Distribution and Immunomodulatory mechanisms of Programmed death(PD)-1 molecule , its ligand PD-L1and related cytokines in Cervical Cancer

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Abstract

PD1(programmed cell death protein 1), a molecule negatively regulated by immunity,is highly expressed in a wide variety of human malignancies making it an attractive immunotherapeutic target. PD-L1 is one of the ligands for PD-1 and expressed both on T cells and tumor cells. The purpose of this study was to investigate the expression levels of PD1, PD-L1, regulatory T cells (Tregs), identified as CD4+CD25+CD127-/low, and related cytokines during the development and progression of cervical cancer(CC). Through different groups to simulate the process of the occurrence and development of CC, peripheral blood of each group was collected, and different indicators were detected by ELISA and flow analysis. It was found that except for INF-γ, PD1, PD-L1, IL-2, IL6, IL-10, TGF-β expression increased gradually with the development of CC, and the differences between groups were statistically significant (P<0.05). This situation also appears on the tracking of preoperative CC patients and postoperative follow-up on test results. And Tregs in peripheral blood, compared with normal control(NC) group, the expression increased CC group, the PD1 expression in the Tregs membrane also increases at the same time. In CC tissues, we found an interesting phenomenon through multiplex immunohistochemical staining that CD8+ T cells mainly infiltrated in the tumor cell aggregation area, while CD4+ T cells mainly appeared around. Therefore, we speculated that CD8+ T cells mainly played an anti-tumor immune role in CC tumor tissues.

Abstract

PD1(programmed cell death protein 1), a molecule negatively regulated by immunity,is highly expressed in a wide variety of human malignancies making it an attractive immunotherapeutic target. PD-L1 is one of the ligands for PD-1 and expressed both on T cells and tumor cells. The purpose of this study was to investigate the expression levels of PD1, PD-L1, regulatory T cells (Tregs), identified as $CD4^+CD25^+CD127^{-/low}$, and related cytokines during the development and progression of cervical cancer(CC). Through different groups to simulate the process of the occurrence and development of CC, peripheral blood of each group was collected, and different indicators were detected by ELISA and flow analysis. It was found that except for INF-γ, PD1, PD-L1, IL-2, IL6, IL-10, TGF-β expression increased gradually with the development of CC, and the differences between groups were statistically significant (P<0.05). This situation also appears on the tracking of preoperative CC patients and postoperative follow-up on test results. And Tregs in peripheral blood, compared with normal control(NC) group, the expression increased CC group, the PD1 expression in the Tregs membrane also increases at the same time. In CC tissues, we found an interesting phenomenon through multiplex immunohistochemical staining that $CD8^+$ T cells mainly infiltrated in the tumor cell aggregation area, while $CD4^+$ T cells mainly appeared around. Therefore, we speculated that $CD8^+$ T cells mainly played an anti-tumor immune role in CC tumor tissues.

INTRODUCTION

Cervical cancer(CC) is the third most common cancer in women worldwide and the most frequent gynecological cancer in developing countries(1-3). The prime causal factor for this cancer is persistent infection with human papillomavirus(HPV). In most cases, the human immune system can be aware of HPV infection, and only a small number of women with persistent high-risk HPV, such as type 16 or 18, will lead to cervical precancerous lesions and develop into cervical cancer(2,4). CC, like all solid tumors, can be treated surgically in early or isolated recurrence. The vast majority of CC is squamous cell carcinoma, which is sensitive to radiation. Through in vitro and intracavitary radiotherapy, local cervical cancer reaches the maximum radiation dose of tumor death. And mid- and late-stage CC tumors are treated with chemoradiotherapy. Immunotargeted therapy is a new method to treat advanced recurrent or metastatic CC in recent years(5-8). According to the report, the prognosis of patients with metastatic CC is poor with a median survival of 8-13 months(9).

PD1/PD-L1, as a key molecule of immune checkpoint, is an important target of immunotherapy and has changed the pattern of tumor treatment(10-11). Especially for patients with terminal and critical cancer, such as melanoma(12), non-small-cell lung cancer(13), ovarian cancer(14), breast cancer(15), including CC(9,16). PD1, a member of the CD28 receptor family, is a co-inhibitory receptor expressed on the cell surface. PD1 and its ligand PD-L1 bind as an immunocheckpoint to play an immunosuppressive role(17-18). PD1 and PD-L1 signal pathway activation contributes to tumor immune escape and provides important negative signals to limit, terminate or impair T cell immune response, and blocking the pathway can reduce tumor inhibition of the anti-tumor immunity effect, to better improve the body's own immune system to resist the ability of the tumor. Thereby the signal pathway as anti-tumor immune and autoimmune diseases such as novel targets for therapeutic intervention, theoretically PD1 and PD-L1 signal pathway with ability for the treatment of various types of tumors(19-21). Therefore, the application value of PD1 and PD-L1 in patients with cervical cancer is also worth expecting.

