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Resveratrol reduces lactate production and modifies the ovarian cancer immune microenvironment

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Tumor cells show deregulated metabolism leading to an enrichment of lactate in the tumor microenvironment (TME). This lactate-rich environment has been reported to impair effector T cells. However, T-regulatory cells (Tregs) show metabolic advantages in lactate-rich TME that maintain a strong suppression of effector T cells, which leads to tumor immune evasion. Therefore, the glycolytic process of tumors could represent a therapeutic target, and agents that modify the energy metabolism of tumor cells have therapeutic potential. Resveratrol is a naturally occurring polyphenol that has been confirmed to suppress tumor cells' glycolytic metabolism. In this study, we show that resveratrol induces metabolic reprogramming in ovarian cancer cells. Resveratrol increases oxidative and decreases glycolysis, in association with decreased lactate production both *in vitro* and *in vivo*. Lactate reduction in TME weakens the suppressive function of Tregs, and subsequently restores anti-tumor immunity. Significantly, combined resveratrol and PD-1 blockade promote anti-tumor efficacy. These data suggest that resveratrol's anti-tumor actions in ovarian cancer could be explained, in part, through modification of the anti-tumor immunity, and indicate a novel treatment strategy for improving immune checkpoint blockade therapy using resveratrol.

Key words: resveratrol, lactate, regulatory T cells, PD-1 blockade

Tumors arise from healthy tissues and acquire properties that enable growth independently from the host's environment. Energy metabolism reprogramming, which promotes rapid cell growth and proliferation by regulating energy metabolism, is thought to be a unique hallmark of tumor cells. Even under sufficient oxygen conditions, tumor cells still preferentially utilize glycolysis to produce ATP, characterized by increased glycolysis flux and lactate production, which is well known for aerobic glycolysis (Warburg effect) [1, 2]. This altered metabolism leads to lactate enrichment in the tumor microenvironment, impairing effector T cells, but not Tregs [3, 4]. Tregs have a distinct metabolic profile from effector T cells: resist T cell function and proliferation mediated by lactate suppression [5, 6]. Such metabolic adaptations enable Tregs to impair anti-cancer immunity, allowing cancer cells to evade immune destruction in the TME.

Resveratrol, a natural polyphenol mainly found in grapes, has been known for different anti-cancer effects. Its anti-cancer properties have been extensively described, including cell proliferation inhibition, apoptosis induction, metastatic potential suppression, metabolic program alteration, as well as direct cytotoxicity [7]. Many studies have proposed that resveratrol can reduce tumor cell energy expenditure, mimicking caloric restriction effects [8–10]. Resveratrol reduces glucose utilization by negatively regulating proteins and enzymes involved in glucose metabolism, thereby decreasing lactate production.

Preclinical studies have emphasized resveratrol's beneficial actions in cancer prevention and treatment and several phases I and II clinical trials are also confirming its pharmacological effects on colon and rectal cancer [11, 12]. Resveratrol combination with conventional chemotherapeutic or novel targeted therapy agents have also suggested its possibilities for refractory cancer treatment [13–15].

In this study, we showed that resveratrol modifies the tumor immune microenvironment (TIME) by reducing lactate production in tumor cells, thereby restraining Tregs' function and reversing anti-tumor immunity. We also found an improvement of immune checkpoint blockade therapy with resveratrol.

Materials and methods

Cell lines and cell culture. Ovarian cancer cell line ID8 (RRID: CVCL_VA22) and SK-OV-3 (RRID: CVCL_0532) were acquired from the American Type Culture Collection (ATCC). ID8 cells were cultured in DMEM supplemented with 10% FBS. SK-OV-3 cells were cultured in RPMI-1640 containing 10% FBS. The cells were cultured in an incubator at 37 °C with 5% CO_2.

Mice. Mice were maintained in specific pathogen-free conditions under protocols approved by the Animal Care Committee of Guangdong Provincial People's Hospital, in compliance with all relevant ethical regulations. Throughout the entire project, the mice were housed in standard polypropylene cages, at optimum density, and in standard laboratory conditions (temperature 25±1 °C, relative humidity 55±5%, and 12 h light/dark cycle). They were allowed free access to a standard granular diet and water. 5- to 21-week-old animals were euthanized by carbon dioxide asphyxiation followed by cervical dislocation, and lymph nodes or spleens were harvested postmortem.

The following mice were used: C57BL/6J and C57BL/6J-*Foxp3*$^{YFP.cre}$ [16]. In this study, tumor-bearing C57BL/6J-*Foxp3*$^{YFP.cre}$ mice were used to analyze and isolate tumor-infiltrating Tregs ($CD45^+CD3^+CD4^+YFP^+$) for Treg suppression assays. These mice were purchased from the International Mouse Knockout Consortium and kindly provided by Prof Zheng [17].

Quantitative real-time polymerase chain reaction analysis. The total RNA of tissues was extracted by TRIzol reagent (Invitrogen; #15596026). After the determination of the RNA concentration, a PrimeScript RT reagent kit (TaKaRa; #RR047A) was used to synthesize complementary DNA. qRT-PCR was performed using a TB Green Fast qPCR Mix (TaKaRa; #RR430A). GAPDH was used to normalize mRNA. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes. Each test was repeated 3 times. The primer sequences were as follows: PKM2 forward, 5'-GCCGCCTGGACATTGACTC-3' and reverse 5'-CCATGAGAGAAATTCAGCCGAG-3'; GLUT1 forward, 5'-TCTCGGCTTAGGGCATGGAT-3' and reverse 5'-TCTATGACGCCGTGATAGCAG-3'; GAPDH forward, 5'-TGGATTTGGACGCATTGGTC-3' and reverse 5'-TTTGCACTGGTACGTGTTGAT-3'.

