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Arsenic Trioxide Exerts Antitumor Activity through Regulatory T Cell Depletion Mediated by Oxidative Stress in a Murine Model of Colon Cancer

Audrey Thomas-Schoemann,*†‡ Céline Mongaret,† Carole Nicco,* Christiane Chéreau,† Maxime Annereau,† Alain Dauphin,‡ François Goldwasser,§ Bernard Weill,† François Lemare,*†‡ and Jérôme Alexandre†‡§

Immunotherapy is a promising antitumor strategy that can successfully be combined with current anticancer treatment. In this study, arsenic trioxide (As2O3) was shown to increase the antitumor immune response in CT26 colon tumor-bearing mice through the modulation of regulatory T cell (Treg) numbers. As2O3 induced Treg-selective depletion in vitro. In vivo, tumor-bearing mice injected with 1 mg/kg As2O3 showed a significant decrease in the Treg/CD4 cell ratio and in absolute Treg count versus controls. As2O3 exerted antitumor effects only in immunocompetent mice and enhanced adoptive immunotherapy effects. Inhibition of immunotherapy strategies in human cancer.

The differential effect of As2O3 on Treg versus other CD4 cells may be related to differences in the cells’ redox status, as indicated manganese [III] tetrakis-(5, 10, 15, 20)-benzoic acid porphyrin suggested that it was mediated by oxidative and nitrosative stress. Tregs through oxidative and nitrosative bursts, and suggest that As2O3 could be used to enhance the antitumor activity of adoptive immunotherapy strategies in human cancer. The Journal of Immunology, 2012, 189: 000–000.

Although immunotherapy represents a promising non-toxic anticancer strategy, the various treatment modalities used so far in the clinical setting have resulted in only limited and sporadic success (1). Several reports have suggested that combination therapies may produce synergistic antitumor responses, and that some chemotherapeutic agents, rather than being immunosuppressive, can act as potent adjuvants to either adoptive or active immunotherapy under certain conditions (2). Currently, cyclophosphamide (CYP) represents the gold standard immunomodulatory chemotherapeutic drug, and the antitumor efficacy of its combination with immunotherapy has long been studied in preclinical models (3–5) and clinical trials (6).

Several mechanisms have been investigated to explain this paradoxical phenomenon, and considerable importance has been attributed to the reduction of regulatory T cell (Treg) numbers and function, as observed in both mouse models (7, 8) and cancer patients (9). Increased Treg numbers have been observed in the blood and tumor tissues of a high proportion of cancer patients, a phenomenon that promotes tumor progression and adversely affects prognosis (10, 11). The adverse effects of Tregs on the immune response are underscored by the observation that depletion of Tregs can enhance the efficacy of passive or active immunotherapy strategies. Use of low-dose CYP, anti-CD25 Abs, denileukin difitox, or ipilimumab (12) to deplete or functionally inactivate Tregs is currently in development or under clinical evaluation.

Arsenic trioxide (As2O3) has shown substantial efficacy in the treatment of patients with newly diagnosed or relapsed acute promyelocytic leukemia (13). In addition, preclinical studies have shown that a wide variety of malignancies, including hematologic cancer and solid tumors derived from several tissue types, are susceptible to therapy with As2O3. Although the exact mechanisms of its antitumoral effect remain unclear, As2O3 has been recognized as a powerful inducer of oxidative stress in tumor cells (14, 15). Recently, immunomodulatory properties of As2O3 have been described. For instance, As2O3 exerts therapeutic effects on lymphoproliferative and severe autoimmune disorders manifested in MRL/lpr mice (16) and enhances the immune response against myeloma cells or breast cancer cells (17, 18).

In this report, we demonstrate that As2O3 increases antitumor immune response in colon tumor-bearing mice through the modulation of Treg numbers. We also examine the consequences of As2O3-induced reactive oxygen species (ROS) and reactive nitrogen species (RNS) production on Treg numbers and the antitumor immune response in colorectal tumor-bearing mice.

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Abbreviations used in this article: As2O3, arsenic trioxide; CYP, cyclophosphamide; DAF2-DA, 4,5-diaminofluorescein diacetate; H2DCFDA, 2’7’ dichlorodihydrofluorescein diacetate; L-NAMe, N6-nitro-L-arginine methyl ester; MnTBAP, manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin; ONOO–, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, reverse transcription; Treg, regulatory T cell; TV, tumor volume.

