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_J Immunol_ published online 9 April 2012
http://www.jimmunol.org/content/early/2012/04/09/jimmunol.1103538
Arsenic Trioxide Prevents Murine Sclerodermatous Graft-versus-Host Disease

Niloufar Kavian,*‡,1 Violeta Marut,*1 Amélie Servettaz,*† Hélène Laude,*§ Carole Nicco,* Christiane Chéreau,* Bernard Weill,*† and Frédéric Batteux*†

Chronic graft-versus-host disease (GVHD) follows allogeneic hematopoietic stem cell transplantation. It results from alloreactive processes induced by minor MHC incompatibilities triggered by activated APCs, such as plasmacytoid dendritic cells (pDCs), and leading to the activation of CD4 T cells. Therefore, we tested whether CD4+ and pDCs, activated cells that produce high levels of reactive oxygen species, could be killed by arsenic trioxide (As2O3), a chemotherapeutic drug used in the treatment of acute promyelocytic leukemia. Indeed, As2O3 exerts its cytotoxic effects by inducing a powerful oxidative stress that exceeds the lethal threshold. Sclerodermatous GVHD was induced in BALB/c mice by body irradiation, followed by B10.D2 bone marrow and spleen cell transplantation. Mice were simultaneously treated with daily i.p. injections of As2O3. Transplanted mice displayed severe clinical symptoms, including diarrhea, alopecia, vasculitis, and fibrosis of the skin and visceral organs. The symptoms were dramatically abrogated in mice treated with As2O3. These beneficial effects were mediated through the depletion of glutathione and the overproduction of H2O2 that killed activated CD4+ T cells and pDCs. The dramatic improvement provided by As2O3 in the model of sclerodermatous GVHD that associates fibrosis with immune activation provides a rationale for the evaluation of As2O3 in the management of patients affected by chronic GVHD. The Journal of Immunology, 2012, 188: 000–000.

Chronic GVHD often mimics autoimmune diseases (7). Sclerodermatous-GVHD (Scl-GVHD) makes up 10–15% of cases of chronic GVHD (8). This clinical form of GVHD resembles systemic sclerosis, because it includes fibrotic changes and chronic inflammation of the skin, lung, and gastrointestinal tract. Several animal models have been developed to help define the pathophysiology of chronic GVHD. One of them is based on the transfer of donor immune cells into sublethally irradiated host mice with mismatched minor MHC histocompatibility Ags, resulting in full donor lymphoid chimerism (9). This model recapitulates the clinical features of Scl-GVHD, with fibrosis of the skin and visceral organs 14 d following the graft.

Activated T cells with a high rate of production of reactive oxygen species (ROS) play a pivotal role in the development of Scl-GVHD. Therefore, we investigated whether a cytotoxic molecule that acts by enhancing ROS production beyond a lethal threshold could be of any help in treating chronic GVHD.

Arsenic trioxide (As2O3) is an inorganic trivalent salt that exhibits potent antitumor effects in vitro and in vivo, especially in the treatment of hematological malignancies, such as acute promyelocytic leukemia refractory to all-trans retinoic acid (10). Several reports suggested that As2O3 can affect many cellular functions, such as proliferation, apoptosis, differentiation, and angiogenesis, in various cell lines. An important cellular event occurring after As2O3 treatment is the elevation of intracellular ROS levels (11). This ROS generation appears to be regulated through several pathways, including NADPH oxidase, mitochondrial electron transport chain, and inhibition of antioxidant enzymes (12–14). The ROS-mediated apoptosis triggered by As2O3 could be of any help in treating chronic GVHD.

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servation of the immune system and fibrosis in mice with Scl-GVHD. As$_2$O$_3$ induces apoptosis in allogeneic CD4$^+$ T cells and activated pDCs, thus limiting the development of GVHD reaction in mice.

