Isoform-Selective HDAC Inhibition for the Treatment of Lupus Nephritis

Nicole L. Regna

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biomedical and Veterinary Sciences

Christopher M. Reilly, Chair
David L. Caudell
Yong W. Lee
Rujuan Dai

June 3, 2014
Blacksburg, VA

Keywords: Histone deacetylase, T cells, B cells, systemic lupus erythematosus

Isoform-Selective HDAC Inhibition for the Treatment of Lupus Nephritis

Nicole L. Regna

Abstract

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease requiring a genetic predisposition coupled with an environmental trigger in order for initiation of disease. While the exact pathoaetiology has yet to be determined, both B and T cell dysregulation are thought to contribute to disease. Histone deacetylases (HDACs) are a class of enzymes that hydrolyze the lysine bound acetyl group in both histone and non-histone proteins thereby altering protein structure and function. While the use of pan-HDAC inhibitors has proven to be effective for the treatment of a number of acute diseases, they may not be viable as therapeutics for chronic disease due to cytotoxicity and adverse side effects following long term treatment. We sought to determine whether treatment with a class I and II HDAC inhibitor (HDACi) or a specific HDAC6i would be able to ameliorate disease in lupus-prone NZB/W mice. We found that both the class I and II HDACi (ITF2357) and the HDAC6i (ACY-738) were able to decrease SLE markers of disease including splenomegaly, proteinuria, and anti-dsDNA and IgG production in the sera. Treatment with ITF2357 resulted in an increase in the number of immunosuppressive regulatory T (T_{reg}) cells and a decrease in the pro-inflammatory Th17 phenotype. Furthermore, ITF2357 was found to increase Foxp3 acetylation leading to increased Foxp3 stability allowing for differentiation into the T_{reg} phenotype. ACY-738 treatment was able to correct aberrant bone marrow B cell differentiation while also increasing the number of splenic T_{reg} cells in NZB/W mice. These results suggest that HDAC inhibition is able to ameliorate SLE in NZB/W mice by altering aberrant T and B cell differentiation. Additional studies were performed to further examine the expression and function of different HDAC

isoforms in immune cells. Due to the ability of HDAC inhibition to decrease markers of SLE disease as well as alter B and T cell development and differentiation, we sought to determine if specific HDAC isoforms are altered in lupus vs non lupus mice in early and late disease states. We determined the level of class IIb HDAC (HDACs 6, 9, and 10) expression in bone marrow B cells, splenic B and T cells, and glomerular cells from early- and late-disease MRL/lpr lupus-prone mice compared to healthy, age-matched C57BL/6 control mice. Expression of HDAC6 and HDAC9 were significantly increased in all of the tissues tested from MRL/lpr mice. Furthermore, both cytoplasmic and nuclear HDAC activity was increased in diseased MRL/lpr mice, and HDAC activity and expression continued to increase as disease progressed. *In vitro* treatment with ACY-738, a selective HDAC6i, was able to decrease cytoplasmic HDAC activity and inhibit iNOS production. Furthermore, ACY-738 was able to alter apoptosis through increased Bax expression in B cells. Treatment with ACY-738 was also able to inhibit Hsp90 expression and decrease NF-κB nuclear translocation, which are both upregulated during active SLE. Our studies indicate that HDAC activity contributes to SLE pathogenesis and that the use of isoform-selective HDAC inhibitors may be a viable treatment for SLE.

Acknowledgements

This work was supported by grants from the National Institutes of Health/National Institute of Allergy and Infectious Diseases (R15 AR062883).

Attributions

Several colleagues aided in the development of project ideas, research, writing, and editing of all of the chapters of this dissertation.

Chapter 1:

Christopher M. Reilly, PhD (Autoimmunity Lab, Department of Physiology) is currently a professor at VCOM. Dr. Reilly is a co-author on this paper and helped with the writing and editing.

Chapter 2 and 3:

Cristen B. Chafin, PhD (Autoimmunity Lab, Department of Biomedical Sciences & Pathobiology) is currently a research scientist and the Virginia State Lab. Dr. Chafin is a co-author on this paper and helped with the molecular techniques used as well as the editing of this manuscript.

Sarah E. Hammond, DVM (Autoimmunity Lab, Department of Biomedical Sciences & Pathobiology) is currently a PhD student at Virginia Tech. Dr. Hammond is a co-author on this manuscript and contributed to the treatment of animals.

Abdul G. Puthiyaveetil, DVM, PhD (Department of Biotechnology) is currently a research scientist at American University of Al Khaimah. Dr. Puthiyaveeitl is a co-author on this manuscript and helped with flow cytometric analysis.

David L. Caudell, DVM, PhD (Department of Pathology/Comparative Medicine) is currently an assistant professor of Pathology at Wake Forest University. Dr. Caudell is a co-author on this paper and contributed to the scoring of renal pathology.

Christopher M. Reilly is a co-author on this paper and aided in the project development and writing of this manuscript.

Chapter 4:

Dr. Hammond is a co-author on this manuscript and contributed to the euthanization of mice and collection of tissues.

Dr. Caudell is a co-author on this paper and contributed to the B cell culture methodology.

Dr. Reilly is a co-author on this paper and aided in the project development and writing of this manuscript.

Table of Contents

Page

Chapter 3

Specific HDAC6 Inhibition by ACY-738 Reduces SLE Pathogenesis in NZB/W Mice

Chapter 4

Overexpression of HDAC6 in lupus-prone mice can be inhibited by ACY-738 *in vitro*

List of Tables

Page

Chapter 1: Literature Review

Chapter 2: Class I and II histone deacetylase inhibition by ITF2357 Reduces SLE pathogensis *in vivo*

List of Figures

Page

Chapter 2

Chapter 3

Chapter 4

Chapter 1: Literature Review

Isoform-Selective HDAC Inhibition in Autoimmune Disease

Nicole L. Regna[1]

Christopher M. Reilly[1, 2]

[1]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061

[2]Edward Via College of Osteopathic Medicine, Blacksburg, VA, 24060

Published by the *Journal of Clinical & Cellular Immunology*

1.2. Abstract

Abstract

Histone deacetylases are a class of enzymes that play an important role in protein modification and cellular function. Ongoing research suggests that HDAC inhibitors may be efficacious in the treatment of a wide range of diseases from cancer to autoimmune disease. HDACi therapy has shown promising results both *in vitro* and *in vivo* for the treatment of autoimmune disease. To date, 18 isoforms of HDACs have been identified, which exist in four different classes: class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10) class III (sirtuins1-7), and class IV (HDAC11). The mechanism of action through which HDACs function remains to be fully elucidated. However, the use of isoform-selective HDAC inhibitors has been helpful in determining the physiological role of individual HDACs as well as in decreasing the toxicity of HDACi therapy. This review will focus on isoform-selective HDACs and how they may be effective for the treatment of autoimmune disease.

1.3 Introduction

Regulation of the immune system is dependent upon both genetic and epigenetic factors. Epigenetics control gene packaging and expression through heritable and stable changes without altering the DNA sequence [1, 2]. These changes can be reversible dependent upon environmental factors and thus may provide the link between the environment and genetics that result in autoimmune disease [1]. Epigenetic changes in cellular function include changes in DNA methylation, microRNA (miRNA), and protein acetylation [3]. Proper cellular function requires acetylation of both histone and nonhistone proteins [4]. Abnormal histone acetyl transferase (HAT) and histone deacetylase (HDAC) expression and activity has been associated with a number of autoimmune and inflammatory diseases and may therefore be a potential target to therapeutically modulate disease [5-12].

HATs add an acetyl group to histone proteins allowing for transcriptional activities. Conversely, histone deacetylases (HDACs) are a group of enzymes that catalyze the removal of acetyl groups from lysine residues on histones thereby restricting chromatin availability for gene transcription [13, 14]. Traditionally, HDACs were thought to function solely through epigenetic regulation of histone proteins; however, HDACs have more recently been shown to regulate acetylation of over 50 nonhistone proteins and may be more accurately described as lysine deacetylases (KDACs) [15, 16]. Of particular interest is the ability of HDACs to regulate transcription factors, signaling molecules, and structural proteins thereby exhibiting an immunomodulatory effect [17].

