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IMAGES IN OBSTETRICS AND GYNECOLOGY

Blockade pf CD73/adenosine axis improves the therapeutic efficacy of docetaxel in epithelial ovarian cancer

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Abstract

Background Epithelial ovarian cancer (EOC) is the most common and lethal ovarian cancer which threatens women health. Nowadays, the standard treatment is maximal cytoreductive surgical debulking followed by chemotherapy. However, immunosuppressive environment generated by chemotherapy, which can be reversed by immunotherapy, leads to the failure of standard treatment. Therefore, a combination of chemotherapy and immunotherapy will be a promising strategy for EOC patients.

Method For in vitro study, CD73 expression, CD73 activity, and $CD8^+$ T-cell proliferation were measured after docetaxel (DTXL) treatment with or without CD73 antibody. For in vivo study, tumor growth, tumor weight, and the population of various immune cells in the tumor were analyzed in different drug treatment groups.

Results DTXL can increase both the CD73 expression and enzymatic activity in patient-derived epithelial cell and ID8 cell while causing immunosuppressive response which was reversed by anti-CD73 antibody (aCD73). Moreover, tumor growth and lung metastasis in mouse were significantly diminished after DTXL + aCD73 treatment.

Conclusion Blocking CD73/adenosine pathway could reverse the immunosuppression caused by DTXL, and a combination of DTXL with CD73 inhibitor or anti-CD73 antibody would be a more effective and promising therapy for EOC.

Keywords Epithelial ovarian cancer · Docetaxel · CD73 · Chemotherapy · Patient-derived cancer cell

Introduction

Ovarian cancer is one of the most common gynecological malignancies in the female genital organs, and accounts for the third highest morbidity following carcinoma of uterine cervix and corpus carcinoma [1, 2]. Among all the ovarian cancers, 50–70% originate from epithelial cells, signifying epithelial ovarian cancer (EOC) as the most common and lethal gynecological malignancy in women [3, 4]. EOC is a highly heterogeneous disease characterized by multiple histological subtypes. There are several treatment options including surgery, chemotherapy, molecularly-targeted therapies, and immune checkpoint therapy, which have gained increasing attention in the recent years [5–8]. Currently, maximal cytoreductive surgical debulking followed by the platinum-based chemotherapy is the standard-to-care treatment for EOC. Because surgical techniques do not have the ability to remove small or invisible tumor cells, chemotherapy is still necessary to lower the death rate of recurrent disease [9].

Docetaxel (DTXL) is a member of the taxane family of chemotherapy medications, commonly used to treat several types of cancers including breast cancer, head and neck cancer, stomach cancer, prostate cancer, ovarian cancer, and non-small-cell lung cancer in clinical. It can be administrated by itself or along with other chemotherapy medication such as carboplatin [9, 10]. Docetaxel binds to microtubules reversibly with high affinity, stabilizes microtubules to prevent depolymerization, and terminates cell division [11]. Docetaxel also leads to the phosphorylation of oncoprotein

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B-cell lymphoma 2 (bcl-2) that either inhibiting (pro-apoptotic) or inducing (anti-apoptotic) which reduces cell apoptosis [12].

An increasing number of studies have suggested that there is immunosuppressive microenvironment in ovarian cancers, such as the abnormal activation of Programmed Death 1 (PD-1)/Programmed Death Ligand 1 (PD-L1) signaling, high expression of indolamine 2,3-dioxygenase (IDO) and accumulation of myeloid-derived inhibitory T cells which are all the leading causes of the failure of standard cancer therapies. Moreover, chemotherapy could facilitate the formation of immunosuppressive microenvironment. Therefore, immunotherapy or a combination with chemotherapy will be a more promising and effective therapy for EOC [13–17].

In the current study, we found that treating patient-derived epithelial ovarian cancer cells with DTXL result in the significant upregulation of CD73, a cell surface expressed extracellular nucleosidase that converts 5′-AMP into adenosine. In turn, adenosine binds to its receptor A2AR to inhibit several immune-activated pathways to influence the activation and proliferation of T cells, and to prevent the cytotoxic effects of $CD8^+$ T cells [18–22]. However, combining DTXL and anti-CD73 effectively killed patient-derived EOC cells. Thus, we show that combining DTXL and anti-CD73 is an effective way to treat EOC and provide a promising outcome for EOC patients.

Method

Epithelial ovarian cancer tumor cell isolated from the EOC patient

Five epithelial ovarian cancer tissue samples were obtained from EOC patients and enzymatic digested as described to yield single cells [23, 24]. Single-cell suspensions were cultured in complete Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum (FBS, Invitrogen, USA, Waltham, MA USA), 2% v/v Penicillin Streptomycin at 37 °C, 5% CO_2, and 5–7% O_2. Cell were treated with DTXL (2 μg/mL) or with vehicle for 48 h and collected for analysis. Written consent was derived from the patients. Human study was approved by the Ethical Committee in Heze Municipal Hospital of Shandong Province.

Quantitative real-time PCR

Real-time reverse transcription PCR was performed using ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. SYBR green (Molecular Probes) was used to detect PCR products. All reactions were performed in a 20 μL reaction volume in triplicate.

PCR amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of PCR at 95 °C for 20 s and 60 °C for 30 s. Relative amount of target genes (CD73) was obtained from a standard curve and normalized against relative amount of GAPDH mRNA.

Western blot

Cell lysates were prepared and resolved on a polyacrylamide gel, transferred to PVDF membranes, and probed with mouse monoclonal primary antibody against human CD73 (Santa Cruz, Dallas, TX, USA) and β-actin (Cell Signaling Technology, Danvers, MA, USA). Secondary HRP-conjugated goat anti-mouse IgG secondary antibodies (Santa Cruz) were used at 1:1000 and blots developed by chemiluminescence (Pierce, Waltham, MA).

