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Artemisinin enhances the anti-tumor immune response in 4T1 breast cancer cells *in vitro* and *in vivo*

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ABSTRACT

Background: Breast cancer is a prominent cause of death among women worldwide. Recent studies have demonstrated that artemisinin (ART) displays anti-tumor activity. Using a mouse breast cancer model, we investigated the effects of ART *in vitro* and *in vivo* to determine how it influences the anti-tumor immune response.
Methods: We measured the proliferation and apoptosis of 4T1 cells *in vitro* after ART treatment by MTT assay and FACS. To examine the effects of ART *in vivo*, tumor volumes and survival rates were measured in 4T1 tumor-bearing mice. FACS was used to determine the frequencies of Tregs, MDSCs, $CD4^+$ IFN-γ^+ T cells, and CTLs in the tumors and spleens of the mice. mRNA levels of the transcription factors T-bet and FOXP3 and cytokines IFN-γ, TNF-α, TGF-β, and IL-10 were also determined by real-time RT-PCR. ELISA was used to measure TGF-β protein levels in the cell culture supernatants.
Results: ART supplementation significantly increased 4T1 cell apoptosis and decreased TGF-β levels *in vitro*. ART also impeded tumor growth in 4T1 TB mice and extended their survival. MDSC and Treg frequencies significantly decreased in the 4T1 TB mice after ART treatment while $CD4^+$ IFN-γ^+ T cells and CTLs significantly increased. ART significantly increased T-bet, IFN-γ, and TNF-α mRNA levels within the tumor and significantly decreased TGF-β mRNA levels.
Conclusion: ART supplementation hindered 4T1 tumor growth *in vivo* by promoting T cell activation and quelling immunosuppression from Tregs and MDSCs in the tumor.

1. Introduction

Breast cancer remains one of the leading causes of cancer-related death in women worldwide [1]. Although considerable progress has been made in treating breast cancer and prolonging patient survival, the cancer often reoccurs [2,3]. Additionally, side effects from these treatments can have many adverse effects on the patients' quality of life [4,5]. A greater understanding of breast cancer metastasis and discovery of new drugs are essential for the development of novel treatment strategies to eradicate breast cancer.

Artemisinin (ART) and its derivatives have long been used as antimalarials due to their efficacy and low toxicity [6–8]. However, recent studies have shown that ART derivatives possess profound anti-tumor activity as well. These studies have shown that ART inhibits the growth of numerous types of cancer cells, including breast, lung, prostate, melanoma, renal, gastric, and CNS cancer cells [9–13]. ART can also inhibit the growth of many drug-resistant cancer cell types [9]. Additionally, ART derivatives may also inhibit the growth of human tumor xenografts transplanted into mice [13].

ART inhibits the proliferation of cancer cells through several mechanisms: 1) ART interacts with ferrous iron [14], resulting in accumulation of reactive oxygen species (ROS) [15,16]. ROS then weaken the integrity of the cell [17], inducing cell cycle arrest [18], apoptosis [19], and autophagy [20]; 2) ART inhibits angiogenesis by inhibiting the secretion of VEGF, VEGFR2, and KDR/flk-1 in tumors [21,22]; and 3) ART may affect signaling pathways and transcription factors associated with tumor growth, including the Wnt/β-catenin pathway, the AMPK pathway, nitric oxide signaling, NF-κB, CREBP, MYC/MAX,

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mTOR, and AP-1 [23]. However, the immune mechanisms affected by ART have not been described. In this study, we examined the effects of ART on anti-tumor immunity *in vitro* and *in vivo* to determine whether ART may be used to treat breast cancer.

2. Materials and methods

2.1. Cell lines and mice

Mouse 4T1 mammary carcinoma cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Inc., USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 mg/mL streptomycin, 100 U/mL penicillin, and 1 mM L-glutamine. Cells were grown at 37 °C with 5% CO_2 and 100% humidity. Experiments were performed using cells in the exponential growth phase.

Six to eight-week-old female Balb/c mice were acquired from the Center of Zoology, Chinese Academy of Sciences, Shanghai Branch. The mice were housed under standard conditions for a week prior to the beginning of the experiment and given unimpeded access to food and water. All animal experiments were conducted under the guidelines of the Guide for the Care and Use of Laboratory Animals, China law of animal welfare. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of China National Institutes of Health (permit number GB 14923-2001).

2.2. Reagents

Artemisinin (ART, Sigma, St. Louis, MO, USA) was suspended in dimethylsulfoxide (DMSO, Solon, OH, USA) to create a stock solution of 10 mmol/L. The stock solution was stored at −20 °C until use and further diluted in DMEM to its final concentration for *in vitro* use. For the *in vivo* experiments, ART was diluted with sterile PBS to 5 mg/mL before administration. All flow cytometry antibodies were purchased from BD Pharmingen (San Diego, CA, USA) unless otherwise indicated. A TGF-β enzyme linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA), and RPMI 1640 with 10% FBS was purchased from Life Technologies (Bedford, MA, USA).

