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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,*‡ Frédéric Batteux, † Céline Mongaret, † Carole Nicco, † Christiane Chéreau, † Maxime Annereau, † Alain Dauphin, ‡ François Goldwasser, ‡ Bernard Weill, † François Lenare, ‡ and Jérôme Alexandre †‡

Immunotherapy is a promising antitumor strategy that can successfully be combined with current anticancer treatment. In this study, arsenic trioxide (As$_2$O$_3$) was shown to increase the antitumor immune response in CT26 colon tumor-bearing mice through the modulation of regulatory T cell ($T_{reg}$) numbers. As$_2$O$_3$ induced $T_{reg}$-selective depletion in vitro. In vivo, tumor-bearing mice injected with 1 mg/kg As$_2$O$_3$ showed a significant decrease in the $T_{reg}$/CD4 cell ratio and in absolute $T_{reg}$ count versus controls. As$_2$O$_3$ exerted antitumor effects only in immunocompetent mice and enhanced adoptive immunotherapy effects. Inhibition of As$_2$O$_3$-induced $T_{reg}$ depletion by the NO synthase inhibitor $N_\text{G}$-nitro-$L$-arginine methyl ester and the superoxide dismutase mimic manganese [III] tetrakis-(5, 10, 15, 20)-benzoic acid porphyrin suggested that it was mediated by oxidative and nitrosative stress. The differential effect of As$_2$O$_3$ on $T_{reg}$ versus other CD4 cells may be related to differences in the cells’ redox status, as indicated by significant differences in 2’7’-dichlorodihydrofluorescein diacetate and 4,5-diaminofluorescein diacetate fluorescence levels. In conclusion, these results show for the first time, to our knowledge, that low doses As$_2$O$_3$ can delay solid tumor growth by depleting $T_{reg}$s through oxidative and nitrosative bursts, and suggest that As$_2$O$_3$ could be used to enhance the antitumor activity of adoptive immunotherapy strategies in human cancer. The Journal of Immunology, 2012, 189: 5171–5177.

Several mechanisms have been investigated to explain this paradoxical phenomenon, and considerable importance has been attributed to the reduction of regulatory T cell ($T_{reg}$) numbers and function, as observed in both mouse models (7, 8) and cancer patients (9). Increased $T_{reg}$ 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 $T_{reg}$ on the immune response are underscored by the observation that depletion of $T_{reg}$s can enhance the efficacy of passive or active immunotherapy strategies. Use of low-dose CYP, anti-CD25 Abs, denileukin diftitox, or ipilimumab (12) to deplete or functionally inactivate $T_{reg}$s is currently in development or under clinical evaluation.

Arsenic trioxide (As$_2$O$_3$) 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 As$_2$O$_3$. Although the exact mechanisms of its antitumoral effect remain unclear, As$_2$O$_3$ has been recognized as a powerful inducer of oxidative stress in tumor cells (14, 15). Recently, immunomodulatory properties of As$_2$O$_3$ have been described. For instance, As$_2$O$_3$ 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 As$_2$O$_3$ increases antitumor immune response in colon tumor-bearing mice through the modulation of $T_{reg}$ numbers. We also examine the consequences of As$_2$O$_3$-induced reactive oxygen species (ROS) and reactive nitrogen species (RNS) production on $T_{reg}$ numbers and the anti-tumor immune response in colorectal tumor-bearing mice.

*Univ. Paris Descartes, Sorbonne Paris Cité, Unité de Formation et Recherche des Sciences Pharmaceutiques et Biologiques, 75270 Paris Cedex 06, France;  †Univ. Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, EA 1833, Paris 75014, France;  ‡Univ. Fronctionnelle de Pharmacocinétique et Pharmacothérapie, Assistance Publique-Hôpitaux de Paris, Hôpital Cochin, Paris 75014, France;  Service d’Oncologie, Assistance Publique-Hôpitaux de Paris, Groupeement des Hôpitaux Paris Centre, Paris 75014, France; and  Département de Pharmacologie Clinique, Institut Gustave Roussy, Villejuif 95805, France

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Address correspondence and reprint requests to Dr. Audrey Thomas-Schoemann, Université Paris Descartes, Unité de Formation et Recherche des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l’Observatoire, 75270 Paris Cedex 06, France. E-mail address: schoemann.audrey@gmail.com

