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Arsenic trioxide, a novel promising therapeutic agent for lymphoproliferative and autoimmune syndromes in MRL/lpr mice

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Abbreviations used: As$_2$O$_3$, Arsenic trioxide; DN cells, double-negative (CD4–CD8–) cells; FasL, Fas ligand; GSH, reduced glutathione; RF, rheumatoid factor; SLE, systemic lupus erythematosus

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Abstract

MRL/lpr mice develop a human-lupus-like syndrome and, as in autoimmune lymphoproliferative syndrome (ALPS), massive lymphoproliferation due to inactivation of Fas-mediated apoptosis. Presently, no effective therapy exists for ALPS, and long term, therapies for lupus are hazardous. We show herein that arsenic trioxide (As$_2$O$_3$) is able to achieve quasi-total regression of antibody- and cell-mediated manifestations in MRL/lpr mice. As$_2$O$_3$ activated caspases, and eliminated the activated T lymphocytes responsible for lymphoproliferation, and skin, lung and kidney lesions, leading to significantly prolonged survival rates. This treatment also markedly reduced anti-DNA autoantibody, rheumatoid factor, IL-18, IFN-γ, nitric oxide metabolite, tumor necrosis factor-α, Fas-ligand and IL-10 levels, and immune-complex deposits in glomeruli. As$_2$O$_3$ restored cellular reduced glutathione levels, thereby limiting the toxic effect of nitric oxide, which is overproduced in MRL/lpr mice. Furthermore, As$_2$O$_3$ protected young animals against developing the syndrome and induced almost total disease disappearance in older affected mice, thereby demonstrating that it is a novel promising therapeutic agent for autoimmune diseases.
Introduction

The pathogenesis of systemic lupus erythematosus (SLE) is multifactorial and polygenic. The apoptosis gene *Fas* is a candidate contributory gene in both human SLE and murine models of it. In humans, *FAS* mutations result in a familial autoimmune lymphoproliferative syndrome (ALPS), characterized by non-malignant accumulation of an unusual population of double-negative (DN) B220^+^CD4^−^CD8^−^α/β^+^ T lymphocytes and development of autoimmune diseases. Overexpression of Th2 cytokine IL-10 is associated with the manifestations of ALPS. In mice with the MRL genetic background, the autosomal recessive gene *lpr* (*Tnfrsf6*<sup>lpr</sup>) is responsible for a syndrome characterized by the progressive accumulation of DN T-cell population in peripheral lymphoid tissues. The *lpr* mutation engenders much less expression of the Fas death receptor, which results in the accumulation, in lymph node (LN) and spleen, of numerous activated DN T lymphocytes that are not normally regulated by the Fas-mediated mechanism controlling apoptosis of mature T cells. Therefore, MRL/*lpr* T and B cells massively overexpress Fas ligand (FasL), which renders them able to kill Fas^+^ cells *in vitro* and *in vivo*. However, *lpr* mutation cannot account for the entire autoimmune syndrome of MRL/*lpr* mice and other genes of pathologic importance in the MRL background have been mapped.

MRL/*lpr* mice spontaneously develop a lupus-like syndrome that can affect many organs. The murine cutaneous lesions resemble human discoid lupus erythematosus. Mononuclear cells infiltrate joints, and lachrymal and salivary glands, resulting in rheumatoid arthritis and Sjögren’s syndrome, respectively. Like human SLE patients, MRL/*lpr* mice have neurological manifestations caused by cellular infiltrations into the central nervous system. MRL/*lpr* mice develop hypergammaglobulinemia and high levels of autoantibodies, including anti-DNA antibodies, associated with immune-complex–mediated glomerulonephritis and vasculitis. Glomerulonephritis, interstitial nephritis and
vasculitis begin by 8 weeks of age, and MRL/lpr females die at about 17 weeks of age and males at 22 weeks. 18.

Because of the resemblance between the murine and human diseases, MRL/lpr mice have been used extensively to attempt to determine SLE etiology and to evaluate therapies. Indeed, MRL/lpr mice provide an attractive model because their syndrome is spontaneous, predictable and rapid; it exhibits the characteristic multifaceted tissue destruction and sexual dimorphism; and its severity varies with the individual. Th1 cytokines, including IFN-γ, are present in the tissues of severely affected SLE patients. IFN-γ production by peripheral blood cells and in kidneys of patients with severe lupus glomerulonephritis was higher than that of patients with milder renal disease. Disease severity in MRL/lpr mice is also linked to Th1 cytokines, IFN-γ and IL-12. IFN-γ gene or IFN-γ receptor (IFN-γR) deletion, and less IgG2a and IgG3 but not IgG1 anti-DNA antibody synthesis dramatically limits glomerulonephritis. IL-18 is associated with severe inflammatory conditions, e.g., autoimmune diseases, allergies or neurological disorders, and its concentration is elevated in sera from SLE patients and MRL/lpr mice. IL-18 has pleiotropic immunoregulatory functions: stimulating IFN-γ production, Fas-mediated cytotoxicity and developmental regulation of Th1. Increased nitric oxide (NO) production plays an important role in various inflammatory diseases. Indeed, excessive NO synthesis can be followed by NO interaction with superoxide to form peroxynitrite, which causes severe oxidative damage to lipids, proteins and DNA. Therefore, the intracellular content of reduced glutathione (GSH) appears to play a key role in dictating cell susceptibility to NO and peroxynitrite. MRL/lpr mice overproduce NO in their disease-affected organs, and this overproduction parallels the development of clinical disease manifestations. The major contribution of NO to disease manifestations is further supported by observations that treatment of MRL/lpr mice with NO synthase inhibitor prevents glomerulonephritis, arthritis, and vasculitis. TNF-α, a
pleiotropic cytokine with proinflammatory properties, is also increased in the autoimmune MRL/lpr mice. Enhanced TNF-α production has been clearly associated with several autoimmune and inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease. Paradoxically, the Th2 cytokine IL-10 has also been associated with the lupus disease in MRL/lpr mice. Likewise, patients with lupus produce large amounts of IL-10, and its serum level correlates with disease activity.