HDACs have been implicated in immune cell regulation and may therefore be efficacious in the treatment of autoimmune disease [18, 19]. Due to the large number of HDACs that are targeted, pan-HDAC inhibitors have been associated with deleterious side effects during clinical

trials including fatigue, nausea, thrombocytopenia, and electrocardiograph abnormalities [20, 21]. For this reason, a more targeted approach is warranted if HDAC inhibitors are to be used in the treatment of autoimmune disease. This review will discuss the potential use of isoform-selective HDAC inhibitors as therapeutics for autoimmune disease. Isoform-selective HDAC inhibitors may allow researchers to determine not only the biological functions of particular HDACs, but also provide a more specific target for potential therapeutics without adversely affecting normal physiological functions.

1.4. HDACs and autoimmunity

There are 18 known mammalian HDACs, which are grouped into classes I-IV. The classical HDACs consist of HDACs 1-11, which are grouped into classes I, II, and IV [22]. Class III HDACs are comprised of 7 members called seven mammalian silent information regulator two proteins (sirtuins or Sirt) which differ from classical HDACs in that they require NAD^+ as a cofactor and are not dependent upon Zn^{2+} as a catalytic mechanism [23, 24]. HDACs are found in both the nucleus and cytoplasm, with some shuttling between the two and others confined to a specific compartment [25].

A nuclear localization signal (NLS) allows HDACs to localize within the nucleus and therefore exert their function on nuclear proteins. HDAC1 and 2 lack a nuclear export signal (NES) and are unable to leave the nucleus [22]. HDAC3 has both a NLS and a NES; however, it is almost always found within the nucleus [22, 26]. Conversely, class II HDACs, particularly HDACs 4, 5, 7, 9, and 10, are known to travel back in forth between the nucleus and the cytoplasm and are thought to play an important role in the function of both nuclear and cytoplasmic proteins [22]. HDAC6 is predominantly found within the cytoplasm and mainly

influences cytosolic proteins [27]. Similarly to class II HDACs, HDAC11 (class IV), can be found in both the nucleus and the cytoplasm and has been demonstrated to colocalize with HDAC6 in the cytoplasm [28]. Due to the specificity of HDACs, selective therapeutic targeting may allow for modulation of specific histones or other non-nuclear proteins.

Autoimmunity is characterized by an abnormal immune response during which the body perceives a normal substance as foreign leading to autoantibody production and inflammation [29]. Studies of monozygotic twins discordant for systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and dermatomyositis; suggest a role of non-genetic factors in disease pathogenesis [30, 31]. HDAC inhibitors have been shown to modulate a number of key regulators of the immune system including B cells, T cells, and APCs [5, 32-36]. During autoimmune disease it is thought that HDAC activity is upregulated leading to increased nuclear translocation and binding of the transcription factors, particularly STAT3 and NF-κB, which promote gene expression of pro-inflammatory genes [37]. HDAC inhibitors have proven to have an anti-inflammatory effect, which may be helpful in the treatment of autoimmune disease during which prolonged inflammation results in tissue destruction and organ failure [36, 38].

Due to the anti-proliferative effect that HDAC inhibitors exhibit; they may be effective agents of immunosuppression for the treatment of autoimmune disease. Treatment with pan-HDAC inhibitors including ITF2357, SAHA, and TsA have shown efficacy in treating autoimmune diseases including SLE, RA, and inflammatory bowel disease (IBD) in murine models [34, 36, 39-43]. We have shown that treatment with ITF2357 is able to reduce disease in lupus-prone mice while increasing the number of T_{reg} cells and decreasing the number of $CD4^+$ T cells [34]. SLE is thought to involve aberrant B and T cell regulation [6, 44-47]. Studies showing

the regulatory effect of HDAC inhibitors on both B and T cell populations make selective HDACi therapy of particular interest in the treatment of SLE ([34, 40, 48].

Selective HDAC inhibitors are able to provide a more targeted approach to treating autoimmune disease and reduce the risk of complications from unwanted side effects. Pan-HDAC and class I-selective inhibitors, currently undergoing clinical trials, alter physiological functions that require protein deacetylation [20, 49]. There are currently two HDAC inhibitors, SAHA (pan-HDACi) and FK228 (selective-class I HDACi), approved by the FDA for the treatment of cutaneous T cell lymphoma (CTCL). Both of these drugs have also been tested for their efficacy in the treatment of autoimmune diseases. SAHA has shown efficacy in the treatment of lupus-prone mice; however, long term treatment resulted in unwanted side effects including possible drug toxicity [41]. SLE is a chronic disease requiring long-term treatment and these results indicate that inhibition of class I and II HDACs by a pan-HDACi may not be optimal [41].

Currently undergoing phase III clinical trials are panobinostat (LBH589) and Valproic acid (VPA) [50, 51]. LBH589 is being tested for its use as a CTCL therapeutic and a number of other cancers [50]. VPA is currently in phase III clinical trials for the treatment of cervical and ovarian cancer, but it has recently shown therapeutic potential in the treatment of autoimmune disease [51, 52]. HDAC inhibitors currently undergoing phase II clinical trials include Mocetinostat (MGCD0103), Entinostat (MS-275), Belinostat (PXD101), and Givinostat (ITF2357) for the treatment of various cancers [53]. CUDC-101, ACY-1215, CHR-2845, and CG200745 have begun phase I clinical trials for the treatments of cancer [54, 55]. Dokamanovic et. al provide a more extensive review of specific HDAC inhibitors currently undergoing clinical trials [15].

Due to the ubiquitous nature of HDACs, not only are the cellular pathways involved with autoimmunity affected, but HDAC inhibition also disrupts the pathways involved with normal cellular function [24]. Furthermore, pan-HDAC inhibitors can be cytotoxic, and it may prove important in clinical treatment for HDAC inhibition to be more selective [56]. The mechanisms through which HDAC inhibitors regulate the immune response are not fully understood. Currently ongoing studies of HDAC inhibitors both *in vivo* and *in vitro* are working to determine the mechanism of both pan-and isoform-selective HDAC inhibitors.

1.5. Selective Class I Inhibitors

Class I HDACs (HDACs 1, 2, 3, and 8) play an important role in cell survival and proliferation [57]. While insight has been gained about the function of HDACs through various knockout mouse studies, gene deletion of HDACs 1, 2, and 3, has proven to be embryonic lethal in mice [24]. HDAC1 has been demonstrated to be overexpressed in SLE, RA, multiple sclerosis (MS), and juvenile idiopathic arthritis (JIA) [5]. Furthermore, HDAC3 and HDAC7 have also been shown to play a role in immune regulation during SLE, suggesting the potential importance of targeting these HDACs for treatment of disease [5].

In regard to class I HDACs it is interesting to note that HDAC2 is able to regulate the binding ability of p53, which controls transcription. HDAC2 has been shown to increase p53 binding activity and consequently increase cellular proliferation [58]. HDAC2 was demonstrated to be involved with an anti-apoptotic function following HDAC2 knockdown in cancer cells [59]. Furthermore, p53 activation has been linked to inhibition of autoimmune disease. Studies suggest that p53 expression is able to induce T_{reg} differentiation leading to suppression of the autoimmune response [60]. P53 activation is further thought to inhibit autoimmune disease

through downregulation of STAT1 resulting in decreased proinflammatory cytokine production [61]. Autoimmune diseases have been shown to be more severe on a p53-deficient background in mice [60, 62]. The studies explain why targeting HDAC2 may be a viable approach for treating autoimmune diseases such as lupus in which T_{reg} cell function may be important to modulate the immune response.

MS-275 is a benzamide-derived selective class I inhibitor currently undergoing Phase I-II clinical trials that has shown promising anti-rheumatic activities including prevention of bone erosion and delayed onset of collagen-induced arthritis (CIA) [24, 63]. Studies have demonstrated MS-275 treatment suppressed LPS-induced pro-inflammatory cytokine production in monocytic cells. Treatment led to phase arrest at G_0/G_1 without increasing apoptosis [64]. Treatment with MS-275 after the onset of arthritis in rodents has been demonstrated to halt disease progression suggesting its potential as a therapeutic. Furthermore, MS-275 may have potential as a therapeutic in the treatment of other inflammatory autoimmune diseases based off of its anti-inflammatory effect *in vitro* and in the CIA induced mouse model [63, 64]. Following treatment with MS-275, E11 cells and monocytic cells had decreased LPS-induced NF-κB nuclear translocation, decreased production of IL-6, IL-18, NO, VEGF, MMP-2, and MMP-9 [64]. Furthermore, MS-275 has been shown to decrease sera production of pro-inflammatory cytokines IL-6 and IL-1β, which are overproduced during a number of autoimmune diseases including RA, SLE, autoimmune encephalomyelitis, and IBD [65-71].

