Selective histone deacetlyase inhibition decreases disease in lupus-prone mice

Adrian L. Castaneda

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Christopher Reilly, Chair
Xin Luo
Liwu Li
Yong Woo Lee

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Abstract

Histone deacetylase 6 (HDAC6) is a cytoplasmic enzyme that acetylates several proteins that are involved in the immune response. HDAC6 inhibition has been shown in various models to decrease inflammation by altering various proteins involved in the dysregulation of B and T cell responses. In our current studies we sought to determine if HDAC6 inhibition would decrease disease in lupus-prone mice using two murine mouse models of SLE: MRL/lpr mice and NZB/W F1 mice. Both mouse models were fed a rodent diet formulated with the selective HDAC6 inhibitor ACY-738 (N-hydroxy-2-(1-phenylcycloproylamino) pyrimidine-5-carboxamide). NZBW mice received 18 weeks of treatment starting at 16-weeks-of-age and had an average of 57.3 +/- 14.6 ng/mL of ACY-738 in the plasma. MRL/lpr mice received 7 weeks of treatment starting at 11-weeks-of-age and had an average of 78.5 +/- 17.3 ng/mL of ACY-738 in the plasma. Controls received either dexamethasone 5x a week or were left untreated. As the mice aged, body weight, urine protein, and blood sera was collected weekly. Spleen cells were isolated following euthanasia for flow cytometry and kidneys were also collected for histological analyses. We found that in both mouse models that mice treated with ACY-738 had reduced splenic weight and IgG immunoglobulin isotypes. MRL/lpr mice that were treated with ACY-738 had a reduction in the number of IL-17+, RORγ-t TH17 cells. NZBW/ F1 mice that received ACY-738 treatment also had a reduction in the TH17 cells and we observed a significant reduction in kidney pathology. Selective HDAC6 targeting may warrant future investigations as a potential therapeutic target for the treatment of SLE.

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Introduction

Inflammation and regulation of the immune system involve complex regulatory pathways that are dependent on environmental, hormonal, genetic and epigenetic factors. Epigenetic factors are stable and heritable modifications to gene expression that do not involve altering the DNA sequence (Cedar & Bergman, 2009, Bayarsaihan, 2011). These factors are dependent upon environmental stimuli and can alter cellular function that can result in autoimmune disease (Feinberg, 2007, Lu, 2013). These epigenetic changes include DNA methylation, microRNA (miRNA) expression, and protein acetylation. The acetylation status of a protein can have a profound effect on its functional implication that affect a wide variety of cellular processes (Batta *et al.*, 2007, Bannister & Kouzarides, 2011). Proper cellular function requires that both histone acetyl transferases (HATs) and histone deacetylases (HDACs) work in conjugation to control proper gene regulation (Spange *et al.*, 2009). Abnormalities in these enzyme's expression and activity has been linked to multiple inflammatory and autoimmune diseases (Elsheikh *et al.*, 2009, Reilly *et al.*, 2008, Samant *et al.*, 2015, Xu *et al.*, 2011, Rahman *et al.*, 2004, Gonneaud *et al.*, 2014, Volmar & Wahlestedt, 2015). Specific targeting of these enzymes can potentially have therapeutic value when it comes to modulating and treating disease.

HATs add acetyl groups to protein residues which allow for gene expression and protein transcription. HDACs counteract HATs and are a class of enzymes that alter histone proteins by removing acetyl groups from lysine resides (Yang & Seto, 2007). Traditionally, HDACs were thought to only be able to regulate histone proteins; however, HDACs have also been shown to regulate acetylation of over 60 non-histone proteins that are important signal regulators for a vast variety of cellular processes (Peng & Seto, 2011, Sterner & Berger, 2000, Yang & Seto, 2007). These enzymes are not only able to regulate proteins at an epigenetic level, but they are also able

to regulate transcription factors, signaling molecules, and structural proteins showing immunomodulatory function (Spange *et al.*, 2009). Due to their immunomodulatory effect, the pharmacological targeting of HDACs has been implemented as a potential target in the treatment of autoimmune disease (Hancock *et al.*, 2012, Reilly *et al.*, 2014, Falkenberg & Johnstone, 2014).

HDACs are classified into different classes (I-IV) based on their cellular location and target protein specificity (de Ruijter *et al.*, 2003). The classical HDACs consist of HDACs 1-11 and are grouped into classes I, II, and IV. These three classes of HDACs are dependent upon the zinc ion as a catalytic mechanism, whereas class III HDACs require NAD+ as a cofactor (de Ruijter *et al.*, 2003, Imai *et al.*, 2000). Class III HDACs are referred to as sirtuins (Dai & Faller, 2008). Sirtuins are structurally distinct from the other HDAC classes and contains 7 members that are ubiquitously expressed in mammals (Schemies *et al.*, 2010). The majority of HDACs are found in either the nucleus or the cytoplasm, while others are confined to specific cellular compartments (de Ruijter *et al.*, 2003, Delcuve *et al.*, 2012).

HDACs contain a nuclear localization signal (NLS) that allow them to stay within the nucleus of a cell and exert their function on nuclear proteins (Reilly, 2014). Class I HDACs consist of HDACs 1, 2, 3 and 8 (Marks, 2010). HDACs 1 and 2 stay strictly in the nucleus of the cell because they lack a nuclear export signal (Brunmeir *et al.*, 2009). HDAC3 contains both types of signals, but its activity is strictly nuclear (Zhang *et al.*, 2005, Bhaskara *et al.*, 2010). HDAC8 is both nuclear and cytoplasmic and expressed in specific cell types such as smooth muscle cells and prostate stromal cells (Waltregny *et al.*, 2004). Class II HDACs are further divided into two subclasses, class IIa and class IIb. Class IIa HDACs consist of HDACs 4,5,7, and 9 while class IIb HDACs consist of HDACs 6 and 10 (Verdin *et al.*, 2003, Martin *et al.*,

2007). HDACs 4, 5, 7, 9, and 10 can shuttle between the nucleus and the cytoplasm and play an important role in the in regulation of nuclear and cytoplasmic proteins (de Ruijter *et al.*, 2003). While HDAC6 is primarily found in the cytoplasm, it does have a nuclear localization signal (NLS) that can affect multiple cytosolic proteins (Valenzuela-Fernandez *et al.*, 2008). HDAC11 is the only member of the class IV HDACs. It is located in both the nucleus and cytoplasmic and is known to have an association with HDAC6 (Gao *et al.*, 2002, Voelter-Mahlknecht *et al.*, 2005). Due to the specificity and wide variety of cellular targets that HDACs can affect, selective targeting of these enzymes may be beneficial in the context of combatting inflammation and autoimmune disease.

Autoimmune disease is characterized by an abnormal immune response in which the body perceives a normal substance as foreign leading the body to attack itself (Aslani *et al.*, 2016, Gupta & Hawkins, 2015). In the context of systemic lupus erythematosus (SLE), the body launches an attack against nuclear antigens such as: double-stranded DNA (dsDNA) and histones (Reilly *et al.*, 2011). The most common manifestation of the disease is glomerulonephritis which is seen in about 60% of human patients (Alsuwaida, 2013, Burnett *et al.*, 2004, Hanrotel-Saliou *et al.*, 2011). While the exact etiology of the disease remains unclear, studies of monozygotic twins have shown that epigenetic factors may play a crucial role disease onset and maintenance (Ballestar, 2010, Javierre *et al.*, 2010, Lu, 2013). HDACs have been shown to not only regulate histone proteins at an epigenetic level, but they are also capable of regulating transcription factors, signaling molecules, and structural proteins showing immunomodulatory function (Dokmanovic *et al.*, 2007, Spange *et al.*, 2009). Inhibition of the activity of HDACs may be effective in treating autoimmune disease and reducing inflammation.

HDAC inhibitors have been shown to have an anti-inflammatory effect (Hancock *et al.*, 2012, Tan *et al.*, 2010, Xu *et al.*, 2011, Mishra *et al.*, 2003). This may be helpful in treating autoimmune diseases like SLE in which prolonged inflammation can lead to tissue damage and organ failure. Non-selective HDAC inhibitors such as: givinostat, vorinostat, trichostatin A, and valproate have been shown to be effective in treating several types of cancer, inflammatory bowel disease, rheumatoid arthritis (RA), and SLE (Gryder *et al.*, 2012, Reddy *et al.*, 2013, Reilly *et al.*, 2014, Zhang *et al.*, 2008). Due to the ubiquitous nature of HDACs, not only are the cellular pathways involved with autoimmunity affected, but HDAC inhibition also affects normal cellular function (Regna, *et al.*, 2015, Yang & Seto, 2007, Waltregny *et al.*, 2004). While non-selective HDAC inhibitors have been shown to treat a wide variety of disease, they also have adverse side effects (Gryder *et al.*, 2012, Hancock *et al.*, 2012, Reilly *et al.*, 2014, Tan *et al.*, 2010). Due to these side effects, a more targeted approach is necessary in order to treat autoimmune disease.

Due to the specificity of HDACs, selective targeting may allow for the modulation of histone and non-nuclear proteins for the treatment of autoimmune disease. Selective HDAC inhibitors provide a more targeted approach to treating autoimmune disease and may reduce the risk of complications from unwanted side effects (Regna *et al.*, 2016, Reilly *et al.*, 2011). HDAC6 is a primarily cytoplasmic and it represents a more suitable target for HDAC inhibition. It has been shown to regulate the acetylation of cytoplasmic and nuclear proteins that are involved in cytoskeletal protein regulation, cell migration, and degradation of proteins through deacetylation of HSP90 and α -tubulin (de Zoeten *et al.*, 2011, Regna *et al.*, 2016, Valenzuela-Fernández *et al.*, 2008). Previous work from our laboratory and others has shown that inhibition

of HDAC6 can ameliorate autoimmune disease and in transplantation (de Zoeten *et al.*, 2011, Hancock *et al.*, 2012, Regna *et al.*, 2015, Regna *et al.*, 2016, Valenzuela-Fernandez *et al.*, 2008).

Dysregulated B and T cells are thought to contribute to SLE disease pathogenesis (Alunno *et al.*, 2012, Nagy *et al.*, 2005, Ohl & Tenbrock, 2015). In addition, an imbalance in T-cell subsets also plays a role in disease maintenance (Anand *et al.*, 2002). In both lupus-prone and in human SLE patients, T-regulatory cells are diminished which is thought to contribute to immune dysregulation (Alvarado-Sanchez *et al.*, 2006, Horwitz, 2008). Selective HDAC6 inhibition treatment has been shown to increase T-regulatory cell percentages both *in vivo* and *in vitro* (de Zoeten *et al.*, 2003, Regna *et al.*, 2016). Previous work in our laboratory has been testing the selective HDAC6 inhibitor, ACY-738, in lupus-prone mice. We have shown in that treatment with ACY-738 was able to decrease specific hallmarks of SLE disease including: splenomegaly, immune complex-mediated glomerulonephritis, and sera anti-dsDNA levels in lupus-prone NZB/W mice (Regna *et al.*, 2016). Our studies have also showed that ACY-738 treatment has the ability to alter B cell differentiation within the bone marrow (Regna *et al.,* 2016). This suggests that HDAC6 inhibition is able to ameliorate SLE altering aberrant B and T cell differentiation.

