ORIGINAL ARTICLE

Macrophages participate in the immunosuppression of condyloma acuminatum through the PD-1/PD-L1 signaling pathway

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Abstract

Background: The aim of this study was to investigate whether macrophages can participate in the immunosuppression of condyloma acuminatum (CA) by expressing PD-1/PD-L1.

Methods: The infiltration of macrophages and expression of programmed death-1 (PD-1) and PD-1 ligand 1 (PD-L1) on wart lesions and in normal skin tissues were detected by immunohistochemistry assay. The amounts of M1- and M2-type macrophages derived from THP-1 cells were measured by flow cytometry. The expression of cytokines on macrophages was examined by using enzyme-linked immunosorbent assay (ELISA). The protein expression of PD-1 and PD-L1 on macrophages was detected by western blotting.

Results: The macrophages were significantly increased, while PD-1 and PD-L1 were highly expressed on wart lesions compared to normal controls. More M2-like macrophages than M1-type macrophages were present on wart lesions. The M2-like macrophages in the CA groups showed high expression of interleukin-10 (IL-10), transforming growth factor (TGF-β), PD-1, and PD-L1 compared to normal controls.

Conclusion: Macrophages participate in the immunosuppression of CA by expressing PD-1/PD-L1.

Keywords: Condyloma acuminatum; Immunosuppression; Macrophages; PD-1; PD-L1

1. INTRODUCTION

Condyloma acuminatum (CA) is a sexually transmitted disease that manifests as epidermal wart-like hyperplasia of the genital and anal areas caused by human papilloma virus (HPV).[1] The incidence of CA is only secondary to that of gonorrhea, and CA is generally characterized by strong infectivity and a high recurrence rate,[2] which is refractory to modern therapy. Studies have reported that the recurrence of CA after treatment was mainly related to the microenvironment of local immunosuppression after HPV infection and HPV escapes from the immune system destruction; notably, recurrence is repeatedly caused by CA attack.[3,4]

Macrophages are important natural medullary immune cells that play a key role in the control of HPV infection and CA development. Macrophages mainly inhibit viral infection by devouring virus particles and releasing cytokines. The functional phenotype of macrophages can produce different polarizations, M1 and M2 macrophages, which participate in different functional immune reaction processes under different local microenvironments.[5] The wart lesions of CA caused by HPV infection are similar to those of tumor tissues. Tumor-associated macrophages (TAMs) have been reported to manifest an M2 macrophage function phenotype that promotes the occurrence and malignant progression of tumors and is unfavorable to the prognosis of tumors in numerous studies.[6] Chen et al.[7] clearly demonstrated a positive association between the expression of M2-polarized TAMs and HPV-related cervical carcinogenesis. Another study showed that interleukin-10 (IL-10) produced by TAMs induces a regulatory phenotype on T cells in the HPV16 TC-1 tumor mouse model, an immune escape mechanism that facilitates tumor growth.[8] These findings demonstrate that macrophages have immunorepressive roles in HPV infection, including CA.

As a coinhibitory receptor molecule, programmed death-1 (PD-1) is induced by activated T and B cells and subsequently modulates peripheral tolerance.[9] PD-1 ligand 1 (PD-L1) is primarily expressed by macrophages and is capable of inhibiting immune responses through interactions with PD-1.[10] Reports have suggested that the suppression of the PD-1/PD-L1 signaling pathway restricts the proliferation of cryptococcal meningitis by suppressing macrophage activation.[11] PD-1/PD-L1 therapies may also function through a direct effect on macrophages in cancer.[12] Lyford-Pike et al.[13] provided evidence for a role of the PD-1/PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. However, no studies to date have confirmed the role of the PD-1/PD-L1 signaling pathway in the macrophage-mediated immunosuppression of CA.

In the present study, we evaluated the relationship between the infiltration of macrophages and the expression of PD-1 and PD-L1 on the wart lesions of CA patients. Consequently, we found that macrophages are involved in the immunosuppression of CA via PD-1 and PD-L1 expression.