Measurement of intracellular ROS concentration. Intracellular ROS levels were quantified with 2',7'-dichlorofluorescein-diacetate (H_2DCFDA; Sigma; #D6883). Briefly, the culture medium was removed from the cells in a 96-well black plate, and 100 μl of 10 μM H_2DCFDA in PBS was added. The cells were incubated in the dark for 30 min at 37 °C with 5% CO_2, then were washed with warmed PBS, and 100 μl of PBS was added to each well. Fluorescence was measured on a microplate reader using 490 nm excitation and 510 to 570 nm emission wavelengths.

Measurement of glucose uptake. Cells were prepared 48 h before experiments. Glucose uptake was measured using a glucose uptake assay kit according to the manufacturer's protocol (Abcam; #ab136955).

Measurement of lactate production. L-lactate production was measured from culture medium or interstitial fluid from spleen or tumors using a Cobas assay kit (Roche; #03183700190) following the manufacturer's instructions. Lactate concentration was calculated from a standard curve of serially diluted standards and expressed in mU/mg.

Quantification of interstitial lactate. Tumors and spleens were collected and cut up with scissors, wrapped with a 5 μm nylon filter paper, and stuffed into a 1.5 ml conical tube, making sure that the tissue did not touch the bottom. Tissues were centrifuged at 4,000×g for 2 h. Interstitial fluid was assayed for L-lactate concentration using a colorimetric detection kit according to the manufacturer's protocol (Abcam; #ab65331).

Cell isolation and flow cytometry. Single-cell suspensions were prepared from tumors upon sacrifice. Tumors were minced and digested with Collagenase IV (Sigma, #C5138) for 30 min at 37 °C. Cells were filtered through 70 μm strainers, washed with PBS, and stained for flow cytometry. Cells of interest were analyzed using surface markers, and for Foxp3, TNF-α, or Ki67 staining, surface marker-stained cells were fixed, permeabilized, and labeled with Foxp3-, TNF-α-, or Ki67-specific mAb. All flow cytometry data were captured using FASCcan Flow Cytometer and analyzed using the FlowJo software. APC/Cyanine7 anti-mouse CD45 Antibody (#157203), PerCP/Cyanine5.5 anti-mouse CD3 Antibody (#100217), Alexa Fluor700 anti-mouse CD4 Antibody (#116021), PE/Cyanine7 anti-mouse CD8a Antibody (#100721), APC anti-mouse B220 Antibody (#103211), PE anti-mouse NK1.1 Antibody (#108707), FITC anti-mouse PD-1 Antibody (#135213), APC anti-mouse NRP-1 Antibody (#145205), PE anti-mouse CD44 Antibody (#103024), PE/Cyanine7 anti-mouse CD11b Antibody (#101215), APC anti-mouse Ly-6G Antibody (#127613), PE/Dazzle 594 anti-mouse F4/80 Antibody (#123145), PerCP/Cyanine5.5 anti-mouse I-A/I-E Antibody (#107625), Alexa Fluor700 anti-mouse CD11c Antibody (#117319), APC anti-mouse Ki67 Antibody (#652405), APC anti-mouse TNF-α Antibody (#506307) were purchased from Biologend Group, Inc. PE/Cyanine7 anti-mouse Foxp3 Antibody (#25-5773-82) was obtained from Invitrogen Group, Inc.

Treg isolation. Tumor-infiltrating Tregs were isolated from tumor-bearing C57BL/6J-*Foxp3*$^{YFP.cre}$ mice. Tumors of C57BL/6J-*Foxp3*$^{YFP.cre}$ mice with or without resveratrol treatment were minced and digested with Collagenase IV (Sigma, #C5138) for 30 min at 37 °C. Cells were filtered through 70 μm strainers and washed with PBS. Treg cell populations ($CD45^+CD3^+CD4^+YFP^+$) were isolated by flow-assisted sorting.

iTreg preparation. For conversion to $Foxp3^+$ Tregs, Tconv cells were purified from the lymph node of the WT

C57BL/6 mice, and incubated for 3 days with CD3ε/CD28 mAb beads (Thermo Fisher; #11453D), plus TGF-β (PeproTech; #AF-100-21C-10; 3 ng/ml) and IL-2 (R&D; #402-ML-020; 25 U/ml), and analyzed by flow cytometry for Foxp3$^+$ iTreg.

Microsuppression and proliferation assays. For Treg suppression assays, Treg cells were cocultured for 72 h with APCs and carboxyfluorescein succinimidyl ester (CFSE; eBioscience; #65-0850-84)-labeled conventional T cells (Tconv) cells at ratios 1:5 in complete RPMI medium with CD3ε mAb (Biolegend; #100340; 1 μg/ml). The proliferation of Tconv cells was determined by flow cytometric analysis of CFSE.

***In vivo* ovarian cancer xenograft model.** Animal protocols were approved by the animal care committee of Guangdong Provincial People's Hospital. ID8 cells (1×10^6) were injected subcutaneously into the back of 4-week-old C57BL/6 female mice. Resveratrol (20 mg/kg body weight) suspended in PBS was administered to these mice via oral gavage every 2 days starting a week before tumor inoculation until the end of the study. One week after engraftment, αPD-1 (BioXCell; #BE0273; 250 μg) was intraperitoneally administered to mice daily. The control group received vehicle (PBS) only. Tumor sizes were measured at the indicated times. Tumor volumes were estimated according to the following formula: volume = (longest diameter × shortest diameter^2)/2.