B cells originate from hematopoietic stem cells in the bone marrow. As these cells mature, they develop and differentiation in a series of stages known as Hardy Fractions (Hardy R.R *et al.*, 2001). B cells start as pro-B cells ($B220^{+}CD43^{+}$) and pass through 4 developmental phases: A ($CD24^{-}BP1^{-}$), B ($CD24^{+}BP1^{-}$), C ($CD24^{lo}BP1^{+}$), and C′ ($CD24^{hi}BP1^{+}$), respectively as they undergo surface receptor editing and V(D) J rearrangement (Hardy R.R *et al.*, 2012, Alt F.W. *et al.,* 1984). Once B cells pass through the pro-B cell fractions, they downregulate CD43 and progress into the pre-B cell ($B220^{+}CD43^{-}$) phase. Pre-B cells pass through 3 fractions: D

(IgM^-IgD^-), E (IgM^+IgD^-), and F (IgM^+IgD^+) (Hardy R.R *et al.*, 2001). Once the cells begin to express IgM (fraction E), they are considered immature B cells (Ehlich *et al*., 1993, Melchers *et al.,* 2015). B cells in fraction E exit the bone marrow and continue to mature in the spleen or periphery lymphoid tissue (Melchers *et al.,* 2015). As these immature B cells begin to express IgD, they progress into fraction F and are considered mature B cells (Hardy R.R *et al.*, 2001).

As B cells undergo development within the bone marrow, there are several essential checkpoints. During these checkpoints, B cells up and down-regulate certain cell surface markers as well as undergo light and heavy chain rearrangement (Carsetti *et al.,* 2000, von Boehmer & Melchers, 2015). B cells that are to survive and continue to become mature lymphocytes receive a positive survival signal, while approximately 50% of B cells lacking this signal will undergo apoptosis (Lu L. *et al.,* 1999, Lu L. *et al.,* 2000). During SLE and autoimmune disease, B cells can become self-reactive. These cells are able to bypass these negative selection processes and are able to differentiate into autoantibody secreting plasma cells (Huang *et al*. 2011). In lupus mice we have observed that autoreactive B cells fail to either become anergic or removed; in fact we and others have found significantly altered B cell Hardy fractions within the bone marrow between NZB/W mice that are clinically diseased compared to NZB/W mice that are in a pre-diseased state (Regna et al., 2016, Lian *et al.,* 2002). This indicates that B cells are bypassing important checkpoints for negative selection that occur within the BM during normal B cell development. HDAC6 inhibitors have been shown to increase acetylation of α-tubulin, which increases microtubule stabilization (Hubbert *et al*., 2002). Studies using the HDAC6 inhibitors ACY-738 in HDAC6 siRNA knockdown experiments has shown that HDAC6 inhibition is able to increase stabilization of microtubules which slows cell proliferation and/or increases DNA damage, which can contribute to apoptosis

of autoreactive cells (Namdar *et al*., 2010). To date, there is no published research investigating the relationship between HDAC6 inhibition and B cell development within the bone marrow.

T cells develop in the thymus and differentiate into four different phenotypes which play a huge role in cell mediated immunity (Appay *et al*., 2008, Smith *et al.,* 2009). Naïve T cells can be stimulated by various cytokines to differentiate into a certain phenotype. Traditionally, it was thought that once a naïve T cell has differentiated into a certain phenotype that it was an irreversible event, but recent research has shown that there is plasticity among T cell phenotypes (Hirahara *et al*., 2013, Zhou *et al*., 2006). In the context of SLE, there is an imbalance among the T-regulatory and Th17 cell phenotype (Alunno *et al.,* 2012, Koga *et al*., 2014). Th17 cells are pro-inflammatory while T-regulatory cells are anti-inflammatory and are responsible for maintaining immune homeostasis. Our laboratory and others has found that HDAC6 inhibition has the ability to increase Foxp3 expression that resulted in an increase in the T-regulatory cell percentages (Regna *et al.,* 2016, Ooi J.D. *et al*., 2011). From this, we sought to determine if selective HDAC6 inhibition could decrease lupus pathogenesis in mouse models of SLE by reducing kidney pathology and Th17 cells.

Selective histone deacetlyase inhibition decreases disease in lupus-prone mice

Adrian Castaneda[1]

Miranda Vieson[1]

Raven Brinkley[1]

Alexander Gojermac[1]

Matthew Jarpe[2]

Christopher Reilly[1,3]

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[1]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, United States

[2]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, United States

[3]Edward Via College of Osteopathic Medicine, Blacksburg VA, 24060, United States

Correspondence address: Virginia-Maryland College of Veterinary Medicine, 205 Duck Pond Drive, Blacksburg, VA, 24061, Phone: (540) 250-2181, Fax: (540) 231-5252 chreilly@vt.edu

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown origin that is characterized by antinuclear antibody production and can involve multiple organs. The exact molecular mechanism is unknown, but it is thought that dysregulated B and T cells contribute to disease pathogenesis (Kil & Hendriks, 2013). B cells are primarily responsible for autoantibody production in SLE and recent evidence suggests that their interaction with other immune cells is important for self-tolerance to nuclear antigens and maintenance of disease (Nagy *et al.,* 2003, Sang *et al.,* 2014). An imbalance in T-cell subsets also plays a role in disease pathogenesis. CD4+ T cells help contribute to disease by facilitating autoreactive B-cell activation which leads to immune complex formation that get deposited in the kidneys (Anand *et al.,* 2002, Desai-Mehta *et al.,* 1996). In addition, T-regulatory cells are also diminished (Horwitz, 2008). This reduction in both T-regulatory cell numbers and function has been seen in SLE patents and in lupus-prone mice and is thought to contribute to immune dysregulation (Alvarado-Sanchez *et al.,* 2006).

MRL/MpJ-*Fas*^{lpr} (MRL/lpr) mice are an appropriate mouse model to study SLE as these mice develop autoimmunity similar to human SLE. These mice develop severe lymphadenopathy, autoantibody production against nuclear antigens, proteinuria, skin lesions, and immune complex glomerulonephritis (Kevil *et al.,* 2004, Swenson *et al.,* 2007). The F1 generation cross of New Zealand Black (NZB) and New Zealand White (NZW) mice generate New Zealand Black/White (NZB/W) mice that develop autoimmunity that mimics human SLE (Burnett *et al.,* 2004). These mice and humans with SLE produce autoantibodies against nuclear antigens such as: double-stranded DNA (dsDNA) and histones. They also develop immune

complex-mediated glomerulonephritis (Rottman & Willis, 2010, Theofilopoulos & Dixon, 1985).

Hyperactive B and T cells are thought to be important for both the maintenance and initiation of SLE, although the underlying mechanism remains unclear (Dorner *et al.,* 2011, Grammer & Lipsky, 2003, Mak & Kow, 2014, Ohl & Tenbrock, 2015). This hyper-reactivity is thought to be the result of defective negative selection, altered cytokine production, and decreased immune-regulatory function (al-Janadi & Raziuddin, 1992, Dorner *et al.,* 2011, Sanz, 2014). Research in both SLE patients and murine models has shown that B cell differentiation has been reported to be altered during the development in the bone marrow, spleen, and periphery (Anolik & Sanz, 2004, Gatto *et al.,* 2013, Korganow *et al.,* 2010, Matsushita, 2014). Due to the ability of a B cell to secret cytokines, antibodies, or act as an antigen-presenting cell (APC), modulation of B cell activity can have potential therapeutic value in the treatment for SLE (Cambridge *et al.,* 2008, Mok, 2014, Reddy *et al.,* 2013).

Epigenetic alteration through DNA methylation, histone modification, and microRNA signaling can alter cellular function and play a role in the pathogenicity of various autoimmune diseases (Aslani *et al.,* 2016, Gupta & Hawkins, 2015, Romanoski *et al.*, 2015). Histone deacetylases (HDACs) are a class of enzymes that alter histone and non-histone proteins by removing acetyl groups from lysine resides. Not only are these enzymes able to regulate histone proteins at an epigenetic level, but they are also able to regulate transcription factors, signaling molecules, and structural proteins showing immunomodulatory function (Dokmanovic *et al.,* 2007, Spange *et al.,* 2009). Non-selective HDAC inhibitors have been used to treat a number of autoimmune and inflammatory diseases, but can have serious side effects (Gryder *et al.,* 2012, Hancock *et al.,* 2012, Tan *et al.,* 2010). Due to these side effects, a more targeted approach is

necessary in order to treat autoimmune disease. Genetic knockout of histone deacetylase 6 (HDAC6) in mice has that these mice are viable and develop only mild abnormalities in their immune response (Zhang *et al*. 2008). Research knocking out HDAC6 has shown that HDAC6 is able to regulate the acetylation of cytoplasmic and nuclear proteins that can affect cytoskeletal protein regulation, cell migration, and degradation of proteins through deacetylation of HSP90 and α -tubulin (de Zoeten *et al.,* 2011, Valenzuela-Fernandez *et al.,* 2008, Zhang *et al.*, 2010, Zhang *et al.*, 2008). This makes HDAC6 a suitable target for inhibition.

The current studies were designed to investigate whether treatment with a selective HDAC6i would decrease disease in two mouse models of murine lupus: MRL/lpr mice and NZB/W mice. Previous work from our lab has shown that the selective HDAC6 inhibitor, ACY-738, can decrease SLE by inhibiting immune complex-mediated glomerulonephritis, decreasing sera anti-dsDNA levels, inflammatory cytokine production and increasing splenic T-regulatory cells (Regna*, et al.,* 2015, Regna *et al.,* 2016). Past work from ourselves and others has also shown that selective HDAC6 inhibition is not only able to reduce specific hallmarks of disease, but it is also capable of increasing Foxp3 expression and upregulate T-regulatory cells (de Zoeten *et al.,* 2011, Regna *et al.,* 2016, Reilly *et al.*, 2008). The current studies were done to further examine the role of HDAC6 inhibition on lupus disease. In these present studies we sought to determine if the the selective HDAC6 inhibitor ACY-738 could be administered orally to decrease disease in lupus-prone mice.

Methods and Materials

Mice

Female MRL/MpJ-Fas^{lpr}/J (MRL/lpr) and NZBW/F1 mice were purchased from Jackson laboratory and housed in the animal facility at the Virginia-Maryland College of Veterinary Medicine (Blacksburg, VA, USA). All mice were used in accordance the Institutional Animal Care and Use Committee of Virginia Tech.

In vivo treatment

NZBW mice received 18 weeks of treatment starting at 16-weeks-of-age. MRL/lpr mice received 7 weeks of treatment starting at 11-weeks-of-age. Mice in both mouse models were injected intraperitoneally 5 days a week with dexamethasone (reconstituted in hydroxypropyl methylcellulose (HPMC) so each mouse received a 2 mg/kg dose). The total volume of each injection was 50 µl. Mice chow formulated with ACY-738 at a 100mg/kg/day dose (achieving an estimated 100nm plasma concentration) and was provided by Acetylon Pharmaceuticals (Boston, MA, USA). The concentrations of ACY-738 in the plasma was determined at different time intervals by mass spectrometry detection (LC/MS) by Agilux (Worcester, MA, USA). The average ACY-738 concentration for the NZB/W mice was calculated to be 57.3 ng/mL 57.3 +/- 14.6 ng/mL of ACY-738 in the plasma. MRL/lpr mice had an average of 70.6 ng/mL +/- 17.3 ng/mL of ACY-738 in the plasma. Food and water was available ad libitum. Proteinuria and weight was measured on a weekly basis and blood was collected once a month for sera analysis. Proteinuria was assessed by using Siemens Uristix dipsticks. Results were quantified and scored according to the manufacturer's instructions. The dipstick scores were as followed: 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.