Regulatory T cells (Tregs) and PD-1/PD-L1 pathways are both key to terminate the immune response(22). It is report that targeting endoglin expressing Tregs in the tumor microenvironment enhances the effect of PD1 checkpoint inhibitor immunotherapy(23). Tregs are mainly divided into natural regulatory T cells(n-Tregs) and adaptive regulatory T cells(a-Tregs). N-Tregs are mainly $CD4^+CD25^+CD127^{-/low}$or $Foxp3^+$ T cells. Foxp3 is not only a marker molecule for $CD4^+CD25^+$ Treg, but also a key gene that determines the function of $CD4^+CD25^+$ Treg. A-Tregs are developed from peripheral naive T cells induced by low doses of antigens or immunosuppressive cytokines, including Tr1(type 1 regulatory T cell) and Th3(T helper 3) cells, which mainly secrete IL-10 and TGF-β to play a negative role in immune regulation(24-29). In this paper, the expression of n-Tregs in cervical cancer and its correlation with the expression of PD1/PD-L1 were mainly discussed.

In this study, groups were set to simulate the occurrence and development of cervical cancer caused by long-term infection of high-risk HPV. In addition, during the development of cervical cancer, the expression levels of PD1, PD-L1, regulatory T cells and related cytokines in the peripheral blood of patients were investigated. At the same time, the changes of PD1, PD-L1 and related cytokines before and after operation in patients with cervical cancer treated by surgery were followed up. This study laid the foundation for further research on the immune regulation mechanism and immunotherapy of the immunoregulatory molecule PD1 and its ligand PD-L1 in cervical cancer.

2. MATERIALS AND METHODS

2.1. Study patients.

The cohort for this study included 140 female patients with CC diagnosed by cervical and cervical biopsyranging from age 32 to 68 years, CIN from age 31 to 63 years, Chronic Cervicitis from age 28 to 58 and HPV+ diagnosed by HPV genotyping detection from age 28 to 52 years(Fig.1-2). Take peripheral blood, separate plasma or serum for ELISA analysis, and peripheral blood mononuclear cell(PBMC) for flow cytometry detection. Other than a single patient, to the best of our knowledge no patients with CC, CIN, Chronic Cervicitis and HPV+ received any treatment with surgery or chemotherapy prior to the collection of

specimens. In addition, samples from five participants of CC were follow-up monitoring detected by ELISA with separated plasma or serum until about 180 days after surgical treatment.

2.2. Human blood and tissue.

PBMCs were isolated from healthy donor, CC, CIN, Chronic Cervicitis and HPV+ patient peripheral blood. CC lesion tissues were separated after surgical treatment, sourced ethically, and their research use was in accord with the terms of the informed consents.

2.3. ELISA detection.

Detection of human PD1, PD-L1, IL-2, IL-6, IL-10, TGF-β and INF-γ concentrations in serum- plasma by ELISA kit(MLBio). The blood specimen was placed at room temperature for 2 hours or 4 overnight, and then 1000×g centrifuged for 20 mins to collect the supernatant. A sample, standard sample and HRP labeled antibody were added to the micropores of pre-coated antibody, incubated and washed thoroughly. The Stop Solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm using a enzyme labeling(Bio-Rad). The color depth is positively correlated with the antigen in the sample.

2.4. Flow cytometry.

The following reagents were from Biolegend and BD Horizon. Cells were labeled with anti-human surface antibodies as follows: CD4-FITC (RPA-T4; BioLegend), CD25-APC (BC96; BioLegend), CD127-PE (A019D5; BioLegend), PD1-BB700 (EH12.1; BD Horizon). Flow cytometry analyses were performed using BD FACS Calibur. Flow cytometric data were analyzed using FlowJo software. Our gating strategy primarily identified $CD4^{+}CD25^{+}CD127^{-/low}$and PD-1.

2.5. Immunohistochemistry.

IHC(immunohistochemistry) samples were held at 4@C in formalin fixator after surgical surgery treatment. The immunohistochemical method was Dako REAL EnVision detection system, and the specific steps were stained according to the instructions of the kit. Primary antibodies used were monoclonal rabbit anti-human IL-2(Abcam), polyclonal rabbit anti-human IL-6(Abcam), monoclonal rabbit anti-human IL-10(Abcam), monoclonal rabbit anti-human TGF-β(CST), polyclonal rabbit anti-human INF-γ(Bioworlde), monoclonal rabbit anti-human Foxp3(CST), monoclonal mouse anti-human PD1(Abcam) and monoclonal rabbit anti-human PD-L1(Abcam).

2.6. Multiplex immunohistochemistry.

Archival formalin fixed paraffin embedded tissue blocks from CC patients were deposited as sections onto microscope slides. Primary antibodies used were polyclonal rabbit anti-human CD4 (CST), monoclonal mouse anti-human CD25 (Novus), monoclonal mouse anti-human Foxp3 (CST), monoclonal mouse anti-human CD8 (Dako) and polyclonal rabbit anti-human PD1 (CST). Primary CD4, CD25, Foxp3, CD8 and PD1 stains were labeled with EnVisionTM FLEX HRP (Dako) conjugated secondary antibodies followed by tyramide signal amplification (TSATM - plus fluorescein system; Perkin-Elmer) as per manufacturer's instructions. All immunohistochemistry images were captured using an Evos FL Auto 2 cell imaging system (Thermo Fisher Scientific) and analyses were completed using Image J software . For antigen retrieval, slides were boiled by microwaving in EnVisionTM FLEX target retrieval solution, pH 9.0 (Dako) antigen retrieval buffer for 15 mins. Prior to primary antibody labeling, slides were blocked in a PBS solution containing 2 % goat serum for 30 min. Prior to HRP labeling, slides were blocked using EnVisionTM FLEX peroxidase blocking reagent (Dako). For washing, slides were rinsed with EnVisionTM FLEX wash buffer (Dako) then immersed in wash buffer and agitated for 3 min, then placed into a second wash buffer and agitated for a further 3 min. Each incubation took place in an agitated humid chamber with protection from light where necessary. Following the completion of all antibody labeling, slides were incubated with DAPI for 5 mins, then washed and coverslips were mounted using ProLong GoldTM antifade mountant (Thermo Fisher Scientific) and allowed to set in the dark.