2.3. Establishment of the 4T1 breast cancer model

Sub-confluent 4T1 cells were harvested, washed once in serum-free media, and resuspended in PBS at a concentration of 5×10^5 cells/0.1 mL PBS. 0.1 mL of the cell suspension was then implanted into the abdominal mammary fat pad of female BALB/c mice subcutaneously. Once the tumor was palpable (5–7 days after implantation), the mice were randomized into either the control group (n = 7; intraperitoneal injection with 200 μl sterile PBS daily for 20 days) or the ART group (n = 10; intraperitoneal injection with 100 mg/kg ART dissolved in 0.2% DMSO daily for 20 days). Tumor volume was measured every 2–3 days using the following formula: $V = 0.5236 \times {d_1}^2 \times d_2$, where d_1 is the shortest diameter and d_2 is the longest diameter.

2.4. MTT assay

4T1 cells were cultured at a density of 5×10^3 cells per well in 96-well plates. After 24 h, ART was added to the cultures at the following concentrations: 0, 0.01, 0.1, 1, 10, and 100 μM in a final volume of 100 μL for 24, 48, or 72 h. Ten μL of 5 mg/mL MTT was then added to each well and incubated for 4 h at 37 °C. One hundred μL of DMSO was then added for 10 min and incubated with light shaking. Optical densities (OD) at 570 nm were measured using a spectrophotometer. All experiments were repeated five times. Tumor cell proliferation rate is expressed as the percentage of cell growth inhibition using the following formula: % cells = (OD of ART-treated sample / OD of untreated sample) × 100%.

Table 1
Primer sequences for RT-PCR.

Primer name	Sequence (5′–3′)
β-actin_F	GATTACTGCTCTGGCTCCTAGC
β-actin_R	GACTCATCGTACTCCTGCTTGC
IFN-γ_F	GTTACTGCCACGGCACAGTCATTG
IFN-γ_R	ACCATCCTTTTGCCAGTTCCTCCAG
TNF-α_F	GCAAGCTTCGCTCTTCTGTCTACTGAACTTCGG
TNF-α_R	GCTCTAGAATGAGATAGCAAATCGGCTGACGG
T-bet_F	TCAACCAGCACCAGACAGAG
T-bet_R	AAACATCCTGTAATGGCTTGTG
TGF-β_F	TGACGTCACTGGAGTTGTACGG
TGF-β_R	GGTTCATGTCATGGATGGTGC
Il-10_F	ACCTGCTCCACTGCCTTGCT
Il-10_R	GGTTGCCAAGCCTTATCGGA
Foxp3_F	GGCCCTTCTCCAGGACAGA
Foxp3_R	GCTGATCATGGCTGGGTTGT

2.5. Apoptosis assay

4T1 cells were cultured in the presence or absence of ART (100 μM) for 48 h in 96-well plates at a concentration of 1×10^6 cells/mL. To detect apoptosis, the cells were stained using an Annexin-V Apoptosis Detection kit I (BD Biosciences) and subsequently evaluated by flow cytometry. The results were analyzed using FlowJo v7.6.2 and GraphPad Prism 6.0.1 software.

2.6. Flow cytometry

Spleens and tumors were excised from the 4T1 tumor-bearing mice. Single cell suspensions were produced by homogenizing the spleen samples, and the tumors were minced and subsequently digested with 500 U/mL collagenase type IV (Sigma) for 1 h at 37 °C with agitation. The resulting single cell suspensions were suspended in RPMI 1640 with 10% fetal calf serum (FCS, Gibco, England).

To stain myeloid-derived suppressor cells (MDSCs), anti-CD11b FITC (clone M1/70, BD Biosciences) and anti-Gr-1 APC (clone RB6-8C5, BD Biosciences) were added to cells suspended in PBS with 3% FCS and incubated for 30 min. Anti-CD4 FITC (clone H129.19, BD Biosciences) and anti-CD25 PE (clone PC61, BD Biosciences) antibodies were used to stain the cell surface of regulatory T cells (Tregs). The cells were then fixed and permeabilized for intracellular staining, and an anti-Foxp3 APC (clone FJK16s, eBioscience) antibody was added. To analyze $CD4^+$ IFN-γ^+ T cells and cytotoxic T lymphocytes (CTLs) expressing granzyme B, anti-CD4 FITC (clone H129.19, BD Biosciences), anti-CD8a PerCP (clone 53–6.7, BD Biosciences), and anti-IFN-γ APC (clone XMG1.2, BD Biosciences) were added to the cells and incubated for 30 min. Cells were then fixed and permeabilized, and intracellular staining was performed using an anti-granzyme B PE antibody (clone NGZB, eBioscience). All staining reactions were performed in a final volume of 100 μl at 4 °C. Data were acquired using a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using FlowJo v7.6.2 software (Tree Star Inc., Ashland, OR, USA).

