Accumulation and suppressive function of regulatory T cells in malignant ascites: Reducing their suppressive function using arsenic trioxide *in vitro*

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Abstract. Although adoptive cell therapy (ACT) has demonstrated effective and remarkable clinical responses in several studies, this approach does not lead to objective clinical responses in all cases. The function of ACT is often compromised by various tumor escape mechanisms, including the accumulation of immunoregulatory cells. As a result of peritoneal metastasis in the terminal stage, malignant ascites fluid lacks effectiveness and is a poor prognostic factor for gastric cancer. The present study assessed T-cell subsets in lymphocytes derived from malignant ascites, and investigated the effects of arsenic trioxide (As_2O_3) on regulatory T cells (Tregs) and ascites-derived tumor-infiltrating lymphocytes (TILs) *in vitro*. In this study, lymphocytes were separated from malignant ascites and T-cell subsets were detected via flow cytometry. Forkhead box P3 (FoxP3) expression was assessed by immunohistochemistry and reverse transcription-quantitative polymerase chain reaction. In addition, cytokines, including interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and interferon-γ (IFN-γ), were measured by enzyme-linked immunosorbent assay (ELISA). Abundant Tregs were observed in ascites lymphocytes, which and exhibited a significantly increased frequency compared with that in the peripheral blood of patients. Furthermore, As_2O_3 treatment significantly reduced Treg numbers and Foxp3 mRNA levels *in vitro* ($P<0.05$). IFN-γ levels in the supernatant of ascites-derived TILs were increased by As_2O_3, whereas IL-10 and TGF-β levels were significantly reduced ($P<0.05$). As_2O_3 may induce selective depletion and inhibit immunosuppressive function of Tregs, and may enhance the cytotoxic activity of ascites-derived TILs.

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Introduction

Immunotherapy has been widely applied in a clinical setting and has achieved success in treating several malignant tumors. Nevertheless, the effectiveness of adoptive cell therapy (ACT) is often compromised by various tumor escape mechanisms, including the accumulation of immunoregulatory cells. Regulatory T cells (Tregs) comprise a highly immunosuppressive subset of $CD4^+$ T cells, which was first identified by Sakaguchi *et al* (1) in 1995. This subpopulation plays a critical role in tumor immunological escape; Tregs are responsible for a weakened immune response (2,3). An increase in Tregs has been demonstrated in a variety of different cancers including melanoma, ovarian, glioblastoma, breast, colorectal, and lung cancers (4,5). Previous studies have shown that, in addition to those found in the tumor site and peripheral blood, Tregs isolated from malignant ascites were equally able to exhibit potent suppressive effects on tumor-antigen specific immunity (6). Tregs are thought to contribute to tumor progression because of their recruitment in the tumor microenvironment. Therefore, reducing the number of Tregs and blockading their immune inhibitory role has become a critical issue for improving cancer immunotherapy.

Arsenic trioxide (As_2O_3) has been used as the standard treatment for acute lymphoblastic leukemia in adults. In many solid tumors, As_2O_3 shows antitumor effects by inhibiting proliferation and inducing apoptosis of tumor cells (7-9). As_2O_3 can reportedly modulate the immune response by promoting apoptosis of $CD4^+$ T lymphocytes. It was demonstrated that low doses of As_2O_3 can inhibit tumor growth by reducing the number of Tregs through oxidative and nitrosative bursts. These researchers suggested that As_2O_3 is useful in enhancing the antitumor activity of adoptive immunotherapy (10,11). Malignant ascites, which results from peritoneal metastasis in advanced gastric cancer, has an extremely poor prognosis and lacks an effective treatment. High Treg levels have been found in malignant ascites and are seen play a key role in tumor progression and metastasis (12). In the present study,

we assessed the proportion of T-cell subsets in ascites-derived tumor-infiltrating lymphocytes (TILs) and peripheral blood mononuclear cells (PBMCs) obtained from gastric cancer patients. The effects of As_2O_3 on the suppressive function of Tregs and the cytotoxicity of ascites-derived TILs *in vitro* were also examined.

Materials and methods

Reagents and cell lines. As_2O_3 (Sigma, St. Louis, MO, USA) was stored at 4˚C after purchase. Human gastric cancer BGC823 cells were obtained from the Institute of General Surgery, Chinese PLA General Hospital (Beijing, China). These cells were cultured in RPMI-1640 medium (Gibbon, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin, at 37˚C in a 5% CO_2 incubator (Thermo Scientific, Waltham, MA, USA).

