International Immunopharmacology 88 (2020) 106831

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Oridonin inhibits 4T1 tumor growth by suppressing Treg differentiation via TGF-β receptor

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ARTICLE INFO

Keywords:
Triple-negative breast cancer
Oridonin
Treg
TGF-β receptor
PD-1

ABSTRACT

The Chinese herbal medicine oridonin has potent anti-inflammatory and antitumor activities. In addition, oridonin treatment effectively suppresses breast cancer growth. However, the underlying mechanisms are poorly defined. Here, we reported that oridonin decreased Treg differentiation *in vitro* and *in vivo*. Oridonin inhibition of Treg differentiation was dependent on decreasing TGF-β receptor expression. Oridonin attenuated Tregs' immunosuppressive ability; thus, oridonin did not inhibit $CD8^+$ T cell proliferation very well *in vitro*. Oridonin greatly delayed the progression of 4T1 tumors *in vivo*. In addition, oridonin combined with anti-PD-1 activated a robust antitumor immune response and suppressed 4T1 tumor growth. Therefore, our results indicate that oridonin inhibits TNBC growth by modulating Treg differentiation, which provides new directions for the clinical treatment of TNBC.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer deaths among women worldwide [1,2]. Triple-negative breast cancer (TNBC), which is characterized by negative expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor factor receptor 2 (HER-2), accounts for approximately 15%-20% of breast cancers [3,4]. TNBC is more prevalent in younger women under the age of 40 years old compared with that of other breast cancer subtypes. TNBC is also an aggressive breast cancer and spreads to the lung, brain, liver and bone at an early stage [5]. Chemotherapy is currently the main means of clinical treatment for TNBC [6], but many TNBC patients treated with chemotherapy have a high risk of recurrence along with poor response, toxicity and multidrug resistance [7,8]. Because TNBC is a heterogeneous disease that is associated with high metastasis and poor prognosis, TNBC currently lacks specific treatments. Therefore, exploring new therapies for TNBC patients is urgent.

Traditional Chinese Medicine (TCM) features extensive philosophical and medical concepts and has become a comprehensive healthcare-focused medical system. TCM applies herbal medicine, acupuncture, moxibustion, massage, food therapy and physical exercise to alleviate illnesses and improve human health [9,10]. Oridonin is the major active ingredient isolated from the Chinese herbal medicine *Rabdosia rubescens* and possesses considerable anti-inflammatory and antitumor activity [11,12]. Oridonin inhibits breast cancer growth by suppressing the EMT and the HIF-1α/VEGF signaling pathway [13]. Oridonin suppresses breast cancer proliferation by inducing apoptosis [14]. Although some mechanisms by which oridonin protects against breast cancer have been proposed, there is still much that needs to be elucidated.

Accumulating clinical trials have revealed that antagonists of the programmed cell death-1/programmed death ligand-1 (PD-1/PD-L1) pathway can extend the overall survival and progression-free survival of TNBC patients [15,16]. However, immune-related adverse drug reactions to anti-PD-1 antibodies are often seen in tumor patients [17,18]. In addition, approximately 20% of tumor samples from TNBC patients are PD-L1 positive [19]. The treatment effect of PD-1/PD-L1 blockade is

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https://doi.org/10.1016/j.intimp.2020.106831
Received 15 February 2020; Received in revised form 23 June 2020; Accepted 22 July 2020
1567-5769/ Published by Elsevier B.V.

not satisfactory. Oridonin combined with capecitabine inhibits the proliferation of breast cancer cells [20]. However, the effect of oridonin combined with PD-1/PD-L1 blockade in TNBC remains unknown.

Here, we demonstrated that oridonin decreased Treg differentiation *in vitro*, which depended on TGF-β receptor. In parallel, oridonin limited Treg induction *in vivo* and greatly suppressed tumor progression via Tregs. Oridonin combined with PD-1 blockade synergistically attenuated breast cancer progression *in vivo*. Thus, our findings shed light on the crucial functions of oridonin-mediated Treg differentiation and suggest that oridonin combined with PD-1 blockade could be used for TNBC therapy.

2. Materials and methods

2.1. Mice and cell lines

Female BALB/c mice and thymus-deficient BALB/c nude mice (6- to 8-week-old) were purchased from Joint Ventures Sipper BK Experimental Animal Co (Shanghai, China). The mice were housed in a specific pathogen-free facility, and experimental protocols were approved by the Animal Care and Use Committee of the School of Medicine of Zhejiang University. Murine 4T1 breast cancer cells and human MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). 4T1 cells and MDA-MB-231 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (Thermo Fisher Scientific, MA, USA). Cells were routinely tested for mycoplasma contamination using a mycoplasma detection kit (Lonza, Basel, Switzerland) and were found to be negative.