Flow cytometry

ID8 murine ovarian surface epithelial cell line (ATCC, Manassas, VA) was maintained in DMEM supplemented with 10% v/v FBS, 2% v/v PSG. Cells were collected after 12 h and stained with fluorescent-labeled antibodies against CD73 (clone), CD8 (clone), and CD45 (clone). Cells were washed twice in phosphate-buffered saline (PBS) buffer and resuspended for flow cytometry.

CD73 activity assay

CD73 activity was measured by 5′ nucleotidase (CD73) activity assay kit (ab235945, Abcam, Cambridge, MA, USA). Briefly, samples and standard curve were prepared, and then added into 96 wells along with reaction mix at 37 °C for 20 min. Next, stop solution added, and then developed I and II. After incubating at room temperature for 20 min, record absorbance at 670 nm.

Co-culture of ID8 cell and splenocytes

10^5 ID8 cells were seeded into each well of a 12-well plate. After 24 h, DTXL (2 μg/mL) or vehicle was added together with either anti-CD73 (Clone TY/23, BioXcell) or control IgG2a antibody (50 μg/mL) for 48 h. Fresh medium supplemented with 5′-AMP (20 μmol/L) was changed for 4 h at 37 °C. Next, medium was collected for culturing splenocytes isolated from fresh spleen of C57 mice. Splenocytes were seeded into 96-well plate as a density of 5×10^5 cells per well. At the same time, CD3 antibody (1 μg/mL) was supplemented for 72 h. Then, medium or cells were collected for further studies.

Carboxyfluorescein succinimidyl ester (CFSE)

Splenic lymphocytes isolated from a C57BL/6J mice were mixed with CFSE (1 μmol/mL, ThermoFisher Scientific, Waltham, MA, USA) for 9 min at 37 °C. Cells were washed three times and then seeded into 96-well plate for further studies [22].

ELISA

After splenocytes were kept in conditioned medium for 72 h, medium was collected for measuring IFN-γ expression by ELISA using mouse IFN-γ ELISA kit (ALPCO).

Tumor-load studies

Tumor-bearing BALB/C mice (9 weeks old) were given a 0.5 mL subcutaneous injection of 5×10^6 ID8 cells prepared as a single-cell suspension in PBS mixed with an equal volume of the cold Matrigel (10 mg/mL of protein) [25]. When the tumor volume reached 50–100 mm^2, mice were randomly divided into four groups, PBS, DTXL (2.5 mg/kg), aCD73 (5 mg/kg), and DTXL + aCD73. Drugs were administrated every 3 days and five times in total, and then, tumor and lung were harvested. Tumor volumes were measured every 2 days using the formula $V = 1/2$ (length × width). Lung was washed in PBS, paraffin-embedded, and sectioned for H&E staining. Lung metastasis was measured by enumerating the number of tumor nodules. Tumors were isolated and single cells prepared by enzyme digestion for flow cytometric analysis. All animal studies were in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, NIH), and approved by the Institutional Animal Care and Use Committee in Heze Municipal Hospital of Shandong Province.

Statistical analysis

Data are shown as means ± SEM, and were analyzed by one or two-way ANOVA analysis followed by a Bonferroni post hoc test.

Results

DTXL increases CD73 expression in EOC patient-derived epithelial ovarian cancer cell

Five epithelial ovarian cancer samples were obtained from EOC patients and then digested into single cells. Cells were treated for 2 days with 2 μg/mL DTXL, and collected and analyzed for the expression of Programmed Death 1 (PD-1), Programmed Death Ligand 1 (PD-L1), Cytotoxic T-Lymphocyte-Associated protein-4 (CTLA-4), Signal Transducer and Activator of Transcription 3 (STAT3), Cluster of Differentiation 39 (CD39), Cluster of Differentiation 73 (CD73), Indolamine 2.3-DiOxygenase (IDO-1), and Colony Stimulating Factor-1 Receptor (CSF-1R). Among all these genes, only CD73 mRNA were highly expressed in DTXL-treated groups (Fig. 1a). CD73 (also known as 5′-nucleotidase, 5′-NT) is a novel target for cancer immunotherapy. It is a key molecule of regulating the degradation of Adenosine monoPhosphate (AMP) into adenosine, endorsing the generation of an immunosuppressed and pro-angiogenic niche in the tumor microenvironment that promotes the onset and progression of cancer. Moreover, CD73 protein also significantly increased after DTXL administration in five different EOC samples (Fig. 1b). These results suggest that CD73 might be involved in the DTXL-activated immunosuppressive pathway.

DTXL elevates CD73 expression in murine ID8 epithelial ovarian cancer cell

Next, we analyzed the relevance of CD73 in a mouse model of ID8 epithelial ovarian cancer cell in which ID8 cell line is a syngeneic mouse model for testing Ovarian Cancer immunotherapies. Flow cytometry (Fig. 2a), RT-PCR, and Western blot (Fig. 2b) analysis indicated that CD73 expression significantly increased after DTXL treatment compared with vehicle-treated cells. CD73 is a key enzyme that serves to convert AMP to adenosine, and thus, we analyzed the enzyme activity of CD73 which was also elevated (Fig. 2c).

