Specific HDAC6 inhibition by ACY-738 reduces SLE pathogenesis in NZB/W mice

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We sought to determine if a selective HDAC6 inhibitor (ACY-738) decreases disease in NZB/W mice. From 22 to 38 weeks-of-age, mice were injected intraperitoneally with 5 or 20 mg/kg of ACY-738, or vehicle control. Body weight and proteinuria were measured every 2 weeks, while sera anti-dsDNA, Ig isotypes, and cytokine levels were measured every 4 weeks. Kidney disease was determined by evaluation of sera, urine, immune complex deposition, and renal pathology. Flow cytometric analysis assessed thymic, splenic, bone marrow, and peripheral lymphocyte differentiation patterns. Our results showed HDAC6 inhibition decreased SLE disease by inhibiting immune complex-mediated glomerulonephritis, sera anti-dsDNA levels, and inflammatory cytokine production and increasing splenic Treg cells. Inhibition of HDAC6 increased the percentage of cells in the early-stage developmental fractions of both pro- and pre-B cells. These results suggest that specific HDAC6 inhibition may be able to decrease SLE disease by altering aberrant T and B cell differentiation.

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect nearly every organ in the body [1]. A pathognomonic feature of lupus nephritis is B cell dysregulation leading to autoantibody production and immune-complex mediated glomerulonephritis [2]. Hyperactive B cells contribute to SLE pathogenesis by inducing CD4+ T helper cells, inhibiting regulatory T (Treg) cells, secreting proinflammatory cytokines, and producing autoantibodies [3]. Reduced Treg cell numbers and function have been reported during active SLE in both mice and humans, which contributes to immune dysregulation and a lack of self-tolerance [4]. Furthermore, TH17/Treg imbalance has been associated with the development of inflammatory disorders and renal dysfunc-

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Numerous studies have shown that the ability of an organism to respond to its environment is dependent upon its capacity to modulate gene expression through chromatin remodeling [25,26]. Cellular function can be altered by DNA methylation, histone modification, and microRNA signaling [27]. Epigenetic alterations have been noted in the pathogenesis of various autoimmune diseases [28,29,30,31]. Histone deacetylases (HDACs) catalyze the removal of acetyl groups from histones restricting chromatin availability for transcription factor binding. Furthermore, HDACs have been implicated in immune cell regulation and may therefore be efficacious as diagnostic or prognostic biomarkers or as targets for the treatment of autoimmune diseases including SLE [32,33,34,35]. Traditionally, HDACs were thought to function solely through epigenetic regulation of histone proteins [36]. However, it is now known that HDACs can also regulate acetylation of over 50 nonhistone proteins, including transcription factors, signaling molecules, DNA repair enzymes, and structural proteins [36,37]. Due to the large number of HDACs that are targeted, pan-HDAC inhibitors have been associated with many deleterious side effects during clinical trials including fatigue, nausea, and electrocardiograph abnormalities [38,39]. For this reason, selective HDAC inhibitors are being pursued as more suitable treatments for autoimmune disease. Multiple class I and II HDACs have been implicated in the regulation of Foxp3 expression, including HDAC6, HDAC7 and HDAC9 [40,41,42]. HDAC6 is a class IIb HDAC that is located primarily in the cytoplasm and has been demonstrated to regulate the acetylation of α-tubulin and HSP90 [43,44]. Treatment with class I and IIb pan-HDAC inhibitors has been shown to increase Treg populations [45,46], however treatment with selective class I HDAC inhibitors has been shown to have minimal effect on Treg function or numbers [47]. Therefore, it can be reasoned that specific class IIb HDAC inhibition is responsible for Treg function.

The current studies were designed to investigate whether treatment with a selective HDAC6i would decrease disease in lupus-prone mice through the regulation of B and/or T cell differentiation. Inhibition of HDAC6 was chosen due to research in HDAC6-deficient mice showing an increase in expression of Foxp3 and Treg cells compared to wild type (WT) mice [39,48]. Furthermore, HDAC6 is known to be expressed at higher levels in Treg cells, which are downregulated during SLE, than in conventional T cells [43]. HDAC6 inhibition has also been associated with increased DNA-damage leading to a greater apoptotic rate and index [48]. HDAC6 may be able to regulate B cell differentiation through regulation of apoptosis. For this study we used the selective HDAC6i, ACY-738 (N-hydroxy-2-(1-phenylcyclopropylamino) pyrimidine-5-carboxamide).

2. Materials and methods

2.1. ACY-738 bioavailability

Male C57BL/6 mice were injected intraperitoneally (i.p.) with ACY-738 (5 mg/kg) in 10% DMAC/10% HS15/80% saline. Plasma was obtained from blood collected at various time points. The concentration of ACY-738 in the plasma was determined by mass spectrometric detection from blood collected at various time points. The concentration of ACY-738(5 mg/kg) in 10% DMAC/10% HS15/80% saline. Plasma was obtained from blood collected at various time points. The concentration of ACY-738(5 mg/kg) in 10% DMAC/10% HS15/80% saline. Plasma was obtained from blood collected at various time points. The concentration of ACY-738(5 mg/kg) in 10% DMAC/10% HS15/80% saline. Plasma was obtained from blood collected at various time points. The concentration of ACY-738(5 mg/kg) in 10% DMAC/10% HS15/80% saline. Plasma was obtained from blood collected at various time points.

