, and PpIX was detected in both right and left sebaceous gland. (F) Red fluorescence of PpIX was accumulated in follicle sebaceous gland units on back of golden hamster. All the results were shown as mean \pm SD ($n = 3$), which were three separate experiments performed in triplicate. * = $p < 0.05$ significantly different from control.

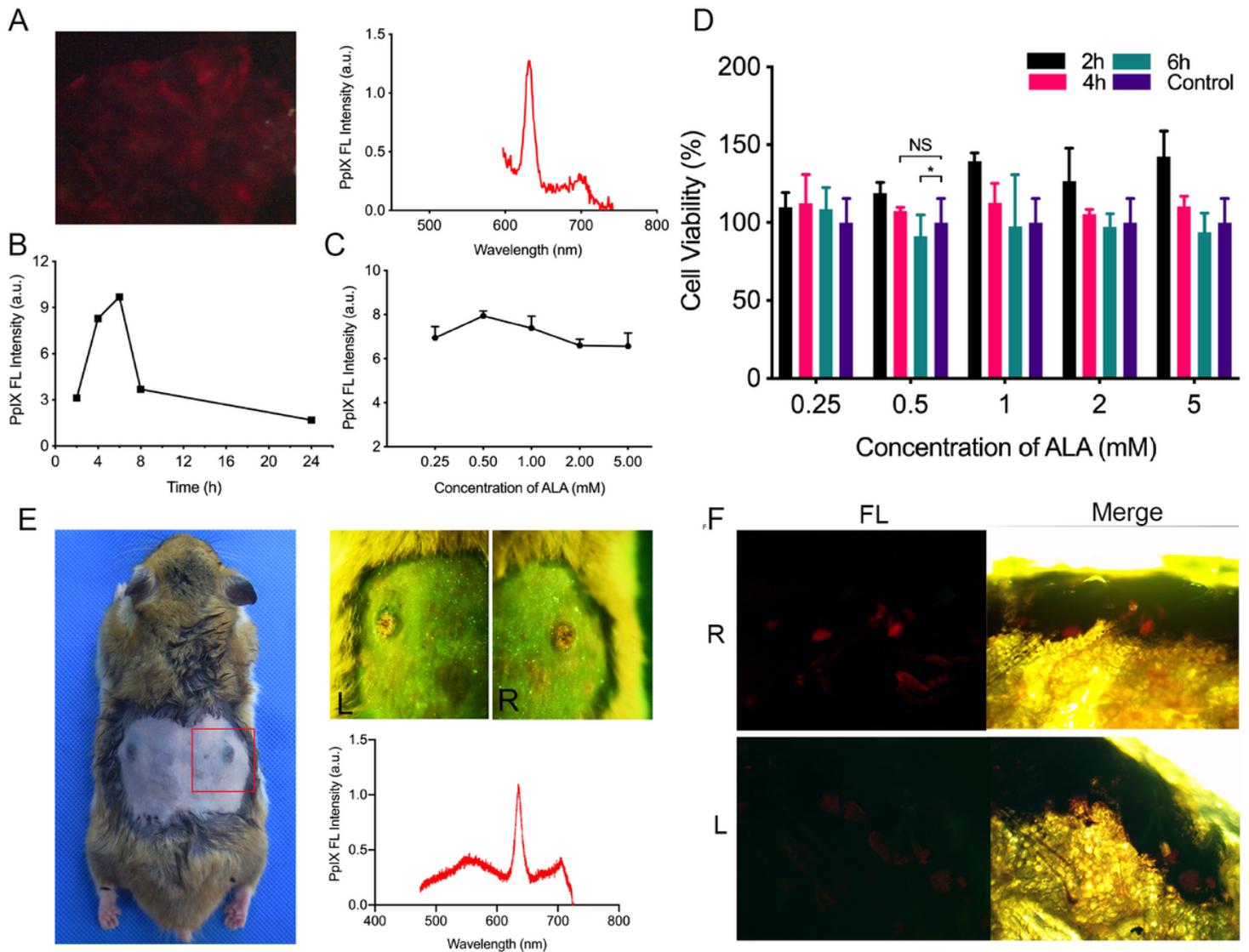


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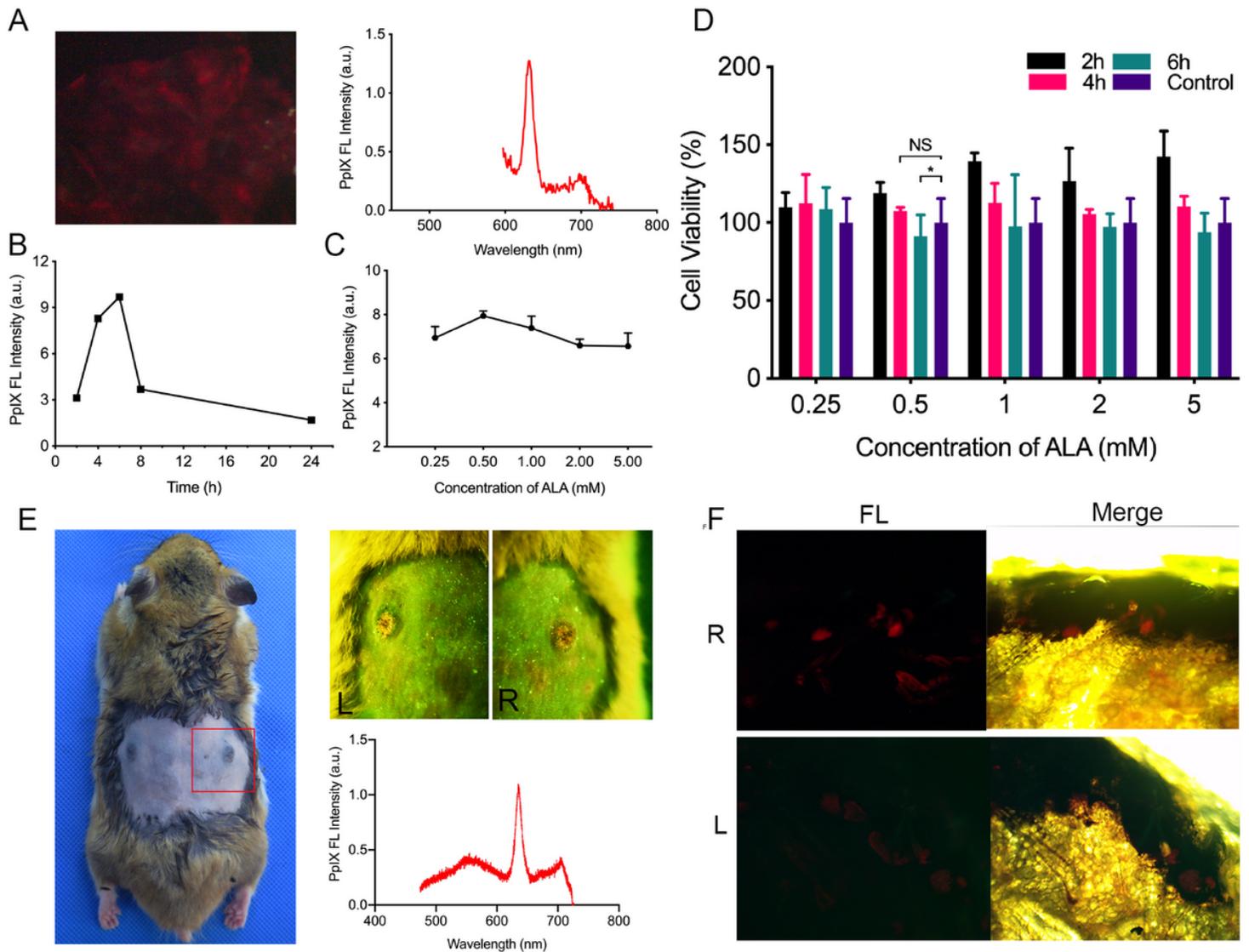