2.7. Ethics Statement

The study was approved by the Ethics Committee of Fuzhou First Hospital, Fujian Province, China. All patients provided written informed consent.

2.8. Statistics.

We analyzed data using GraphPad Prism6. To determine whether parametric or non-parametric statistical analyses were required, D'Agostino and Pearson omnibus normality tests were conducted. Statistical significance was based on p=0.05.

1. **Result**
2. **Increased percentages of PD1, PD-L1 and related cytokines in serum in the development of CC patients compared with NCs.**

We investigated the expression of PD1, PD-L1, IL-2, IL-6, IL-10, TGF-β and INF-γ in serum in CC, CIN, Chronic Cervicitis, HPV+ patients and NC group simulated the development of CC(**Fig.1A-H**). Through comparative analysis, we found that in each detection group, except for the INF-γ group, the expression level of CC group was the highest, and the difference was significant compared with the NCs, which was statistically significant. In addition to CC group, CIN, Chronic Cervicitis, and HPV+ group, independent t-test analysis was also conducted respectively with NC group, and the results showed differences, some with significant statistical differences, such as Figure1A, B, C, G and H. Some showed no significant difference, as shown in Figure1D, E and F. One-way ANOVA was performed for each test group, and the differences were statistically significant. Therefore, we concluded that, except for INF-γ, PD1, PD-L1, IL-2, IL6, IL-10 and TGF-β expression in CC was significantly higher than that in normal healthy people, in which IL-2, IL-6, IL-10, PD1 and PD-L1 showed a significant upward trend in the occurrence and development of CC, and the expression levels were all higher than that in the NC group, with significant differences.

The expression of tregs and PD1 in peripheral blood in CCs higher than NCs.

Peripheral blood samples of CC, CIN, Chronic Cervicitis, HPV+ and NC group were collected, PBMC was isolated, CD4, CD25, CD127 and PD1 antibodies were stained, and analyzed by flow cytometry(**Fig.2A-C**). The main objective was to investigate whether the expression level of PD1 in tregs($CD4^+CD25^+CD127^{-/low}$), PD1 in total cells and tregs changed during the development of CC. The expression of these three indexes was statistically different by one-way ANOVA analysis(**Fig.2D-F**). The independent sample T-test analysis showed that the expression of CC was significantly increased compared with that of the NC group, and the difference was statistically significant, while the Chronic Cervicitis and HPV+ group had no significant difference(**Fig.2D-F**).

The expression level of PD1, PD-L1 and related cytokines in CC patients before and after surgery.

Follow-up tracking five in the surgical treatment of CC patients, from preoperative to within six months after the operation. Plasma was collected in stages and detected by ELISA for PD1, PD-L1, IL-2, IL-6, IL-10, TGF-β and INF-γ (**Fig.3a-h**). According to the curve analysis, the expressions of PD1, PD-L1, IL-2, IL-6, IL-10, and TGF-β gradually decreased and tended to be flat with the time after the operation. However, the expression of INF-γ did not change significantly in relation to time. Therefore, we conclude that the occurrence and development of cervical cancer are associated with increased expression levels of PD1, PD-L1, IL-2, IL-6, IL-10, TGF-β and no definitive association with INF-γ was found in this study.

In situ localization of related cytokines,tregs , PD1 and PD-L1 in CC tissues.

Two parts of CC tissue samples were selected, one was tumor tissue, and the other was normal tissue at the edge of the surgically removed tissue. Firstly, histoimmunochemical staining was performed to detect CD4, CD25, Foxp3, PD1, PD-L1, IL-2, IL-6, IL-10, TGF-β and INF-γ, respectively, to understand the expression position and level of these indicators in CC tissues(**Fig.4**). According to histoimmunochemical analysis, a large number of $CD4^+$, $CD8^+$ and $Foxp^+$ lymphocyte infiltration in CC tissue(**Fig.4I,III,IV**), PD-L1, TGF-β shows diffuse distribution(**Fig.4VI,X**), IL-6 mainly expressed in extracellular(**Fig.4VIII**),

IL-10 and INF-γ mainly expressed in the cell membrane (**Fig.4IX,XI**). Secondly, we performed multicolor immunofluorescence staining to detect CD4, CD25, CD8, Foxp3, PD1, PD-L1, DAPI, separately. **Fig.5A-B** shows that $CD8^+Foxp^+$ and $CD4^+Foxp^+$ tregs are diffusently expressed in tumor tissues. As shown in **Fig.5C-D** , PD1 was expressed in $Foxp^+$ cells, and it was found that a very small number of $Foxp^+$ cells were coincident with $PD\text{-}L1^+$ cells, that is, a small number of $Foxp^+$ cells could express PD-L1. Comparison with the control tissues showed that the expression levels of $CD4^+$, $CD8^+$, $CD4^+PD1$, $CD8^+PD1$, $Foxp3^+PD1$ and $Foxp^+PD\text{-}L1$ in tumor tissues were all higher than those in the control tissues analyzed by imageJ software and the differences were statistically significant.**Fig.6** shows the combined images of six fluorescence channels taken under the same field of view. It was found a large number of cell clusters with irregular nuclei in the three regions of A, B and C in the figure, preliminarily judging the cervical tumor cells, which were highly consistent with the regions where PD-L1 was expressed, further confirming the conclusion that a large number of tumor cells expressed PD-L1. In addition, a large number of $CD8^+$ cells in tumor cell clusters field infiltration, and a small amount of $CD4^+$ infiltration among them, the majority of $CD4^+$ cells in tumor cells surrounded gathered.