$CD8^+$ T cells (cytotoxic T cell, also known as killer T cell) play an important role in T-cell-mediated tumor immunosuppressive response and $CD8^+$ T-cell numbers in tumor site are a key denominator for overall survival in patients. We first incubated ID8 cells with or without DTXL in the supplement of 5′-AMP for 4 h, and then collected the medium and used them to culture CFSE labeled $CD8^+$ T cells to study the effects of adenosine generated by CD73 on $CD8^+$ T-cell proliferation. Compared with complete medium, conditioned medium without treatment inhibited T-cell proliferation due to the adenosine generated by CD73, and the inhibitory effect will further be enhanced in the existence of DTXL (Fig. 2d). These results suggest that induction of CD73 further increases the production of adenosine to inhibit tumor suppressive CD8 T cells.

Anti-CD73 antibody inhibits CD73 enzymatic activity and relieves CD73-mediated suppressive factors of T-cell proliferation in vitro

To test the efficacy of a CD73 neutralizing antibody, we measured CD73 activity after CD73 blockade by anti-CD73 antibody in ID8 cells (Fig. 3a). CD73 blockade

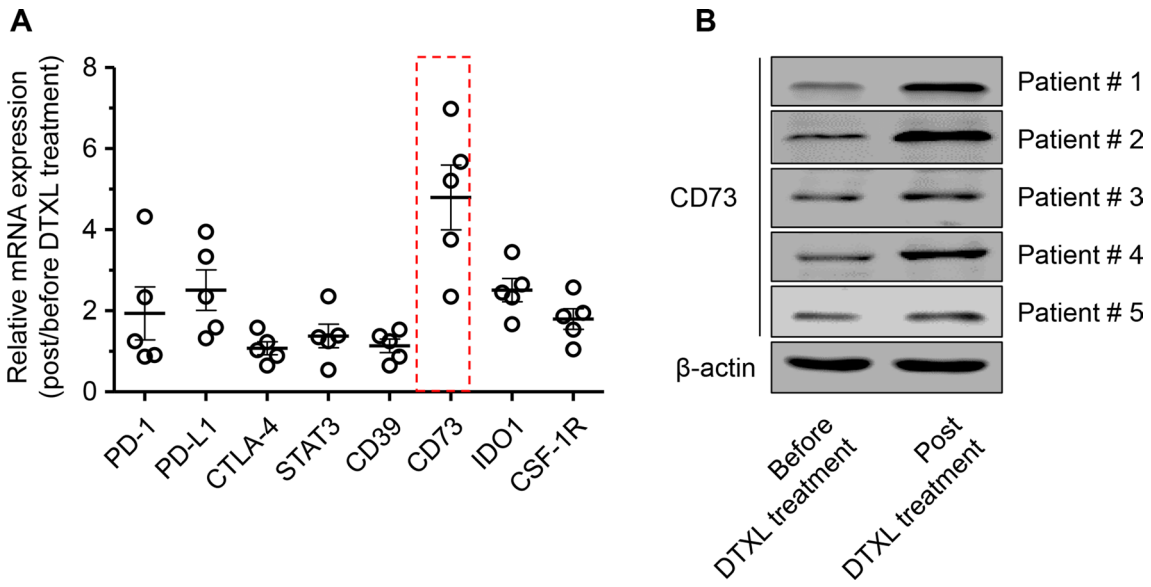

Fig. 1 DTXL treatment induces upregulation of CD73 in patient-derived epithelial ovarian cancer cells. **a** Gene expression in patient-derived epithelial ovarian cancer cells post-DTXL treatment. Five epithelial ovarian cancer tissue samples were obtained from EOC patients and enzymatically digested into single cells. Cells were cultured in complete DMEM medium-containing 2 μg/mL DTXL for 2 days, and then harvested for measuring the immunosuppression-related gene expressions (including *PD-1*, *PD-L1*, *CTLA-4*, *STAT3*, *CD39*, *CD73*, *IDO1*, and *CSF-1R*) by real-time PCR. *GAPDH* was detected as control. **b** The protein level of CD73 in patient-derived epithelial ovarian cancer cells after DTXL treatment was examined by western blot. β-actin was used as housekeeping control

caused increased $CD8^+$ T-cell proliferation (Fig. 3b) which was previously suppressed by the conditioned medium with or without DTXL treatments, suggesting that anti-CD73 relieves CD73-mediated suppression of $CD8^+$ T-cell proliferation.

Interferon gamma (IFN-γ) plays important roles in inhibiting cancer growth through its anti-proliferation and autophagic and non-autophagic apoptosis inducing properties. We hypothesized that IFN-γ may also play a role in CD73-mediated inhibition of T-cell proliferation. We measured the level of IFN-γ in ID8 medium treated with or without DTXL, and found that IFN-γ was decreased after DTXL-treated (Fig. 3c). Treatment with anti-CD73 abolished DTXL induced suppression of IFN-γ, suggesting that DTXL inhibits IFN-γ expression by increasing CD73 and this inhibition is reversible by CD73 blockade.

In parallel to inhibiting proliferation of cytotoxic $CD8^+$ T cell, inducing cancer cell death is another promising choice for cancer therapy. To test whether anti-CD73 and DTXL can induce apoptosis in ID8 cancer cell lines, we co-cultured ID8 cells with anti-CD73 or a control IgG with or without DTXL for 48 h and measured apoptosis by Annexin V and propidium iodine. While DTXL alone does not cause cancer cell death, a combination of aCD73 and DTXL potently induced apoptosis in ID8 cancer cells (Fig. 3d), suggesting a cooperative action between anti-CD73 and DTXL.