Arsenic trioxide (As$_2$O$_3$) has shown substantial efficacy in the treatment of both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL). Side effects are more likely to occur with intravenous than with oral administration of As$_2$O$_3$. As$_2$O$_3$ induces remissions in APL patients in part through degradation of the aberrant promyelocytic leukaemia-retinoic acid receptor α (PML-RAR) fusion protein. Although numerous studies have been conducted on the molecular mechanisms accounting for PML/RAR degradation, neither the precise basis for the selectivity of As$_2$O$_3$ for PML-RAR that it targets, nor the exact molecular pathway through which As$_2$O$_3$ induces remission in APL patients were understood. As$_2$O$_3$ acts on signalling, caspases and apoptosis, cellular redox and cellular responses to stress. Although mostly focused on the APL response to As$_2$O$_3$, investigators using many different experimental systems concluded that As$_2$O$_3$ may be beneficial against hematopoietic malignancies and solid tumors. We examined the therapeutic impact of As$_2$O$_3$ on the lymphoproliferative and severe autoimmune disorders manifested in MRL/lpr mice.
Materials and Methods

Mice and As₂O₃ treatment. Wild-type MRL/MpJ (MRL⁺/⁺), MRL/MpJ-Tnfrsf6lpr/J mutant (MRL/lpr) and C57BL/6J mice originally from The Jackson Laboratory were maintained in our animal facilities. All experiments were performed in accordance with institutional Animal Research Committee guidelines. A stock solution was prepared by dissolving As₂O₃ powder (Sigma, St. Louis, MO) in 1 M NaOH, then further diluting it in PBS and mice were injected i.p. daily with 2.5, 5, 7.5, 10 or 15 µg/g body weight; controls received a daily i.p. injection of PBS (volume weight-determined).

Phenotyping. Spleen and LN cell suspensions were phenotyped by flow cytometry using either FITC-, phycoerythrin- or biotin-conjugated mAb: rat anti-Thy-1.2, anti-CD4, anti-CD8, anti-B220 (all from PharMingen, San Diego, CA), and rat IgG2a mAb as the isotype control ((Serotec France, Cergy Saint-Christophe, France). Use of mAb to mouse Fcγ receptor (PharMingen) avoided non-specific antibody binding.

Western blotting. Lymphoid cell pellets were directly lysed in Laemmli sample buffer and boiled for 5 minutes. Protein samples (30 µg each) were subjected to 10% SDS–PAGE followed by Western blotting using rabbit polyclonal anti-FasL (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin antibodies (Sigma). Antibody-recognized proteins-labeled were detected using ECL kit (Amersham Pharmacia biotech, Orsay, France).

Histopathology and Immunohistochemistry. Kidney, liver, skin and lung samples were fixed overnight in GlyoFix (Shandon Lipshaw, Pittsburgh, PA), then dehydrated, paraffin-embedded and 4-µm-thick sections were cut. After hematoxylin, eosin and saffron staining, sections were examined. For immunohistochemical labeling, kidney samples were frozen in
OCT compound, and immune complexes deposited in glomeruli were detected by incubating cryosections with horseradish peroxidase-conjugated goat-anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA). Sections were then counterstained with hemalun.

**ELISA detection of anti-DNA autoantibodies, rheumatoid factor and cytokines.** To detect serum IgG (IgG1, IgG2a, IgG2b, IgG3) anti-DNA autoantibodies, 96-well plates were coated with 10 µg/ml calf-thymus DNA (Sigma). For detection of IgM and IgG rheumatoid factor (RF), plates were coated with rabbit IgG (1µg/ml; Sigma), and to detect IgG3 RF, plates were coated with mouse IgG2a (1µg/ml; Sigma) \(^{43}\). After blocking with 1% BSA, serial serum dilutions (starting at 1/400) were added in triplicate for 2 h at room temperature, then washed; bound IgG anti-DNA was detected with biotin-labeled rat anti-mouse IgG, streptavidin–alkaline phosphatase and pNPP (Sigma). Bound RF was detected with alkaline phosphatase-labeled goat anti-mouse IgM, IgG (both Sigma), or IgG3 (Caltag Laboratories, Burlingame, CA) and pNPP. OD was determined at 405 nm.

Serum levels of IFN-γ, soluble FasL, IL-18, TNF-α and IL-10 were assayed using the Mouse Interferon-gamma ELISA Ready-SET-Go (eBioscience, San Diego, CA), Mouse Fas Ligand Immunossay (Quantikine M kit; R&D systems, Minneapolis, MN), Mouse IL-18 ELISA (R&D Systems), Mouse TNF-α Immunoassay (R&D systems), and Mouse IL-10 Immunoassay (R&D systems) respectively, following the manufacturer’s instructions.

