The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice

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ABSTRACT
We sought to determine if the histone deacetylase inhibitor (HDI), trichostatin A (TSA), would alter systemic lupus erythematosus (SLE) in NZB/W mice. Fourteen to sixteen-week-old female NZB/W F1 mice were given TSA (1.0 mg/kg body weight (BW)) intraperitoneally (i.p.) daily, TSA (1.0 mg/kg BW) i.p. + anti-CD25 (250 mg/mouse) i.p. every third day, only anti-CD25 (250 mg/mouse) i.p., DMSO or isotype IgG. Disease progression was assessed as they aged. Mice were sacrificed at 26 or 38 weeks of age, tissues collected and evaluated. At 36 weeks, TSA-treated animals had decreased anti-double stranded DNA (dsDNA) autoantibodies and decreased protein excretion compared to controls. Spleen size and the percentage of CD4+CD69+ cells were decreased, with an increase in CD4+CD25+ T cells in the TSA-treated mice. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of T cells showed a decrease in IL-6 production but an increase in TGF-β1 and Foxp3 in the TSA-treated animals. Kidney analysis showed a decrease in IgG and C3 deposition, decrease in pathologic glomerular disease and renal MCP-1, MMP-9, and IL-6 mRNA expression. Anti-CD25-treated mice euthanized at 26 weeks of age showed decreased Foxp3+ CD4+CD25+ T cells compared to TSA-treated mice. These data suggest TSA administration modulates lupus-like disease, in part, by increasing T regulatory cells.

1. Introduction
The equilibrium between opposing activities of histone acetylase transferases and histone deacetylase enzymes determines global cellular acetylation status. The acetylation status of lysine residues in nuclear core histones is an important factor in allowing the chromatin superstructure to be accessed by transcriptional factors to initiate gene activation [1]. Histone deacetylase inhibitors (HDIs) are grouped into three classes; TSA is a potent and specific inhibitor for class I and class II HDIs and has been shown to have efficacy at low nanomolar concentrations. TSA given i.p. to mice is rapidly absorbed and detectable in the plasma within 2 min and has a half-life of 10 min [2]. TSA has a potent broad spectrum antitumor activity against virally transformed cells in culture without apparent toxicity in preclinical studies and inhibits tumor cell growth in vitro [3–6]. We have previously shown that the histone deacetylase inhibitor (SAHA) can modulate renal disease in MRL/lpr mice and TSA can decrease renal disease in MRL/lpr mice [7–9]. TSA has been shown to inhibit the stimulation of toll-like receptors (TLRs) [10] and has been proposed to be used as therapy in autoimmune disorders [11].

CD4+CD25+ regulatory T cells (Treg), constitute 5–10% of peripheral CD4+ T cells in normal naive mice and humans. Deletion of Treg from healthy animals can break self-tolerance, leading to the development of autoimmune disease, whereas repopulation of these cells can re-establish self-tolerance and prevents autoimmune diseases [12–14]. This observation indicates that Treg play a critical role in the maintenance of self-tolerance and the
prevention of organ-specific autoimmunity [15]. Our current studies were designed to show effacy of TSA in another model of lupus, the NZB/W mouse, and to identify the mechanism(s) of how TSA acts to decrease lupus nephritis.

2. Materials and methods

2.1. Animals

NZB/NZW F1 female mice were purchased from Jackson Laboratories (Bar Harbor, MA) and housed under specific pathogen free conditions at the Center for Molecular Medicine and Infectious Diseases animal facility at Virginia Polytechnic and State University, and provided autoclaved food and sterile water ad libitum. At 14–16 weeks of age mice were given TSA (0.5 or 1.0 mg/kg BW) by i.p. injection in 40 μl of DMSO for 7 days/wk or anti-CD25 derived from PC61 every third day (250 μg/mouse), or both 1.0 mg/kg BW TSA and anti-CD25 for up to a 20 week period. The anti-CD25 injections were based on previous publications showing this regime depletes CD25+ cells [16]. Control mice received DMSO or IgG isotype control (40 μl). All animal protocols were approved by the University Committee on Use and Care of Animals at the Virginia Polytechnic Institute and State University.

2.2. Measurement of proteinuria

Urine was collected every two weeks and tested for proteinuria by a standard semiquantitative test using Bayer Multistix dipsticks (Bayer, Fernwald, Germany). Results were graded according to the manufacturer’s instructions.