2.7. TGF-β ELISA

A commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to analyze TGF-β levels in the cell culture supernatants. All steps were performed according to the manufacturer's instructions, and OD values were measured at 450 nm using a microplate reader. A standard curve was generated using known concentrations of recombinant cytokines, and this was used to calculate the TGF-β concentrations of the

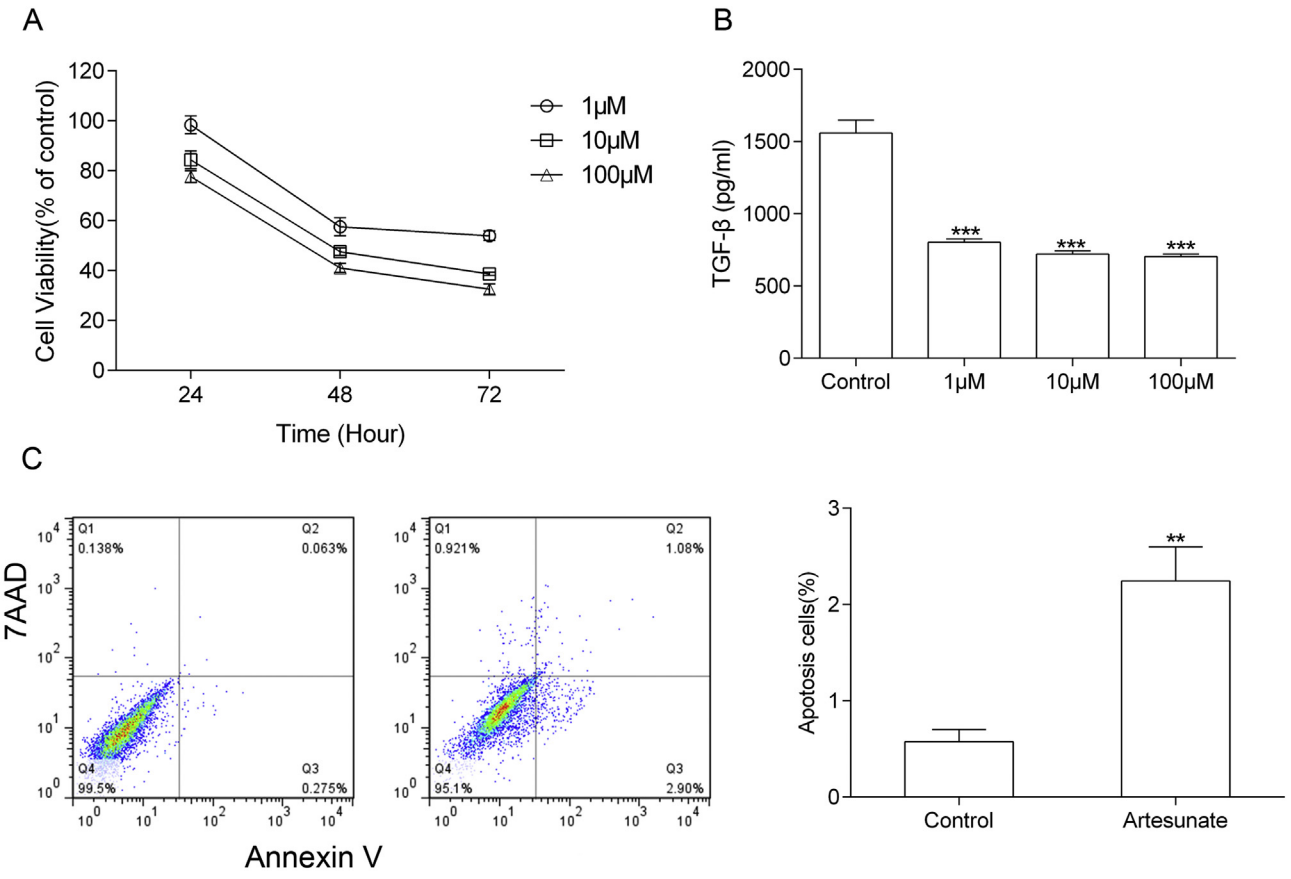

Fig. 1. ART treatment inhibited the proliferation of 4T1 cells *in vitro*. 4 T1 cells were treated with various concentrations of ART for 24, 48, and 72 h. Cell proliferation was then was determined by MTT assay. TGF-β levels in the supernatant were detected by ELISA after treatment with ART for 48 h. Apoptosis was measured by flow cytometry. Results are shown as mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group.