Measurement of Autoantibodies

Sera was collected prior to initiation of treatment in both mouse models. Sera was collected every 4-6 weeks until and at the time euthanasia. Mice were anesthetized using isoflurane and blood was collected from the retro-orbital sinus. Blood was allowed to clot for 2 hours and then centrifuged for 20 minutes at 16,000 x g. Sera dsDNA antibody levels were measured by semi-quantitative ELISA. High binding 96-well plates were coated with 100 µL/well of calf thymus DNA dissolved in saline-sodium citrate (SCC) buffer. Plates were covered and allowed to incubate overnight at 37°C. Plates were washed the next day with 200 µL of wash buffer (PBS + 0.05% Tween 20) 3 times. Plates were then blocked with 200 µL/well of PBS + 1% BSA and incubated at room temperature for 45 minutes. The plate was then washed three times with wash buffer and 100 µL of incubation buffer was added to the plate. Sera was diluted 1:200 in incubation buffer (PBS + 1% BSA + 0.05% Tween 20) and transferred to the plate where it was serial diluted (1:2) down the plate. The plate was covered and incubated at room temperature for 45 minutes. The plates were washed 3 more times as described above and then incubated with HRP-conjugated goat anti-mouse IgG antibody in incubation buffer (at a 1:4000 concentration). A 1:200 serial dilution was conducted down the plate and allowed to incubate for 45 minutes and washed 3 times as previously described. SureBlue Reserve TMB substrate was added and the plate was read at 380 nm on a microplate spectrophotometer. A 1:1600 dilution was reported.

Kidney Pathology

One kidney was removed at the time of euthanization and fixed in formalin. The kidney was then embedded in paraffin, sectioned, and stained for Periodic-acid Schiff (PAS). Kidneys were scored (0-4) for renal pathology by two pathologists in a blinded fashion (Drs. David Caudell and Miranda Vieson, DVM). Kidneys were graded and scored based on the following criteria: glomerular proliferation, inflammation, crescent formation, and necrosis. Kidneys were also scored according to the International Societies of Nephrology/Renal Pathology Society (ISN/RPS) pathological classification criteria of interstitial nephritis (Alsuwaida, 2013).

Splenocyte Isolation

A single-cell suspension was obtained from the spleens of 17-week-old MRL/lpr mice. Cells were dissociated using frosted slides in cold PBS + 1% BSA and centrifuged for 10 minutes at 300 x g. RBC lysis buffer was added to the cells and allowed to incubate for 5 minutes. Culture media was added to dilute the buffer and the cells were centrifuged at 300 x g. Cells were then washed 3 times and resuspended in 100 µL/well of Flow Buffer. Cells were then fixed by adding 100 µL/well of cold IC Fixation Buffer, incubated in the fridge for 1 hour, washed with cold flow buffer, and resuspended in cold PBS.

C3 and IgG Immunostaining

After euthanization, one kidney was placed in OCT media and flash frozen in a petri dish containing a small amount of dry ice and 2-methylbutane. Frozen kidneys were cut into 4 µM sections and allowed to thaw/dry at room temperature prior to staining. Slides were fixed in cold acetone and washed 3 times with PBS before being placed in a humid incubation chamber. 100 µL of antibody (at a 1:100 dilution) was added to cover each kidney section and allowed to

incubate for 1 hour. The antibody solution was then aspirated off and the slides were washed with PBS 3 times. VectaShield mounting medium was added to coverslip the slides. The edges of the coverslip were sealed with nail polish and allowed to dry overnight at 4°C. Kidneys were then scored blindly (1-4) based on the following criteria: how much of the glomeruli is affected, the type of deposits that are there (membranous or granular), and the amount of deposition present.

Measurement of sera IgG levels

Total serum IgG was evaluated by ELISA according to the manufacturer's protocol (eBiosciences Ready-SET-Go!). Briefly, 100 µL/well of coating buffer (40 µL of capture antibody in 10 mL of PBS) was used to coat the plate and incubated overnight at 4°C. The plate was washed twice with %0.05 Tween and PBS and blocked by adding 250 µL/well of blocking buffer (2.5 mL Assay Buffer A in 25 mL diH_2O) which was allowed to incubate overnight at 4°C. The plate was washed twice and the standards (200 ng/mL concentration) were added and serially diluted down the plate. Serum samples that were 10,000-fold diluted were added to the plate and 90 µL of Assay Buffer A was added to them. Detection antibody solution (50 µL/well) was added to all wells and the plate was allowed to incubate at room temperature for 3 hours. Lastly, the plate was washed 4 times and 100 µL/well of substrate solution was added and allowed to incubate for 15 minutes. Stop solution was added and the plate was read at 450 nm on spectrophotometer.

Serum IgG2a levels were evaluated by ELISA (eBiosciences Ready-SET-Go!). The plate was coated with coating buffer and incubated overnight at 4°C. The plate was washed twice as previously described and blocking buffer was added which was allowed to incubate overnight at

4 °C. The plate was washed twice and the standards were serially diluted down the plate. Serum samples were 10,000-fold diluted were added to plate along with 50 µL of Assay Buffer A. Detection antibody was added to all the wells and the plate was incubated at room temperature for 3 hours. The plate was washed 4 times and incubated for 15 minutes with substrate solution. Stop solution was added and the plate was read at 450 nm on a spectrophotometer.

Serum IgG2b levels were also evaluated by ELISA according to the manufacturer's protocol (eBiosciences Ready-SET-Go!) (eBioscience, San Diego, CA, USA). The methodology was the same as described above and the plate was read at 450nm on a spectrophotometer.

TGF-B quantification

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.

Statistical Analyses

All statistical analyses were conducted in GraphPad Prism 6. ANOVA and multiple comparison tests were used to determine the statistical significant of the data. A p-value of less than 0.05 was considered to be statistically significant.

Results

Selective HDAC6 inhibition reduces spleen size in MRL/lpr mice

Previous pharmacokinetic studies performed by our laboratory has shown that an intraperitoneal injection of ACY-738 is well tolerated in mice and is at a biologically active concentration for several hours after injection (Regna *et al.,* 2016). To investigate the effects of an oral dosing administration of ACY-738, formulated rodent diet was provided from Acetylon Pharmaceuticals (Boston, MA, USA). Thirty female MRL/lpr mice were obtained at 8 weeks of age from Jackson Laboratory. Ten mice were control in which they received no treatment, ten mice received ACY-738 formulated rodent diet, and ten mice received intraperitoneal dexamethasone injections 5 days/week. Treatments started at 11 weeks-of-age and continued for 7 weeks until euthanasia at 18 weeks-of-age. Proteinuria and body weight were monitored weekly and serum was collected for analyses monthly (Figure 1). Throughout treatment, MRL/lpr mice in all treatment groups gained weight over time (Figure 1A). Compared to controls, mice treated with dexamethasone and ACY-738 gained significantly less weight (Figure 1A). There were no significant differences in proteinuria scores between treatment groups (Figure 1B). Spleen weight was also recorded at the time of euthanasia and compared to body weight. Treatment with ACY-738 significantly decreased spleen size when compared to controls (Figures 1C & 1D).

ACY-738 treatment reduces the percentage of IL-17+, RORγ-t Th-17 cells in MRL/lpr mice

Th-17 and T-regulatory cell phenotypes were assessed because both SLE patients and lupus-prone mice exhibit an imbalance between these two subsets (Alunno *et al.,* 2012, Tucci *et al.,* 2010). After euthanasia, splenocytes were isolated, and fixed. Flow cytometry was

performed to determine if ACY-738 altered T cell profiles as mice aged (Figure 2). T-regulatory cell subsets were considered to be CD4+, CD8-, CD25+, and FOXP3+ and Th17 cell subsets were classified as CD4+, CD8+, IL-17+, and ROR-gamma+. We found that mice that were treated with ACY-738 had a significantly decreased number of Th17 cells when compared to controls (Figure 2A). No significant differences in the percentages of T-regulatory cells after treatment with ACY-738 were observed (Figure 2B).

Treatment with ACY-738 decreases serum IgG isotypes and increases TGF-beta levels in MRL/lpr mice

An important hallmark of disease in both humans and lupus-prone mice is ds-DNA antibody levels (Rottman & Willis, 2010, Mandik-Nayak *et al.*, 1999). Ig isotype levels were also assessed due to previous studies linking elevated IgG levels to glomerulonephritis in both SLE patients and MRL/lpr mice (Gupta & Hawkins, 2015, Field *et al.*, 1985, Bond *et al.*, 1990). Lymphocyte production of TGF-β has been shown to be decreased during active SLE (Ohtsuka *et al.,* 1998). We collected serum once a month until the time of euthanasia. Measurements of anti-ds DNA antibody, TGF-beta, total IgG, IgG2a, and IgG2b were evaluated via ELISAs (Figure 3). At the time of euthanasia, we observed no significant differences in the serum levels of anti-ds DNA antibody in between treatment groups (Figure 3A). However, mice that were treated with ACY-738 had significantly higher concentrations of TGF-beta in the serum when compared to controls (Figure 3B). Throughout the course of treatment, total IgG levels decreased although not statistically significant. At the time of euthanasia, total serum IgG levels were decreased in the mice that were treated with ACY-738 (Figure 3C). We also measured IgG2a levels at the same time intervals. We found no significant differences in IgG2a levels at

either time point in the experiment (Figure 3D). Lastly, we observed a decrease in IgG2b concentrations through the course of treatment and at the time of euthanasia (Figure 3E).

ACY-738 treatment reduces renal pathology in MRL/lpr mice

At the time of euthanasia, both kidneys were removed. One kidney was placed in a cassette in buffered formalin that would later be submitted to Histology for PAS staining while the other kidney was flash frozen in OCT media (Figure 4). Representative micrographs of the glomeruli were taken and scored in a blinded fashion by two pathologists (Figure 4A). ACY-738 treatment reduced glomerular pathology scores when compared to controls, albeit not statistically significant (Figure 4B). Kidneys were also given a score based on interstitial inflammation (Figure 4F). Mice that were treated with dexamethasone had a significantly lower interstitial inflammation score when compared to controls (Figure 4C). Frozen kidney sections were stained with C3 and IgG. Representative images were taken (Figures 4D & 4E) and both C3 and IgG immunostained kidneys were scored (0-4). Both C3 and IgG kidney scores were reduced in mice that received ACY-738 treatment, although not statistically significant (Figure 4E).

ACY-738 treatment decreases proteinuria and splenic weight in NZB/W mice

Previous work in our laboratory has shown that treatment with ACY-738 can ameliorate disease in NZB/W mice (Regna *et al.,* 2016). To investigate the effects of an oral dosage of ACY-738 in NZB/W mice, formulated rodent diet was provided to us from Acetylon Pharmaceuticals (Boston, MA, USA). Ten mice were control in which they received no treatment, ten mice received ACY-738 formulated rodent diet, and ten mice received IP dexamethasone injections 5 days/week. Treatments started at 16 weeks-of-age and continued for

18 weeks until euthanasia at 34 weeks-of-age (Figure 5). Throughout treatment mice in all treatment groups gained weight over time, although not significantly different (Figure 5A). Mice that were treated with either dexamethasone or ACY-738 had significantly reduced proteinuria scores when compared to untreated controls (Figure 5B). Spleen weight was also recorded at the time of euthanasia and compared to body weight. Treatment with ACY-738 significantly decreased spleen size when compared to controls (Figures 5C & 5D). Gross images were also taken of the spleen at the time of euthanasia. These images showed a significant reduction in spleen size of mice that were treated with dexamethasone and ACY-738 when compared to untreated controls (Figure 5E).