2.3. Reagents

TSA was purchased from (Sigma, St. Louis, MO). The protein assay kit was purchased from Bio-Rad (Hercules, CA). All other reagents, including LPS were purchased from Sigma (St. Louis, MO). PC61 Hybridoma B Lymphocytes used to produce anti-CD25, were purchased from ATCC, Manassas, VA.

2.4. Flow cytometric analysis

Flow cytometric analysis was performed using fluorescein–isothiocyanate (FITC)-conjugated CD69; R-phycocerythrin (R-PE)-conjugated CD4, and PerCP-Cy5.5-conjugated CD25 rat anti-mouse monoclonal antibodies (BD Pharmingen, San Diego, CA). Splenic cells were isolated as previously described [17]. Briefly, spleen lymphocytes from NZB/W mice at 36 weeks of age were aseptically dissociated, treated with Tris–ammonium chloride-lysis buffer (pH 7.2) to remove erythrocytes, washed and resuspended with magnetic beads coated with sheep anti-rat IgG and sheep anti-mouse IgG (11007, 11001, Dynal Biotech). Cells were placed in a magnetic field and the non-magnetized cell suspension was aspirated and placed in complete media. Cells were stained with monoclonal antibodies or appropriate fluorochrome-tagged isotype anti-rat IgG2a control antibodies then analyzed on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL).

2.4.1. Isolation of CD4+CD25+ T cells

For isolation of CD4+CD25+ T regulatory cells, splenocytes were isolated as described above. MagCellect mouse CD4+CD25+ regulatory T cell isolation kit (R&D system (Minneapolis, MN)) was used to collect cells according to the manufacturer’s protocol. Flow cytometric analysis of isolated cells demonstrated 92–95% purity for CD4+CD25+ staining.

2.5. Sera collection

From 14 to 36 weeks of age, mice were bled from the retro-orbital sinus following inhalation of isofluroane anesthesia. Serum was tested for anti-dsDNA levels by ELISA as previously described [18]. Anti-dsDNA antibodies were also measured using the Crithidia luciliae kinetoplast staining assay according to the manufacturer’s instructions (Antibodies Inc., Davis, CA).

2.6. Pathology

At 38 weeks of age mice were sacrificed for pathologic evaluation. At the time of sacrifice, the mice were weighed; kidneys were removed and divided into sections. One portion was placed in buffered formalin for subsequent embedding in paraffin, sectioning, H&E and PAS staining. Sections were assessed via light microscopy for glomerular proliferation, glomerular inflammation, glomerular size, number of nuclei per glomerulus, crescents, necrosis and fibrosis. Each of these parameters was graded for 0–3+ and an overall glomerular score was derived. The pathology and morphometric analysis were performed by a pathologist blinded to the groups (Dr. Arben Santo). One portion of the kidney was frozen in OCT media and cut into 5 μm and stained with fluorescein–isothiocyanate (FITC)-conjugated antibodies: goat anti-mouse IgG dilutes 1:100 (Pierce, Rockford, IL); goat anti-mouse C3 diluted 1:100. The severity of Ig and C3 deposition was determined in a blind manner. Scores ranged from 0 to 3+, where 0 corresponded to a non-autoimmune healthy mouse and 3+ the maximal alteration observed in the study.

2.7. Isolation of RNA

Total RNA was isolated using TRIZOL (Invitrogen Corp., Carlsbad CA) from isolated T cells and from renal cortex according to the manufacturer’s protocol. One microgram of total RNA was converted to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions.

2.8. Real-time PCR

Real-time PCR was performed on 20 ng of cDNA using the TaqMan mouse IL-2 (Mm_00434256_m1), TGF-β1 (Mm_00441724_m1), IL-4 (Mm_00445259_m1), IL-6 (Mm_00446190_m1), IL-10 (Mm_00445259_m1), MCP-1 (Mm_011333_m1), Foxp3 (Mm_00446190_m1), and MMP-9 (Mm_00443256_m1).
using Pre-Developed TaqMan Assay Reagents supplied by PE Applied Biosystems, CA. As a control we used GAPDH. RT-PCR was performed and amplified in a 7000 Sequence Detection System (PE Applied Biosystems). Relative quantization of gene was determined by calculating the fold difference after normalizing to the GAPDH endogenous control (User Bulletin #2, PE Applied Biosystems). All samples were run in duplicate.

2.9. Statistics

Statistical significance was calculated by Student’s t-test or analysis of variance (ANOVA) followed by post-hoc analysis. p Values less than 0.05 were considered significant.