Preparation of PBMCs. Density gradient centrifugation and lymphocyte separation medium (Xinminglitai, Beijing, China) were used to prepare a mononuclear cell suspension. A 20-ml volume of peripheral blood was obtained from eight gastric cancer patients and eight healthy donors. Samples were collected in a 50-ml centrifuge tube, and the serum was removed after centrifuging at 3,000 rpm/min for 30 min. A 50-ml centrifuge tube was filled with normal saline (NS), and another 50-ml centrifuge tube containing 20-ml lymphocyte separation medium was also prepared. The blood was mixed with the NS and added to the surface of the lymphocyte separation medium. After the supernatant was removed, mononuclear cells were collected from the corresponding layer and placed into another 50-ml centrifuge tube. The serum was removed after centrifugation, and NS was added to the tube. The PBMCs were obtained after centrifuging at 1,700 rpm/min for 10 min and removal of the serum.

Preparation of ascites-derived TILs. Diagnosis of gastric cancer was determined by tumor biopsy; all patients consented to provide ascites fluid through a correlative science protocol approved by the institutional review board. A volume of 500-800 ml ascites fluid was obtained aseptically from each patient with malignant ascites. The supernatant was removed after centrifugation at 1,500 rpm/min for 20 min, and the red blood cells were lysed in a lysis buffer. Adherent cells, including fibrocytes and macrophages, were removed and resuspended in a medium supplemented with inactivated fetal bovine serum (Gibco Inc., Grand Island, NY, USA). To this, 20 ml human lymphocyte separation medium (Chinese Academy of Medical Sciences, Beijing, China) was added. After centrifugation at 2,000 rpm/min for 20 min, the nonadherent floating cells were collected to obtain the ascites lymphocytes (ALs): $4x10^5$ cells were acquired for flow cytometric detection. Next, the ALs were cultured in 25-mm T-type flasks in RPMI-1640 medium, supplemented with 20% inactivated FBS, at 37˚C in 5% ChO_2. After 24 h, 2 μg/ml anti-CD3 McAb (BD Biosciences, NJ, USA) and 1,000 U/ml recombinant human interleukin-2 (IL-2; R&D Systems, Inc., Minneapolis, MN, USA) were added. On day 5, the medium was replaced with Cellix 602 serum-free medium (Xinminglitai). The media and recombinant human IL-2 were supplemented, if necessary. On day 14, TILs were harvested. In our *in vitro* studies, $2x10^7$ TILs were treated with different doses of As_2O_3 (1, 5 or 10 μM) for 48 h. The percentage of $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+/CD25^+/CD127^{low}$ Tregs were detected by flow cytometry. Forkhead box P3 (FoxP3) expression, interferon-γ (IFN-γ) levels in the supernatant, and cytotoxic activity of TILs were also evaluated and analyzed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection of Foxp3. Total RNA was extracted from target cells using TRIzol reagent (Life Technologies, Rockville, MD, USA), according to the manufacturer's directions. The amount of total RNA was determined by spectrophotometrically measuring the absorbance at 260 nm. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the PrimeScript 1st Strand cDNA Synthesis kit (Takara, Beijing, China). The reactions were run with SYBR-Green on a Corbett Rotor-Gene 3000 (Corbett Research, Concorde, Australia) real-time thermal cycler. Data were expressed by the comparative threshold cycle method and normalized to the β-actin. The sequences of primers were synthesized as follows: Human β-actin primer sequences H-β-actin-F, 5'TAG TTGCGTTACACCCTTTCTTG-3' and H-β-actin-R, 5'-TCA CCTTCACCGTTCCAGTTT-3'; and human Foxp3 primer sequences HFoxp3-F, 5'-AGGAAAGGAGGATGGACGAA-3' and HFoxp3-R, 5'-GCAGGCAAGACAGTGGAAAC-3'.

Flow cytometry. At least $4x10^5$ events per sample were acquired for detection by flow cytometer. Phenotypic characterization of T-cell subsets in ALs, PBMCs of GC (gastric cancer patients), and PBMCs of HD (healthy donors) were performed using antibodies against CD3, CD4, CD8, CD25, CD56, and CD127 (BD Biosciences), according to the manufacturer's protocol. All samples were examined using a FACSCalibur, and the data were analyzed using FlowJo 7.6.1 software (both BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA). Supernatant from the TILs were isolated from ascites-derived TILs after treated with As_2O_3 for 48 h. The concentrations of interleukin-10 (IL-10), IFN-γ, and tumor necrosis factor-α (TNF-α) in the supernatant were determined in triplicate using commercial ELISA kits (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions.