2.2. Mice

Female NZB/W F1 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were used in accordance with the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University and housed in the animal facility at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM, Blacksburg, VA, USA).

2.3. In vivo treatment

Mice were injected i.p. 5 days/week with the vehicle control (DMSO), ACY-738 treatment at 5 mg/kg (low-dose), or ACY-738 treatment at 20 mg/kg (high-dose) beginning at 22-weeks-of-age until euthanasia at 38 weeks-of-age. The total volume injected was 80 μL. ACY-738 was received as a generous donation from Acetylon Pharmaceuticals for use in all studies. Proteinuria and weight were measured every 2 weeks and blood was collected every four weeks for sera analysis. Proteinuria was measured by a standard semi-quantitative test using Siemens Uristix dipsticks (Siemens Healthcare, Deerfield, IL, USA). Results were quantified according to the manufacturer’s instructions and scored as follows: dipstick reading of 0 mg/dL = 0, trace = 1, 30–100 mg/dL = 2, 100–300 mg/dL = 3, 300–2000 mg/dL = 4, and 2000+ mg/dL = 5.

2.4. Measurement of autoantibodies

Sera were collected prior to initiation of treatment at 22 weeks-of-age and every 4 weeks until euthanasia. The mice were anesthetized using isoflurane (Piramal Healthcare, Mumbai, Maharashtra, India) and bled from the retro-orbital sinus. Blood was allowed to clot for 2 h and then centrifuged for 15 min at 10,000 × g. The levels of sera antibodies to dsDNA were measured by ELISA. Sera samples were added to the plate at a 1:100 dilution, followed by a two-fold serial dilution. The plate was read at 380 nm on a Spectrax 3400C microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A final dilution of 1:800 was reported.

2.5. Isolation of B cells from the BM

BM cells were flushed in PBS with 1% BSA from the femurs of NZB/W mice following euthanasia. Red blood cells (RBCs) were lysed using ammonium chloride potassium (ACK) lysing solution. Single-cell suspensions were then washed and stained with Allo-Phycocyanin (APC)-conjugated anti-mouse B220 and Fluorescein Isothiocyanate (FITC)-conjugated anti-mouse CD43 mAbs to identify pro-B cell (B220+CD43+) and pre-B cell (B220+CD43+) populations. Pre-B cell populations were further stained with Phycoerythrin (PE)-conjugated anti-mouse B220 and PerC/5 or Peridinin-chlorophyll proteins (PerCP)-conjugated anti-mouse CD44 mAbs to identify fractions A (B220+CD43+CD44-BP1+), B (B220+CD43+CD44-BP1+), C (B220+CD43+CD44-BP1+), and D (B220+CD43+CD44-BP1+). Pre-B cells fractions were further stained with PE-conjugated anti-mouse IgD and PE-Cy5-conjugated anti-mouse IgM mAbs to identify fractions E (B220+CD43+IgM+B1-), F (B220+CD43+IgM+B1+). Fractions were measured by flow cytometric analysis. All antibodies were purchased from ebioscience (San Diego, CA, USA). Flow cytometry was performed at the College of Veterinary Medicine Flow Cytometry Core Facility using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry data was analyzed using FlowJo Software (Tree Star, Ashland, OR, USA).

2.6. Isolation of splenic B cells

Following euthanasia, spleens were removed and single-cell suspensions of splenocytes were incubated with PerCP710 conjugated IgM, FITC conjugated AA4.1, PE conjugated CD23, and APC conjugated CD21 anti-mouse mAbs (ebioscience, San Diego, CA, USA). IgM+ cells were analyzed for the expression of AA4.1, CD23 and CD21 and divided into the following developmental stages using flow cytometry: T1 (IgM+CD23−AA4.1+CD21+), T2 (IgM+CD23−AA4.1+CD211), T3 (IgM+CD23−AA4.1+CD21+) , T0 (IgM−CD23−AA4.1−CD21−), MZ
Fig. 1. Evaluation of pro- and pre-B cells in NZB/W mice. BM cells were harvested from pre-diseased and diseased NZB/W mice and labeled with fluorescently tagged antibodies specific for pro- and pre-B cells. (A) Representative images of B cells labeled with CD43 and B220. (B) There were no significant differences in the percentage of B cells in the pro- or pre-B subsets between pre-diseased and diseased NZB/W mice. (C) The pro-B cell subset was further divided into fractions A (CD24$^{-}$BP1$^{-}$), B (CD24$^{+}$BP1$^{-}$), C (CD24$^{+}$BP1$^{+}$) and C$'$ (CD24$^{+}$BP1$^{+}$). Representative flow cytometry image of pro-B cell fractions A, B, C, and C$'$ from pre-diseased and diseased NZB/W mice. (D) There were significantly fewer pro-B cells in the C and C$'$ fractions in diseased NZB/W mice. (E) The pre-B cell subset was further divided into fractions D (IgM$^{-}$IgD$^{-}$), E (IgM$^{+}$IgD$^{-}$), and F (IgM$^{+}$IgD$^{+}$). Representative images of pre-B cell subsets from NZB/W mice. (F) Diseased NZB/W mice had significantly decreased numbers of cells in fraction D and E, yet significantly increased percentages of cells in fraction F ($n=3$; $^*$p < 0.05, $^{**}$p < 0.005, $^{***}$p < 0.0005).
Kidney sections were scored (0–4) for glomerular proliferation, inflammation, crescent formation, necrosis, and by a pathologist, in a blinded manner.

