Arsenic trioxide inhibits lung metastasis of mouse colon cancer via reducing the infiltration of regulatory T cells

Lei Wang¹,² · Xiang Hu¹ · Yingxin Xu³ · Zhong Liu¹,⁴

Abstract The purpose of this study was to investigate the effects of arsenic trioxide (As₂O₃) on the infiltration of regulatory T cells (Tregs) in the local lung metastasis of mouse colon cancer in vivo and the regulation of Tregs in cytokine-induced killer cells (CIKs) in vitro. A high Tregs infiltration mouse colon cancer lung metastasis model was established by intravenous injection of CT26 murine colon carcinoma cells. Tumor-bearing mice were randomly divided into three groups: control group, low-dose As₂O₃ group, and high-dose As₂O₃ group. For in vitro studies, CIKs were treated with vehicle control or 0.1, 1, or 5 μM As₂O₃. The level of Tregs was detected via flow cytometry, Foxp3 expression was assessed by immunohistochemistry and reverse transcription–polymerase chain reaction (RT-PCR), the level of interferon gamma (IFN-γ) was evaluated by enzyme-linked immunosorbent assay (ELISA), and the cytotoxic activity of As₂O₃-treated CIKs was assessed through a lactate dehydrogenase (LDH) release assay. Obvious lung metastasis was observed 3 days after CT26 murine colon carcinoma cell injection. The numbers of Tregs in the lungs and spleens of tumor-bearing mice were significantly higher than those of the normal group (p < 0.01). As₂O₃ treatment increased the mouse weight as well as reduced the number of metastatic lung nodules and the lung/body weight ratio (p < 0.01). Moreover, As₂O₃ treatment significantly reduced the Tregs proportion and the Foxp3 messenger RNA (mRNA) levels in metastatic lung tissues (p < 0.01). In vitro, As₂O₃ significantly reduced the Tregs proportion and the Foxp3 mRNA levels (p < 0.01) and significantly increased the cytotoxic activity of CIKs and the IFN-γ levels in the supernatant of cultured CIKs (p < 0.01). As₂O₃ might inhibit lung metastasis of colon cancer by reducing the local infiltration of Tregs and increase the cytotoxic activity of CIKs by suppressing Tregs.

Keywords Arsenic trioxide · Colon cancer · Lung metastasis · Regulatory T cells · Cytokine-induced killer cells

Introduction

Arsenic trioxide (As₂O₃) has been applied for the treatment of acute lymphoblastic leukemia. Emerging evidence has suggested that As₂O₃ has a therapeutic effect in many solid tumors by inducing apoptosis and inhibiting the invasion and migration of tumor cells [1–4]. Despite a limited anti-tumor effect of As₂O₃ monotherapy, it can enhance the anti-tumor effects of metformin [5] and sorafenib [6]. It has been well documented that immunity plays a key role in cancer therapy response [7]. Some studies have found that As₂O₃ can modulate the body’s immune response via CD4⁺ T/CD8⁺ T cells [8, 9], which suggests that As₂O₃ may be useful in tumor immunotherapy.
The number of regulatory T cells (Tregs) is highly increased in cancer patients, and Tregs promote tumorigenesis by reducing the number of T helper cells [10]. In addition, a high proportion of Tregs is one of the bottlenecks that affect the efficacy of adoptive immunotherapy [11]. Therefore, reducing the number of Tregs has become a critical issue for successful tumor immunotherapy. Cyclophosphamide, CD25 monoclonal antibodies, and cytotoxic T lymphocyte-associated protein 4 monoclonal antibodies have been shown to reduce the number of Tregs [12], but these drugs only transiently reduce the number of Tregs [13]. Hernandez et al. first reported that As2O3 could increase the Treg ratio in the spleen of myelitis rats [14]. Subsequently, Thomas et al. found that As2O3 reduced the proportion of Tregs in the spleen of a colon cancer subcutaneous rat model [15]. These findings suggest that As2O3 may act as a sensitizer of other therapeutic modules by reducing the number of Tregs. However, the molecular pharmacology of suppression of Tregs by As2O3 and whether As2O3 can reduce Tregs infiltration in the local metastatic tumor tissues remain to be determined.