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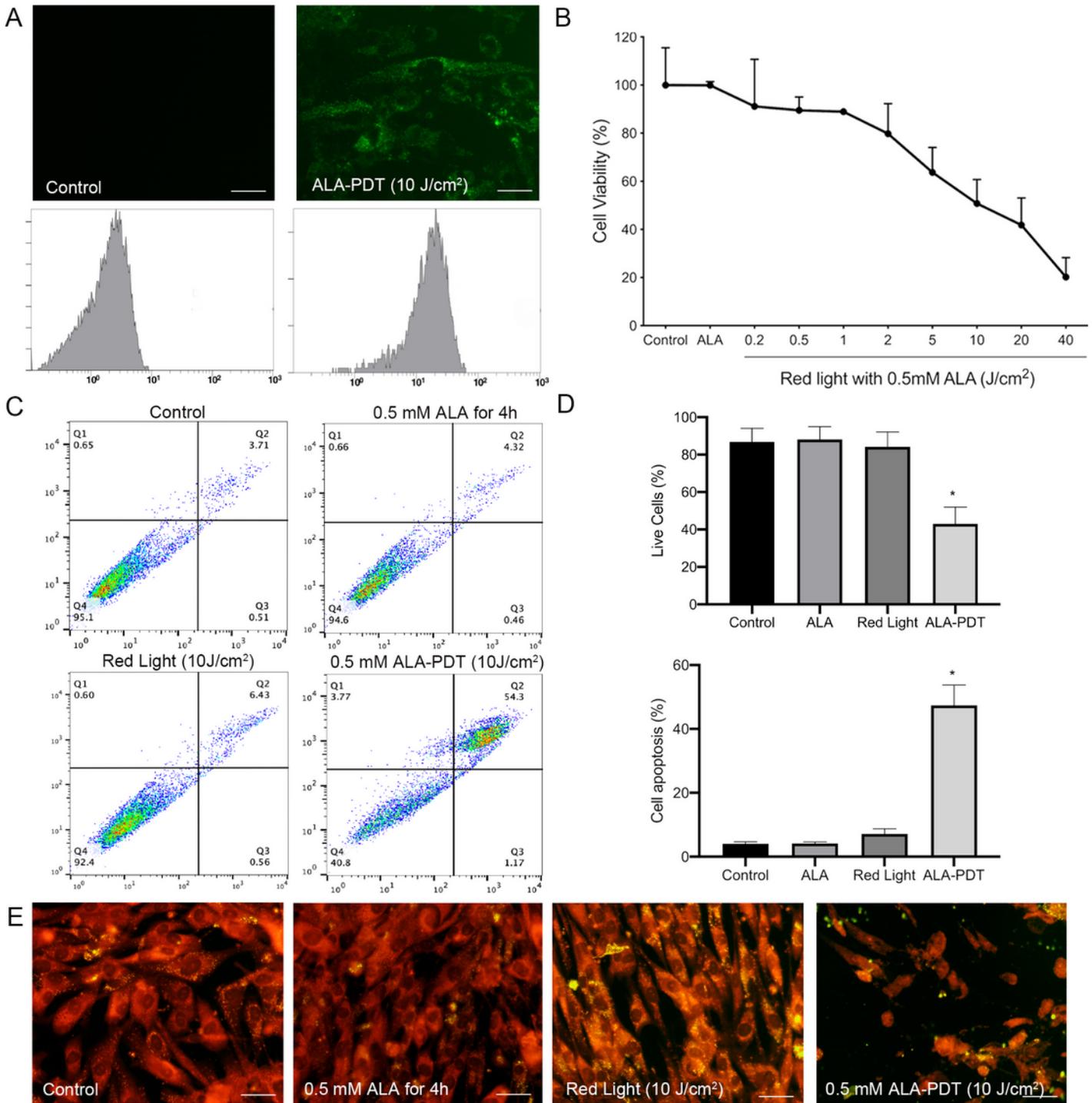


Figure 2

ALA-PDT promotes apoptosis rate and suppresses proliferation and lipid secretion of sebocytes. (A) ROS production in sebocytes were observed by fluorescence microscope and detected by flow cytometry. (B) After 24h post-treated with different energy intensity of red light, CCK8 was used to detect the viability of sebocytes incubated with 0.5mM ALA for 4h. (C-D) Flow cytometry was used to detect the apoptosis in sebocytes. (E) The secretion of lipids in sebocytes were observed by fluorescence microscope after Nile

red staining. All the results were shown as mean \pm SD (n = 3), which were three separate experiments performed in triplicate. * = p < 0.05 significantly different from control.