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Materials and Methods

Chemicals, Abs, and cell line
All chemicals were obtained from Sigma (Saint Quentin Fallavier, France) except CYP, which was obtained from Baxter (Endoxan; Baxter, Deerfield, IL). mAbs against mouse CD4-allophycocyanin (GK1.5) and CD25-PE (PC61.5), and a Treg staining kit (no. 88-1188-40) including Abs against mouse CD4-FTTC (GK1.5), CD25-PE (PC61.5), foxp3-allophycocyanin (FJK-16s), and a permeabilization buffer, were purchased from eBioscience (San Diego, CA). The kit for mouse Treg isolation was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The mouse colon carcinoma cell line CT26 was obtained from ATCC (Manassas, VA) and was cultured in DMEM (Life Technologies Invitrogen, Cergy Pontoise, France) supplemented with 10% FBS and antibiotics (Life Technologies Invitrogen, Cergy Pontoise, France).

Animal studies
BALB/c female mice between 6 and 8 wk old were used (Iffa Credo, L’Arbresles, France). Eight-wk-old female nude mice were purchased from Charles River Laboratories (L’Arbresles, France). Animals received humane care in compliance with institutional guidelines.

Adoptive cell transfer experiments
In adoptive cell transfer experiments, donor mice were CT26 tumor-bearing BALB/c animals. Those mice were sacrificed 10 d after CT26 injection, and splenocytes were isolated as described later. The donor mice were given i.p. PBS or As2O3 7 d after tumor implantation.

Tumor growth
BALB/c or nude mice were injected s.c. with 5 × 10⁶ CT26 cells diluted in DMEM without FBS. When tumors reached 30 mm³ in size (7 d later for nude mice and 10 d later for BALB/c mice), mice were injected once i.p. with 1 mg/kg As2O3, 50 mg/kg CYP, or with PBS.

Preparation of splenocytes from mouse spleens
Spleens were collected, crushed, hemolyzed with 0.15 mM ammonium chloride, and centrifugated at 1200 rpm for 10 min. Pellets were then suspended in RPMI 1640 medium supplemented with 10% FBS. The total number of splenocytes from each spleen was counted using a Malassez cell. For in vitro experiments, splenocytes from healthy BALB/c mouse were seeded (3 × 10⁵/well) on 12-well culture plates and incubated for 2 h with As2O3 (0.125, 0.5, or 1 μM).

Detection of Treg by flow cytometry
Cell suspensions from spleens were prepared after hypotonic lysis of erythrocytes. Tregs were labeled with anti–CD4-FTTC (GK1.5), anti–CD25-PE (PC61.5), and anti–Foxp3-FTTC (FJK-16s) from the Treg staining kit obtained from eBiosciences, using manufacturer’s recommendations. Cells were subjected to analysis on a FACS Canto flow cytometer (BD Biosciences, San Jose, CA). Tregs were recognized as CD4+CD25+Foxp3⁺ cells.

Immunohistochemistry
Four-micrometer sections were cut from formalin-fixed, paraffin-embedded tissue and subjected to an Ag retrieval treatment performed in 10 mM citrate buffer (pH 6.0) in a pressure cooker. Next, sections were incubated with the rat anti-Foxp3 mAb clone FJK-16s (eBioscience), followed by peroxidase-conjugated goat anti-rat IgG (Invitrogen). Peroxidase was developed with a highly sensitive dianobenzidine chromogenic substrate for ∼10 min. Negative controls were performed by omitting the primary Ab. For each animal, the average number of positive stained cells within at least five independent high-power fields (∼400 magnification) was determined microscopically.

Assays of ROS and RNS production in splenocytes and Treg
Levels of cellular peroxynitrite (ONOO⁻) and/or H₂O₂ were assessed using flow cytometry by oxidation of the specific probe 2′,7′ dichlorodihydrofluorescein diacetate (H₂DCFDA). Cellular NO was measured by using 4,5-diaminofluorescein diacetate (DAF-2-DA).