**Determination of NO production.** Serum levels of nitrite were measured using the Nitric Oxide quantification kit (Active Motif, Rixensart, Belgium), according to the manufacturer’s instructions.
Apoptosis analysis and GSH detection. Activated caspase levels in spleen and LN cells were measured using the CaspaTag kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. GSH levels were detected in spleen and LN cells using 5 µM CellTracker probe CMFDA, following the manufacturer’s instructions (Molecular Probes, Eugene, OR) and flow cytometry.

Statistical analysis. Data are reported as the mean ± SE. Comparisons between experimental and control groups were made by Student's t-test. Statistical difference was accepted at $P < 0.05$. 
Results

\textbf{As}_2\text{O}_3\text{ prevents and reduces lymphoproliferation in MRL/lpr mice}

Accumulation of DN B220^{+}\text{CD4}^{-}\text{CD8}^{-} T lymphocytes in MRL/lpr LN and spleen starts at 2 months and this lymphoproliferation peaks at 4–5 months. In experiments starting at 2 months of age, MRL/lpr and MRL^{+/+} mice were treated for 2 months with As2O3 daily (2.5–10 µg/g body weight) or PBS. With 2.5 µg/g of As2O3 the lymphoproliferation was significantly reduced ($P < 0.05$) when compared to PBS-treated MRL/lpr mice. However, with 5, 7.5 or 10 µg/g, no lymphoproliferation was seen in the spleen and LN of treated MRL/lpr mice (Figure 1, data not shown). Because 5 µg/g/day was the lowest dose of As2O3 efficient to block the lymphoproliferation, this dose was used for subsequent experiments.

To assess As2O3 activity on non-lymphoid organs, MRL^{+/+} and MRL/lpr mice received 5 µg/g of As2O3 daily for 2 months and then their spleens, mesenteric LN, axillary LN, livers, hearts, lungs and kidneys were weighed (Table 1). In MRL/lpr mice, mean spleen weight increased 6-fold and their mean axillary and mesenteric LN weights rose 45- and 48-fold, respectively, versus MRL^{+/+}. Mean MRL/lpr liver and lung weights were also higher than MRL^{+/+} values, at ~1.5- and ~1.7-fold, respectively. Under As2O3, the abnormally high MRL/lpr lymphoid organ, liver and lung weights were sharply reduced, while kidney and heart weights remained unaffected (Table 1). In As2O3-treated MRL^{+/+} mice, spleen- and LN-cell numbers as well as liver, lung, kidney and heart weights were unchanged (Table 1).

To determine whether As2O3 could also be curative, 4-month-old MRL/lpr mice, with severe lymphoproliferation were given daily As2O3 (5 µg/g) injections for 2 months; lymphoproliferation in their spleens and LN regressed under therapy (Figure 2A). Importantly, As2O3 had no effect on lymphoid organs from MRL^{+/+} mice (Figure 2A). Flow-cytometry quantification of spleen cell subpopulations from MRL/lpr mice treated with PBS or As2O3 using mAb specific to CD3, CD4, CD8, CD19 and B220 cell-surface antigens
yielded (mean ± SE), for 4-month-old PBS-treated MRL/lpr mice, $7.2 ± 0.7 \times 10^8$ splenocytes ($n = 8$), among which $77 ± 1.5\%$ and $13 ± 0.5\%$ were CD3$^+$ T- and CD19$^+$ B-lymphocytes, respectively. DN represented $75 ± 7\%$ of the CD3$^+$ T cells, while CD4$^+$ and CD8$^+$ subsets accounted for $13 ± 2\%$ and $12 ± 1.5\%$, respectively. The number of splenocytes in As$_2$O$_3$-treated MRL/lpr mice was reduced about 15-fold to $0.49 ± 0.6 \times 10^8$ ($n = 10$), among which $51 ± 2\%$ and $49 ± 3\%$ were CD3$^+$ T- and CD19$^+$ B-lymphocytes, respectively. Abnormal DN T lymphocytes represented $23 ± 1.5\%$ of CD3$^+$ T lymphocytes, with $50 ± 4\%$ and $25 ± 2\%$ normal CD4$^+$ and CD8$^+$ subpopulations, respectively. Similarly, mesenteric LN cell counts were 10-fold lower. These data demonstrate the ability of As$_2$O$_3$ to specifically eliminate abnormal DN T lymphocytes. To completely exclude that As$_2$O$_3$ acts as a cytotoxic agent by causing generalized immunosuppression, we determined bone marrow-, thymus-, blood-, spleen- and LN-cell numbers in autoimmune MRL/lpr and non-autoimmune C57BL/6J mice treated with As$_2$O$_3$ (5 µg/g/day) for 2 months. In As$_2$O$_3$-treated C57BL/6J mice, bone marrow-, thymus-, red blood-, spleen- and LN-cell numbers were unchanged. White blood cell numbers from these mice were slightly, but not significantly, decreased (Table 2). In As$_2$O$_3$-treated MRL/lpr mice, the number of white blood cell is normalized and the lymphoproliferation in their spleen and LN regressed, while bone marrow-, thymus- and red blood-cell numbers remained unchanged (Table 2). Taken together our results suggest that As$_2$O$_3$ has a selective effect on DN T lymphocytes which accumulate in autoimmune MRL/lpr mice.