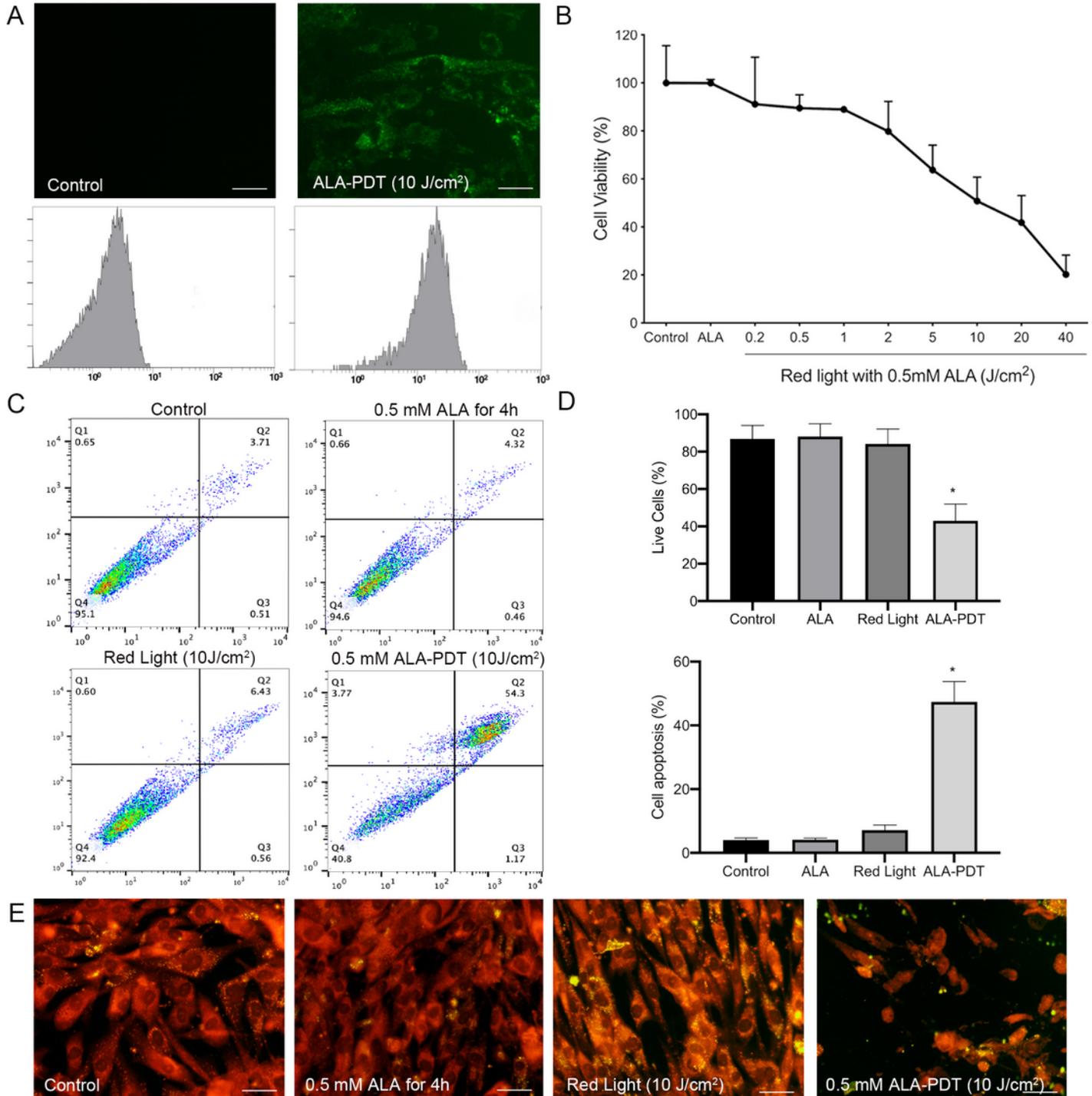


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sebocytes incubated with 0.5mM ALA for 4h. (C-D) Flow cytometry was used to detect the apoptosis in sebocytes. (E) The secretion of lipids in sebocytes were observed by fluorescence microscope after Nile red staining. All the results were shown as mean \pm SD (n = 3), which were three separate experiments performed in triplicate. * = p < 0.05 significantly different from control.