3. Results

Animals receiving daily injections of TSA (either 0.5 or 1.0 mg/kg BW) showed no adverse effects, although at 5 mg/kg BW adverse effects were noted after two weeks of treatment including poor grooming and weight loss. As a result, the 5 mg/kg BW dose was discontinued. There were no differences observed in the weight of the animals, skin involvement, and food or water consumption between 0.5 mg/kg BW TSA, 1.0 mg/kg BW TSA, and DMSO-treated mice during 20 weeks of treatment.

To determine whether TSA altered the progression of renal disease, we quantified protein excretion in the urine as the animals aged. At 16 weeks, the protein levels in both groups were low but as
the mice aged, protein concentrations in the urine began to increase in the control group. By 36 weeks of age, five of the eight control animals had urinary protein concentrations of 200 mg/dL or greater compared to zero of six in the 1.0 mg/kg TSA group (Fig. 1).

To determine whether TSA treatment modified autoantibody production, serum levels of anti-dsDNA antibody levels were quantified. As the animals aged, the levels of anti-dsDNA antibodies increased. Compared to vehicle-treated controls, the levels of anti-dsDNA in the serum of TSA-treated mice were significantly decreased at 36 weeks of age (data not shown). To assess further the specificity of antibody production we used the Crithidia assay (Fig. 2). Our results showed TSA treatment decreased the dsDNA specific antibody titers levels judged by the Crithidia assay.

At age 36 weeks, the animals were euthanized and the spleen weights were measured. Compared to vehicle-treated controls, the 1.0 mg/kg TSA-treated mice had significantly smaller spleens (Control 0.14 ± 0.03 g; 0.5 TSA 0.13 ± 0.02 g; 1.0 TSA 0.095 ± 0.03 g; p < 0.05; n = 6). To characterize further the splenic phenotype, we isolated the dissociated splenocytes and assessed the T cell populations by flow cytometry. The TSA-treated 36-week-old mice had significantly higher percentages of CD4⁺CD25⁺ T cells and a decreased expression of CD4⁺CD69⁺ T cells compared to the control group (Fig. 3).

Next, we sought to examine the production of specific cytokines produced by lymphocytes associated with lupus since HDIs have been reported to suppress inflammatory cytokine expression [8,9,19]. We performed real-time RT-PCR on T cells isolated from 36-week-old 1.0 mg/Kg BW TSA-treated or DMSO-treated NZB/W F1 mice for IL-4, IL-6, IL-10, IL-2, TGF-β and Foxp3 (Fig. 4). Our results showed that IL-2 and IL-4 expression were increased in TSA-treated animals compared to the DMSO controls. TGF-β1 and Foxp3 expression were significantly increased in the TSA mice and IL-6 expression was suppressed in the spleens from the TSA-treated mice as compared to the DMSO controls (n = 3).

To assess the effect of TSA administration on renal disease progression in 36-week-old NZB/W F1 mice, we conducted histopathology analysis of the kidneys. Light microscopy of the kidney sections from the DMSO-treated control mice revealed severe glomerulonephritis and interstitial inflammation (Fig. 5). The mice also had extensive proteinaceous tubular casts, consistent with proteinuria. Kidney sections from the mice treated with TSA revealed a significant decrease in renal lesions. Findings included glomerular hypercellularity, cellular crescents, and tubular dilation with cast formation. In addition to examining the renal tissue pathology, we conducted immunofluorescent analysis of kidney sections for IgG and C3 deposition (Fig. 5a). Our results showed extensive IgG and C3 deposition in the glomerulus of the control mice, however, the TSA-treated animals showed greatly reduced immune complex and complement deposition. When these results were tabulated, a significant decrease in renal disease was evident in the TSA-treated mice (Fig. 5b).

To assess the mechanism of disease inhibition in the kidneys of TSA-treated NZB/W F1 mice, we performed real-time RT-PCR on kidney homogenates from TSA-treated and control mice for MCP-1,

![Graphs of TGF-1, Foxp3, IL-2, IL-4, IL-6, and IL-10 expression](figures)

Fig. 4. Real-time RT-PCR measurement of mRNA levels for TGF-β1, Foxp3, IL-2, IL-4, and IL-6 levels in T cells isolated from 38-week-old female NZB/NZW F1 mice receiving daily injections of either TSA (1.0 mg/kg BW in DMSO) or vehicle (DMSO) for 20 weeks beginning at 16 weeks of age (n = 3).
MMP-9, IL-6, and TGF-β (Fig. 6). We found that TSA-treated mice showed no change in TGF-B1 expression, however, a decrease in MCP-1, MMP-9, and a significant decrease in IL-6 mRNA expression in the kidney were observed.