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Abbreviations used in this article: As$_2$O$_3$, arsenic trioxide; CYP, cyclophosphamide; DAF$_2$-DA, 4,5-diaminofluorescein diacetate; H$_2$DCFDA, 2’7’-dichlorodihydrofluorescein diacetate; L-NAME, $N_\text{G}$-nitro-$L$-arginine methyl ester; MnTBAP, manganese [III] tetakis-(5,10,15,20)-benzoic acid porphyrin; ONOO$^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, reverse transcription; $T_{reg}$, 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. 88-8118-40) including Abs against mouse CD4-FITC (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 human care in compliance with institutional guidelines. BALB/c or NUDE mice were injected s.c. with 5 × 10^5 CT26 cells diluted in DMEM without FBS. When tumors reached 30 mm^2 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. In experiments testing the effects of oxidative stress modulators, mice were injected i.p. either with a superoxide dismutase mimic, manganese [III] tetrakis-(5, 10, 15, 20)-benzoic acid porphyrin (MnTBAP; 10 mg/kg/d) or were given per os an NO synthesize inhibitor, N-(nitro-arginine methyl ester (L-NAME; 100 mg/kg/d) for 3 d.

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.

The recipient mice were a second set of BALB/c mice carrying a 10-d tumor, given As2O3 or PBS 5 h before receiving donor spleen cells (i.v. (3 × 10^5/mice). Transferred splenocytes were isolated from either untreated donor mouse (control splenocyte group) or As2O3-treated donor mice (Treg-depleted splenocyte group). After a procedure previously described for determination of the immunomodulating effects of CYP (19), recipient mice were treated with As2O3 before receiving donor spleen cells to remove Tregs.

As negative controls, mice were injected with DMEM i.v. and PBS i.p. (control group), or with DMEM i.v. and As2O3 i.p. (As2O3 alone group), or with Treg-depleted splenocytes i.v. and PBS i.p. (Treg-depleted splenocyte alone group).

Tumor growth

BALB/c or NUDE mice were injected s.c. with 5 × 10^5 CT26 cells diluted in DMEM without FBS. When tumors reached 30 mm^2 in size, mice were injected once i.p. with either 1 mg/kg As2O3 or with PBS. Tumor size was measured with a Vernier caliper every 3 d. Tumor volume (TV) was calculated as follows: TV (mm^3) = (L × W^2)/2, where L is the longest and W the shortest radius of the tumor. Results are expressed as the mean of TV within each group. TVs were compared across treatment groups after each tumor size measurement, that is, every 3 d. Five mice were treated in each group.

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 × 10^7/well) on 12-well culture plates and incubated for 2 h with As2O3 (0.125, 0.5, or 1 μM).

Detection of Tregs by flow cytometry

Cell suspensions from spleens were prepared after hypotonic lysis of erythrocytes. Tregs were labeled with anti-CD4-FITC (GK1.5), anti-CD25-PE (PC61.5), anti-CD8-PE-Cy7 (53-6.7), and anti-foxp3-PE-Cy5 (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 mmol/l 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 diaminobenzidine 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 Tregs

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 (DAF2-DA).

As previously described by Gupta et al. (20), splenocytes were isolated from healthy BALB/c mice and suspended (1 × 10^7 cells/ml) in RPMI 1640 medium containing 5 μM H2DCFDA or DAF2-DA for 20 min at 37°C. Then 3 × 10^5 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 bioin-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 ∼10^6 isolated CD4^+CD25^+ cells or CD4^+CD25^- cells using a RNAeasy 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 ascorbic acid-treated 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 proportion of Tregs was first investigated in splenocytes obtained from healthy mice. As depicted in Fig. 1A, treating splenocytes with 0.5 or 1 μM As2O3 for 2 h decreased the ratio of Tregs/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 splenocytes 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.
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. (**p < 0.001; **p < 0.01; *p < 0.05; p > 0.05). (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 × 10^5 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. *p < 0.05, **p < 0.01, ***p < 0.001.

Because Treg depletion induced by As2O3 enhanced the antitumor effects in immunocompetent mice and enhances adoptive immunotherapy effects

Because 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 immunosuppressive 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 significantly decreased in the Treg/CD4+ cell ratio (Fig. 1B) and the absolute number of Treg cells (Fig. 1C) compared with mice injected with PBS. However, CYP induced a severe splenocyte depletion that was not observed with As2O3 (data not shown). Higher doses of As2O3 were not associated with more powerful effects on Treg number. Therefore, the dose of 1 mg/kg was used for the subsequent experiments. As2O3-induced Treg depletion was observed similarly in a 4T1 breast tumor model (Supplemental Fig. 1).

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 immunosuppressive 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.