HDAC6 inhibition decreased T-regulatory T-cell subsets in NZB/W mice

Splenic T-cell subsets were classified and quantified as previously described in MRL/lpr mice (Figure 6). Mice that were treated with dexamethasone were found to have an increase in the percentage of Th-17 cells (Figure 6A). No statistical differences were found between the mice that were treated with ACY-738 and control groups. Interestingly, mice that were treated with ACY-738 had a decrease in the number of T-regulatory cells when compared to controls (Figure 6B). The mice that were given dexamethasone had an increase in the number of T-regulatory cells.

ACY-738 treatment decreases autoantibody and serum IgG levels in NZB/W mice

Diseased NZB/W mice have elevated production of autoantibodies against ds-DNA to go along with an increase in production in IgG isotypes, especially IgG2a (Ehlers *et al.,*). These immunoglobulins are considered pathogenic as they can form immune complexes that can

accumulate various organs and cause inflammation which may ultimately lead to organ failure. Serum was collected at the time of euthanasia. Measurements of ds DNA antibody, total IgG, IgG2a, and IgG2b were evaluated via ELISAs (Figure 7). A 1:1600 dilution was reported for the ds-DNA ELISA (Figure 7). Serum autoantibody levels were significantly decreased in mice that were treated with ACY-738 and dexamethasone when compared to untreated controls (Figure 7A). Total serum IgG levels were also significantly decreased throughout the experiment in mice that received treatment when compared to untreated mice (Figure 7B). We also observed a decrease among all groups in IgG2a levels throughout the course of treatment, although not statistically significantly different among groups (Figure 7C). Lastly, serum IgG2b levels were significantly increased in mice that received ACY-738 treatment when compared to untreated controls (Figure 7D).

HDAC6 inhibition reduces renal pathology in NZB/W mice

At the time of euthanasia, both kidneys were sectioned and stained. One kidney was placed in a cassette in buffered formalin for PAS staining while the other kidney was flash frozen in OCT media for IgG and C3 immunostaining (Figure 8). Representative micrographs of PAS staining of the glomeruli were taken and scored in a blinded fashion by two pathologists (Figure 8A). Kidneys were scored and we found that treatment with ACY-738 and dexamethasone significantly reduced glomerular pathology scores when compared to controls (Figure 8B). Frozen kidney sections were stained with C3 and IgG and given a representative score (0-4), respectively. Representative images were taken on at a 40x magnification (Figures 8C & 8D) and there were no significant differences in C3 kidney scores between treatment groups (Figure 8E). Mice that were treated either with dexamethasone or ACY-738 had a significantly lower

IgG kidney score when compared to untreated controls (Figure 8E). Lastly, mice that were treated with either dexamethasone or ACY-738 were found to have significantly lower interstitial inflammation scores when compared to untreated controls (Figure 8F).

Discussion

These studies were done to further examine the role of selective HDAC6 inhibition on lupus disease. We sought to determine if the selective HDAC6 inhibitor, ACY-738, could be used be administered orally to reduce hallmarks of disease in both MRL/lpr mice and NZB/W mice. We have shown in the past that HDAC inhibition can decrease disease in both MRL/lpr and NZB/W mouse models (Reilly *et al.,* 2014, Mishra *et al.*, 2003, Reilly *et al.*, 2008). Both MRL/lpr and NZB/W mice develop spontaneous SLE and glomerulonephritis. However, each mouse model disease duration and mechanism by which disease initiates and maintains itself is different between models (Rottman & Willis, 2010, Perry *et al.*, 2011). NZB/W are F1 generated from crossing NZB and NZW mice. NZB mice display mild hemolytic anemia, mild glomerulonephritis and have a relatively short lifespan while NZW develop anti-nuclear antibodies and glomerulonephritis later in life (Perry *et al.*, 2011). NZB/W F1 mice develop autoimmunity more quickly in life. These mice and humans with SLE produce autoantibodies against nuclear antigens such as: double-stranded DNA (dsDNA) and develop severe immune-complex glomerulonephritis that becomes apparent at 5-6 months (Theofilopoulos & Dixon, 1985, Perry *et al.*, 2011). Disease in this model is also strongly biased towards females due to estrogen levels which very closely mimics human disease (Fan *et al., 2*014, Grimaldi, 2006, Reilly *et al.,* 2008, Zhang, 2010, Zhang *et al*., 2010).

MRL/lpr mice are generated from crossing several inbred mouse strains and both male and female mice exhibit disease, unlike NZB/W mice. These mice also have elevated levels of autoantibodies such as: anti-dsDNA, and rheumatoid factors that result in immune complex deposition in the kidneys (Andrews *et al.,* 1978, Perry *et al.*, 2011). Lastly, these mice have extremely accelerated mortality rates due to an accumulation of double negative ($CD4^-$ $CD8^-$) T-

cells that are not eliminated through normal apoptosis (Theofilopoulos & Dixon, 1985). This aberrant T-cell differentiation is due to a mutation in the Fas ligand receptor. This mutation in the Fas ligand receptor is responsible for the SLE symptoms that these mice develop (Jabs *et al.,* 1994, Takahashi *et al.,* 1994). In humans, a mutation in the Fas ligand receptor may cause autoimmune lymphoproliferative syndrome (ALPS), but these patients do not develop glomerulonephritis (Teachey *et al.,* 2010). Understanding the mechanism for action for how each mouse model develops and maintains disease may account for the difference seen between the models.

Anti-dsDNA antibodies are a hallmark of SLE and it is produced by autoreactive B cells that escape apoptotic selection pathways during development (Dorner *et al.,* 2011, Kil & Hendriks, 2013). Previous work in our lab has shown that as NZB/W mice age, there is an increase in auto-antibody production that corresponds with an increase in proteinuria (Regna *et al.,* 2016). IgG is the primary immunoglobulin that forms autoantibodies complexes that accumulate and can lodge in the kidneys. This is mostly responsible for the glomerulonephritis associated with SLE (Shimp *et al.,* 2012, Bond *et al.*, 1990). These autoantibodies are able to bind to antigen to form immune complexes that become may become lodged in the glomeruli. This can result in inflammation, increased activation of the immune system, and damage to the kidneys which leave them unable to properly filter proteins (Hanrotel-Saliou *et al.,* 2011, Mok, 2014). Our studies were able to show that in both mouse models, treatment with ACY-738 was able to reduce both spleen weight and proteinuria. We were also able to observe significant decreases in both mouse models in serum autoantibody and several IgG isotypes when compared to untreated control mice. In addition, we observed a reduction in kidney pathology in both mouse models from mice that received ACY-738 treatment.

Patients with SLE and lupus-prone mice exhibit an imbalance of TH17 and T-regulatory cells (Alunno *et al.,* 2012, Koga *et al.,* 2014). An increase in TGF-β stimulation promotes FoxP3 expression which allows for the induction of naïve $CD4^+$ T cells to differentiate into T-regulatory cells (Horwitz, 2008, Zhou *et al.,* 2008). Our studies showed an increase in TGF-β serum levels and a decrease in TH-17 cell percentages. The physiological and molecular differences between each model mouse could explain the inconsistencies that we observed in T-regulatory cell populations. The disease that the MRL/lpr mice develop is mainly caused by a T cell defect. The disease is caused and maintained by the Fas ligand receptor mutation that allows aberrant T cells to proliferate uncontrollably. Studies are currently underway to define the mechanism through which ACY-738 is able to modulate various protein signaling pathways to decrease disease.

Due to HDACs being expressed ubiquitously and having a wide variety of cellular targets, selective targeting them may be effective in treating a wide variety of diseases (Dokmanovic *et al.,* 2007, Gryder *et al.,* 2012, Xu *et al.,* 2011, Delcuve *et al.*, 2012). Class I non-selective HDAC inhibitors including: vorinostat, romidepsin, and panobinostat have been demonstrated to be effective in treating multiple forms of cancer and have recently become compounds of interest in treating autoimmune disease (Fang *et al.,* 2016, Hancock *et al.,* 2012, Li *et al.,* 2014, Shin *et al.,* 2015, Mishra *et al.*, 2003). These compounds also come with adverse side effects for many patients that may include: nausea, vomiting, diarrhea, and thrombocytopenia (Wagner *et al.*, 2010, Falkenberg & Johnstone, 2014). SLE is a multifactorial that can manifest itself in any organ and cause damage to the surrounding tissue (Petri *et al.,* 2012). Difficulties arise in creating a safe and effective treatment due to the fact that it encompasses many inflammatory pathways (Vieson & Reilly, 2015).

Selective HDAC inhibition may allow effective targeting of HDAC activity while reducing side effects for patients. Current treatments for SLE rely heavily on the use of immunosuppression drugs, corticoids, nonsteroidal anti-inflammatory drugs and anti-malarial agents (Tsokos, 2011, Vieson & Reilly, 2015). Ongoing research on the molecular mechanism underlying SLE pathogenesis has led to the development and use of monoclonal antibody therapy designed to target B cells, T cells, and cytokines (Robak & Robak, 2009). While the development of these biological agents provides some encouragement, several clinical trials of these agents have resulted in disappointment (Postal *et al.,* 2012). Better selective targeting may provide a balance between treating SLE more effectively and minimizing harmful side-effects.

HDAC6 is a class IIb cytoplasmic HDAC that can modulate a variety of different proteins that are important in cellular signaling, apoptosis, and the immune response (Kovacs *et al.,* 2005, Valenzuela-Fernández *et al.*, 2008, Li *et al.*, 2011). HDAC6 has been demonstrated to also increase acetylation many cellular proteins including: SMAD7, HSP90, and FOXP3 (de Zoeten *et al.,* 2011, Guo *et al.,* 2012, Kovacs *et al.,* 2005, Simonsson *et al.,* 2005). Furthermore, while genetic knockouts of other HDACs are embryonic lethal, HDAC6 knockout mice are viable and have few alterations in their immune response (Zhang*, et al.,* 2008). This makes HDAC6 a potential candidate for selective targeting that could prove to be both effective and safe. This study and previous work from our laboratory has shown that selective HDAC6 inhibition is able to ameliorate SLE disease in lupus-prone mice (Regna *et al.,* 2016, Reilly *et al.*, 2008, Reilly, 2014). Future studies are warranted to unveil the exact molecular mechanism behind HDAC6 inhibition and how it is able to correct the aberrant immune regulation involved SLE without having deleterious side effects.