**As$_2$O$_3$ suppresses skin lesions in MRL/lpr mice**

Human lupus exhibits several clinical phenotypes. Discoid lupus affects the skin, causing rashes and lesions, usually on the face and upper torso, and the cutaneous lesions of MRL/lpr
mice resemble it. MRL/lpr mice develop necrotic skin lesions on their ears, hair loss and scab formation, typically on the upper back (Figure 2B). Light microscopy examination of the skin lesions revealed hyperkeratosis, acanthosis, liquefaction, vasodilation and mononuclear cell infiltrates in the dermis (Figure 2B, left panel). Unexpectedly, injecting As$_2$O$_3$ (5 µg/g/day, for 1–2 months) into MRL/lpr mice led to the quasi-total disappearance of cervical cutaneous lesions and hair grew back (Figure 2B). The quasi-total regression of skin lesions in As$_2$O$_3$-treated mice was confirmed by light microscopy examination of skin sections (Figure 2B, right panel). Thus, in arsenic-treated animals, dermal lymphoid infiltration was dramatically attenuated, and hyperkeratosis, acanthosis, hypergranulosis, liquefaction and dermal vasodilatation disappeared.

**As$_2$O$_3$ returns FasL levels to normal in MRL/lpr mice**

FasL is synthesized by lymphoid cells in membrane-associated and soluble forms$^{44,45}$. Both forms were studied in MRL$^{+/+}$ and MRL/lpr mice treated with PBS or As$_2$O$_3$. Cell surface-anchored FasL was analysed by Western blotting on lymphoid cell lysates, while soluble FasL in mouse sera was quantified by ELISA. As expected, in Fas-deficient MRL/lpr, but not MRL$^{+/+}$ mice, both FasL forms were overexpressed (Figure 3A and B). As$_2$O$_3$ treatment of MRL/lpr mice sharply decreased soluble and membrane-associated FasL levels to approach those found in MRL$^{+/+}$ mice (Figure 3A and B). Administering As$_2$O$_3$ to MRL$^{+/+}$ mice did not affect FasL levels, suggesting that FasL-overexpressing T lymphocytes are the main target of this agent.

**IFN-$\gamma$, IL-18, TNF-$\alpha$ and IL-10 syntheses decline in As$_2$O$_3$-treated MRL/lpr mice**

Because IFN-$\gamma$, IL-18 and TNF-$\alpha$ are major mediators of several autoimmune and inflammatory diseases$^{23,31,32,46}$, we compared their concentrations in the sera of MRL$^{+/+}$ and
MRL/lpr mice treated with PBS or As$_2$O$_3$. In MRL$^{+/+}$ mice, IFN-$\gamma$, IL-18 and TNF-$\alpha$ syntheses were not affected by As$_2$O$_3$ treatment (Figure 4A, B and C). As expected, PBS-treated MRL/lpr mice had very high serum IFN-$\gamma$, IL-18 and TNF-$\alpha$ levels but As$_2$O$_3$ treatment maintained normal cytokine concentrations, comparable to those of MRL$^{+/+}$ mice (Figure 4A, B and C).

Like patients with ALPS$^2$ and human SLE patients$^{35}$, untreated MRL/lpr produce large amounts of IL-10 when compared to MRL$^{+/+}$ mice (Figure 5A). As expected, PBS-treated MRL/lpr mice had very high serum IL-10 levels but As$_2$O$_3$ treatment significantly reduced IL-10 concentrations, in a dose-dependent manner (Figure 5B). However, even at the highest dose of As$_2$O$_3$ tested (10 µg/g), serum from MRL/lpr mice contained significantly higher concentrations of IL-10 than those from MRL$^{+/+}$ mice. The DN T cells are primary producers of IL-10 in ALPS patients$^2$, we therefore quantified these cells in MRL/lpr mice treated with PBS or As$_2$O$_3$ by flow-cytometry using mAb specific to CD3, CD4, CD8, CD19 and B220 molecules. In LN from PBS-treated MRL/lpr mice, DN T cells represented 66.8 ± 4.35% of CD3$^+$ T cells. In MRL/lpr mice treated with 2.5, 5, 7.5 or 10 µg/g of As$_2$O$_3$, DN T cells represented 48 ± 5.4%, 19.33 ± 3.8%, 15 ± 2.34% or 8.51 ± 2.75% of CD3$^+$ T lymphocytes, respectively. Importantly, the reduction of serum IL-10 paralleled the decrease in DN T-cell numbers.

**Concentrations of nitrites decline in As$_2$O$_3$-treated MRL/lpr mice**

Measuring NO synthase (NOS) activity by monitoring the accumulation of nitrite, a stable oxidative end-product of NO, is a standard assay for NOS activity. MRL/lpr mice overproduce NO as autoimmune disease progresses$^{29}$. Therefore, serum from MRL/lpr mice contained significantly higher concentrations of nitrite than those from MRL$^{+/+}$ mice (Figure 5D). While As$_2$O$_3$ did not modified nitrite levels in serum from MRL$^{+/+}$ mice, it sharply
decreased nitrite levels in serum from MRL/lpr mice to approach those found in MRL\textsuperscript{+/+} mice (Figure 5D).

