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CCL8 enhances sensitivity of cutaneous squamous cell carcinoma to photodynamic therapy by recruiting M1 macrophages

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ABSTRACT

Background: Antitumor immunity induced by photodynamic therapy (PDT) is believed to depend on the degree of local and systemic inflammation. The recruitment of leukocytes, in particular by the chemokine CCL8, to the sites of tissue damage has been strongly associated with the initiation of inflammatory reactions.
Objective: To evaluate whether and how CCL8 enhances the immune response against tumors in 5-aminolevulinic acid (ALA)-mediated PDT.
Methods: In this study, we investigated the effect of ALA-PDT-induced CCL8 expression on the recruitment and polarization of macrophages using immunohistochemistry, western blot and Transwell cell migration assay. We evaluated CCL8 expression following ALA-PDT *in vitro* and *in vivo* by using RT-PCR, western blot, and ELISA in clinical cutaneous squamous cell carcinoma (cSCC) samples, a mouse model of cSCC, tumor cells, and macrophages. The effect of the combination of ALA-PDT with CCL8 treatment on anti-tumor immunity was tested in the mouse model.
Results: We found that ALA-PDT enhanced CCL8 expression, increased the number of macrophages in tumor, and stimulated their M1 pro-inflammatory phenotype characterized by high expression levels of CD16 and CD80, low expression level of CD163, and absence of CD206 expression. Furthermore, CCL8 enhanced the effect of ALA-PDT on cSCC in mice, such a combination of CCL8 and ALA-PDT had a stronger positive effect in the treatment of mouse cSCC than PDT alone and suppressed tumor volume regrowth.
Conclusion: ALA-PDT induces CCL8 expression and recruits M1 macrophages, thus suppressing tumor growth.

1. Introduction

Cutaneous squamous cell carcinoma (cSCC) is an increasingly common skin cancer worldwide, particularly in the elderly [1–3]. Patients with invasive cSCC constitute a group under significant risk for tumor recurrence, impact on quality of life, and death [1,3]. Body's own immunosuppression is a key impact factor in each progression step of cancer, inducing favorable consequences for tumorigenesis [4]. Therefore, the most promising therapy for cSCC should stimulate body's own anti-tumor immunity to destroy tumors.

Photodynamic therapy (PDT) has emerged as a non-invasive or minimally invasive treatment modality for solid tumors, using a combination of light and photosensitizers to produce reactive oxygen species at the sites of photosensitizer accumulation, which directly cause photodamage and subsequent leading to target cell death [5,6]. PDT has been reported to have good effects on cSCC in situ and believed to be an excellent additional option for cSCC patients with special lesion locations, such as the head, face, and genitals [7]. In addition, it has been demonstrated that PDT-killed tumor cells stimulate strong anti-tumor immunity responses [8]. As a result, PDT-generated tumor vaccines have been developed and showed promising results in preclinical models [9,10]. Our previous studies indicated that topical 5-aminolevulinic acid-mediated PDT (ALA-PDT) induced rapid apoptosis and necrosis, inhibited cSCC growth, and caused tumor volume shrinkage in a mouse cSCC model [11,12]. Furthermore, we noted a marked increase in the numbers of dendritic cells, $CD4^+$ T cells, and $CD8^+$ T cells, as well as enhanced TNF-α expression in the tumor tissues after ALA-PDT [12]. More importantly, our previous experiments in an animal model revealed that ALA-PDT-induced apoptotic cSCC cells provided complete protection against cSCC cell challenge *in vivo* [13,14].Our studies also indicated that ALA-PDT causes immunogenic cell death of tumor cells by inducing damage-associated molecular patterns [14,15].

Moreover, it is now known that the long-term efficacy of PDT is strongly associated with host intact adaptive immune system [16–18].

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Numerous studies that examined the mechanisms of the PDT-generated antitumor immune response, suggested that the induction of this antitumor immunity relies on the extent of local and systemic inflammation [19–21].

The recruitment of leukocytes to damaged tissue plays an important role in the initiation of inflammatory responses. The process is tightly regulated by the chemokine superfamily, particularly by "inflammatory" chemokines. Representatives of the monocyte-chemoattractant protein (MCP) family have mainly proinflammatory activities [22]. CCL8/MCP-2, a member of the MCP family, is crucial for the recruitment of monocytes to the sites of inflammation [22,23]. Experiments in animals have confirmed that CCL8 exhibits antitumor/antimetastatic effects in a subcutaneous transplantation model of B16F10 melanoma [23]. It has been reported that CCL8 expression stimulated migration of monocytes and T lymphocytes and induced anti-tumor response [22–24]. However, whether and how CCL8 affects the immune response against tumors during the treatment of ALA-PDT for cSCC remain unknown.

Macrophages are a type of antigen-presenting cells that play a prominent active role in the clearance of dying and dead cells by their inherent phagocytic and cytotoxic properties. It has been demonstrated that macrophages can work as both immune activators and negative regulators of immune responses. Tumor-associated macrophages (TAMs) are key prototypic components of the tumor microenvironment, which is crucial for inflammatory reactions promoted by tumors. The high concentration of TAMs is closely related to the poor prognosis of many malignant tumors in humans [25,26]. Therapeutic strategies targeting the opposite sides of the macrophage balance should therefore take into account the dual potential of these cells [23]. TAMs play an important role from early carcinogenesis to tumor progression including metastasis by modulating immune responses. TAMs may have two distinct phenotypes, M1 and M2. M1 macrophages are mainly regarded as tumor-killer cells; they predominantly exert anti-tumor and pro-inflammatory activity by secreting cytokines such as IL-6. In contrast, M2 subpopulation promotes tumor growth by producing cytokines such as IL-10 [26,27]. The role of macrophage recruitment, activation, and polarization in tumor treatment by ALA-PDT has not been analyzed in detail.