As previously described by Gupta et al. (20), splenocytes were isolated from healthy BALB/c mice and suspended (1 × 10⁶ cells/ml) in RPMI 1640 medium containing 5 μM H₂DCFDA or DAF-2-DA for 20 min at 37°C. Then 3 × 10⁶ cells/well were seeded on 12-well culture plates and incubated with or without 1 μM As2O3 for an additional 2-h period at 37°C. Cells were then washed twice with PBS, stained with mAbs against mouse CD4-allophycocyanin (GK1.5) and CD25-PE (PC61.5), and analyzed on a BD FACS canto flow cytometer.

Treg purification
Purification of CD4⁺CD25⁻ Tregs was performed in a two-step procedure, according to the manufacturer’s instructions. Non-CD4⁺ cells were indirectly magnetically labeled with a mixture of biotin-conjugated Abs and anti-biotin microbeads. Labeled cells were subsequently depleted by separation over a MACS column. In the second step, CD4⁺CD25⁻ Tregs were directly labeled with anti-CD25-coated microbeads and isolated by positive selection from the pre-enriched CD4⁺ T cell fraction. The magnetically labeled CD4⁺CD25⁻ Tregs were retained on the column and eluted after removal of the column from the magnetic field.

Analysis of RNA isolation and NO signaling pathway
RNA was purified from ∼10⁶ isolated CD4⁺CD25⁻ cells or CD4⁺CD25⁺ cells using a RNeasy mini kit (Qiagen). RNA integrity was assessed spectrophotometrically and by electrophoresis on agarose gel. Reverse transcription (RT) was done using a RT2 First Strand Kit (SA Biosciences, Frederick, MD). PCR was performed using RT2 Profiler PCR Array PAMM-062, according to the manufacturer’s instructions on a Roche LightCycler 480 using RT2 Real-Time SYBR Green PCR Master Mix. The array evaluated the expression of 84 genes whose expression is controlled by or involved in signaling by NO. Samples from untreated and arsenic pretreated splenocytes were compared. For data analysis, the ΔΔCt method was used with the aid of a Microsoft Excel spreadsheet containing algorithms provided by the manufacturer.

Statistical analysis
Statistical analysis was performed on the means of data obtained from at least three independent experiments. All results are presented as means ± SEM. The Student t test was used to compare statistical differences in the experimental values between two samples (GraphPad Prism; GraphPad Software, San Diego, CA). A P value of 0.05 was considered statistically significant.

Results

As2O3 induces selective Treg depletion in mice
The in vitro effect of increasing concentrations of As2O3 on the ratio of Treg/CD4 cells was observed compared with healthy mice. As depicted in Fig. 1A, treating splenocytes with 0.5 or 1 μM As2O3 for 2 h decreased the ratio of Treg/CD4 cells from 14.67% in untreated cells to 10.33% (P = 0.0044) and 6.67% (P = 0.0011), respectively. Of note, 1 μM As2O3 did not induce in vitro cytotoxicity against mouse colon carcinoma CT26 cells (data not shown).

The proportion of Tregs in spleens obtained from CT26 tumor-bearing mice was then evaluated, and a significant increase in the ratio of Treg/CD4 cells was observed compared with healthy mice.
delayed in BALB/c mice receiving a single dose of 1 mg/kg As2O3 assessed in immunocompetent mice. Tumor growth was found to be cant decrease in the Treg/CD4 cell ratio (Fig. 1B) and the absolute number of CD4+CD25+foxp3+ cells/CD4+ T cells (B) and on CD4+CD25+foxp3+ absolute cell numbers (C). BALB/c mice were injected with 5 × 10^6 CT26 cells diluted in DMEM without FBS (except for mice from the control group that were injected s.c. with DMEM without FBS only). Ten days later, the mice were injected i.p. with a single dose of 1 mg/kg As2O3, 50 mg/kg CYP, or PBS. After 3 d, spleen cells were isolated, stained with a mouse CD4–allophycocyanin and mouse CD25-PE, and analyzed by flow cytometry. (A) In vitro effects of As2O3 on the ratio of CD4+CD25+foxp3+ cells/CD4+ T cell ratio. Splenocytes isolated from healthy BALB/c mice were incubated for 2 h with As2O3 (0.125, 0.5, and 1 μM). Cells were then washed twice with PBS, stained with mAb against mouse CD4- allophycocyanin and mouse CD25-PE, and analyzed by flow cytometry. (B and C) In vivo effects of As2O3 in CT26 tumor-bearing mice on the ratio of CD4+CD25+foxp3+ cells/CD4+ T cells (B) and on CD4+CD25+foxp3+ absolute cell numbers (C). BALB/c mice were injected with 5 × 10^6 CT26 cells diluted in DMEM without FBS (except for mice from the control group that were injected s.c. with DMEM without FBS only). Ten days later, the mice were injected i.p. with a single dose of 1 mg/kg As2O3, 50 mg/kg CYP, or PBS. After 3 d, spleen cells were isolated, stained with a mouse Treg staining kit, and analyzed by flow cytometry. Five mice were treated in each group, and similar results were obtained in two independent experiments. *0.01 < p < 0.05, **0.001 < p < 0.01, ***0.0001 < p < 0.001.