**As\textsubscript{2}O\textsubscript{3} prevents inflammatory infiltrates in lungs and kidneys and inhibits immune-complex deposition in kidneys of MRL/lpr mice**

SLE, usually more severe than discoid lupus, can attack any organ. Pleurisy is a common pulmonary manifestation of SLE and, more rarely, patients develop acute lupus pneumonitis. Similarly, the MRL/lpr disease is characterized by massive accumulation of leukocytes in the lungs \textsuperscript{47}, with widespread perivascular and peribronchiolar mononuclear cell infiltrates (Figure 6A). In contrast, in As\textsubscript{2}O\textsubscript{3}-treated MRL/lpr mice, the pulmonary architecture was normal, with mononuclear cells rarely seen around vessels and never around the airways (Figure 6A).

Lupus nephritis or glomerulonephritis is another severe manifestation of human SLE. The kidney disease developing in MRL/lpr mice is fatal, and caused by lymphoid cells infiltrating into glomeruli, interstitium and perivascular compartments (Figure 6B). In contrast, MRL/lpr mice treated with As\textsubscript{2}O\textsubscript{3} for 2 months had normal kidney structure, with a few mononuclear cells around glomeruli (Figure 6B). As in humans, MRL/lpr lupus nephritis is caused by the deposition of immune complexes (Figure 6C) in kidney parenchyma. Treating 2-month-old MRL/lpr mice with As\textsubscript{2}O\textsubscript{3} for 2 months completely prevented immune-complex deposits in their glomeruli (Figure 6C).

**As\textsubscript{2}O\textsubscript{3} inhibits autoantibody and rheumatoid factor production in MRL/lpr mice**

Anti-DNA antibodies and rheumatoid factor (RF), commonly found in SLE patients, are thought to play important pathogenic roles in lupus nephritis \textsuperscript{17,48,49}. IgG1, IgG2a, IgG2b and DNA-reactive IgG3, and IgM, IgG and IgG3 RF were quantified by ELISA in sera from
MRL+/+ and MRL/lpr mice treated with PBS or As$_2$O$_3$. IgG1 and IgG3 anti-DNA, and IgG and IgG3 RF levels were markedly lower in As$_2$O$_3$-treated mice, and IgG2a and IgG2b anti-DNA, and IgM RF were moderately lower (Figure 7).

**As$_2$O$_3$ significantly improves survival of MRL/lpr mice**

As$_2$O$_3$ significantly prolonged survival ($P < 0.001$) of male and female MRL/lpr mice with established disease (Figure 8; data not shown). Indeed, 18 weeks after starting treatment, all As$_2$O$_3$-treated female mice were alive, whereas all PBS-treated MRL/lpr mice had died. After 45 weeks of treatment, As$_2$O$_3$-treated MRL/lpr mice were still alive with no sign of lymphoproliferation. Finally, 75 weeks after starting treatment, all As$_2$O$_3$-treated MRL/lpr mice had died. Likewise, most of the MRL$^{+/+}$ mice died by 77 weeks. Therefore, the survival curves of As$_2$O$_3$-treated MRL/lpr are similar to those of MRL$^{+/+}$ mice.

**As$_2$O$_3$ activates caspases and normalizes glutathione levels in MRL/lpr mice**

Because As$_2$O$_3$ was shown to induce apoptosis of various cell types, we determined the levels of activated caspase-2, -8 and -9 in lymphocyte populations from mice treated with As$_2$O$_3$ for 1 up to 90 days. Surprisingly, as early as day 4 of treatment, ~50% of MRL/lpr B220$^+$ DN T lymphocytes contained activated caspases, as opposed to only a few MRL/lpr B cells, and MRL/lpr B220$^+$CD4$^+$ and B220$^+$CD8$^+$ T cells (Figure 9A). No activated caspases were detected in B lymphocytes or the B220$^+$CD4$^+$ and B220$^+$CD8$^+$ T-cell subpopulations from MRL$^{+/+}$ mice treated with As$_2$O$_3$ for 4 days or longer (data not shown).

Because NO is overproduced in the diseased organs of MRL/lpr mice, we measured levels of GSH, a critical antioxidant in cellular defenses against free radical-induced damage. Notably, GSH concentrations were 4–5 times lower in MRL/lpr spleen and LN cell populations than in those from MRL$^{+/+}$ mice (Figure 9B, and data not shown). While MRL$^{+/+}$
GSH levels were not significantly modified by As$_2$O$_3$, intracellular GSH levels in MRL/lpr spleen and LN cell populations had increased significantly ($P < 0.001$) 3–5-fold as of day 4 of treatment to reach the levels found in MRL$^{+/+}$ mice (Figure 9B, and data not shown).
Discussion

Our data presented herein demonstrate that As$_2$O$_3$ is a novel therapeutic agent to delete autoreactive lymphocytes and block the progression of autoimmune diseases. Arsenic therapy strongly limited or even abrogated, depending on the mouse, the following autoimmune manifestations of MRL/lpr disease: cytokine and autoantibody production, lymphoid hyperplasia, skin lesions, and mononuclear cell infiltration into lungs and kidneys, immune-complex deposition in glomeruli and early mortality. Remarkably, As$_2$O$_3$ administration achieved the quasi-total disappearance of the disease, even when it was initiated at an advanced stage, and its prevention when initiated at the predisease stage. This model of disease reversal makes As$_2$O$_3$ application in humans with lupus or other autoimmune diseases highly promising.

