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D-MT Prompts the Anti-Tumor Effect of Oxaliplatin by Inhibiting IDO Expression in A Mouse Model of Colon Cancer

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ABSTRACT

Colon cancer is one of the most common malignant tumors in the digestive system. Although oxaliplatin, a chemotherapy drug, has been clinically used to treat colon cancer, its therapeutic effect is unsatisfactory. It has been proved that indoleamine dioxygenase 2,3 (IDO), an immune checkpoint, is a result of tolerance to chemotherapy. Herein, an IDO inhibitor, D-MT (indoximod, 1-Methyl-D-tryptophan), was combined with oxaliplatin to treat colon cancer in mice. T cell infiltration in tumor tissues, the ratios of immune cells in the spleens, and the tumor growth and survival of the mice were detected and recorded. The results showed that the combination of oxaliplatin and D-MT significantly inhibited tumor growth and prolonged the survival of tumor-bearing mice. More importantly, the combination treatment increased the ratios of CD4+ T, CD8+ T and NK cells from the spleen in tumor-bearing mice, and prompted T cell infiltration in tumor tissues. This study provided a new therapeutic strategy for colon cancer treatment in the clinic, especially for patients with oxaliplatin resistance.

Keywords:

colon cancer · IDO · oxaliplatin · anti-tumor immune response

Introduction

Colon cancer is one of the most common malignant tumors of the gastrointestinal tract, with its incidence rising. Worldwide, colon cancer ranks the third in the incidence of male malignancies and fourth in mortality, and ranks the second in the incidence of female malignant tumors and third in mortality[1,2]. In 2018, about 1.8 million colon cancer cases were diagnosed, with 881,000 patients dying of the disease. In the last decade, the incidence and mortality rates of colon cancer have been increasing in certain countries, such as China[1]. Colon cancer is a major burden for patients worldwide. At present, the main treatment for colon cancer is surgery, with chemotherapy often performed following surgery for patients with metastasis. The response rate of chemotherapy could reach up to 50%, but drug resistance has been reported in nearly all patients with colon cancer[3]. Therefore, it is important to find a more effective treatment.

Oxaliplatin is a new anticancer platinum-based drug commonly used in the treatment of metastatic colorectal and liver cancer. Due to the heterogeneity of a tumor, a single drug often does not achieve satisfactory results. As reported, oxaliplatin treatment would lead to a reduction in macrophages and raised the expression of HMGB1 related to immunosuppression in the colon[4]. Therefore, studies have been trying to combine oxaliplatin with other therapies to treat tumors, improving the therapeutic effect of oxaliplatin[5-7]. Studies have shown that Huaier can effectively improve the anti-tumor effect of oxaliplatin by downregulating the expression of YAP protein which located in cell nucleus and related with cell proliferation, apoptosis and migration[8]. With the development of immunology, tumor immunotherapy has received more and more attention[9]. It has been found that anti-tumor immunity in colon cancer patients is inhibited to varying degrees, and thus enhancing the anti-tumor immune response may become an effective means of treating colon cancer. Consequently, chemical and immune combination treatment has become a new approach to cancer treatment[10].

Studies have confirmed that the tumor microenvironment plays an important role in the tumor development stage[11,12]. Tumor cells have the ability to evade host immune surveillance, which is one of the most important pathological features of tumor

formation and metastasis. For cancer patients, it is critical to reactivate and enhance the anti-tumor capabilities of their immune cells. The occurrence and development of tumors are highly correlated with the host immune response. Clinical studies have found that, although tumor-bearing patients produce potent anti-tumor immune responses, malignant tumors can continue to proliferate and metastasize. Tumor formation and development are associated with multiple factors, such as tumor immune escape, activation of inhibitory tumor immune response and failure of effective tumor immunity. There is a large number of immunosuppressive factors in the tumor microenvironment. These immunosuppressive factors play an important role in inhibiting T cells, such as indoleamine dioxygenase 2,3 (IDO)[13-15].

IDO is a rate-limiting enzyme of the immunosuppressive tryptophan (Trp)/kynurenine (Kyn) metabolic pathway, including IDO1 and IDO2, which are highly expressed by tumor tissues, thereby, inhibiting T cell anti-tumor immunity. IDO has been found to be highly expressed in several types of human cancer[16,17], such as melanoma[18-20], and colon[21,22], brain[23] and ovarian cancer[24]. In tumors such as acute myeloid leukemia [25-27], the high expression of IDO inhibits anti-tumor immunity; therefore, IDO can serve as a new target for the treatment of tumors[28,29]. A variety of IDO inhibitors have been approved for clinical trials by the US FDA. D-MT has been shown to be effective in inhibiting the expression of IDO[30]. A previous study found that the inhibition of the IDO expression in melanoma-bearing mice enhances the anti-tumor effect of pimozide[31], but it remains unclear whether IDO inhibition can promote the anti-tumor effect of oxaliplatin by increasing the anti-tumor immune response in tumor-bearing mice.

The present study explored the therapeutic effect of oxaliplatin combined with D-MT, an IDO inhibitor, on colon cancer, as well as the underlying mechanism. This study will provide a new method and experimental and theoretical basis for the clinical treatment of colon tumor.

Results

Combination treatment with D-MT + oxaliplatin significantly inhibits IDO and p-Stat3 expression in CT-26 cells

To detect the effectiveness of D-MT + oxaliplatin on the expression levels of IDO and p-Stat3 in colon cancer cells, the dosage of D-MT or oxaliplatin was 2.5 μg/ml (the data not shown). As shown in Fig. 1, both of the IDO and p-Stat3 expression levels were significantly inhibited in the CT26 cells after treatment with D-MT for 24 or 48h, while those of expression levels were increased by treatment with oxaliplatin only. Interestingly, with the combination treatment of D-MT and oxaliplatin, the IDO and p-Stat3 expression levels in the cells were comparable with those of the D-MT group.

Combination treatment with D-MT + oxaliplatin inhibits tumor growth and prolongs the survival of CT-26 cell-bearing mice

In order to determine the therapeutic effect of D-MT + oxaliplatin on tumor-bearing mice, mouse survival and tumor weight were recorded. As shown in Fig 2a,b and d, 7 days after the last treatment, tumor growth was significantly inhibited in the oxaliplatin group, or D-MT + oxaliplatin group. Of note, the average tumor weight of mice treated with D-MT + oxaliplatin was the lowest. In addition, treatment with D-MT + oxaliplatin significantly prolonged the survival time of colon tumor-bearing mice, as compared with that of mice in other groups (Fig. 2c).

Combination treatment with D-MT + oxaliplatin promotes cell apoptosis in tumor tissues

Next, in order to determine whether treatment with D-MT + oxaliplatin damaged tumor cells, cell apoptosis was examined in tumor tissues by TUNEL, and the expression of cleaved caspase-3 by western blotting. First, the TUNEL results showed that the number of cells with positive staining significantly increased in D-MT or oxaliplatin-treated tumor cells, indicating that increased cell apoptosis. Of note, the combination treatment induced the greatest number of apoptotic cells in the tissues (Fig. 3a and b). In addition, the western blotting results revealed a similar trend. As

compared with other groups, combination treatment significantly increased the expression of cleaved caspase-3 (Fig. 3 c and d).