Discussion

The data from many articles show that the increased tumor infiltrating lymphocyte cells(TILs) is associated with increased PD1/PD-L1 levels in diverse tumors suggesting that these factors are biologically linked(30-33).

Firstly, in order to analyze the expression of PD1, PD-L1 and related cytokines in the occurrence and development of cervical cancer, this study was divided into five groups: normal control group(NC), HPV-positive group(HPV+), chronic cervicitis group, cervical intraepithelial neoplasia group(CIN) and cervical cancer group(CC), to simulate the development process of cervical cancer caused by continuous infection of high-risk HPV. And plasma samples were collected from each group, and the expressions of IL-2, IL-6, IL-10, TGF-β, INF-γ, Foxp3, PD1 and PD-L1 were detected by ELISA. As shown in Figure 1: **(1)**With the continuous progress of high-risk HPV infection, the expression of PD1 and PD-L1 in peripheral blood gradually increased, and compared with NC, $p<0.05$ (independent t-test analysis), the difference was statistically significant. **(2)** Except for INF-γ, the expression level of other cytokines was higher than that of NC, especially CC. **(3)** INF-γ results are different, there are no INF-γ detected in the healthy control group, and HPV positive group is much higher than other groups as the peak parabolic decline trend. At the same time, the peripheral blood of the five groups was collected for flow cytometry analysis to analyze the expression of $CD4^+CD25^+CD127^{-/low}$and PD1 in the peripheral blood. It was found that the number of $CD4^+CD25^+CD127^{-/low}$, PD1 and $CD4^+CD25^+CD127^{-/low}PD1^+$cells in the peripheral blood was significantly higher than that in the NC group, with statistical difference, while there was no significant difference or individual difference in the other groups. Secondly, the patients with cervical cancer were followed up before and after surgery to understand the changes in the expression levels of various factors in the peripheral blood of patients. Results As shown in Figure 3:**(1)** The levels of PD1 and PD-L1 were significantly changed before and after surgery, and gradually leveled off after surgery.**(2)** In addition to INF-γ, the expression of other related cytokines also showed a trend of decreasing and gradually leveling off after surgery, which was consistent with the experimental data presented in **Figure 1** . Finally, histoimmunochemical tests of tumor tissues surgically removed from patients with cervical cancer showed a large number of lymphocyte infiltration, such as $CD4^+$and $CD8^+$, and $Foxp3^+$ cells, including regulatory T-lymphocyte infiltration of $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$. PD1, PD-L1 and related cytokines were also abundantly expressed in the cancer tissues. The pattern was as follows: **(1)** PD1 was mainly expressed in infiltrating lymphocytes. **(2)** The distribution characteristics of PD-L1 and $CD8^+$ cells were diffusively distributed throughout the whole field of vision, especially clustered in tumor cells. **(3)** The interesting phenomenon is that we found that the expression of $CD4^+$ and $Foxp3^+$ cells was around the tumor cell population, but partially scattered in the tumor cell population.

From what has been discussed above, we draw the conclusion: **(1)**the occurrence and development of cervical cancer are closely related to the expression of PD1, PD-L1, and IL-2, IL-6, IL-10, the expression

of TGF-β, that is, with the development of the disease, the expression is on the rise, while the relationship between the expression of INF-γ is not clear from the current experimental data, which needs further study.**(2)** Increased $CD4^+$ lymphocytes were found in the peripheral blood of patients with CC, including $CD4^+CD25^+CD127^{-/low}$cells, and the expression level of PD1 was increased in $CD4^+CD25^+CD127^{-/low}$cells. **(3)** In CC tissues, there are a large number of infiltrating lymphocytes, mainly $CD8^+$ cells, and a large number of them infiltrate into the tumor cell aggregation area, while $CD4^+$ cells gather around the tumor cells. At the same time, the corresponding hypothesis was proposed: **(1)**Although an increase in $CD4^+CD25^+CD127^{-/low}$cells was detected in the CC group, no significant increase was detected in other groups. We know that $CD4^+CD25^+CD127^{-/low}$cells are naturally regulated T cells, and we hypothesized that with the development of CC, the body can be induced to produce adaptive regulatory T cells (aTreg), such as Tr1(type 1 regulatory T cell), Th3(T helper 3) cells. The secretion of IL-10 by Tr1 cells and TGF-β by Th3 cells can further promote the proliferation of $CD8^+$regulatory T cells, further promote the secretion of IL-6, IL-10, INF-γ, and play an immunosuppressive role. **(2)** $CD4^+$and $CD8^+$ T cells can infiltrate tumor areas, and it may be that T cells dominated by $CD8^+$ cells play an anti-tumor role. The distribution of $Foxp3^+$ regulatory T cells is similar to that of $CD4^+$ T cells, mainly surrounding tumor cell population, and may play a role of regulating tumor immunity mainly by secreting some cytokines, such as IL-10 and TGF-β, etc. The above assumptions lay the foundation for the further design of experimental scheme, and at the same time depends on further experiments to explore the truth.