Here, we studied the effects of CCL8 during ALA-PDT for cSCC. We specifically focused on the effects of ALA-PDT on macrophage recruitment and polarization, and the role of CCL8 induction by ALA-PDT in this setting. We also explored whether CCL8 can enhance the effect of ALA-PDT against cSCC tumors in mice. Here we present our findings that ALA-PDT induced the expression of the CCL8, which amplified anti-tumor action of ALA-PDT via recruiting M1 macrophages.

2. Materials and methods

2.1. Animals and cell lines

Female, hairless, immunocompetent SKH-1 mice (6–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). In this study, we used two mouse models, in which cSCC was induced either by UV irradiation or by implantation of cSCC cells. For the former, tumors were induced by solar-simulated ultraviolet irradiation (Solar UV Simulator, SIGMA, Shanghai, China) as described previously [12]. Murine cSCC cell line XL50, established from UV-induced cSCC in SKH-1 hairless mice, is stored at the China Center for Type Culture Collection (CCTCC No. C201827, Wuhan, China). For the cSCC model induced by implantation, 5×10^6 XL50 cells were injected subcutaneously into the back of SKH-1 mice.

Murine cSCC PECA cell line and murine macrophage RAW264.7 cell line were also used in this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA USA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA USA), penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}) (Gibco, Carlsbad, CA USA), at 37 °C in the atmosphere of 95% air and 5% CO_2.

2.2. Reagents

ALA hydrochloride powder was from Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Co, Ltd. (Shanghai, China). The following reagents and commercially available assay kits were used: mouse anti-CD80 antibody (60460-1-Ig, Proteintech, Rosemont, USA), rabbit anti-CD206 antibody (ab64693, Abcam, Cambridge, MA, USA), anti-CD16 antibody (ab203883, Abcam, Cambridge, MA, USA), anti-CD163 antibody (ab182422, Abcam, Cambridge, MA, USA), mouse monoclonal anti-CCL8/MCP-2 antibody for neutralization or western blot (ab10391, Cambridge, MA, USA), recombinant murine CCL8/MCP-2 (Lot#120196, Peprotech, Rocky Hill, USA), and mouse CCL-8/MCP-2 ELISA kit (LS-F2848, LifeSpan BioSciences, WA, USA).

2.3. ALA-PDT treatment

For the *in vitro* study, PECA cells or RAW264.7 cells were incubated with ALA (0.5 mM) in serum-free medium for 5 h at 37 °C, then washed twice with phosphate buffered saline (PBS) and exposed to a red LED light at 633 nm at 10 mW/cm^2, the total light dose was 0.5 J/cm^2.

For the *in vivo* study, twelve mice with ultraviolet light (UV)-induced cSCC were divided into two groups: untreated (n = 3) and those undergoing ALA-PDT (n = 9). In mice from the ALA-PDT group, 8% ALA cream was topically applied onto the tumor surface. After 3 h of incubation in the dark, excess ALA cream was removed, and the tumors were irradiated by a red LED light (633 nm) at a power density of 100 mW/cm^2 and energy density of 30 J/cm^2. Samples were obtained at 6 h, and 12 h, and 24 h after ALA-PDT (n = 3 each group).

In the human study, three patients (two females, 78 and 85 years old; one male, 91 years old) diagnosed with invasive cSCC received ALA-PDT treatment. First, 20% ALA cream was applied to the lesion with 2 cm margins for 3 h. Then, the lesions were irradiated by 126 J/cm^2 red LED light (633 nm) at 100 mW/cm^2. Tissue samples were obtained before the treatment as well as in 3 h and 6 h after the treatment for subsequent RT-PCR analysis. The study protocol and informed consent were approved by the research ethics committee of the Shanghai Skin Diseases Hospital (The approval number: 2016-06). All subjects were fully informed about the study and written consent was obtained from all participants.

2.4. RT-PCR

Total RNA of tissue samples from UV-induced cSCC in mice and from human clinical samples was harvested using TRIzol (Invitrogen Corp.) according to the manufacturer's instruction and reverse transcribed to complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher, Loughborough, UK). 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). CCL8, CD16, CD32, and CD206 mRNA levels were detected by SYBR Green qPCR (Life Technologies) using the following primers: 5′-CCA TTC CAA TCA CCT GCT GCT T-3′ (human CCL8 forward primer), 5′-CCA GAT AAG GCT CCA GTC ACC-3′ (mouse CCL8 forward primer), 5′-TGT TCC TGG AGC CTC AAT GGT-3′ (human CD16 reverse primer), 5′-TCT CCA TCC CAC AAG CAA ACC A-3′ (human CD32 forward primer), 5′-TTG GAC GGA TGG ACG AGG A-3′ (human CD206 reverse primer). GAPDH mRNA level was used as control.

2.5. Quantification of CCL8 release

In order to evaluate CCL8 release, PECA cells, RAW264.7 cells, and co-cultured PECA and RAW264.7 cells were cultured in six-well tissue culture plates and treated with ALA-PDT. After centrifugation, the

supernatants were collected at 12 h post-treatment and analyzed using an ELISA-based CCL8 detection kit according to the manufacturer's instructions.

2.6. Cell migration assays

Matrigel-coated Transwell inserts were used for monitoring cell migration. PECA cells in 0.5 mL of serum-free DMEM (200,000 cells/well) were added into the lower chamber and either treated by ALA-PDT or left untreated. Pure DMEM was used as negative control, whereas DMEM containing 50 μg/mL recombinant murine CCL8 was used as positive CCL8 control. The blocking or enhancing agents, i.e., an anti-CCL8 neutralizing antibody or recombinant CCL8, were added to PECA cells that underwent ALA-PDT. Then, RAW264.7 cells were plated onto the upper side of the Transwell inserts (500,000 cells/well) in 0.1 mL of a serum-free DMEM. After 12 h, the non-invading cells on the upper surface of the membrane were removed by wiping, and the invading cells were fixed and stained with hematoxylin. The number of invading or migrating cells was counted under a microscope in five predetermined fields for each membrane at ×400 magnification.