Given our results showing that TSA upregulated CD4^+ CD25^+ T cells and reduced inflammatory mediator production and renal pathology in NZB/W F1 mice, we sought to define the mechanism of how TSA modulated disease. We treated NZB/W F1 mice with TSA, anti-CD-25, anti-CD25 + 1.0 mg/kg BW TSA, or an IgG isotype control antibody for eight weeks beginning at 14 weeks of age. The rationale being that if TSA was increasing the Treg cells, then the addition of anti-CD25 would deplete the Treg population. Mice receiving injections of TSA and/or anti-CD25 showed no adverse effects of the treatments. There were no significant differences in weight gain, water intake, diet, or grooming habits observed over the 12 weeks of treatment. At 14, 18, 22, and 26 weeks of age blood was collected by retro-orbital bleeding and the sera were assessed for dsDNA autoantibody production. At 26 weeks of age, the animals treated with anti-CD25 antibody had elevated dsDNA antibody production compared to the control or the TSA only treated animals (Fig. 7).

To assess changes in kidney function, we measured urine protein levels every other week beginning at 14 weeks of age (Fig. 8). Anti-CD25-treated mice at 18 weeks of age had increased proteinuria compared to the TSA-treated group indicating that depletion of the Treg cells hastened the development of renal disease.
At 26 weeks of age the animals were sacrificed and splenic tissue was removed and weighed. The anti-CD25 and TSA + anti-CD25 treated mice had significantly larger spleens 0.19 ± 0.04 g and 0.18 ± 0.03 g, respectively, compared to the TSA-treated or control animals 0.11 ± 0.02 g and 0.12 ± 0.02 g. Next, the splenocytes were dissociated and T cells isolated by negative selection. Following tissue dissociation, the cells were stained with various antibodies and examined by flow cytometry (Fig. 9). Our results showed that TSA significantly increased the number of CD4+ cells stained positive for Foxp3 compared to the anti-CD25 + TSA-treated mice (Fig. 9c). TSA also significantly increased the number of CD4+CD25+ T cells compared to the anti-CD25 treated mice (Fig. 9d). When the CD4+ gated cells were stained for CD25+ and Foxp3+, both TSA and TSA + anti-CD25 treated mice showed significantly elevated CD4+CD25+Foxp3+ (Treg) cells (Fig. 9e).

4. Discussion

Numerous studies have shown HDIs reduce the proliferation and exert pro-apoptotic effects on cellular function of transformed cells in vitro and in vivo [4,20,21]. Additionally, HDIs reduce inflammatory mediator production [22,23]. SLE is a disorder of generalized autoimmunity characterized by pathogenic autoantibodies and immune complexes that are attributed to inappropriate regulation of hyper-activated B and T cells, defective clearance of apoptotic cells and immune complexes, loss of immune tolerance and unregulated inflammatory mediator production [24,25]. Remission or improvement may occur spontaneously in the course of the disease suggesting dysregulation of immune cells is transient. Treg cells (CD4+CD25+Foxp3+) are critical in maintaining self-tolerance and preventing organ-specific autoimmunity. However, the role of Treg cells in SLE is perplexing. Data showing reduced numbers of CD4+CD25+ T cells in adults with SLE have been reported. Studies in humans have demonstrated the frequency of CD4+CD25+ T cells in SLE patients were inversely related to both the serum level of anti-dsDNA and the disease activity [26]. However, others have shown a depletion of active Treg cells in SLE and that Treg cells increase in number following B cell depletion therapy indicating that an increase in T regulatory cells could act to inhibit disease [27,28]. Recently, TSA was shown to promote the generation and action of Treg cells [29]. In MRL/lpr mouse studies, the numbers of Treg cells were increased but the functionality of the Tregs was decreased suggesting impaired Treg function in SLE [30]. In irradiated MRL/lpr mice, regulatory T cells were significantly increased [31]. We have previously shown that HDIs decrease inflammation in vitro and in vivo in lupus [32,33]. The present studies evaluated the effect of daily administration of TSA to NZB/W F1 mice. We examined the mechanism of TSA on inhibition of inflammatory mediator production both in the spleen and kidney and the ability of TSA to increase the functionality of Treg cells.