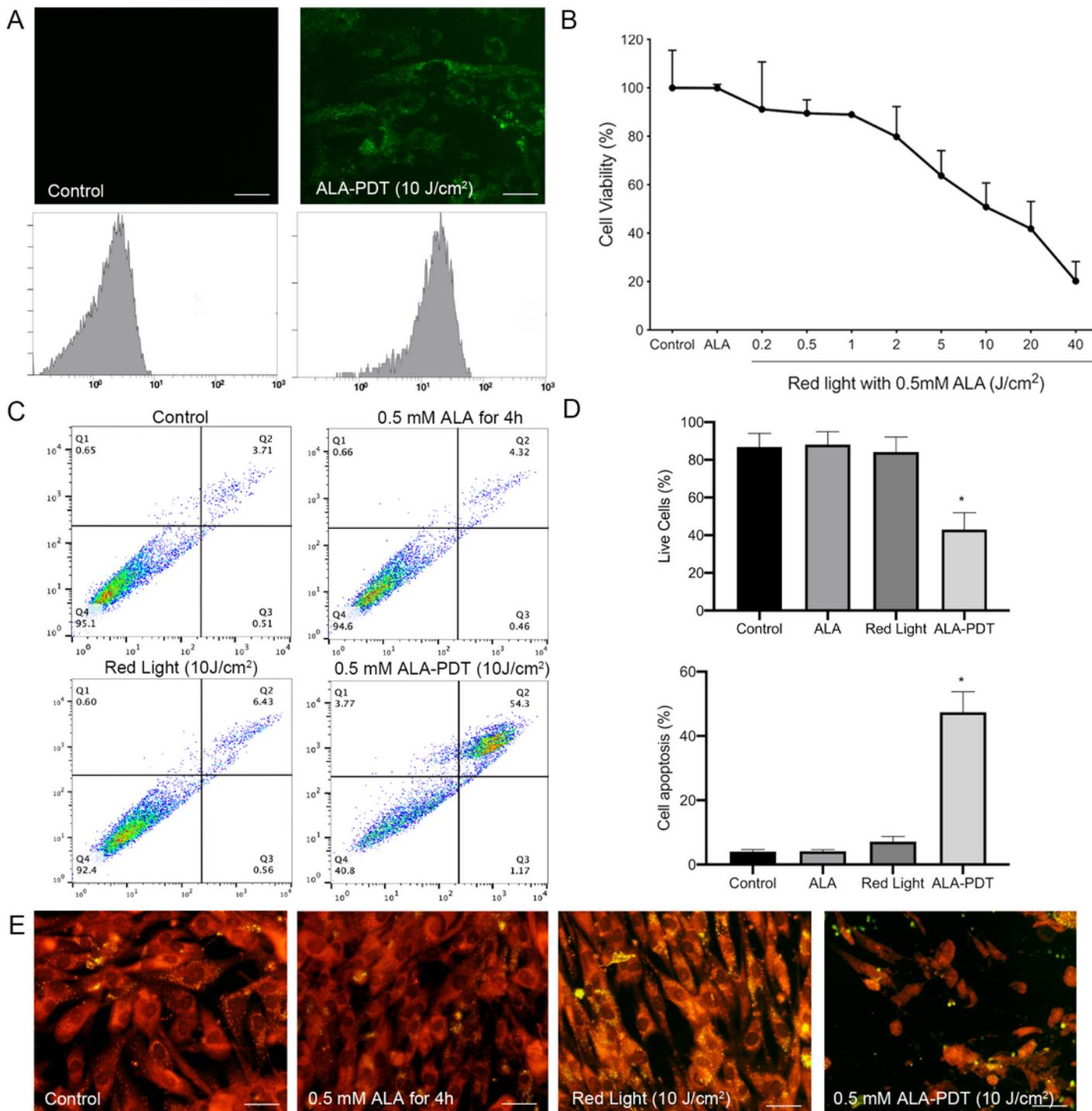


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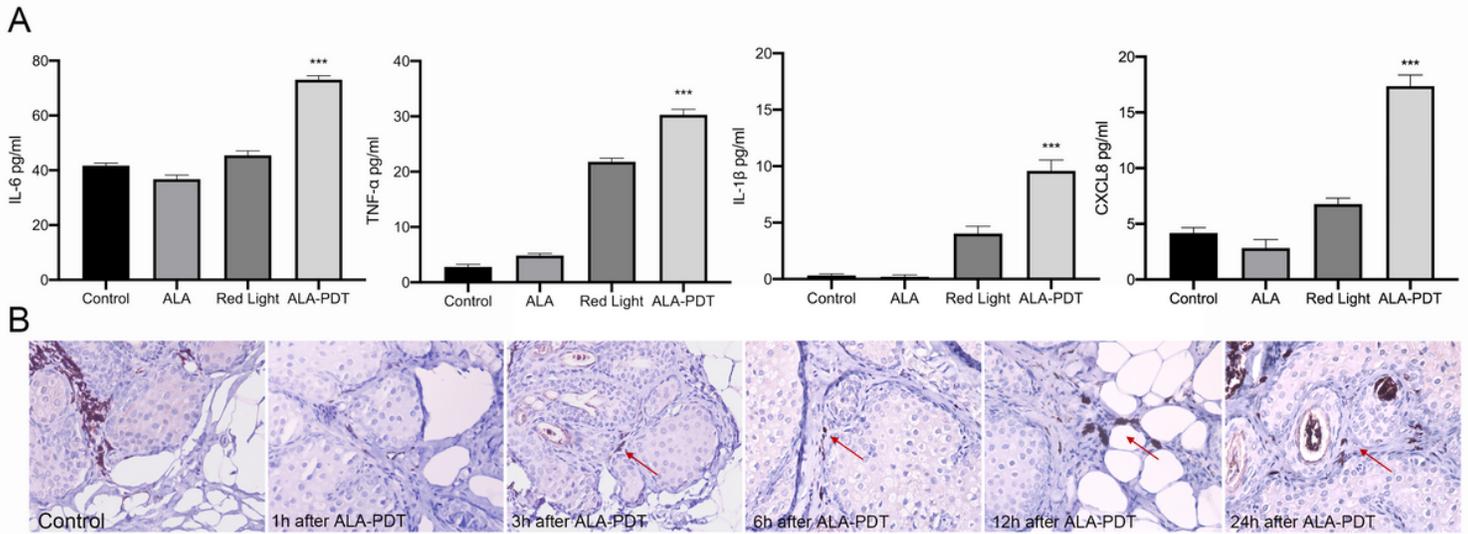


Figure 3

ALA-PDT increased the level of CXCL8 in sebocytes and sebaceous gland of golden hamster. Treated cells were collected for ELISA (A) 24h after ALA-PDT. (B) CXCL8 in sebaceous gland of golden hamster. Tissues were taken at 1h, 3h, 6h, 12h, 24 h after ALA-PDT and untreated tissues were used for comparisons. It was observed markedly increased expression of CXCL8. All the results were shown as mean \pm SD (n = 3), which were three separate experiments performed in triplicate. ***= p < 0.001 significantly different from control.