Combination treatment with D-MT + oxaliplatin influences the expression of tumor-related proteins

The results showed that compared with the PBS group, single-agent therapy with D-MT or oxaliplatin significantly inhibited the expression of MMP2 and combination treatment showed the similar trends (Fig. 4). In addition, compared with the PBS group, treatment with D-MT inhibited the expression of p-Stat3, but treatment with oxaliplatin prompted the expression of p-Stat3 significant (Fig. 4). Interestingly, treatment with oxaliplatin significantly increased the expression of IDO. Treatment with D-MT or D-MT+oxaliplatin showed a reduction of IDO expression (Fig. 4), indicating that combination treatment has the synergic action.

Combination treatment with D-MT + oxaliplatin significantly increases T cell infiltration in tumor tissues

It has been shown that the activation of IDO in the tumor microenvironment can impair the survival and function of T cells[32]. it was therefore detected herein that the T cells infiltrated tumor tissues by IF. The data showed that single-agent treatment with oxaliplatin or D-MT increased $CD4^+$ and $CD8^+$ T cell infiltration in tumor tissues (Fig. 5a-d). Of note, as compared with other groups, combination treatment with D-MT + oxaliplatin could significantly increase $CD4^+$ and $CD8^+$ T cell infiltration, indicating that combination treatment could significantly prompts the survival of T cells in tumor-bearing mice.

Combination treatment with D-MT + oxaliplatin significantly increases the ratio of immune cells in spleens from tumor-bearing mice

In order to determine whether combination treatment improved the whole anti-tumor effect of tumor-bearing mice, the ratio of immune cells in the spleen, the largest peripheral immune organ in the body, was detected. The results of flow cytometry

showed that the ratio of $CD8^+$ T and NK cells were increased in the spleens of mice treated with D-MT (Fig. 6b-f). Although treatment with oxaliplatin did not significantly raised the ratio of immune cells (Fig. 6 a-f), but the ratio of $CD4^+$ T, $CD8^+$ T and NK cells were significantly raised in mice treated with D-MT and oxaliplatin, as compared with the D-MT or oxaliplatin group (Fig. 6 a-f).

Combination treatment with D-MT + oxaliplatin significantly increases the concentration of TNF-α or IFN-γ in the sera of tumor-bearing mice

Finally, the concentration of TNF-α or IFN-γ was detected to play an anti-tumor role. The ELISA results showed that, as compared with the PBS group, the concentration of TNF-α or IFN-γ in the sera of tumor-bearing mice in the D-MT, oxaliplatin and combination groups was raised. Of note, the concentration of TNF-α or IFN-γ in the combination group was higher than that in other groups (Fig. 7).

Discussion

Colon cancer is a malignant tumor that seriously endangers human health in several countries. Despite the existence of certain drugs for the treatment of colon cancer, their therapeutic effect is generally low. Colon cancer treatment has therefore been attracting more and more attention. In the present study, it was determined that the D-MT, an IDO inhibitor, prompted an anti-tumor effect of oxaliplatin and strengthened the immune response against tumor in mice.

Oxaliplatin, a chemotherapy drug, is the standard first-line treatment for colon cancer in the clinic[33,34]. Unfortunately, clinical treatment often fails due to oxaliplatin resistance, which is associated with complex mechanisms, such as DNA adduct repair, cell death mechanisms and autophagy[35]. In addition, it was found shown that immunosuppressive mechanisms, such as the immune checkpoint or immunosuppressive factors, were also important reasons for drug resistance[36]. It has also been shown that oxaliplatin could effectively inhibit the growth of colon cancer, and even increase cell apoptosis, which would release a number of antigens in tumor tissues. It has been proven that chemotherapy could induce immunogenic cell death[37-39], thereby increasing cancer immunogenicity by promoting dendritic cell maturation and T cell infiltration in tumor tissues[40]. Furthermore, oxaliplatin could also increase T cell infiltration[41], which would favour a good prognosis for tumor patients[42]. The present results showed that oxaliplatin significantly increased T cell infiltration and the ratio of T cells in the spleen, indicating that oxaliplatin could play an anti-tumor effect against colon cancer.

However, despite the several factors that affect the occurrence of tumors, the therapeutic effect of oxaliplatin was limited and unsatisfactory[43]. One of the reasons is that the tumors that develope to cause immune suppression in the body, such as overexpression of the immune checkpoint[36]. IDO has been proven to catalyze the oxidative catabolism of Trp to Kyn, and regulate immune responses by impairing the survival and activity of T cells[44,45]. IDO has therefore become a therapeutic target for cancer that could either be used alone or in combination with other treatments for tumors[46,47]. However, our results showed that oxaliplatin could inhibit tumor growth

and increase the apoptosis of tumor cells, but could also increase the expression of IDO, which might interfere with the therapeutic effect of oxaliplatin. We therefore detected the therapeutic effect of combination treatment with D-MT and oxaliplatin. D-MT has been proven to effectively inhibit the IDO expression[48] and enhance the anti-tumor immune response. D-MT might play a role in disrupting tumor immune escape[30,49,50]. In fact, the present study determined that, even though oxaliplatin increased the IDO expression, the protein level of IDO could also be reduced in mice treated with D-MT and oxaliplatin. The reason might be that D-MT prompted the anti-tumor effect of oxaliplatin. In addition, the IDO expression could be upregulated by the activation of Stat3[51]. Stat3 activation would promote the development of colon tumor and related with the poor prognosis[52]. It was therefore found that D-MT also inhibited the expression of p-Stat3 in tumor cells. In addition, study showed that prevention of Stat3 activation could inhibition the expression of MMP2[53] which could promote the metastasis in colon tumor cells[54]. Our results also confirmed that D-MT reduced the expression of MMP2 in CT26 cells or tumor-tissues. It might be related with that D-MT significantly inhibited the migration of CT26 cells.

Furthermore, the present results showed that the combination treatment with D-MT + oxaliplatin not only increased T cell infiltration in tumor tissues, but also raised the ratio of T cells in the spleen. They also indicated that this strategy could strengthen the anti-tumor immune response in tumor-bearing mice. T cells were found to play an important role in tumor immunity[55] and inhibit the expression of IDO, which could reverse $CD8^+$ T cell suppression in breast cancer cell-bearing mice[56]. Interfering with the activity of IDO could lead to strengthening the anti-tumor immune response by increasing the amount of $CD8^+$ T lymphocyte infiltration[57]. In addition, the present data showed that combination treatment with oxaliplatin and D-MT increased the ratio of NK cells, which was also an important reason against tumor. This might be associated with the inhibition of IDO, whose activation could lead to the downregulation of the function of NK cells[58,59].

In the present study, it was confirmed that D-MT could strengthen the anti-tumor effect of oxaliplatin, which might be associated with the inhibition of IDO.

Combination treatment with oxaliplatin and D-MT significantly inhibited tumor growth and prolonged the survival of tumor-bearing mice. Of note, the combination strategy could prompt the anti-tumor immune response by increasing T cell infiltration in tumors. This study might provide a new therapeutic strategy for colon cancer treatment in the clinic, particularly in patients treated with oxaliplatin, in which treatment was ineffective.

Methods

All animal studies were carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org/) as detailed below.

Cell lines, mice and drugs

A mouse colon cancer CT-26 cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and kept in the laboratory. IDO inhibitor D-MT was purchased from Merck KGaA. Oxaliplatin was purchased from Hengrui Pharmaceutical Co., Ltd. The Balb/c mice (Female, 6-8 weeks old) were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were fed in pathogen-free conditions, housed under a 12 h light/dark cycle at a temperature of 25±2˚C. The animal study was approved by the Ethics Committee of Xinxiang Medical University.