Activation of anti-dsDNA-producing autoreactive B cells in lupus mice requires help from CD4+ helper T cells and the overcoming of the suppression of Treg cells. The activated CD4+ helper T cells presumably rescue self-reactive B cells from apoptosis and stimulate them to produce autoantibodies. Foxp3 expression programs the development of the suppressive function of Treg cells. Our studies demonstrate that NZB/W F1 mice treated with TSA have an increased percentage of Treg cells. Furthermore, we demonstrate that TSA upregulates Foxp3 expression in these CD4+CD25+ T cells. The increase in the percentage of Treg showing Foxp3 expression results in either direct or indirect effects to delay disease pathogenesis. Although the suppressive effects of Treg cells have been widely reported, recently Lee and coworkers reported a decrease in Foxp3 expression in humans with active lupus compared to normal controls and patients with inactive lupus [26]. The authors did note that the overall number of Treg cells in humans with lupus was decreased suggesting that a defect in the T regulatory population may contribute to the pathogenesis of disease. Treg cells are potent suppressors of CD4+ and CD8+ T cell responses in vitro and inhibit several organ-specific autoimmune diseases [34]. Treg cell-derived TGF-β1 is essential for this suppressive function [35–38]. In our studies we found that in TSA-treated mice, TGF-β1 mRNA was increased compared to vehicle-treated mice. We also observed increased Foxp3 mRNA expression compared to controls with little change in IL-2 expression. Taken together, these studies suggest that HDAC inhibitors upregulate Treg cell function by increasing TGF-β1.

We demonstrated a significant increase in CD4+CD25+ T cells with TSA treatment. The precise mechanism of suppression by CD4+CD25+ T regulatory cells is not clear, but includes direct cell-cell contact and production of inhibitory cytokines, such as TGF-β1 [38,39]. Further phenotype analysis of splenocytes showed that the TSA decreased the T cell early activation marker CD69+ on CD4+ cells. In lupus patients with active proliferative nephritis, T cell CD69 expression was shown to be decreased in partial and complete remission [40]. Similarly, Crispin and coworkers reported increased CD4+CD69+ T cells in patients with active lupus [41].
Many animal studies using TSA or various other histone deacetylase inhibitors have employed dosing regimens composed of only a single dose or a short-term treatment [42]. Since the half-life of TSA has been shown to be relatively short (6 min at 0.5 mg/kg) the need for daily administration is justified [43]. Furthermore, since SLE is a chronic disease, the use of long-term immunosuppressive drugs is common treatment. In the present studies, we have shown that administration of 1.0 mg/kg TSA daily for a 20 weeks period did not adversely impact the health of the animal.

TSA induces hyperacetylation of core histone proteins and leads to global hyperacetylation of histone proteins but not a generalized increase in transcription [44]. The observation that rather small number of genes are affected by HDACs suggests HDAC's regulation is a gene specific event. In our current studies, we show that TSA administration upregulates Foxp3 expression and propose that this is one mechanism by which TSA acts to modulate SLE.

Several different studies have shown that lupus nephritis can be modulated at several different times during disease progression. Inhibiting IgG anti-dsDNA autoantibody production reduces complement activation and lupus nephritis. Richards and co-workers demonstrated that IL-6 knockout mice have reduced anti-dsDNA antibody production in pristine induced lupus [45]. IL-6 production has been implicated in both anti-DNA antibody production and the pathogenesis of nephritis in NZB/W mice [46,47]. Others have shown that IL-6 production is mediated through a NFκB mechanism and histone acetylation [48]. Our studies support these findings showing the beneficial effects of modulating IL-6 production and reveals a possible mechanism of how IL-6 may be altered at the histone level either directly or indirectly through Treg cell activation. To this end, we show that IL-6 mRNA expression was reduced in the spleen and kidney in TSA-treated NZB/W mice.

In summary, our data demonstrate that daily TSA (1.0 mg/kg BW) administration to NZB/W F1 mice over a 20-week period is well tolerated. TSA administration increases the levels of Foxp3 expression in pre-disease NZB/W mice and increases the percentage of Treg cells. TSA decreases inflammatory mediator production, autoantibody production and indices of disease in NZB/W F1 mice over a 20-week treatment. Taken together, these results suggest,
TSA may have therapeutic efficacy in treating autoimmune diseases such as SLE and the use of HDIs to treat SLE warrants further investigation.

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