2. METHODS

2.1. Patients and sample collection

Twenty-four CA patients, including 13 females and 11 males, aged 18 to 60 years (mean age: 34.15 years), with a sick course

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of 19 to 165 days (average 52.36 days), were recruited from the Anhui Provincial Hospital Dermatology Clinic. All the clinical manifestations of the patients were typical, the acetic acid white test was positive, and the histopathological diagnosis was definite. Fragments of existing HPV DNA in skin lesions were detected by polymerase chain reaction. All of the wart lesions were located in the anal area, external genitals, distal urethra, or external genitals plus anal area. The maximum diameter of a single lesion was ≤ 4 cm. Excluded cases were gonorrhea, nongonococcal urethritis, cervicitis, autoimmune diseases, HIV infection, and/or patients with systemic application of glucocorticoids. All cases met the clinical and laboratory diagnostic criteria of CA. All patients had no other treatments prior to 5-aminolevulinic acid-photodynamic therapy (ALA-PDT) and no other systematic diseases.

Three kinds of samples were taken from each patient: wart tissues before ALA-PDT treatment, wart tissues after ALA-PDT treatment, which were obtained from the same position as the sample collected before treatment, and normal skin tissues, which served as a control. All samples were resected during the operation and immediately placed in liquid nitrogen for the following study. Additionally, pathological examinations of tissue samples from 24 patients were performed to ensure the accuracy of tissue sampling and trimming. This study was approved ethically by Anhui Provincial Hospital, and written informed consent was obtained from every participant.

2.2. Cell culture

The wart lesions and normal skin tissues were collected and rinsed eight times with phosphate buffered saline (PBS), and the cleaned tissues were placed in culture dishes containing 5 mL of 1640 culture medium supplemented with penicillin and streptomycin. The tissues were cut up by using surgical scissors and cultured in 5% CO_2 at 37°C for 24 hours. Then, the suspension was collected and centrifuged at 1500 rpm for 8 min, and the centrifugal suspension was collected.

THP-1 cell lines (Shanghai Institute of Cellular Biology of Chinese Academy of Sciences) were differentiated into macrophages. THP-1 cells were dissolved in a water bath at 37°C and transferred to a centrifuge tube containing roswell park memorial institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and centrifuged for 5 min at 4°C at 1200 rpm. The supernatant was discarded, 5 mL RPMI 1640 culture medium containing 10% FBS was added, and the cells were cultured at 37°C. Normal cultured THP-1 cells were collected, 10^6 cells were transferred into a six-well plate, and 100 ng/mL of the differentiation inducer phorbol myristate acetate was added to induce differentiation for 24 hours. Then, 1640 medium containing 20% tissues suspension was added to the complete medium, and the supernatant and fresh macrophages were collected for flow cytometry, enzyme-linked immunosorbent assay (ELISA), and western blotting.

2.3. Immunohistochemistry assay

The isolated wart lesions and normal skin tissues were fixed with formalin, dehydrated with ethanol and xylene, embedded in paraffin and sliced into 4-μm sections. Then, the tissue sections were deparaffinized in xylene and hydrated through a graded ethanol series. Antigen retrieval was carried out with citrate buffer by heating for 15 min in a microwave. The sections were incubated with H_2O_2 deionized water and rinsed with PBS. The primary antibodies, including anti-CD86 antibody (1:100, Santa Cruz Biotechnology, CA, USA) and anti-CD163 antibody (1:100, AbD Serotec, Düsseldorf, Germany), PD-1 antibody (1:100, eBioscience, San Diego, CA, USA) and PD-L1 antibody (1:100, eBioscience, San Diego, CA, USA), were added, incubated overnight at 4°C and rinsed with PBS. The secondary antibodies were conjugated with horseradish peroxidase, which was added to the sheep anti-rat/rabbit IgG polymer enzyme marker (MXB, FuJian, China), incubated at 37°C for 2 hours and rinsed with PBS. The sections were dipped into DAB chromogenic agent, washed with water, dehydrated in a graded ethanol series, and then covered with xylene. The immunohistochemistry results were observed.