Combined therapy significantly inhibits tumor growth and reduces lung metastasis in murine ID8 epithelial ovarian cancer

Since DTXL and anti-CD73 was effective in ID8 cancer cell lines in vitro, we further explored this combined therapy in vivo in a mouse model. ID8 cells were injected subcutaneously into the right flank of female BALB/C mice. Once the tumor volume reached 50–100 mm^2, mice were randomly divided and treated with PBS, DTXL (2.5 mg/kg), aCD73 (5 mg/kg), and DTXL + aCD73 once every 3 days for 15 days. Tumor volume was measured every 2 days as showed in Fig. 4a. While DTXL or anti-CD73 alone could reduce the tumor growth, a combination of DTXL and aCD73 dramatically slowed tumor growth compared to single agent alone (Fig. 4b).

Lung metastasis is the most common metastasis symptom in late ovarian cancer patients, and therefore, we also measured lung metastasis in mice. H&E staining in paraffin-embedded sections of the lung showed that DTXL + aCD73 effectively diminished the lung metastasis (Fig. 4c). Pulmonary metastatic nodules which are a marker of lung metastasis also verified that DTXL did not change the lung metastasis, which might be the cause of recurrent disease in clinical for treating ovarian cancer patients, but DTXL + aCD73 combination significantly reduced the number of pulmonary metastasis nodules (Fig. 4d) compared with aCD73 alone.

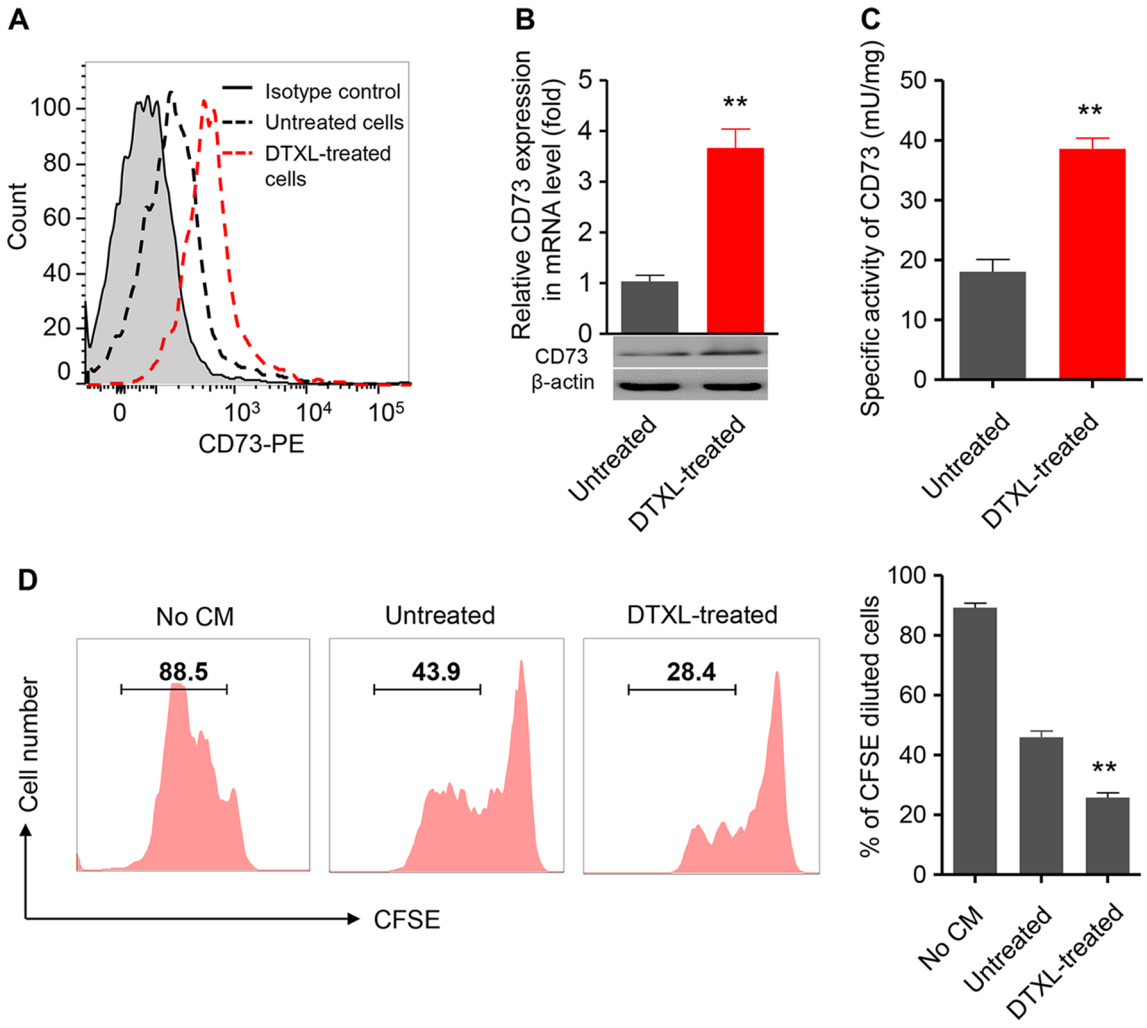

Fig. 2 DTXL treatment induces upregulation of CD73 in murine ID8 epithelial ovarian cancer cell. ID8 epithelial ovarian cancer cells treated with or without DTXL (2 μg/mL) for 48 h and then analyzed CD73 expression by flow cytometry (**a**), and RT-PCR and Western blot (**b**). Data represent as means ± SEM. $^{**}p < 0.005$ (versus untreated group). **c** The CD73 enzymatic activity in untreated or DTXL-treated ID8 cells examined by 5′-nucleotidase (CD73) Activity Assay Kit. **d** Untreated and DTXL-treated ID8 cells were incubated with conditioned medium in the presence of 20 μmol/L 5′-AMP for 4 h at 37 °C. Fresh isolated splenocytes labeled with CFSE were incubated with conditioned medium collected from the above ID8 cultures or regular complete medium as a control, and 1 μg/mL anti-CD3 monoclonal antibody (mAb) was added for 72 h. CFSE dilution was measured by flow cytometry by gating on $CD8^+$ T cells. Data represent as means ± SEM. $^{***}p < 0.005$ (versus untreated group)

Although DTXL alone is not effective in treating EOC patients, our data suggest that DTXL + anti-CD73 could effectively inhibit lung metastasis in EOC patients.