Western blot. SK-OV-3 and ID8 cells with or without resveratrol treatment were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein quantification was performed by a BCA Protein Assay (Thermo Fisher;

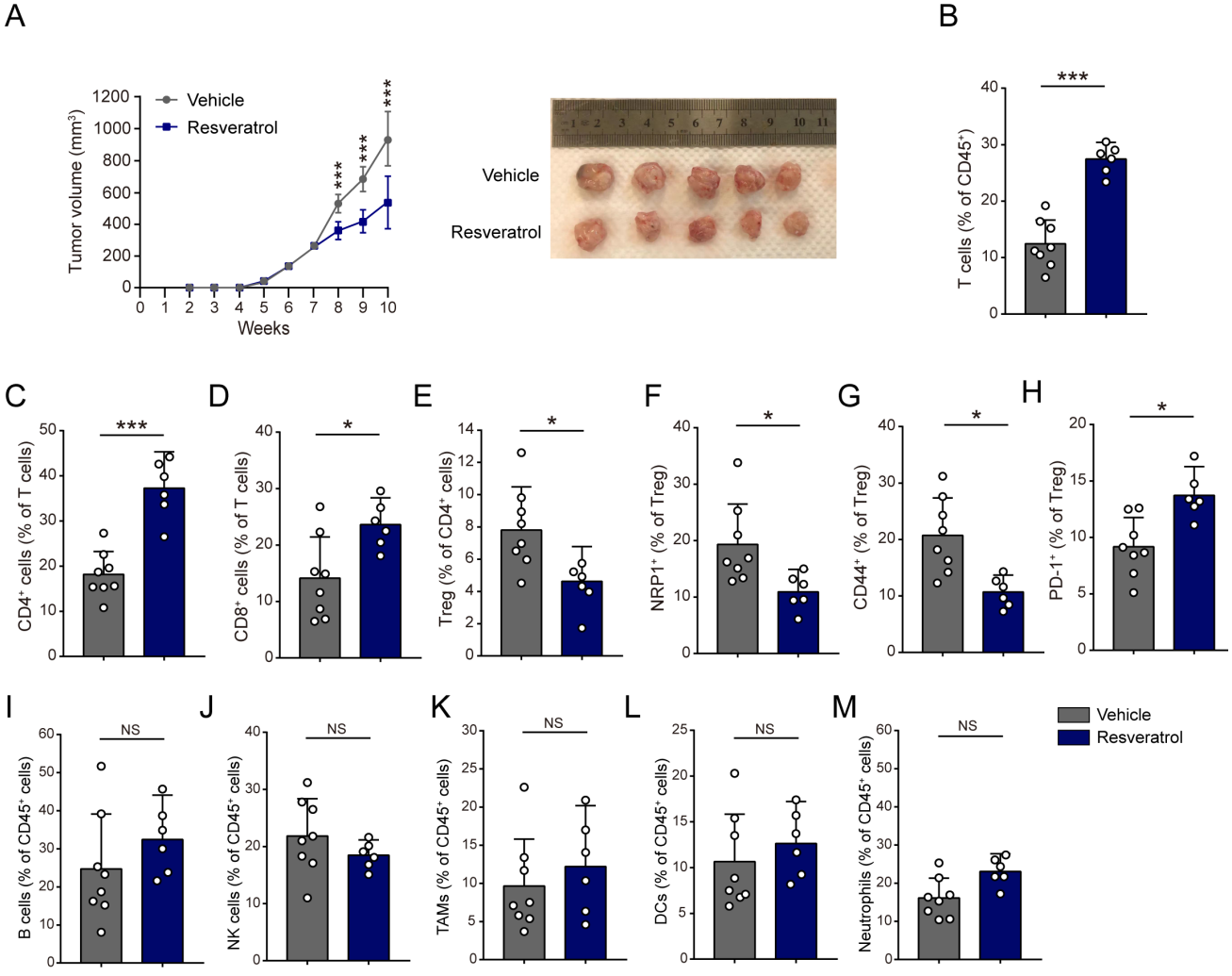

Figure 1. Resveratrol suppresses tumor growth and modifies the tumor immune microenvironment (TIME). A) Oral gavage of resveratrol (20 mg/kg) every 2 days starting 1 week prior to subcutaneous injections of ID8 tumor cells into the back of mice. Left, at the indicated times, tumors were measured with vernier calipers. Right, all the mice were euthanized at week 13, a representative image of tumor excised from mice is shown. n=5, each group. B–M) Flow cytometry analysis of the immune infiltrate of tumors with or without resveratrol treatment. Vehicle-treated group, n=8; resveratrol-treated group, n=6.

#23227). Then 20 μg of soluble protein were loaded onto each lane of 8–12% Bis-Tris gel. The proteins were transferred to polyvinylidene fluoride (PVDF; Merck Millipore; #IPVH00010) membrane. For the immunoblot, the membranes were blocked with 5% skimmed milk (Bio-Rad; #1706404) in TBST for 1 h. Primary antibodies (1:1,000 dilution) in 5% bovine serum albumin (BSA; Aladdin; #9048-46-8) were added and incubated overnight at 4 °C on a shaker. The membranes then were washed with TBST and incubated with secondary antibody (1:5,000 dilution) in 5% skimmed milk at room temperature for 1 h. The membranes then were washed with TBST and incubated with ECL mix (Epizyme; cat. no. SQ202). Rabbit antibody against PKM2 (cat. no. 15822), GLUT1 (cat. no. 21829), mouse antibody against GAPDH (cat. no. 60004), and horseradish peroxidase-conjugated secondary antibodies (cat. no. SA00001-1 and SA00001-2) were obtained from ProteinTech Group, Inc.