2.2. In vitro Treg differentiation

Naïve $CD4^+$ T cells were obtained from the spleen and lymph nodes of mice using a mouse naïve $CD4^+$ T cell isolation kit (#19765, Stemcell, Vancouver, BC, V6A 1B6, Canada). Sorted naïve $CD4^+$ T cells were routinely 98% pure. T_H0 and Treg-skewing conditions are as follows: the sorted naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (145-2C11, 2 μg/ml, Bio X Cell, West Lebanon, NH, USA) and anti-CD28 (PV-1, 2 μg/ml, Bio X Cell) antibodies and polarized into effector $CD4^+$ T lymphocyte subsets with anti-IFN-γ (BE0054, 10 μg/ml, Bio X Cell) and anti-IL-4 (BE0045, 10 μg/ml, Bio X Cell) for T_H0 cells or with TGF-β1 (10 ng/ml, 240-B, R&D Systems, Minneapolis, MN, USA), anti-IFN-γ (10 μg/ml), and anti-IL-4 (10 μg/ml) for Tregs. In some experiments, oridonin (S2335, 0.1 μM, Selleck, Houston, TX, USA), SB431542 (S1067, 0.5 μM, Selleck), anti-mouse TGF-β1 antibody (19D8, 10 μg/ml, BioLegend, San Diego, CA, USA), and mouse IgG1 isotype antibody (MOPC-21, 10 μg/ml, BioLegend) were added at the beginning of cell culture. Cells were classically harvested for the detection of Foxp3 by flow cytometry and real-time PCR. The supernatant was collected for detection of TGF-β1 (BioLegend) and IL-10 level (BioLegend) by ELISA.

2.3. Cell staining and FACS analysis

PE-anti-CD4 (GK1.5), FITC-anti-CD8 (53-6.7), Pacific Blue-anti-CD45 (30-F11), APC-anti-Foxp3 (FJK-16s), APC-anti-IFN-γ (XMG1.2), APC-anti-Granzyme B (NGZB), FITC-anti-CD69 (H1.2F3), FITC-anti-CTLA4 (UC10-4B9) and isotype antibodies were purchased from eBioscience (San Diego, CA, USA). For surface staining, the cells were stained with fluorescence-conjugated antibodies against surface antigens at room temperature for 20 min. For detecting cytokines, the cells were stimulated with the cell stimulation cocktail (00-4975-03, eBioscience) for 6 h and fixed with IC fixation buffer (00-8222-49, Invitrogen, Carlsbad, Cal, USA) at room temperature for 20 min, permeabilized (00–8333-56, Invitrogen) and stained with fluorescence-conjugated antibodies against intracellular cytokines at room temperature for 30 min. For detecting Foxp3 expression, fixation/permeabilization diluent and concentrate (00-5521-00, Invitrogen) were used. The data were acquired on a Beckman Coulter DxFLEX flow cytometer equipped with CytExpert experiment-based software (Beckman Coulter, Inc.), and data were analyzed using FlowJo software (TreeStar).

To detect cell proliferation, the cells were stained with CFSE (C34554, Thermo Fisher Scientific) and seeded into 96-well plates for 3 days. Then, the cells were harvested and washed with PBS twice. To detect apoptosis, the cells were collected. The apoptosis assay was conducted using an Annexin V/PI apoptosis kit (Multi Sciences Biotech, Hangzhou, Zhejiang, China) according to the manufacturer's instructions.

2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted from T cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Complementary DNAs (cDNAs) were synthesized using a cDNA synthesis kit (TaKaRa, Kusatsu, Shiga, Japan) following the manufacturer's instructions. Real-time PCR was conducted using SYBR Green (TaKaRa). The following PCR conditions were used: 1 cycle at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Real-time PCR was performed on an Applied Biosystems 7500 real-time PCR system. The primers were as follows: *Actb* 5′-atggaggggaatacagccc-3′ and 5′-ttctttgcagctccttcgtt-3′; *Foxp3* 5′-ctcgtctgaaggcagagt-3′ and 5′-tggcagagaggtattgaggg −3′; *Tgfb1* 5′-tgatacgcctgagtggctgtct-3′ and 5′-cacaagagcagtgagcgctgaa-3′; *TgfbrI* 5′-tgctccaaaccacagagtaggc-3′ and 5′-cccagaacactaagcccattgc-3′; *TgfbrII* 5′-cctactctgtctgtggatgacc-3′ and 5′-gacatccgtctgcttgaacgac-3′.