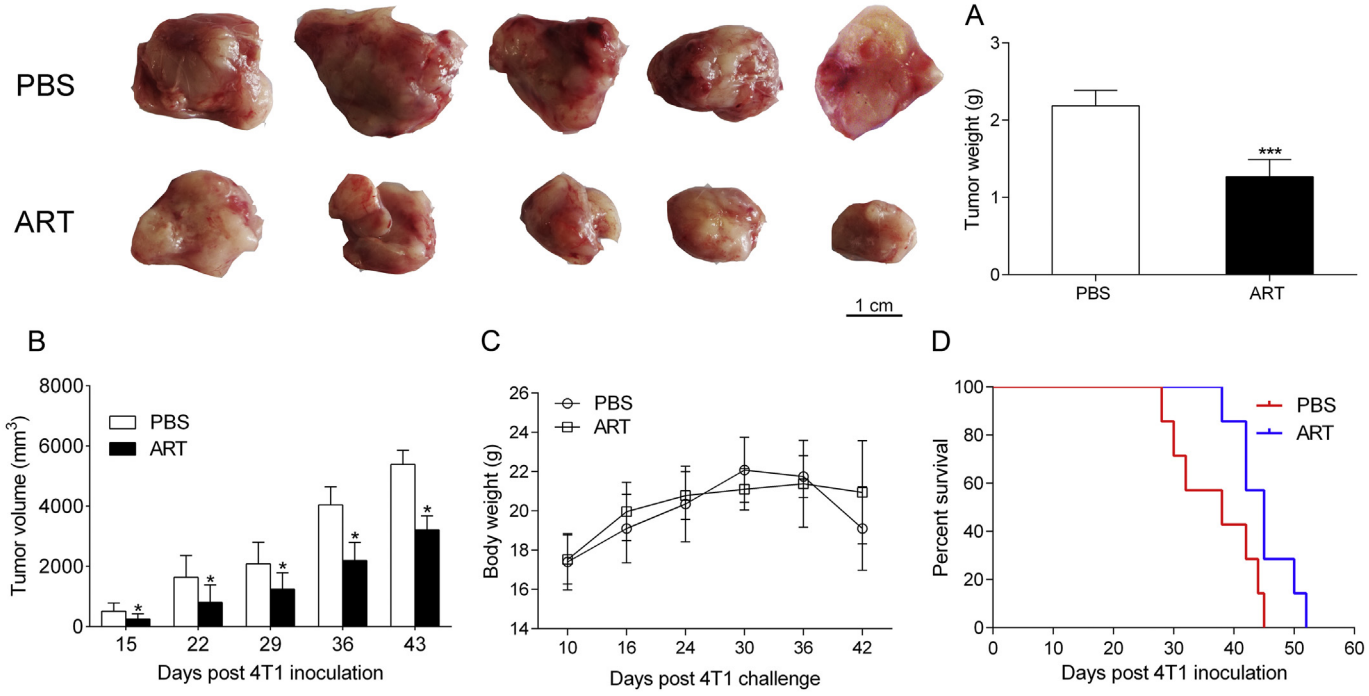

Fig. 2. ART inhibited the growth of 4T1 tumors and prolonged the survival of 4T1 TB mice. Tumors were removed from mice after ART treatment and body weights were compared (A). Tumor volume (B) and body weight (C) curves at the end of treatment. Survival curves (D) of 4T1 TB mice (n = 7). The results are shown as mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to the control group.