Animal experiments

The CT-26 cells were adjusted to $1x10^7$ cells/ml, and a 0.1-ml cell suspension was injected subcutaneously into the upper part of the right leg of mice to establish the colon cancer-bearing mouse model. Next, 24 mice were randomly divided into the PBS, D-MT, oxaliplatin and D-MT + oxaliplatin groups. On the 7th day of the establishment of the model, PBS, D-MT (1 mg/mouse), oxaliplatin (50 μg/mouse), D-MT (1 mg/mouse) and oxaliplatin (50 μ g/mouse) were injected once a day for 7 days. A further 7 days after the last treatment, the tumors were separated and weighted. Finally, each tumor was divided into two parts, one was used to extract the protein for western blotting, and another was fixed in 4% formalin for immunofluorescence (IF) detection. The spleens were also separated from the mice for flow cytometry. In addition, 7 days after establishing the mouse model of colon cancer, another 40 mice were randomly divided into the PBS, D-MT, oxaliplatin and D-MT + oxaliplatin groups and administered the appropriate treatment. Mouse survival was observed and recorded every day. All the mice were giving euthanasia by cervical dislocation after sera collection. All animal studies were performed according to protocols approved by the Ethics Committee of Xinxiang Medical University.

Western blotting

The protein of cells or tumor tissues was extracted in lysis buffer (Beyotime Institute of Biotechnology), and then the concentrations were quantified according to the instructions of the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Based on the concentration, 30 μg protein was isolated by 10% SDS-polyacrylamide separation gel before being transferred onto PVDF membranes. The membranes were then incubated with the primary antibodies [Tubulin, IDO, p-signal transducer and activator of transcription 3 (p-Stat3), signal transducer and activator of transcription 3 (Stat3), matrix metallopeptidase 2 (MMP2), cleaved caspase-3 or lc3b; Cell Signaling Technology, Inc.)], diluted according to the manufacturer's instructions. After 2 h, the membranes were washed with 1xTBST and incubated with the secondary antibodies for 1 h. Finally, the specificity of antigen-antibody complexes was detected using enhanced chemiluminescence reagent (Cell Signaling Technology, Inc.) and the images were visualized by Fusion FX Spectra imaging system.

Immunofluorescence (IF) detection

Indirect IF was performed as previously described [60]. Briefly, the tumor slides were incubated with the appropriate antibody (CD3, CD4 and CD8) at 4˚C overnight in a wet box. The tumor slides were restored to 25˚C at least 30 min after being incubated with the secondary antibody for 30 min. Finally, the slides were stained with DAPI and the images were captured using a fluorescence or a laser confocal microscope. The intensities of positive cells were analyzed using a scale of 0-3+: 0, no staining identified; 1+, <25% positive cells; 2+, 25-75% positive cells; 3+, >75% positive cells.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

The cell apoptosis in tumor tissues was detected by TUNEL assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Briefly, the TUNEL detection solution was dropwise added onto the surface of tumor section.

Following incubation in the dark for 60 min at 37˚C, the sections were washed with PBS for 10 min, 3 times. Finally, the sections were dried and sealed with anti-fluorescence quenching solution, and the positive cells were observed using the fluorescence microscope. In addition, Hoechst 33342 solution (Beyotime Institute of Biotechnology) was used for staining living cells, according to the manufacturer's instructions.

Flow cytometry

The concentration of spleen cells was adjusted to $1x10^7$ cells/ml, and each tube was added into 100 µl cell suspension. The cells were incubated with antibodies for CD3, CD4, CD8 and CD49b (BioLegend, Inc.) at 4˚C for 30 min. The ratio of cells was detected using flow cytometry (CytoFLEX; Beckman Coulter, Inc.).

Enzyme-linked immunosorbent assay (ELISA).

One week after the last treatment, the mice in each group were sacrificed and the sera collected. The concentration of tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) was analyzed by ELISA kits (RayBiotech), according to the manufacturer's instructions.

Statistical analysis

Measurement data are expressed as the mean ± SD of three independent experiments. Data were analyzed by SPSS 19.0 (IBM Corp.). One-way ANOVA was performed to test the difference among the different groups, and the Kaplan-Meier method with a log-rank test was used to analyze survival. $P<0.05$ were considered to indicate a statistically significant difference.

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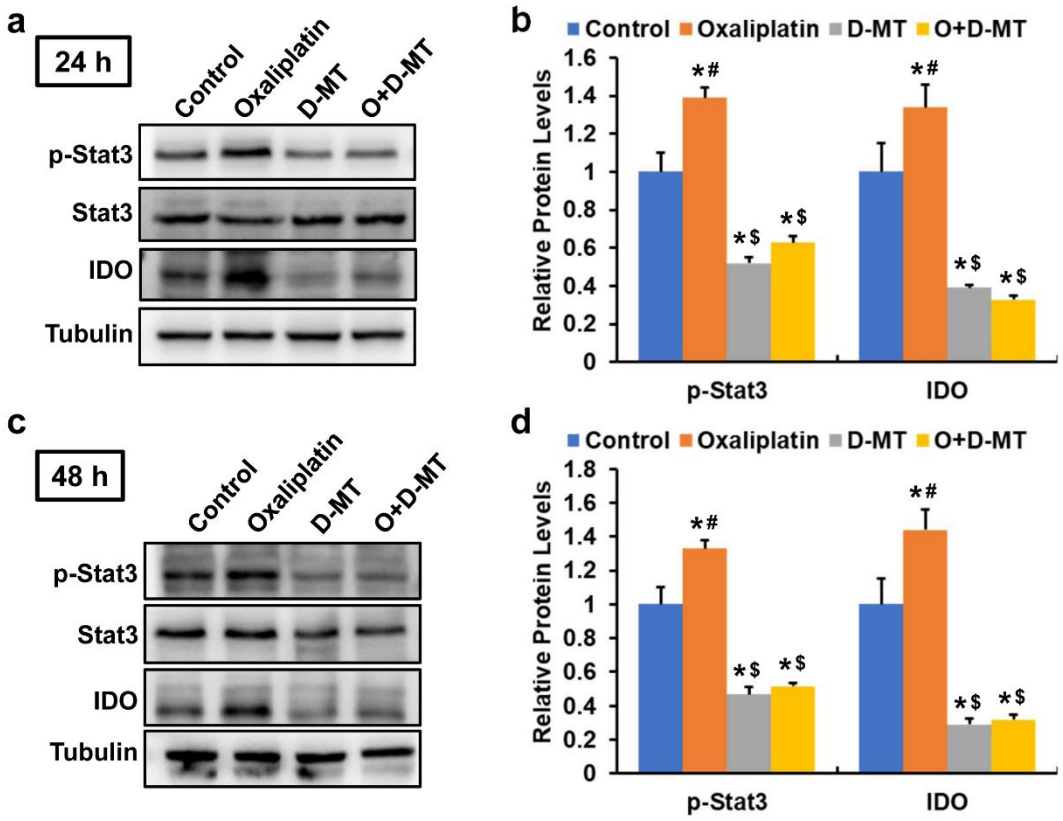
Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

All authors have read and approved the manuscript. TSZ and CXD designed the experiments. YXZ and YL participated in designing the experiment, analysing the data and revising the manuscript. YXZ, YL, ZAL, YJ, JG, WWR, RPL, GZZ, JL, MJL, XYL and SGW carried out the experiments. YXZ wrote the manuscript.