Materials and Methods

Animals, cells, and chemicals
Specific pathogen-free, 6–week-old female BALB/c and male B10.D2 mice were purchased from Harlan (Gannat, France) and maintained with food and water ad libitum. They were given humane care, according to the guidelines of our institution (Université Paris Descartes). All cells were cultured as previously reported (18). All chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Experimental procedure in Scl-GVHD mice

Induction of GVHD in BALB/c mice. GVHD following bone marrow transplantation (BMT) was induced in BALB/c mice (H-2d; Janvier Laboratory, Le Genest Saint Isle, France) by grafting cells from 7–8-week-old female B10.D2 mice (H-2d; Janvier Laboratory), as previously described by Jaffe and Claman (19). Briefly, recipient mice were lethally irradiated with 750 cGy from a Gammacel $^{137}$Cs source. Three hours later, they were injected i.v. with donor spleen cells ($2 \times 10^7$/mouse) and bone marrow cells ($1 \times 10^7$/mouse) suspended in RPMI 1640. A control group of BALB/c recipient mice received BALB/c spleen and bone marrow cells (syngeneic BMT, referred to as control animals). Transplanted animals were maintained in sterile microisolator cages (Lab Products, Langen- selbold, Germany) and supplied with autoclaved food and sterile water. Animals were sacrificed by cervical dislocation 4 wk after BMT.

Treatment of Scl-GVHD mice with As$_2$O$_3$. Scl-GVHD and control mice were randomized and treated for 3 wk with i.p. injections of either As$_2$O$_3$ or vehicle alone beginning on day 7 post-BMT (10 mice/group). A stock solution was prepared extemporaneously, as described above. As$_2$O$_3$ was given 5 dwk at a dose of 5 mg/g body weight, as described by Bobé et al. (17). Control mice received i.p. injections of PBS 5 dwk. Four weeks after BMT, the animals were sacrificed by cervical dislocation.

Assessment of collagen accumulation
Skin thickness. Skin thickness of the shaved back of mice was measured 1 d before sacrifice with a caliper and expressed in millimeters.

Histopathological analysis. Fixed lung and skin pieces were embedded in paraffin. A 5-μm-thick section was stained with H&E or Picrosirius Red. Slides were examined by standard bright-field microscopy (Olympus BX60, Rungis, France) by a pathologist who was blinded to the assignment of the animal group.

Collagen content in skin and lung. Skin and lung pieces were diced using a scalpel, put into tubes, thawed, and mixed with pepsin (1:10 weight ratio) and 0.5 M acetic acid overnight at room temperature under stirring. The assay of collagen content was based on the quantitative dye-binding Sircol method (Biosciel, Belfast, Ireland).

Disease severity score
To determine the incidence and severity of disease, we assigned a score to each mouse using the following criteria: 0; no external sign; 1; piloeruption on back and underside, 1; hunched posture or lethargy; 1; weight loss >10%; 0.5; alopecia <1 cm$^2$; 1; alopecia >1 cm$^2$; 1; vasculitis (one or more purpural lesions); and 1; eyelid swelling (blepharophymosia). The severity score is the sum of these values and ranges from 0 (unaffected) to a maximum of 6. The incidence and severity score was recorded every week by two blinded scientists.

Flow cytometric analysis of spleen cell subsets
Cell suspensions from spleens were prepared after hypotonic lysis of erythrocytes in potassium acetate solution. Cells were incubated with the appropriate labeled Ab at 4˚C for 30 min in PBS with 0.1% sodium azide and 4 mM NAC for 24 h. Cells were then washed and labeled with anti-CD4 mAb. Levels of anti-DNA topoisomerase 1 IgG Abs were detected by ELISA on microtiter plates (ImmuoVision, Springdale, AR). A 1:50 serum dilution was used for the ELISA.

Effects of As$_2$O$_3$ on B10.D2 CD4 T cells in vitro
Suspensions of spleen cells from a male B10.D2 mouse and a female BALB/c mouse were prepared after hypotonic lysis of erythrocytes. The BALB/c cell suspension was irradiated at 30 Gy. A B10.D2 cell suspension was also prepared and irradiated at 30 Gy for syngeneic control.

B10.D2 cells were labeled with PKH26 dye, according to the manufacturer’s instructions (Sigma-Aldrich).