In MRL/lpr mice, DN T-lymphocyte accumulation in LN and spleen starts at 2 months and peaks at 4–5 months. In MRL/lpr mice treated with As$_2$O$_3$ more than 50% of DN T-lymphocytes contained activated caspases-2, -8 and -9, and the number of DN T-cells was sharply diminished, suggesting that these cells were eliminated by apoptosis. In contrast, in As$_2$O$_3$-treated MRL$^{+/+}$ mice only a few T cells contained activated caspases, and the number of T-cells was unchanged. Therefore, we showed herein that As$_2$O$_3$ specifically activated caspases in the abnormal FasL-overexpressing DN T-cells, thereby maintaining normal FasL levels.

Under As$_2$O$_3$, IgG1, IgG2a, IgG2b and IgG3 anti-DNA autoantibodies, as well as IgM, IgG and IgG3 RF concentrations were much lower. Pathogenic autoantibodies in lupus mice generally belong to the IgG2a, IgG2b, and IgG3 subclasses, which are predominantly regulated by IFN-γ. IFN-γ, which is produced primarily by Th1 lymphocytes and NK cells, is a pleiotropic cytokine that modulates immune function, cell proliferation, apoptosis and numerous other cellular responses. In the immune system, IFN-γ modulates
macrophage-effector functions, influences isotype switching and potentiates Ig secretion by B cells.

IFN-γ synthesis by peripheral blood cells and in the kidneys of patients with severe lupus glomerulonephritis was elevated, compared to individuals with milder renal disease. Although, this enhanced production was recently ascribed a beneficial role in murine lupus, the authors of several studies accorded it a major pathogenic role. In MRL/lpr mice, the IFN-γ concentration gradually rises over a prolonged period. Moreover, IFN-γ administration exacerbates the disease both in humans and mice, whereas MRL/lpr mice, with defective IFN-γ or IFN-γR expression, develop less severe forms. Indeed, IFN-γ is responsible of an overproduction of NO in diseased organs, and this overproduction parallels the development of clinical disease manifestations. Pertinently, we showed herein that antioxidant GSH levels were much lower in MRL/lpr than MRL+/+ spleen and LN cells. This GSH depletion most likely amplifies the toxic effect of NO and peroxynitrite on tissues. Interestingly, As2O3 treatment rapidly (4 days) restored intracellular GSH levels in MRL/lpr mice, thus limiting the deleterious effects of NO and/or peroxynitrite. Finally, we showed that the quasi-total disease regression observed in MRL/lpr mice treated with As2O3 corresponded to normal IFN-γ secretion, and consequently NO production, comparable to that found in MRL+/+ mice. We hypothesize that Th1 lymphocytes and NK cells are the targets of As2O3, because IFN-γ and FasL, molecules predominantly expressed by these cells, were maintained at near-normal levels by As2O3 therapy. Because activated caspase-2, -8 and -9 are not detected in CD4+ T cells from As2O3-treated MRL/lpr mice, these cells might have been eliminated by a caspase-independent mechanism. Our hypothesis is in agreement with a recent study showing that As2O3 triggers a caspase-independent necrotic cell death.

IL-18 also participates in the progression of this autoimmune syndrome. This multifunctional cytokine is expressed by various cell types, including antigen-presenting cells (macrophages and dendritic cells (DC)) and cells not primarily involved in immune
responses, e.g. keratinocytes, articular chondrocytes, synovial fibroblasts and osteoblasts, and also within the adrenal cortex and pituitary gland. IL-18 induces IFN-γ synthesis by T lymphocytes and NK cells. Enhanced IL-18 synthesis at the site of inflammation has been described in numerous inflammatory disorders, but the factors influencing its production are still poorly understood. In human SLE, elevated levels of circulating IL-18 paralleled disease activity. MRL/lpr lymphoid cells are hypersensitive to IL-18 and express high levels of it. Pertinently, IL-18 levels, which were abnormally high in MRL/lpr mice, returned to normal MRL+/+ levels in As2O3-treated MRL/lpr mice. This observation suggests that, in addition to eliminating DN T lymphocytes, activated Th1 and NK cells, As2O3 can also eradicate activated macrophages and DC. As2O3-mediated regulation of IL-18 is probably involved in the maintenance of normal IFN-γ levels and minimal renal damage. Our results are in agreement with a study showing that lower IL-18 activity protects against autoimmune diseases. Because IL-18 had been shown to up-regulate FasL expression on NK cells, less IL-18 also contributes to maintaining normal levels of membrane-bound and soluble forms of FasL in As2O3-treated MRL/lpr mice. Finally, TNF-α concentrations, which were abnormally high in MRL/lpr mice, returned to normal MRL+/+ levels in MRL/lpr mice treated with As2O3. The major source of this proinflammatory cytokine is the cells of the monocyte/macrophage lineage, with T lymphocytes, neutrophils, mast cells, and endothelium also contributing under different circumstances. This observation is in agreement with our hypothesis that As2O3 eliminate activated macrophages.