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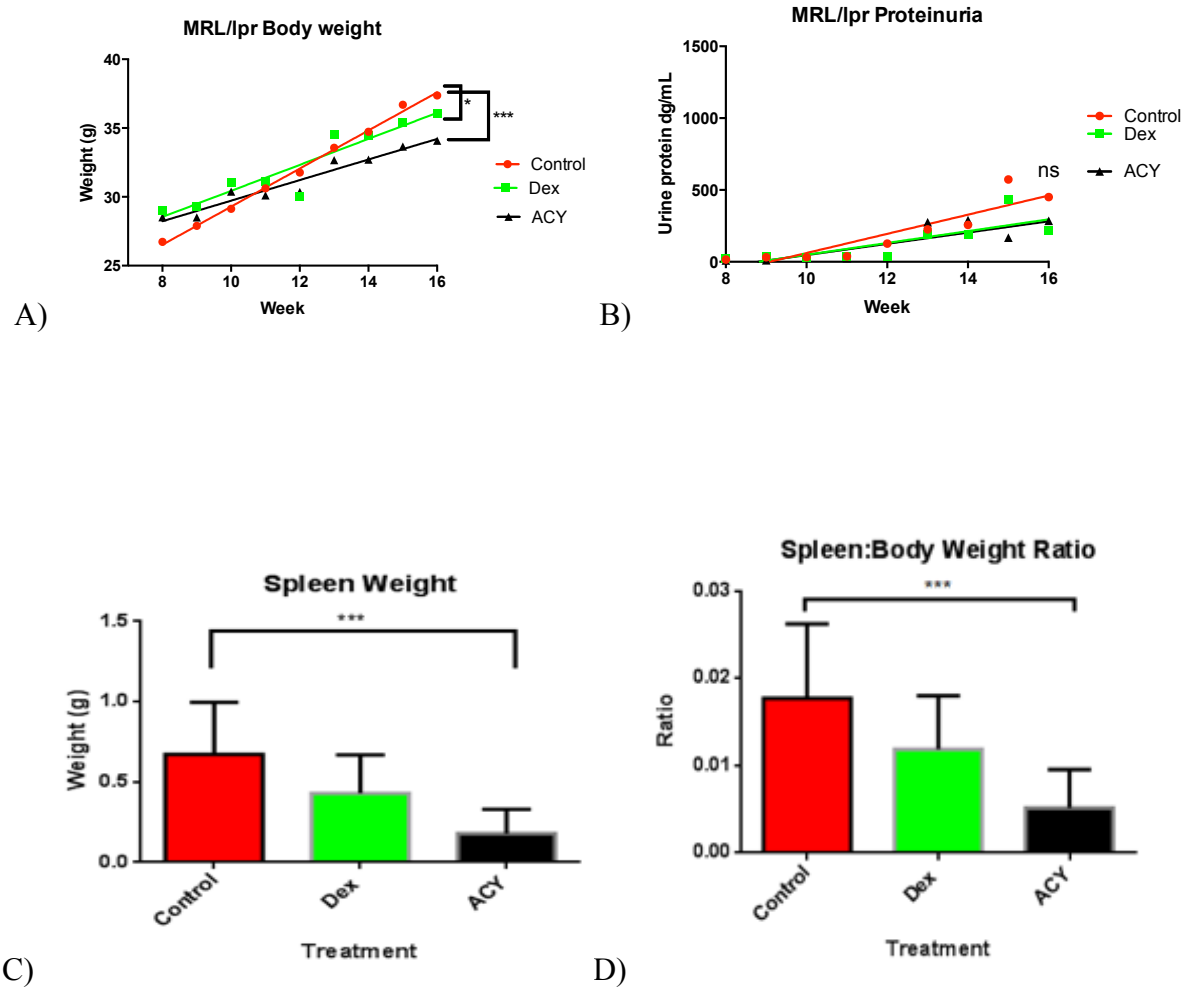
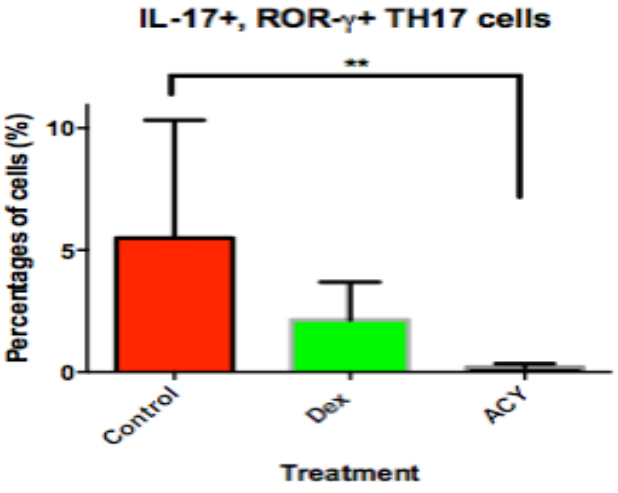
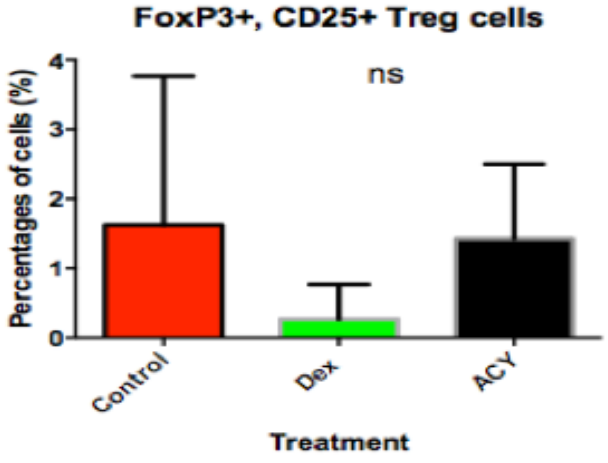


Figure 1 Selective HDAC6 inhibition reduces MRL/lpr spleen size

(A)MRL/lpr mice in all treatment groups continued to gain weight over time. Compared to controls, mice treated with ACY-738 and DEX gained less weight. (B) There were no significant differences in proteinuria scores between treatment groups. (C and D) ACY-738 significantly decreased spleen size compared to controls (*$p < 0.05$, ***$p < 0.001$).



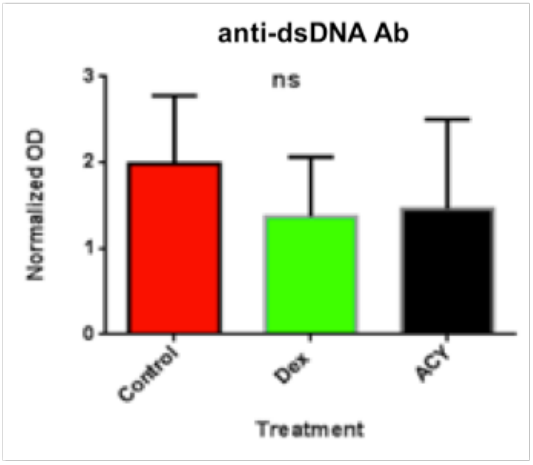
A)



B)

Figure 2 ACY-738 treatment reduces the percentage of splenic Th-17 cells

(A and B) There was a significant decrease in TH17 cells in mice that were treated with ACY-738. No differences in cell percentages were observed for T-regulatory cells (** $p < 0.01$).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Normalized OD
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Control
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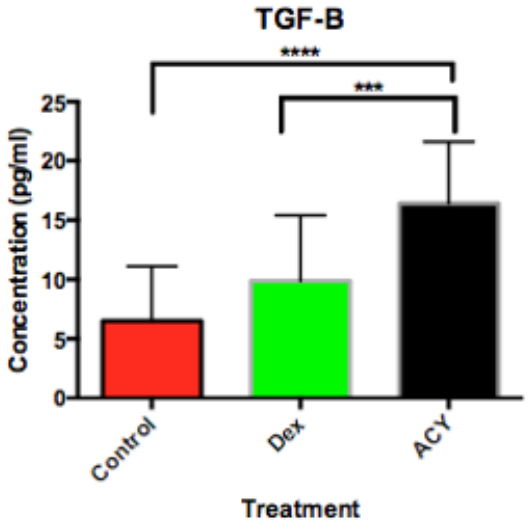
ACY
Treatment

A)


TGF-B
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Concentration (pg/ml)
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15
10
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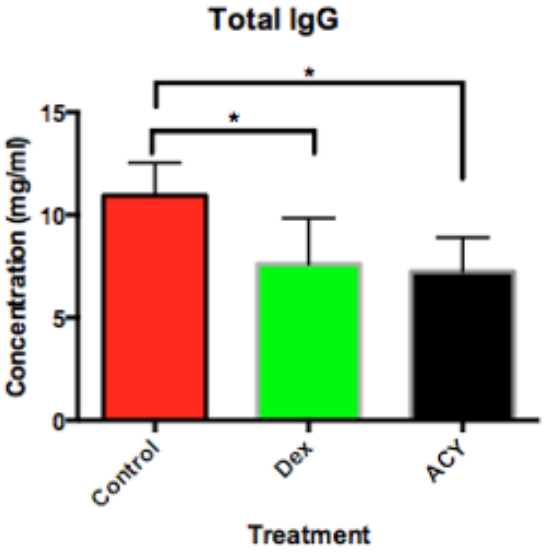
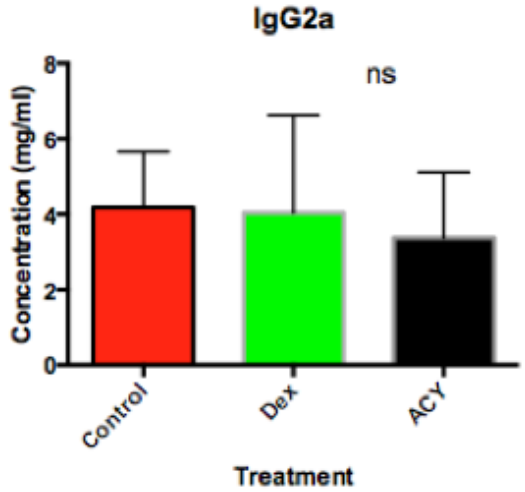
Control
Dex
ACY
Treatment

B)


Total IgG
*
*
Concentration (mg/ml)
15
10
5
0
Control
Dex
ACY
Treatment

C)


IgG2a
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Concentration (mg/ml)
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Control
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ACY
Treatment

D)

E)

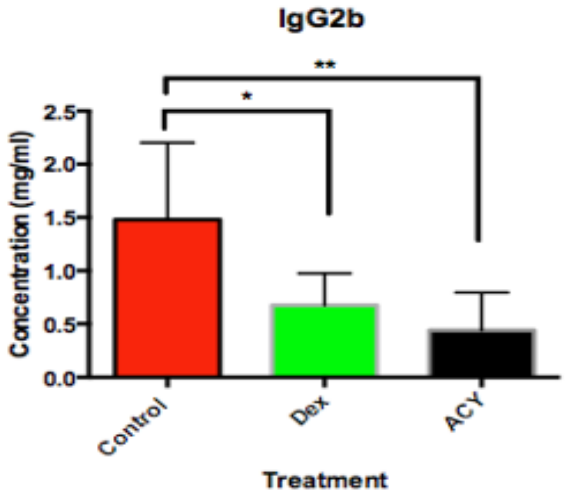
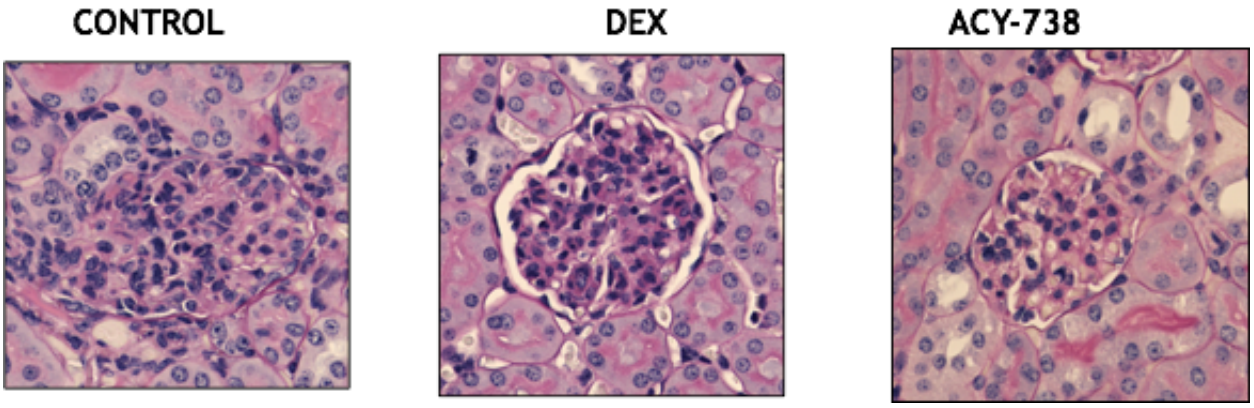