2.7. Immunohistochemical studies

Freshly isolated tissue from UV-induced cSCC lesions was obtained in 24 h from mice that received the treatment or were left untreated. The tissue was stored in formalin and 5 μm sections were de-waxed (30 min 56 °C, 2 × 10 min xylene), followed by rehydration, antigen unmasking, and blocking. Then, the samples were stained with anti-CD16, anti-CD80, anti-CD163, and anti-CD206 primary antibodies at 1 μg/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.

2.8. Western blot

The regulation of macrophage polarization by ALA-PDT was inferred from changes in CD16, CD80, CD163, and CD206 protein levels. At designated time point (6 to 24 h) after ALA-PDT or following incubation with the supernatants of PECA cells that underwent ALA-PDT for different periods (6 to 24 h) for 24 h, RAW264.7 cells were collected and lysed with radioimmunoprecipitation assay buffer (Tris base 50 mM, NaCl 150 mM, NP40 1%, sodium deoxycholate 0.25%, EDTA 1 mM) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (pH 7.4) containing 0.1% (v/v) Tween 20 (TBST) and then incubated with primary antibodies against CD16, CD80, CD163, and CD206 overnight at 4 °C. The membranes were then washed three times (15 min each) with TBST and incubated with a secondary antibody conjugated with alkaline phosphatase (1:2000) for 1 h at room temperature. The color reaction was developed using *p*-Nitro-Blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma).

2.9. Detection of CCL8 influence on the effectiveness of ALA-PDT in cSCC

Forty mice were injected with cSCC cells and developed cSCC tumors. When tumors were about 5–7 mm in diameter, the mice were randomly divided into four groups: ALA-PDT, ALA-PDT + anti-CCL8, ALA-PDT + CCL8, and control group (10 mice per group). Treatment of mouse cSCC with ALA-PDT was performed as described in detail above. In ALA-PDT + anti-CCL8 and ALA-PDT + CCL8 groups, before ALA-PDT, fine PBS suspensions containing, respectively, a neutralizing antibody against CCL8 or recombinant CCL8, were injected intratumorally (volume 50 μL) at a dose of 0.5 mg/mouse. All treatments were repeated three times at weekly intervals. To assess treatment efficacy, baseline tumor volume was measured and digital photographs were taken. After the third treatment, tumor volume was measured every other day and photographs were taken every third day. The maximal and minimal diameters of the tumor were measured with a precision electronic caliper, and tumor volume was calculated by using the following formula:

$V = ab^2/2$, where V indicates volume, "a" is the maximum diameter, and "b" is the minimum diameter.

2.10. Tumor volume regrowth

Twenty mice with implanted cSCC that had tumors of about 5 mm in diameter were randomly divided into four groups (5 mice per group). The four groups were named as described above: ALA-PDT, ALA-PDT + anti-CCL8, ALA-PDT + CCL8, and control. Mice in all the groups received similar treatment as mentioned above but only once. After the treatment, tumor volume was measured every other day for 28 days, and the results were compared between different groups of mice.

2.11. Histopathology

Twenty mice with implanted cSCC that had tumors of about 5 mm in diameter were randomly divided into four groups: ALA-PDT, ALA-PDT + anti-CCL8, ALA-PDT + CCL8, and control (five mice per group). Treatments were performed only once. Tissues from treated tumor sites were obtained at 1 week after ALA-PDT. The tumor specimens were fixed with a 10% formalin solution, embedded in polyester wax, and sectioned at 5 μm. The sections were subjected to hematoxylin and eosin staining.

2.12. Statistical analysis

Data are presented as the mean ± standard deviation, unless otherwise specified. Data were analyzed with GraphPad Prism 5 software. All statistical analyses were performed using the Student's *t*-test with a significance level of $\alpha = 0.05$ ($P < 0.05$).

3. Results

3.1. Induction of CCL8 expression by ALA-PDT

To determine the effect of ALA-PDT on mRNA expression of CCL8, tissues taken from clinically treated cSCC tumor sites in 3 h and 6 h after ALA-PDT as well as mouse cSCC model tumor samples collected in 6 h and 12 h after ALA-PDT were examined by PT-PCR. mRNA levels of CCL8 in untreated tumor tissues were used as control. As shown in Fig. 1A, ALA-PDT elevated CCL8 mRNA expression in both clinical cSCC patient samples and cSCC mouse model samples ($P < 0.05$).

To explore the sources of CCL8, the mouse cSCC cell line PECA and mouse macrophage cell line RAW264.7 were used in this study. As shown in Fig. 1B, the expression of intracellular CCL8 in PECA, RAW264.7, and co-cutured PECA and RAW264.7 cells was also markedly increased at 12 h after ALA-PDT. CCL8 was detected in cell culture supernatant at 12 h after ALA-PDT, using CCL8 ELISA (Fig. 1C). In accordance with this observation, we observed that ALA-PDT stimulated the release of CCL8 in both PECA and RAW264.7 cell lines ($P < 0.05$).