The exact mechanism through which MS-275 treatment results in anti-rheumatic and anti-inflammatory effects remains to be elucidated. One proposed mechanism suggests MS-275 increases the stability of histone acetylation associated with the c-Fos promoter which plays an important role in cellular functions including proliferation, differentiation and survival [72]. MS-

275 has been shown to increase acetylation of NF-κB p65 leading to decreased nuclear translocation and inhibition of gene transcription [64]. NF-κB activation and nuclear translocation is required for c-Fos expression [72]. These studies suggest that inhibition of NF-κB nuclear accumulation by MS-275 treatment, results in decreased cellular proliferation of osteoclasts induced by c-Fos expression [72, 73]. Furthermore, MS-275 has been shown to decrease the chaperone activity of Hsp90 [74]. The ability of MS-275 to inhibit Hsp90 is of particular interest in the treatment of SLE, which has been found to have elevated hsp90 sera levels [75]. Furthermore, use of an Hsp90 inhibitor in lupus-prone mice has shown therapeutic potential [76].

VPA is a selective class I HDACi, effective against HDACs 1-5 and HDAC 7 that has been used as a treatment for seizures and mental disorders [77]. More recently VPA has been tested for its efficacy in the treatment of autoimmune disease using the Fas-deficient MRL/MPJ-Fas^{lpr}/J (MRL/$lpr^{-/-}$) mouse model. MRL/$lpr^{-/-}$ mice injected intraperitoneally with 500 mg/kg VPA for 8 weeks had decreased lymphoid organ weight and cellularity, decreased DN T cells in the spleen, lymph nodes, and blood, and a reduced number of WBCs, particularly lymphocytes, in the peripheral blood compared to vehicle-treated control mice. VPA treatment was found to induce caspase- dependent and independent apoptosis in PBMCs *in vitro* [52]. Furthermore, treatment of glomerulosclerosis in the adriamycin nephropathy mouse model with VPA reduced proteinuria in early phase renal disease [78]. VPA has been demonstrated to inhibit TNF-α, NF-κB, and IL-6 pathways, which have been shown to be dysregulated during many autoimmune diseases [78]. The mechanism of action for VPA in the treatment of autoimmune diseases has yet to be identified. However, treatment of ADR nephropathy with VPA was found to increase glomerular H3K9 acetylation and decrease glomerular apoptosis [78].

MGCD0103 is a selective class I HDAC (HDACs 1, 2, and 3) inhibitor that has also shown selectivity for HDAC11 and is currently undergoing phase I/II clinical trials [79]. MGCD0103 has been demonstrated to have antiproliferative activity in Hodgkin lymphoma cell lines and B-cell chronic lymphocytic leukemia [79-81]. Previous studies indicate that MGCD10103 increases caspase-dependent apoptosis while inhibiting autophagy through the activation of the PI3K/AKT/mTOR pathway [81, 82]. Furthermore, MGD10103 increased NF-κB activation and resulted in increased TNF-α expression and production [80]. For these reasons, MGCD0103 may not be optimal for treatment for autoimmune diseases, including SLE and RA, which are characterized by increased PI3K/AKT/mTOR signaling and NF-κB activation [83-85].

Selective HDAC3 inhibition has also been explored for its use in treating inflammatory autoimmune disease. HDAC3 expression has been shown to be elevated in PBMCs from MS patients when compared to healthy controls [86]. MI192 is a selective HDAC3i that has been shown to regulate cytokine production from PBMCs. IL-6 production by PBMCs was decreased in a dose-dependent manner following treatment with MI192; however, the mechanism remains to be elucidated [87]. Studies indicate that overexpression of HDAC3 causes apoptotic-resistant autoreactive lymphocytes that contribute to autoimmune disease [86]. These data suggest that HDAC3 inhibition may be beneficial in the treatment of autoimmunity.

Romidepsin (Depsipeptide, FK288) is a selective HDACi of HDACs 1, 2, 3, and 4 currently undergoing clinical trials for the treatment of T cell lymphoma [88]. Treatment of autoantibody-mediated arthritis (AMA) mice with FK228 reduced inflammation, joint swelling, and bone destruction. Pro-inflammatory cytokines IL-1β and TNF-α were reduced following treatment with FK228 [89]. TNF-α is known to play an important role in the pathogenesis of a number of autoimmune diseases including SLE, RA, and Crohn's disease and anti-TNF-α

therapies have proven to be an effective clinical treatment for people with these diseases [38, 90-93]. The molecular mechanism through which FK288 reduces inflammation has yet to be determined.

1.6. Class IIa HDAC inhibitors

Class II HDACS are not as ubiquitous as class I, but they are still thought to be essential for regulatory functions of the cell. Class IIa HDACs include HDACs 4, 5, 7, and 9 [24]. Expression of class IIa HDACs is thought to be more tissue specific with increased expression in the brain, muscle, and T lymphocytes [94]. While deletion of HDAC7 is embryonic lethal in mice, deletion of HDACs 4, 5, and 9 produce viable mice, but with defects in cellular hypertrophy, stress response, cardiovascular function, and bone development [24]. Studies suggest a role for class IIa HDACs (HDAC4, 5, and 7) in pro-inflammatory gene expression [95]. Given the pro-inflammatory environment associated with many autoimmune diseases, class IIa HDACs could serve as promising targets for autoimmune therapies. While class IIa HDACs are able to move back and forth between the nucleus and the cytoplasm, they are currently thought to have limited deacetylase function; rather functioning through the recruitment of HDAC3 [56, 96].

HDAC9 has been found to be overexpressed in T cells from lupus patients and SLE murine models. HDAC9 deficient MRL/lpr mice had prolonged survival and decreased lymphproliferation, autoantibody production, inflammation, and kidney disease [97]. Furthermore, HDAC9 deficient mice have decreased colitis following dextran sodium sulfate (DSS) treatment compared to wild type (WT) mice [48]. HDAC9 deficiency increased site specific lysine histone acetylation of H3K9, H3K14, and H3K18 localized to IL-4, roquin, and

PPAR-γ, respectively in MRL/lpr mice [97]. These results indicate that inhibition of HDAC9 may be able to decrease the inflammatory response through hyperacetylation and stabilization of IL-4 and PPAR-γ.

Inhibition of HDAC9 has also been associated with an increased T_{reg} suppressive function, and studies have shown that HDAC9 is exported from the nucleus upon T_{reg} activation [12, 48]. These studies suggest that when located within the nucleus HDAC9 suppresses Foxp3 function and HDAC9 nuclear exportation is required for an effective T_{reg} response [12, 48]. SiRNA knockdown of HDAC9 in WT T_{regs} resulted in increased Foxp3 expression and enhanced T_{reg} suppressive function *in vitro*. HDAC9 knockdown in T_{reg} cells caused increased HSP70 expression; however, when HDAC9$^{-/-}$ T_{regs} were treated with triptolide (an HSP70 inhibitor) suppressive function was decreased to levels comparable by WT T_{regs} [48]. Similarly to HDAC9 inhibition, knockdown of HDAC7 increased T_{reg} suppressive ability [98]. These data suggest the potential of inhibiting HDACs 7 and 9 with isoform-selective inhibitors to decrease autoimmune disease. T_{regs} function to suppress the proliferation of immune cell subsets and regulate cytokine production and the response to self-Ags. T_{reg} deficiency has been associated with a number of autoimmune diseases including SLE, MS, RA, and IBD [99-103]. Furthermore, deletion of T_{regs} in animals has been demonstrated to cause autoimmunity [104-107].

1.7. Class IIb HDAC inhibitors

Class IIb HDACs (HDAC6 and 10) are found in both the nucleus and the cytoplasm [24]. The role of HDAC10 has yet to be determined; however, HDAC6 has been shown to regulate acetylation of cytoplasmic and nuclear proteins as wells as deacetylase-independent functions. HDAC6 is thought to play an integral role in a number of cellular functions including regulation

of the cytoskeleton, cell migration, and degradation of misfolded proteins through deacetylation of α-tubulin, HSP90, and cortacin [108-110].