2.5. Immunoblotting analysis

Purified naïve T cells were differentiated into Tregs for different times in the presence or absence of oridonin. Cell pellets were incubated in five volumes of RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and protease inhibitors) for 30 min on ice. After centrifugation at 16,000*g* for 15 min at 4 °C, the supernatant was recovered for WB analysis. Finally, cell lysates were separated by SDS–PAGE, transferred onto PVDF membranes (Millipore) and probed with primary antibodies against the target proteins. Antibodies against p-Smad2 (E8F3R), Smad2 (D43B4), p-Smad3 (C25A9), Smad3 (C67H9) and β-actin (8H10D10) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against TGF-βRI (ab31013) was purchased from abcam (Cambridge, MA, USA) and TGF-βRII antibody (2D5H7) was from Proteintech Group, Inc (Wuhan, Hubei, China). The membranes were scanned using a Tanon 4500 gel imaging system.

2.6. T Cell suppression assay

A total of 4×10^5 splenocytes from mice were labeled with CFSE (Invitrogen) and seeded into a flat-bottom 48-well plate that was precoated with anti-CD3 (2 μg/ml, Bio X cell) plus anti-CD28 (2 μg/ml, Bio X cell) antibodies. Control Tregs or oridonin-treated Tregs were added to some wells as indicated. Cells were harvested 3 days later and analyzed by flow cytometry.

2.7. Tumor growth experiments

A total of 5×10^6 4T1 cells were injected subcutaneously into WT BALB/c mice. To evaluate the role of oridonin in tumors, the mice received intraperitoneal injections of oridonin (5 mg/kg) every three days following tumor implantation. To evaluate the role of T cell in tumor, a total of 5×10^6 4T1 or MDA-MB-231 cells were injected subcutaneously into thymus-deficient BALB/c nude mice. For the depletion of $CD8^+$ T cells, mice were intraperitoneally injected with anti-CD8

antibodies (100 μg, 2.43, Bio X Cell) on days 1, 5, 9 and 13. To detect the therapeutic effect of combining oridonin with anti-PD-1, the mice received intraperitoneal injections of oridonin (5 mg/kg) every three days, along with anti-PD-1 (100 μg, RMP1-14, Bio X Cell) or rat IgG2a isotype control (2A3, Bio X Cell) every three days following tumor implantation. Tumor size was monitored every three days by Vernier calipers. Animals with ulcerated tumours or with tumours above 2000 mm^3 in size were killed for ethical reasons. TILs were prepared by enzymatic digestion with 1 mg/ml collagenase (Sigma-Aldrich), 0.5 mg/ml DNase I and 25 μg/ml hyaluronidase (Sigma-Aldrich) at 37 °C for 30 min, followed by Percoll (GE Healthcare) gradient purification.

2.8. Tumor metastasis experiments

A total of 5×10^5 4T1 cells were injected into the mammary fat pad of female BALB/c mice. Primary tumours were surgically resected on day 15. Then, mice received intraperitoneal injections of oridonin (5 mg/kg) on day 15, 18, 21, 24. Mice were sacrificed to analyse lung metastases with H&E staining on day 25. TILs were also isolated to detect Treg and IFN-γ^+CD8^+ T cells on day 25.

2.9. Statistical analysis

The data are expressed as the mean ± s.d. Comparisons between 2 groups were performed using 2-tailed Student's *t*-test, and comparisons between multiple groups were performed by one-way ANOVA and Tukey-Kramer multiple test, using GraphPad Prism 8. Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Oridonin suppresses breast cancer progression

To assess the effect of oridonin (Fig. 1a) on the tumor growth of 4T1 murine TNBC *in vivo*, we injected 4T1 cells subcutaneously into WT BALB/c mice. We found that tumor progression in control mice was superior to that in oridonin-treated mice (Fig. 1b). Oridonin has been reported to attenuate mouse colitis by suppressing Th1/Th17 cells [21]. Therefore, we wondered whether oridonin inhibits tumor progression in a T cell-dependent manner. Next, we analyzed tumor-infiltrating lymphocytes (TILs). TILs from oridonin-treated mice had a significantly reduced frequency of Tregs (Fig. 1c) but an increased proportion of $CD8^+$ T cells (Fig. 1d). In addition, we found that treatment with oridonin largely increased the frequency of IFN-γ^+CD8^+ T cells in TILs of oridonin-treated mice (Fig. 1e). In addition, the spleen in the oridonin-treated mice also demonstrated a decreased percentage of Tregs and an enhanced level of IFN-γ^+CD8^+ T cells (Fig. 1f). However, oridonin did not affect tumor growth when 4T1 cells were subcutaneously into nude mice (Fig. 1g), indicating the essential role of T cells in oridonin-mediated tumor suppression. Besides, oridonin could not inhibit the progression of MDA-MB-231 in nude mice too (Fig. 1h). Tregs inhibit tumor growth via $CD8^+$ T cells [22]. To elucidate whether oridonin inhibition of tumor growth was $CD8^+$ T cell dependent, we treated mice with anti-CD8 T antibody. After the depletion of $CD8^+$ T cells, oridonin had no effect on 4T1 tumor suppression (Fig. 1i). Then, we examined the role of oridonin in 4T1 tumor metastasis. Oridonin substantially decreased the lung metasis of 4T1 tumours (Fig. 1j). Additionally, we observed reduced Tregs and increased IFN-γ^+CD8^+ T cells in TILs of lung tumors from mice that received oridonin injections (Fig. 1k). Thus, these results suggest that oridonin functions in its antitumor activity by targeting T cells.