Overexpression of Th2 cytokine IL-10 is associated with the manifestations of ALPS, and has a disease-promoting effect in humans and mice with lupus. IL-10 can be secreted by many cell types, with multiple biological effects. For example, IL-10 can inhibit inflammatory responses as well as promote B cell proliferation and antibody production. IL-10 contributed to the abnormal production of Ig and of autoantibodies in SLE.
Administration of anti-IL-10 antibodies delayed onset of autoimmunity in NZB/W F1 mice. Moreover, anti-IL-10 monoclonal antibodies administration to SLE patients with active disease led to a reduction in disease activity. However, the precise role of IL-10 in the pathogenesis of lupus remains uncertain. IL-10 levels, which were abnormally high in MRL/lpr mice, decreased significantly in As2O3-treated MRL/lpr mice. Importantly, the reduction of serum IL-10 paralleled the decrease in DN T-cell numbers in peripheral lymphoid tissues. These observations suggest that like DN T cells from ALPS patients, the DN T cells are primary producers of IL-10 in MRL/lpr mice. As2O3-mediated regulation of IL-10 probably reduced serum Ig and autoantibodies, and renal immune complex deposition.

The prototypical autoimmune disease, SLE is currently treated with corticosteroids and cytotoxic or immunosuppressive drugs. These therapies prolong survival but are associated with severe side effects, particularly infections. The therapeutic intraperitoneal route of arsenic in our mice model of autoimmune disease is different from intravenous or oral administration used in APL patients. It would be interesting to compare the three routes of As2O3 administration in MRL/lpr mice. As2O3 significantly prolonged survival of MRL/lpr mice by preventing young mice from developing the syndrome and quasi-totally reversing established disease in older animals, thereby demonstrating its potential as a novel therapeutic agent for autoimmune diseases.

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References


58. Neumann D, Del Giudice E, Ciaramella A, Boraschi D, Bossu P. Lymphocytes from autoimmune MRL/lpr/lpr mice are hyperresponsive to IL-18 and overexpress the IL-18 receptor accessory chain. J Immunol. 2001;166:3757-3762.


Table 1: \( \text{As}_2\text{O}_3 \) effects on MRL\(^{+/+}\) and MRL/\(lpr\) mouse tissue/organ weights

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>PBS((n = 5)^*)</th>
<th>(\text{As}_2\text{O}_3)((n = 5)^*)</th>
<th>PBS((n = 6)^*)</th>
<th>(\text{As}_2\text{O}_3)((n = 10)^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axillary LN†</td>
<td>10 ± 1</td>
<td>10 ± 1</td>
<td>450 ± 10</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Mesenteric LN†</td>
<td>50 ± 3</td>
<td>45 ± 3</td>
<td>2410 ± 100</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>Spleen†</td>
<td>100 ± 3</td>
<td>120 ± 10</td>
<td>630 ± 40</td>
<td>170 ± 3</td>
</tr>
<tr>
<td>Liver†</td>
<td>2460 ± 50</td>
<td>2620 ± 80</td>
<td>3760 ± 90</td>
<td>2750 ± 35</td>
</tr>
<tr>
<td>Lung†</td>
<td>320 ± 30</td>
<td>330 ± 20</td>
<td>550 ± 20</td>
<td>400 ± 7</td>
</tr>
<tr>
<td>Kidney†</td>
<td>400 ± 3</td>
<td>420 ± 10</td>
<td>510 ± 10</td>
<td>540 ± 2</td>
</tr>
<tr>
<td>Heart†</td>
<td>220 ± 10</td>
<td>210 ± 3</td>
<td>290 ± 7</td>
<td>240 ± 3</td>
</tr>
</tbody>
</table>

*Two-month-old MRL\(^{+/+}\) and MRL/\(lpr\) mice had been treated daily with PBS or \(\text{As}_2\text{O}_3\) (5\(\mu\)g/g) for 2 months, \(n\) represents the number of mice per group.

† Results are expressed as mean organ weights in mg ± SE from \(n\) mice. LN = lymph node.
Table 2. Lymphoid cell numbers in MRL/lpr and C57BL/6J mice treated by As$_2$O$_3$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment*</th>
<th>Thymus**</th>
<th>Bone Marrow**</th>
<th>Blood**</th>
<th>Spleen**</th>
<th>Mesenteric LN**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRL/lpr</td>
<td>PBS</td>
<td>5.4 ± 1.54</td>
<td>3.2 ± 0.2</td>
<td>6.55 ± 1</td>
<td>14.92 ± 4.4</td>
<td>37 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>As$_2$O$_3$</td>
<td>5.45 ± 0.83</td>
<td>3.23 ± 0.69</td>
<td>6.16 ± 0.40</td>
<td>6.15 ± 0.55</td>
<td>11 ± 3.12</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>PBS</td>
<td>6.28 ± 0.59</td>
<td>4.1 ± 0.45</td>
<td>5.98 ± 0.81</td>
<td>9.1 ± 0.46</td>
<td>7 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>As$_2$O$_3$</td>
<td>5.16 ± 0.55</td>
<td>4.76 ± 0.41</td>
<td>5.5 ± 0.59</td>
<td>7.28 ± 0.78</td>
<td>7.15 ± 0.75</td>
</tr>
</tbody>
</table>

*Two-month-old mice were treated for 2 months with As$_2$O$_3$ (5µg/g) or PBS daily, n represents the number of mice per group.