In clinical practice, some colorectal cancer patients have lung metastases at diagnosis; moreover, lung metastasis also frequently occurs in colorectal cancer patients postoperation. It has been found that significantly increased numbers of tumor-infiltrating Tregs are seen in postoperation. It has been found that significantly increased numbers of tumor-infiltrating Tregs are seen in colorectal cancer patients [11]. Therefore, reducing the number of Tregs has become a critical issue for successful tumor immunotherapy. Cyclophosphamide, CD25 monoclonal antibodies, and cytotoxic T lymphocyte-associated protein 4 monoclonal antibodies have been shown to reduce the number of Tregs [12], but these drugs only transiently reduce the number of Tregs [13]. Hernandez et al. first reported that As2O3 could increase the Treg ratio in the spleen of myelitis rats [14]. Subsequently, Thomas et al. found that As2O3 reduced the proportion of Tregs in the spleen of a colon cancer subcutaneous rat model [15]. These findings suggest that As2O3 may act as a sensitizer of other therapeutic modules by reducing the number of Tregs. However, the molecular pharmacology of suppression of Tregs by As2O3 and whether As2O3 can reduce Tregs infiltration in the local metastatic tumor tissues remain to be determined.

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**Materials and methods**

**Reagents and cell lines**

As2O3 was purchased from Sigma (St. Louis, MO, USA) and stored at 4 °C. Human colon cancer SW-620 cells and mouse colon cancer CT26 cells were obtained from the Institute of General Surgery, Chinese PLA General Hospital (Beijing, China), and cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10 % inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5 % CO2 incubator (Thermo Scientific, Waltham, MA, USA). Bouin’s solution was prepared by mixing 75 mL of saturated picric acid (Beijing Taiyu Tiangong Biotechnology, Beijing, China), 25 mL of 40 % formaldehyde, and 5 mL of acetic acid.

**Animal models and in vivo experiments**

BALB/c female mice of 6–8 weeks old were purchased from the Beijing Experimental Animal Center of the Academy of Military Medical Sciences (Beijing, China). A total of 1 × 105 mouse CT26 cells in 100 μL of phosphate-buffered saline (PBS) were slowly injected into mice through the tail vein to establish the mouse lung metastasis model of colon cancer. Three days after injection, tumor-bearing mice were randomly divided into three groups: control group (treated with saline, i.v.), low-dose group (treated with As2O3 at 3 mg/kg/day, i.v.), and high-dose group (treated with As2O3 at 6 mg/kg every other day, i.v.), and the treatment continued for 2 weeks. The weight of the mice was recorded every day. When the experiment was terminated, a mononuclear cell suspension was prepared from eyeball blood. Then, the lungs were removed with a sterile technique to calculate the lung/body weight ratio, and enriched lymphocytes were prepared from the left lungs (described below). A mononuclear cell suspension was prepared by grinding the spleen tissue. Aliquots of the peripheral blood, spleen fluid, and lung metastasis lymphocytes were analyzed by flow cytometry to determine the proportion of Tregs. Parts of the right lobe of the lungs were processed for hematoxylin and eosin (HE) staining, immunohistochemical staining of Foxp3, and reverse transcription–polymerase chain reaction (RT-PCR) analysis of Foxp3 messenger RNA (mRNA). Another batch of mice was used to evaluate the effects of As2O3 on the survival of the CT26 mice.

**Count of metastatic lung nodules**

Lung tissues were first fixed and stained with Bouin’s solution, and then the metastatic lung nodules were classified under a microscope as described previously [16]. Metastatic nodule diameters of less than 0.5, 0.5–1, 1–2, and greater than 2 mm were classified as grade I, II, III, and IV metastasis, respectively. Then, the total number of metastases was calculated according to the following formula: total metastasis number = (grade I metastasis number) + (grade II metastasis number × 2) + (grade III metastasis number × 3) + (grade IV metastasis number × 4).