3.2. Oridonin decreases Treg differentiation in vitro

To further elucidate the role of oridonin in Treg differentiation *in vitro*, we differentiated naïve $CD4^+$ T cells from WT BALB/c mice into Tregs *in vitro*. Consistent with the above *in vivo* results, the oridonin-treated $CD4^+$ T cells generated substantially lower numbers of $Foxp3^+CD4^+$ T cells compared to that of control $Foxp3^+CD4^+$ T cells (Fig. 2a). Decreased Foxp3 mRNA levels were also observed in $CD4^+$ T cells in response to oridonin treatment (Fig. 2b). In addition, oridonin decreased the number of Tregs in a dose-dependent manner (Fig. 2a, b). As oridonin mediates cell proliferation and apoptosis, we detected the proliferation and apoptosis of Tregs with or without oridonin treatment. Oridonin did not affect Tregs proliferation (Fig. 2c) or apoptosis (Fig. 2d). These results abundantly indicate that oridonin attenuates the induction of $Foxp3^+CD4^+$ T cells *in vitro*.

3.3. Oridonin-treated Tregs have attenuated immunosuppressive ability

Tregs have potent immunosuppressive capacity and express many inhibitory molecules, such as CTLA4 and CD69 [23,24]. We found that oridonin inhibited the expression of CTLA4 (Fig. 3a) and CD69 (Fig. 3b) on oridonin-treated Tregs. Besides, Tregs also exert their inhibition function by producing TGF-β1 and IL-10 cytokines. We therefore compared the capacity of the two subsets of Tregs to produce such cytokines. We observed that the secretion levels of TGF-β1 and IL-10 protein were lower in oridonin treated Tregs than in control Tregs (Fig. 3c). Next, a suppression assay was performed to evaluate the suppressive potential of oridonin-treated Tregs. Splenocytes were co-cultured with control Tregs or oridonin-treated Tregs, and T cell proliferation was determined by CFSE dilution. Tregs treated with oridonin displayed a reduced ability to suppress the proliferation of $CD8^+$ T cells (Fig. 3d). These results prove that oridonin restrains the suppressive ability of Tregs.

3.4. Oridonin represses Treg polarization by decreasing TGF-β receptor

Next, we investigated how oridonin suppressed Treg differentiation. As TGF-β1 signaling is necessary for the induction of Tregs, we detected molecules associated with TGF-β1 signaling. We discovered that the phosphorylation of Smad2 and Smad3 was notably decreased in oridonin-treated Tregs (Fig. 4a). In addition, oridonin did not influence the mRNA expression of TGF-β1, TGF-βRI and TGF-βRII (Fig. 4b), while it inhibited the protein level of TGF-βRI and TGF-βRII (Fig. 4c). There were no differences between oridonin-treated Tregs and control Tregs in the presence of oridonin and anti-TGF-β1 (Fig. 4d). Meanwhile, we also utilized TGF-β receptor inhibitor SB431542. After blocking TGF-β receptor, oridonin could not further suppress Treg differentiation (Fig. 4e). Therefore, oridonin suppressed Treg differentiation by decreasing the protein level of TGF-β receptor.

3.5. Oridonin is effective in combination with anti-PD-1 therapy in 4T1 tumors

To detect the effect of oridonin and anti-PD-1 on breast cancer, we established subcutaneous 4T1 tumors in WT BALB/c mice. Combination therapy with oridonin and anti-PD-1 resulted in superior tumor regression compared to that of treatment with either oridonin or anti-PD-1 (Fig. 5a). We observed more $CD8^+$ T cells in TILs from mice that received oridonin and anti-PD-1 injections (Fig. 5b). In addition, increased numbers of IFN-γ^+CD8^+ T cells and granzyme B^+CD8^+ T cells were observed in mice treated with oridonin and anti-PD-1 (Fig. 5c, d). Therefore, oridonin improves the antitumor activity of anti-PD-1 in breast cancer.