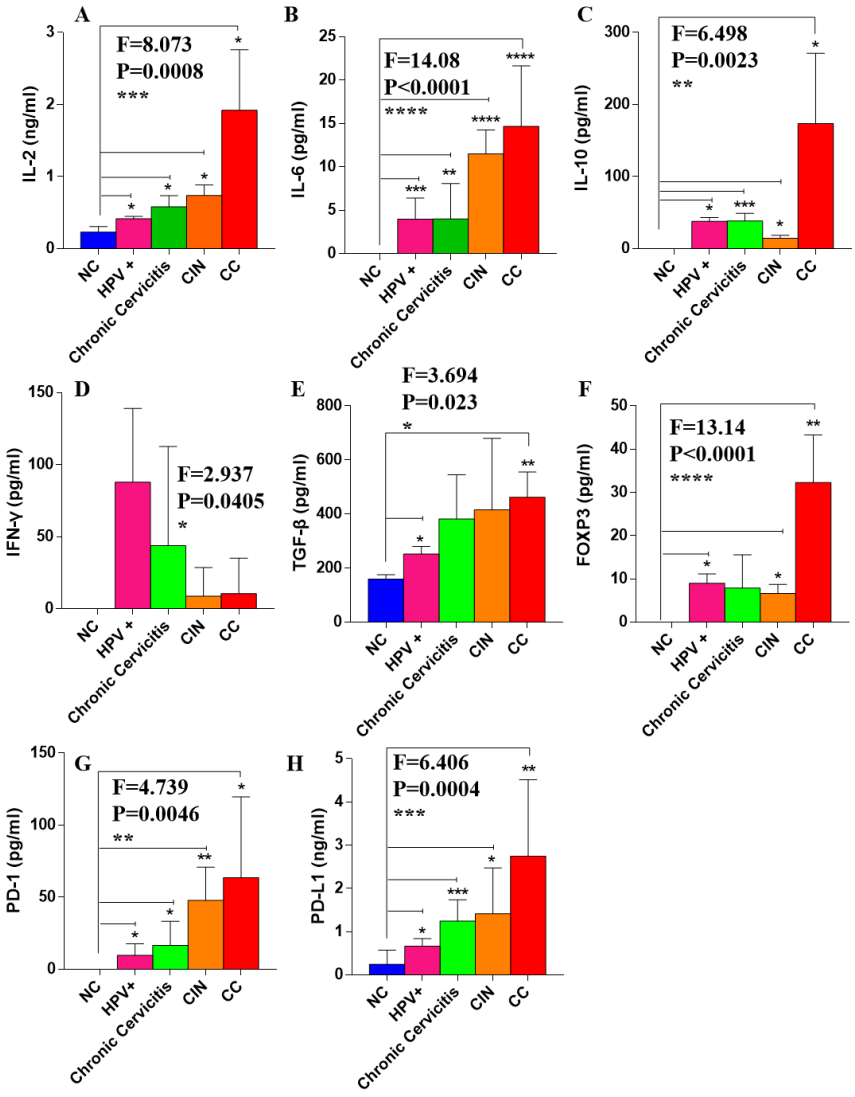

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Fig1. Expression of PD1 , PD-L1 and related cytokines in the development of cervical cancer(serum/plasma). The serum levels of IL-2(**A**), IL-6(**B**), IL-10(**C**),INF-γ(**D**),TGF-β(**E**), Foxp3(**F**), PD-1(**G**), and PD-L1(**H**) in**CC** , cervical **CIN** , chronic cervicitis, **HPV+**and **NC** were measured with commercial ELISA kits. Data are reported as means±SEM.*P <0.05;**P <0.01;***P <0.001;****P <0.0001(Student's t-test and ordinary one-way ANOVA).