DTXL + aCD73 activates immune response by stimulating/inhibiting immune cells in tumor

To look at whether DTXL and anti-CD73 treatment also modulated immune response in our ID8 mouse model, we analyze several immune cells population in ID8 cell-injected tumor tissues under various treatments. Specifically, we analyzed three immune cells, cytotoxic T cells defined by $CD45^+CD3^+CD8^+$, T-regulatory cells defined by $CD45^+CD3^+CD4^+Foxp3^+$, and myeloid-derived $CD45^+CD11b1Gr\text{-}1$ cells. $CD45^+CD3^+CD8^+$ T cells (Fig. 5b) are cytotoxic cells that can kill cancer cell, which is beneficial to cancer therapy. DTXL + aCD73 remarkably stimulated $CD8^+$ cell infiltration in the tumor, which was not obvious under the treatment of DTXL or aCD73, respectively. On the contrary, another two immune cells that suppress the immune responses, $CD45^+CD4^+CD4^+Foxp3^+$ T-regulatory cell (T reg) and $CD45^+CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs) were both significantly reduced after DTXL + aCD73 treatment (Fig. 5c, d). aCD73 alone decreased the infiltration of T regs in the tumor, consistent with a functional role of CD73 on T regs.

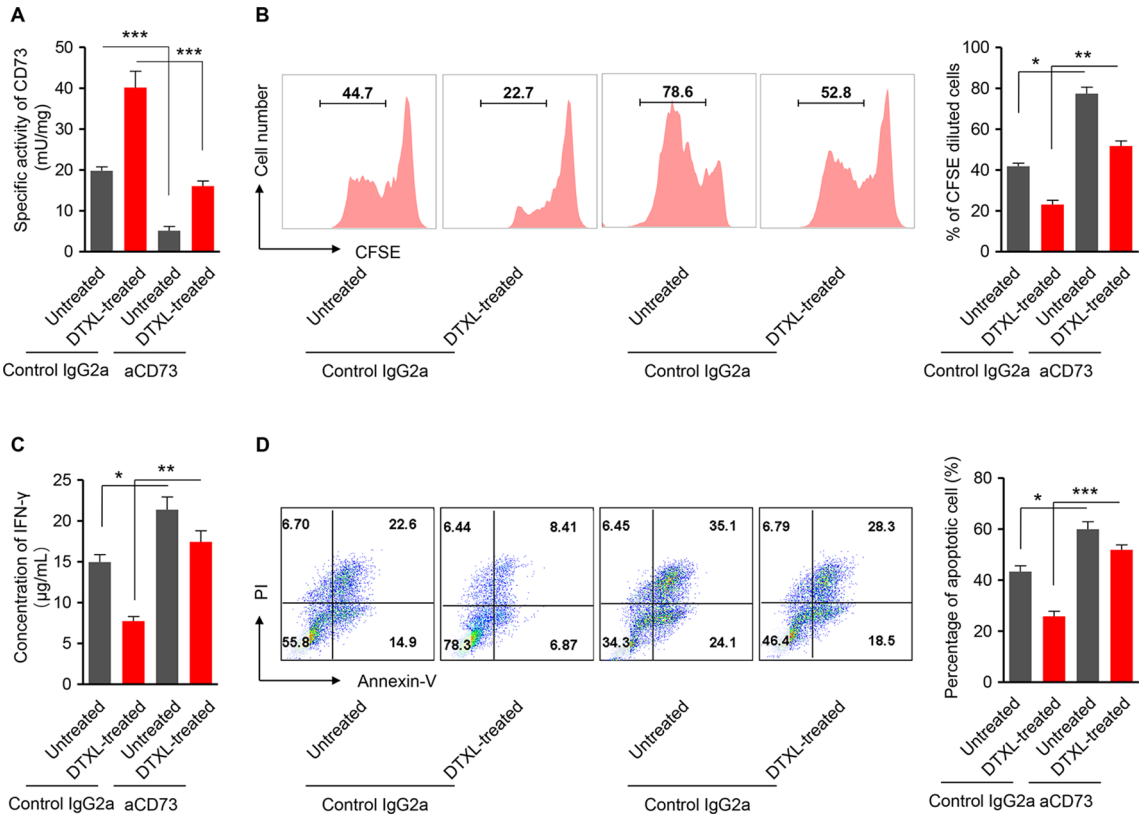

Fig. 3 Anti-CD73 antibody inhibits the CD73 enzyme activity and relieves CD73-mediated suppression of T-cell proliferation in vitro. **a** ID8 cells were treated with or without DTXL (2 μg/mL) in the presence of 50 μg/mL aCD73 (Clone TY/23) or control IgG2a for 24 h, and then, the enzymatic activity of CD73 was analyzed. **b** aCD73 relieves CD73-mediated suppression of T-cell proliferation. ID8 cells were treated with DTXL and antibodies as above mentioned, and then incubated with conditioned medium in the presence of 20 μmol/L 5′-AMP for 4 h at 37 °C. Splenocytes labeled with CFSE were then incubated with conditioned medium from the above ID8 cultures, and 1 μg/mL anti-CD3 monoclonal antibody was added for 72 h. CFSE dilution was measured by flow cytometry by gating on $CD8^+$ T cells. Data represent means ± SEM. $^*p < 0.05$, $^{***}p < 0.005$. **c** Release of IFN-γ by splenocytes after incubated with the conditioned medium. Splenocytes were incubated with above-conditioned medium from ID8 cell culture in the presence of anti-CD3 monoclonal antibody for 72 h. The concentration of released IFN-γ was examined by ELISA. Data represent means ± SEM. $^{**}p < 0.005$. **d** Splenocytes were incubated with above-conditioned medium in the presence of anti-CD3 monoclonal antibody, and then co-cultured with ID8 cells. Two days later, cells were harvested for the detection of apoptosis by double staining with Annexin V and propidium iodide (PI) using flow cytometry by gating on $CD45^-$ cells. Data represent as means ± SEM. $^*p < 0.05$. $^{****}p < 0.001$