Cell proliferation analysis. Ascites-derived TILs were plated in 96-well plates at $1x10^4$ cells/well in complete medium with 20% FBS. The cells were incubated overnight at 37˚C, and the next day, As_2O_3 was added in complete growth media to a final dose of 10 μmol/l. The cells were incubated with test compounds at 37˚C in complete growth medium, and cell proliferation was evaluated using a Cell Counting kit-8 (CCK-8; Beyotime, Shanghai, China).

Cytolytic assay. The cytotoxicity of TILs against BGC823 cells *in vitro* was tested by the lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega Corp., Madison, WI, USA), as described previously (13). The target BGC823 cells were mixed with TILs treated with different concentrations of As_2O_3 at a ratio of 5:1

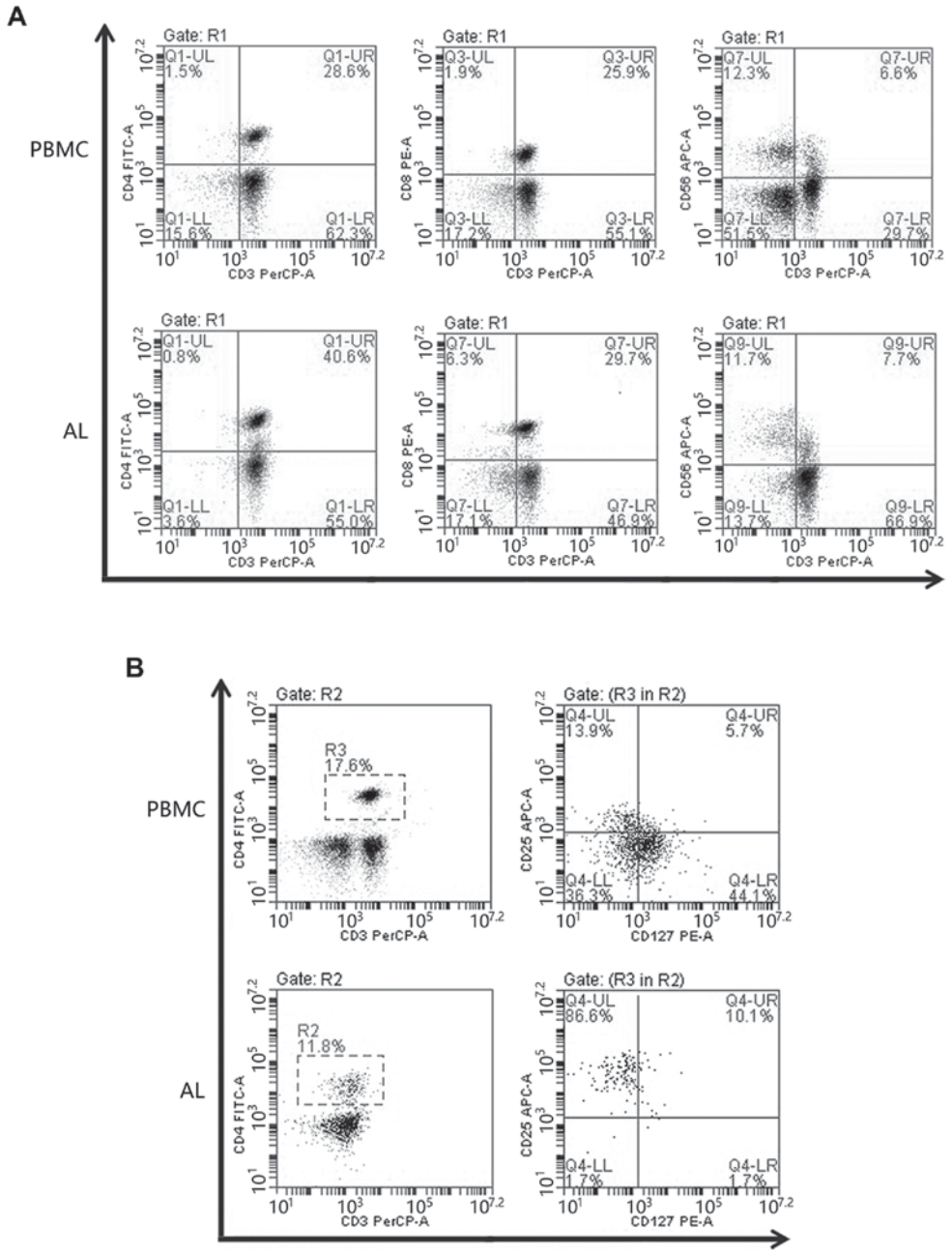

Figure 1. The phenotypic characteristics of ALs and PBMCs were all detected by flow cytometric analysis using antibodies against CD3, CD4, CD8, CD56, CD25, and CD127 (A and B). ALs, ascites lymphocytes; PBMCs, peripheral blood mononuclear cells.

or 10:1 and cultured for 24 h. Cytotoxicity was calculated using the following formula: %Cytotoxicity = [A(Experimental) -A (Effector Spontaneous) - A(Target Spontaneous)] x100/[A(Target maximum) - A(Target spontaneous)].