**Cell number × 10$^7$ ± SE.
Legends to Figures

Figure 1 Absence of lymphoproliferation in As$_2$O$_3$-treated MRL/lpr mice. Mesenteric LN weights from 2-month-old MRL/lpr mice that had been treated daily for 2 months with 2.5, 5, 7.5 or 10 µg/g of As$_2$O$_3$ or PBS.

Figure 2 Absence of lymphoproliferation and regression of skin lesions in As$_2$O$_3$-treated MRL/lpr mice. Two-month-old mice had been treated daily for 2 months with PBS or As$_2$O$_3$ (5µg/g). (A) Spleens and mesenteric LN from 4-month-old MRL/lpr and MRL$^{+/+}$ mice. (B) Skin lesions on the back and ears from 4-month-old MRL/lpr mice. Light microscopy histology of these skin lesions (lower panel, original magnification, 50 ×).

Figure 3 FasL levels return to normal in As$_2$O$_3$-treated MRL/lpr mice. (A) Level of cell surface-anchored form of FasL from 4-month-old MRL$^{+/+}$ (□) and MRL/lpr (■) mice that had been treated daily with PBS (–) or As$_2$O$_3$ (5µg/g) (+) for 2 months were analyzed by Western blotting of lymphoid cell lysates from 8 individual mice using anti-FasL mAb. The blot was stripped and reprobed with anti-actin mAb. (B) ELISA were used to determine their serum FasL concentrations. Black squares represent data from individual animals and bars correspond to the mean for 6–12 mice per group.

Figure 4 IFN-γ, IL-18, nitrites and TNF-α levels return to normal in As$_2$O$_3$-treated MRL/lpr mice. 4-month-old MRL$^{+/+}$ (□) and MRL/lpr (■) mice had been treated daily with PBS or As$_2$O$_3$ (5µg/g) for 2 months. ELISA were used to determine their serum IFN-γ (A), IL-18 (B) and TNF-α (C) concentrations, and Griess reagents to measure their serum nitrite concentrations (D). Black squares represent data from individual animals and bars correspond to the mean for 6–9 mice per group.
Figure 5  Reduction of serum IL-10 in As₂O₃-treated MRL/lpr mice. Serum IL-10 levels were quantified by ELISA in the sera of (A) untreated 4-month-old MRL⁺/⁺ and MRL/lpr (B) 2-month-old MRL/lpr mice that had been treated daily for 2 months with 2.5, 5, 7.5 or 10 µg/g of As₂O₃ or PBS. Black squares represent data from individual animals and bars correspond to the mean for 3–4 mice per group.

Figure 6  As₂O₃-induces regression of pulmonary and renal inflammatory infiltrates and inhibits immune-complex deposition in kidneys of MRL/lpr mice. Histological examination of mononuclear cell infiltrates (arrows) in lung (original magnification, 50×) (A) and kidney (original magnification, 200×) (B) sections from a 4-month-old MRL/lpr mouse that had been treated daily for 2 months with As₂O₃ (5µg/g) or PBS. (C) Glomerular IgG deposits in frozen kidney sections labeled with HRP-conjugated goat anti-mouse IgG (original magnification, 200 ×).

Figure 7  As₂O₃ inhibits anti-DNA autoantibody and RF production in MRL/lpr mice. IgG1, IgG2a, IgG2b and IgG3 anti-DNA autoantibodies, and IgM, IgG and IgG3 RF levels were quantified by ELISA in the sera (dilution, 1/1600) of 4-month-old MRL⁺/⁺ and MRL/lpr mice that had been treated daily for 2 months with As₂O₃ (5µg/g) or PBS.

Figure 8  As₂O₃ dramatically prolongs survival of MRL/lpr mice. Starting at 2 months of age, MRL/lpr mice (15/group) were treated daily with As₂O₃ (5 µg/g) (–) or PBS (---) and monitored to establish mortality rates.

Figure 9  As₂O₃ activates caspases and restores cellular levels of GSH in MRL/lpr mice. (A) Activated caspase-2 (■), -8 (□) and -9 (■) were detected by flow cytometry in the spleen
cell subpopulations of 4-month-old MRL/lpr mice (4/group) that had been treated daily for 4 days with As$_2$O$_3$ (5µg/g). (B) Intracellular levels of GSH were detected by flow cytometry in the spleen cell populations of 4-month-old MRL$^{+/+}$ and MRL/lpr mice (5/group) that had been treated daily for 4 days with As$_2$O$_3$ (5µg/g) (■) or PBS (□).
Figure 1
Figure 3
Figure 4

A. Serum IFN-γ (ng/ml)

B. Serum IL-18 (ng/ml)

C. Serum TNF-α (ng/ml)

D. Serum nitrite (μM)
Figure 5
Figure 6
Figure 7

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

OD 405 nm

IgG anti-DNA

- IgG1
- IgG2a
- IgG2b
- IgG3

RF

- IgM
- IgG
- IgG3

- MRL** + PBS
- MRL** + $\text{As}_2\text{O}_3$
- MRL/lpr + PBS
- MRL/lpr + $\text{As}_2\text{O}_3$
Figure 9
Arsenic trioxide, a novel promising therapeutic agent for lymphoproliferative and autoimmune syndromes in MRL/lpr mice

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