Figure legends



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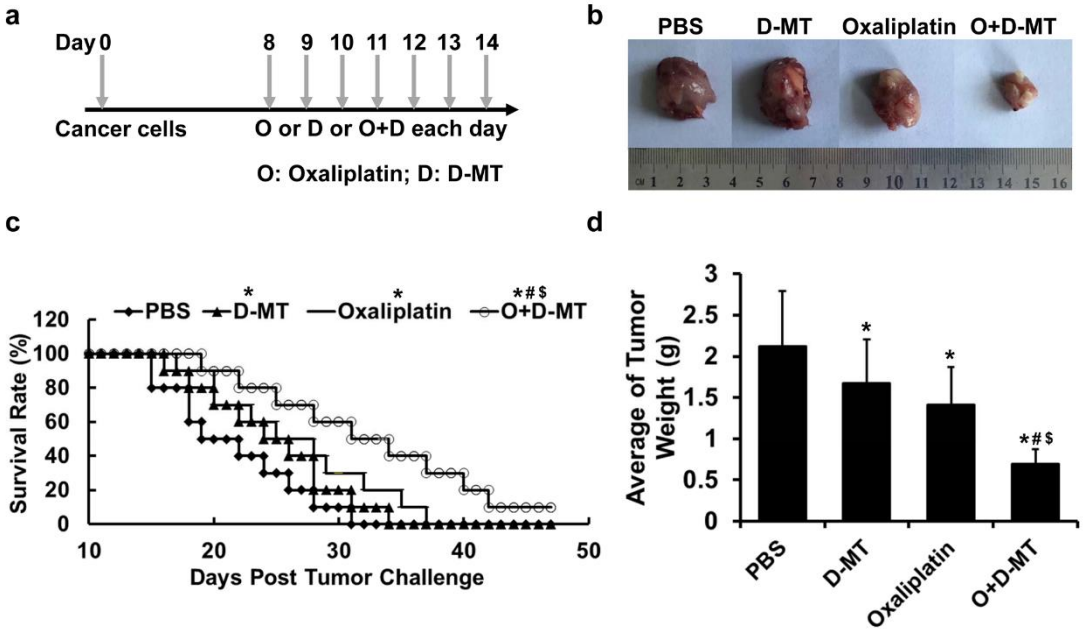

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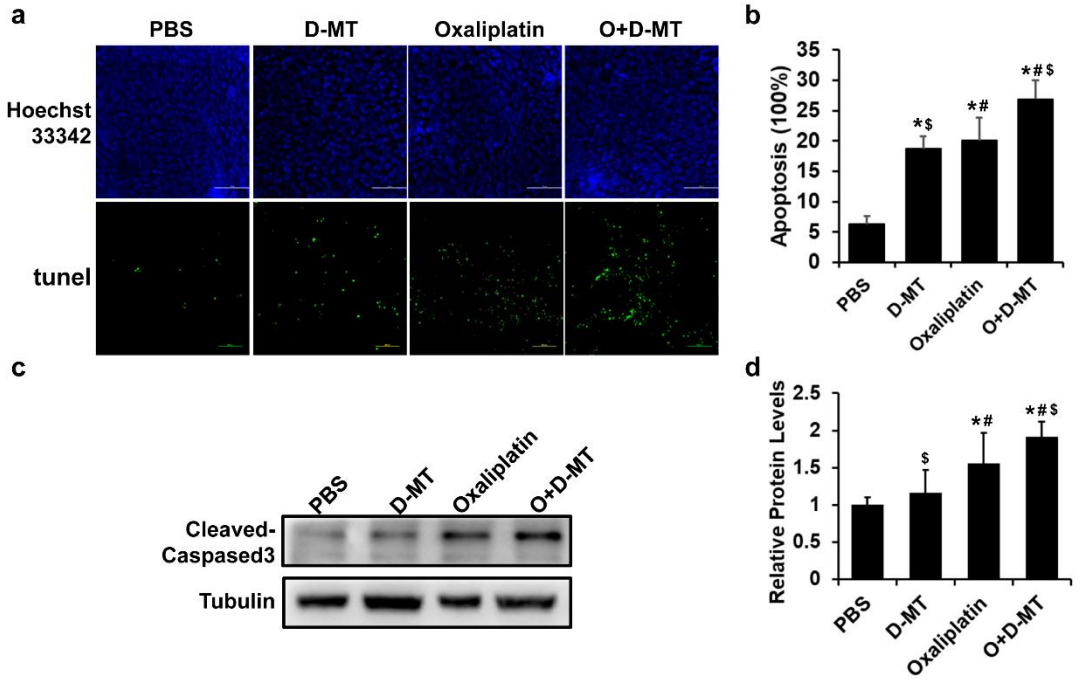

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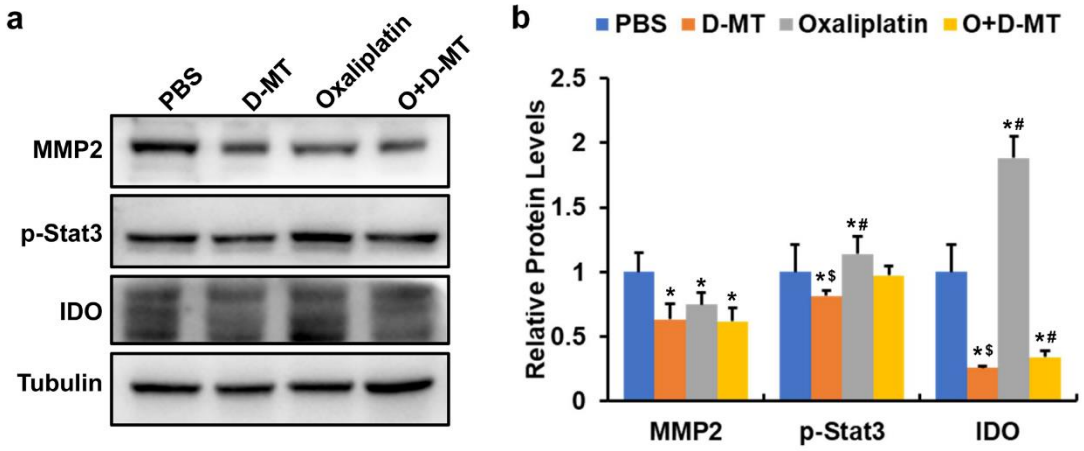