2.9. Immunofluorescence staining

One half of each kidney was placed in OCT media and snap-frozen in a slurry containing dry ice and 2-methylbutane (Fisher Scientific, Hampton, NH, USA). Frozen kidney sections were cut into 3 μm sections and stained with goat anti-mouse IgG conjugated to FITC (Pierce) diluted 1:100 or goat anti-mouse C3-FITC (Pierce, Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted 1:100. Kidney sections were examined by fluorescent microscopy. Sections were scored (0–4) for immune complex deposition by a pathologist in a blinded manner.

2.10. Isolation of T cells

A single-cell suspension was obtained from the thymuses and spleens of treated NZB/W mice at 38 weeks-of-age. Briefly, the thymus was removed from each NZB/W mouse and dissociated across a sterile wire mesh in a petri dish containing ice-cold RPMI 1640 medium (Thermo Scientific). RBCs were lysed using RBC lysis buffer and cells were pelleted and washed with PBS. Splenocytes were stained with APC-conjugated anti-mouse CD3 (APC-CD3), FITC-conjugated anti-mouse CD4 (FITC-CD4), eFluor450 (eF450)-conjugated anti-mouse CD8a, PerCP-CY5.5-conjugated anti-mouse CD25, and PE-conjugated anti-mouse Foxp3. Cells were fixed and permeabilized prior to staining with Foxp3. Thymocytes were stained with APC-CD3, FITC-CD4, and PE-conjugated anti-mouse CD8 mAbs (eBioscience, San Diego, CA, USA). Fluorescence was measured using a FACScan flow cytometer and data was analyzed by FlowJo software.

2.11. Glomerular isolation

The cortical tissue was isolated from one kidney of each mouse and pooled by treatment group. Briefly, cortical tissue was pressed through grading sieves and resuspended in 750 U/mL Worthington type I collagenase at 37 °C for 20 min. Glomerular cells were pelleted, resuspended in RNAlater (QIAGEN, Valencia, CA, USA), and stored at −20 °C until RNA isolation.

2.12. Isolation of RNA

RNA was isolated using the mirVana miRNA isolation kit according to the manufacturer’s protocol (Applied Biosystems, Carlsbad, CA, USA). The eluates were quantified on a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA). An aliquot was taken and

![Fig. 2. ACY-738 PK parameters. PK data following i.p. injection of 5 mg/kg ACY-738. ACY-738 reached a maximum plasma concentration of 1310 ng/ml at 0.0830 h following treatment.]
diluted to 1 ng/μL for real-time RT-PCR. The eluted RNA was stored at −80 °C.

2.13. Real-time RT-PCR

IL-6, IL-10 and TGF-β mRNA expressions were measured using TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA, USA). The ΔΔCt was calculated using the endogenous control GAPDH, and then the ΔCt was determined by calculating the fold change in expression between the NZB/W mice treated with ACY-738 and the DMSO-treated controls. All samples were run in triplicate.

2.14. ELISA

IL-1β and TGF-β levels were measured from the sera by ELISA according to the manufacturer’s protocol (eBioscience, San Diego, CA, USA). The plate was read at 450 nm on a microplate spectrophotometer.

2.15. Statistics

Statistical analysis was performed using Student’s unpaired t-test (two-tailed). A linear regression analysis was used to determine the relationship between age and cytokine production following treatment...
with ACY-738. \( P \) values less than 0.05 were considered statistically significant.

3. Results

3.1. BM differentiation of B cells is altered in diseased NZB/W mice

During B cell development in the BM, autoreactive B lymphocytes can be removed by a number of mechanisms. In SLE, one or more of these mechanisms may be defective at each of the B cell development stages. To determine if B cell development was altered in the BM of lupus mice, pro- and pre-B cell differentiation was evaluated in pre-diseased and diseased NZB/W mice. BM cells were harvested from NZB/W mice at 8 (pre-diseased) or 38 weeks-of-age (diseased) and sorted into pro-B cell (CD43+ B220+) and pre-B cell (CD43−B220+) populations (Fig. 1A–B). There were no significant differences in the percentages of pro- or pre-B cells between diseased and pre-diseased mice (Fig. 1B).