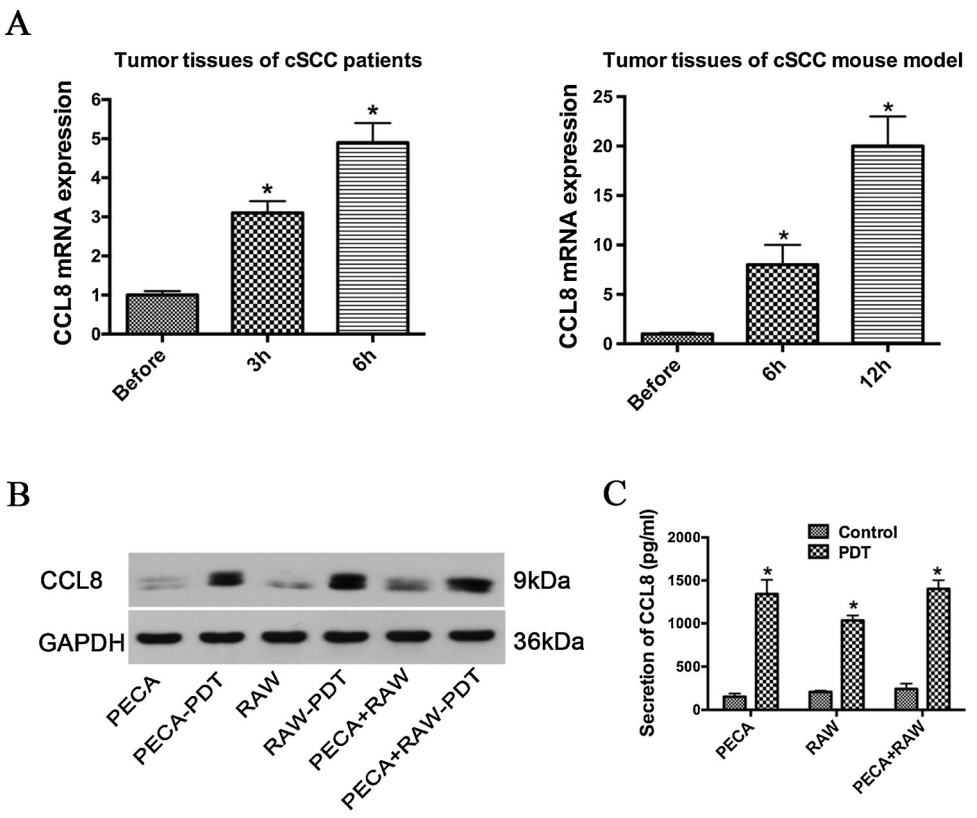

Fig. 1. Induction of CCL8 expression by ALA-PDT. (A) Tissues taken from clinically treated cSCC sites at 3 h and 6 h after ALA-PDT as well as mouse model cSCC samples collected at 6 h and 12 h after ALA-PDT were examined by RT-PCR, with tumor tissues berore treatment used for control (n = 3). ALA-PDT elevated CCL8 mRNA expression in both clinical cSCC patient samples and cSCC mouse model samples. (B) Expression of intracellular CCL8. Mouse cSCC cell line PECA, mouse macrophage cell line RAW264.7 and co-cultured PECA and RAW246.7 were treated by ALA-PDT, and CCL8 expression was analyzed by Western blot at 12 h after ALA-PDT, with cells of no treatment used for negative control. Intracellular CCL8 in PECA and RAW264.7 cells was markedly increased after ALA-PDT. (C) Extracellular release of CCL8. PDT-stimulated release of CCL8 from PECA cells, RAW264.7 cells, PECA + RAW264.7 cells at 12 h after ALA-PDT using ELISA assay. ALA-PDT stimulated the release of CCL8 in both PECA and RAW264.7 cell lines. Statistical analysis was performed by *t*-test; $^*p < 0.05$. Means ± SD are shown from independent experiments.

3.2. ALA-PDT increases the number of tumor macrophages and confers M1 pro-inflammatory phenotype on them

To confirm that ALA-PDT induced macrophage infiltration and phenotypic changes, expression levels of the M1 markers CD16, CD32, and CD80 as well as those of M2 markers D163 and CD206 were determined. Higher expression levels of the genes encoding CD16, CD32 and CD206 were detected by RT-PCR in clinical cSCC patient samples at 3 h and 6 h after ALA-PDT. Compared with their levels in untreated cSCC tissue, CD16, CD32 and CD206 expression levels were significantly increased after ALA-PDT at the two time points, as shown in Fig. 2A ($P < 0.05$). The M1/M2 ratio judged from the CD16/CD206 ratio was significantly increased at 6 h after PDT, whereas CD32/CD206 was significantly increased at 3 h after PDT ($P < 0.05$).

Then, we examined TAMs infiltration in a mouse model of cSCC induced by UV radiation. Tissues were taken from treated tumor sites in 24 h after ALA-PDT and untreated tumor tissues were used for comparisons. Immunohistochemistry methods were employed to observe the expression of CD16, CD80, CD163 and CD206 in tumors treated by ALA-PDT. As shown in Fig. 2B, positive staining for CD16 and CD80 was observed after ALA-PDT, but not in the untreated tumor tissue. CD163 expression slightly increased after ALA-PDT. No marked increase in CD206 expression was seen (Fig. 2B).

To investigate re-polarization of TAMs during the emergence of M1 phenotype by ALA-PDT, CD16, CD80, CD163, CD206 expression in the macrophage cell line RAW264.7 was analyzed by western blot. We found that expression levels of CD16, CD80, CD163, CD206 of macrophage markedly decreased in 6 h, 12 h, and 24 h after ALA-PDT, compared with those in untreated cells. However, when co-cultured with PDT-treated PECA supernatants between 6 h–24 h, CD16 and CD80 expression levels increased considerably, whereas the expression of CD163 and CD206 decreased (Fig. 2C).

3.3. Effect of CCL8 on the migration of macrophages

Cell migration ability was measured using the Transwell cell migration assay. Macrophages were cultured with recombinant CCL8 or PECA cells treated by ALA-PDT or untreated PECA cells. Untreated macrophages were used as negative control. To inhibit or stimulate CCL8 function, a neutralizing antibody against wild-type CCL8 or recombinant CCL8 was added to ALA-PDT treated PECA cells, respectively. RAW264.7 cells were allowed to migrate for 12 h. As shown in Fig. 3, macrophages cultured with ALA-PDT-treated tumor cells had enhanced migration abilities compared with migration of control cells or macrophages cultured with untreated tumor cells. When CCL8 was blocked, macrophage migration ability was significantly suppressed. However, adding recombinant CCL8 increased the number of migrating cells. These results indicate that CCL8 plays a pivotal role in macrophage infiltration induced by ALA-PDT.