Data analysis. The descriptive data is shown as mean ± standard deviation. Statistical analyses, including Chi-square tests, Student's *t*-tests, and Fisher's test were performed, where applicable. One-way ANOVA and post hoc test (Newman-Keuls) were applied to compare the multiple groups. For all tests, $P<0.05$ was considered to indicate a statistically significant difference. Statistical Package for the Social Science (SPSS) v.23.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) were used for the data analysis.

Results

Phenotypic characteristics of ALs and PBMCs. To explore the proportion of T-cell subsets, the phenotypic characteristics of ALs and PBMCs derived from gastric cancer patients were detected by flow cytometry (Fig. 1). NK cells ($CD3^- CD56^+$) accounted for 10.5±3.6% in ALs compared to 13.1±4.1% in PBMCs of GC and 12.9±4.6% in PBMCs of HD. Furthermore, the amount of $CD8^+$ T cells in PBMCs of HD, PBMCs of GC, and ALs were 26.7±6.5, 25.9±5.5, and 28.3±7.2%, respevtively. With regard to NK cells and $CD8^+$ T cells, there were no significant difference between the three groups ($P>0.05$) (Fig. 2A and B). However, the percentage of $CD4^+$ T cells was significantly increased in ALs (41.1±7.7%) compared to PBMCs of GC (28.7±6.2%) and PBMCs of HD