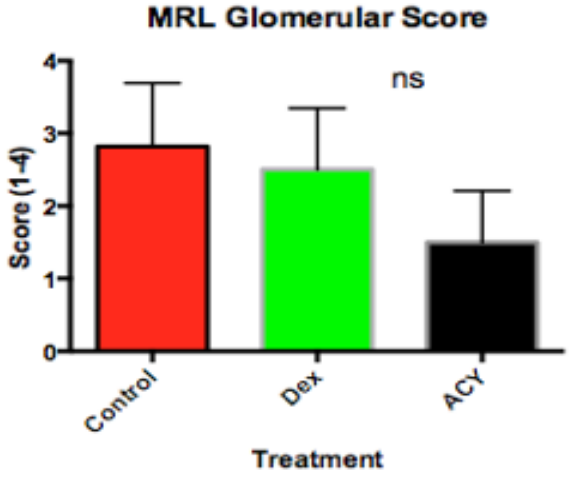
Figure 3 Treatment with ACY-738 decreases serum IgG isotypes and increases TGF-beta levels

All measurements were conducted at the time of euthanasia. (A) No significant differences in serum ds-DNA were observed. (B)Treatment with ACY-738 increased TGF-B levels when compared to controls. (C) Total IgG decreased in mice that were treated with ACY-738. (D)Treatment with ACY-738 did not affect IgG2a concentration levels. (E) Mice that received ACY-738 treatment had significantly lower IgG2b levels when compared to control.(*p < 0.05, ** p < 0.01,***p < 0.001, ***p< 0.0001).

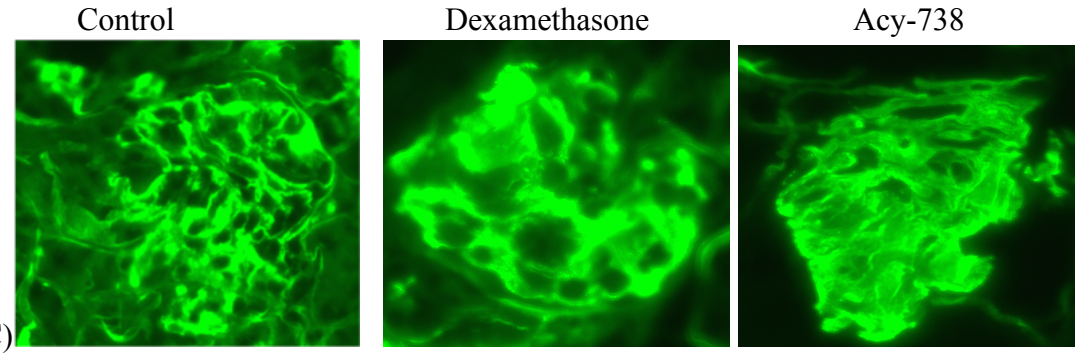
A)


CONTROL
DEX
ACY-738

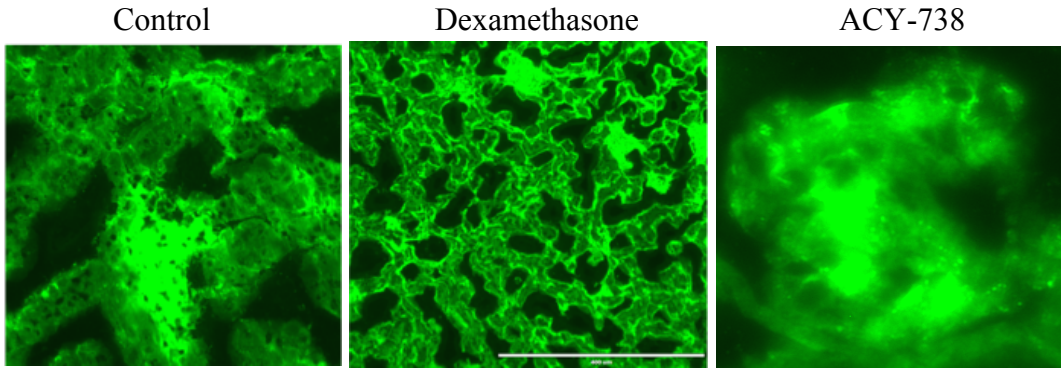
B)


MRL Glomerular Score
ns
Score (1-4)
0
1
2
3
4
Control
Dex
ACY
Treatment

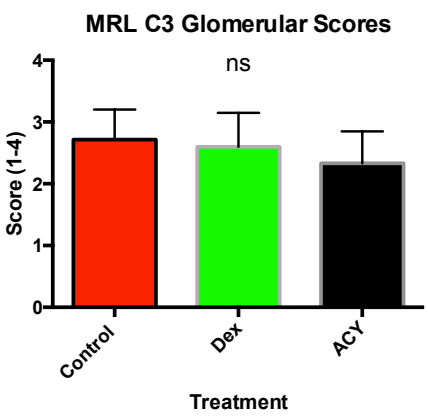
C)


Control
Dexamethasone
Acy-738

D)


Control
Dexamethasone
ACY-738

E)


MRL C3 Glomerular Scores
ns
Score (1-4)
0
1
2
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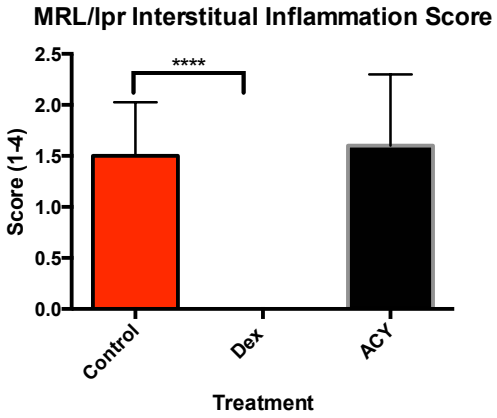
4
Control
Dex
ACY
Treatment


MRL/lpr Interstitual Inflammation Score
****
Score (1-4)
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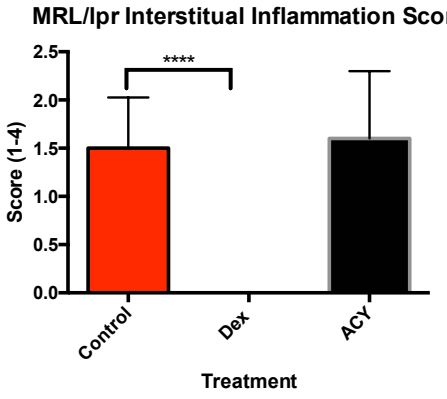
0.5
1.0
1.5
2.0
2.5
Control
Dex
ACY
Treatment

F)


MRL/lpr Interstitual Inflammation Score
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Score (1-4)
0.0
0.5
1.0
1.5
2.0
2.5
Control
Dex
ACY
Treatment

Figure 4 ACY-738 treatment reduces renal pathology

(A) Representative micrographs at 40x of glomeruli from MRL/lpr mice treated with ACY-738, DEX, or control. (B) ACY-738 and DEX significantly decreased glomerular pathology scores compared to untreated controls. (C) Insterstial inflammation scores were significantly decreased just in mice that received dexamethasone treatment. (D and E) Representative images of MRL/lpr frozen kidney sections at 40x magnification after C3 and IgG immunostaining. (F) There was a slight decrease in both C3 and IgG kidney scores, although neither were statistically significant ($p^{****} < 0.0001$).

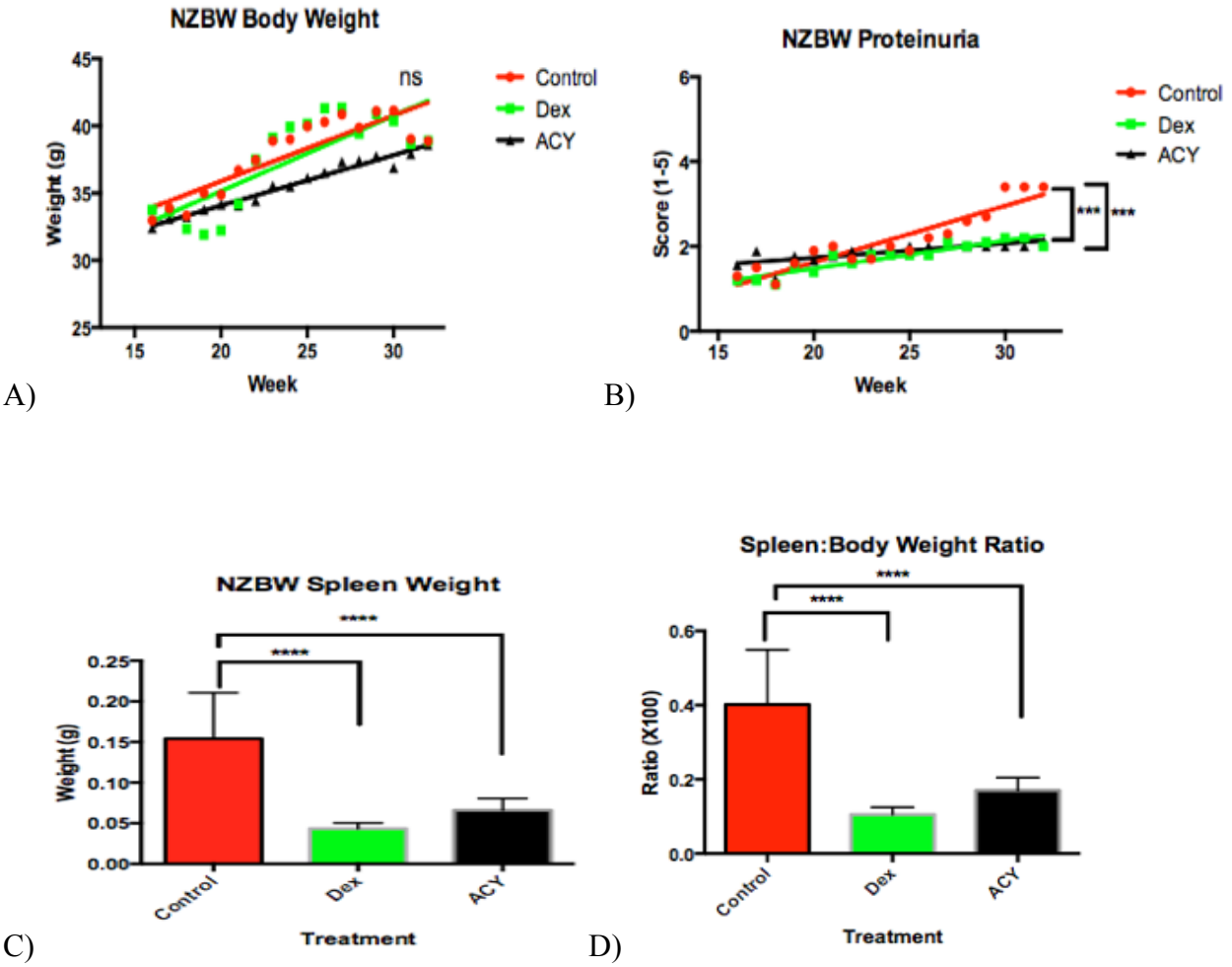
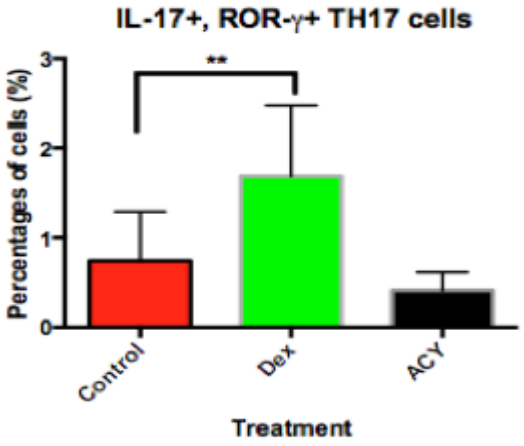