Furthermore, we observed by immunohistochemistry that As2O3 treatment caused a slight but significant decrease in foxp3+ cells infiltrating the tumor with 0.75 ± 0.18 foxp3+ cells per field in untreated mice versus 0.25 ± 0.13 in As2O3-treated mice (p = 0.03). As2O3 did not significantly change the ratio of other immune-suppressive cells (i.e., myeloid-derived suppressor cells [CD11b+GR1+ cells] and plasmacytoid dendritic cells [CD11c+ B220+ cells]) or effector cells including CD4, CD8, and B cells (data not shown). These results show that low-dose As2O3 induces selective depletion of Tregs in tumor-bearing mice.

Antitumor effect of low-dose As2O3 is observed only in immunocompetent mice and enhances adoptive immunotherapy effects

Because Treg depletion induced by As2O3 enhanced the antitumor immune response, the antitumor effect of low-dose As2O3 was assessed in immunocompetent mice. Tumor growth was found to be delayed in BALB/c mice receiving a single dose of 1 mg/kg As2O3 compared with mice treated with PBS (p = 0.0023 on day 26; Fig. 2A). In contrast, 1 mg/kg As2O3 had no significant antitumor effect in athymic NUDE mice (Fig. 2B), suggesting that a competent immune system was necessary for the As2O3 antitumor effect.

In tumor-bearing recipient mice pretreated with As2O3, adoptive transfer of splenocytes from untreated tumor-bearing donor mice (As2O3 + control splenocytes group; Fig. 2C) prevented the antitumoral effect of As2O3 treatment because no significant difference to the control group was observed (p = 0.35). In contrast, in tumor-bearing recipient mice pretreated with As2O3, adoptive transfer of splenocytes from As2O3-treated, tumor-bearing donor mice (As2O3 + Treg-depleted splenocytes group) resulted in a significant decrease in tumor growth (p = 0.007 versus control group; p = 0.5 versus As2O3 + control splenocytes group), which was more pronounced than the effect obtained after treatment with As2O3 alone. Therefore, the spleens of As2O3-treated tumor bearers had an increased level of antitumor immunity compared with the spleens of control tumor bearers.

As2O3 induces a specific gene expression profile in Tregs

The expression of 84 genes involved in oxidative and nitrosative stress pathways was assessed in CD4+CD25+ and CD4+CD25− cells with or without As2O3 treatment for 2 h. In CD4+CD25+ cells, treatment with As2O3 was associated with downregulation of 50 genes (60%; Fig. 3A). The pro-oxidative genes Nos2 (NO synthase 2), Sod1 (superoxide dismutase 1), Ccs (copper chaperone for superoxide dismutase) and Noxo1 (NADPH oxidase organizer 1), and the proapoptotic gene Fas were among the few genes that were upregulated (fold upregulation > 1.3).

In CD4+CD25− cells treated with As2O3, 31 genes were downregulated (37%) and 41 genes were upregulated (49%). Ccng1 (cyclin G1), Pkaca (protein kinase cAMP dependent), and Med4 (mediator of RNA polymerase II transcription) were among the upregulated genes whose expression showed more than a 3-fold increase, whereas pro-oxidative genes were not upregulated. Thus, As2O3 selectively induces the expression of pro-oxidative genes in Tregs.