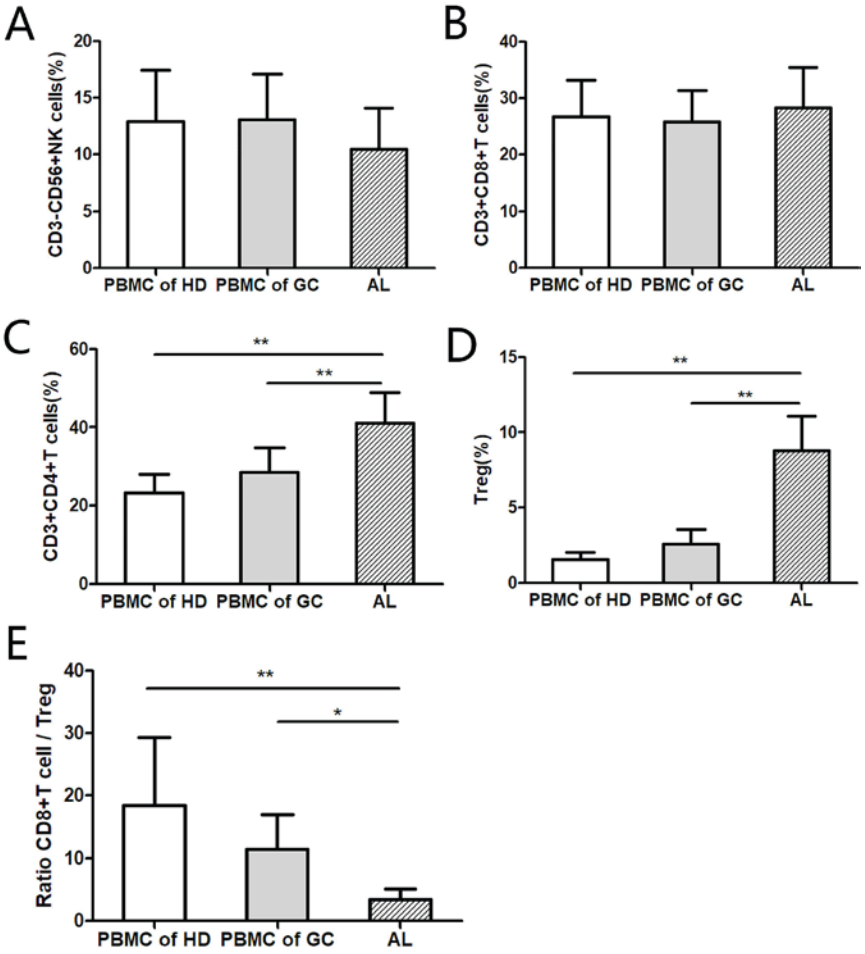

Figure 2. The percentage of T-cell subsets including helper T cells ($CD3^+/CD4^+$), Cytotoxic T lymphocytes ($CD3^+/CD8^+$), NK cells ($CD3^-/CD56^+$), and regulatory T cells ($CD4^+/CD25^+/CD127^{low}$) were measured by flow cytometry. With regard to NK cells and $CD8^+$ T cell, there were no significant differences among the groups (A and B). The results revealed a high proportion of $CD4^+$ T cells (C) and $CD4^+/CD25^+/CD127^{low}$ Tregs (D) in ascites lymphocytes compared with PBMCs. The $CD8^+$ T cell/Treg ratio was lower in TILs than in the PBMCs of patients (E) ($^*P<0.05$, $^{**}P<0.01$ by a one-way ANOVA and post hoc test).

(23.2±4.9) (P<0.01, Fig. 2C). To assess the proportion of Tregs, we analyzed the $CD4^+/CD25^+/CD127^{low}$ T-cell subset in T cells. The result show that $CD4^+/CD25^+/CD127^{low}$ Treg made up a significantly larger proportion of T cells in ALs compared to PBMCs of GC and PBMCs of HD (P<0.01) (Fig. 2D). In addition, the $CD8^+$ T cell/Treg ratio was lower in TILs than in the PBMCs of GC and PBMCs of HD (P<0.05) (Fig. 2E). The immunosuppressive microenvironment was reflected by this low $CD8^+$ T cell/Treg ratio.

As_2O_3 treatment inhibits FoxP3 expression and cytokines secretion in ascites-derived TILs. Because expression of the transcription factor FoxP3 is critical to the immunosuppressive function of Tregs, we assessed FoxP3 mRNA expression in ascites-derived TILs by RT-qPCR. Our results revealed that both low and high doses of As_2O_3 treatment could significantly reduce FoxP3 mRNA expression, with a greater effect by doses of 5 and 10 μM (P<0.01) (Fig. 3A). In addition, Tregs are able to secrete immunosuppressive cytokines such as IL-10 and transforming growth factor-β (TGF-β) (14,15). In the present study, we found that IL-10 and TGF-β levels in the ascites-derived TILs supernatants of the As_2O_3 treatment groups decreased when compared with the control group (P<0.01) (Fig. 3B and C). Meanwhile, the result of cell survival assay by CCK-8 show that As_2O_3 treatment did not induce significant apoptosis of TILs (P>0.05) (Fig. 3D). Analysis of cytokine secretion revealed a trend toward lower amounts of IL-10 and TGF-β in As_2O_3-treated TILs than in the control group. These results suggest that As_2O_3 may functionally inhibit the immunosuppression in TILs and enhance the antitumor immune response.

As_2O_3 treatment decreases the proportion of Tregs and improved the cytotoxic activity of ascites-derived TILs in vitro. The As_2O_3 treatment groups described above were incubated at 37˚C with 5% CO_2 for 48 h. Phenotypic characteristics of TIL and effector cytokine (IFN-γ) secretions were examined by flow cytometry and ELISA. The results revealed that the percentage of $CD4^+$ T cells was significantly decreased in As_2O_3 treatment groups compared with the control group (P<0.05, Fig. 4A). Moreover, the result showed a decreased $CD4^+/CD25^+/CD127^{low}$ Treg percentage in As_2O_3 treatment groups and that 5 μM As_2O_3 treatment is associated with a greater effect than the other concentrations of As_2O_3 (P<0.01) (Fig. 4B).