Measurement of H$_2$O$_2$ concentration in C4D T cells. B10.D2 cells labeled with PKH26 were incubated with irradiated BALB/c cells and complete medium for 24 h. After this incubation period, cells were washed without serum and incubated with 5 μM CM-H$_2$DCFDA (Sigma-Aldrich) at 37˚C for 20 min. After washing, cells were incubated with 10 μM As$_2$O$_3$, with or without 4 mM N-acetylcycteine (NAC), for 5 h at 37˚C. Cells were then washed and labeled with anti-CD4 mAb.

Preparation of Scl-GVHD mice for in vitro experiments. Scl-GVHD mice were prepared after hypotonic lysis of erythrocytes. The BALB/c cell suspension was irradiated at 30 Gy. A B10.D2 cell suspension was also prepared and irradiated at 30 Gy for syngeneic control.

B10.D2 cells were labeled with PKH26 dye, according to the manufacturer’s instructions (Sigma-Aldrich).

Measurement of glutathione concentration in C4D T cells. B10.D2 cells labeled with PKH26 were incubated with or without irradiated BALB/c cells and complete medium, with or without 10 μM As$_2$O$_3$ and with or without 4 mM NAC for 24 h. Cells were washed and labeled with 100 μM monochlorobimane at 37˚C for 20 min, followed by labeling with anti-CD4 mAb.

Determination of apoptosis in C4D T cells. B10.D2 cells labeled with PKH26 were incubated with or without irradiated BALB/c spleen cells and complete medium alone, with 10 μM As$_2$O$_3$ alone, or with 4 mM NAC for 24 h. Cells were then washed and stained with anti-CD4 mAb (eBio-science) at 4˚C for 20 min and Yopro-1 (Sigma-Aldrich) at room temperature for 10 min.

Effects of As$_2$O$_3$ on B10.D2 pDCs in vitro
A suspension of spleen cells from a male B10.D2 mouse was prepared after hypotonic lysis of erythrocytes.

Measurement of H$_2$O$_2$ concentration in pDCs. B10.D2 cells were incubated in complete medium, washed without serum, and incubated with 5 μM CM-H$_2$DCFDA (Sigma-Aldrich) at 37˚C for 20 min. After washing, cells were incubated with 10 μM As$_2$O$_3$, with or without 4 mM NAC for 24 h. Cells were then washed and stained with anti-CD11c mAb (eBio-science) at 4˚C for 20 min and Yopro-1 (Sigma-Aldrich) at room temperature for 10 min.

For all flow cytometry analyses, pDCs were defined as B220$^+$CD11c$^+$CD11b$^-$low. Data were acquired on a FACSCanto II flow cytometer (BD Bioscienes) and analyzed with FlowJo software (Tree Star).

Measurement of in vitro IFN-α production by pDCs
pDCs were isolated from the spleen of a BALB/c mouse using the pDC isolation kit and MS columns (Milltenyi Biotec), pDCs were then coated in six-well plates and incubated with 10 μg/ml Gardiquimod (InvivoGen, Toulouse, France) and increasing doses of As$_2$O$_3$ (from 0 to 25 μM) with medium alone for 24 h. Supernatants were harvested, and IFN-α was assayed as described by Dubois et al. (20). Briefly, L929 cells (5 × 10$^4$/well) were seeded kindly provided by P. Lebon, Laboratoire de Virologie, Hôpital...
Cochin, Paris, France) were coated in 96-well plates and cultured in RPMI 1640 at 37°C with 5% CO2 until confluence. Culture media were discarded, and plates were incubated for an additional 24 h with 50 μl 2-fold serial dilutions (from 1:2 to 1:256 in RPMI 1640) of supernatant samples or with serial dilutions of a standard solution of murine IFN-α. Thereafter, vesicular stomatitis virus was added; the final concentration was 1:400 of a stock solution previously shown to cause complete lysis of L929 cells at a dilution of 1:2000. We considered the dilution that destroyed half of the cell layer at 24 h, and IFN-α units were determined by comparison with cells incubated with 2-fold serial dilutions of mouse IFN-α (kindly provided by P. Lebon).