Discussion

Our study discovered that DTXL can induce both the mRNA and protein e expression and enzymatic activity of CD73 not only in patient-derived epithelial ovarian cancer cells, but also in murine ID8 epithelial ovarian cancer cells. CD73 can catalyze 5′-AMP into adenosine which forms immunosuppressive environment through inhibiting $CD8^+$ T-cell proliferation, reducing IFN-γ secretion, and decreasing $CD45^+$ cell apoptosis. All these effects can be reversed by blocking CD73 using anti-CD73 antibody. Furthermore, we used mouse model of subcutaneous tumors by injecting ID8 rumor cells into the mouse back, and confirmed that a combination therapy of DTXL + aCD73 treatment could most effectively slow tumor growth, diminish tumor weight, and inhibit lung metastasis than either DTXL or aCD73 alone, and flow cytometry further verified that this combined therapy could increase the infiltration of $CD8^+$ T cells, at the same time, reducing the proportion of $CD45^+$ $CD3^+$ $CD4^+$ $Foxp3^+$ T-regulatory cells and $CD45^+$ $CD11b^+$ $Gr\text{-}1^+$ myeloid-derived suppressor cells. Our study discovered that blocking CD73/adenosine pathway could partially or totally reverse immunosuppressive effects caused by DTXL treatment, and suggest a combination of DTXL and small molecule inhibitor targeted CD73 or CD73 antibody could be a much more effective and promising therapy for EOC patients.

However, much knowledge still undiscover. It is not clear that how DTXL forms the immunosuppressive environment. DTXL was reported to be accumulated in ovarian adenocarcinoma cells than kidney carcinoma cells that might explain the reason that DTXL contributes more effective for OC