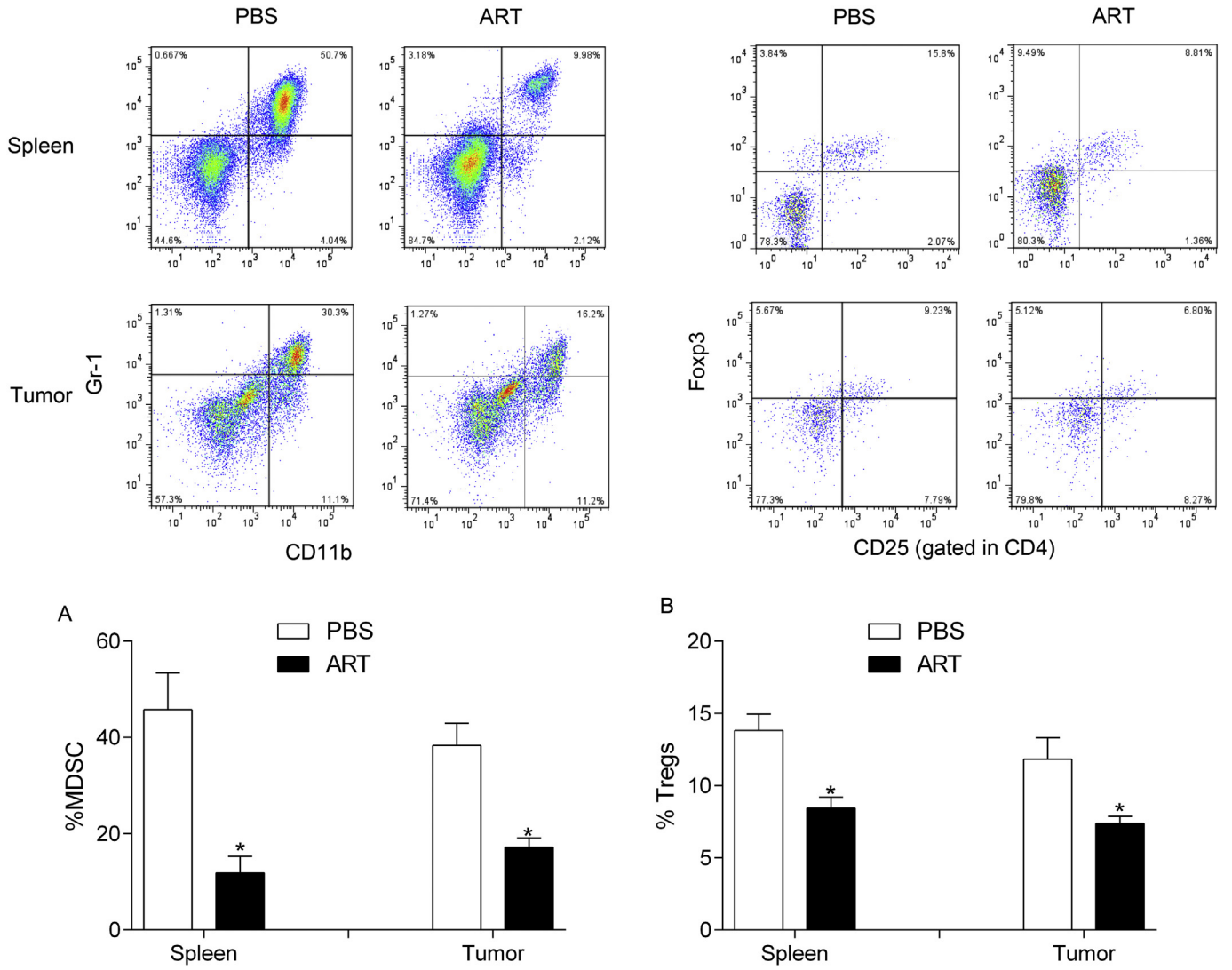

Fig. 3. ART treatment decreased the frequencies MDSCs and Tregs in the spleens and tumors of 4T1 TB mice. Flow cytometric analysis of MDSCs ($CD11b^+$ $Gr\text{-}1^+$) and Tregs ($CD4^+$ $CD25^+$ $Foxp3^+$) in both the spleen and tumor (A and B). $^*P < 0.05$ compared to the control group.

samples.

2.8. RNA isolation and real-time RT-PCR

Trizol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA from excised tumors (~100 mg) per the manufacturer's instructions. The RNA was then quantified at 260 nm using a UV–VIS spectrophotometer (PYE-UNICAM, USA). To remove any contaminant genomic DNA, the RNA was treated with DNaseI and cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser kit (Takara, China). PCR was then performed using the cDNA as a template. Table 1 shows the specific oligonucleotide primers used for the PCR reaction. Quantitative PCR was performed on an ABI 7500 real-time PCR system (ABI, USA) using a SYBR® Premix Ex Taq™ reagent kit (Takara) according to the manufacturers' instructions. β-actin amplification served as an internal control. Gene expression was quantified using the $2^{-\Delta\Delta}$CT method.

2.9. Statistical analysis

Results are presented as the mean ± standard deviation of three experiments. One-way ANOVA and two-tailed Student's *t*-tests were used to test for statistical significance. The Kaplan–Meier method was used to calculate survival analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ART inhibits proliferation of 4T1 cells

ART has been shown to considerably inhibit the proliferation of certain breast cancer cells, but this effect has not been demonstrated in 4T1 cells. Therefore, we performed an MTT assay to examine 4T1 cell proliferation after ART treatment. High doses (10 μM and 100 μM) of ART significantly inhibited the proliferation of 4T1 cells, beginning 24 h post treatment. Lower doses (0.01 μM, 0.1 μM, and 1 μM) of ART also inhibited proliferation, but not until 48 h post treatment. Furthermore, ART treatment also induced apoptosis of 4T1 cells, as 100 μM ART significantly increased the rate of tumor cell apoptosis ($P < 0.01$). Additionally, transforming growth factor β (TGF-β) levels in the supernatant significantly decreased following ART treatment (concentrations ranging from 1 to 100 μM, $P < 0.001$) (Fig. 1).