To characterize B cell development further, pro-B cells were divided into developmental fractions A (B220+ CD43+ CD24+ BP1−), B (B220+ CD43+ CD24+ BP1−), C (B220+ CD43+ CD24+ BP1−), and C′ (B220+ CD43+ CD24+ BP1−) (Fig. 1C–D). Diseased NZB/W mice had significantly fewer cells in fractions C and C′ when compared to pre-diseased mice (Fig. 1D). Pre-B cells were further divided into fractions D (B220+ CD43− IgM− IgD−), E (B220+ CD43− IgM− IgD−) and F (B220+ CD43− IgM+ IgD+)(Fig. 1E–F). Diseased NZB/W mice had markedly fewer cells in fractions D and E, but a significant increase in the percentage of cells in fraction F (Fig. 1F). Diseased NZB/W mice have increased survival of cells during BM B cell differentiation leading to an accumulation of cells in the late pre-B cell fraction F. These results suggest that BM B cell development progresses more rapidly through developmental stages during SLE disease, without the proper removal...
3.2. Selectivity and bioavailability of ACY-738 were determined

ACY-738 is a HDAC6 selective inhibitor [49]. To determine the dose that would achieve a plasma concentration of approximately 2 nM for up to 4, we performed a pharmacokinetic study using a single dose of ACY-738 (5 mg/kg) by i.p. injection. We found that the plasma levels 4 h after the dose average 5.6 nM (Fig. 2). A dose four fold higher than this was also well tolerated in mice and would be expected to extend the biologically active concentration for several hours longer. These doses would be expected to induce the acetylation of histones through class I HDAC inhibition, although for a shorter time period [50].

3.3. Treatment with ACY-738 alters BM B cell differentiation in vivo

We tested whether pharmacologic inhibition HDAC6 would be able to alter B cell BM differentiation in NZB/W mice. Following treatment with ACY-738, the percentage of pro- and pre-B cells was determined using flow cytometric analysis. The percentage of pre-B cells was not significantly altered following HDAC6 inhibition (Fig. 3A–B). However, the treatment did alter the percentage of cells in developmental fractions A, B, C, and C' by increasing the percentage of cells in fractions A and C (Fig. 3C–D). The pre-B cell population was significantly decreased in a dose-dependent manner following HDAC6 inhibition in NZB/W mice (Fig. 3A, E). The developmental pre-B cell stages were also significantly altered. At both the 20 mg/kg BW dose and 5 mg/kg BW dose of ACY-738, there was a significant increase in the percentage of cells in fractions D and E that corresponded with a decrease in cells in fraction F (Fig. 3F–G).

3.4. Splenic and peripheral B cell populations were not significantly altered by HDAC6 inhibition

Previous studies have shown abnormal numbers of splenic B cells from SLE patients in the transitional and MZ developmental stages [51]. We tested whether treatment with ACY-738 was able to correct the abnormal populations of splenic B cells in lupus-prone mice. Splenic B cells were sorted into their developmental stages T1, T2, T3, F0, B1, and MZ. However, treatment with ACY-738 did not significantly affect these populations of B cells at either the 20 mg/kg BW dose or 5 mg/kg BW dose (Fig. 4A).

We investigated whether HDAC6 inhibition was able to alter peripheral B cell populations since previous reports have shown that there are...
major differences in peripheral B cells between SLE patients and healthy controls [19,52]. A single-cell suspension was obtained from the blood of 38-week-old NZB/W mice and stained for IgM and B220. Treatment had no effect on the percentage of peripheral B cells (IgM+ B220+) in 38-week-old NZB/W mice (Fig. 4B–C).

3.5. Inhibition of HDAC6 alters thymic T cell development

SLE patients and lupus-prone murine models have been reported to have abnormal expression of T cells [7,53,54]. Of particular interest is an increase in the DN (CD3+ CD4− CD8−) T cell population. It is believed that the DN T cell population can lead to the induction of pathogenic autoantibodies. We tested whether treatment with ACY-738 was able to alter the percentage of DN T cells. HDAC6 inhibition resulted in a substantial decrease in CD4− CD8− T cells coupled with a significant increase in DP (CD3+ CD4+ CD8+) T cells (Fig. 4B, D). There were also significant decreases in the number of CD3+ CD4+ CD8− and CD3+− CD4− CD8+ single positive (SP) T cells, but an increase in the percentage of double positive (CD3+ CD4+ CD8+) T cells (Fig. 5B, C).

3.6. Inhibition of HDAC6 increased the number of regulatory T cells in the spleen

The Treg phenotype was assessed due to its role in the maintenance of self-tolerance and the prevention of autoimmune disease [55]. During active SLE the overall number and function of Treg cells is reduced [56]. Splenocytes were obtained from 38-week-old NZB/W mice and stained with CD4, CD25, and Foxp3. Treatment with ACY-738 resulted in a significant increase in the percentage of Treg cells at the 20 mg/kg BW dose and 5 mg/kg BW dose compared to mice treated with vehicle control alone (Fig. 6A–B).