Statistical analysis

All of the quantitative data are expressed as mean ± SEM and were analyzed with Prism 5 (GraphPad), using one-way ANOVA or the Student t test, as appropriate. A p value <0.05 was considered statistically significant.

Results

As₂O₃ prevented clinical symptoms of systemic sclerosis induced by GVHD

We investigated the effects of As₂O₃ on the development of Scl-GVHD, a fibrotic variant of GVHD. As₂O₃ was administered daily from day 7 following BMT, and mice were sacrificed 21 d later. Lethally irradiated BALB/c mice transplanted with bone marrow and spleen cells from B10.D2 mice developed skin fibrosis, as shown by the measurement of ear skin thickening in Fig. 1A. Control BALB/c animals with syngeneic grafts did not develop skin fibrosis or GVHD (Fig. 2). In addition to skin thickening, the engrafted animals displayed alopecia (100% of mice), vasculitis (>80% of mice), and diarrhea (100% of mice). On day 21, the disease severity score of Scl-GVHD mice was ≥5, whereas that of control animals (syngeneic graft) was 0 (Fig. 2B). As₂O₃ effectively prevented severe GVHD; the mean severity score of treated mice at day 14 was 2.5 ± 0.2 versus 5 ± 0.4 in untreated Scl-GVHD mice (p < 0.001, Fig. 2B). Scl-GVHD mice treated with As₂O₃ displayed a reduction in skin thickness >40% compared with untreated Scl-GVHD mice (p = 0.007, Fig. 1B). Moreover, type 1 collagen content in the skin of Scl-GVHD mice treated with As₂O₃ was 45% lower than in untreated Scl-GVHD mice (p = 0.002, Fig. 1C). Picrosirius Red staining of ear sections showed important collagen deposits in the ears from Scl-GVHD mice but not in those of Scl-GVHD mice treated with As₂O₃ (Fig. 1A). Also, type 1 collagen concentration was higher in the lungs of Scl-GVHD mice than in Scl-GVHD mice treated with As₂O₃ (data not shown). Altogether, these results show that As₂O₃ limits the deposition of collagen and prevents the development of alopecia, vasculitis, and diarrhea in Scl-GVHD mice.

As₂O₃ altered spleen cell subsets in Scl-GVHD mice

GVHD is caused by a donor T cell antihost reaction. We investigated whether the clinical improvement observed in Scl-GVHD mice treated with As₂O₃ correlated with quantitative or qualitative alterations in spleen cell subsets. Flow cytometric analysis of splenocytes showed that As₂O₃ decreased the percentage of CD4⁺ T cells (p = 0.049 versus untreated Scl-GVHD mice). This reduction involved the CD4⁺CD62L⁺ effector memory CD4⁺ subset, because the ratio of CD4⁺CD44highCD62Llow/CD4⁺CD44lowCD62Lhigh cells was 15.7 ± 4.6 in Scl-GVHD mice versus 3.6 ± 0.7 in Scl-GVHD mice treated with arsenic (p = 0.001, Fig. 3A–C). Furthermore, the percentage of splenic pDCs, defined as B220⁺CD11c⁺CD11blow was three times lower in arsenic-treated Scl-GVHD mice compared with untreated Scl-GVHD mice (p = 0.021, Fig. 3D).

As₂O₃ modified the splenic production of cytokines and the serum levels of autoantibodies in Scl-GVHD mice

In addition, we explored the splenic production of IL-4 and IL-17, two cytokines implicated in the development of GVHD in mice (21). Scl-GVHD mice produced more IL-4 and IL-17 than did control mice (IL-4: 0.24 ± 0.023 for Scl-GVHD mice and 0.12 ± 0.021 for control mice, p = 0.011; IL-17: 0.64 ± 0.083 for Scl-GVHD mice and 0.37 ± 0.088 for control mice, p = 0.042; Fig. 3E, 3F). As₂O₃ significantly reduced the production of the two cytokines (p = 0.043 and p = 0.022 for IL-4 and IL-17, respectively, versus nontreated mice, Fig. 3E, 3F). We then investigated...
the presence of anti-DNA topoisomerase 1 Abs, a hallmark of the Scl-GVHD model, which are generally detected 3–9 wk following the onset of disease (9). On the day of sacrifice, 80% of Scl-GVHD mice and only 20% of Scl-GVHD mice treated with As2O3 were positive for anti–DNA-topoisomerase 1 Abs ($p = 0.048$, Fig. 3G).

