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Arsenic Trioxide Prevents Murine Sclerodermatous Graft-versus-Host Disease

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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: 5142–5149.

C hronic graft-versus-host disease (GVHD) is a major factor of morbidity following allogeneic hematopoietic stem cell transplantation, with variable clinical presentations (1, 2). Chronic GVHD emerges from alloreactive processes between donor-derived immune cells and host cell populations induced by minor MHC incompatibilities between donor and recipient. Its pathophysiology is poorly understood in contrast to that of acute GVHD (1, 3). Donor CD4+ T cells are involved in the induction of chronic GVHD, but the effector mechanisms through which they mediate tissue inflammation are unclear; the recognition of MHC class II alloantigens on host dendritic cells (DCs) is sufficient to prime donor CD4+ T cells and induce GVHD (3–5). Moreover, among APCs, plasmacytoid DCs (pDCs) were shown to be pathogenic in GVHD. In the absence of other APCs, pDCs alone can stimulate donor T cells to trigger GVHD, and total-body irradiation is crucial for their maturation and the subsequent priming of alloreactive CD4+ T cells (6).

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 can impact hematological tumor cells; under certain circumstances, it can also affect nontumor cells, such as keratinocytes, fibroblasts, or activated autoimmune lymphocytes (15–17).

In this study, we tested the therapeutic effects of As2O3 in a murine model of Scl-GVHD generated by grafting B10.D2 (H-2b) bone marrow and spleen cells into sublethally irradiated BALB/c mice (H-2d). We show that As2O3 limits both the acti-

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Abbreviations used in this article: As2O3, arsenic trioxide; BMT, bone marrow transplantation; cDC, conventional dendritic cell; mDC, dendritic cell; GSH, glutathione; GVHD, graft-versus-host disease; MFI, mean fluorescence intensity; NAC, N-acetylcysteine; pDC, plasmacytoid dendritic cell; ROS, reactive oxygen species; Scl-GVHD, sclerodermatous graft-versus-host disease.
vation of the immune system and fibrosis in mice with Scl-GVHD. As$_2$O$_3$ induces apoptosis in alloreactive 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-2$d$; Janvier Laboratory, Le Genest Saint Isle, France) by grafting cells from 7–8-week-old female B10.D2 mice (H-2$d$; Janvier Laboratory), as previously described by Jaffée 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 d/wk 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 d/wk. 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 tissue 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 (Bioscor, 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: piloerection 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 sclerosis (blepharophymosis). 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 2% FCS. The mAbs used in this study were anti-B220-PE, anti-CD11b-biotin, anti-CD11c-PE-FITC, anti-CD4-PerCP, and anti-CD8-PE-Cy7 (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Determination of IL-4 and IL-17 production by splenocytes

Spleen cells were isolated by gentle disruption of the tissues, and the erythrocytes were lysed by hypotonic shock in potassium acetate solution. Spleen cells were cultured in RPMI 1640 supplemented with antibiotics, GluMax (Invitrogen Life Technologies), and 10% heat-inactivated FCS (Invitrogen Life Technologies) (complete medium). CD4 T cells were isolated from spleen cell suspensions by positive selection using CD4 microbeads and LS columns (Millenyi Biotec, Paris, France), according to the manufacturer’s instructions. CD4 T cell suspensions were then seeded in 96-well flat-bottom plates and cultured (2 $\times$ 10$^5$ cells) in complete medium for 48 h in the presence of 5 μg/ml Con A. Supernatants were collected, and cytokine concentrations were determined by ELISA (for IL-4: eBioscience, San Diego, CA; for IL-17: DuoSet; R&D Systems). Results are expressed in ng/ml.

Assays of serum anti-DNA topoisomerase 1 autoantibodies

Levels of anti-DNA topoisomerase 1 IgG Abs were detected by ELISA on microtiter plates (ImmunoVision, Springfield, 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 mouse spleen 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 CD4 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 supernatant, 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-acyctelysine (NAC), for 5 h at 37˚C. Cells were then washed and labeled with anti-CD4 mAb.

Measurement of glutathione concentration in CD4 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 for 20 min.

Determination of apoptosis in CD4 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 Yopor-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 labeled with anti-CD4 mAb. For all flow cytometry analyses, pDCs were defined as B220$^+$CD11c$^{hi}$, and anti-CD11c mAb (BD, Le Pont de Claix, France).

Measurement of glutathione concentration in pDCs. B10.D2 cells were incubated with complete medium with or without 10 μM As$_2$O$_3$, and with or without 4 mM NAC for 24 h. Cells were then washed and stained with anti-CD11b and anti-CD11c mAb (BD, Le Pont de Claix, France).