**Enrichment of lymphocytes from lung tissues**

The lungs were flushed three times with PBS in order to wipe off the lymphocytes resided in the branchial alveolus. Next, using sterile techniques, the left lung was removed, cut into pieces, and digested with collagenase II (Gibco,
Preparation of human CIKs for in vitro experiments

A mononuclear cell suspension from 20 mL of healthy human peripheral blood was prepared by density gradient centrifugation with human lymphocyte separation medium (MP Biomedicals, Santa Ana, CA, USA), after incubation for 3 h, the nonadherent floating cells were collected and cultured in 75-mm T-type flasks with 10 mL of Cellix 601 serum-free medium (Beijing Xin Ming Thai Biotechnology, Beijing, China) supplemented with 1000 U/mL recombinant human interferon gamma (IFN-γ) (R&D, MN, USA) at 37 °C in a humidified incubator containing 5% CO₂ for 24 h. The nonadherent floating cells were collected, stained and sliced into 4-μm sections. HE staining was performed to check for necrosis of the tumor cells. Infiltrating Foxp3⁺ Tregs in the local lung metastasis were analyzed by immunohistochemistry. Elivision immunohistochemistry was performed using the standard streptavidin peroxidase complex technique on 4-μm-thick paraffin-embedded tissue sections. After pretreatment and blocking, Foxp3 immunostaining was performed using a rabbit anti-mouse Foxp3 monoclonal antibody (Abcam, Cambridge, UK) as the primary antibody and streptavidin-biotinylated anti-rabbit immunoglobulin G as the secondary antibody. This procedure was followed by incubation with a streptavidin-horseradish peroxidase enzyme conjugate. The sections were visualized using 3,3′-diaminobenzidine and finally counterstained with hematoxylin. Negative controls were subjected to immunohistochemistry by replacing the primary antibody with PBS solution. Foxp3⁺ cells were stained tan or brown. The number of Foxp3⁺ cells in five random fields was counted under a light microscope at high magnification (×400, Olympus BX53, Japan). The average number of cells from five fields was defined as the number of Tregs.

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ELISA detection of cytokines

CIKs were treated with different concentrations of As₂O₃ for 48 h. The IFN-γ content in the supernatant was determined using an IFN-γ ELISA kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s protocol. The OD value was detected with a microplate reader at a wavelength of 492 nm. The in vitro cytotoxicity of CIKs to SW-620 cells was performed by an LDH release assay (Sigma-Aldrich, St. Louis, MO, USA), as described previously [17]. The target SW-620 cells were mixed with CIKs treated with different concentrations of As₂O₃ at a ratio of 5:1 or 10:1 and cultured for 24 h. The optical density (OD) value was detected with a microplate reader at a wavelength of 492 nm. The killing rate was calculated as follows: Cytotoxicity % = [OD(Experimental) − OD(Target spontaneous)] / [OD(Target maximum) − OD(Target spontaneous)] × 100.