$As_2O_3$ triggered apoptosis of activated B10.D2 CD4+ T cells by enhancing ROS production

In vitro, ROS production was higher in B10.D2 CD4+ T cells stimulated with BALB/c splenocytes than in B10.D2 CD4+ T cells stimulated with B10.D2 splenocytes (syngeneic controls) (mean fluorescence intensity [MFI] = 1721 ± 40 versus 1186 ± 92, $p = 0.032$). Treatment of stimulated B10.D2 CD4+ T cells with $As_2O_3$ further increased their production of H2O2 that reached an MFI of 2172 ± 103 ($p = 0.031$ versus stimulated B10.D2 CD4+ T cells without $As_2O_3$, Fig. 4A). The enhancement of ROS production was abrogated by incubation with 4 mM NAC (MFI: 1636 ± 87, $p = 0.021$ versus $As_2O_3$ alone, Fig. 4A). The level of glutathione (GSH) in B10.D2 CD4+ T cells was in accordance with those results. Syngeneic stimulated cells displayed elevated levels of GSH (mean of 45 ± 2% of GSH+ cells), whereas allogeneic stimulated cells had a slight decrease in their GSH content (mean 31 ± 2.5% of positive cells) ($p = 0.041$, Fig. 4B). Incubation with 10 μM $As_2O_3$ dramatically decreased the GSH content in stimulated B10.D2 CD4+ T cells (Fig. 4B). In addition, Yopro-1 staining of activated B10.D2 CD4+ T cells indicated that arsenic dramatically triggered apoptosis in those cells. Apoptosis correlated with ROS production measured by flow cytometry and was downregulated by incubation with 4 mM NAC ($p = 0.0009$, Fig. 4C).

**FIGURE 2.** $As_2O_3$ improves the clinical symptoms of Scl-GVHD in mice. GVHD was induced by allogeneic transplant of B10.D2 bone marrow and spleen cells into irradiated BALB/c mice. Control mice received syngeneic transplants. $As_2O_3$ was administered from day 7 post-BMT at a dose of 5 μg/g. Mice were sacrificed on day 28. Data from two independent experiments were pooled. (A) Representative photographs of mice on day 28 post-BMT ($n = 10/group$). (B) Disease severity scores (mean ± SEM).

**FIGURE 3.** Treatment of Scl-GVHD mice with $As_2O_3$ altered the balance between memory and naive CD4+ T cells and decreased numbers of splenic pDCs. Mice were treated with $As_2O_3$ from day 7 post-BMT at a dose of 5 μg/g ($n = 10/group$). Spleen cells were harvested on day 28 post-BMT. (A) Numbers of CD4+ naive T cells in untreated and treated Scl-GVHD mice. (B) Numbers of CD4+ effector memory T cells. (C) Effector memory/naive CD4+ T cell ratio. (D) Numbers of splenic pDCs in untreated and treated Scl-GVHD mice. (E) Numbers of splenic cDCs in untreated and treated Scl-GVHD mice. (F) Percentages of CD86+ pDCs in untreated and treated Scl-GVHD mice. Values are mean ± SEM of data from all mice in the experimental or control groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired Mann–Whitney U test.
As$_2$O$_3$ also triggered apoptosis of pDCs by enhancing H$_2$O$_2$ production