For all flow cytometry analyses, pDCs were defined as B220$^+$CD11c$^{hi}$, and anti-CD11c mAb (BD, Le Pont de Claix, France).

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 (Millenyi Biotec). pDCs were then cultured 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 or without medium alone for 24 h. Supernatants were harvested, and IFN-α was assayed as described by Dubois et al. (20). Briefly, L929 cells (5 $\times$ 10$^7$ well) kindly provided by P. Lebon, Laboratoire de Virologie, Hôpital
As$_2$O$_3$ displayed a reduction in skin thickness in GVHD mice (kindly provided by P. Lebon).

Type 1 collagen content in the skin of Scl-GVHD mice treated with As$_2$O$_3$ 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 As$_2$O$_3$ (Fig. 1A). Also, type 1 collagen concentration was higher in the lungs of Scl-GVHD mice than in Scl-GVHD mice treated with As$_2$O$_3$ (data not shown). Altogether, these results show that As$_2$O$_3$ limits the deposition of collagen and prevents the development of alopecia, vasculitis, and diarrhea in Scl-GVHD mice.

As$_2$O$_3$ altered spleen cell subsets in Scl-GVHD mice

GVHD is caused by a donor T cell antithost reaction. We investigated whether the clinical improvement observed in Scl-GVHD mice treated with As$_2$O$_3$ correlated with quantitative or qualitative alterations in spleen cell subsets.

Flow cytometric analysis of splenocytes showed that As$_2$O$_3$ decreased the percentage of CD4$^+$ T cells ($p = 0.049$ versus untreated Scl-GVHD mice). This reduction involved the CD4$^+$CD62L$^-$ effector memory CD4$^+$ T cell subset, because the ratio of CD4$^+$CD44$^{hi}$CD62L$^{lo}$/CD4$^+$CD44$^{lo}$CD62L$^{hi}$ cells was $15.7 \pm 4.6$ in Scl-GVHD mice versus $3.6 \pm 0.7$ in Scl-GVHD mice treated with arsenic ($p = 0.001$, Fig. 3A–C). Furthermore, the percentage of splenic pDCs, defined as B220$^+$CD11c$^+$CD11b$^{lo}$, was three times lower in arsenic-treated Scl-GVHD mice compared with untreated Scl-GVHD mice ($p = 0.021$, Fig. 3D).

As$_2$O$_3$ 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: $0.24 \pm 0.023$ for Scl-GVHD mice and $0.12 \pm 0.021$ for control mice, $p = 0.011$; IL-17: $0.64 \pm 0.083$ for Scl-GVHD mice and $0.37 \pm 0.088$ for control mice, $p = 0.042$; Fig. 3E, 3F). As$_2$O$_3$ 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 As$_2$O$_3$ were positive for anti–DNA-topoisomerase 1 Abs ($p = 0.048$, Fig. 3G).

As$_2$O$_3$ 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 ± 640 versus 1186 ± 92, $p = 0.032$). Treatment of stimulated B10.D2 CD4$^+$ T cells with As$_2$O$_3$ further increased their production of H$_2$O$_2$ that reached an MFI of 2172 ± 103 ($p = 0.031$ versus stimulated B10.D2 CD4$^+$ T cells without As$_2$O$_3$, Fig. 4A). The enhancement of ROS production was abrogated by incubation with 4 mM NAC (MFI: 1636 ± 87, $p = 0.021$ versus As$_2$O$_3$ 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$_2$O$_3$ 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).
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 ± 39 for cDCs alone, 1,445 ± 86 for cDCs + As$_2$O$_3$, 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 auto-immune 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.
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 As2O3 does in our model (29).

**FIGURE 5.** As2O3 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 As2O3 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 H2O2 generation by As2O3, measured by flow cytometry using CM-H2DCFDA. (B) GSH content in B10.D2 CD4 T cells, measured by flow cytometry using monochlorobimane staining. (C) Induction of apoptosis by As2O3, 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 As2O3 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 As2O3 in our model. Similar effects of As2O3 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 As2O3...
prevents the development of an autoimmune reaction in ScI-
GVHD mice by specifically targeting CD4+ T cells.

In contrast, the role of APCs in chronic GVHD was recently emphasized by the observation that costimulation of donor T cells through CD80 or CD86 on either host or donor APCs is necessary to induce chronic GVHD (32). pDCs are especially involved in the physiopathology of GVHD because the adoptive transfer of mature pDCs exacerbates GVHD, and they can stimulate donor T cells to trigger GVHD in the absence of other APCs (6, 33). Yet, the state of maturation of pDCs seems to be crucial in their ability to trigger GVHD. On the one hand, total-body irradiation induces inflammation and maturation of pDCs, which can subsequently activate T cells (6). 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 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).

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