RT-PCR detection of Foxp3

Mouse and human Foxp3 as well as β-actin primers were designed and synthesized by Shanghai Sangon (Shanghai, China). The sequences of the primers were as follows: mouse β-actin primer sequences, M-β-actin-F: 5’-GTGCTATG-3’, M-β-actin-R: 5’-GGAGTCCAGTCAGGACAA-3’; human Foxp3 primer sequences, H-Foxp3-F: 5’-GACGAGTCCAGTCAGGACAA-3’, H-Foxp3-R: 5’-GTGCTATG-3’.
TTGCTCTAGACTTCG-3'; M-β-actin-R: 5'-ATGCCACA GGATTCCATAACC-3'; mouse Foxp3 primer sequence,
M-Foxp3-F: 5'-TGGTTTACTCGATGTTCCG-3', M-
Foxp3-R: 5'-CCCACCTTTTCTTGGTTTTGA-3' human
β-actin primer sequences, H-β-actin-F: 5'-TAGTGGC
TTACACCCTTTCTTG-3', H-β-actin-R: 5'-TCACCTTC
ACGTCCAGTGT-3'; human Foxp3 primer sequence, H-
Foxp3-F: 5'-AGGAAAAGGAGATGGACGAA-3', H-
Foxp3-R: 5'-GCAGGCAAGACAGTGGAAC-3'. The
lungs were flushed three times with PBS, and then the homog-
enates were prepared by rapidly grinding the frozen tissues in
liquid nitrogen. ATO-treated CIKs were washed three times
and resuspended at 1.0 × 10⁷/mL. Total RNA was extracted
using TRIzol reagent (Life Technologies, Rockville, MD,
USA), according to the manufacturer’s instructions. The
amount of total RNA was determined by measuring the absorb-
cance at 260 nm with a spectrophotometer. One microgram of
total RNA was reverse-transcribed to the complementary
DNA (cDNA) using the PrimeScript 1st Strand cDNA
Synthesis Kit (Takara, Dalian, China), according to the manu-
facturer’s protocol. Real-time PCR was conducted by the
SYBR Green method on a Corbett Rotor-Gene 3000 (Corbett Research, Concorde, Australia) real-time thermal cy-
cler. Data were expressed using the comparative threshold
cycle method and normalized to the housekeeping gene β-
actin.

Statistical analysis

SPSS 21.0 statistical software was used for statistical analysis
of the relevant data. Data are expressed as the mean ± standard
deviation. Differences between two groups were compared
using the t test. Differences among several groups were ana-
yzed using one-way analysis of variance. The Kaplan-Meier
test was performed for survival analysis. P < 0.05 was con-
sidered statistically significant.

Results

Establishment of the mouse colon cancer lung metastasis
model with high Treg infiltration

BALB/c mice were randomly divided into two groups: normal
group and model group. Metastatic lung nodules appeared
3 days after intravenous injection of CT26 cells (Fig. 1a).
The number and volume of nodules gradually increased and
fused with time (Fig. 1b). A significantly higher number of
infiltrating Tregs in the model group was detected compared
with that of normal group (p < 0.01, Fig. 1b). Moreover, the
number of infiltrating Tregs in the spleen of the model group
was also significantly higher than that of the normal group
(p < 0.01, Fig. 1c). However, there was no significant
difference in the number of infiltrating Tregs in the peripheral
blood between model group and normal group (Fig. 1c).

AS₂O₃ inhibited lung metastasis of mouse colon cancer

Three days after the injection of CT26 cells, tumor-bearing
mice were randomly divided into three groups: control group
(treated with saline, i.v.), low-dose group (treated with AS₂O₃
at 3 mg/kg/day, i.v.), and high-dose group (treated with AS₂O₃
at 6 mg/kg every other day, i.v.). After AS₂O₃ treatment, the
mouse body weight increased significantly, with the most ob-
vious effect in the high-dose group (p < 0.01). However, there
was no significant difference of body weight between these
two treatment groups (Fig. 2a). Physical analysis showed that
both the lung volume and the number of metastatic lung nod-
ules were decreased after AS₂O₃ treatment (Fig. 2b). HE stain-
ing demonstrated increased tumor cell debris and tumor inter-
stitial fibrosis in the AS₂O₃ treatment groups, compared with
the control group (Fig. 2c). Quantitative analysis showed that
AS₂O₃ treatment significantly reduced the number of meta-
static lung nodules (Fig. 2d) and the lung/body weight ratio
(Fig. 2e) (p < 0.01). The survival times of mice in the low-
and high-dose AS₂O₃ groups were significantly prolonged
(p < 0.01, Fig. 2f), and the survival rates were 37.5 and
50% at 40 days after CT26 injection, respectively (Fig. 2f).