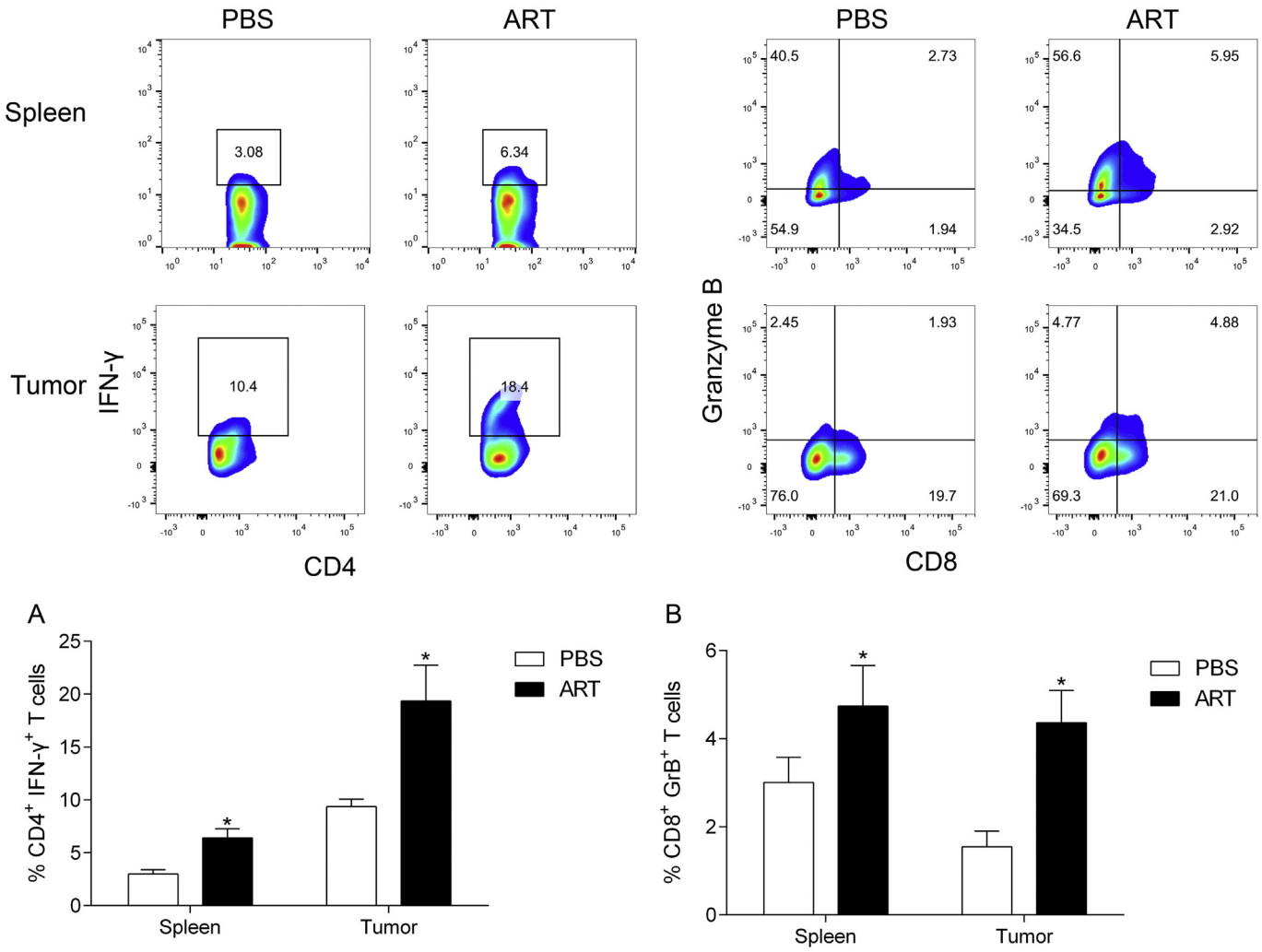

Fig. 4. ART promoted T cell-mediated anti-tumor immune responses. Flow cytometric analysis of $CD4^+$ IFN-γ^+ T cells and CTLs in the spleen and tumor from 4T1 mice treated with ART or PBS (control). $^*P < 0.05$ compared to the control group.

3.2. ART inhibits the growth of 4T1 breast cancer cells and prolongs the survival of 4T1 TB mice

To determine whether ART could suppress tumor development and enhance the survival of 4T1 TB mice, mice were given ART daily for 20 days. We found that the tumor volume in the ART group was reduced compared to that of the control group (Fig. 2B). Similarly, the weight and size of the tumors were significantly decreased in the ART group ($P < 0.001$) compared to the control group (Fig. 2A). No differences in body weights were seen between the two groups (Fig. 2C). All the control mice died 28–45 days post 4 T1 cell inoculation. However, ART supplementation significantly extended the survival of 4T1 mice, with no deaths seen before 38 days post inoculation ($P < 0.05$; Fig. 2D).