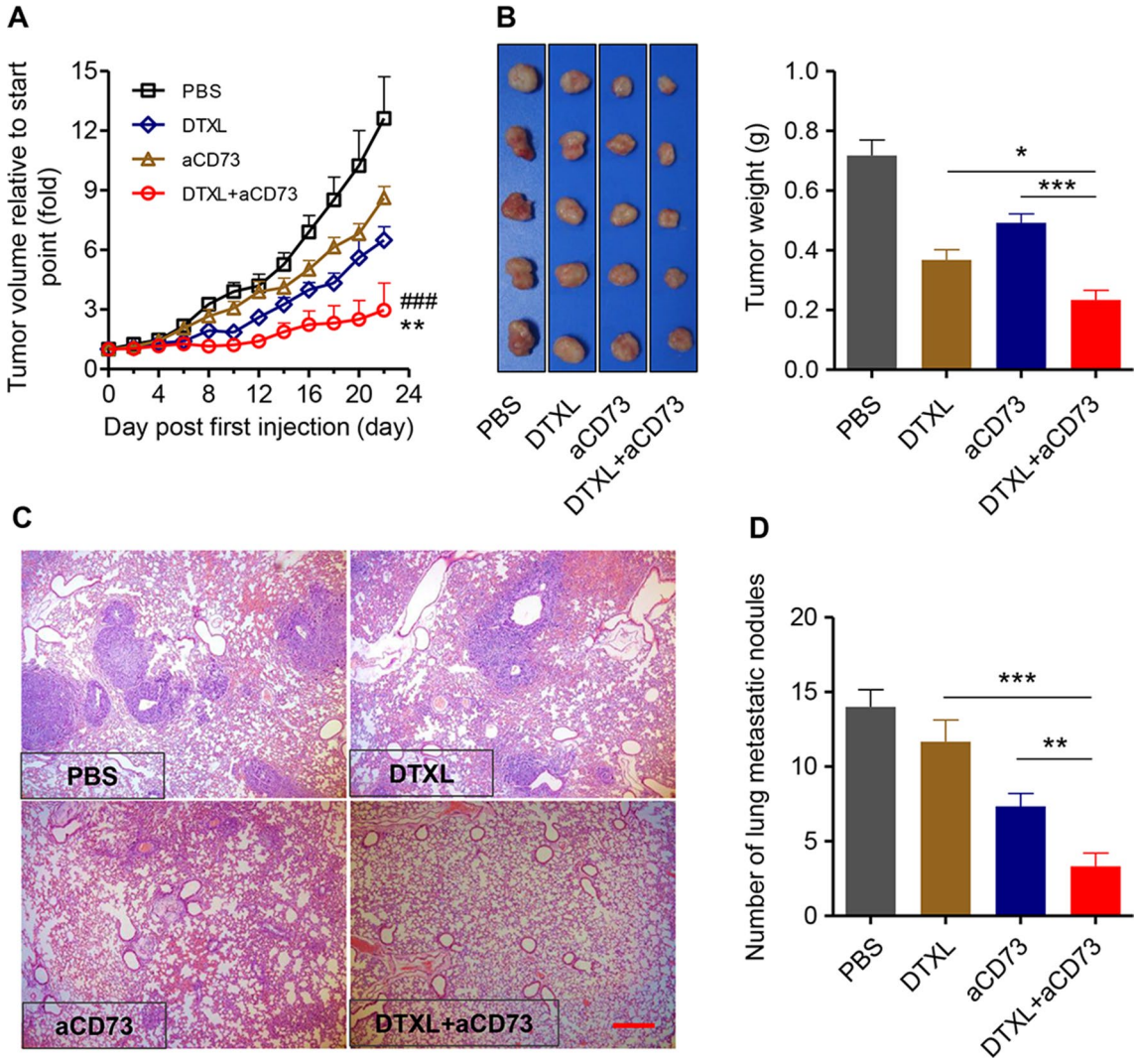

Fig. 4 Combination therapy significantly inhibits tumor growth and reduce lung metastasis in murine ID8 epithelial ovarian cancer. **a** Inhibition of tumor growth by various treatments (PBS, DTXL, aCD73, and DTXL + aCD73) in ID8 tumor-bearing BALB/c mice ($n = 5$). The injection dose of DTXL and aCD73 was 2.5 mg/kg and 5 mg/kg, respectively. DTXL + aCD73 versus DTXL, $^{***}p < 0.005$; $^{****}p < 0.001$. **b** Images of ID8 xenograft tumors (left) and tumor weight (right) at the end point of treatment. Data are shown as means ± SEM ($n = 5$ per group). $^{*}p < 0.05$, $^{****}p < 0.001$. **c** H&E staining of lung metastases from mice treated with different drugs. Scale bar, 20 μm. **d** The average numbers of the pulmonary metastatic nodules in ID8 tumor-bearing mice at the end of treatment ($n = 5$ per group)

patients than other cancer patients, especially combined with other chemotherapy medication like carboplatin. Nevertheless, our study demonstrated that DTXL alone could increase immunosuppressive responses by activating CD73/adenosine pathway which leads to the activation of downstream cascade reactions, such as inhibiting $CD8^+$ T-cell infiltration and proliferation, reducing IFN-γ secretion, and decreasing $CD45^+$ cell apoptosis. Another reason for the ineffectiveness of DTXL to cure EOC patients might be the failure of well-controlling lung metastasis. In late ovarian cancer patients, lung metastasis is the most common metastasis symptom. Even DTXL can effectively suppress the development of epithelial ovarian cancer, if lung metastasis could not be well controlled or inhibited, the cancer cells still existed and would quickly grew and develop in the lung; eventually, the patients got recurrent disease. Therefore, DTXL or chemotherapy medication itself has its limitation for treating EOC patients, even with other chemotherapy medication together. Moreover, DTXL, like most of the chemotherapy medication, can cause some side effects. Some common side effects include hair loss, low blood cell counts, numbness, shortness of breath, vomiting, and muscle pains; other severe ones include allergic reactions and future cancers. Moreover, it was reported that side effects are more popular in people