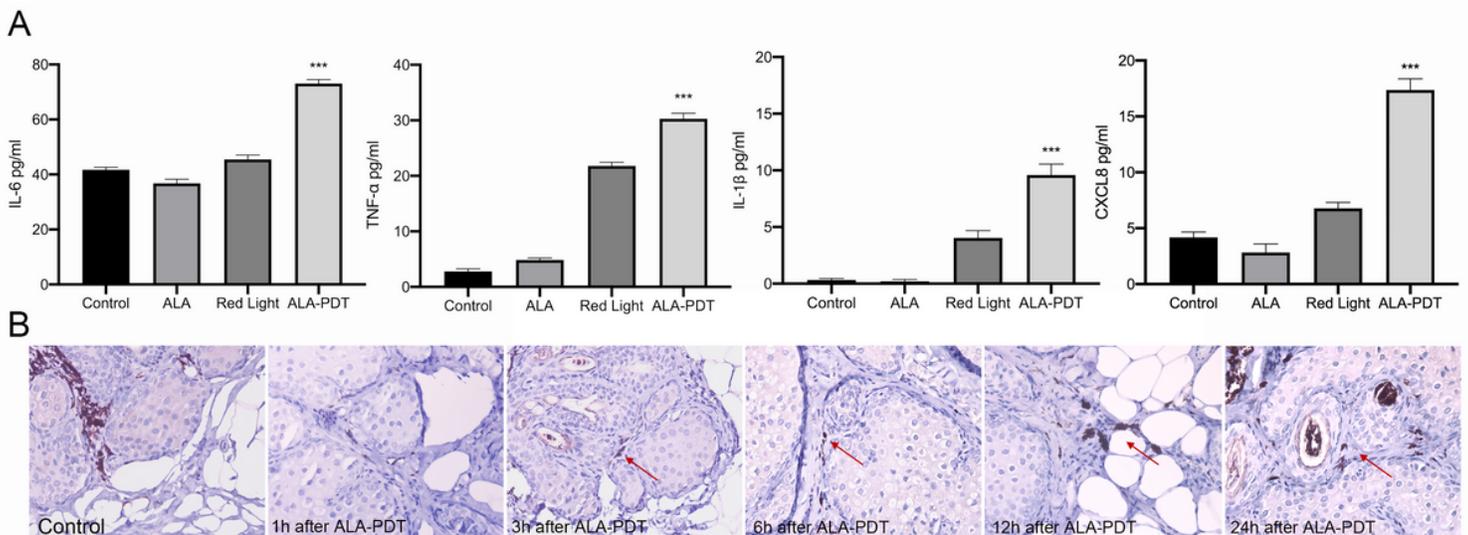


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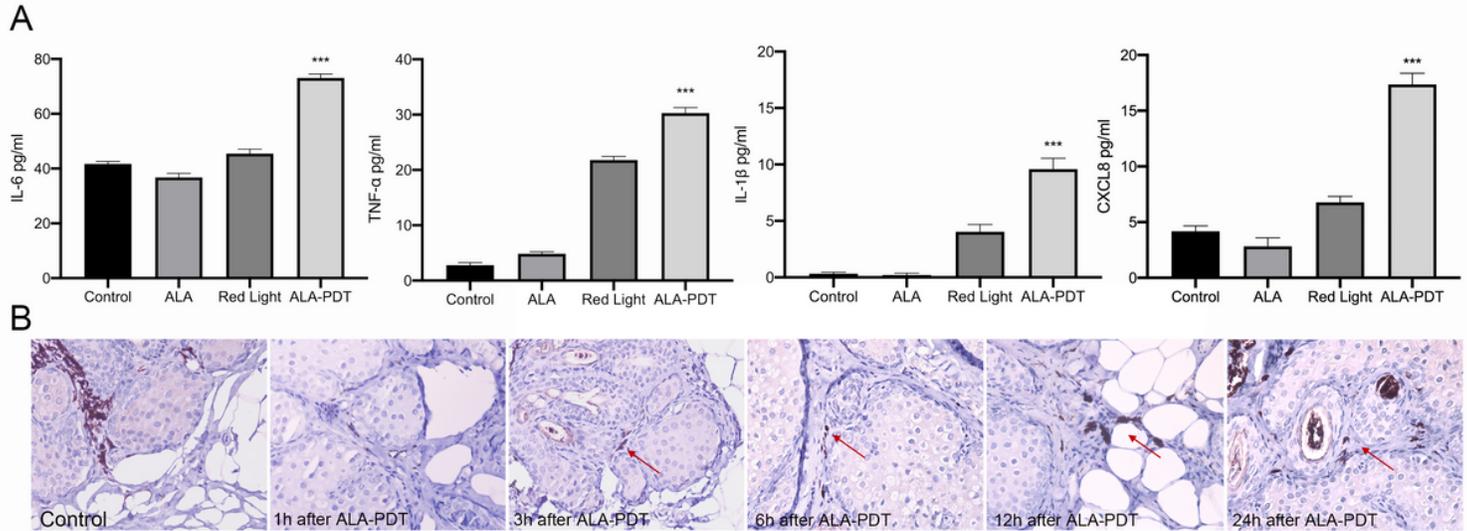


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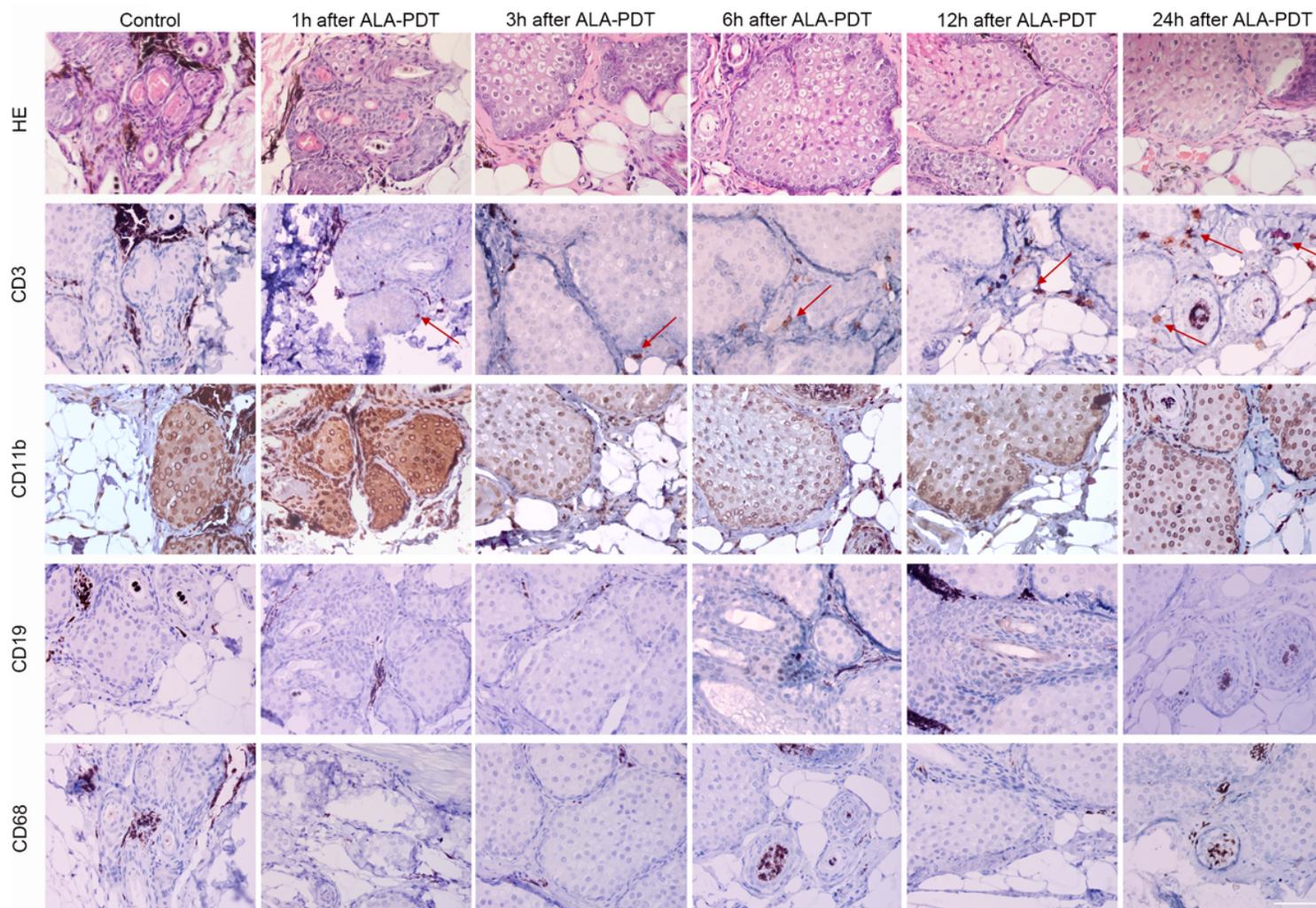


Figure 4

HE and immunohistochemical staining of T cell marker CD3, neutrophils marker CD11b, B cell marker CD19 and macrophages marker CD68 in sebaceous gland of golden hamster. Tissues were taken at 1h, 3h, 6h, 12h, 24 h after ALA-PDT and untreated tissues were used for comparisons. It was observed markedly increased expression of CD3 and CD11b, moderately increased expression of CD19 and CD68 from 3h after ALA-PDT.