2.4. Flow cytometry

For intracellular staining, cultured macrophages were first stained for surface markers and then fixed and permeabilized with Fix/Perm solution (eBioscience). The cells were then resuspended in Perm buffer and incubated with APC-rat anti-human CD68 (BioLegend, CA) or PE-rat anti-human CD163 (BD, USA) at room temperature in the dark for 20 min. After washing, the cells were used for flow cytometric analysis.

2.5. ELISA

The macrophage supernatant from differentiated THP-1 cells was collected to detect the expression of TNF-α, IL-6, TGF-β, and IL-10 by using ELISA kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. The concentration of cytokines was calculated per the corresponding OD value.

2.6. Western blotting

The expression of PD-1 and PD-L1 on macrophages was detected by western blotting. Total proteins were extracted from the macrophages and quantified with a Bio-Rad protein assay. Equal volumes of the proteins were added to each well, fractioned by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. The membrane was blocked with 5% skim milk for 30 min, incubated with the primary antibodies (50 ng to 200 ng/mL) for 1 hour at room temperature, followed by incubation with the secondary antibodies (conjugated with horseradish peroxidase). The membranes were washed with Tris-buffered saline with Triton X-100 after each antibody incubation. The immune complexes in the membrane were developed with enhanced chemiluminescence. The results were recorded on X-ray film.

2.7. Statistical analysis

All data were analysed with SPSS 22.0, and Pearson's correlation analysis was used to explore the relationship between the parameters of the samples. Student's *t*-test was used to analyse the differences between two groups. One-way ANOVA was used to determine the multisample analysis. All statistical tests were two-sided, and a p value less than 0.05 was considered significant.

3. RESULTS

3.1. The macrophages are significantly elevated in the wart lesions of patients with CA

We obtained fresh tissues from surgically excised wart lesions from 24 patients with CA before and after a week of treatment with ALA-PDT. The normal skin tissues from 24 patients excised during dermatological surgery served as the control group and the phenotype of the macrophages was examined. First, we investigated the expression of CD86, a M1-like macrophage marker,[14] in different groups by immunohistochemical assay. CD86 (M1 macrophage marker) was significantly overexpressed in the CA group compared to the normal group and showed low expression after treatment with ALA-PDT (Fig. 1A). We also investigated the expression of CD163, a phenotypic marker of M2 macrophages.[15] Similarly, the expression of CD163 was markedly higher than that of the normal control group, and treatment with ALA-PDT reduced the amount of M2 macrophages (Fig. 1B).