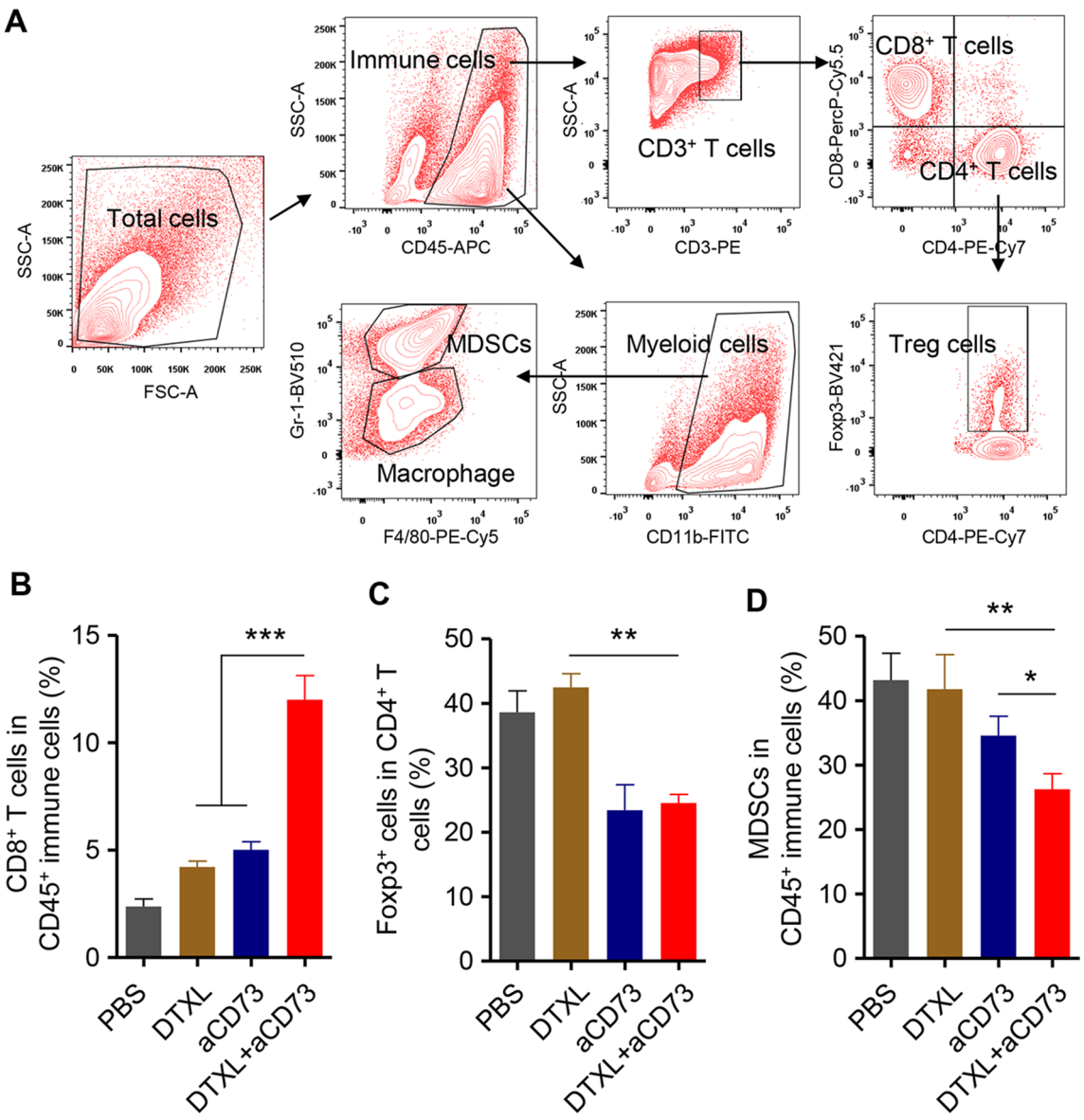

Fig. 5 Analysis of immune cells in tumor tissues after treatment. **a** Gating strategy to identify $CD45^+CD3^+CD8^+$ (or $CD4^+$) T cells, $CD45^+CD3^+CD4^+Foxp3^+$ regulatory T cells, and $CD45^+CD11b^+Gr\text{-}1^+$ MDSCs. Cell populations were gated sequentially as arrows indicated. **b–d** Relative abundance of various immune cells in ID8 tumor tissues at the end of treatment by flow cytometry. These cells included $CD45^+CD3^+CD8^+$ T cells (**b**), $CD45^+CD3^+CD4^+Foxp3^+$ regulatory T cells (**c**), and $CD45^+CD11b^+Gr\text{-}1^+$ MDSCs (**d**). The error bars represent as means ± SEM (*$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$; $n = 5$ per group)

with liver problem. All these reasons impel us to explore a more effective, safe, and promising therapy for EOC, and might be a hint for other cancers.

CD73 is a novel target for cancer immunotherapy. It facilitates 5′-AMP degrading into adenosine, which generates an immunosuppressed and pro-angiogenic niche so as to promote the cancer development. Therefore, blocking CD73/adenosine pathway can inhibit immunosuppressive responses, facilitate immune responses, and benefit for immunotherapy for cancer. In parallel to chemotherapy, immunotherapy has some advantages, including less side effects, easily activated immune response. In this study, anti-CD73 remarkably reduced the two major immunosuppressive cells (T-regulatory cells and myeloid-derived suppressive cells) in the tumor, while DTXL alone had no effect on it at all. However, immunotherapy also has its limitation. As Fig. 4b showed, anti-CD73 antibody treated itself was not as effective as DTXL alone on inhibiting tumor growth and tumor weight, even the former was more effective in suppressing lung metastasis. Altogether, either chemotherapy or immunotherapy administration alone has their disadvantages and advantages, it is better to combine these two therapies

together, as to a great extend come into play in epithelial ovarian cancer, or maybe other more cancer therapy.

Our results discovered a much more promising and effective therapy for treating epithelial ovarian cancer both in vitro and in vivo. In brief, chemotherapy medication DTXL alone could inhibit tumor growth but not lung metastasis, and cause immunosuppressive response, while an immunotherapy target CD73 was improved to restrain immunosuppressive environment and lung metastasis, and hence, a combination of both can compensate for each other. However, the mechanism was still not fully understood. For example, how the expression and enzymatic activity of CD73 was upregulated without altering its upstream regulator CD39 gene expression by DTXL is still unknown.

Therefore, there are several possibilities that how DTXL affects CD73 without altering CD39. One possibility is that DTXL directly promotes anti-apoptosis or suppresses pro-apoptosis of CD73 or CD39 without changing its mRNA expression; otherwise, DDTXL indirectly acts on CD73 through some mediators, which is not relevant to CD39. All these hypotheses are still in the box that needs to be explored.

Nevertheless, even a combined therapy of chemotherapy medication and immunotherapy medication was improved effective for treating tumor growth and lung metastasis in the mouse, it is unknown that whether this combined therapy will work or not in the EOC patients.

Another challenge is that there is no small molecule inhibitor targeted CD73 nowadays, it still takes time to design, develop, and testify the small molecular inhibitory of CD73, because administration CD73 antibody to the patients in clinical for treating EOC patients is not ideal, due to its high dose of anti-CD73 antibody and also its expensive cost.

In total, our study demonstrated that a combined therapy of chemotherapy medication DTXL and immunotherapy medication (anti-CD73 antibody) remarkably inhibits the tumor growth and its lung metastasis in mouse, and provides a much more effective and promising therapy for treating epithelial ovarian cancer. Furthermore, this might be a hint for other cancer therapy.

Conclusion

Our study demonstrated that a combined therapy of chemotherapy medication DTXL and immunotherapy medication (anti-CD73 antibody) remarkably inhibits the tumor growth and its lung metastasis in mouse, and provides a much more effective and promising therapy for treating epithelial ovarian cancer. Furthermore, this might be a hint for other cancer therapy.

Author contributions Data collection and analysis: HL, ML, BQ, and XL; study designed and manuscript writing: XL. All authors approved the final submission.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Research involving human participants and/or animals Human study was approved by the Ethical Committee in Heze Municipal Hospital of Shandong Province. All animal studies were in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, NIH), and approved by the Institutional Animal Care and Use Committee in Heze Municipal Hospital of Shandong Province.

Informed consent Written consent was derived from the participants.

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