Statistical analysis. GraphPad Prism 7 was applied for statistical data analysis. Unpaired t-test was utilized to analyze the data conforming to normal distribution and homogeneity of variance between two groups. All experiments were repeated 3 times unless stated otherwise. Data are presented as mean values with a standard error of mean (SEM). The two-sided p-value <0.05 were defined as statistically significant.

Results

Resveratrol suppresses tumor growth and modifies TIME. In this study, we first evaluated *in vivo* resveratrol's treatment effect on ovarian cancer using the xenograft C57BL/6 mice model. Mice received vehicle (control) or resveratrol by oral gavage every 2 days, starting 1 week before ID8 tumor cells subcutaneous injections into the mice's back. Throughout the entire experiment, tumor growth increased exponentially in the control, but in resveratrol-treated mice, the growth was significantly reduced (Figure 1A). Then, we

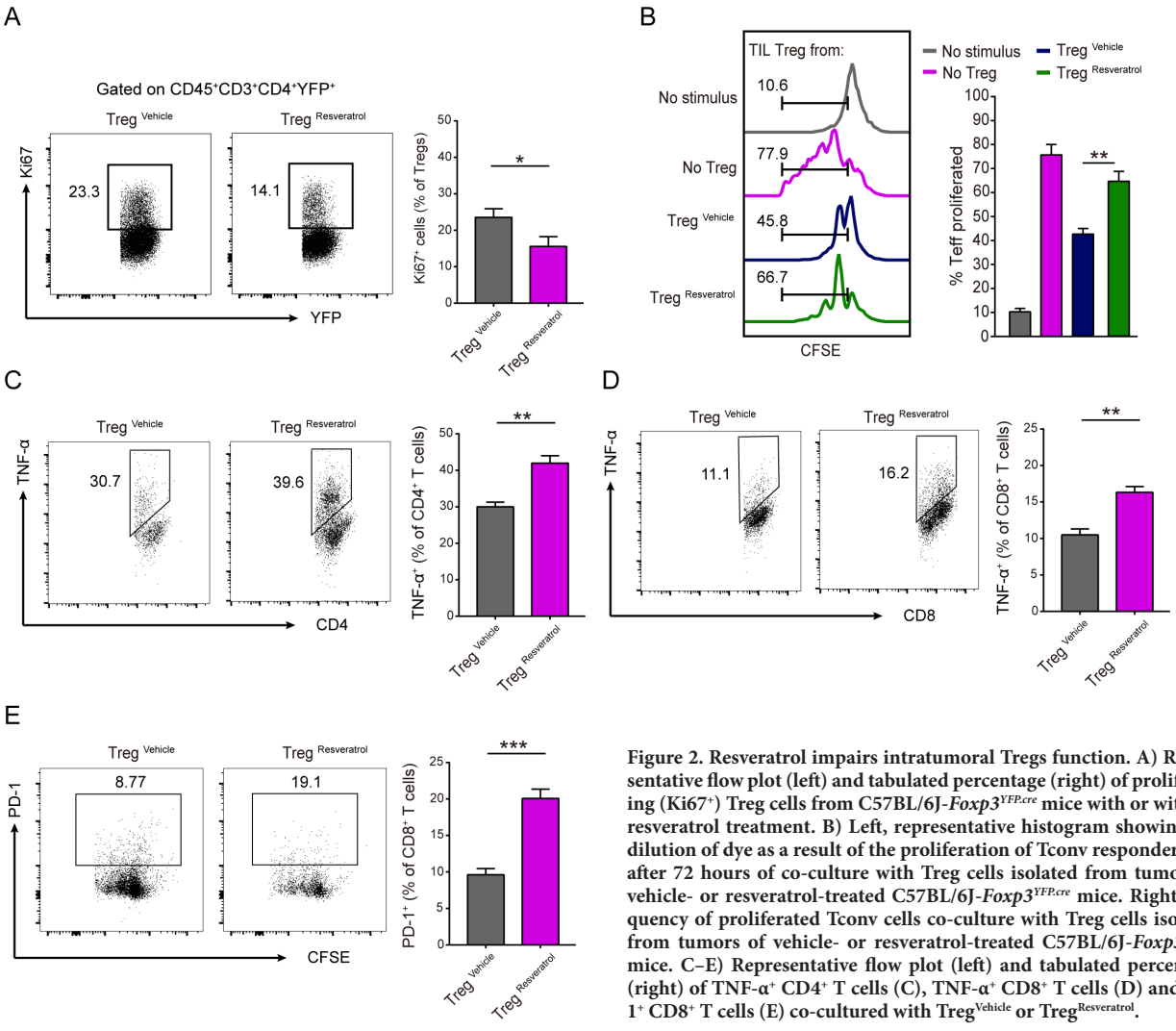

Figure 2. Resveratrol impairs intratumoral Tregs function. A) Representative flow plot (left) and tabulated percentage (right) of proliferating (Ki67+) Treg cells from C57BL/6J-*Foxp3*$^{YFP.cre}$ mice with or without resveratrol treatment. B) Left, representative histogram showing the dilution of dye as a result of the proliferation of Tconv responder cells after 72 hours of co-culture with Treg cells isolated from tumors of vehicle- or resveratrol-treated C57BL/6J-*Foxp3*$^{YFP.cre}$ mice. Right, frequency of proliferated Tconv cells co-culture with Treg cells isolated from tumors of vehicle- or resveratrol-treated C57BL/6J-*Foxp3*$^{YFP.cre}$ mice. C–E) Representative flow plot (left) and tabulated percentage (right) of TNF-α+ CD4+ T cells (C), TNF-α+ CD8+ T cells (D) and PD-1+ CD8+ T cells (E) co-cultured with Treg^{Vehicle} or Treg^{Resveratrol}.