We observed the same effects on ROS production and apoptosis in B10.D2 pDCs incubated with 10 µM As$_2$O$_3$. Indeed, the basal detection MFI of CM-H$_2$DCFDA by flow cytometry was 5405 ± 12 for B10.D2 pDCs and 6527 ± 25 when treated with As$_2$O$_3$. Adding NAC reduced H$_2$O$_2$ production by pDCs, as shown by the CM-H$_2$DCFDA MFI of 3675 ± 15 (Fig. 5). Monochlorobimane staining was also reduced in pDCs treated with arsenic compared with untreated pDCs (MFI = 707 ± 10 versus 1583 ± 80), whereas coaddition of NAC and As$_2$O$_3$ increased the intracellular content of GSH (MFI = 1541 ± 8). The effects of As$_2$O$_3$ were also studied on conventional DCs (cDCs). There was a tendency toward an increase in H$_2$O$_2$ production and a decrease in GSH content in cDCs upon treatment with arsenic, but those results did not reach significance (for H$_2$O$_2$ levels: MFI = 1,118 ± 25 when treated with As$_2$O$_3$, 86 for cDCs + As$_2$O$_3$ versus 1,325 ± 76 for cDCs + As$_2$O$_3$ + NAC; p = 0.08 for cDCs versus cDCs + As$_2$O$_3$; p = 0.09 for cDCs + As$_2$O$_3$ + NAC versus cDCs + As$_2$O$_3$; for GSH levels: MFI = 24,308 ± 395 for cDCs alone, 21,719 ± 384 for cDCs + As$_2$O$_3$, 23,000 ± 376 for cDCs + As$_2$O$_3$ + NAC; p = 0.089 for cDCs versus cDCs + As$_2$O$_3$, p = 0.10 for cDCs + As$_2$O$_3$ + NAC versus cDCs + As$_2$O$_3$).

As$_2$O$_3$ blocked IFN-α production of B10.D2 pDCs

To assess the specificity of As$_2$O$_3$ against pDCs, we investigated its effect on the production of IFN-α by splenic pDCs, with and without activation by the TLR7 agonist Gardiquimod. Fig. 6 shows that, in the absence of stimulation of pDCs, As$_2$O$_3$ has a significant effect on the production of IFN-α only at the highest concentrations tested (10 and 25 µM). In contrast, after stimulation of TLR7 and incubation with 10 and 25 µM As$_2$O$_3$, the levels of IFN-α in cell supernatants were strongly decreased (p = 0.002 for 10 µM As$_2$O$_3$). Incubation with 5 µM As$_2$O$_3$ decreased the concentration of IFN-α in the supernatants >2-fold, whereas lower doses of As$_2$O$_3$ had no effect on IFN-α production (Fig. 7).

Discussion

This study shows that As$_2$O$_3$ selectively deletes activated CD4$^+$ T cells and pDCs that have low levels of GSH and overproduce H$_2$O$_2$ and, thus, ameliorates Scl-GVHD in mice.

We tested the effects of As$_2$O$_3$, a chemotherapeutic drug used in hematological malignancies, on the development of Scl-GVHD in mice. This model of chronic GVHD shares typical features with systemic sclerosis, including skin and visceral fibrosis and autoimmune manifestations (22). As$_2$O$_3$ dramatically improved the clinical outcome of sublethally irradiated BALB/c mice transplanted with B10.D2 hematopoietic cells; weight loss, fibrosis, vasculitis, and alopecia were markedly reduced in treated versus untreated mice.