To evaluate the As_2O_3 treatment effect on cytotoxic activity of ascites-derived TILs, we detected and compared the percentage of $CD8^+$ T cells among the different concentrations in As_2O_3 treatment groups. The percentage of $CD8^+$ T cells

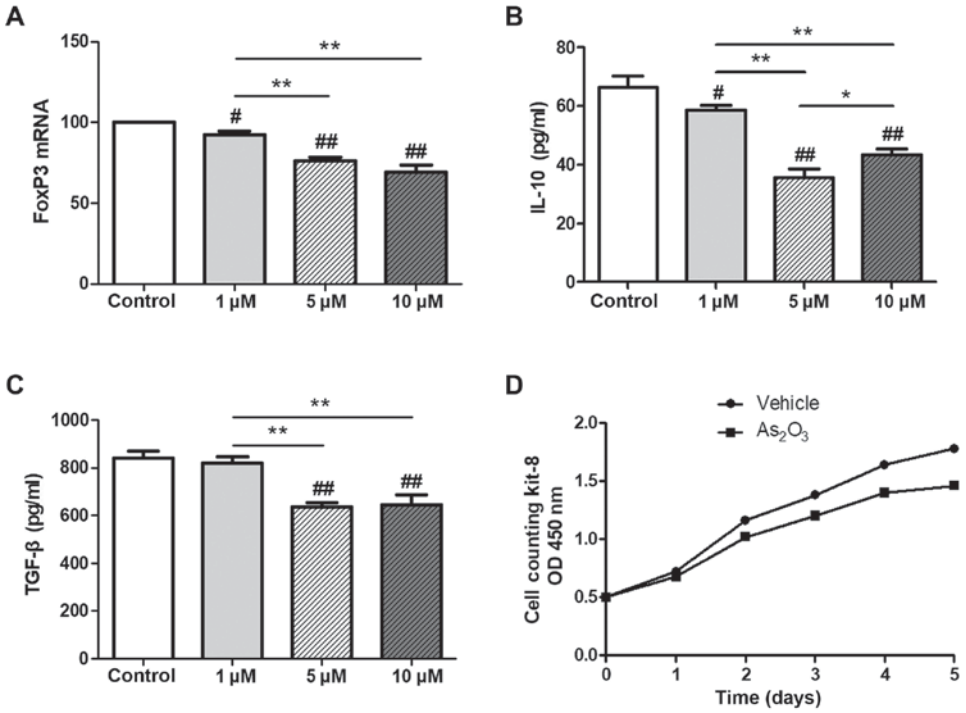

Figure 3. Effect of low-dose, medium-dose, and high-dose As_2O_3 on FoxP3 expression and cytokine secretion of ascites-derived TILs *in vitro*. The ELISA results showed that As_2O_3 treatment significantly reduced FoxP3 mRNA expression, with a greater effect by 5 and 10 μM (P<0.01) (A). The level of IL-10 decreased significantly by treatment with As_2O_3, with a greater effect by 5 μM (P<0.05) (B), and the level of TGF-β harvested from the supernatant of ascites-derived TILs dereased significantly, with a greater effect by 5 and 10 μM (P<0.01) (C). Proliferation of TILs treated with vehicle or As_2O_3 was evaluated by CCK-8 assay. As_2O_3 treatment did not induce significant apoptosis of TILs (P>0.05) (D). ($^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the control group;*P<0.05, **P<0.01 compared between the experimental groups).

increased in As_2O_3 treatment groups, with the greater effect using a dose of 5 μM As_2O_3 (P<0.01) (Fig. 4C). Furthermore, ELISA detection revealed that IFN-γ levels in the TIL supernatants of the As_2O_3 treatment groups increased when compared with those in the control group (P<0.01) (Fig. 4D). In our next experiment, cytotoxicity of TILs toward BGC823 cells was assessed in an *in vitro* killing experiment by the LDH release assay at 5:1 and 10:1 effector-target ratios. It was found that the cytotoxic activity of ascites-derived TILs significantly enhanced As_2O_3 treatment (P<0.01) (Fig. 4E).

Discussion

Although immunotherapy has achieved a certain effectiveness in treating diverse malignant tumors (16-18), it frequently has to contend with immune-escape mechanisms induced by the tumors.