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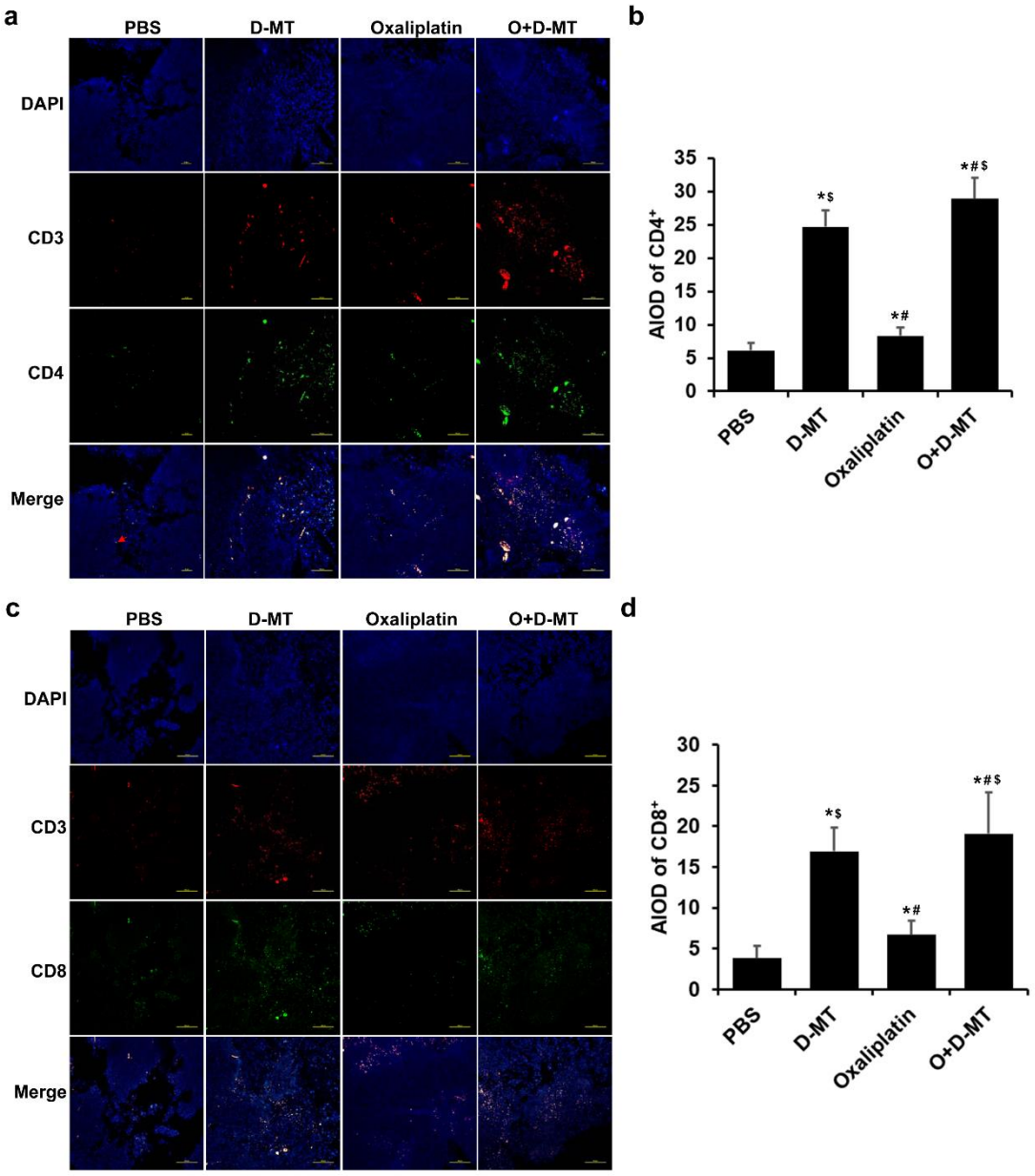

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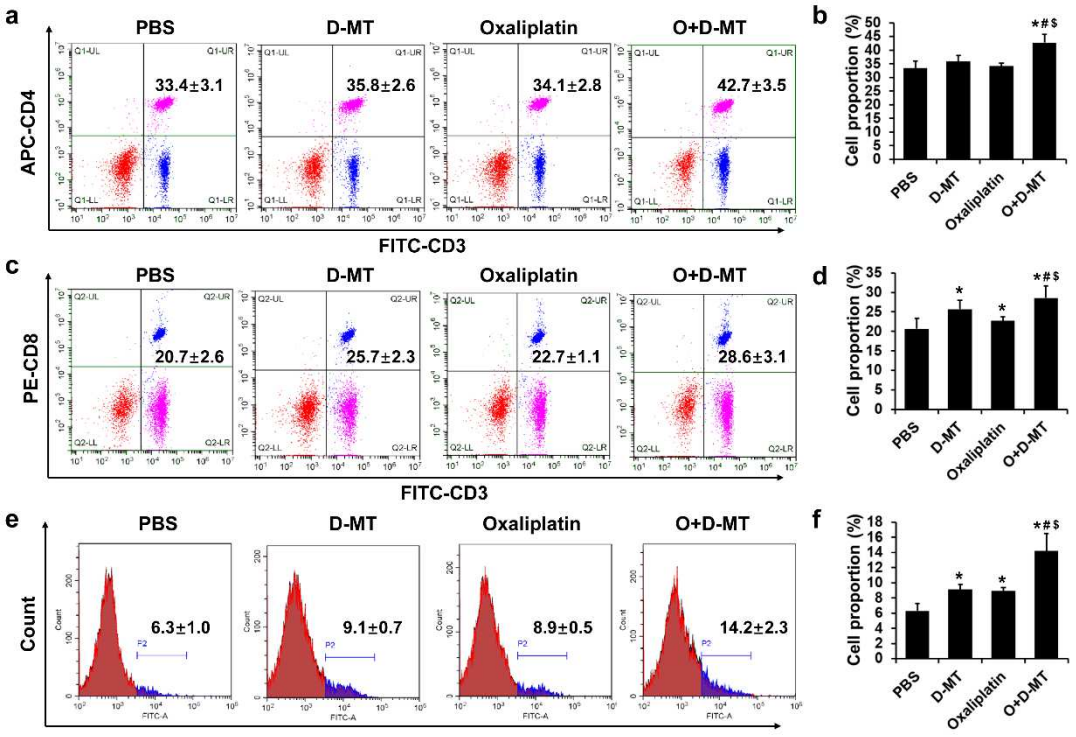

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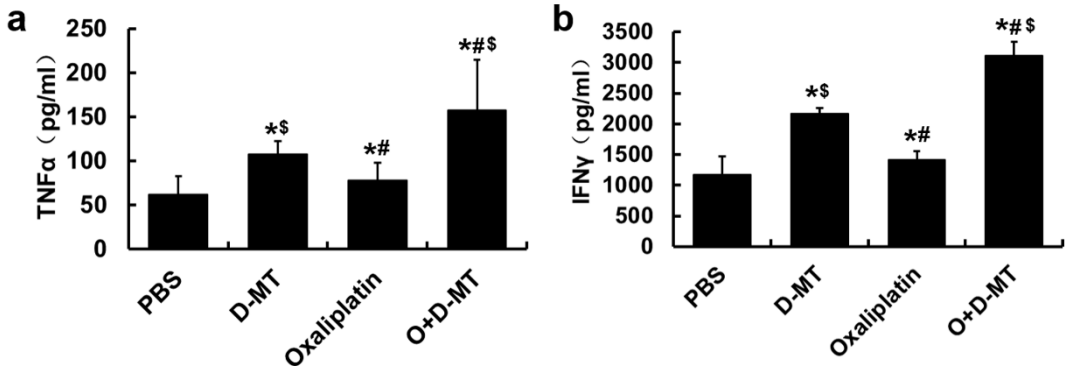