3.4. CCL8 enhances the effect of ALA-PDT on cSCC

To test the role of CCL8 in ALA-PDT of cSCC *in vivo* and to determine whether CCL8 enhances the effect of ALA-PDT, SKH-1 mice were injected with viable XL50 cells and developed cSCC subsequently. The tumors in ALA-PDT group were topically treated with 8% ALA cream for 3 h and then irradiated by LED red light at a dose of 30 J/cm^2. In ALA-PDT + anti-CCL8 group and ALA-PDT + CCL8 group, a neutralizing antibody against CCL8 or recombinant CCL8 was injected intratumorally before ALA-PDT, respectively. All treatments were repeated three times with 1-week intervals. Tumor volume regressed gradually and complete clearance of tumors was observed in the ALA-PDT + CCL8 group after 21 days of treatment (Fig. 4). In contrast, tumor volume in other three groups increased in size and the control group had the largest tumor (> 1000 mm^3). After 21 days, tumor volumes in ALA-PDT + anti-CCL8 and ALA-PDT groups were about 700 mm^3 and 300 mm^3, respectively.

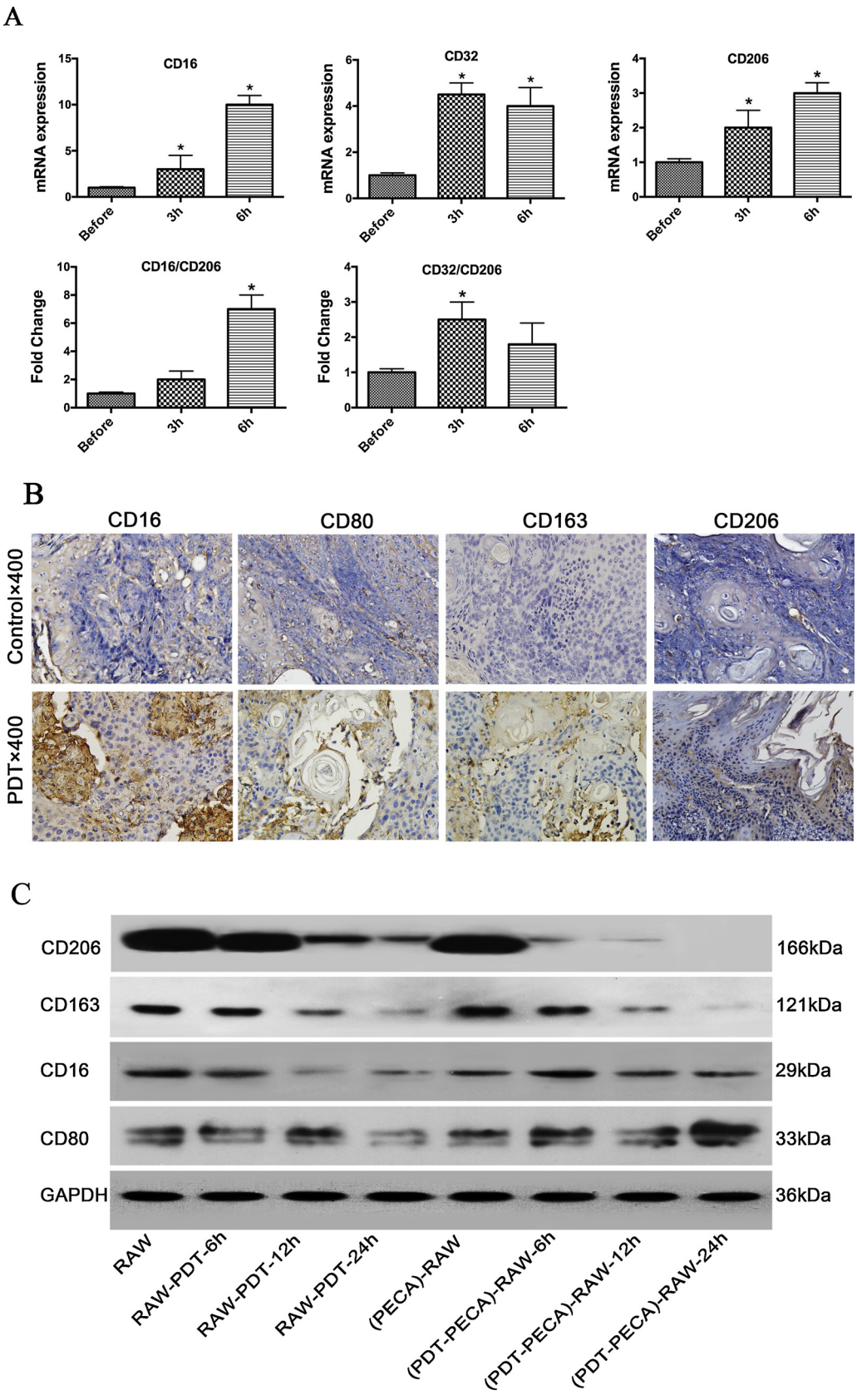

Fig. 2. ALA-PDT induced macrophage infiltration and phenotypic changes. (A) Expression of the M1 markers CD16, CD32, and M2 marker CD206 in cSCC. Clinical cSCC tissues were collected at 3 h and 6 h after ALA-PDT, mRNA expression of the CD16, CD32, and CD206 were determined by RT-PCR, with tumor tissues berore treatment used for control (n = 3). CD16, CD32 and CD206 expression levels as well as CD16/CD206, CD32/CD206 was significantly increased after ALA-PDT. Statistical analysis was performed by *t*-test; $^*p < 0.05$. (B) Representative immunohistochemical staining of M1 markers CD16, CD80, and M2 marker CD163 and CD206 in mouse cSCC model. Tissues were taken from treated tumor sites at 24 h after ALA-PDT and untreated tumor tissues were used for comparisons. It was observed markedly increased expression of CD16, CD80, moderately increased expression of CD163, and no expression of CD206 after ALA-PDT. (C) Expression of CD16, CD80, CD163, and CD206 in macrophage cell. RAW264.7 cells were treated by ALA-PDT or co-culture with the supernatant of ALA-PDT-treated PECA cells, and Western blot was performed at different time points (6 h to 24 h) after treatment. ALA-PDT-treated PECA cells had ability to stimulate RAW264.7 cells to exhibit a distinct M1 phenotype characterized by high expression levels of CD16 and CD80, low expression level of CD163, and absence of CD206 expression.