examined the ID8 tumors' immune infiltrates in vehicle- and resveratrol-treated groups by flow cytometry (Supplementary Figures S1A–S1C). Results showed that the T cells relative proportion, as well as $CD4^+$ T and $CD8^+$ T cells, significantly increased, while Treg cells reduced in resveratrol-treated infiltrates (Figures 1B–1E). Characterization of intratumoral resveratrol-treated Treg cells revealed decreases in Nrp1 and CD44 staining, as well as elevated PD-1 staining, potentially indicating dysfunctional Treg cells (Figures 1F–1H). B cells, natural killer (NK) cells, neutrophils, tumor-associated macrophages (TAMs), and dendritic cells were equally represented in control and resveratrol-treated groups' tumor infiltrates (Figures 1I–1M). Collectively, these data suggested that resveratrol treatment modified the tumor immune microenvironment.

Resveratrol impairs intratumoral Tregs function. To further identify resveratrol's TIME modulation in ovarian cancer, we measured the Tregs suppressor activity, isolated from C57BL/6J-*Foxp3*$^{YFP.cre}$ mice bearing tumors, with vehicle or resveratrol treatment. Results show that intratumoral Tregs from resveratrol-treated mice ($Treg^{Resveratrol}$) were less proliferative than that from vehicle-treated mice ($Treg^{Vehicle}$) (Figure 2A). Next, we co-cultured T cells with $Treg^{Vehicle}$ or $Treg^{Resveratrol}$ and used a CFSE dilution assay as the readout for T cell proliferation. We found that $Treg^{Resveratrol}$ had reduced suppressive function *ex vivo* than $Treg^{Vehicle}$ (Figure 2B). Also, increased numbers of TNF-α^+ $CD4^+$ T cells, TNF-α^+ $CD8^+$ T cells and PD-1^+ $CD8^+$ T cells were found in $Treg^{Resveratrol}$ co-culture groups (Figures 2C–2E).

Resveratrol decreases intratumoral lactate production. Lactic acid is highly enriched in the TME and is known to be immunosuppressive. Indeed, lactate promotes the function of Treg cells. Resveratrol can suppress tumor cells' lactate production through several signaling pathways, such as PI3K/Akt, mTOR, and AMPK [8]. PKM2 and GLUT1 are also involved in tumor cells' reduced glucose uptake and

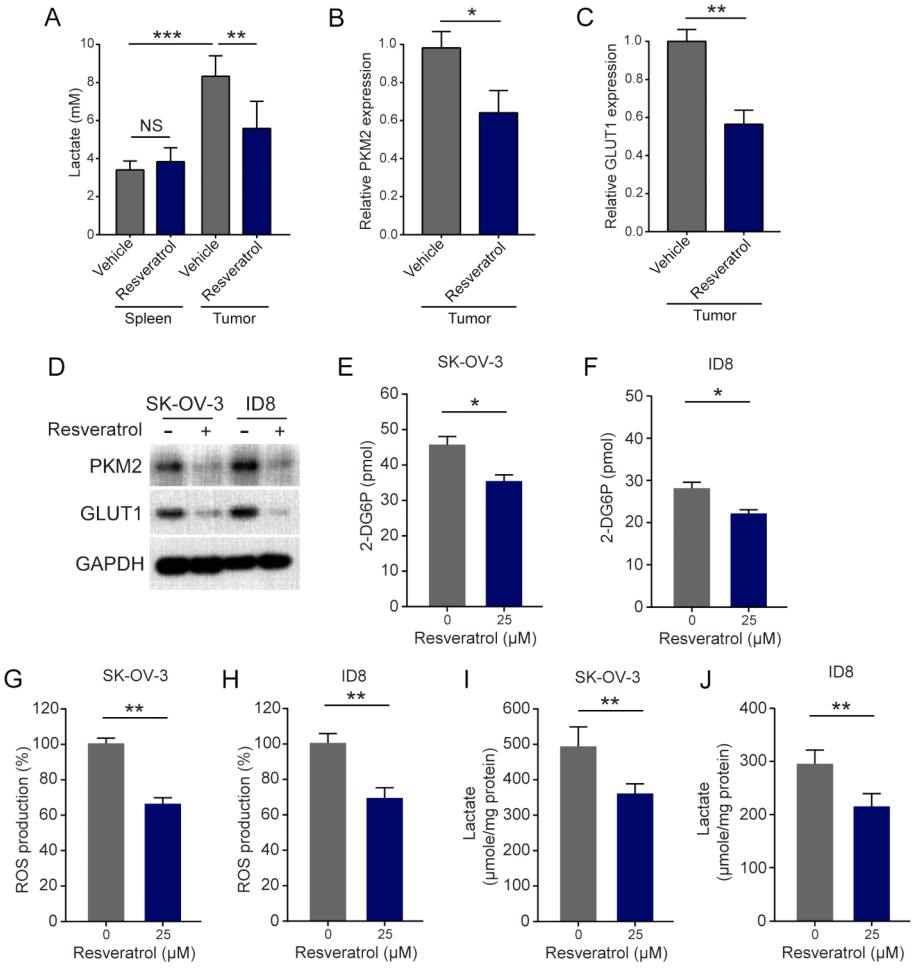

Figure 3. Resveratrol decreases intratumoral lactate production. A) Lactate concentration in interstitial fluid from spleen or tumors with or without resveratrol treatment. B, C) PKM2 (B) and GLUT1 (C) expressions in tumors from vehicle- or resveratrol-treated mice were evaluated by RT-qPCR. D) PKM2 and GLUT1 expression in control or resveratrol-treated ovarian cancer cell lines were evaluated by western blot. E, F) 2-DG6P uptake in vehicle- or resveratrol-treated SK-OV-3 (E) and ID8 cells (F). G, H) ROS production in vehicle- or resveratrol-treated SK-OV-3 (G) and ID8 cells (H). I, J) Lactate secretion of vehicle- or resveratrol-treated SK-OV-3 (I) and ID8 cells (J).