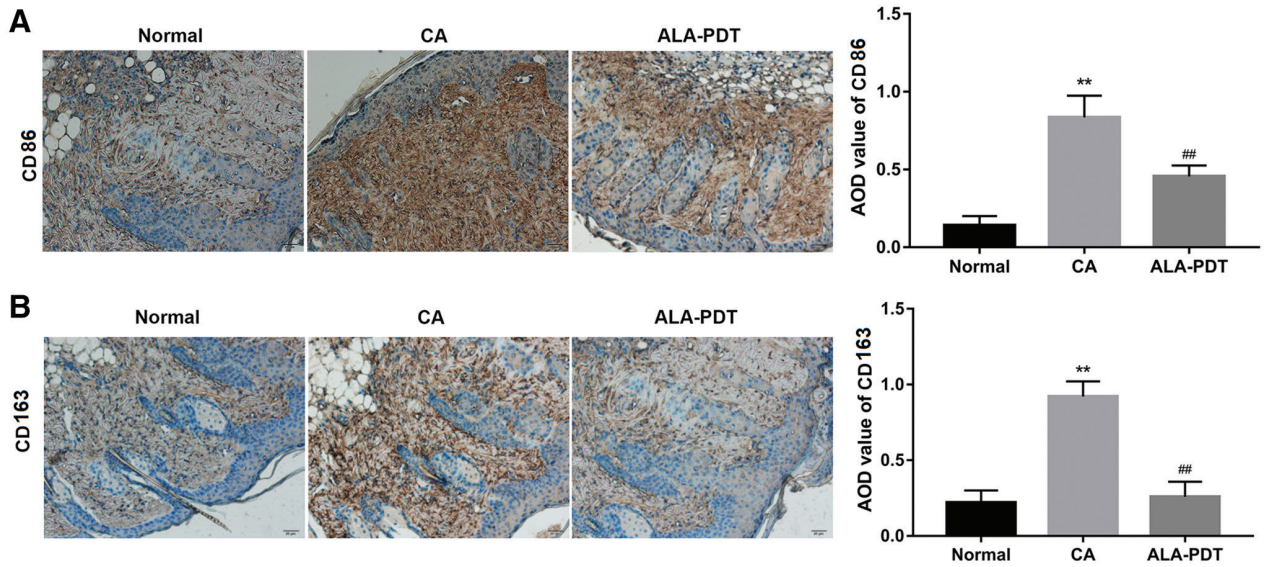

Fig. 1 Immunohistochemical detection of CD86+ and CD163+ macrophages expression in tissues from CA patients. The wart lesions before treatment (CA group) showed stronger immunohistochemical staining with the increased CD68+ (A) and CD163+ (B) macrophages population compared with control normal skin tissues(normal group), and wart lesions after treatment with ALA-PDT (ALA-PDT group) displayed weaker positive staining with few CD68+ (A) and CD163+ (B) macrophages compared to CA group. ALA-PDT, 5-aminolevulinic acid-photodynamic therapy; CA, condyloma acuminatum.

3.2. PD-1 and PD-L1 are highly expressed in the wart lesions of patients with CA

The immunohistochemical results indicated that the expression of PD-1 and PD-L1 was markedly upregulated in the CA group compared with the control group and downregulated by ALA-PDT treatment (Fig. 2A, B). The protein levels of PD-1 and PD-L1 were analysed by western blotting. As shown in Fig. 2C, D, the expression levels of PD-1 and PD-L1 were markedly higher in the CA group, in contrast to the control group, in which the expression was very low.

3.3. More M2 macrophages are present in the wart lesions of patients with CA

To detect the amounts of macrophages in the tissues, wart lesions from patients with CA were collected before and after one-week treatment with ALA-PDT, and the normal skin tissue was treated as a control. The membrane macrophage CD68 marker has been shown to identify M1 macrophages in human tissues.[16] The expression levels of CD68+ macrophages in the three histological groups were analysed by using flow cytometry. Statistical analysis revealed that there was no significant difference in the CD68+ M1-type macrophage amount between the CA group and the ALA-PDT-treatment group compared to the normal control group (Fig. 3A, C). Interestingly, an obvious upregulation of CD163 (M2 macrophages) was observed in the CA group compared to the normal control group, and treatment with ALA-PDT suppressed the regulation of CD163 (M2 macrophages) (Fig. 3B, D), leading to a significantly decreased ratio of M1/M2 macrophages (<1.0) in the CA group (Fig. 3E).

3.4. Macrophages from wart lesions highly expressed IL-10, TGF-β, PD-1, and PD-L1

The expression of cytokines in histological tissues was detected by using ELISA. There was no significant difference in TNF-α and IL-6 expression among the control normal group, CA group, and ALA-PDT-treatment group (Fig. 4A, B). In contrast, the levels of IL-10 and TGF-β in the CA group were prominently higher than those in the control group (Fig. 4C, D). In addition, the expression of PD-1 and PD-L1 on macrophages isolated from three tissues was detected by western blotting. As shown in Fig. 4E, F, the macrophages from wart lesions strongly expressed PD-1 and PD-L1, although the macrophages from normal skin tissues did not.

4. DISCUSSION

The viral clearance obstacle caused by immune escape is key to persistent HPV infections in patients with CA.[17] PD-1 and PD-L1 are vital molecules influencing immunosuppression in chronic viral infections.[18] The present study established a correlation between macrophage activation and the PD-1/PD-L1 signaling pathway in the immunosuppression of CA, thus allowing the verification of the potential of the PD-1/PD-L1 signaling pathway as a target of immunotherapy for CA.