3.5. CCL8 suppresses cSCC volume regrowth

To detect the tumor-suppressive effect, tumor volume regrowth was assessed after a single treatment. After initial regression, tumor volume curves showed faster regrowth of the tumor in ALA-PDT + anti-CCL8 and ALA-PDT groups compared to that in ALA-PDT + CCL8 group. The comparison of tumor volume regrowth between ALA-PDT + anti-CCL8 and ALA-PDT groups showed that tumors grew faster and larger in the

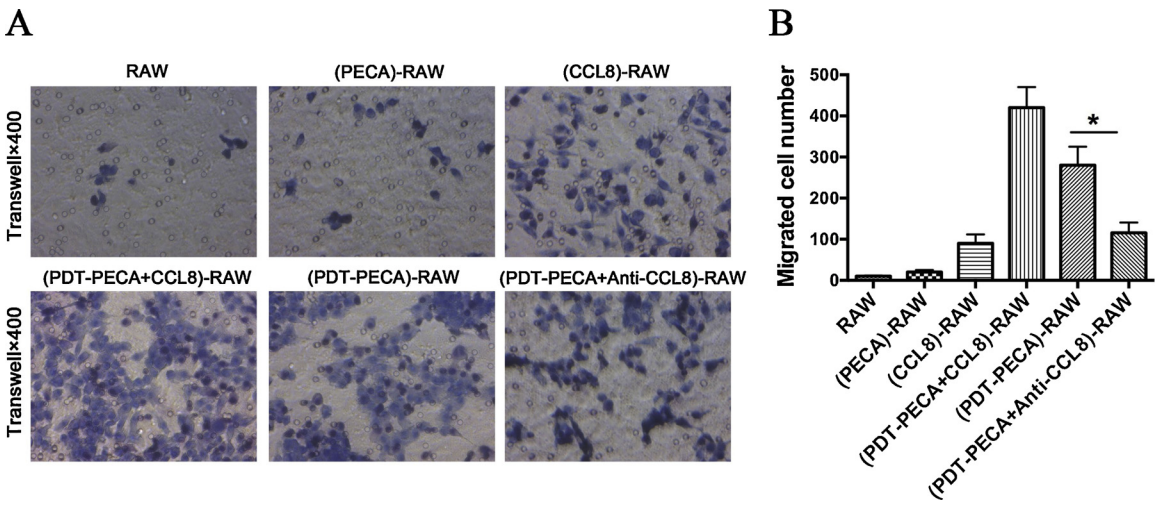

Fig. 3. Effect of CCL8 on migration of macrophages. (A) RAW264.7 cells were co-cultured with recombinant CCL8, untreated PECA cells (PECA), PECA cells treated by ALA-PDT (PDT-PECA), PDT-PECA plus recombinant CCL8 or CCL8 neutralizing antibody, untreated RAW264.7 cells were uses as control. Cell migration ability was measured using a Transwell cell migration assay. (B) Quantitative analysis of migrated RAW264.7 cells. Data are expressed as means ± SD, n = 3; $^*p < 0.05$.