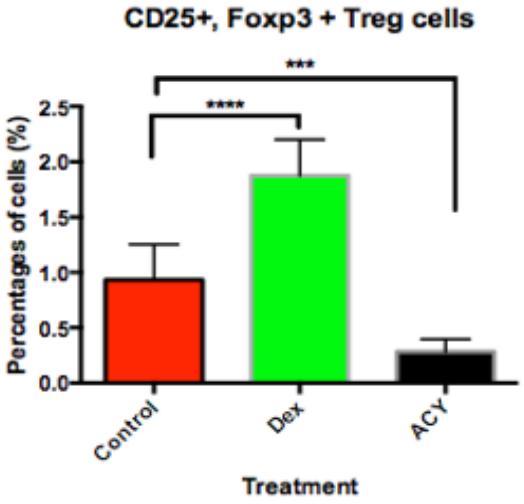
Figure 5 ACY-738 treatment decreases proteinuria and splenic weight

(A) NZB/W F1 mice continued to gain weight over time in all treatment groups. (B) Proteinuria over time was significantly decreased in mice treated with ACY-738 and DEX

compared to untreated controls. (C and D) Gross images and weight showed that treatment with ACY-738 significantly reduced spleen weight (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$).



A)



B)

Figure 6 HDAC6 inhibition did not have an effect on T-cell subsets

(A and B) NZBW splenic T cell subsets. There was an increase in the percentages of TH17 cells in mice that were treated with dexamethasone. There was a significant decrease in T-regulatory cells in mice that were treated with ACY-738 (*$p < 0.05$,** $p < 0.01$,***$p < 0.001$).

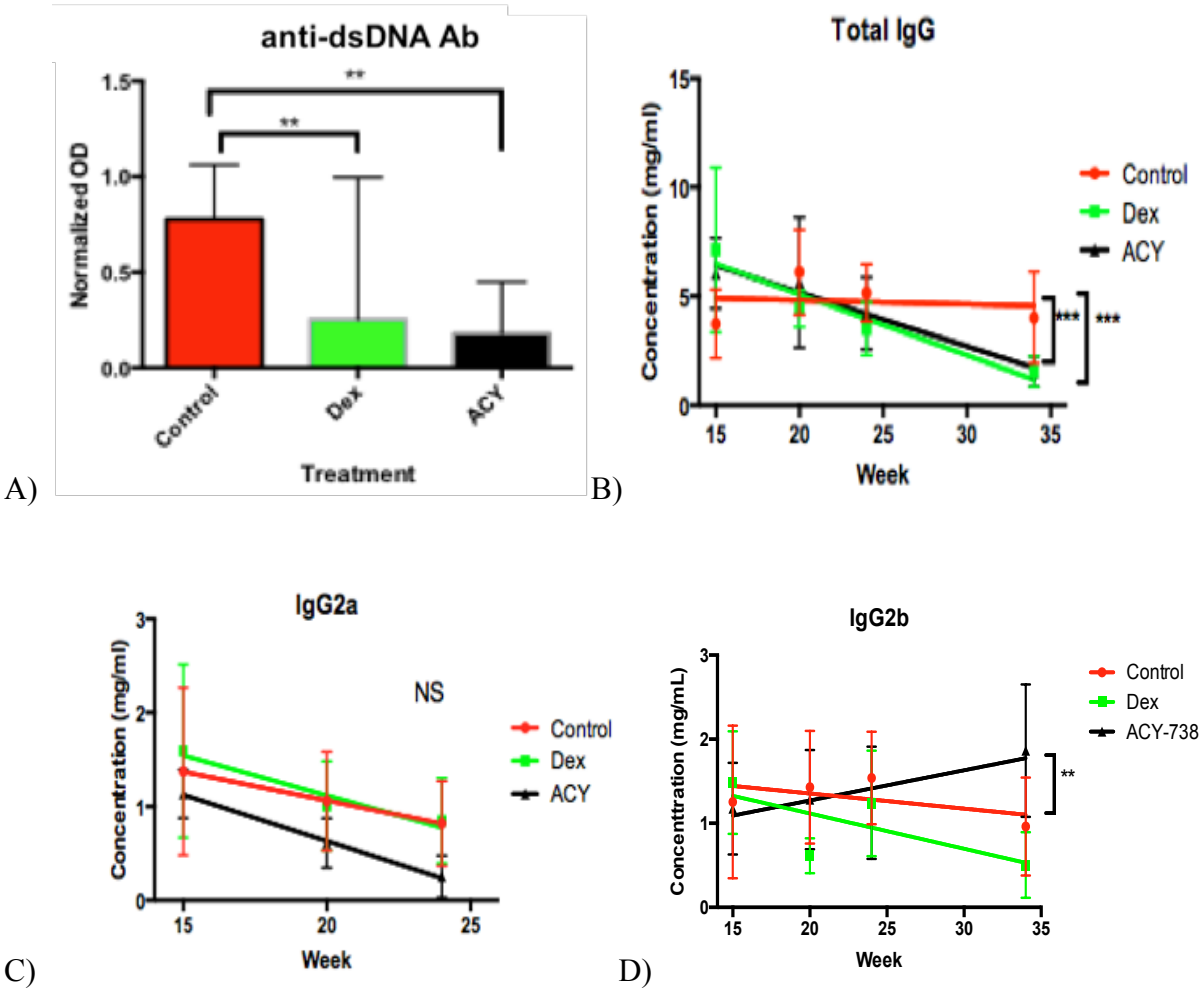
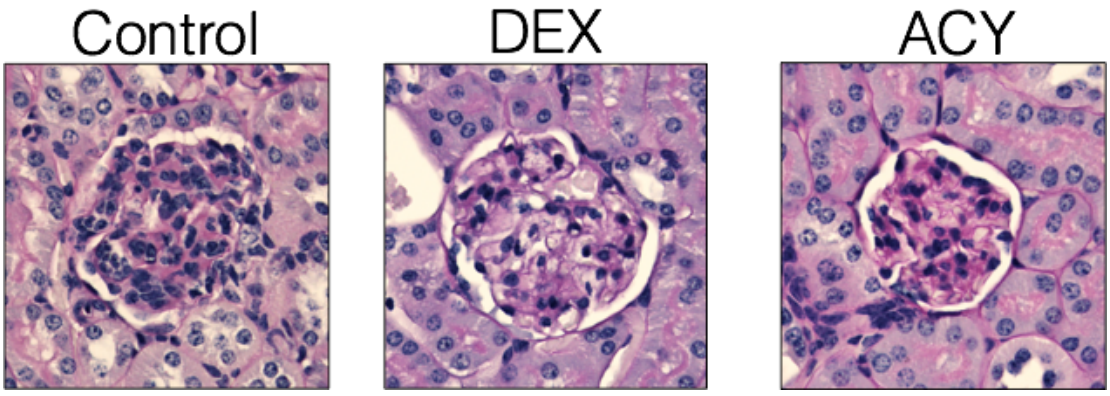


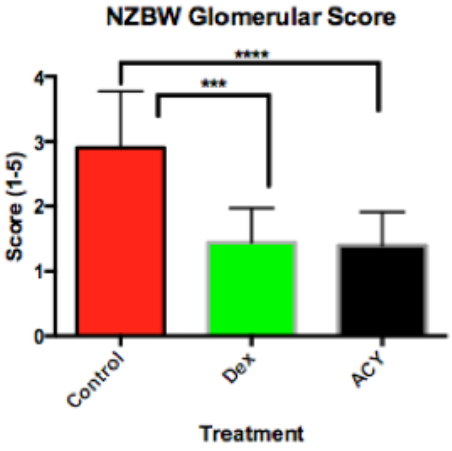
Figure 7 ACY-738 treatment decreases autoantibody and serum IgG cytokine levels

(A)Treatment with ACY-738 significantly reduced serum ds-DNA. (B)Total IgG decreased throughout the the course of treatment. (C) No statistical differences in IgG2a levels were observed throughout the course of treatment. (D)Treatment with ACY-738 significantly increased IgG2b levels (** $p < 0.01$,***$p < 0.001$).

A)


Control
DEX
ACY

B)


NZBW Glomerular Score
****
***
Score (1-5)
0
1
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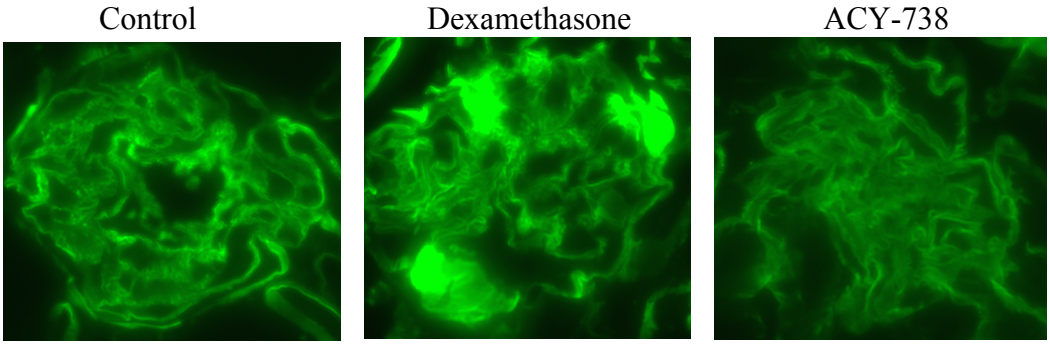
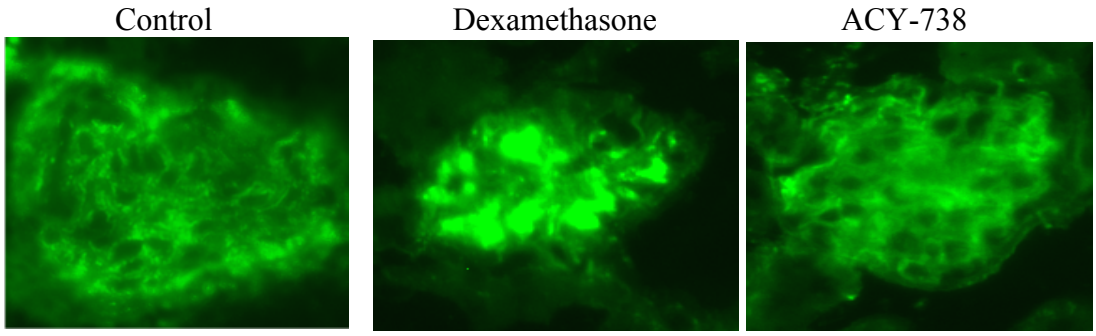
Control
Dex
ACY
Treatment