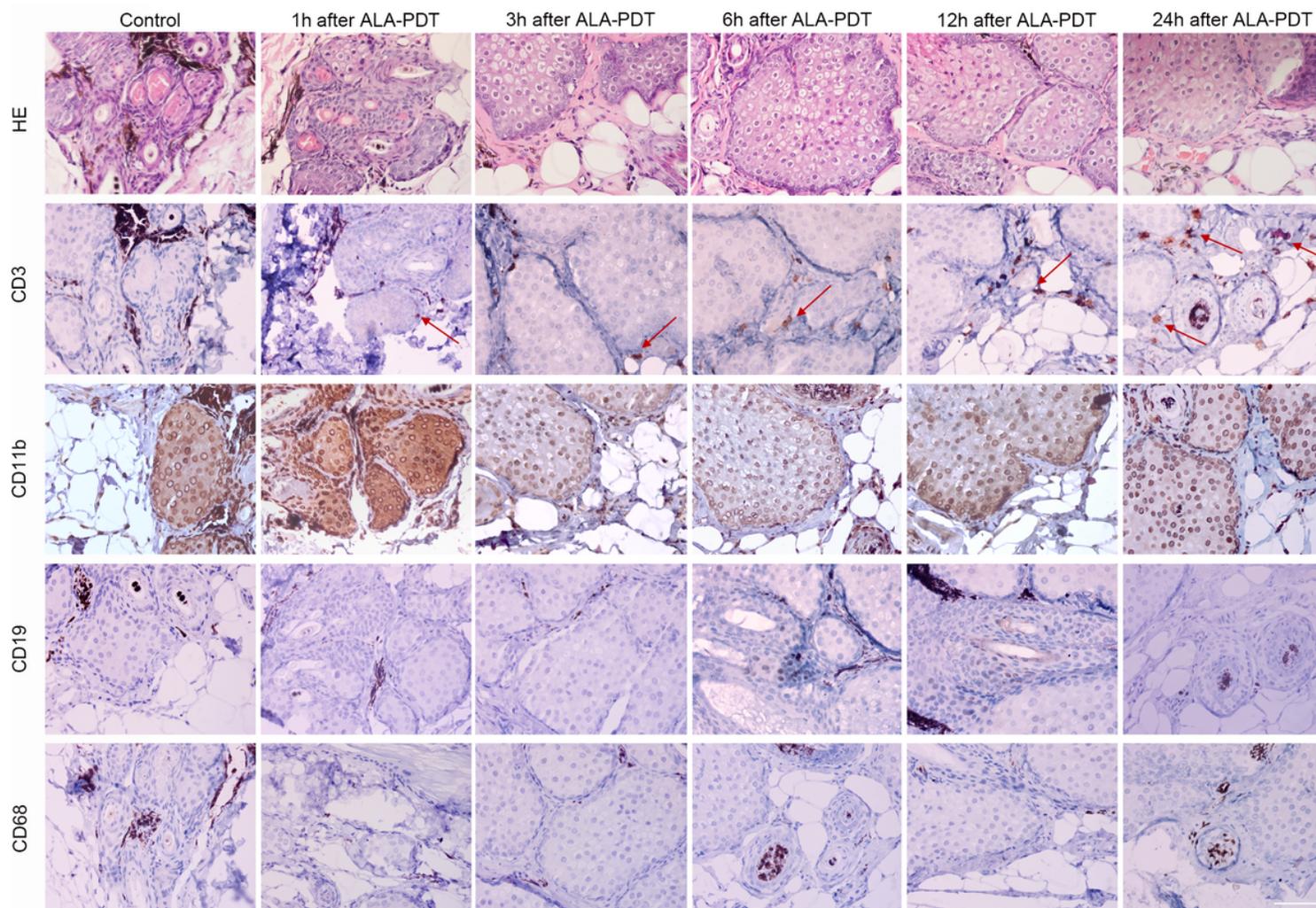


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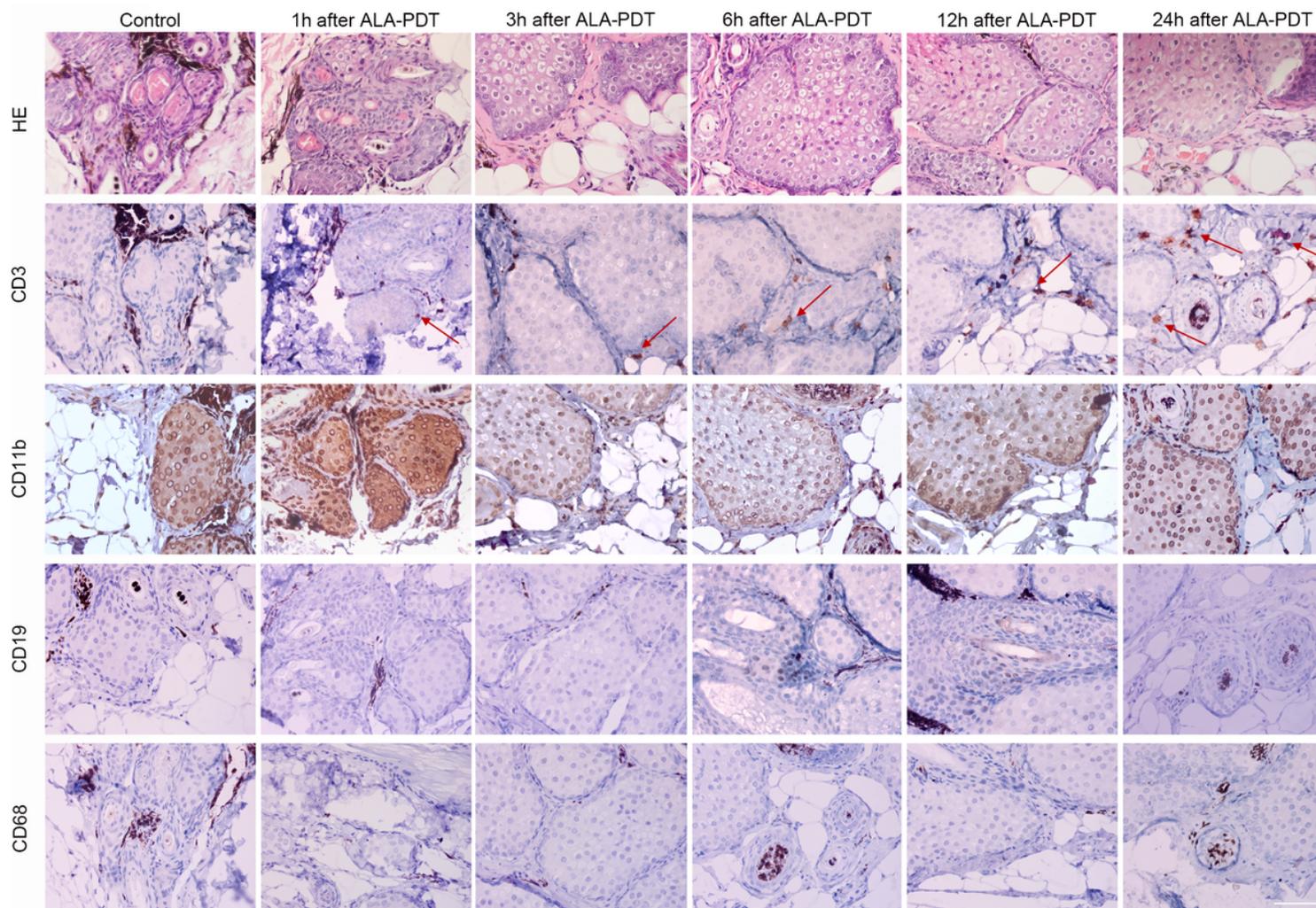