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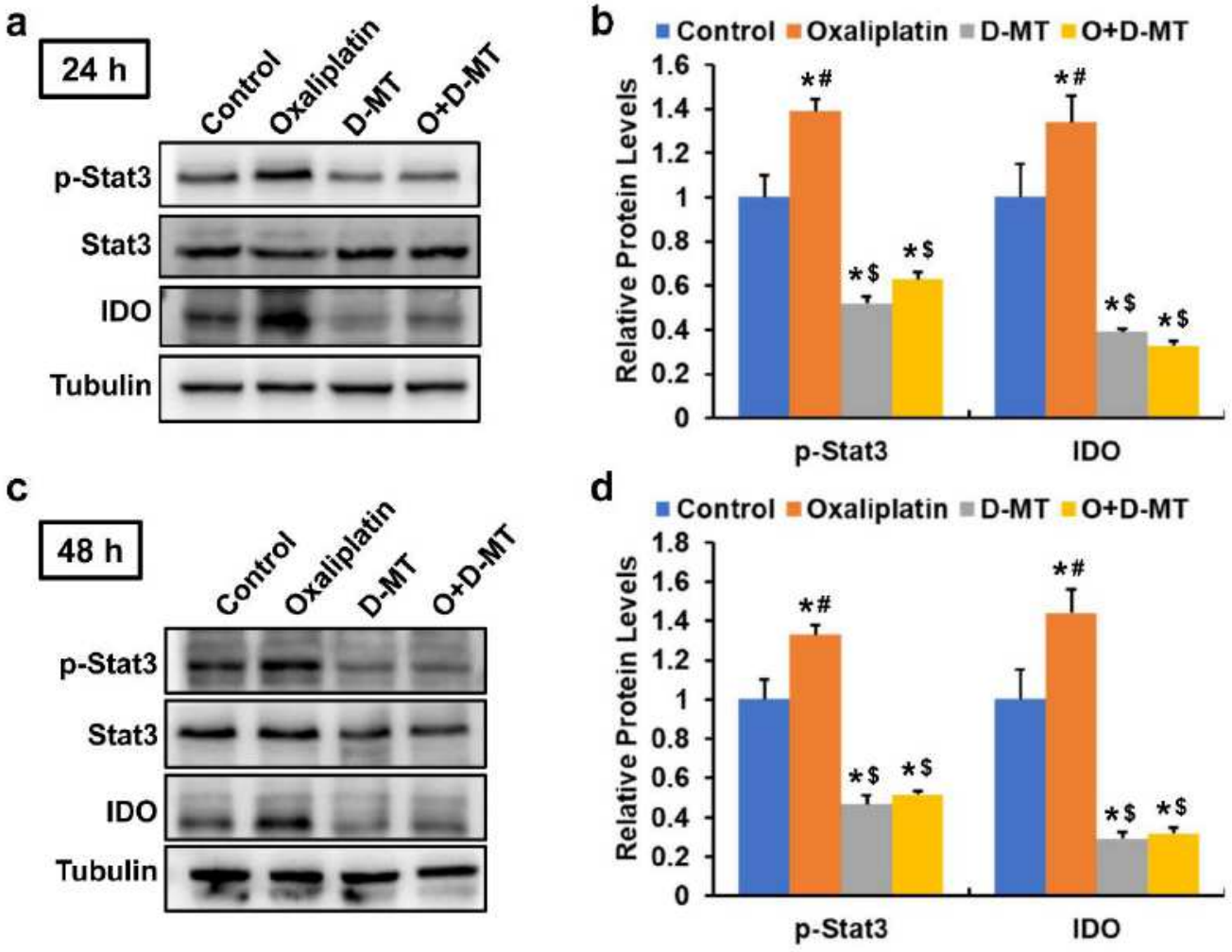
Figures



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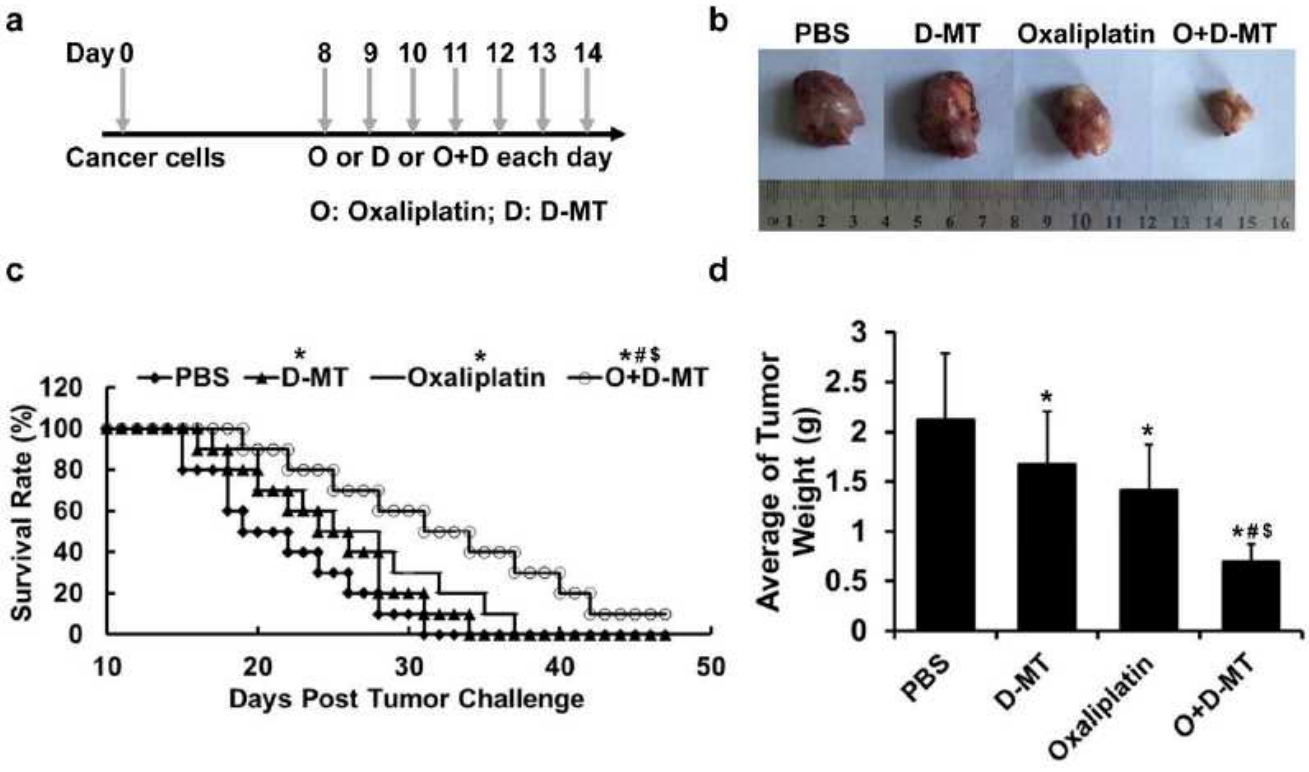