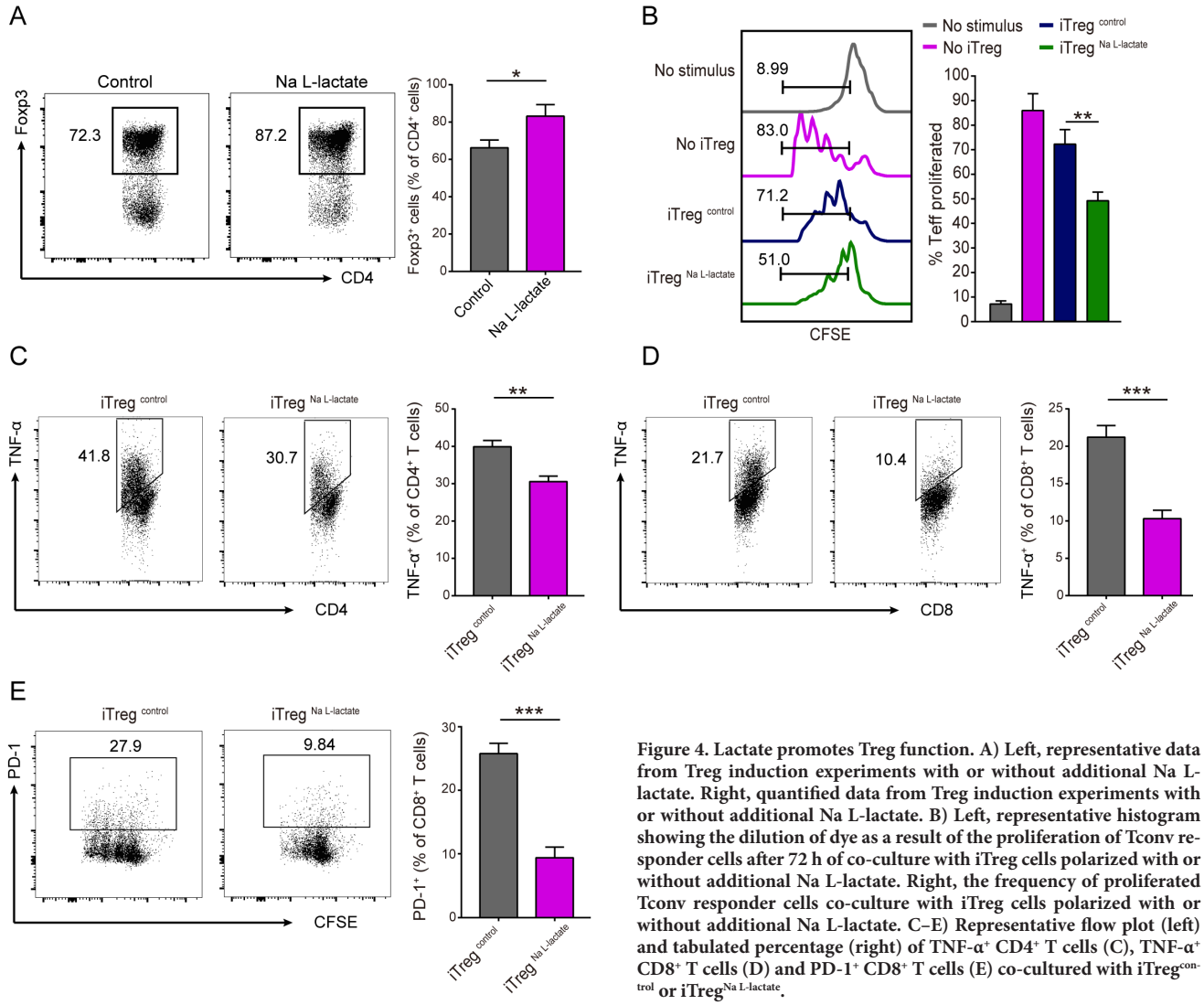

Figure 4. Lactate promotes Treg function. A) Left, representative data from Treg induction experiments with or without additional Na L-lactate. Right, quantified data from Treg induction experiments with or without additional Na L-lactate. B) Left, representative histogram showing the dilution of dye as a result of the proliferation of Tconv responder cells after 72 h of co-culture with iTreg cells polarized with or without additional Na L-lactate. Right, the frequency of proliferated Tconv responder cells co-culture with iTreg cells polarized with or without additional Na L-lactate. C–E) Representative flow plot (left) and tabulated percentage (right) of TNF-α$^+$ CD4$^+$ T cells (C), TNF-α$^+$ CD8$^+$ T cells (D) and PD-1$^+$ CD8$^+$ T cells (E) co-cultured with iTreg^{control} or iTreg$^{Na\ L\text{-}lactate}$.

glycolysis by resveratrol [9, 18–20]. In this study, we found that the lactate concentration in the TME, but not spleen, was reduced in resveratrol-treated mice (Figure 3A), along with PKM2 and GLUT1 downregulation in tumors (Figures 3B, 3C) and ovarian cancer cell lines (Figure 3D). In SK-OV-3 and ID8 cells, glucose uptake and ROS concentration were diminished by resveratrol treatment, in association with decreased lactate production (Figures 3E–3J). These results supported that resveratrol suppresses ovarian cancer cell glucose uptake and lactate production.