The transformation of the macrophage phenotype can be induced by a virus infection.[19] Macrophages mostly show an M2-like phenotype, which helps viruses to escape from immune attack and promotes tumor-induced immunosuppression.[6,20] M1 macrophages are characterized by the high expression of IL-6 and TNF-α,[21] while M2 macrophages are characterized by the high expression of IL-10 and TGF-β.[22] IL-10-producing monocytes differentiate into M2 macrophages[23] and play a central nonredundant role in immunosuppression.[24] A large body of evidence supports the role of M2-like macrophages in immune regulation, including the inhibition of the immune activity of T cells and natural killer cells.[25,26] In this study, a significantly decreased ratio of M1/M2 macrophages (<1.0) and the high expression of IL-10 and TGF-β by M2 macrophages were observed in the wart lesions of patients with CA (Figs. 3, 4C, D), which is similar to the results of other studies.[27,28] The findings indicated that macrophages from the M1 phenotype switch to an M2-like phenotype during HPV infection to participate in the immunosuppression process of CA. Macrophages can polarize into different functional phenotypes due to different microenvironments but the polarization mechanism has remained unclear until now. However, increasing data have confirmed that signaling molecules, transcription factors, histone modifications, and microRNAs have important regulatory effects on the polarization and plasticity of macrophages.[29,30] In addition, it has been reported that CD4+CD25+Foxp3+ regulatory T cells (Tregs) can induce the activation of M2-type macrophages. Tregs upregulated the expression of CD163 by secreting IL-10 and induced the alternative activation of monocytes/macrophages.[31] Treg cells regulate innate immunity by promoting the differentiation