During SLE, the number and function of T_{reg} cells is diminished [111, 112]. Pan-HDAC inhibitors have been shown to increase the number and suppressive effects of T_{regs}, but treatment with class-I specific HDAC inhibitors, such as MS-275, have been unable to produce the same result suggesting a role of class II HDACs [12, 109]. Treatment with a specific HDAC6i leads to increased T_{reg} function. Furthermore, T_{regs} from HDAC6 deficient mice have been demonstrated to have increased suppressive T_{reg} function [109]. T_{reg} cells from HDAC6$^{-/-}$ mice had a T_{reg} effector/memory phenotype with decreased expression of CD44 and CD62L, but increased expression of CD103 [109]. Regulatory effector-memory T cells (T_{REM}) are T_{reg} cells capable of activation, expansion, and memory that function to control the immune response in inflamed tissues [113]. Furthermore, T_{regs} isolated from HDAC6 deficient mice had increased function *in vitro* suppressive of CFSE-labeled WT conventional T (T_{con}) cells [109]. Similarly, treatment with the HDAC6 specific inhibitors tubacin and tubastatin A, resulted in increased suppression of *in vitro* proliferation of T_{con} cells by T_{reg} cells. Although Tubastatin A and tubacin inhibit HDAC6, tubacin is more selective for HDAC6 and may have greater efficacy at lower doses [114].

Crohn's diseases and ulcerative colitis are two forms of IBD and are modeled by the DSS model of colitis. Similarly to other autoimmune diseases, IBD requires a genetic susceptibility coupled with environmental factors leading to an inflammatory response [115]. Studies have demonstrated treatment with tubacin is able to prevent weight loss and diarrhea in the DSS model of colitis in a T_{reg} dependent fashion [109].

Another selective HDAC6i, ACY-738, has minimal reactivity against other class II HDACs and 100-fold less selectivity against class I HDACs [116]. ACY-738 was tested for its efficacy in the treatment of SLE in NZB/W mice. We found that HDAC6 inhibition with ACY-738 was able to decrease a number of hallmarks of SLE disease including splenomegaly, immune complex-mediated glomerulonephritis, and sera anti-dsDNA levels. ACY-738 treatment altered BM B cell differentiation by increasing the percentage of cells in the late pro-B cell and early pre-B cell fractions while decreasing the accumulation of cells in the late pre-B fraction F. Furthermore, ACY-738 also increased the percentage of T_{reg} cells with a concomitant decrease in SLE-associated markers of disease (unpublished data). Studies have shown that treatment with ACY-738 (1μM) increased the suppressive function of T_{regs} alone and in combination with a sirtuin1 inhibitor, Ex-527 [117].

1.8. Class IV HDAC inhibitors

HDAC11 is the most recently identified member of HDAC proteins and is the sole member of class IV [24]. The role of HDAC11 in normal cell function still remains to be fully elucidated and no isoform-selective HDACi has yet been developed [118]. However, HDAC11 has been identified as a potential molecular target for the treatment of autoimmune disease due to its role as a negative transcriptional regulator of *IL10* [119]. Overexpression of HDAC11 in a mouse macrophage cell line prevented an increase in *IL10* mRNA expression following LPS-stimulation. Furthermore, knocking down HDAC11 using shRNA in human APCs resulted in an increase in expression of *IL10* mRNA following immune stimulation. Given the role IL-10 plays in the induction of tolerance, these results suggest targeting HDAC11 in the treatment of autoimmune disease may be beneficial. IL-10 is an anti-inflammatory cytokine with wide-

ranging effects from B cell stimulation to limiting the immune response and action of pro-inflammatory cytokines. Dysregulation of IL-10 production contributes to an increased risk for autoimmune diseases including SLE, IBD, and allergic asthma [120]. Specifically during SLE, high sera levels of IL-10 correlate with disease activity [121].

HDAC11 has also been identified as a potential target for regulating APC- mediated immune activation. Primary mouse macrophages overexpressing HDAC11 showed enhanced production of IL-2 and IFN-γ following clonotypic T cell encounter. Conversely, clonotypic T cells that were introduced to APCs with knocked down HDAC11 had reduced IL-2 and IFN-γ production [119].

1.9. Summary

Previous studies suggest a complex mechanism of action for HDAC inhibitors; the use of isoform-selective HDAC inhibitors will be helpful in determining the specific roles of individual HDACs. Questions remain about the long-term safety of HDAC inhibitor use for the treatment of chronic diseases. The identification of aberrant HDAC specific isoforms to each autoimmune disease may be important in reducing toxicity. Isoform-selective HDAC inhibition has the potential to correct aberrant immune regulation by altering the function of components of the inflammatory cascades without the deleterious side effects associated with traditional pan-HDAC inhibitors (Table 1).

1.10. References

[1] Brooks WH, Le Dantec C, Pers JO, Youinou P, Renaudineau Y. Epigenetics and autoimmunity. Journal of autoimmunity, 2010;34**:**J207-19.

[2] Meda F, Folci M, Baccarelli A, Selmi C. The epigenetics of autoimmunity. Cellular & molecular immunology, 2011;8**:**226-36.

[3] Portela A, Esteller M. Epigenetic modifications and human disease. Nature biotechnology, 2010;28**:**1057-68.

[4] Batta K, Das C, Gadad S, Shandilya J, Kundu TK. Reversible acetylation of non histone proteins: role in cellular function and disease. Sub-cellular biochemistry, 2007;41**:**193-212.

[5] Woan KV, Sahakian E, Sotomayor EM, Seto E, Villagra A. Modulation of antigen-presenting cells by HDAC inhibitors: implications in autoimmunity and cancer. Immunology and cell biology, 2012;90**:**55-65.

[6] Reilly CM, Regna N, Mishra N. HDAC inhibition in lupus models. Molecular medicine, 2011;17**:**417-25.

[7] Leoni F, Fossati G, Lewis EC, Lee JK, Porro G, Pagani P *et al.* The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo. Molecular medicine, 2005;11**:**1-15.

[8] Kawabata T, Nishida K, Takasugi K, Ogawa H, Sada K, Kadota Y *et al.* Increased activity and expression of histone deacetylase 1 in relation to tumor necrosis factor-alpha in synovial tissue of rheumatoid arthritis. Arthritis research & therapy, 2010;12**:**R133.

[9] Faraco G, Cavone L, Chiarugi A. The therapeutic potential of HDAC inhibitors in the treatment of multiple sclerosis. Molecular medicine, 2011;17**:**442-7.

[10] Huber LC, Brock M, Hemmatazad H, Giger OT, Moritz F, Trenkmann M *et al.* Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients. Arthritis and rheumatism, 2007;56**:**1087-93.

[11] Perl A. Pathogenic mechanisms in systemic lupus erythematosus. Autoimmunity, 2010;43**:**1-6.

[12] de Zoeten EF, Wang L, Sai H, Dillmann WH, Hancock WW. Inhibition of HDAC9 increases T regulatory cell function and prevents colitis in mice. Gastroenterology, 2010;138**:**583-94.

[13] Karberg S. Switching on epigenetic therapy. Cell, 2009;139**:**1029-31.

[14] Willyard C. The saving switch. Nature medicine, 2010;16**:**18-21.

[15] Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. Molecular cancer research : MCR, 2007;5**:**981-9.

[16] Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. Gene, 2005;363**:**15-23.

[17] Spange S, Wagner T, Heinzel T, Kramer OH. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. The international journal of biochemistry & cell biology, 2009;41**:**185-98.

[18] Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. Trends in immunology, 2011;32**:**335-43.

[19] Akimova T, Beier UH, Liu Y, Wang L, Hancock WW. Histone/protein deacetylases and T-cell immune responses. Blood, 2012;119**:**2443-51.

[20] Gryder BE, Sodji QH, Oyelere AK. Targeted cancer therapy: giving histone deacetylase inhibitors all they need to succeed. Future medicinal chemistry, 2012;4**:**505-24.

[21] Tan J, Cang S, Ma Y, Petrillo RL, Liu D. Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. Journal of hematology & oncology, 2010;3**:**5.