As2O3 induces NO and ROS accumulation in the Treg population, but not in other T cells

To assess the role of oxidative stress in Treg depletion induced by As2O3, we first examined the effect of As2O3 on the production of NO and ROS. As2O3 increased NO and ROS production by splenocytes from As2O3-treated mice compared with control splenocytes (Fig. 4A). As2O3-induced NO and ROS accumulation was associated with increased expression of iNOS and the proapoptotic genes Bcl2l11 (pre-B cell leukemia/lymphoma 11) and Mgst2 (magnesium transport 2) (Fig. 4B). These results suggest that As2O3-induced NO and ROS accumulation contribute to Treg depletion.

In summary, our results indicate that As2O3 induces selective depletion of Tregs in tumor-bearing mice, leading to enhanced antitumor immunity. This effect is mediated by the induction of pro-oxidative genes in Tregs, which results in the depletion of Tregs and an increase in antitumor immunity.

**FIGURE 1.** Effect of low-dose As2O3 on Treg numbers in mice in vitro and in vivo. (A) In vitro effects of As2O3 on the CD4+CD25+foxp3+ cells/CD4+ T cell ratio. Splenocytes isolated from healthy BALB/c mice were incubated for 2 h with As2O3 (0.125, 0.5, and 1 μM). Cells were then washed twice with PBS, stained with mAb against mouse CD4- allophycocyanin and mouse CD25-PE, and analyzed by flow cytometry. (B and C) In vivo effects of As2O3 in CT26 tumor-bearing mice on the ratio of CD4+CD25+foxp3+ cells/CD4+ T cells (B) and on CD4+CD25+foxp3+ absolute cell numbers (C). BALB/c mice were injected with 5 × 10^6 CT26 cells diluted in DMEM without FBS (except for mice from the control group that were injected s.c. with DMEM without FBS only). Ten days later, the mice were injected i.p. with a single dose of 1 mg/kg As2O3, 50 mg/kg CYP, or PBS. After 3 d, spleen cells were isolated, stained with a mouse CD4–allophycocyanin and mouse CD25-PE, and analyzed by flow cytometry. Five mice were treated in each group, and similar results were obtained in two independent experiments. *0.01 < p < 0.05, **0.001 < p < 0.01, ***0.0001 < p < 0.001.

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ROS and RNS in splenocytes by flow cytometry. After a 2-h exposure to As2O3, the cellular accumulation of NO as assessed by DAF2-DA fluorescence was increased in CD4+CD25+ cells (p = 0.025), but not in CD4+CD25- cells (Fig. 4A). Similarly, As2O3 induced a nearly 2-fold increase of H2DCFDA fluorescence in CD4+CD25+ cells, indicative of H2O2 and/or ONOO- accumulation, but not in CD4+CD25- cells (Fig. 4B). Representative dot plots of DAF2-DA and H2DCFDA fluorescence in Tregs with or without As2O3 treatment are shown in Fig. 4C and 4D, respectively. Furthermore, H2DCFDA and DAF2-DA basal levels were lower in CD4+CD25- cells than in CD4+CD25+ cells and remained lower after As2O3 treatment (Fig. 4A, 4B).

These results suggest that As2O3 could be more toxic to Tregs than conventional T cells because of an increased basal oxidative and nitrosative stress in this population, and that specific As2O3 Treg cytotoxicity could be mediated by oxidative and nitrosative stress.

As2O3-induced Treg depletion is mediated by ROS and RNS accumulation

To further investigate the role of ROS and RNS in mediating As2O3-induced Treg depletion, CT26 tumor-bearing BALB/c mice previously injected with As2O3 were treated with the oxidative stress modulators MnTBAP and L-NAME. In mice treated with MnTBAP, a superoxide dismutase mimic that prevents superoxide and ONOO- accumulation (Fig. 5C), As2O3-induced Treg depletion was inhibited (p = 0.0055; Fig. 5A). Similarly, L-NAME, an NO synthase inhibitor that decreases NO accumulation, inhibited As2O3-induced Treg depletion (Fig. 5B). Together, these results suggest a key role for ONOO- in Treg depletion induced by As2O3 (Fig. 5C).