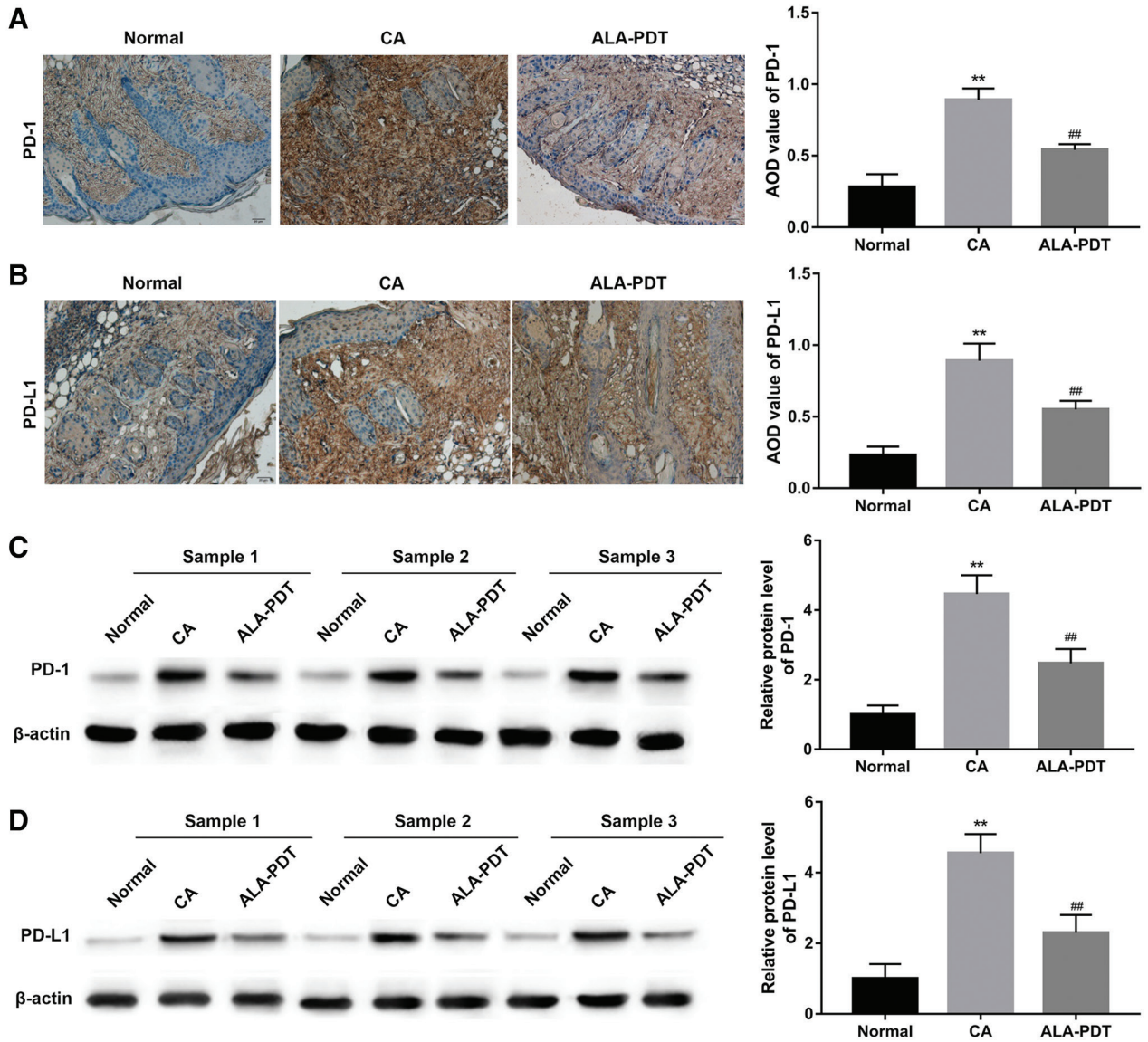

Fig. 2 PD-1 and PD-L1 are highly expressed in wart lesions of patients with CA. Three histological tissues were isolated from patients, including normal skin tissues (normal group), wart lesions before treatment (CA group), and wart lesions after treatment with ALA-PDT (ALA-PDT group). A, B: Immunohistochemical results of PD-1 and PD-L1 in three groups. C, D: Western blotting results of PD-1 and PD-L1 in three groups. ALA-PDT, 5-aminolevulinic acid-photodynamic therapy; CA, condyloma acuminatum; PD-L1, PD-1 ligand 1; PD-1, programmed death-1.

of M2 macrophages and by inhibiting M1 macrophage induction in mice in part through arginase, IL-10, and TGF-β pathways.[32] There are many Treg cells in CA wart lesions that play a positive role in the local immune escape of CA.[33] Therefore, we speculated that Treg cells may be involved in the transformation of local macrophages into M2-type macrophages in CA tissue but further research is needed to be performed.

The PD-1/PD-L1 signaling pathway is involved in immunosuppressive regulation in various virus-induced diseases, including HPV infection. Kawachi et al.[34] found that a higher density of stroma-infiltrating macrophages was observed in PD-L1-positive tumors induced by HPV than in negative tumors. Other studies have reported similar observations showing 7% of the CD68 cells (macrophages) in the tumor area of HPV+ expressing PD-L1.[35] Lyford-Pike et al.[13] provided evidence that PD-L1 is expressed by CD68+ TAMs in human papillomavirus-associated head and neck squamous cell carcinomas. Gordon et al.[12] demonstrated that the specific inhibition of the PD-1/PD-L1 axis in TAMs is responsible for tumor immunity in primary human cancers, and the M2 population expresses significantly more PD-1 than the M1 population. The findings presented here suggest that the macrophages from wart lesions in CA highly expressed PD-1 and PD-L1 (Fig. 4), which is consistent with the above-mentioned reports. The CA group in the current study reportedly showed a significant elevation of M1 and M2 macrophages and a significant increase in the expression of PD-1 and PD-L1 (Figs. 1, 2), which indicated that the expression of PD-1 and PD-L1 was positively related to M1 macrophages and M2 macrophages in wart lesions and contributed to an immunosuppressive CA microenvironment.[36]