4. Discussion

TCM has been widely accepted in Western countries. Some studies have reported that TCM is beneficial for cancer treatments. Oridonin inhibits breast cancer via multiple mechanisms. Almost all reports

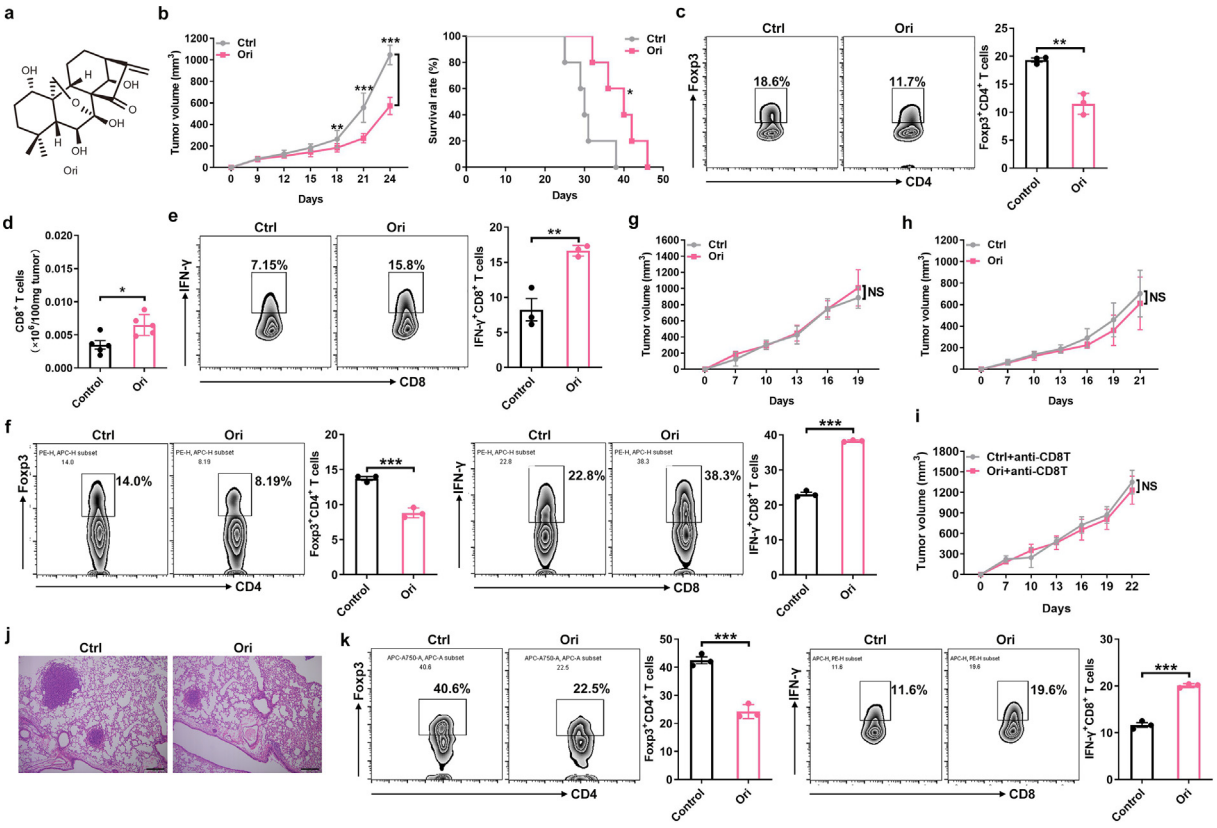

Fig. 1. Oridonin suppresses breast cancer progression. (a) Oridonin (Ori) structure. (b) A total of 5 × 10^6 4T1 cells were injected subcutaneously into WT BALB/c mice on day 0. Then, the mice were intraperitoneally injected with 5 mg/kg Ori on days 0, 3, 6, 9, 12 and 15. Tumor size was monitored every three days and the survival rate was recorded. (c-f) Flow cytometric analysis of Foxp3$^+$CD4$^+$ T cells (c), CD8$^+$ T cells (d), IFN-γ$^+$CD8$^+$ T cells (e) and the corresponding statistical analysis in the TILs of tumor tissues from tumor-bearing mice; Foxp3$^+$CD4$^+$ T cells, IFN-γ$^+$CD8$^+$ T cells and the corresponding statistical analysis (f) in the spleen from tumor-bearing mice were analyzed by flow cytometry. (g, h) A total of 5 × 10^6 4T1 cells (g) or 5 × 10^6 MDA-MB-231 cells (h) were injected subcutaneously into nude mice on day 0. Tumour size was monitored every three days. (i) A total of 5 × 10^6 4T1 cells were injected subcutaneously into C57BL/6 mice on day 0. 100 μg anti-CD8 antibodies were intraperitoneally injected into mice on days 1, 5, 9 and 13. Tumour size was monitored every three day. (j, k) A total of 5 × 10^5 4T1 cells were injected into the mammary fat pad of female BALB/c mice. Primary tumours were surgically resected on day 15. Then, the mice were intraperitoneally injected with 5 mg/kg Ori on days 15, 18, 21 and 24. Lung metastases were visualized in hematoxylin and eosin-stained pathological sections (j). Tregs and IFN-γ$^+$CD8$^+$ T cells in TILs of tumour tussues were detected by flow cytometry (k). NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.005 (unpaired Student's t test). Scale, 200 μm. Representative results from three independent experiments are shown (mean and s.d.) (n = 3–5).