AS₂O₃ reduced the infiltration of Tregs and suppressed
the expression of Foxp3 in lung metastases of colon cancer
in mice

To test whether AS₂O₃ reduces the infiltration of Tregs, the
Treg ratios in the lung, spleen, and peripheral blood were
assessed by flow cytometry. The results showed that both
the low and high doses of AS₂O₃ could significantly reduce
the number of infiltrating Tregs in lung metastases, with a
greater inhibitory effect by the low dose (p < 0.01, Fig. 3a).
The high dose but not the low dose of AS₂O₃ significantly
reduced the number of infiltrating Tregs in the peripheral
blood (p < 0.01, Fig. 3a). Neither the low dose nor the high dose
of AS₂O₃ reduced the number of infiltrating Tregs in the per-
ipheral blood (Fig. 3a). We determined the expression of
Foxp3 in the lung by RT-PCR and immunohistochemistry.
Immunohistochemical analysis showed lots of dark brown
Foxp3+ stained cells in the lung tissues before treatment,
while in the low-dose and the high-dose AS₂O₃ groups, obvi-
ously reduced numbers of Foxp3+ stained cells in the lungs
were seen (Fig. 3b). Further semiquantitative analysis demon-
strated that AS₂O₃ treatment significantly decreased the
number of Foxp3+ cells in the lung tissues (p < 0.01, Fig. 3c),
but there was no significant difference between these two treat-
ment groups (Fig. 3c). RT-PCR results showed the same trend,
in which AS₂O₃ treatment significantly decreased Foxp3
mRNA expression in the lung tissues (p < 0.01, Fig. 3d),
and there was no significant difference between these two treatment groups (Fig. 3d).

**As2O3 dose-dependently decreased the percentage of Tregs in CIKs and improved the cytotoxic activity of CIKs in vitro**

To extend our in vivo observation and explore the potential pharmacology of As2O3 in reducing the tumor burden of the mouse colon cancer, we treated CIKs with different concentrations of As2O3 for 48 h. The expression of CD25 and Foxp3 was double-stained in the pooled CD4+ T cells (Fig. 4a). The flow cytometry results showed that As2O3 treatment significantly decreased the percentage of CD4+ T cells, with a greater effect by 1 μM As2O3 ($p < 0.01$, Fig. 4b). As2O3 treatment significantly decreased the ratio of Tregs, with a greater effect by 5 μM As2O3 ($p < 0.01$, Fig. 4c).

Further studies showed that the expression of Foxp3 in the pooled CD4+ T cells was reduced in a dose-dependent manner ($p < 0.01$, Fig. 4d), which was confirmed with respect to the Foxp3 mRNA levels by RT-PCR ($p < 0.01$, Fig. 4e). The cytotoxic activity of As2O3-treated CIKs was evaluated through different ways in vitro. First, the percentage of CD8+ T cells increased according to the different concentrations of As2O3, with a greater effect by 5 μM As2O3 ($p < 0.05$, Fig. 4f). Second, ELISA analysis detected a significant increase of IFN-γ levels in the CIK supernatants of the As2O3-treated groups ($p < 0.01$, Fig. 4g). Finally, we determined the in vitro cytotoxicity of CIKs toward SW-620 cells by the LDH release assay. The target SW-620 cells were mixed with As2O3-treated CIKs at a ratio of 5:1 or 10:1, and the cells were cultured for 24 h. The results showed that As2O3 treatment significantly improved the cytotoxic activity of CIKs ($p < 0.01$, Fig. 4h).
Discussion

In this study, we demonstrated that As$_2$O$_3$ potently decreased the tumor burden and inhibited the lung metastasis of colon cancer in a mouse model. Importantly, we showed that As$_2$O$_3$ reduced the infiltration of Tregs and suppressed the expression of Foxp3 in lung metastases of colon cancer. In vitro, As$_2$O$_3$ significantly improved the cytotoxic activity of CIKs, accompanied with a decreased percentage of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in CIKs.