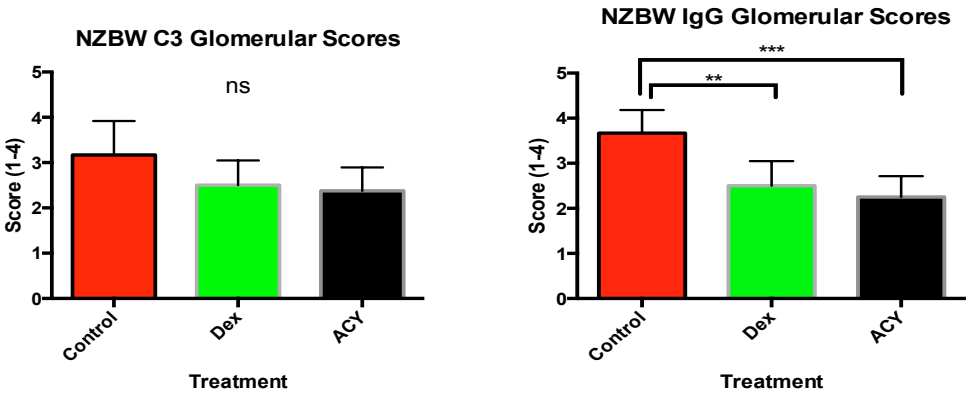
C)


Control
Dexamethasone
ACY-738

D)



E)



F)

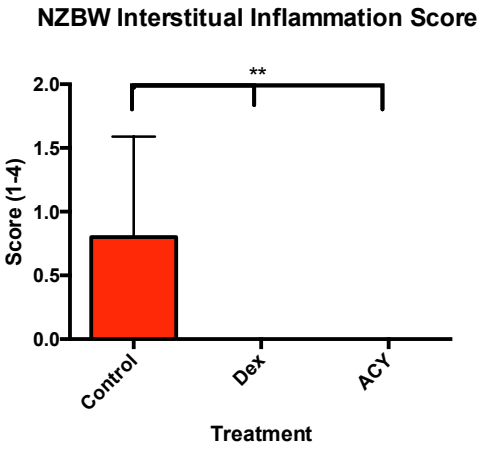


Figure 8 HDAC6 inhibition reduces renal pathology

(A) Representative micrographs at 40x of glomeruli from NZB/W mice treated with ACY-738, DEX, or control. (B) Treatment with ACY-738 and dexamethasone significantly

reduced glomerular kidney pathology scores when compared to untreated controls. (C and D) Representative images of NZBW frozen kidney sections at 40x magnification after C3 and IgG immunostaining. (E) C3 kidney scores were decreased in mice that received ACY-738 and dexamethasone treatment, although not statistically significant. IgG kidneys scores were significantly decreased in mice both treatment groups. (F) Treatment with ACY-738 and dexamethasone significantly reduced interstitial inflammation scores when compared to untreated controls.

Future Directions

We have recently shown that oral administration of the selective HDAC6 inhibitor, ACY-738, is able to reduce hallmarks of SLE in both MRL/lpr and NZB/W mice. Furthermore, we also observed a decrease in both spleen and proteinuria scores in both mouse models that received ACY-738 treatment. In addition, ACY-738 treatment also led significant decreases in serum autoantibody levels and total IgG and IgG2b isotypes. Lastly, treatment with ACY-738 was able to increase serum TGF-beta levels as well as increase in T-regulatory cell percentages. Future experiments will aim to elucidate the mechanism(s) through which HDAC6 inhibition is able to correct aberrant B and T cell differentiation.

Previous data from our laboratory has shown that HDAC6 is increased in the bone marrow (BM) in lupus-prone mice and that inhibition of HDAC6 leads to increased acetylation of α -tubulin (Regna *et al.,* 2016). A-tubulin is a major protein in microtubules and it plays a role in cell structure and shape (Regna *et al.*, 2015, Valenzuela-Fernandez *et al.*, 2008, Li *et al.*, 2011). Hyperacetylation of α -tubulin through HDAC6 inhibition may affect stromal cell-B cell interaction. Due to the importance of stromal cell contact with B cells during BM development, we believe that altered microtubule stability may affect the ability of B cells to make contact with stromal cells (Takemura *et al.*, 1992). This is important for both the maturation and differentiation of a B cell within the BM (Melchers, 2015, Regna *et al.*, 2015, Regna *et al.*, 2016). Future experiments will help define the changes that occur in α -tubulin acetylation during BM B cell differentiation in lupus-prone mice and define how α -tubulin is able to stabilize microtubule structure for B-cell to stromal cell interaction. Future experiments will measure whether α-tubulin hyperacetylation alters the ability of B cells to bind to and interact with stromal cells. B-cells will be cultured in direct contact with stromal cells to determine this

interaction and ELISAs will be ran on the media to determine cytokine production. These studies will show whether or not HDAC6 inhibition is able to alter B-cell development through stromal cell contact and help understand the role that α -tubulin acetylation plays in this interaction.

B cells originate from hematopoietic stem cells in the bone marrow. As these cells mature, they undergo stepwise development known as Hardy Fractions (Hardy R.R *et al.*, 2001, Hardy R.R *et al*., 2012). . As these B cells differentiate, they up and down-regulate certain cell surface markers as well as undergo light and heavy chain rearrangement. During B cell development there are several essential checkpoints; without a positive survival signal at each checkpoint for further differentiation, B cells will undergo apoptosis (Lu *et al.*, 2000, Melchers, 2015). During SLE, these B cells bypass normal apoptotic pathways and continue to develop into autoreactive autoantibody producing cells (Dorner *et al.,* 2011). We believe that α-tubulin acetylation may affect gene expression involved with various stages of B cell development. Preliminary experiments conducted have indicated that there is an altered distribution of B cells in developmental Hardy fractions following HDAC6 inhibition (Regna*, et al.,* 2016). Future experiments will determine seek to determine how α -tubulin acetylation regulates genes affecting B cell development in BM cells from NZB/W and MRL/lpr mice using mRNA sequencing. *In vitro* experiments measuring B-cell and stromal cell interaction will be stimulated with HDAC6 inhibitor. Hardy fractions will be measured via flow cytometry 4 days after stimulation to determine whether HDAC6 inhibition is able to alter B cell development in the BM through affecting the rate of apoptosis.

HDAC6 is cytoplasmic and its activity is known to affect multiple substrates (Batta *et al.,* 2007). HDAC6 inhibition has been demonstrated to also increase acetylation of other proteins

including: SMAD7, HSP90, and FOXP3 which can directly regulation cells of the immune system (de Zoeten *et al.,* 2011, Guo *et al.,* 2002, Kovacs *et al.,* 2005, Simonsson *et al.,* 2005). Future experiments using cell sorting and antibody staining will determine the acetylation status of the proteins with respect to HDAC6 inhibition. The results of these experiments could warrant other experiments to investigate the role that these proteins on cell function. These experiments will help us define and understand how deacetylation of these proteins has an influence on lupus disease.

SMAD7 belongs to the TGF-beta family of proteins and is involved in cell signaling where its function is to inhibit TGF-beta and activin signaling by associating with their receptors (Yan *et al.,* 2016). It has also been shown that SMAD7 is able to decrease renal inflammation by inducing IκB which can inhibit certain inflammatory responses (Wang *et al.,* 2005). HDACS have been found to affect SMAD7's stability, which is a competition between ubiquitination and acetylation (Gronroos *et al.,* 2002, Simonsson *et al.,* 2005). Our laboratory has previously shown that HDAC6 expression and activity is increased in glomerular cells of MRL/lpr mice which may contribute to disease progression due to the decreased Smad7 stability (Regna *et al*., 2015). Further studies are warranted to elicit the possible mechanism behind this and to test if this hypothesis is responsible for the decrease in renal inflammation we observe following HDAC6 inhibition in NZB/W mice.

HSP90 is a chaperone protein that helps other proteins fold correctly, stabilizes them against heat/stress, and aids protein degradation (de Zoeten *et al.,* 2011, Kovacs *et al.,* 2005). HSP90 can play a conflicting role in the context of autoimmune disease. Elevated expression of HSP90 has been found in the kidneys and serum of SLE patients, as well as in MRL/lpr mice (Shimp *et al.,* 2012). We found in the past that MRL/lpr mice that were treated with HSP90

inhibitor (17-DMAG) had decreased proteinuria and serum anti-dsDNA production. Interestingly, we found that there were no differences glomerular pathology despite reductions in proteinuria following treatment with 17-DMAG (Shimp *et al.,* 2012). Acetylation of HSP90 has also been shown to to prevent its chaperoning function which is critical for its immunological activation (de Zoeten *et al.,* 2011, Giustiniani *et al.,* 2009). We have also shown in the past that HDAC6 inhibition is able to inhibit HSP90 expression which decreases NF-κB nuclear translocation in NZB/W mice (Regna*, et al.,* 2016). Future experiments are needed to explain how HSP90 modulation can affect disease pathogenesis.

T-regulatory cells are considered anti-inflammatory and are responsible for maintaining immune homeostasis. These cells comprise about 3-5% of the CD4+ T cell population and function to suppress deleterious and unwanted immune responses (Sakaguchi *et al.,* 2008). In the context of SLE, T-regulatory cells suppress kidney inflammation. Patients with SLE and lupus-prone mice exhibit an imbalance in TH17 and T-regulatory cells (Alunno *et al.,* 2012). FOXP3 is the master regulation and lineage specific factor for T-regulatory cells (Rudensky, 2011). There is a decrease in T-regulatory cell percentages and function in both human SLE patients and NZB/W mice (Koga *et al.,* 2014, Ooi *et al.,* 2011). This reduction results in decreased renal function and accelerated glomerulonephritis. Previous work from our laboratory and others has shown that HDAC6 inhibition is able to increase T-regulatory cell by acetylating FOXP3, which results in an increase in its stability (Loosdregt *et al.*, 2010, Reilly, 2014, Vieson, 2015). Future experiments using *in vitro* T-cell differentiation assays can better help us understand the role and define the molecular mechanism in which HDAC6 is able to interact with FOXP3 to generate T-regulatory cells.

Future work will help define the proteins in signaling pathways that lead to autoreactive B-cell development and T-cell differentiation. One major limitation during the experiment was the amount of variability seen in the MRL/lpr mice. These mice may exhibit relatively few autoimmune-like symptoms one week and progress very rapidly in disease severity. Some alternative approaches to the current studies would be longer treatment time regime for the MRL/lpr mice. Another alternative approach would be to examine the effects of different dosing groups (low, medium, and high) for both mouse models. Lastly, *in vitro* cell culture experiments of splenocytes from diseased mice could be treated with HDAC6 inhibitor to help confirm our findings.

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