indicate that oridonin suppresses tumors by targeting breast cancer itself. Immunotherapy activates the immune system to fight tumors. Tregs have high suppressive potential in the immune system and sustain immune homeostasis and self-tolerance. High numbers of Tregs exist in the circulation and tumor microenvironment of cancer patients. Tregs inhibit tumor growth via different immune cells. Thus, removing Tregs to promote the efficacy of antitumor immunity has broad prospects. An important question is raised by this study. In addition to oridonin directly targeting the breast tumor itself, can it directly influence the immune system to protect against the tumor? Surprisingly, in our study, we found that oridonin decreased Treg differentiation *in vitro* and *in vivo*. These findings are important in understanding the relationship between oridonin and Tregs.

Multiple factors determine naïve CD4$^+$ T cell differentiation into specific T cell subsets. TGF-β1 signaling is the most important factor for Treg differentiation *in vitro*. Oridonin-mediated inhibition of Treg differentiation disappeared when TGF-β1 signaling was blocked, which indicates that oridonin participates in TGF-β1 signaling to regulate Treg differentiation. In this study, we found that oridonin decreased the phosphorylation of smad2 and smad3 proteins. In addition, oridonin did not affect TGF-β1, TGF-βRI and TGF-βRII mRNA, but it did decrease TGF-βRI and TGF-βRII protein. Thus, we speculate that oridonin inhibits TGF-β1 signaling by decreasing the protein levels of TGF-βRI and TGF-βRII. Specifically, oridonin could promote TGF-βRI and TGF-βRII protein degradation. However, whether TGF-βRI and TGF-βRII are the only targets of oridonin is not clear. In addition, how oridonin regulates TGF-βRI and TGF-βRII protein degradation requires further study.

Tumor immunotherapy, especially the immunological checkpoint inhibitor PD-1/PD-L1 monoclonal antibody, has been widely used in the treatment of clinical tumors. The application of anti-PD-L1 in the treatment of clinical TNBC patients greatly prolongs patient survival. However, the effect of treatments is not satisfactory. Therefore, the monoclonal antibody atezolizumab that targets PD-L1 plus chemotherapy Nab-paclitaxel has made great progress in phase III clinical trials [25]. Immunological checkpoint inhibitors primarily resolve the problem of tumor-mediated inhibition of CD8$^+$ T cell activation. The inhibition of CD8$^+$ T cell function by Tregs, which are the most important inhibitory cells in the immunosuppressive tumor microenvironment, has not been well resolved. Therefore, in this paper, the combination of anti-PD-1 and oridonin not only eliminated the inhibition of CD8$^+$ T cell activation by tumors but also removed the inhibition of CD8$^+$ T cell function by Tregs.

However, our results demonstrated that oridonin suppressed Treg differentiation *in vitro* and *in vivo*. Oridonin restrained the suppressive ability of Tregs, which effectively increased CD8$^+$ T cell responses. Oridonin improved the antitumor effect of anti-PD-1 in breast tumors. Thus, our study reveals a novel mechanism for oridonin-mediated tumor immunity.