Fig. 7. Assessment of survival rate and disease progression in NZB/W mice. (A) By 38 weeks-of-age, half of the NZB/W mice receiving the vehicle control alone had died. No mice receiving the HDAC6i died before termination of the study. (B) Body weight increased as NZB/W mice aged, until mice reached the terminal stage of disease. There was significant weight loss in NZB/W mice treated with the vehicle control alone. There was no significant weight loss in mice that received ACY-738 treatment. (C) Measurement of proteinuria every 2 weeks in NZB/W mice being treated with ACY-738 (5 mg/kg in DMSO), ACY-738 (20 mg/kg in DMSO) or vehicle control (DMSO) from 22 to 38 weeks-of-age. Proteinuria gradually increased as the NZB/W mice treated with the vehicle control or the low-dose of ACY-738 aged. However, treatment with the high-dose of ACY-738 prevented proteinuria from increasing in NZB/W mice. (D) Average spleen weight by group was determined following euthanization of mice at 38 weeks-of-age. HDAC6 inhibition significantly decreased spleen weight in NZB/W mice. (E) Spleen:body weight ratio was calculated and multiplied by 100. Treatment with ACY-738 decreased the spleen:body weight ratio in a dose-dependent manner (n ≥ 5; *p < 0.05, **p < 0.005).
3.7. HDAC6 inhibition prolonged survival of NZB/W mice and decreased urinary markers of SLE and splenomegaly

HDAC6 inhibition was evaluated for its efficacy in decreasing SLE markers of disease and prolonging the survival of NZB/W mice. All mice receiving either the 20 mg/kg BW dose and 5 mg/kg BW dose of ACY-738 survived to the completion of the study. However, half of the NZB/W mice receiving the vehicle control alone died before termination of the study at 38 weeks-of-age (Fig. 7A). Body weight increased as NZB/W mice aged following treatment with ACY-738. Vehicle control-treated NZB/W mice experienced weight loss concomitantly with increased proteinuria (Fig. 7B). Throughout the treatment period, NZB/W mice were monitored for changes in proteinuria and body weight. Treatment with 20 mg/kg ACY-738 significantly attenuated the severity of proteinuria in NZB/W mice (Fig. 7C). Because SLE is characterized by enlargement of the spleen, splenomegaly was assessed in mice following euthanasia by determining the total spleen weight along with the spleen:body weight ratio. Both spleen weight and spleen:body weight ratio were decreased following HDAC6 inhibition in a dose-dependent manner (Fig. 7D–E).

3.8. Treatment with ACY-738 reduced serum anti-dsDNA and altered Ig isotype levels

Ig isotype levels were assessed due to previous studies linking elevated IgG levels to glomerulonephritis in both NZB/W mice and human SLE [57]. Diseased NZB/W mice have elevated production of anti-dsDNA as well as increased production of IgM and IgG isotypes (specifically IgG2a) [58]. To determine the effect of specific HDAC6 inhibition on disease in NZB/W mice, serum anti-dsDNA and Ig isotype levels were measured every 4 weeks beginning at 22 weeks-of-age. Anti-dsDNA, IgG2a, and total IgG levels gradually increased as the mice aged (Fig. 8A–C). There were no significant differences between the groups in anti-dsDNA at 22 weeks-of-age. Treatment with the high dose of ACY-738 was able to prevent an increase of anti-dsDNA as the mice aged. NZB/W mice that received 5 mg/kg ACY-738 showed a significant decrease in anti-dsDNA production as they aged when compared to vehicle control-treated mice (p < 0.05). Following treatment with the 20 mg/kg dose, the decrease in autoantibody production was more pronounced compared to NZB/W mice that received the lower dose or vehicle control alone (Fig. 8A). The HDAC6 treatment significantly decreased levels of IgG2a and total IgG (Fig. 8B–C).

3.9. HDAC6 inhibition prevented TGF-β and IL-1β production from being altered as NZB/W mice aged

Elevated IL-1β levels have been reported to play a role in the pathogenicity of a number of autoimmune diseases including SLE [59,60]. Conversely, lymphocyte production of TGF-β has been shown to be decreased during active SLE [61]. Beginning at 22 weeks-of-age, cytokine levels were measured in the sera every 4 weeks until euthanasia at 38 weeks-of-age (Fig. 9A–B). As the NZB/W mice aged, sera levels of TGF-β decreased whereas levels of IL-1β increased (Fig. 9A–B). HDAC6 inhibition attenuated the reduction of TGF-β production as the mice aged in a dose-dependent manner (Fig. 9A). Treatment with both the low and high dose of ACY-738 attenuated sera IL-1β production as the NZB/W mice aged (Fig. 9B).