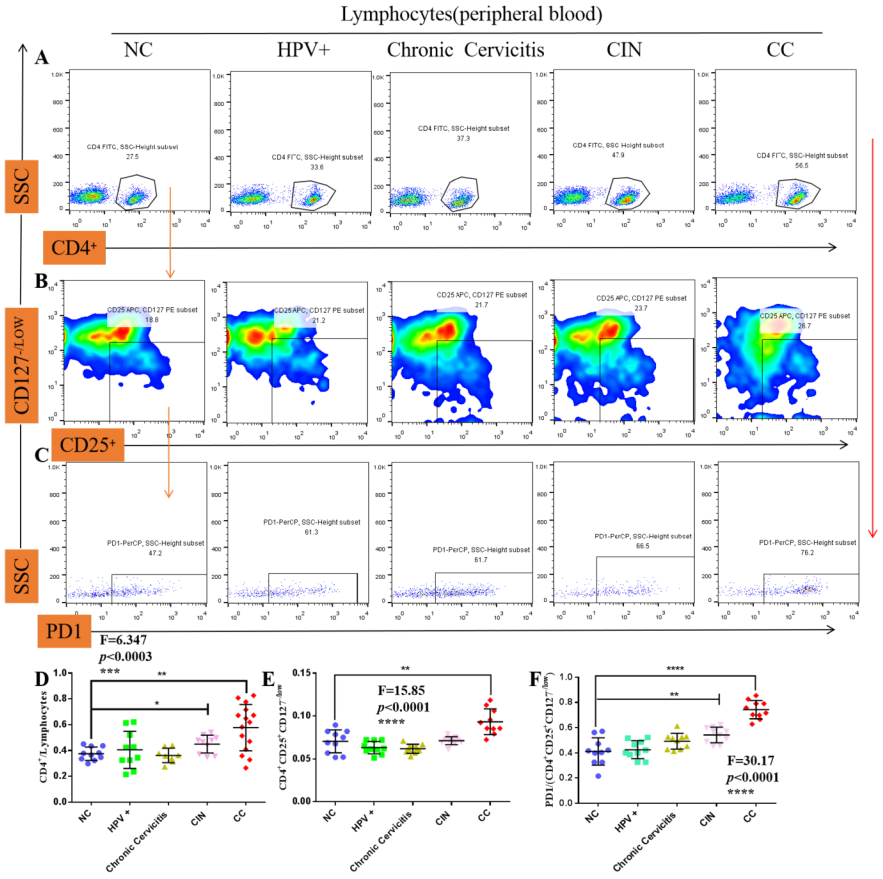

Fig2. Cell surface PD1 expression by $CD4^+CD25^+CD127^{-/low}$Tregs in the development of cervical cancer(peripheral blood). Gating strategy to identify PD1 on $CD4^+CD25^+CD127^{-/low}$Tregs(regulatory T cells) of CC, CIN, Chronic Cervicitis, HPV+ and NC in peripheral blood(**A-C**).The plots show combined date for flow cytometric analysis of levels of $CD4^+$ T cell(D), $CD4^+CD25^+CD127^{-/low}$Tregs(**E**) and PD1 expression on $CD4^+CD25^+CD127^{-/low}$Tregs(**F**) in CC, CIN, Chronic Cervicitis, HPV+ and NC. Scatter plot: Each dot represents one individual. Data are reported as mean±SEM. $^*P$ <0.05;$^{**}P$ <0.01;$^{***}P$ <0.001;$^{****}P$ <0.0001(Student's t test and ordinary one-way ANOVA).

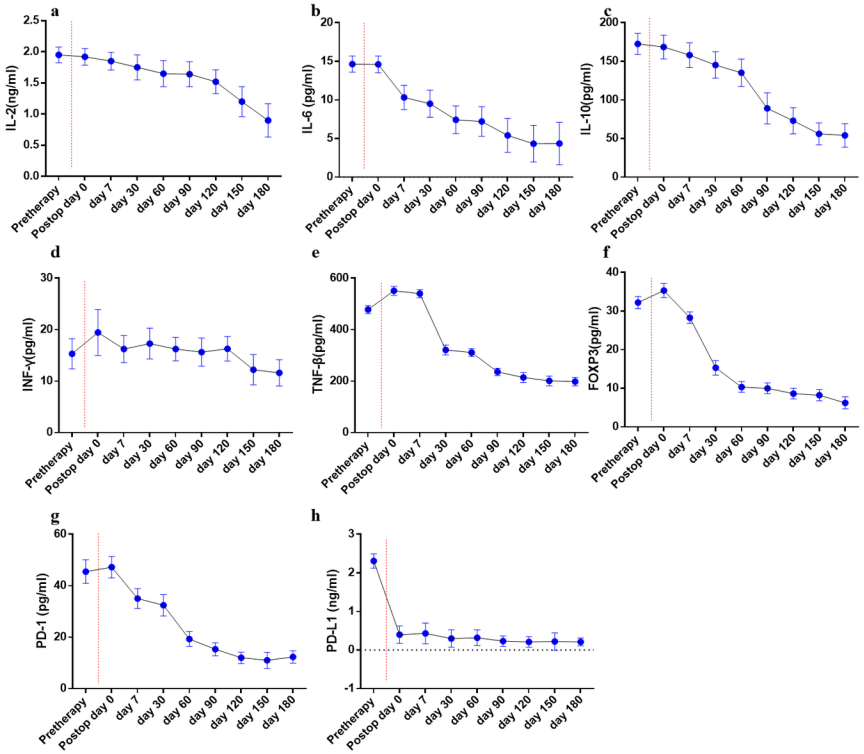

Fig3. Levels of PD1, PD-L1 and related cytokines before and after cervical cancer treatment. (serum) Five patients with cervical squamous cell carcinoma were followed up for 6 months to find out the changes of IL-2(**a**), IL-6(**b**), IL-10(**c**), INF-γ(**d**), TNF-β(**e**), Foxp3(**f**), PD1(**g**), PD-L1(**h**) in peripheral blood plasma before and after surgery measured with ELISA kits. And tested on the day after treatment, 7, 30, 60, 90, 120, 150 and 180 days. Data are reported as mean±SEM.