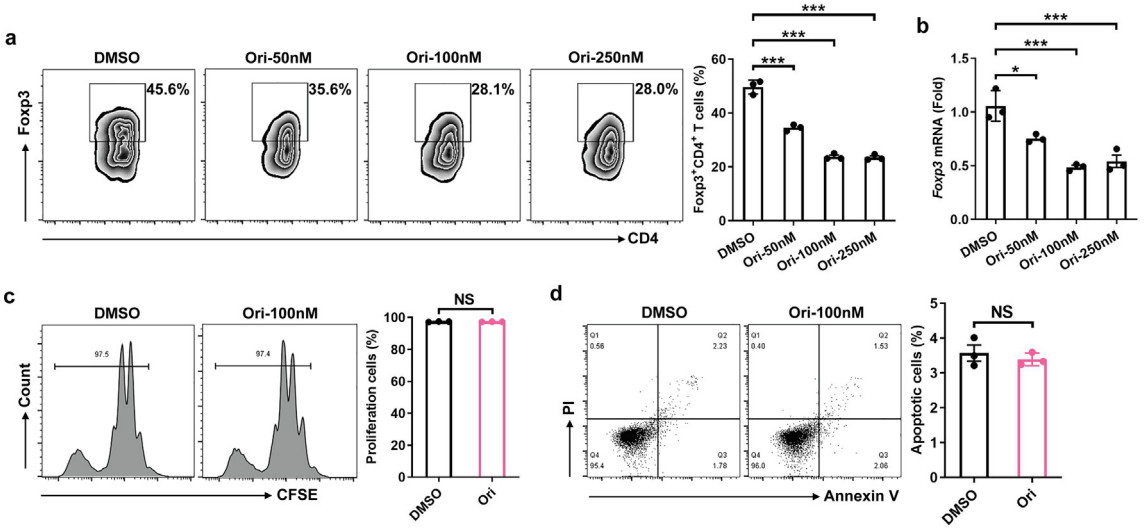

Fig. 2. Oridonin decreases Treg differentiation *in vitro*. (a-b) Naïve CD4$^+$ T cells were sorted from WT BALB/c mice and differentiated into Tregs with or without the indicated concentration of Ori for 3–4 days. Flow cytometric analysis of Foxp3$^+$ cell frequency among CD4$^+$ T cells and the corresponding statistical analysis on day 4 (a) and real-time PCR analysis of the expression of *Foxp3* in Tregs on day 3 (b). (c-d) Naïve T cells from WT mice with or without CFSE labeling were differentiated into Tregs under Treg-skewing conditions with or without 100 nM oridonin for 3 days. Flow cytometric analysis of Treg proliferation, as measured by CFSE dilution and the corresponding statistical analysis (c); flow cytometric analysis of apoptotic cells and the corresponding statistical analysis (d). NS, not significant; * $P < 0.05$, *** $P < 0.001$ (unpaired Student's *t* test). Representative results from three independent experiments are shown (mean and s.d.) (n = 3).

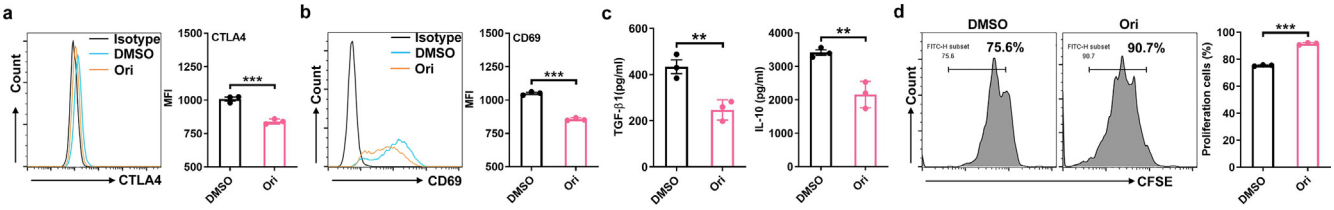

Fig. 3. Oridonin-treated Tregs have attenuated immunosuppressive ability. (a-c) Naïve CD4$^+$ T cells were sorted from WT BALB/c mice and differentiated into Tregs with or without 100 nM Ori for 4 days. Flow cytometric analysis of CTLA4 expression in Tregs and the corresponding statistical analysis (a); flow cytometric analysis of CD69 expression in Tregs and the corresponding statistical analysis (b); analysis of TGF-β1 and IL-10 cytokines in the supernatants of Tregs with or without Ori treatment by ELISA (c). (d) Naïve CD4$^+$ T cells were sorted from WT BALB/c mice and differentiated into Tregs with or without Ori for 4 days. Different Tregs were cocultured with CFSE-labeled, anti-CD3/CD28-activated splenocytes at a 1:5 ratio (Tregs: splenocytes). The proliferation of CD8$^+$ T cells was analyzed by flow cytometry 3 days later and the corresponding statistical analysis is shown. *** $P < 0.001$ (unpaired Student's *t* test). Representative results from three independent experiments are shown (mean and s.d.) (n = 3).