In the present study, we compared and analyzed T-cell subsets in ascites-derived TILs of patients, PBMCs of patients with gastric cancer, and PBMCs of healthy donors. In agreement with previous studies, we showed that ascites-derived TILs displayed an increased proportion of Tregs. Moreover, we observed that the $CD8^+$ T cell/Treg ratio decreased in TILs and PBMCs of patients compared with the PBMCs of healthy donors. Indeed, the increased proportion of Tregs and a low $CD8^+$ T cell/Treg ratio reflect an immunosuppressive microenvironment and may be a factor that contributes to immune escape in patients with malignant ascites. Indeed, the microenvironment in malignant tumors is suppressive for immune responses, and successful cancer immunotherapy can only be effective when associated with the elimination of suppressive cells. Because of their highly suppressive functions, Tregs have been seen as a major obstacle to successful immunotherapy. In addition, previous studies also suggest that a high concentration of Tregs in a tumor site is associated with poor prognosis (19,20). It can impede antitumor cytotoxic activity in adoptive immunotherapy because of Tregs recruitment and accumulation in the tumor site and in malignant ascites (21-23). Therefore, it is of great clinical significance to explore therapeutic interventions that eliminate immunosuppressive factors.

We also observed that As_2O_3 induces selective depletion of Tregs in ascites-derived TILs *in vitro*. This is consistent with previous reports. Thomas-Schoemann *et al* (11) suggested that As_2O_3 treatment can inhibit solid tumor growth by reducing the number of Tregs, thereby leading to better survival outcomes. The exact mechanisms underlying signaling pathway of As_2O_3 function on Tregs have not been fully elucidated. It has shown that NO signaling pathway play a role in Treg depletion by As_2O_3 (11). It is related to superoxide and nitrite oxide production resulting in $ONOO^-$ accumulation. As_2O_3 is able to induce the intracellular accumulation of superoxide anions and interfere with the mitochondrial respiratory chain and membrane nicotinamide adenine dinucleotide phosphate oxidase activation (24,25). Importantly, we also showed that the transcription factor FoxP3 expression in TILs was inhibited by As_2O_3 treatment. It has been proven that FoxP3 is substantially and stably expressed in $CD4^+CD25^+$ Tregs. FoxP3 is a crucial regulatory gene for the development and function of Tregs because the retroviral gene transfer of Foxp3 converts naïve