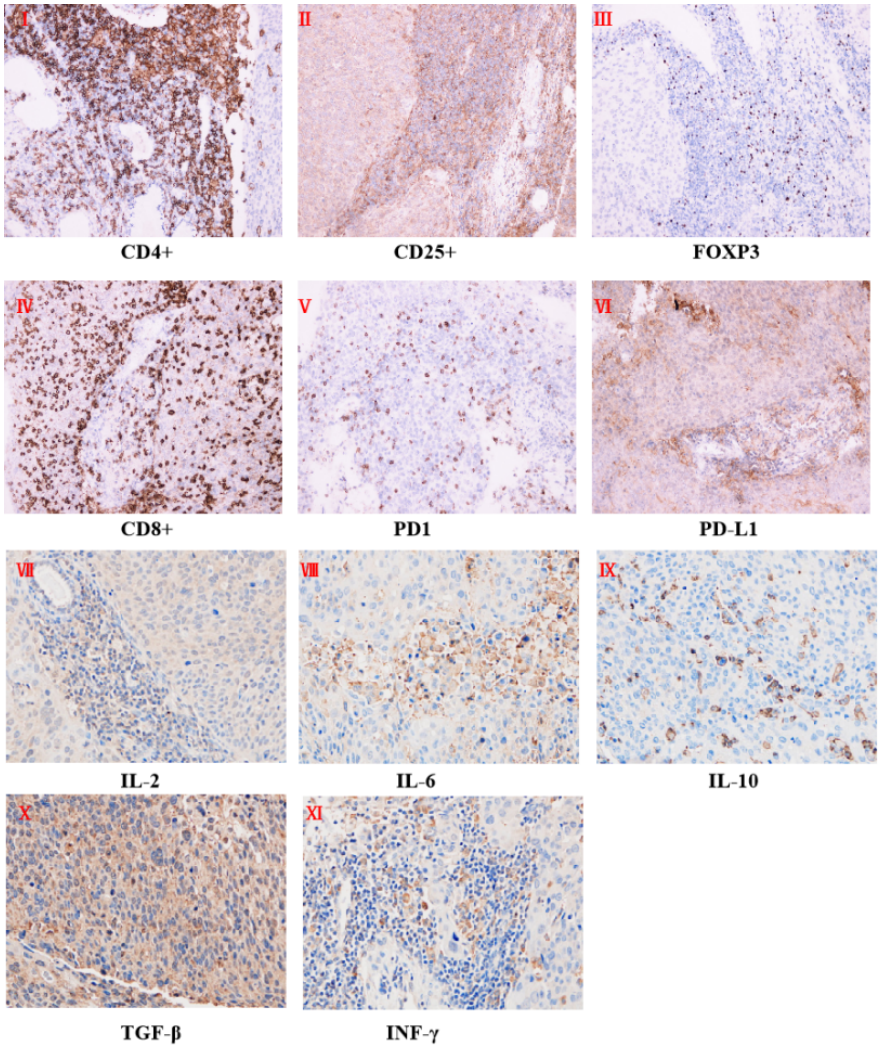

Fig4. Expression of PD1, PDL1 and related cytokines in CC tissues(Immunohistochemistry). $CD4^+$(**I**), $CD25^+$(**II**), Foxp3(**III**), $CD8^+$(**IV**), PD1(**V**), PD-L1(**VI**), IL2(**VII**), IL6(**VIII**), IL10(**IX**), TNF-β(**X**) and INF-γ(**XI**) were detected by immunohistochemical staining of the cervical cancer tissue. And pick out some representative tissue images to show. Images(**I -VI**) are from the same specimen, while (**VII-XI**) are from different representative patients.