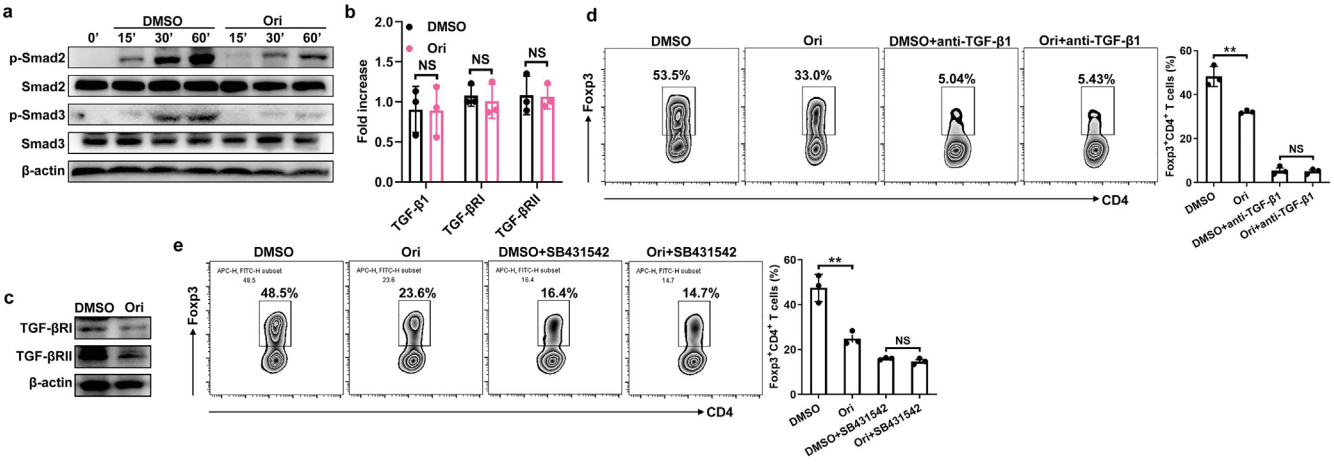

Fig. 4. Oridonin represses Treg polarization by decreasing TGF-β receptor. (a) Western blot analysis of p-Smad2, Smad2, p-Smad3 and Smad3 in naïve CD4$^+$ T cells stimulated with or without 100 nM Ori under Treg-skewing conditions for 15 min, 30 min or 60 min. (b) The mRNA expression of TGF-β1, TGF-βRI and TGF-βRII in naïve CD4$^+$ T cells stimulated with or without 100 nM Ori under Treg-skewing conditions for 3 days by RT-PCR. (c) Western blot analysis of TGF-βRI and TGF-βRII in naïve CD4$^+$ T cells stimulated with or without 100 nM Ori under Treg-skewing conditions for 24 h. (d, e) Flow cytometric analysis of Foxp3$^+$ cell frequency among CD4$^+$ T cells stimulated with 100 nM Ori and with isotype antibody or anti-TGF-β1 antibody at 10 μg/ml (d), or with 500 nM SB431542 (e) under Treg-skewing conditions for 4 days and the corresponding statistical analysis. NS, not significant; ** $P < 0.01$ (one-way ANOVA test (**c**)). Representative results from three independent experiments are shown (mean and s.d.) (n = 3) or from one experiment that is representative of three independent experiments (a, b).

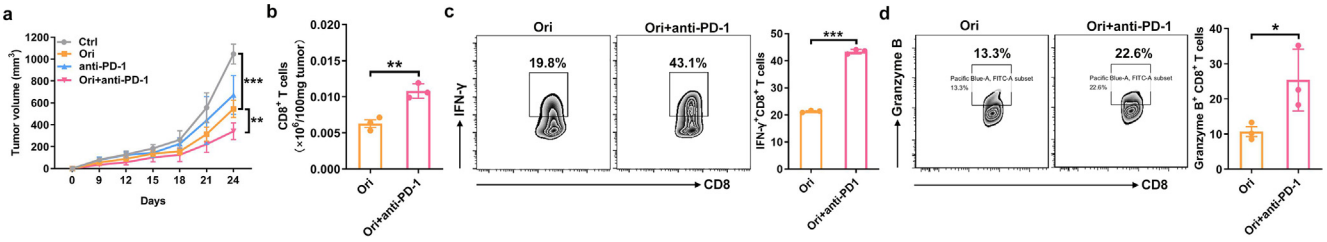

Fig. 5. Oridonin is effective in combination with anti-PD-1 therapy in tumors. (a–d) A total of 5 × 10^6 4T1 cells were injected subcutaneously into WT BALB/c mice on day 0. Then, the mice were intraperitoneally injected with 5 mg/kg oridonin on days 6, 9, 12 and 15, along with anti-PD-1 on days 6, 9, 12 and 15. Tumor size was monitored every three days (a). $CD8^+$ T (b), IFN-γ^+CD8^+ T (c), Granzyme B^+CD8^+ T cells (d) in the TILs of tumor tissues from tumor-bearing mice were analyzed by flow cytometry and the corresponding statistical analysis is shown. ** $P < 0.01$, ***$P < 0.005$ (unpaired Student's *t* test). Representative results from three independent experiments are shown (mean and s.d.) (n = 3–5).

Funding

This work was supported by grants from the Provincial Natural Science Foundation of Zhejiang (LQ20H160018) and the Hangzhou Health Science and Technology plan (OO20191170).

CRediT authorship contribution statement

Jufeng Guo: Conceptualization, Methodology, Writing - original draft. **Tao Chen:** Conceptualization, Methodology, Writing - original draft. **Zeyu Ma:** Investigation, Software. **Chenxiao Qiao:** Investigation, Software. **Fanli Yuan:** Investigation, Methodology, Visualization. **Xintian Guo:** Investigation, Methodology, Visualization. **Jian Liu:** Supervision, Software. **Yingying Shen:** Supervision, Software. **Lei Yu:** Writing - original draft, Writing - review & editing. **Aizhai Xiang:** Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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