Discussion

We report in this article for the first time, to our knowledge, that As2O3 can deplete Tregs through oxidative and nitrosative bursts, thus improving the antitumor immune response. Multiple immunotherapy strategies for colorectal carcinoma are currently under preclinical and clinical evaluation (21). Immunotherapy usually has to face strong mechanisms of immune escape induced by tumors. To increase the efficacy of immunotherapy strategies, it is potentially interesting to explore therapeutic interventions that exploit tolerogenic processes such as those induced by Tregs in tumor-bearing hosts (22). In this context, it has been shown that low-dose CYP decreases the number of Tregs (8, 21) and can enhance the antitumor activity of adoptively transferred T cells, as well as tumor vaccines (23). However, CYP may exert a toxic effect on other lymphocyte populations that could attenuate its immunostimulatory effect. Indeed, we observed that CYP induced a severe splenocyte depletion that was not observed with As2O3. These data suggest that the effect of As2O3 could be more specific to Treg than CYP. In addition, long-term administration of CYP has been associated with an increased risk for acute leukemia and bladder cancer.

We observed that As2O3 induces selective depletion of Tregs in vitro and in vivo. In agreement with previous studies, we first showed that tumor-bearing mice displayed an increased proportion of Tregs within the CD4 cell population, contributing to immune escape (24). Indeed, in a CT26 colon cancer mice model, As2O3 induced depletion in Tregs in the spleen and the tumor to levels observed in healthy mice. Low-dose As2O3 was found to have an antitumor effect that is related to Treg depletion, in a CT26 colon cancer mice model as well as in a 4T1 breast cancer mouse.
model. Adoptive cell transfer experiments showed that As$_2$O$_3$ is able to restore the activity of adoptive immune cells from donor mice, and thus enhance their antitumor potential. At least two previous studies have shown that As$_2$O$_3$ is able to enhance the immune response against myeloma and breast cancer cells (17, 18). In these studies, As$_2$O$_3$ increased the cytotoxicity of lymphokine-activated killer cells. Tregs were not specifically explored, but they are well-known to effect the antitumor activity of effector cells (24).

In our hands, the immunostimulatory effects of As$_2$O$_3$ were observed with concentrations of 0.5–1 μM, levels that were not cytotoxic to CT26 colon carcinoma cells. Similarly, previous studies have shown that 1 μM As$_2$O$_3$ is able to induce antitumor immune response, whereas only doses >2 μM can induce tumor cells apoptosis (17, 18). In another study, the IC$_{50}$ of As$_2$O$_3$ against prostate and ovarian cancer cell lines was around 5 μM (25). In vivo, doses of As$_2$O$_3$ ranging from 2 to 6.5 mg/kg/d for 1–6 wk were found necessary to treat solid tumors (26). Such high doses are probably not clinically achievable without severe adverse effects caused by As$_2$O$_3$ toxicity. In contrast, we showed in this study that a single dose of 1 mg/kg As$_2$O$_3$ delayed tumor growth by enhancing the antitumor immune response. The immunologic effect of low-dose As$_2$O$_3$ appears to be related to the high sensitivity of T$_{reg}$ to the drug.

Finally, we showed that T$_{reg}$ depletion induced by As$_2$O$_3$ was mediated by the generation of ROS and RNS. Previous studies have observed that As$_2$O$_3$ was able to induce the intracellular accumulation of superoxide anion. Disruption of the mitochondrial respiratory chain and membrane NADPH oxidase activation by As$_2$O$_3$ have been proposed as sources of superoxide anion (27–29). It has also been reported that As$_2$O$_3$ is able to increase cellular production of nitrite oxide (30). As illustrated in Fig. 5C, superoxide dismutases catalyze the dismutation of superoxide in H$_2$O$_2$, which is converted in H$_2$O by catalase and glutathione peroxidase. Superoxide anions may also react with NO to form peroxynitrite, one of the most toxic radicals. We showed that in T$_{reg}$, As$_2$O$_3$ increases the fluorescence of H$_2$DCFDA, which could be related to the accumulation of H$_2$O$_2$ and/or ONOO$^−$. Our data strongly suggest that T$_{reg}$ depletion induced by As$_2$O$_3$ is related more to ONOO$^−$ production than to H$_2$O$_2$ production, because it was inhibited by the NO synthase inhibitor L-NAME and by the superoxide dismutase mimic MnTBAP. Thus, T$_{reg}$ depletion...
induced by As$_2$O$_3$ is related to superoxide and nitrite oxide production resulting in ONOO$^-$ accumulation. In contrast, As$_2$O$_3$ failed to induce significant NO and ONOO$^-$ accumulation in non-T$_{reg}$ CD4 cells. Differences in the effect of As$_2$O$_3$ on T$_{reg}$ and other CD4 cells could be related to differences in the redox status of these cells. The threshold of toxicity could be more easily reached in T$_{reg}$ after As$_2$O$_3$ exposure because of greater basal levels of NO and ONOO$^-$ in these cells and overwhelming of antioxidant defenses. This redox-based differential effect of As$_2$O$_3$ has previously been observed in acute promyelocytic leukemia cells (31). It has been proposed that the high susceptibility of PML cells versus other cancer cells to arsenic cytotoxicity is related to higher basal activity of NADPH oxidase, inducing basal oxidative stress.