The percentages of effector memory CD4$^+$ T cells (CD4$^+$ CD44$^{high}$CD62L$^{low}$) decreased in mice with Scl-GVHD that were treated with arsenic. The pathophysiology of chronic GVHD remains poorly understood, although a large amount of evidence suggests that, in contrast to acute GVHD, which is dependent on CD8$^+$ T cells, the manifestations observed in chronic GVHD are dependent on the activation of minor histocompatibility Ag-specific donor CD4$^+$ T cells (4, 23–26). After transplantation of B10.D2 lymphoid cells into irradiated BALB/c mice, naive donor CD4$^+$ T cells initiate the disease. As a result, donor CD4$^+$ T cells infiltrate the skin, recruit macrophages and monocytes, and induce fibrosis and destructive changes. Because activated CD4$^+$ T cells play a pivotal role in the induction of the disease and are decreased by in vivo treatment with arsenic, we investigated the mechanism of action of arsenic on those cells. We show that B10.D2 CD4$^+$ T cells stimulated with irradiated BALB/c spleen cells display lower GSH contents and produce higher levels of H$_2$O$_2$ than do unstimulated CD4$^+$ T cells. These results are in agreement with previous studies showing that, upon activation, T cells overproduce ROS (23, 24). Then, we showed that the high levels of ROS production by activated CD4$^+$ T cells make them hypersensitive to arsenic-induced apoptosis. Indeed, in vitro treatment with arsenic induced an important decrease in GSH content and a subsequent increase in H$_2$O$_2$ levels beyond a lethal threshold, inducing cell apoptosis. These data confirm the role of the oxidant/antioxidant balance as a crucial factor that determines cell susceptibility to arsenic (25). Several studies demonstrated that GSH can bind arsenic from attacking its target by formation of a transient As(GS)$_3$ complex and that GSH depletion in acute promyelocytic leukemia cells synergizes with As$_2$O$_3$ in the induction of apoptosis (27).

We next investigated whether an alteration in the profile of cytokine production by splenocytes from mice with Scl-GVHD and treated with As$_2$O$_3$ could reflect changes in splenic T cell populations. Splenic IL-17 production was lower in arsenic-treated Scl-GVHD mice than in untreated mice. These data are consistent with a large number of recent studies conducted in mice that concluded that Th17 cells are implicated in GVHD development.

FIGURE 4. In vivo treatment with As$_2$O$_3$ reduced the production of IL-4, IL-17, and autoantibodies in Scl-GVHD mice. Mice were treated with As$_2$O$_3$ from day 7 post-BMT at a dose of 5 µg/g (n = 10/group). Spleen cells were harvested on day 28 post-BMT, and CD4 T cells were purified as described in Materials and Methods. (A) IL-4 secretion measured in supernatants of CD4 T cells by ELISA (ng/ml). (B) IL-17 secretion measured in supernatants of CD4 T cells by ELISA (ng/ml). (C) Anti-DNA-topoisomerase 1 autoantibody concentrations in the sera (A.U.). Values are mean ± SEM of data from all mice in the experimental or control groups. *p < 0.05, paired Mann–Whitney U test.
in mice. Among them, a study reported that amplification of IL-17 production by the use of the stem cell mobilization factor G-CSF leads to a cutaneous fibrosis occurring late after the graft, as in Scl-GVHD (21). Consistent with those data, another recent article stated that the use of an anti–IL-17 mAb can ameliorate skin symptoms in chronic GVHD (20, 26). Moreover, Nishimori et al. (28) recently showed the beneficial effects of the synthetic retinoid Am80, which belongs to the same family as all-trans retinoic acid, on chronic GVHD. These effects are mediated through the downregulation of Th17 cells. Because other synthetic retinoids, such as N-(4-hydroxyphenyl)retinamide, can induce apoptosis through increased production of ROS, it is possible that Am80 also acts on Th17 cells through the induction of ROS, because As$_2$O$_3$ does in our model (29).

**FIGURE 5.** As$_2$O$_3$ induced apoptosis of activated B10.D2 CD4$^+$ T cells in culture through ROS production. B10D2 spleen cells were incubated with irradiated B10.D2 splenocytes (↓B10, syngeneic control) or BALB splenocytes (↓BALB) and treated or not with 10 μM As$_2$O$_3$ with or without NAC. Flow cytometry analysis was gated on CD4$^+$ T cells. Results are representative of four experiments carried out in duplicates. Data were analyzed with FlowJo software. (A) Increase in H$_2$O$_2$ generation by As$_2$O$_3$, measured by flow cytometry using CM-H$_2$DCFDA. (B) GSH content in B10.D2 CD4$^+$ T cells, measured by flow cytometry using monochlorobimane staining. (C) Induction of apoptosis by As$_2$O$_3$, measured by flow cytometry using Yopro staining. Mean values were compared using paired Mann–Whitney U tests.
The decrease in splenic CD4+ effector memory cells in Scl-GVHD mice treated with As$_2$O$_3$ also correlates with a reduction in the Th2 cytokine IL-4 produced in vitro by activated splenocytes. Following the graft of B10.D2 spleen and bone marrow cells into sublethally irradiated BALB/c mice, Zhou et al. (30) observed an increase in the expression of type 2 cytokines in the skin of Scl-GVHD mice compared with syngeneic grafts. Moreover, in other chronic GVHD models, type 2 polarized immune responses are required for the induction of skin GVHD in mice and the development of fibrosis in the skin and visceral organs (31). Thus, the decreased production of IL-4 observed in our study probably contributes to the improvement of skin fibrosis.