[22] de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. The Biochemical journal, 2003;370**:**737-49.

[23] Saunders LR, Verdin E. Sirtuins: critical regulators at the crossroads between cancer and aging. Oncogene, 2007;26**:**5489-504.

[24] Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: What are the cancer relevant targets? Cancer letters, 2009;277**:**8-21.

[25] Delcuve GP, Khan DH, Davie JR. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clinical epigenetics, 2012;4**:**5.

[26] Yang WM, Tsai SC, Wen YD, Fejer G, Seto E. Functional domains of histone deacetylase-3. The Journal of biological chemistry, 2002;277**:**9447-54.

[27] Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A *et al.* HDAC6 is a microtubule-associated deacetylase. Nature, 2002;417**:**455-8.

[28] Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. The Journal of biological chemistry, 2002;277**:**25748-55.

[29] Davidson A, Diamond B. Autoimmune diseases. The New England journal of medicine, 2001;345**:**340-50.

[30] Javierre BM, Fernandez AF, Richter J, Al-Shahrour F, Martin-Subero JI, Rodriguez-Ubreva J *et al.* Changes in the pattern of DNA methylation associate with twin discordance in systemic lupus erythematosus. Genome research, 2010;20**:**170-9.

[31] Ballestar E. Epigenetics lessons from twins: prospects for autoimmune disease. Clinical reviews in allergy & immunology, 2010;39**:**30-41.

[32] Simmons JK, Patel J, Michalowski A, Zhang S, Wei BR, Sullivan P *et al.* TORC1 and class I HDAC inhibitors synergize to suppress mature B cell neoplasms. Molecular oncology, 2013.

[33] Akimova T, Ge G, Golovina T, Mikheeva T, Wang L, Riley JL *et al.* Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs. Clinical immunology, 2010;136**:**348-63.

[34] Regna NL, Chafin CB, Hammond SE, Puthiyaveetil AG, Caudell DL, Reilly CM. Class I and II histone deacetylase inhibition by ITF2357 reduces SLE pathogenesis in vivo. Clinical immunology, 2014;151**:**29-42.

[35] Dovey OM, Foster CT, Conte N, Edwards SA, Edwards JM, Singh R *et al.* Histone deacetylase 1 and 2 are essential for normal T-cell development and genomic stability in mice. Blood, 2013;121**:**1335-44.

[36] Glauben R, Siegmund B. Inhibition of histone deacetylases in inflammatory bowel diseases. Molecular medicine, 2011;17**:**426-33.

[37] Wang L, de Zoeten EF, Greene MI, Hancock WW. Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3+ regulatory T cells. Nature reviews Drug discovery, 2009;8**:**969-81.

[38] Zhu LJ, Yang X, Yu XQ. Anti-TNF-alpha therapies in systemic lupus erythematosus. Journal of biomedicine & biotechnology, 2010;2010**:**465898.

[39] Mishra N, Reilly CM, Brown DR, Ruiz P, Gilkeson GS. Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. Journal of Clinical Investigation, 2003;111**:**539-52.

[40] Reilly CM, Thomas M, Gogal Jr R, Olgun S, Santo A, Sodhi R *et al.* The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice. Journal of autoimmunity, 2008;31**:**123-30.

[41] Reilly CM, Mishra N, Miller JM, Joshi D, Ruiz P, Richon VM *et al.* Modulation of renal disease in MRL/lpr mice by suberoylanilide hydroxamic acid. Journal of immunology, 2004;173**:**4171-8.

[42] Joosten LA, Leoni F, Meghji S, Mascagni P. Inhibition of HDAC activity by ITF2357 ameliorates joint inflammation and prevents cartilage and bone destruction in experimental arthritis. Molecular medicine, 2011;17**:**391-6.

[43] Nasu Y, Nishida K, Miyazawa S, Komiyama T, Kadota Y, Abe N *et al.* Trichostatin A, a histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society, 2008;16**:**723-32.

[44] Chan VS, Tsang HH, Tam RC, Lu L, Lau CS. B-cell-targeted therapies in systemic lupus erythematosus. Cellular & molecular immunology, 2013;10**:**133-42.

[45] Anolik J, Sanz I. B cells in human and murine systemic lupus erythematosus. Current opinion in rheumatology, 2004;16**:**505-12.

[46] Morimoto C, Reinherz EL, Schlossman SF, Schur PH, Mills JA, Steinberg AD. Alterations in immunoregulatory T cell subsets in active systemic lupus erythematosus. The Journal of clinical investigation, 1980;66**:**1171-4.

[47] Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE *et al.* Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. Journal of immunology, 2008;181**:**8761-6.

[48] Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM *et al.* Deacetylase inhibition promotes the generation and function of regulatory T cells. Nature medicine, 2007;13**:**1299-307.

[49] Kim HJ, Bae SC. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. American journal of translational research, 2011;3**:**166-79.

[50] Ellis L, Pan Y, Smyth GK, George DJ, McCormack C, Williams-Truax R *et al.* Histone deacetylase inhibitor panobinostat induces clinical responses with associated alterations in gene expression profiles in cutaneous T-cell lymphoma. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008;14**:**4500-10.

[51] Coronel J, Cetina L, Pacheco I, Trejo-Becerril C, Gonzalez-Fierro A, de la Cruz-Hernandez E *et al.* A double-blind, placebo-controlled, randomized phase III trial of chemotherapy plus epigenetic therapy with hydralazine valproate for advanced cervical cancer. Preliminary results. Medical oncology, 2011;28 Suppl 1**:**S540-6.

[52] Dowdell KC, Pesnicak L, Hoffmann V, Steadman K, Remaley AT, Cohen JI *et al.* Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, diminishes lymphoproliferation in the Fas -deficient MRL/lpr(-/-) murine model of autoimmune lymphoproliferative syndrome (ALPS). Experimental hematology, 2009;37**:**487-94.

[53] Wagner JM, Hackanson B, Lubbert M, Jung M. Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. Clinical epigenetics, 2010;1**:**117-36.

[54] Ganesan A, Nolan L, Crabb SJ, Packham G. Epigenetic therapy: histone acetylation, DNA methylation and anti-cancer drug discovery. Current cancer drug targets, 2009;9**:**963-81.

[55] Bannerman B, Xu L, Jones M, Tsu C, Yu J, Hales P *et al.* Preclinical evaluation of the antitumor activity of bortezomib in combination with vitamin C or with epigallocatechin gallate, a component of green tea. Cancer chemotherapy and pharmacology, 2011;68**:**1145-54.

[56] Hancock WW. Rationale for HDAC inhibitor therapy in autoimmunity and transplantation. Handbook of experimental pharmacology, 2011;206**:**103-23.

[57] Reichert N, Choukrallah MA, Matthias P. Multiple roles of class I HDACs in proliferation, differentiation, and development. Cellular and molecular life sciences : CMLS, 2012;69**:**2173-87.

[58] Harms KL, Chen X. Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity. Cancer research, 2007;67**:**3145-52.

[59] Huang BH, Laban M, Leung CH, Lee L, Lee CK, Salto-Tellez M *et al.* Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. Cell death and differentiation, 2005;12**:**395-404.

[60] Kawashima H, Takatori H, Suzuki K, Iwata A, Yokota M, Suto A *et al.* Tumor suppressor p53 inhibits systemic autoimmune diseases by inducing regulatory T cells. Journal of immunology, 2013;191**:**3614-23.

[61] Zheng SJ, Lamhamedi-Cherradi SE, Wang P, Xu L, Chen YH. Tumor suppressor p53 inhibits autoimmune inflammation and macrophage function. Diabetes, 2005;54**:**1423-8.

[62] Komarova EA, Krivokrysenko V, Wang K, Neznanov N, Chernov MV, Komarov PG *et al.* p53 is a suppressor of inflammatory response in mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2005;19**:**1030-2.

[63] Lin HS, Hu CY, Chan HY, Liew YY, Huang HP, Lepescheux L *et al.* Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents. British journal of pharmacology, 2007;150**:**862-72.

[64] Choo QY, Ho PC, Tanaka Y, Lin HS. Histone deacetylase inhibitors MS-275 and SAHA induced growth arrest and suppressed lipopolysaccharide-stimulated NF-kappaB p65 nuclear accumulation in human rheumatoid arthritis synovial fibroblastic E11 cells. Rheumatology, 2010;49**:**1447-60.