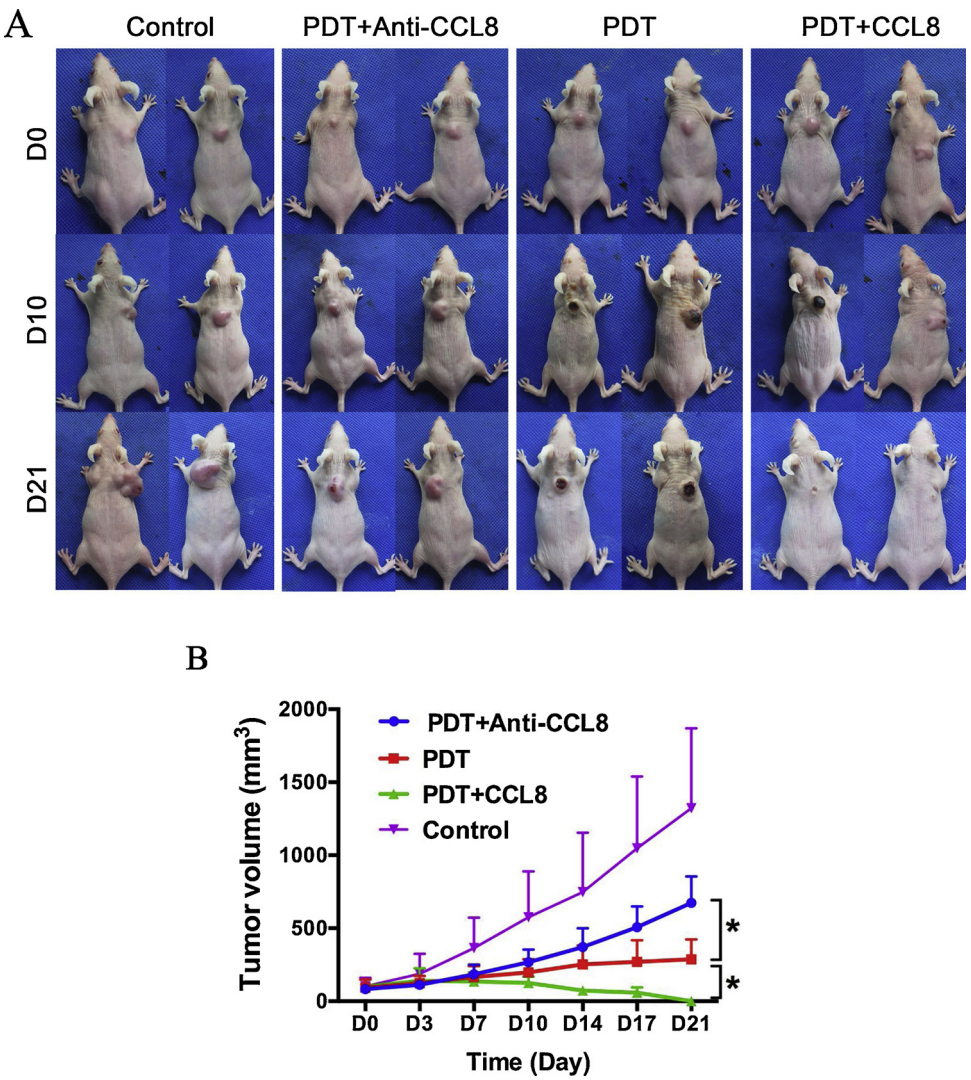

Fig. 4. Inhibition of cSCC growth in mice treated with ALA-PDT combined with CCL8. (A) Mice pictures demonstrating complete clearance of cSCC in PDT + CCL8 group after third treatment while tumor in other group grew bigger in size. (B) Tumor growth curve of different groups. Data are expressed as means ± SD, n = 5.

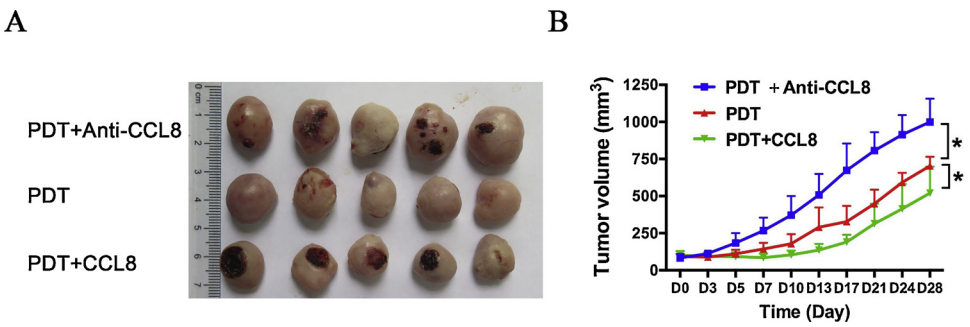

Fig. 5. Tumor volume regrowth after the treatment performed only once. **(A).** Tumor in PDT group grew bigger than that in PDT + CCL8 group, and smaller than that in PDT + Anti-CCL8 group ($p < 0.05$), 28 days after treatment. (B) Volume regrowth curve after initial regression showed faster growth of cSCC in PDT group when compared to PDT + CCL8 group, and slower when compared to PDT + Anti-CCL8 group. Data are expressed as means ± SD, n = 5; *$p < 0.05$.

former group (Fig. 5) ($P < 0.05$).

3.6. CCL8 possesses pro-inflammatory effect

Histological analyses revealed that cSCC tumors in control mice contained atypical squamous cells. There were no marked differences in cell morphology between ALA-PDT + anti-CCL8 group and control group. Many immune cells were seen in cSCC tissues that underwent ALA-PDT; they mainly accumulated between the dermis and epidermis. In the ALA-PDT + CCL8 group, the tumor tissue after treatment was surrounded by a large number of immune cells and dead tumor cells, as the original tumor cell structure was lost (Fig. 6).

4. Discussion

Inflammation is believed to play an important role in tumorigenesis [23]. Many causes and risk factors of cancer are associated with some form of chronic inflammation, such as cSCC. Chronic inflammation has been demonstrated to be associated with increased incidence of malignancy and those two processes share the regulatory mechanisms. The infiltration of innate immune cells, such as macrophages, into tumors promotes tumor development by various mechanisms. Conversely, the elimination of early malignant lesions, which relies on the cytotoxic activity of tumor-infiltrating T cells, is thought to be rate-limiting for the risk of developing cancer [27].

ALA-PDT is a noninvasive or minimally invasive effective method for the treatment of tumors. Given its advantages, such as better aesthetic outcomes, low morbidity, minimal functional disturbance, and the ability to be used topically or repeatedly [28], ALA-PDT is increasingly used for skin tumors. It has been shown that ALA-PDT enhances systemic antitumor immunity, which is characterized by the rapid influx of immune cells into the treated tumor bed. The appropriate antitumor immune response relies on the recruitment of leukocytes/immune cells. As an initial step, the increased expression of proinflammatory cytokines may play a key role.

Using samples of tumor tissues from clinical patients with cSCC and from UV-treated cSCC mice, we observed that ALA-PDT increased CCL8 expression (Fig. 1A). The induction of CCL8 by ALA-PDT was further supported by our findings *in vitro*. As shown in Fig. 1B, expression levels of intracellular CCL8 in PECA and RAW264.7 cells were increased after ALA-PDT. Furthermore, more CCL8 was secreted by PDT-treated tumor cells or macrophages (Fig. 1C).

TAMs derive from circulating nonresident monocytes or tissue-resident macrophages. TAMs differentiate into classically activated macrophages (M1) and alternatively activated macrophages (M2), which possess either tumor growth-inhibiting or tumor growth-promoting capabilities [26,27]. TAMs are thought to represent predominantly M2 polarized macrophages. The patients whose tumor tissues are infiltrated a large number of TAMs are believed to have poor prognosis, because TAMs promote tumor angiogenesis and metastasis by various mechanisms. It has been directly shown that the contribution of macrophages is essential for the long-term maintenance of PDT-treated tumors [29]. Furthermore, it has been demonstrated that tumor-resident or tumor-recruited macrophages differentially influence the response of tumors to PDT [27,29]. Enhancement of PDT cytotoxicity was observed in the presence of nonresident macrophages, which had a strong antitumor phenotype. On the contrary, tumor-resident macrophages exhibited a pro-tumor phenotype, promoting tumor cell migration and endothelial stimulation [27]. In this study, we found that ALA-PDT enhanced the accumulation of TAMs in tumors, because this treatment elevated gene expression of the M1 markers CD16 and CD32, as well as of the M2 marker CD206. According to immunohistochemistry results, no positive staining for M1 or M2 marker was observed in UV-induced mouse cSCC tissue before ALA-PDT, whereas following the treatment,