As a whole, the alterations in splenic production of cytokines in our model are consecutive to a reduced immune activation after treatment with arsenic and, thus, contribute to the amelioration of Scl-GVHD symptoms.

Chronic GVHD is associated with other autoimmune manifestations, such as the production of autoantibodies in relation to the production of Th2 cytokines. As described by others, we observed the production of anti–DNA-topoisomerase 1 Abs in mice with Scl-GVHD. The levels of these autoantibodies were decreased by As$_2$O$_3$ in our model. Similar effects of As$_2$O$_3$ were reported in a lupus mouse model (MRL/lpr mice), with a decrease in the production of autoantibodies (anti-dsDNA and rheumatoid factors) (17). Consistent with these data, we conclude that As$_2$O$_3$

**FIGURE 6.** Effects of in vitro treatment with As$_2$O$_3$ on B10.D2 pDCs. B10.D2 spleen cells were incubated with 10 μM As$_2$O$_3$ with or without NAC for 24 h. Flow cytometry analysis was gated on pDCs subsets defined as B220$^+$CD11c$^{int}$CD11b$^{low}$. Results are representative of four experiments carried out in duplicates. Data were analyzed with FlowJo software. (A) Induction of ROS formation by As$_2$O$_3$, measured by flow cytometry using CM-H$_2$DCFDA. (B) Arsenic induces a decrease in the GSH content in pDCs, measured by flow cytometry using monochlorobimane staining. (C) Induction of apoptosis in pDCs by As$_2$O$_3$, measured by flow cytometry using Yopro staining.

**FIGURE 7.** As$_2$O$_3$ targets pDCs and blocks IFN-α production by those cells. pDCs were seeded in six-well plates and coincubated with 10 μg/ml Gardiquimod and/or increasing doses (0–25 μM) of As$_2$O$_3$. *p < 0.05.
 sachet. In accordance with our results, they reported a cytotoxic effect of As2O3. In our hands, the pDC subset was decreased in treated mice indicative of GVHD, emphasizing the differential functions of pDCs in the state of maturation of pDCs seems to be crucial in their ability to trigger GVHD. On the other hand, Banovic et al. (34) showed that precursors of pDCs, but not mature pDCs, can attenuate the symptoms of GVHD, emphasizing the differential functions of pDCs depending on the environment and the model of GVHD. In our hands, the pDC subset was decreased in treated mice compared with untreated mice. As observed for CD4+ T cells, the increased levels of H2O2 production, along with the decreased GSH content, render pDCs sensitive to As2O3-induced apoptosis. The action of arsenic on pDCs was confirmed in vitro by the decrease in IFN-α production following stimulation of pDCs by a TLR-7 agonist. This selective effect of arsenic on pDCs could also lead to interesting insights about IFN-α-related diseases, such as systemic lupus erythematosus. Indeed, Bobé et al. (17) showed the beneficial effects of As2O3 in MRL/lpr lupus-prone mice. In accordance with our results, they reported a cytotoxic effect of As2O3 on activated CD4+ T cells through the reduction in GSH levels, but the beneficial effects observed in their model could also be mediated through regulation of IFN-α production by pDCs. In summary, our work highlights the beneficial effects of As2O3 in chronic GVHD. As2O3 could be a therapeutic tool in hematologic and solid malignancies, as well as in chronic GVHD (10).

Acknowledgments

We thank P. Lebon for kindly providing L229 cells and IFN-α standard solution and A. Colle for typing the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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