[65] Tackey E, Lipsky PE, Illei GG. Rationale for interleukin-6 blockade in systemic lupus erythematosus. Lupus, 2004;13**:**339-43.

[66] Ohshima S, Saeki Y, Mima T, Sasai M, Nishioka K, Nomura S *et al.* Interleukin 6 plays a key role in the development of antigen-induced arthritis. Proceedings of the National Academy of Sciences of the United States of America, 1998;95**:**8222-6.

[67] Rogler G, Andus T. Cytokines in inflammatory bowel disease. World journal of surgery, 1998;22**:**382-9.

[68] Samoilova EB, Horton JL, Hilliard B, Liu TS, Chen Y. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. Journal of immunology, 1998;161**:**6480-6.

[69] Kay J, Calabrese L. The role of interleukin-1 in the pathogenesis of rheumatoid arthritis. Rheumatology, 2004;43 Suppl 3**:**iii2-iii9.

[70] Coccia M, Harrison OJ, Schiering C, Asquith MJ, Becher B, Powrie F *et al.* IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. The Journal of experimental medicine, 2012;209**:**1595-609.

[71] Voronov E, Dayan M, Zinger H, Gayvoronsky L, Lin JP, Iwakura Y *et al.* IL-1 beta-deficient mice are resistant to induction of experimental SLE. European cytokine network, 2006;17**:**109-16.

[72] Kim HN, Lee JH, Jin WJ, Ko S, Jung K, Ha H *et al.* MS-275, a benzamide histone deacetylase inhibitor, prevents osteoclastogenesis by down-regulating c-Fos expression and suppresses bone loss in mice. European journal of pharmacology, 2012;691**:**69-76.

[73] Yamashita T, Yao Z, Li F, Zhang Q, Badell IR, Schwarz EM *et al.* NF-kappaB p50 and p52 regulate receptor activator of NF-kappaB ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. The Journal of biological chemistry, 2007;282**:**18245-53.

[74] Nishioka C, Ikezoe T, Yang J, Takeuchi S, Koeffler HP, Yokoyama A. MS-275, a novel histone deacetylase inhibitor with selectivity against HDAC1, induces degradation of FLT3 via inhibition of chaperone function of heat shock protein 90 in AML cells. Leukemia research, 2008;32**:**1382-92.

[75] Shukla HD, Pitha PM. Role of hsp90 in systemic lupus erythematosus and its clinical relevance. Autoimmune diseases, 2012;2012**:**728605.

[76] Shimp SK, 3rd, Chafin CB, Regna NL, Hammond SE, Read MA, Caudell DL *et al.* Heat shock protein 90 inhibition by 17-DMAG lessens disease in the MRL/lpr mouse model of systemic lupus erythematosus. Cellular & molecular immunology, 2012;9**:**255-66.

[77] Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer research, 2004;64**:**1079-86.

[78] Van Beneden K, Geers C, Pauwels M, Mannaerts I, Verbeelen D, van Grunsven LA *et al.* Valproic acid attenuates proteinuria and kidney injury. Journal of the American Society of Nephrology : JASN, 2011;22**:**1863-75.

[79] Siu LL, Pili R, Duran I, Messersmith WA, Chen EX, Sullivan R *et al.* Phase I study of MGCD0103 given as a three-times-per-week oral dose in patients with advanced solid

tumors. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2008;26**:**1940-7.

[80] Buglio D, Mamidipudi V, Khaskhely NM, Brady H, Heise C, Besterman J *et al.* The class-I HDAC inhibitor MGCD0103 induces apoptosis in Hodgkin lymphoma cell lines and synergizes with proteasome inhibitors by an HDAC6-independent mechanism. British journal of haematology, 2010;151**:**387-96.

[81] El-Khoury V, Pierson S, Szwarcbart E, Brons NH, Roland O, Cherrier-De Wilde S *et al.* Disruption of autophagy by the histone deacetylase inhibitor MGCD0103 and its therapeutic implication in B-cell chronic lymphocytic leukemia. Leukemia, 2014.

[82] El-Khoury V, Moussay E, Janji B, Palissot V, Aouali N, Brons NH *et al.* The histone deacetylase inhibitor MGCD0103 induces apoptosis in B-cell chronic lymphocytic leukemia cells through a mitochondria-mediated caspase activation cascade. Molecular cancer therapeutics, 2010;9**:**1349-60.

[83] Stylianou K, Petrakis I, Mavroeidi V, Stratakis S, Vardaki E, Perakis K *et al.* The PI3K/Akt/mTOR pathway is activated in murine lupus nephritis and downregulated by rapamycin. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2011;26**:**498-508.

[84] Kim TH, Choi SJ, Lee YH, Song GG, Ji JD. Combined therapeutic application of mTOR inhibitor and vitamin D(3) for inflammatory bone destruction of rheumatoid arthritis. Medical hypotheses, 2012;79**:**757-60.

[85] Foster JG, Blunt MD, Carter E, Ward SG. Inhibition of PI3K signaling spurs new therapeutic opportunities in inflammatory/autoimmune diseases and hematological malignancies. Pharmacological reviews, 2012;64**:**1027-54.

[86] Zhang F, Shi Y, Wang L, Sriram S. Role of HDAC3 on p53 expression and apoptosis in T cells of patients with multiple sclerosis. PloS one, 2011;6**:**e16795.

[87] Gillespie J, Savic S, Wong C, Hempshall A, Inman M, Emery P *et al.* Histone deacetylases are dysregulated in rheumatoid arthritis and a novel histone deacetylase 3-selective inhibitor reduces interleukin-6 production by peripheral blood mononuclear cells from rheumatoid arthritis patients. Arthritis and rheumatism, 2012;64**:**418-22.

[88] Furumai R, Matsuyama A, Kobashi N, Lee KH, Nishiyama M, Nakajima H *et al.* FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer research, 2002;62**:**4916-21.

[89] Nishida K, Komiyama T, Miyazawa S, Shen ZN, Furumatsu T, Doi H *et al.* Histone deacetylase inhibitor suppression of autoantibody-mediated arthritis in mice via regulation of p16INK4a and p21(WAF1/Cip1) expression. Arthritis and rheumatism, 2004;50**:**3365-76.

[90] Aringer M, Houssiau F, Gordon C, Graninger WB, Voll RE, Rath E *et al.* Adverse events and efficacy of TNF-alpha blockade with infliximab in patients with systemic lupus erythematosus: long-term follow-up of 13 patients. Rheumatology, 2009;48**:**1451-4.

[91] Aringer M, Smolen JS. The role of tumor necrosis factor-alpha in systemic lupus erythematosus. Arthritis research & therapy, 2008;10**:**202.

[92] Reimund JM, Ratajczyk J, Sola B, Justum AM, Muller CD. Anti-tumor necrosis factor-alpha (TNF-alpha) treatment strategies in Crohn's disease. Recent patents on inflammation & allergy drug discovery, 2007;1**:**21-34.

[93] Seymour HE, Worsley A, Smith JM, Thomas SH. Anti-TNF agents for rheumatoid arthritis. British journal of clinical pharmacology, 2001;51**:**201-8.

[94] Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nature reviews Genetics, 2009;10**:**32-42.

[95] Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A *et al.* LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2006;20**:**1315-27.

[96] Fischle W, Dequiedt F, Hendzel MJ, Guenther MG, Lazar MA, Voelter W *et al.* Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Molecular cell, 2002;9**:**45-57.

[97] Yan K, Cao Q, Reilly CM, Young NL, Garcia BA, Mishra N. Histone deacetylase 9 deficiency protects against effector T cell-mediated systemic autoimmunity. The Journal of biological chemistry, 2011;286**:**28833-43.

[98] Tao R, Hancock WW. Resistance of Foxp3+ regulatory T cells to Nur77-induced apoptosis promotes allograft survival. PloS one, 2008;3**:**e2321.

[99] Kuhn A, Beissert S, Krammer PH. CD4(+)CD25 (+) regulatory T cells in human lupus erythematosus. Archives of dermatological research, 2009;301**:**71-81.

[100] Crispin JC, Liossis SN, Kis-Toth K, Lieberman LA, Kyttaris VC, Juang YT *et al.* Pathogenesis of human systemic lupus erythematosus: recent advances. Trends in molecular medicine, 2010;16**:**47-57.