In conclusion, we show for the first time, to our knowledge, that low doses of As$_2$O$_3$ are able to delay solid tumor growth by depleting T$_{reg}$. Our results offer a new opportunity to use low doses of As$_2$O$_3$ to enhance the antitumor activity of adoptive immunotherapy strategy in human cancer.

**FIGURE 4.** Cellular ROS and RNS accumulation in splenocytes exposed to As$_2$O$_3$. Cellular H$_2$DCFDA (H$_2$O$_2$ and/or ONOO$^-$) (A) and DAF$_{2}$-DA (NO) (B) fluorescence in CD4$^+$/CD25$^+$ or CD4$^+$/CD25$^-$ cells exposed to 1 mM As$_2$O$_3$ or PBS for 2 h. Spleen cells were incubated for 30 min with either 5 mM H$_2$DCFDA or 5 mM DAF$_{2}$-DA in medium and centrifugated. Cells were then stained with mAb against mouse CD4-allophycocyanin and CD25-PE, and analyzed by flow cytometry. *0.01 < $p$ < 0.05, **0.001 < $p$ < 0.01. Representative dot plots of DAF$_{2}$-DA (C) and H$_2$DCFDA fluorescence (D) in CD4$^+$/CD25$^+$ cells treated or not with As$_2$O$_3$ were shown.

**FIGURE 5.** Oxidative stress modulator effects on As$_2$O$_3$-induced T$_{reg}$ depletion. BALB/c mice were injected s.c. with $5 \times 10^5$ CT26 cells diluted in medium. Ten days later, mice were injected i.p. with a single dose of 1 mg/kg As$_2$O$_3$. Mice were injected i.p. with 10 mg/kg/d MnTBAP (A) or were given per os 100 mg/ kg/d L-NAME (B) for 3 d. After 3 d, mice were sacrificed, splenocytes were isolated and stained with a mouse T$_{reg}$ staining kit, and analyzed by flow cytometry. *0.01 < $p$ < 0.05, **0.001 < $p$ < 0.01. (C) Inhibition of the effect of As$_2$O$_3$ by L-NAME and MnTBAP suggests a potential role of ONOO$^-$ in the effects of As$_2$O$_3$ on T$_{reg}$ depletion.
Acknowledgments
The authors have no financial conflicts of interest.

Disclosures
The authors have no financial conflicts of interest.

References
Figure S1: Effect of low-dose As$_2$O$_3$ on tumor growth and Treg number in 4T1 tumor-bearing mice

(A) Effect of low-dose As$_2$O$_3$ on tumor growth: BALB/c mice were injected SC with 10$^5$ 4T1 cells diluted in medium. Ten days later, the mice were injected IP with a single dose of As$_2$O$_3$ 1 mg/kg. Tumor size was measured with a Vernier caliper every three days.

(B, C) Effects of As$_2$O$_3$ in 4T1 tumor-bearing mice on the ratio of CD4$^+$CD25$^+$Foxp3$^+$ cells/CD4$^+$ T cells (B), and on CD4$^+$CD25$^+$Foxp3$^+$ absolute cell numbers (C). Ten days after 10$^5$ 4T1 cell injection, mice were injected IP with As2O3 1mg/kg or PBS. After three days, spleen cells were stained with a regulatory T-cell staining kit and analyzed by flow cytometry. Five mice were treated in each group and similar results were obtained in two independent experiments. * 0.01< p<0.05.