3.3. ART inhibits Treg and MDSC expansion in the spleen and tumor

$CD11b^+$ Gr-1^+ MDSCs suppress immune responses in the tumor microenvironment and directly stimulate tumorigenesis, thereby promoting tumor development [24]. To assess whether ART treatment could inhibit the expansion of MDSCs, the frequencies of MDSCs in the spleens and tumors of 4T1 TB mice were analyzed by flow cytometry. We found that MDSC frequencies were significantly lower in both the splenic ($P < 0.05$) and tumor samples ($P < 0.05$) from ART-treated 4T1 TB mice compared to the control mice (Fig. 3A).

Previous studies have shown that tumor-induced regulatory B cells (tBregs) can stimulate FoxP3^+ Treg differentiation from resting non-regulatory CD4+ T cells, which suppresses host anti-tumor immunity and promotes metastasis [25]. Therefore, we also measured the frequencies of Tregs in the spleens and tumors of ART-treated and non-treated 4T1 TB mice. ART treatment significantly decreased the percentage of Tregs in TB mice ($P < 0.05$, Fig. 3B), indicating that ART may inhibit immunosuppression in the tumor microenvironment.

3.4. ART promotes the activation of T cell

T cells play an important role in the anti-tumor immune response and therefore in preventing tumor growth. To determine whether ART can induce the activation of T cells, we quantified the percentages of $CD4^+$ IFN-γ^+ T cells and granzyme B-expressing cytotoxic T lymphocytes (CTLs) within the spleens and tumors of 4T1 mice. ART treatment resulted in significantly increased percentages of $CD4^+$ IFN-γ^+ T cells in both the spleen and tumor samples (Fig. 4A, $P < 0.05$). ART treatment also significantly increased the expansion of granzyme B^+ CTLs (Fig. 4B, $P < 0.05$). These results indicate that ART can promote the activation of T cells.

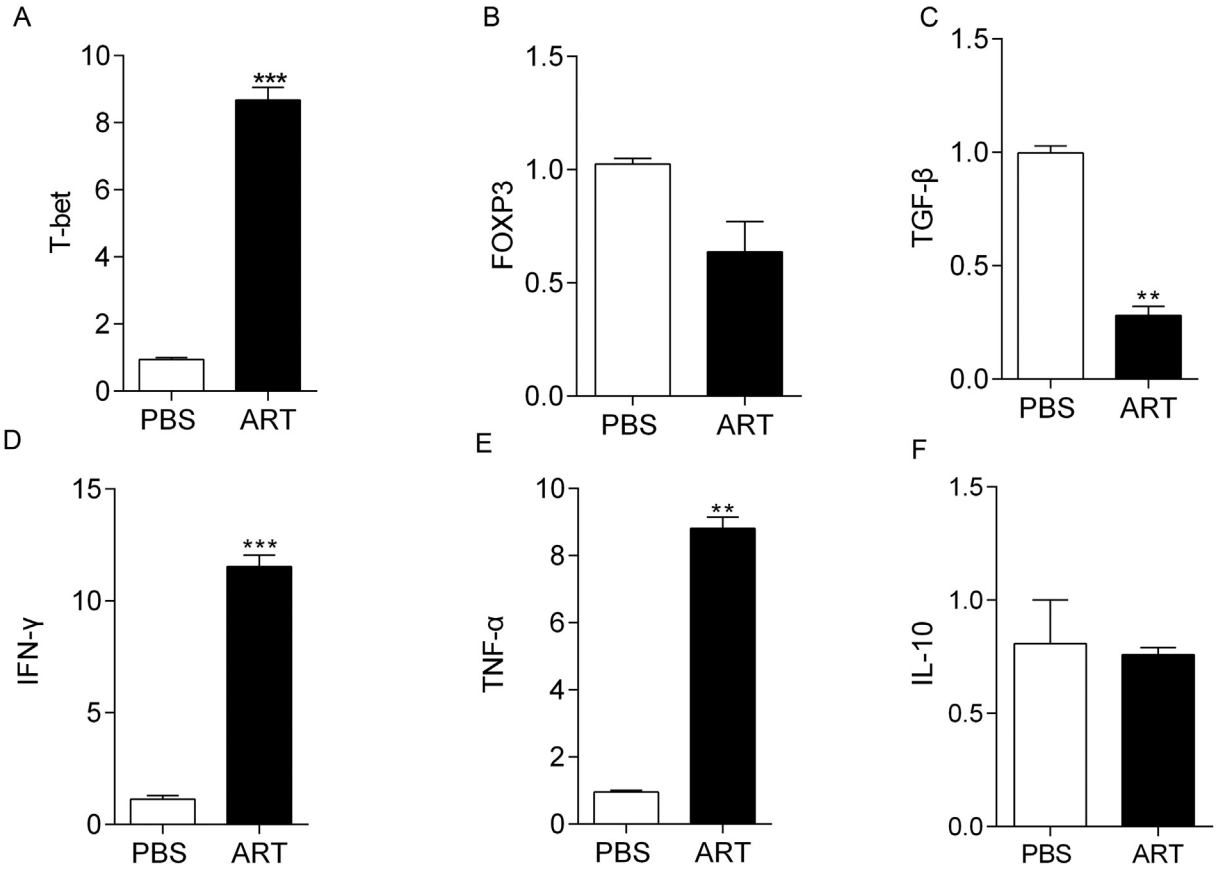

Fig. 5. Transcription factor and cytokine expression induced by ART. T-bet, FOXP3, IFN-γ, TNF-α, TGF-β, and IL-10 mRNA levels within the tumor tissue were measured by qRT-PCR. $^{**}P < 0.01$ compared to the control group.