In the lung metastasis model of mouse colon cancer established in this study, lung metastasis appeared very early, with significant infiltration of Tregs/CD3 in the lung but not in the peripheral blood, suggesting an ideal model for the study of pharmacological inhibition of Tregs in the treatment of colon cancer. With this model, we found that As$_2$O$_3$ significantly inhibited the pulmonary metastasis of colon cancer, without significant toxicity. As$_2$O$_3$-treated mice displayed a significantly increased body weight and reduced the tumor burden and the number of metastatic lung nodules. However, no significant difference was observed between the low and high doses of As$_2$O$_3$. It has been reported that 6.5 mg/kg As$_2$O$_3$ is selectively enriched in tumor tissues rather than in the liver, brain, kidney, and other tissues and does not cause significant toxicity [18]. However, we found that in the first administration, high-dose As$_2$O$_3$ (6.0 mg/kg) induced transient acute toxicity in mice, which lasted for 10 min and returned to normal after 30 min (data not shown), suggesting that As$_2$O$_3$ toxicity in the first dosing should be noted. Moreover, As$_2$O$_3$ has been shown to induce tumor cell tolerance [19] and cumulative toxic effects [20], which may explain the similar effects of the low-dose and high-dose As$_2$O$_3$ treatment. Compared with 5-fluorouracil, As$_2$O$_3$ does not cause deterioration of the nutritional status of tumor-bearing mice [21]. Therefore, these facts suggest that As$_2$O$_3$ might be
more effective than 5-fluorouracil for the treatment of lung metastasis in colon cancer. Previous studies have shown that Tregs are selectively enriched in colon tumor sites and play a key role in tumor immune escape [22–24]. In addition, a high concentration of local tumor-infiltrating Tregs is the most crucial factor affecting the prognosis of patients with colon cancer [25–27]. Thus, reducing the number of local tumor-infiltrating Tregs may be of more clinical significance. Our in vivo study showed that As₂O₃ could significantly reduce local infiltration of Tregs and Foxp3 expression, which suggested that As₂O₃ inhibited lung metastasis of colon cancer via selectively reducing the infiltration of Tregs. In vitro, As₂O₃ not only selectively reduced the proportion of Tregs in CIKs but also increased the cytotoxic activity of CIKs, as demonstrated by the higher proportion of CD8⁺ T cells and the increased IFN-γ levels. Moreover, we found that As₂O₃ reduced the amount of CD4⁺ T cells, which was consistent with the previous reports [28, 29]. Taken together, As₂O₃ might inhibit lymphocyte proliferation largely through selective inhibition of CD4⁺ T cells, thereby increasing the CD8⁺ T cell ratio. Therefore, the appropriate dose of As₂O₃ might be used as an immune adjuvant to provide an improved treatment outcome for colon cancer.

The mechanism by which As₂O₃ reduces Tregs is unclear now. In this study, we found that the order of reduction of Foxp3 expression by As₂O₃ was high dose, medium dose, and low dose; the order of reduction of CD4⁺ T cells by As₂O₃ was medium dose, high dose, and low dose, which...
means that different concentrations of As2O3 reduce Tregs via
different mechanisms: the medium concentration might mainly be
due to decreasing the proportion of CD4+ T cells, while the
high concentration might mainly be through reducing Foxp3
expression. Furthermore, the detailed mechanisms need to be
explored in the future.

In summary, we demonstrated that As2O3 might inhibit
lung metastasis of colon cancer by reducing the local infiltra-
tion of Tregs and increase the cytotoxic activity of CIKs by
suppressing Tregs.

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Compliance with ethical standards Written informed consents were
obtained from the patients and healthy donors. This study was approved
by the Ethics Committee of The First Affiliated Hospital of Dalian
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mice used in this study were obtained from the Laboratory Animal
Center of the Academy of Military Medical Science. All experimental
procedures and postoperative animal care were conducted according to
the protocols approved by the Animal Care and Use Committee of
Chinese PLA General Hospital and the National Institute of Health’s
Guidelines for the Care and Use of Laboratory Animals.

Conflicts of interest None

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References

1. Powell BL. Arsenic trioxide in acute promyelocytic leukemia: poison not poison. Expert Rev Anticancer Ther. 2011;11:1317–9.

2. Zhou J. Arsenic trioxide: an ancient drug revived. Chin Med J. 2012;125:3556–60.

3. Wang X, Jiang F, Mu J, et al. Arsenic trioxide attenuates the invasion potential of human liver cancer cells through the demethylation-activated microRNA-491. Toxicol Lett. 2014;227:75–83.