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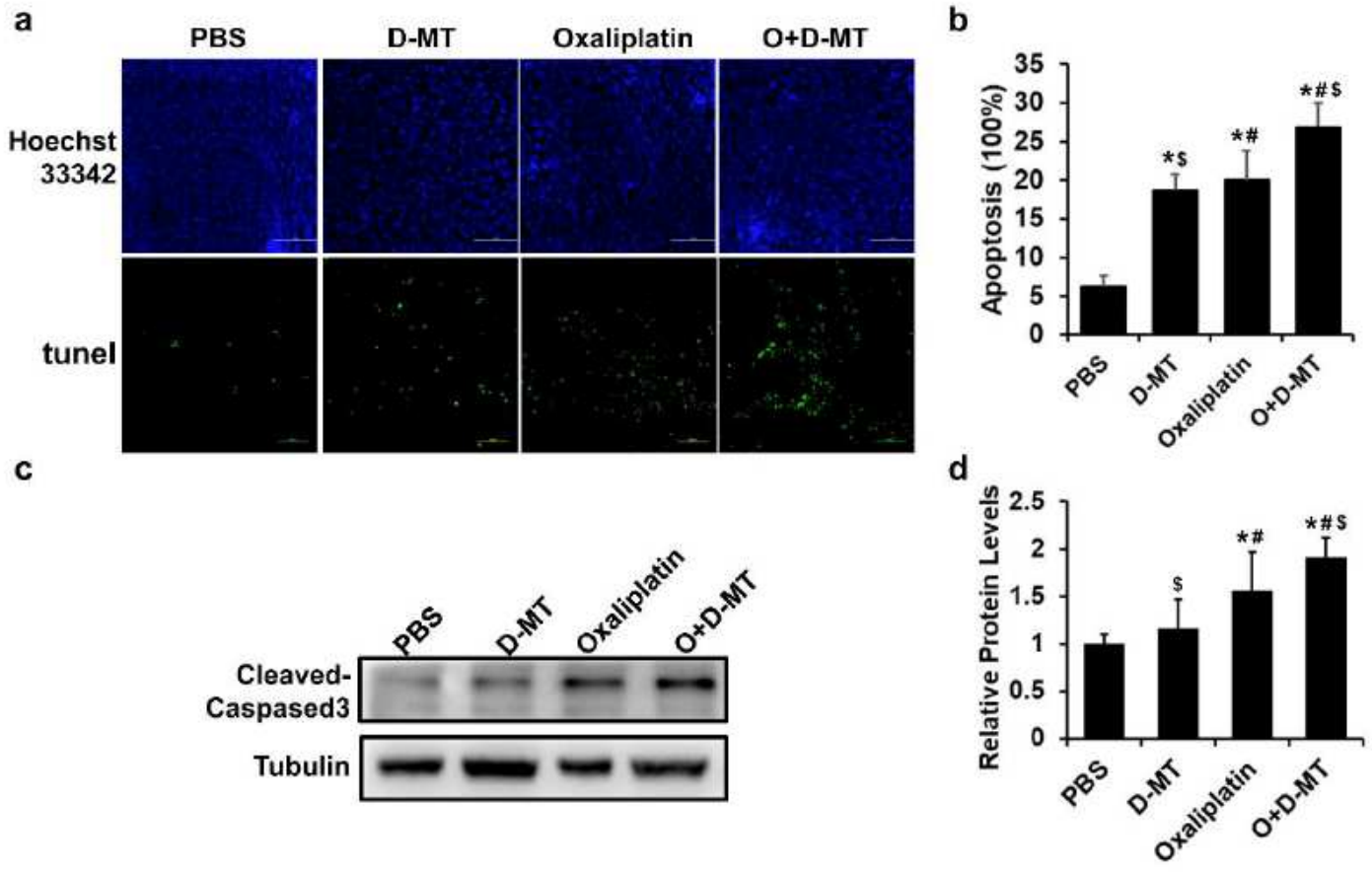

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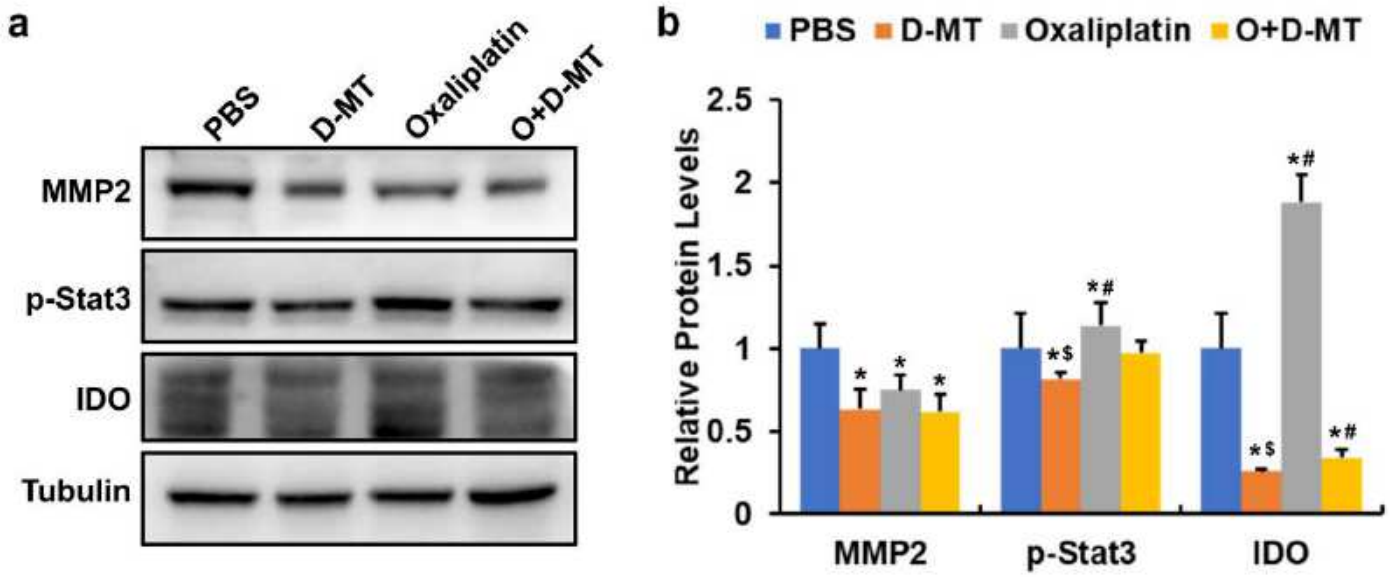

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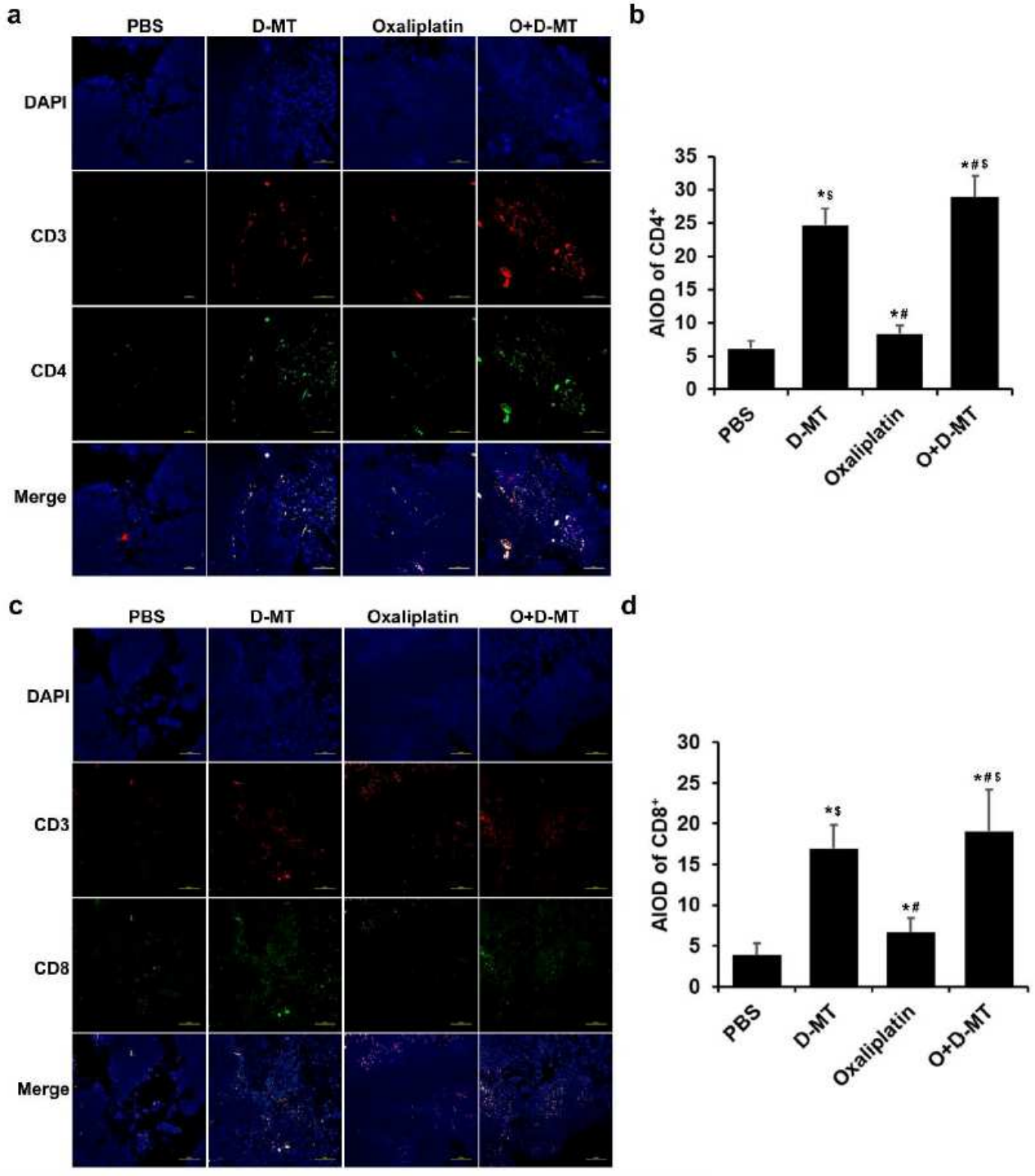