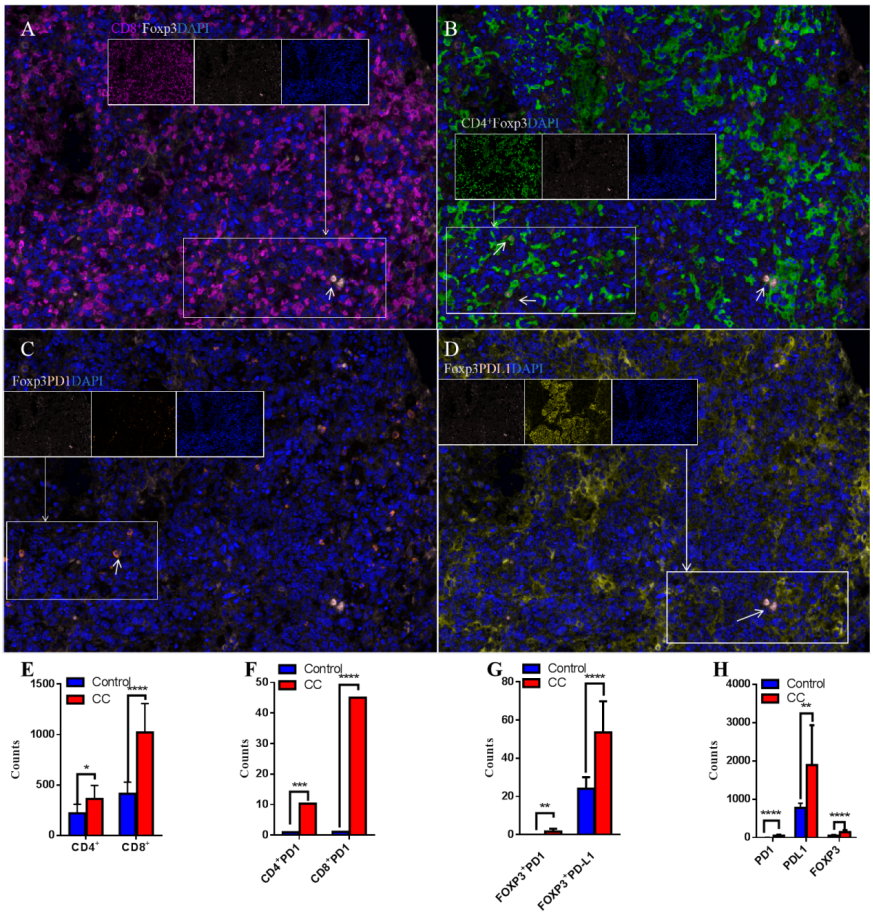

Fig5. In situ localization of CD4$^+$Foxp3$^+$, CD8$^+$Foxp3$^+$, Foxp3$^+$PD1 and Foxp3$^+$PD-L1 inCC tissues(multiple fluorescence immunohistochemical staining). Representative images show tissue sections from CC with (**A**) CD8$^+$-opal650(magenta), Foxp3-opal570(pink), and DAPI(4,6-diamidino-2-phenylindole; blue) labeling, (**B**)CD4$^+$-opal520(green), Foxp3-opal570(pink) and DAPI(blue) labeling, (**C**)Foxp3-opal570(pink), PD1-opal620(orange) and DAPI(blue) labeling, and (**D**)Foxp3-opal570(pink), PD-L1-opal540(yellow) and DAPI(blue) labeling. The graph show combined date for multiple fluorescence immunohistochemical staining analysis of counts of CD4$^+$ /CD8$^+$ T cell(**E**), CD4$^+$PD1/CD8$^+$PD1(**F**) and Foxp3$^+$PD1/Foxp3$^+$PD-L1(**G**) expression on CC and healthy cervical tissues from the operation. Images(**A**), (**B**), (**C**) and (**D**) are from the same specimen and representative of CC patients. All images were captured using an Evos FL Auto 2 cell imaging system(Thermo Fisher Scientific) and merged using image J software. Data are reported as mean±SEM. *P<0.05;**P<0.01;***P<0.001;****P <0.0001(Student's t test).

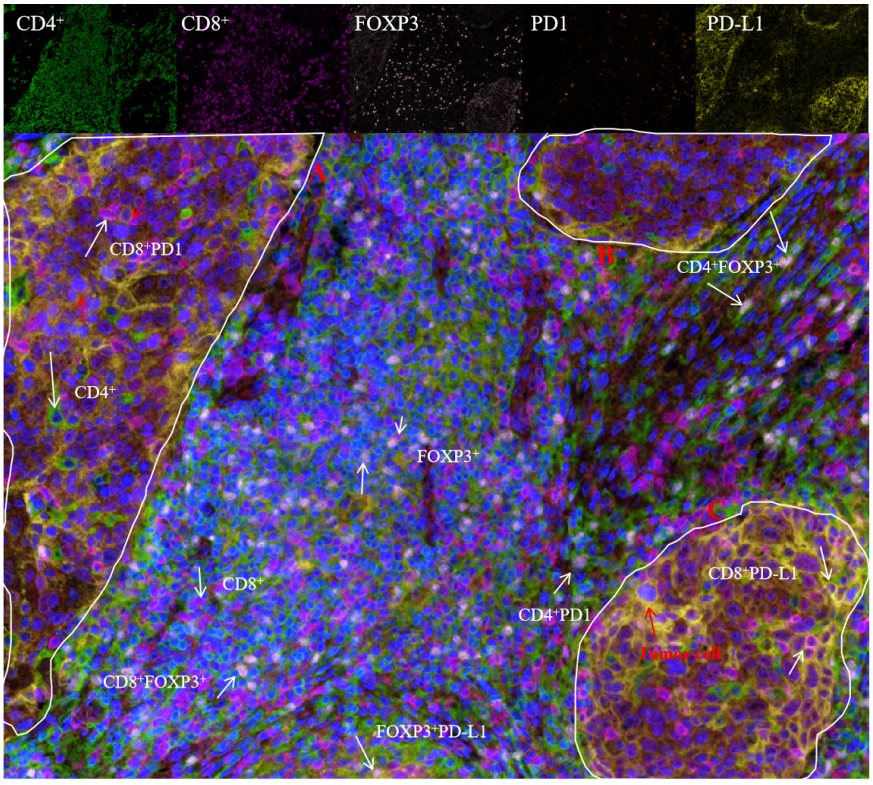

Fig6. In situ localization of CD4$^+$, CD8$^+$, Foxp3, PD1 and PD-L1 in CC tissues(multiple fluorescence immunohistochemical staining). Representative images show tissue sections from CC with CD4$^+$(green), CD8$^+$(magenta), Foxp3(pink), PD1(orange), PD-L1(yellow) and DAPI(blue) labeling. Looking at these images, it was found that the position presented by the Foxp3 staining almost coincides with that of the PD-L1, and the PD-L1 coincides with the location of tumor cell aggregation(**A-C**). CD8$^+$ T cell and PD1 were diffusely distributed, partially infiltrating tumor cell clusters and coincident with PD-L1 expression. Interestingly,the distribution of CD4$^+$ T cell was almost around the tumor cell population. All images were captured using an Evos FL Auto 2 cell imaging system(Thermo Fisher Scientific) and merged using image J software.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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