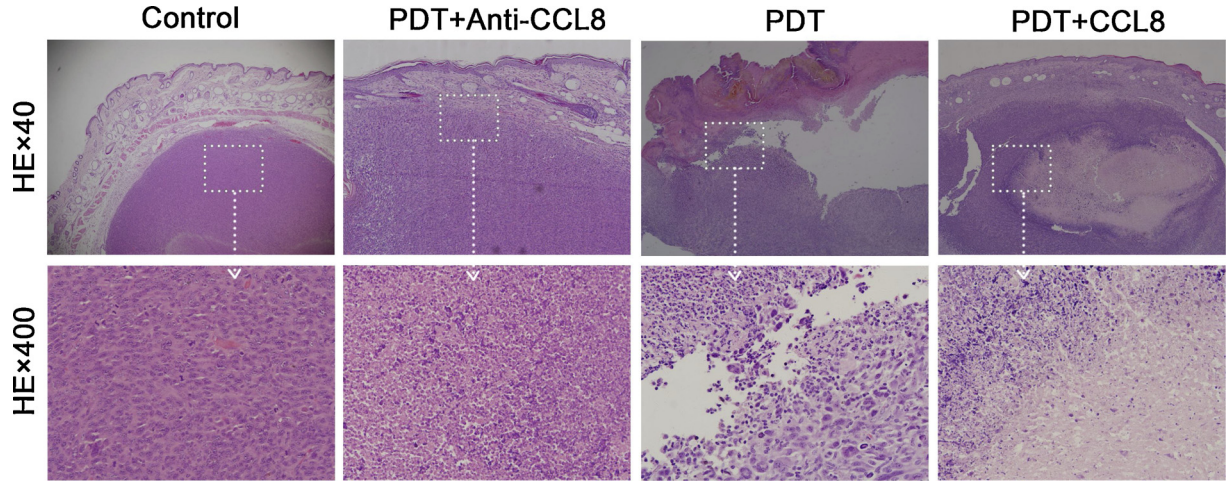

Fig. 6. Histological analyses. CSCC tumors in mice contained atypical squamous cells. Many immune cells were seen in cSCC tissues in PDT group, while tumor tissue in PDT + CCL8 group was surrounded by a large number of immune cells and dead tumor cells, as the original tumor cell structure was lost.

we observed markedly increased expression of CD16, CD80, moderately increased expression of CD163, and no expression of CD206. Our data revealed that macrophages that participate in anti-tumor effects elicited by ALA-PDT are likely the newly infiltrated cells attracted to the tumor by PDT-induced signals, and not those already present in tumors before PDT. We observed that ALA-PDT increased the M1/M2 ratios, such as CD16/CD206 and CD32/CD206, indicating that tumor macrophages recruited by ALA-PDT are mainly of the M1 phenotype.

Due to its functional plasticity, the balance of the macrophage effects from tumor promotion to tumor suppression represents a viable therapeutic target and may be shifted [29,30]. To get further insight into the capability of ALA-PDT to modulate polarization of macrophages toward M1 or M2 phenotype, we examined expression levels of CD16, CD80, CD163, and CD206 in RAW264.7 macrophage cell line after direct exposure to ALA-PDT or following co-culture with the supernatant of ALA-PDT-treated PECA cells. We found that the latter treatment caused RAW264.7 cells to exhibit a distinct M1 phenotype characterized by high expression levels of CD16 and CD80, low expression level of CD163, and absence of CD206 expression (Fig. 2). Interestingly, direct ALA-PDT treatment decreased the expression levels of both M1 and M2 markers.

In the present study, it was demonstrated that the supernatants of ALA-PDT-treated cancer cells also promoted migration of macrophages (Fig. 3). However, blocking CCL8 resulted in a significant reduction of migrating cells. Furthermore, adding recombinant CCL8 significantly promoted macrophage migration. Our results suggest that CCL8 is a very important regulator of the macrophage ability to infiltrate the tumor following ALA-PDT as it has a potential to attract macrophages.

CCL8 expression stimulates monocyte migration and exerts an antitumor effect [22,23]. In a previous study, CCL8 expression, induced via the STAT3 pathway, had an antimetastatic effect in mice as it resulted in fewer lung and liver metastatic tumor nodules in mice intravenously injected with B16F10 melanoma cells [22]. In this study, the antitumor effect of CCL8 was confirmed in SKH-1 mice with implanted cSCC. As shown in Fig. 4, large cSCC lesions of 0.6˜0.7 cm in diameter had poor treatment response to ALA-PDT because of limited light and drug penetration depth. When CCL8 was blocked, ALA-PDT had a weak effect as the tumor grew faster and larger in size. Our study, however, demonstrated that a combination of CCL8 and ALA-PDT had a stronger positive effect in the treatment of mouse cSCC than PDT alone. In addition, such a combination suppressed tumor volume regrowth. It has been previously suggested that CCL8 may directly inhibit tumor cell proliferation or the engraftment of tumor cells [23]. In this study, we detected some immune cells in cSCC tissues treated with ALA-PDT, whereas following the ALA-PDT + CCL8 treatment, the tumor tissue became surrounded by a large number of immune cells and dead tumor cells that apparently lost the original anatomical structure (Fig. 6).

Our data revealed that macrophages infiltrated the tumor tissue, being attracted by ALA-PDT, and killed tumor cells. Further studies are necessary to determine the mechanisms whereby tumor growth is suppressed by CCL8.

In conclusion, this study shows that CCL8 plays a pivotal role in macrophage infiltration into the tumor upon ALA-PDT treatment. Macrophages recruited to tumors by PDT were mostly of the M1 phenotype, and ALA-PDT also shifted macrophage polarization toward M1 phenotype. CCL8 enhanced the effect of ALA-PDT in murine cSCC. Thus, our results support the notion that ALA-PDT increases the expression and release of CCL8 as well as recruits M1 macrophages, thereby suppressing tumor growth.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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