3.10. Glomerular IL-10, TGF-β, and IL-6 mRNA expression is decreased following HDAC inhibition in vivo

We next sought to determine whether ACY-738 would alter glomerular mRNA expression in NZB/W mice. NZB/W mice develop renal disease around 20 weeks-of-age, progressing to severe glomerulonephritis by 36 weeks-of-age [62]. Altered mRNA glomerular expression can lead to fibrosis and irreversable glomerular damage [63]. The balance between Th1 and Th2 cytokines plays a critical role in the immune response and the pathogenesis of autoimmune disease. IL-10 has been reported to be elevated in SLE and plays a critical role in B cell survival, differentiation, and Ig secretion. Inhibiting IL-10 has been demonstrated to decrease disease; while administration of IL-10 has been shown to accelerate disease in lupus-prone mice [64]. TGF-β has been shown to play a dual role in SLE pathogenesis. In NZB/W mice, TGF-β is produced in the kidneys to counter inflammation resulting from autoantibody production [65]. IL-6 induces polyclonal B-cell activation and autoantibody production during SLE. Studies have demonstrated increased expression of the proinflammatory cytokine, IL-6, in lupus kidneys [66].

Following euthanasia, glomeruli were isolated from the kidneys and RNA was extracted. Relative glomerular mRNA expression of IL-10, TGF-β, and IL-6 were determined using real-time RT-PCR. Administration of ACY-738 (5 mg/kg) was able to significantly reduce glomerular IL-6 and IL-10 mRNA levels by more than 50% while treatment with 20 mg/kg ACY-738 reduced IL-6 and IL-10 mRNA to non-detectable levels (Fig. 10). Furthermore, glomerular TGF-β mRNA was decreased following inhibition of ACY-738 in a dose-dependent manner (Fig. 10). Taken together these results suggest that HDAC6 inhibition by ACY-738 reduces production of SLE-associated cytokine mRNA.

3.11. Glomerular immune complex deposition is reduced following HDAC6 inhibition

We sought to determine whether ACY-738 decreased immune complex deposition and complement activation in renal tissue. Treatment with the high-dose of ACY-738 was able to decrease both the number of glomeruli with C3 and IgG deposition as well as the overall level of deposition within each glomerulus (Fig. 11A). Mice that received 5 mg/kg ACY-738 had a slight decrease in C3 and IgG deposition (data not shown). Treatment with 20 mg/kg of ACY-738 resulted in decreased IgG and C3 staining compared to vehicle control-treated mice (Fig. 11B).
Each kidney was scored in a blinded manner for fluorescence intensity. Treatment with the 20 mg/kg dose significantly decreased IgG and C3 deposition, however, 5 mg/kg ACY-738 showed no significant effect on IgG and C3 deposition compared to mice that received vehicle control alone (Fig. 11C–D).

3.12. HDAC6 inhibition decreased SLE renal pathology

In order to assess renal disease, kidney sections were embedded in paraffin and stained by PAS. NZB/W mice have been shown to develop severe renal disease by 32 weeks-of-age. NZB/W mice treated with DMSO alone had thickened, irregular glomerular basement membranes, increased cellularity, fibrosis and crescent formation (Fig. 12A). Treatment with 5 mg/kg ACY-738 did not alter kidney pathology. However, kidneys from mice treated with 20 mg/kg of ACY-738 had significantly reduced SLE renal pathology characterized by the lack of fibrosis and crescent bodies (Fig. 12B). All kidneys were scored (0–4) by a pathologist, in a blinded manner. NZB/W mice that were treated with the vehicle control alone received an average score of 4 compared to an average score of 2 from mice that received the high-dose of ACY-738 (Fig. 12C).

4. Discussion

In our studies, we found altered differentiation of B cells in the BM of diseased NZB/W mice characterized by increased survival of differentiating B cells resulting in the accumulation of cells in the late pre-B cell fraction. Furthermore, diseased NZB/W mice had decreased percentages of cells in the late pro-B cell/early pre-B cell phases when compared to pre-diseased mice. Treatment with ACY-738 was able to reverse these abnormalities by altering percentages of cells in late pro- and early pre-B cells fractions, and more specifically decreasing the accumulation of B cells in fraction F. HDAC6 inhibition was also able to alter T cell differentiation evidenced by increased Treg cells and decreased thymic DN T cells after 16 weeks of HDAC6i therapy. These results suggest that B and T cell differentiation play an important role in SLE pathogenesis and that inhibition of HDAC6 may be effective in correcting aberrant lymphocyte development. Indeed, a B cell...
targeting therapy (belimumab) has received Food and Drug Administration (FDA) approval for its use in SLE [67]. Furthermore, other new drugs inhibit the proteasome and immune cell signaling molecules such as Btk, ROCK and CaMK4 are actively being investigated as lupus therapies [68].