Lactate promotes Treg function. To further identify the lactate effect on Tregs, we cultured conventional T cells (Tconv) under polarizing conditions to form iTregs, with or without additional Na L-lactate. These results indicated that lactate led to an increase in iTreg formation (Figure 4A). Furthermore, lactate increased iTregs suppressor activity (Figure 4B). Moreover, decreased numbers of TNF-α$^+$ CD4$^+$ T cells, TNF-α$^+$ CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells were found in Treg$^{Na\ L\text{-}lactate}$ co-culture groups (Figures 4C–4E). Altogether, our findings supported an immunosuppressive role for lactate promoting Tregs function, and that this can be diminished by resveratrol.

Combined resveratrol and PD-1 blockade promotes anti-tumor efficacy. A recent study has suggested a potential upregulation of PD-L1 *in vivo* by resveratrol [21]. Another study shows that resveratrol interferes the PD-L1 stability and trafficking by targeting PD-L1 glycosylation and dimerization [22]. Both studies indicate that resveratrol may render tumor cells more sensitive to PD-1/PD-L1 blockade. Here, we carried out a treatment study using ID8 xenograft in C57BL/6 mice, with four treatment groups (vehicle, resveratrol, anti-PD-1, and combined resveratrol and anti-PD-1) (Figure 5A). Both resveratrol and anti-PD-1 monotherapies were able to reduce tumor size, a combination of resveratrol

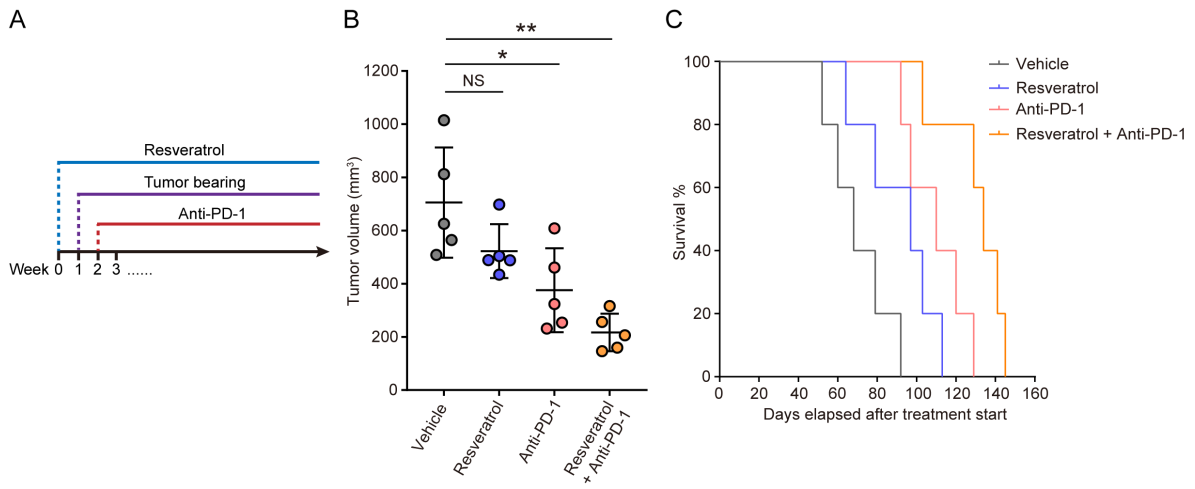

Figure 5. Combined resveratrol and PD-1 blockade promotes anti-tumor efficacy. A) Treatment of ID8 tumors in C57BL/6 mice with both resveratrol and PD-1 blockade, and combination therapy. B) The tumor volumes were measured in week 8. n=5, each group, an experiment was conducted once. Unpaired one-way ANOVA without multiple comparison correction. C) Survival analysis of mice shown in A. n=5, each group, one-tailed log-rank Mantel-Cox test.

and anti-PD-1 showed a great reduction (Figure 5B). This enhancement in anti-tumor efficacy also had an effect on survival, with both resveratrol and anti-PD-1 monotherapy groups living significantly longer than the vehicle control (Figure 5C). Combination therapy increased survival more than either one of the monotherapies (Figure 5C).

Discussion

Solid tumors usually exhibit glucose deprivation and lactate enrichment in TME. This promotes invasion, metastasis, immune evasion, and therapy resistance [23–28]. Lactate selectively impairs cytotoxic and effector T cells. However, Tregs are metabolically better adapted to the lactate-rich TME. Although Tregs seemingly do not require lactate for survival, they have the metabolic flexibility to use this carbon source as fuel and as means to protect their high suppressive capacity from glucose's negative effects [5, 6]. This difference in immune cells suggests that modulating lactate production of tumor cells may be helpful to cancer therapy.

Several signaling pathways have been previously implicated in glucose metabolism downregulation by resveratrol. PI3K/Akt, mTOR, and AMPK signaling are involved in tumor cells' reduced glucose uptake and glycolysis by resveratrol [8]. Decreased glycolytic metabolism by resveratrol can be mediated by downregulated PKM2 expression [18, 19]. GLUT1 downregulation by resveratrol also decreased glucose uptake in tumor cells [9, 20]. Our results are in agreement with these studies, suggesting that PI3K/Akt and mTOR signaling are involved in the resveratrol capacity to suppress ovarian cancer cell glucose metabolism. Glucose uptake reduction by resveratrol in our study was also linked to downregulated PKM2 and GLUT1 expressions and further linked to decreased lactate production.