In this study, we first demonstrated a positive correlation between the expression of PD-1/PD-L1 and amount of M1/M2 macrophages. Furthermore, we found that the macrophages of wart lesions were polarized from an M1 phenotype to an M2 phenotype. Finally, PD-1 and PD-L1 expression on macrophages from wart lesions of CA was examined by western blotting. Accordingly, we speculate that macrophages participate in the immunosuppression of CA by expressing PD-1/PD-L1. Our results suggest that PD-1/PD-L1 should be considered as a potential therapeutic target in the development of novel immunotherapy treatments for patients with CA.

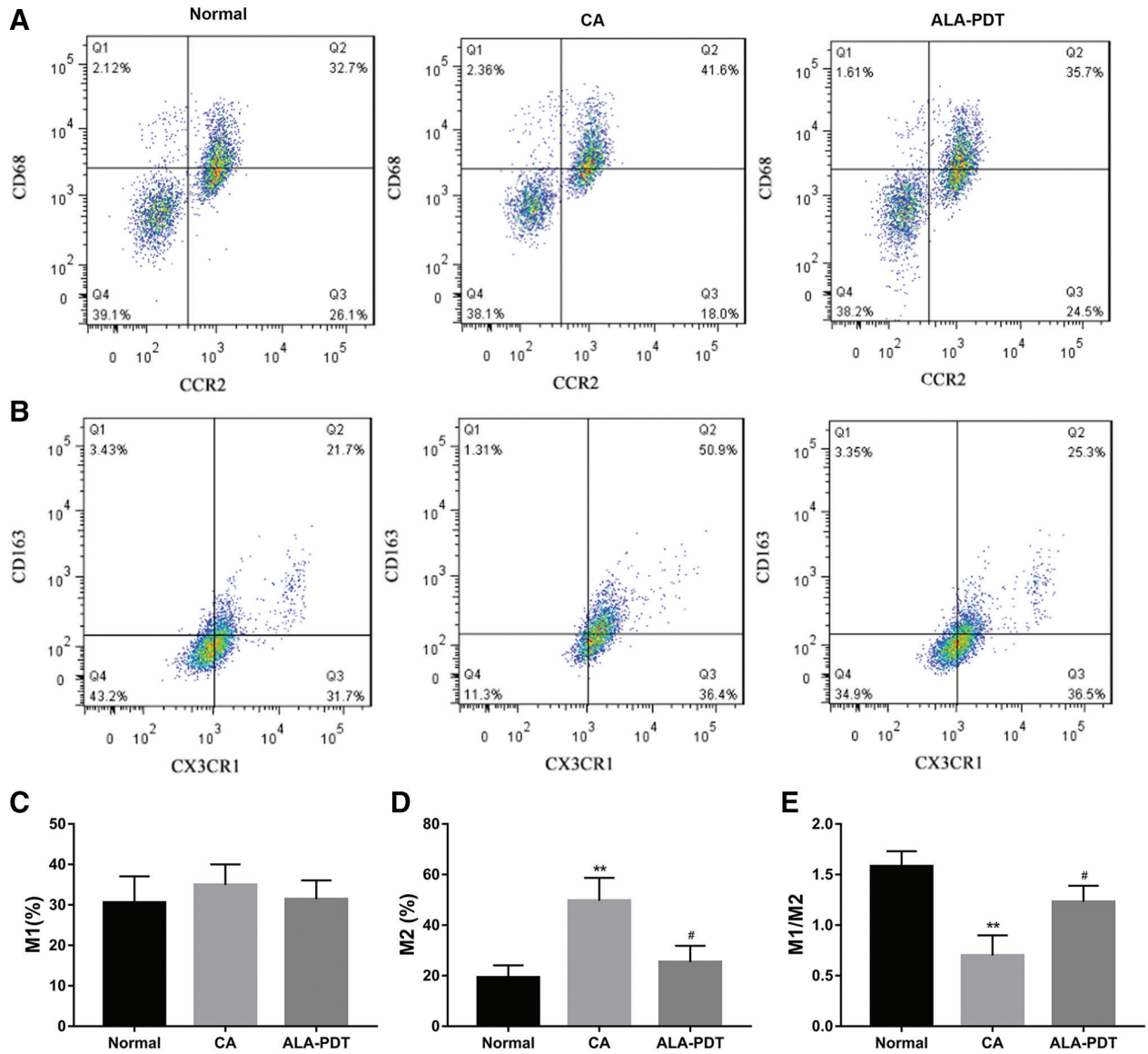

Fig. 3 The amount of M1 and M2 macrophages from tissue suspension of CA patients. Three different tissues were isolated from CA patients, including normal skin tissues (normal group), wart lesions before treatment (CA group), and wart lesions after treatment with ALA-PDT (ALA-PDT group). A, C: The flow cytometry results of CD68 + M1 macrophages in three groups. B, D: The flow cytometry results of CD163 + M2 macrophages in three groups. E: The ratio of M1/M2 in three groups. ALA-PDT, 5-aminolevulinic acid-photodynamic therapy; CA, condyloma acuminatum.

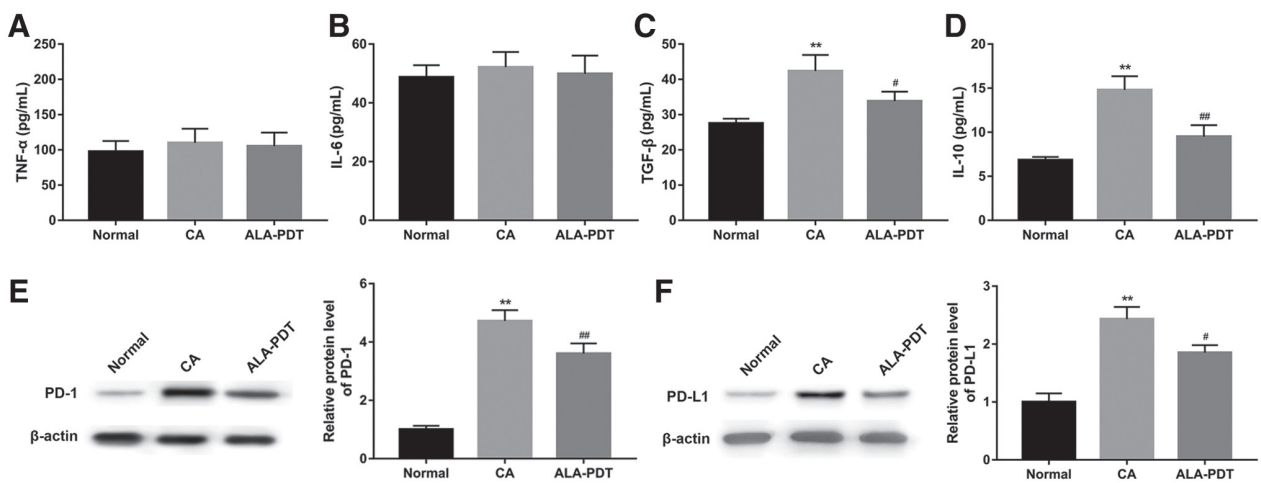

Fig. 4 PD-1 and PD-L1 expression by macrophages and correlation with cytokine production. The macrophages were from normal skin tissues (normal group), wart lesions before treatment (CA group), and wart lesions after treatment with ALA-PDT (ALA-PDT group). A-D: the level of TNF-α, IL-6, transforming growth factor (TGF-β), and IL-10 on macrophages isolated from three tissues was analyzed by enzyme-linked immunosorbent assay (ELISA). E, F: The expression of PD-1 and PD-L1 by macrophages isolated from three tissues were analyzed by western blot. ALA-PDT, 5-aminolevulinic acid-photodynamic therapy; CA, condyloma acuminatum; PD-L1, PD-1 ligand 1; PD-1, programmed death-1.

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