Normally, B cells that develop autoreactive B cell receptors (BCRs) are removed by both positive and negative selection from the BM in three ways: receptor editing, deletion, and anergy [10]. In healthy individuals, approximately 55–75% of the repertoire produced by Ig gene rearrangement in the BM is autoreactive [69]. These autoreactive B cells are removed at two checkpoints [17]. The majority of autoreactive B cells are removed in the BM while the cells are still immature [70]. Previous studies have shown that early checkpoints during B cell development in the BM are abnormal in SLE resulting in increased numbers of self-reactive B cells in circulation [69]. The current studies support the notion that there exists a defect in one or more of the developmental checkpoints during BM B cell development. While our study showed no difference in the overall number of pro- or pre-B cells between pre-diseased and diseased NZB/W mice, there were altered proportions of cells in the pro- and pre-B cell developmental fractions. When compared to pre-diseased mice, diseased NZB/W mice had decreased numbers of B cells in late pro-B cell fractions (C and C′) and early pre-B cell fractions (D and E). There was also a notable increase in the percentage of B cells in the late pre-B stage, fraction F. These data suggest that diseased NZB/W mice are unable to support proper B cell differentiation in the BM possibly due to altered regulation of developmental checkpoints, resulting in failure to remove defective B cells. It was noted that while Fr.'s C and C′ were decreased in diseased mice compared to the control (Fig. 1D), treatment with ACY-738 led to increases of Fr. A and Fr. C in the bone marrow of these mice (Fig. 3D). It is likely that inhibition of HDAC6 blocked two distinct checkpoints of B cell development—i.e., the stages represented by Fr. B and Fr. D, respectively—causing cells of Fr. A and Fr. C/C′ to accumulate. A common feature of Fr. B and Fr. D are enhanced activities of Rag1 and Rag2 that control V(D)J recombination of μH locus and κ locus, respectively [10]. Thus, we speculate that HDAC6i could potentially cause cytoplasmic sequestration of Rag2, which is known to be translocated out of the

Fig. 10. Glomerular mRNA levels were assessed in 38-week-old mice. Relative glomerular levels of IL-6, IL-10, and TGF-β mRNA were determined using real time RT-PCR. Treatment with ACY-738 decreased IL-6, IL-10, and TGF-β in a dose-dependent manner. Following treatment with 20 mg/kg ACY-738, IL-6 and IL-10 were undetectable (n ≥ 5; *p < 0.05, **p < 0.005, ***p < 0.0005).

Fig. 11. HDAC6 inhibition decreased glomerular immune complex deposition. 5 μM kidney sections were stained with FITC-conjugated C3 or IgG and assessed for fluorescence intensity. (A–B) Representative images of glomerular deposition of both C3 and IgG in NZB/W mice treated with the vehicle control or the high dose of ACY-738. (C–D) IgG and C3 deposition were evaluated by a pathologist in a blinded manner and scored (0–4) for the level and frequency of fluorescence. Both IgG and C3 deposition levels were significantly decreased following 16 weeks of ACY-738 treatment (n ≥ 5; *p < 0.05, **p < 0.005).
nucleus and degraded blocking the V(D)J recombination checkpoints in Fr.’s B and D [71]. Under normal conditions, the apoptotic index and the apoptotic rate, for the removal of autoreactive B cells, are greatest around the pro/pre-B cell transition [70,72]. Our results show that the greatest difference in the alteration of B cell development in diseased NZB/W mice occurs at the pre-B/pro-B stages indicating a possible apoptotic defect in the bone marrow.

ACY-738 is a selective HDAC6 inhibitor with minimal reactivity against other class II HDACs and 100-fold less selectivity against class I HDACs [49]. Jochems et al. showed a dose-dependent enzymatic selective inhibition of recombinant HDCA6 over HDACS 1, 2, and 3 following treatment with ACY-738. Class I HDAC inhibition is known to significantly reduce the total number of peripheral lymphocytes [49]. There was no significant difference in the numbers of peripheral lymphocytes, WBCs, RBCs, or platelets between treatment groups, indicating further that Class I HDACs were not inhibited by ACY-738.

After immature B cells leave the BM, they continue to develop in secondary lymphoid organs including the spleen [10]. As immature B cells develop into mature B cells they become antigen-specific [10]. B cells that escape negative selection during BM differentiation may mature in the spleen to become marginal zone or follicular cells [73]. IgM⁺ IgD⁺ B cells leave the BM as transitional cells and enter the spleen where they mature into follicular B cells or marginal-zone B cells [10,73]. ACY-738 did not alter the percentages of B cells in transitional, follicular or

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**Fig. 12.** SLE-associated renal pathology was decreased following ACY-738 therapy. Paraffin-embedded kidneys were sectioned and then stained with PAS. (A) Representative image of severe glomerulonephritis (thickened GBM and crescent formations) from an NZB/W mouse treated with the vehicle control alone. (B) When treated with ACY-738 (20 mg/kg), NZB/W mice had improved renal pathology. (C) Sections were assessed for glomerular proliferation, inflammation, number of nuclei per glomerulus, crescent formation, and fibrosis by a blinded pathologist, and a glomerular score (0–4) was assigned. The ACY-738 treatment significantly decreased SLE kidney pathology (n ≥ 5; *p < 0.05).
marginal zone stages. These results suggest that ACY-738 is acting during early B cell development in the BM and not on peripheral or splenic B cells.