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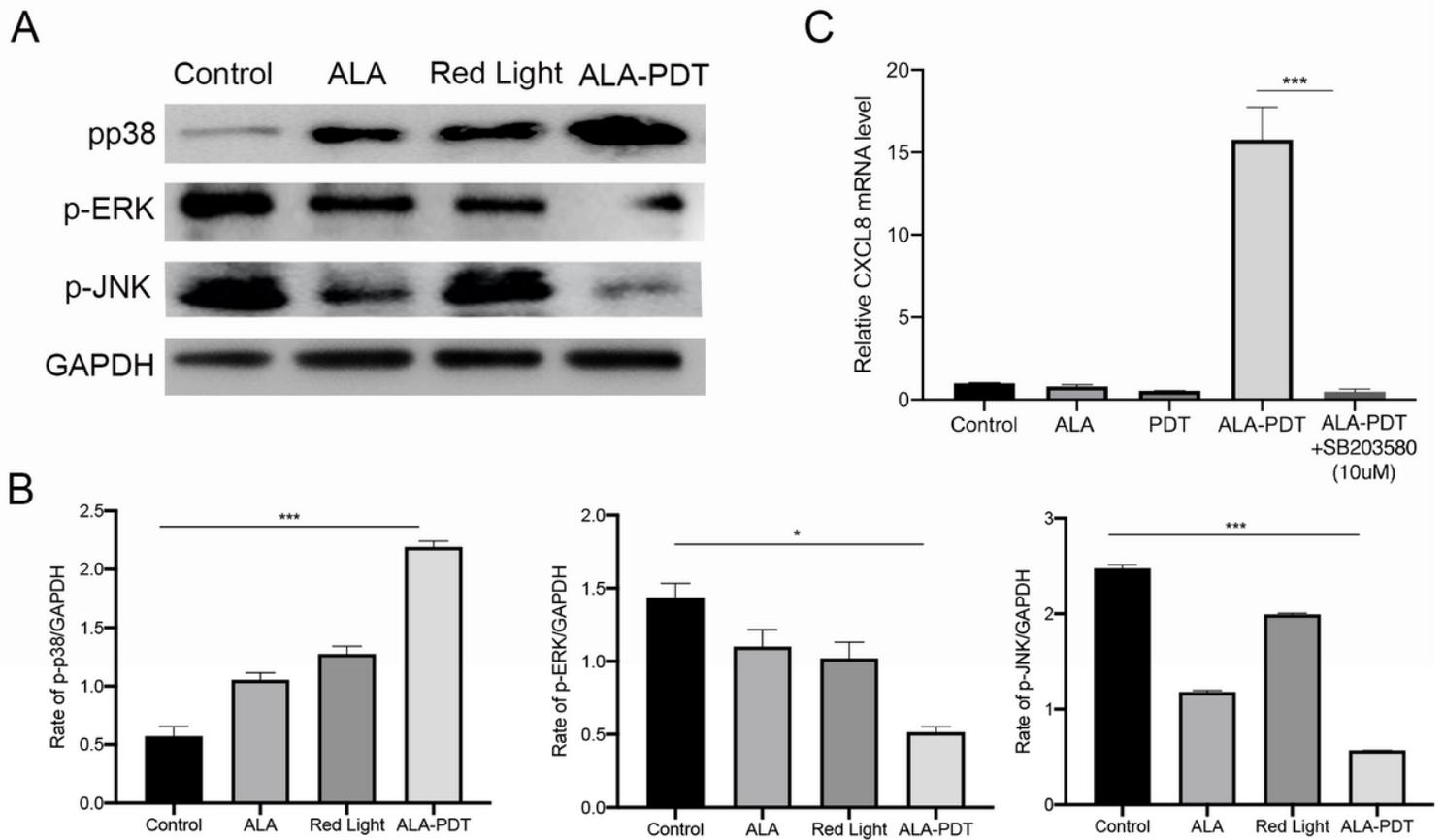


Figure 5

ALA-PDT activated p38 MAPK pathway and p38 inhibitor SB203580 inhibited the level of CXCL8 induced by ALA-PDT in sebocytes. (A) ALA-PDT activated MAPK pathway with up-regulation of pp38 and downregulation of p-ERK and p-JNK. (B) Quantification of the results from panel A. The protein ratios were determined by the relative intensities of the protein bands. (C) p38 inhibitor SB203580 inhibited the level of CXCL8 in sebocytes. Treated cells were collected for qRT-PCR 24h after ALA-PDT. All the results were shown as mean \pm SD ($n = 3$), which were three separate experiments performed in triplicate. *** = $p < 0.001$ significantly different from control.