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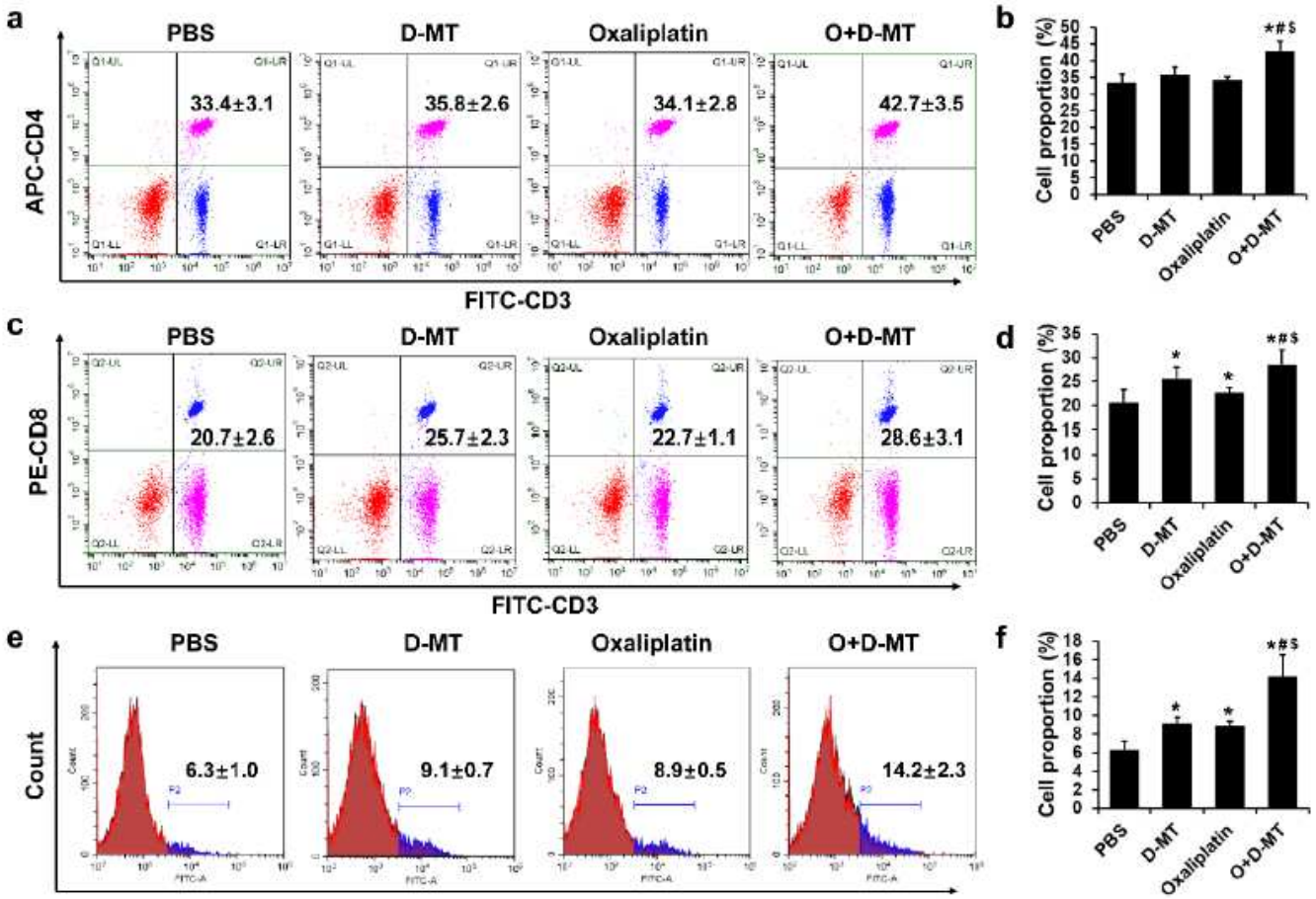

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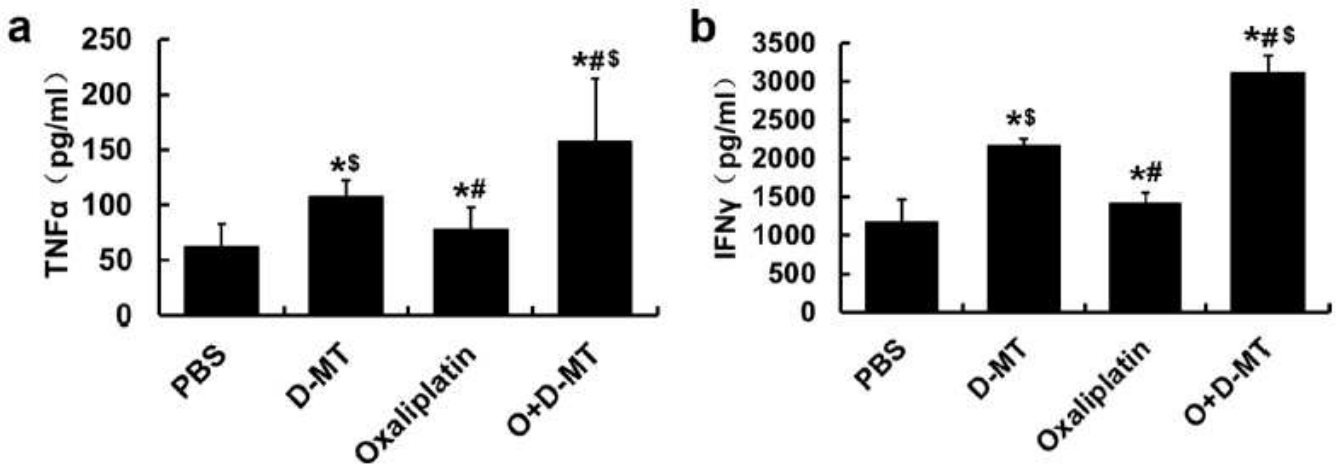

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Supplementary Files

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