[101] Cao D, Malmstrom V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C. Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis. European journal of immunology, 2003;33**:**215-23.

[102] Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. The Journal of experimental medicine, 2004;199**:**971-9.

[103] Boden EK, Snapper SB. Regulatory T cells in inflammatory bowel disease. Current opinion in gastroenterology, 2008;24**:**733-41.

[104] Liu MF, Lin LH, Weng CT, Weng MY. Decreased CD4+CD25+bright T cells in peripheral blood of patients with primary Sjogren's syndrome. Lupus, 2008;17**:**34-9.

[105] Wahl SM, Chen W. Transforming growth factor-beta-induced regulatory T cells referee inflammatory and autoimmune diseases. Arthritis research & therapy, 2005;7**:**62-8.

[106] Wenzel J, Henze S, Brahler S, Bieber T, Tuting T. The expression of human leukocyte antigen-DR and CD25 on circulating T cells in cutaneous lupus erythematosus and correlation with disease activity. Experimental dermatology, 2005;14**:**454-9.

[107] Hayashi T, Hasegawa K, Adachi C. Elimination of CD4(+)CD25(+) T cell accelerates the development of glomerulonephritis during the preactive phase in autoimmune-prone female NZB x NZW F mice. International journal of experimental pathology, 2005;86**:**289-96.

[108] Dallavalle S, Pisano C, Zunino F. Development and therapeutic impact of HDAC6-selective inhibitors. Biochemical pharmacology, 2012;84**:**756-65.

[109] de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H *et al.* Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. Molecular and cellular biology, 2011;31**:**2066-78.

[110] Valenzuela-Fernandez A, Cabrero JR, Serrador JM, Sanchez-Madrid F. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. Trends in cell biology, 2008;18**:**291-7.

[111] Horwitz DA. Regulatory T cells in systemic lupus erythematosus: past, present and future. Arthritis research & therapy, 2008;10**:**227.

[112] La Cava A. T-regulatory cells in systemic lupus erythematosus. Lupus, 2008;17**:**421-5.

[113] Kleinewietfeld M, Puentes F, Borsellino G, Battistini L, Rotzschke O, Falk K. CCR6 expression defines regulatory effector/memory-like cells within the CD25(+)CD4+ T-cell subset. Blood, 2005;105**:**2877-86.

[114] Butler KV, Kalin J, Brochier C, Vistoli G, Langley B, Kozikowski AP. Rational design and simple chemistry yield a superior, neuroprotective HDAC6 inhibitor, tubastatin A. Journal of the American Chemical Society, 2010;132**:**10842-6.

[115] Zhang YZ, Li YY. Inflammatory bowel disease: Pathogenesis. World journal of gastroenterology : WJG, 2014;20**:**91-9.

[116] Jochems J, Boulden J, Lee BG, Blendy JA, Jarpe M, Mazitschek R *et al.* Antidepressant-Like Properties of Novel HDAC6 Selective Inhibitors with Improved Brain Bioavailability. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2013.

[117] Beier UH, Wang L, Han R, Akimova T, Liu Y, Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. Science signaling, 2012;5**:**ra45.

[118] Hancock WW, Akimova T, Beier UH, Liu Y, Wang L. HDAC inhibitor therapy in autoimmunity and transplantation. Annals of the rheumatic diseases, 2012;71 Suppl 2**:**i46-54.

[119] Villagra A, Cheng F, Wang HW, Suarez I, Glozak M, Maurin M *et al.* The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nature immunology, 2009;10**:**92-100.

[120] Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Critical reviews in immunology, 2012;32**:**23-63.

[121] Park YB, Lee SK, Kim DS, Lee J, Lee CH, Song CH. Elevated interleukin-10 levels correlated with disease activity in systemic lupus erythematosus. Clinical and experimental rheumatology, 1998;16**:**283-8.

[122] Zhang ZY, Zhang Z, Schluesener HJ. MS-275, an histone deacetylase inhibitor, reduces the inflammatory reaction in rat experimental autoimmune neuritis. Neuroscience, 2010;169**:**370-7.

[123] Namdar M, Perez G, Ngo L, Marks PA. Selective inhibition of histone deacetylase 6 (HDAC6) induces DNA damage and sensitizes transformed cells to anticancer agents. Proceedings of the National Academy of Sciences of the United States of America, 2010;107**:**20003-8.

[124] Vishwakarma S, Iyer LR, Muley M, Singh PK, Shastry A, Saxena A *et al.* Tubastatin, a selective histone deacetylase 6 inhibitor shows anti-inflammatory and anti-rheumatic effects. International immunopharmacology, 2013;16**:**72-8.

Table I. Isoform-Selective HDAC Inhibitors and Immune Regulation

Compound	Isoform-Specificity	Protein/enzyme/ gene	Cellular response	Disease	Reference
MS-275	HDACs 1,2,3,9	↑Foxp3 ↓nuclear NFκB p65, VEGF	↑IL-10 ↓IL-1β, IFN-γ, IL-17, IL-18, TNF-α, IL-18, NO	RA	[64, 122]
MI192	HDAC3	ND	↓TNF, IL-6, IFN-γ	RA	[87]
MGCD0103	HDACs 1,2,3,11	Jak/STAT ↑NF-κB activation, *TNFSF4, TNFSF9, TNF* ↓*TNFRSF8*	↑TNF-α	ND	[79, 80]
Valproic acid	HDACs 1,2,3,8	PI3K/Akt,mTOR, NF-κB	↓TNF-α	ALPS, SLE, IBD	[52, 78]
FK228	HDACs 1,2,3,4	ND	↓IL-1β, TNF-α	AMA, RA, diabetes	[20, 88, 89]
ACY-738	HDAC6	Foxp3	↑TGF-β ↓IL-1β	SLE	[117] Unpublished data
Tubacin	HDAC6	α-tubulin, HSp90 Foxp3 ↑CTLA-4, PD-1, GITR	↓IL-2, IFN-γ ↑IL-10	RA, IBD	[109, 123]
Tubastatin A	HDAC6	α-tubulin, Foxp3	↓TNF-α, IL-6	RA, IBD	[114, 124]

Chapter 2

Class I and II histone deacetylase inhibition by ITF2357 reduces SLE pathogenesis *in vivo*

N. L.Regna[1]
C. B. Chafin[1]
S.E. Hammond[1]
A.G. Puthiyaveetil[2]
D.L. Caudell[3]
C. M. Reilly[1, 4]

[1]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061

[2] Department of Biotechnology, American University of Ras Al Khaimah, United Arab Emirates

[3] Wake Forest University Primate Center and the Department of Pathology/Comparative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157

[4]Edward Via College of Osteopathic Medicine, Blacksburg, VA, 24060

Accepted by *Clinical Immunology* (PMID 24503172)

2.2. Abstract

We sought to determine if a specific class I and II HDAC inhibitor (ITF2357) was able to decrease disease in lupus-prone NZB/W mice through regulation of T cell profiles. From 22 to 38 weeks-of-age, NZB/W and non-lupus NZW mice were treated with ITF2357 (5 mg/kg or 10 mg/kg), or vehicle control. Body weight and proteinuria were measured every 2 weeks, while sera anti-dsDNA and cytokine levels were measured every 4 weeks. Kidney disease was determined by sera IgG levels, immune complex deposition, and renal pathology. T lymphocyte profiles were assessed using flow cytometric analyses. Our results showed that NZB/W mice treated with the 10 mg/kgof ITF2357 had decreased renal disease and inflammatory cytokines in the sera. Treatment with ITF2357 decreased the Th17 phenotype while increasing the percentage of Tregs as well as Foxp3 acetylation. These results suggest that specific HDAC inhibition may decrease disease by altering T cell differentiation and acetylation.

Keywords: Systemic lupus erythematosus; Histone deacetylase Regulatory T cells

2.3. Introduction

Systemic lupus erythematous (SLE) is an autoimmune disease in which a genetic predisposition coupled with an environmental trigger initiates disease. The major cause of morbidity and mortality is lupus nephritis (LN), which affects over half of all SLE patients [1]. Altered T cell profiles leading to a loss of self-tolerance, an increased immune response, and decreased B cell suppression contribute to glomerular immune complex deposition and kidney dysfunction [2]. Treg cell numbers and function are diminished in patients with SLE [3, 4]. The percentage of Treg cells has been shown to be inversely related to anti-dsDNA serum levels and disease severity in human SLE patients [5].