4. Emadi A, Gore SD. Arsenic trioxide—an old drug rediscovered. Blood. 2010;124:191–9.

5. Gronocka G, Asemissen A, Model F, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Res. 2009;69:60–8.

6. Li K, Zhang L, Xiang X, et al. Arsenic trioxide alleviates airway hyperresponsiveness and promotes apoptosis of CD4+ T lymphocytes: evidence for involvement of the ER stress-CHOP pathway. J Immunol. 2013;182:573–83.

7. Koestler DC, Avissar-Whiting M, Houseman EA, et al. Differential DNA methylation in umbilical cord blood of infants exposed to low levels of arsenic in utero. Environ Health Perspect. 2013;121:971–7.

8. Terzic J, Grivennikov S, Karin E, et al. Inflammation and colon cancer. Gastroenterology. 2010;138:2101

9. Wang X, Jiang F, Mu J, et al. Arsenic trioxide exerts antitumor activity through regulatory T cell depletion mediated by oxidative stress in a murine model of colon cancer. J Immunol. 2012;189:5171–7.

10. Gong H, Lin H, Zhang Y, et al. Establishment and evaluation of a mouse model for breast cancer lung metastasis with 4T1-luc. Modern Oncol. 2015;23:735–7.

11. Yan Y, Xu Y, Zhao Y, et al. Combination of E2F-1 promoter-regulated oncolytic adenovirus and cytokine-induced killer cells enhances the antitumor effects in an orthotopic rectal cancer model. Tumor Biol. 2014;35:1113–22.

12. Kito M, Matsumoto K, Wada N, et al. Antitumor effect of arsenic trioxide in murine xenograft model. Cancer Sci. 2003;94:1010–4.

13. Luo Q, Li Y, Deng J, et al. PARP-1 inhibitor sensitizes arsenic trioxide in hepatocellular carcinoma cells via abrogation of G2/M checkpoint and suppression of DNA damage repair. Chem Biol Interact. 2015;226:12–22.

14. Liu B, Pan S, Dong X, et al. Opposing effects of arsenic trioxide on hepatocellular carcinomas in mice. Cancer Sci. 2006;97:675–81.

15. Xu HY, Yang YL, Liu SM, et al. Effect of arsenic trioxide on human hepatocarcinoma in nude mice. World J Gastroenterol. 2004;10:3677–9.

16. Frey DM, Droeser RA, Viehl CT, et al. High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. J Cancer. 2010;126:2635–43.

17. Liu Z, Huang Q, Liu G, et al. Presence of FOXP3(T) Treg cells is correlated with colorectal cancer progression. Int J Clin Exp Med. 2014;7:1781–5.

18. Salama P, Phillips M, Grieu F, et al. Tumor-infiltrating FOXP3(+) regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol. 2009;27:186–92.

19. Vlad C, Kubelac P, Fetica B, et al. The prognostic value of FOXP3(+) regulatory T cells in colorectal cancer. J BUON. 2015;20:114–9.

20. Lee WS, Park S, Lee WY, et al. Clinical impact of tumor-infiltrating lymphocytes for survival in stage II colon cancer. Cancer. 2010;116:5188–99.

21. Ling A, Edin S, Wikberg ML, et al. The intratumoural subside and relation of CD8(+) and FOXP3(+) T lymphocytes in colorectal cancer provide important prognostic clues. Br J Cancer. 2014;110:2551–9.

22. Yan G, Xi Y, Xu S, et al. Inhibition of accelerated rejection mediated by alloreactive CD4(+) memory T cells and prolonged allograft survival by arsenic trioxide. Immunol Invest. 2013;42:438–54.

23. Liao WT, Yu CL, Lan CC, et al. Differential effects of arsenic on cutaneous and systemic immunity: focusing on CD4(+) cell apoptosis in patients with arsenic-induced Bowen’s disease. Carcinogenesis. 2009;30:1064–72.