Anti-dsDNA is produced by autoreactive B cells that escape negative and positive selection during B cell development [3,17]. Our studies showed that as NZB/W mice aged, auto-antibody production increased, which corresponded with a concomitant increase in proteinuria. IgG autoantibodies are responsible for glomerulonephritis associated with SLE. When IgG autoantibodies encounter antigen, they bind to form immune complexes that become lodged in renal glomeruli, resulting in increased activation of the immune system and inflammation [74]. The damaged kidneys are then unable to properly filter proteins, which pass into the urine and cause elevated levels of proteinuria [3]. Our study showed that decreased IgG production in the sera following HDAC6 inhibition, correlated with reduced glomerular immune complex deposition, SLE-associated kidney pathology, and proteinuria. This indicates that although ACY-738 does not affect numbers of peripheral B cells, it does alter mature plasma cell production of autoantibodies.

Our results showed that inhibition of HDAC6 is able to increase the T_{reg} phenotype, which correlated with a decrease in SLE-associated markers of disease. T_{reg} cells have been shown to directly influence Ab production as depletion of T_{reg} cells in mice leads to overproduction of Abs. Conversely, adoptive transfer of T_{reg} cells to autoimmune prone mice, significantly decreases Ab production and disease pathology [75, 76]. At 38 weeks-of-age, NZB/W mice that received 20 mg/kg ACY-738 had significantly higher numbers of T_{reg} cells and reduced levels of autoantibody production when compared to vehicle-control treated mice. Previous research has shown that treatment with ACY-738 is able to increase the suppressive function of T_{reg} cells in vitro [42]. Research indicates that a decrease in T_{reg} numbers and function may contribute to SLE pathogenesis due to their important role in the regulation of the immune system and the suppression of autoantibody-producing B cells [56,77]. Studies have reported that T_{reg} cell numbers are reduced during active SLE, but numbers are increased following treatment and clinical improvement [56]. It has been demonstrated that pan-HDAC inhibitors, but not class I specific HDAC inhibitors, are able to increase populations of T_{reg} cells [39]. It is possible that HDAC inhibition may stabilize T_{reg} cells and enhance their suppressive function [78].

Naïve CD4^{+} T cells differentiate into T_{reg} cells following TGF-β stimulation, which promotes Foxp3 expression [79]. Our results showed that as NZB/W mice aged, sera TGF-β levels were reduced; however, treatment with ACY-738 was able to ameliorate the reduction of TGF-β in a dose-dependent manner. This correlated with an increase in the T_{reg} population in NZB/W mice treated with ACY-738. Conversely, glomerular mRNA expression of TGF-β was decreased following HDACi treatment indicating a dual role of TGF-β in SLE pathogenesis. Previous studies have shown a correlation between decreased TGF-β levels in lymphoid tissues and an increase in autoantibody production leading to a proinflammatory environment [65]. Anti-inflammatory cytokines including TGF-β are produced in order to combat inflammation within target organs such as the kidneys. The increased production of anti-inflammatory cytokines causes deposition of extracellular matrix and fibrosis [80]. Elevated levels of TGF-β in immune cells can coincide with a reduction in TGF-β in target organs leading to autoimmune disease including lupus [65,80,81]. Our results showed the ability of HDACi inhibition to reverse these trends by increasing the sera levels of TGF-β to induce a T_{reg} phenotype, while decreasing TGF-β in the glomeruli of the kidneys that may lead to fibrosis.

During SLE there is an imbalance between the production of Th1 and Th2, and Th17 cytokines [82,83]. We investigated whether HDACi therapy was able to reverse altered IL-6, IL-10, and IL-1β cytokine production trends that are characteristic of SLE. Following treatment with ACY-738 (20 mg/kg), IL-6 was undetectable in the kidneys of NZB/W mice. The proinflammatory cytokine IL-6 is upregulated in SLE and contributes to overproduction of IgG [84,85]. The reduction in IL-6 correlated with a decrease in total IgG and the IgG2a isotype levels in the sera. Similarly, elevated levels of the proinflammatory cytokine IL-1β have been reported to play a role in the pathogenesis of a number of autoimmune diseases including SLE [86,87]. In lupus-prone mice that are deficient in IL-1β, sera levels of autoantibodies are decreased and the manifestation of disease is much milder [88]. Our results indicate that ACY-738 is able to decrease IL-1β in a dose-dependent manner and correlates with the reduction in dsDNA autoantibody levels.

Our studies showed that ACY-738 decreased several characteristics of SLE in NZB/W mice by dictating B cell development in the bone marrow. We found that there was a decrease in the percentages of cells in early B cell developmental stages and an increase in the number of cells in late B cell BM developmental stages during disease in NZB/W mice. ACY-738 treatment increased the percentage of B cells in early developmental stages, while decreasing the percentage of cells in late B cell fraction F. ACY-738 regulation of BM B cell development could be due to regulation developmental checkpoints known to be dysfunctional during SLE. Studies are currently underway to define the mechanism through which ACY-738 regulates abnormal B cell development observed in SLE.

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