Immune checkpoint blockade (ICB) with antibodies targeting CTLA-4 and PD-1/PD-L1 has led to impressive and durable clinical responses in diverse cancer patients, including melanoma, colorectal cancer, non-small-cell lung cancer, and Hodgkin's lymphoma [29–33]. However, clinical observations showed that only a few patients are able to benefit from this therapy. There are many factors that lead to immunotherapy failure. The tumor microenvironment contains a variety of immune cells that inhibit cytotoxic T cell function, including myeloid cells, tumor-associated macrophages, and regulatory T cells. Signals released by tumor cells play a key role in creating this deep immunosuppressive environment, which limits the anti-tumor immunity. Therefore, effective new strategies that improve ICB therapy are urgently needed.

In this study, we showed that resveratrol induced ovarian cancer cells' metabolic reprogramming, leading to decreases in lactate production, negated Tregs' detrimental metabolic advantages over effector and cytotoxic T cells, thereby enhancing anti-tumor immunity. We also highlighted a novel treatment strategy for improving PD-1 blockade therapy efficacy in ovarian cancer using resveratrol.

Supplementary information is available in the online version of the paper.

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Resveratrol reduces lactate production and modifies the ovarian cancer immune microenvironment

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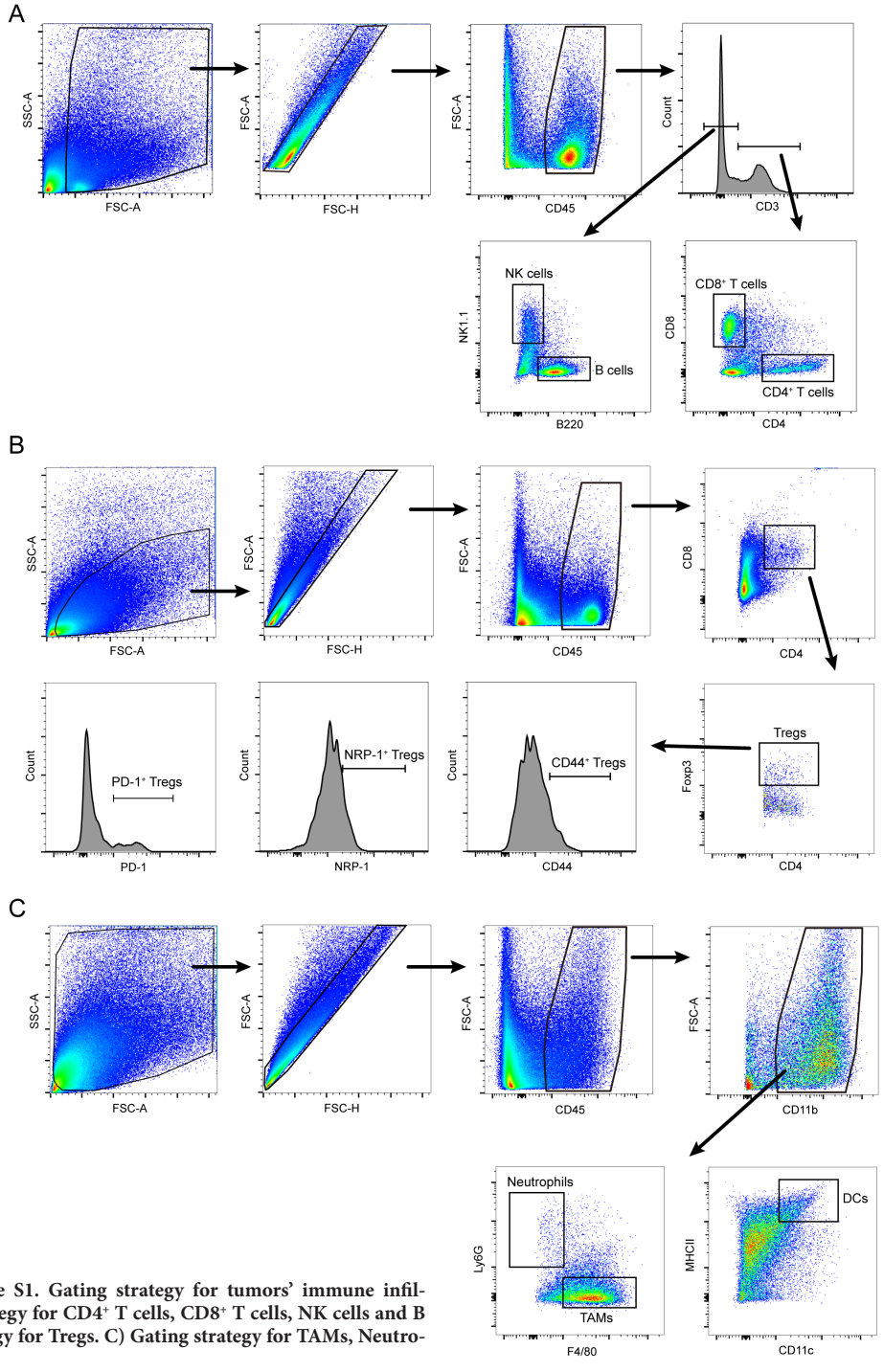
Supplementary Information



Supplementary Figure S1. Gating strategy for tumors' immune infiltrates. A) Gating strategy for CD4⁺ T cells, CD8⁺ T cells, NK cells and B cells. B) Gating strategy for Tregs. C) Gating strategy for TAMs, Neutrophils and DCs.