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

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ROS and RNS in splenocytes by flow cytometry. After a 2-h exposure to As$_2$O$_3$, 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, As$_2$O$_3$ induced a nearly 2-fold increase of H$_2$DCFDA fluorescence in CD4$^+$CD25$^+$ cells, indicative of H$_2$O$_2$ and/or ONOO$^-$ accumulation, but not in CD4$^+$CD25$^-$ cells (Fig. 4B). Representative dot plots of DAF2-DA and H$_2$DCFDA fluorescence in Tregs with or without As$_2$O$_3$ treatment are shown in Fig. 4C and 4D, respectively. Furthermore, H$_2$DCFDA and DAF2-DA basal levels were lower in CD4$^+$CD25$^-$ cells than in CD4$^+$CD25$^+$ cells and remained lower after As$_2$O$_3$ treatment (Fig. 4A, 4B).

These results suggest that As$_2$O$_3$ 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 As$_2$O$_3$ Treg cytotoxicity could be mediated by oxidative and nitrosative stress.

$As_2O_3$-induced T$_{reg}$ depletion is mediated by ROS and RNS accumulation

To further investigate the role of ROS and RNS in mediating As$_2$O$_3$-induced T$_{reg}$ depletion, CT26 tumor-bearing BALB/c mice previously injected with As$_2$O$_3$ 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), As$_2$O$_3$-induced T$_{reg}$ depletion was inhibited ($p = 0.0055$; Fig. 5A). Similarly, L-NAME, an NO synthase inhibitor that decreases NO accumulation, inhibited As$_2$O$_3$-induced T$_{reg}$ depletion (Fig. 5B). Together, these results suggest a key role for ONOO$^-$ in T$_{reg}$ depletion induced by As$_2$O$_3$ (Fig. 5C).

**Discussion**

We report in this article for the first time, to our knowledge, that As$_2$O$_3$ can deplete T$_{reg}$s 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 T$_{reg}$s (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 As$_2$O$_3$. These data suggest that the effect of As$_2$O$_3$ could be more specific to T$_{reg}$ 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 As$_2$O$_3$ induces selective depletion of T$_{reg}$s both in vitro and in vivo. In agreement with previous studies, we first showed that tumor-bearing mice displayed an increased proportion of T$_{reg}$s within the CD4$^+$ cell population, contributing to immune escape (24). Indeed, in a CT26 colon cancer mice model, As$_2$O$_3$ induced depletion in T$_{reg}$s in the spleen and the tumor to levels observed in healthy mice. Low-dose As$_2$O$_3$ was found to have an antitumor effect that is related to T$_{reg}$ depletion, in a CT26 colon cancer mice model as well as in a 4T1 breast cancer mice model.
model. Adoptive cell transfer experiments showed that As₂O₃ 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 As₂O₃ is able to enhance the immune response against myeloma and breast cancer cells (17, 18). In these studies, As₂O₃ 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 As₂O₃ 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 As₂O₃ is able to induce antitumor immune response, whereas only doses >2 μM can induce tumor cells apoptosis (17, 18). In another study, the IC₅₀ of As₂O₃ against prostate and ovarian cancer cell lines was around 5 μM (25). In vivo, doses of As₂O₃ 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 As₂O₃ toxicity. In contrast, we showed in this study that a single dose of 1 mg/kg As₂O₃ delayed tumor growth by enhancing the antitumor immune response. The immunologic effect of low-dose As₂O₃ appears to be related to the high sensitivity of Tregs to the drug.

Finally, we showed that Treg depletion induced by As₂O₃ was mediated by the generation of ROS and RNS. Previous studies have observed that As₂O₃ was able to induce the intracellular accumulation of superoxide anion. Disruption of the mitochondrial respiratory chain and membrane NADPH oxidase activation by As₂O₃ have been proposed as sources of superoxide anion (27–29). It has also been reported that As₂O₃ is able to increase cellular production of nitrite oxide (30). As illustrated in Fig. 5C, superoxide dismutases catalyze the dismutation of superoxide in H₂O₂, which is converted in H₂O 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 Tregs, As₂O₃ increases the fluorescence of H₂DCFDA, which could be related to the accumulation of H₂O₂ and/or ONOO⁻. Our data strongly suggest that Treg depletion induced by As₂O₃ is related more to ONOO⁻ production than to H₂O₂ production, because it was inhibited by the NO synthase inhibitor L-NAME and by the superoxide dismutase mimic MnTBAP. Thus, Treg 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-Treg CD4 cells. Differences in the effect of As$_2$O$_3$ on Treg 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 Treg 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 Treg. 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 DAF2-DA (NO) (B) fluorescence in CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells exposed to 1 $\mu$M As$_2$O$_3$ or PBS for 2 h. Spleen cells were incubated for 30 min with either 5 $\mu$M H$_2$DCFDA or 5 $\mu$M DAF2-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 DAF2-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 Treg 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 Treg 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 Treg depletion.
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Disclosures
The authors have no financial conflicts of interest.

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