3.5. ART treatment enhances the expression of immune mediators in the tumor microenvironment

Various immune mediators in the tumor microenvironment, including cytokines and transcription factors, play crucial roles in inhibiting the progression of breast cancer. We used qRT-PCR to measure the relative mRNA expression levels of the transcription factors T-bet and FOXP3, and the cytokines IFN-γ, TNF-α, TGF-β, and IL-10 in the tumor tissues from 4T1 mice. As shown in Fig. 5, expression of T-bet and the related pro-inflammatory cytokine IFN-γ, as well as TNF-α, increased after ART treatment ($P < 0.01$). TGF-β mRNA levels within the tumor significantly decreased after ART treatment ($P < 0.01$), while IL-10 levels did not change significantly. Expression of Foxp3, a transcription factor active in Tregs, also did not change significantly.

4. Discussion

As a leading cause of death in women, breast cancer remains difficult to control despite advances in treatment. Although many different approaches have been used to treat breast cancer, some of these treatments display significant toxicity and may lead to drug resistance. Thus, there is an urgent need for novel drugs which target cancer cells specifically without side effects. Aside from its anti-malaria activity, ART has shown strong anti-tumor activity in previous studies, especially in aggressive metastatic cancers [26]. In this study, we have demonstrated that reduced growth of breast cancer associated with ART is dependent upon the suppression of MDSCs and Tregs.

TGF-β is involved in the function and development of mammary epithelium and promotes cancer metastasis by inducing the epithelial to mesenchymal transition (EMT) of cancer cells [27,28]. Increasing evidence suggests that TGF-β initially suppresses tumor growth, but that constitutive activation of TGF-β promotes tumor growth [29,30]. Previous clinical studies have shown that excessive TGF-β levels in breast cancer patients are associated with metastases to the bone and regional lymph nodes [31]. In this study, we have demonstrated that TGF-β protein levels in the culture supernatant of 4T1 cells and mRNA levels in the tumor decrease after ART treatment.

MDSCs are a key immunosuppressive cell type and can suppress T cell-mediated anti-tumor immunity in breast cancer [32]. The suppressive abilities of MDSCs are closely related to their continuous expression of ARG-1, which metabolizes l-arginine [33]. In addition, MDSCs are involved in angiogenesis, invasion, and metastasis of cancer cells [34]. Another mechanism which suppresses tumor development is the overexpression of PD1 or CTLA-4 by Tregs [35–37]. $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs are a type of immune cell that play an important role in controlling the balance between pro- and anti-inflammatory immune responses [38,39]. Foxp3, a transcription factor expressed in Tregs, is essential for the development and maintenance of Tregs [40]. IL-10 and TGF-β, as well as sustained expression of inhibitory CTLA-4, are key mediators of Treg-induced immunosuppression [41,42]. Suppression of anti-tumor responses by Tregs was first demonstrated in mice treated with an anti-CD25 monoclonal antibody (clone PC61), which caused regression of the tumor [43]. In the 4T1 tumor model, Treg depletion results in accumulation of CTLs in the tumor [44,45]. In the present study, ART supplementation significantly decreased the frequencies of MDSCs and Tregs in both the tumors and spleens, indicating that ART treatment can halt the immunosuppression caused by MDSCs and Tregs in 4T1 bearing mice.

Anti-tumor immunity mediated by CTLs ($CD8^+$ cytotoxic T cells) and $CD4^+$ T helper 1 (Th1) cells inhibits cancer cell growth [46,47]. $CD4^+$ Th1 cells release cytokines, including IFN-γ and TNF-α. IFN-γ stimulates the production of reactive oxygen species (ROS) and reactive

nitrogen species (RNS) from macrophages, which leads to the destruction of cancer cells [48]. Th1 cells exert their anti-tumor effects through two major mechanisms: 1) they can directly kill tumor cells by engaging the TNF-related apoptosis-inducing ligand (TRAIL) or Fas/Fas ligand (FasL) pathways [49] or 2) they can help generate and enhance CTL responses [50]. We found that ART treatment significantly increased the frequencies of CTL and Th1 cells, as well as IFN-γ and TNF-α expression levels, in the tumor. In conclusion, ART inhibits breast cancer growth *in vivo* by promoting T cell activation as well as the suppression of Tregs and MDSCs in the tumor microenvironment.

Acknowledgements

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