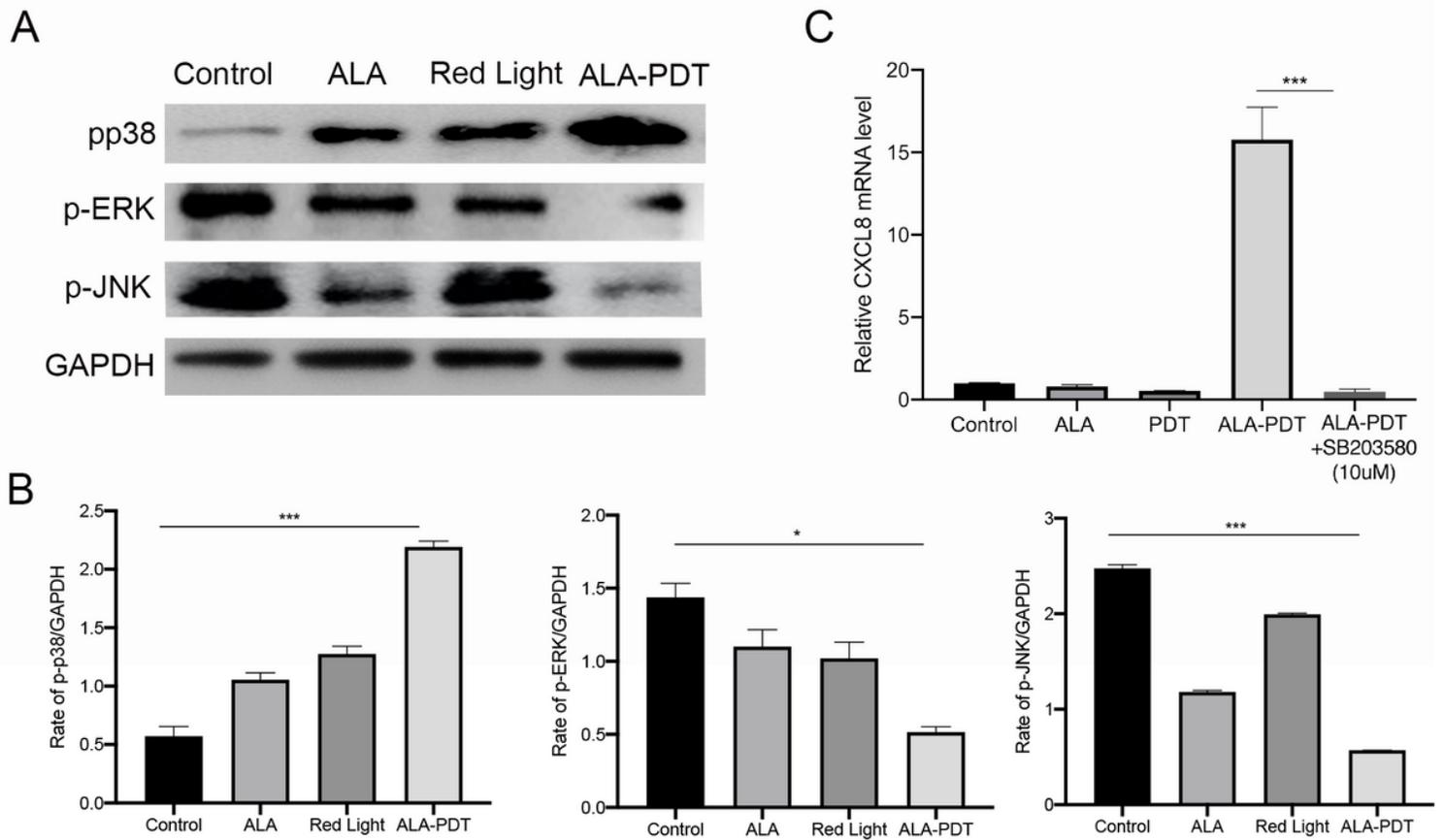


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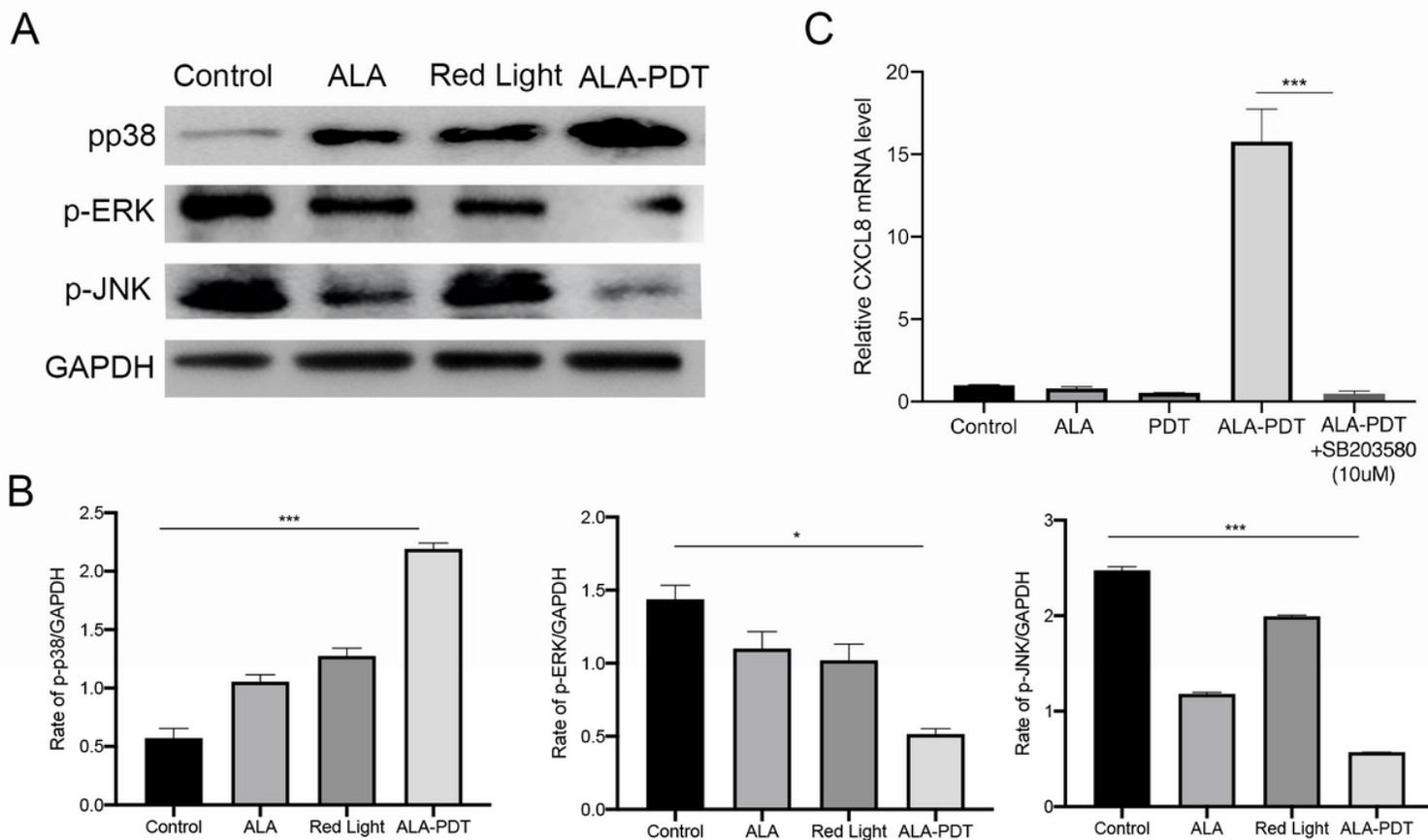


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