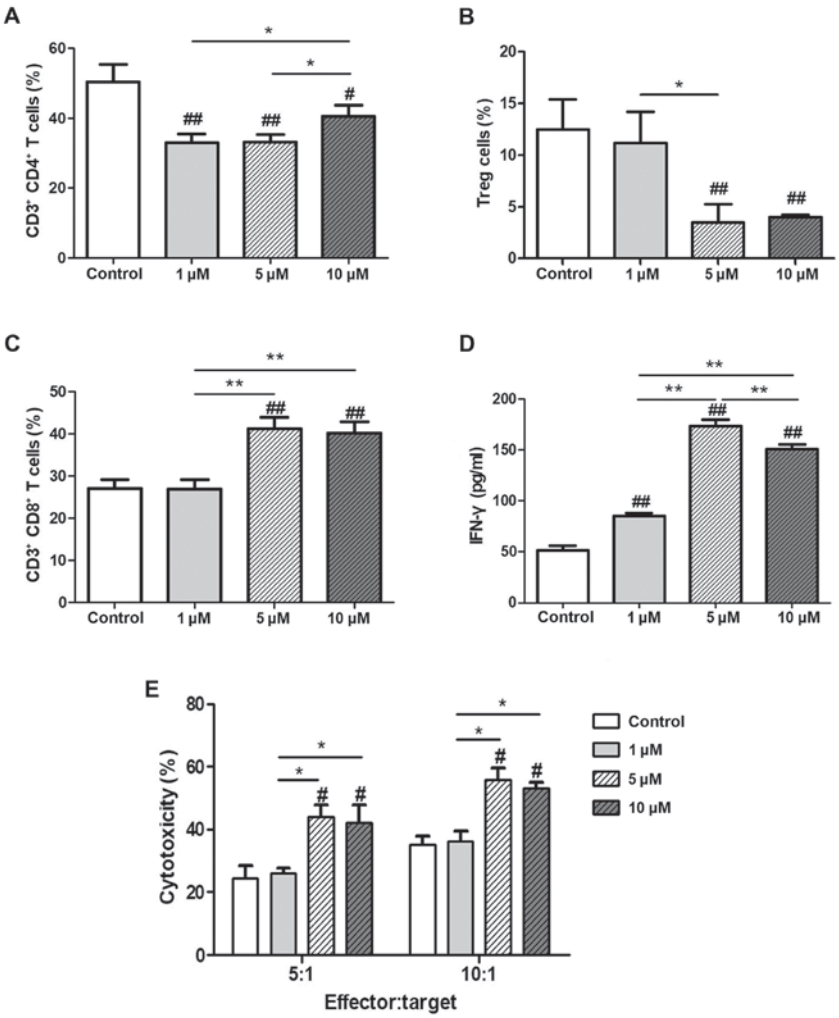

Figure 4. As_2O_3 treatment reduced the proportion of Tregs and improved the cytotoxic activity of ascites-derived TILs. The results showed that As_2O_3 treatment significantly decreased the percentage of $CD4^+$ T cells, with a greater effect by 1 and 5 μM (P<0.01) (A). As_2O_3 treatment significantly decreased the ratio of Tregs, with a greater effect by 5 and 10 μM As_2O_3 (P<0.01) (B). The cytotoxic activity of ascites-derived TILs after As_2O_3 treatment was evaluated through different ways *in vitro*. The percentage of $CD8^+$ T cells increased significantly by treatment with 5 and 10 μM As_2O_3 (P<0.01) (C), and the level of IFN-γ harvested from the supernatant of ascites-derived TILs increased significantly, with a greater effect by 5 μM As_2O_3 (P<0.01) (D). As_2O_3 treatment significantly improved the cytotoxic activity of ascites-derived TILs at 5:1 or 10:1 effector-target ratio (P<0.01, E). ($^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the control group; *P<0.05, **P<0.01 compared between the experimental groups). The *in vitro* experiments were repeated three times).

T cells into a regulatory T-cell phenotype similar to that of naturally occurring $CD4^+$ Tregs (26). In addition, we assessed the effect of As_2O_3 on cytokine secretion and found that As_2O_3 treatment reduced IL-10 and TGF-β levels in TILs, corresponding with Treg levels. IL-10 and TGF-β are indeed significant contributors to Treg-mediator suppression. It has been demonstrated that stimulation of effector T cells by $CD103^+$ dendritic cells in the presence of TGF-β and retinoic acid induces the generation of $FoxP3^+$ T cells (27). TGF-β has an important role in maintaining FoxP3 expression in natural Treg cells. IL-10 can maintain the regulatory activity of Tregs and impair dendritic cell function. Moreover, the tumor microenvironment promotes the generation of Tregs that mediate IL-10-dependent suppression. Indeed, IL-10 production by Treg cells appears to be important for blocking antitumor immunity. These results in our study collectively indicate that As_2O_3 treatment inhibits the function of Tregs through depletion of Tregs and weakens their functions, such as cytokine secretion.

The cytotoxic activity of ascites-derived TILs toward cell line BGC803 was examined by an LDH assay *in vitro* experiment. We found that As_2O_3 increased the cytotoxic activity of ascites-derived TILs at both the 5:1 and 10:1 effector-target ratios. The proportion of $CD8^+$ T cells and effector cytokine IFN-γ levels were also assessed. As_2O_3 reduced the proportion of Tregs in ascites-derived TILs as described above, thereby increasing the $CD8^+$ T cell ratio, with a greater effect by higher doses of As_2O_3. With regard to effector cytokine IFN-γ levels, those in As_2O_3 treatment groups were significantly higher than those in the control group. These results indicate that As_2O_3 can enhance the immune response against gastric cancer cells. Therefore, an appropriate dose of As_2O_3 may be applied as an immune adjuvant to provide an improved therapeutic effect against gastric cancer in patients with malignant ascites. Nevertheless, the exact mechanisms underlying the efficacy of As_2O_3 have not been fully elucidated; further research is needed to explore the detailed mechanisms.

In conclusion, the present study revealed a high proportion of Tregs and low $CD8^+$ T cell/Treg ratio in ascites-derived TILs. We showed that appropriate doses of As_2O_3 can induce selective depletion of Tregs and increase the cytotoxic activity of ascites-derived TILs by suppressing Tregs. These results offer a new strategy for use of As_2O_3 to enhance the antitumor activity of an ACT strategy.

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