New Zealand Black/BinJ (NZB) mice spontaneously develop autoimmune abnormalities including hemolytic anemia, increased levels of Ig, glomerulonephritis, and anti-dsDNA antibodies. New Zealand White/LacJ (NZW) mice do not develop severe autoimmune disease and can be used as non-lupus controls [6, 7]. NZW mice have a normal lifespan, but do develop anti-DNA Abs late in life and have been demonstrated to have increased numbers of Treg cells as early as 5-6 weeks-of-age [8]. The F1 progeny (NZB/W) from the cross between NZB and NZW mice develop lupus-like symptoms including glomerulonephritis, immune complex deposition, activated T and B cells, and autoantibody production to dsDNA [6]. NZB/W mice share many similar symptoms and disease pathologies with human SLE and serve as an acceptable model to study human disease. [9, 10]. NZB/W mice begin to develop disease by 20 weeks-of-age, which progresses to severe renal disease by 36 weeks-of-age [11]. NZB/W mice predominantly overproduce the IgG2a subclass of IgG, which is associated with increased SLE pathogenicity [12].

T cells play a critical role in the adaptive immune response and their dysregulation has been implicated in many autoimmune diseases, including SLE. Cytotoxic T cell ($CD8^+$) activity is known to be reduced in SLE patients, which contributes to increased B cell activity leading to autoantibody production [13, 14]. $CD4^+$ T cells can differentiate into 4 major subsets: Th1, Th2, Th17, and Treg cells [15]. Th2 cells are instrumental to humoral immunity and are responsible for secreting IL-4, IL-5, and IL-10 [16-18]. The balance between Th1 and Th2 subsets is an important regulator of autoimmune disease [19]. Studies of SLE have demonstrated an increase in both Th1 and Th2 cytokines in murine models as well as in humans [19]. Th17 ($CD4^+$ $ROR\gamma^+IL\text{-}17^+$) cells produce IL-17 and have recently been implicated in multiple autoimmune diseases [20]. SLE patients tend to have increased levels of Th17 cells leading to overproduction of IL-17 and increased activation of inflammatory mediators contributing to tissue damage [14, 19, 21, 22]. Treg cells ($CD4^+CD25^+Foxp3^+$) function to suppress the proliferation of other immune cell subsets, regulating cytokine production and self-reactive T cells. Differentiation of $CD4^+$ T cells into Treg cells requires the Foxp3 transcription factor. When Foxp3 is mutated in T cells, autoimmune disease can develop due to the immune system's inability to regulate Th1 pro-inflammatory cytokines including IL-2, IFN-γ, and TNF-α, involved with cell-mediated immunity. Studies in healthy mice have shown that depletion of Tregs leads to the development of autoimmune disease in these animals [23-26]. Histone deacetylases (HDACs) are able to influence the Foxp3 gene directly through histone deacetylation as well as indirectly by altering Foxp3 transcription factors [27, 28].

HDACs have been implicated for their role in autoimmune dysregulation. DNA is packaged into approximately 146 bp and structured around a histone core to form a nucleosome [29]. Histone proteins can be modified through the addition of acetyl groups to lysine residues by

histone acetyl transferases (HATs), regulating gene expression [30, 31]. Conversely, HDACs remove acetyl groups from the lysine residues, condensing chromatin and preventing gene transcription. HDAC inhibitors prevent the removal of acetyl groups from histone proteins leading to hyperacetylation of histones [28, 32]. HDACs are not only able to epigenetically regulate gene transcription, but more recently have been shown to regulate acetylation of non-histone proteins including transcriptional factors, DNA repair enzymes, and structural proteins. HDACs are thereby able to directly influence protein stability, protein-protein interactions, and protein-DNA interactions through post-translational acetylation [33, 34].

HDACs are grouped into four classes: classes I – IV. Class I HDACs, which includes HDAC 1, 2, 3, 6, and 8, are located solely within the nucleus. Class II HDACs, which includes HDAC 4, 5, 7, and 9) are found in both the nucleus and the cytoplasm [27, 32]. Class III HDACs consist of seven mammalian silent information regulator two proteins (sirtuins or Sirt) [32, 35]. Class IV HDACs solely consist of HDAC 11,which modify DNA expression by changing the core histones [32]. HDACi are able to target specific classes of HDAC proteins eliciting various effects on both histone and non-histone proteins.

The current studies were designed to determine whether a class I and II HDACi would decrease lupus nephritis by epigenetically altering the differentiation of splenic T cells. ITF2357 is a known inhibitor of class I and II HDACS with anti-inflammatory properties [36]. Previous studies have shown that ITF2357, a hydroxamic acid-derived compound, is selective against HDACs 1, 2, 3, 4, 6, and 7 and has demonstrated no specificity for class III or IV HDACs [37, 38]. Current research of ITF2357 has indicated it is able to reduce the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, and IFN-γ) at a low dose (1.0 mg/kg) without adverse cytotoxic effects [39-43]. ITF2357 has been demonstrated to be efficacious in cancer

treatment [44] and is in a phase II clinical trial for children with active systemic onset juvenile idiopathic arthritis [45].

2.4. Materials and Methods

2.4.1. Mice

Female NZB/W F1 and NZW 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 (Virginia Tech) and housed in the animal facility at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM, Blacksburg, VA, USA).

2.4.2. *In vivo* treatment

Mice were injected intraperitoneally 5 days/week with the vehicle control (DMSO), ITF2357 treatment at 5mg/kg, or ITF2357 treatment at 10mg/kg. The total volume of each injection was 50 µl. Treatment began at 22-weeks-of-age until euthanization during late stage clinical disease at 38 weeks-of-age. ITF2357 was courtesy of a generous donation from Dr. Paolo Mascagni and Italfarmaco for use in all studies. Proteinuria and weight were measured every two weeks and blood was collected once a month for sera analysis. Proteinuria was measured in a blinded manner 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.

2.4.3. Meausurement of autoantibodies

Sera were collected prior to initiation of treatment at 22 weeks-of-age and every 4 weeks until euthanization. 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 hours and then centrifuged for 15 min at 10,000 x *g*. The levels of sera antibodies to dsDNA were measured by ELISA. High-binding plates were coated with 100 µL of 5 µg/mL Calf Thymus DNA (Sigma, St. Louis, MO, USA) in saline-sodium citrate (SSC) buffer and incubated overnight at 37°C. Plates were washed 3 times with 0.05% Tween-20 in 1X PBS (Thermo Scientific, Waltham, MA, USA) and then blocked with 1% BSA for 1 hour (BSA, Sigma, St. Louis, MO). Sera samples were added to the plate at a 1:100 dilution, followed by a two-fold serial dilution. The plates were incubated for 45 minutes at 37°C. The plates were then incubated with an HRP-conjugated goat anti-mouse IgG gamma chain specific Ab (1:4000, Southern Biotech, Birmingham, AL, USA) and washed as described above. TMB substrate (Pierce, ThermoScientific, Rockford, IL, USA) was added to the wells and the plate was read at 380 nm on a Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A final dilution of 1:1600 was reported.

2.4.4. Pathology

At the time of euthanization, the kidneys were removed and cut in half. One half of the kidney from each mouse was fixed in formalin, embedded in paraffin, sectioned, and stained with Periodic acid-Schiff (PAS). Kidney sections were scored (0 – 4) for glomerular proliferation, inflammation, crescent formation, necrosis, and fibrosis by a pathologist (David Caudell) in a blinded manner.

2.4.5. Immunofluoresence 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) diluted 1:100. Kidney sections were fixed in acetone for 10 minutes and washed 3 times with 1X PBS for 5 minutes each. The sections were then incubated with C3 or IgG antibodies in a humid chamber for 1 hour. Slides were mounted using Vectashield mounting media with DAPI (Vector Labs, Burlingame, CA, USA) and examined by fluorescent microscopy. Sections were scored (0 – 4) for immune complex deposition by a pathologist in a blinded manner.

2.4.6. Fluorescent histone acetylation

Three μM sections were obtained from kidneys frozen in OCT media. Kidney sections were thawed and fixed in acetone for 10 minutes at room temperature. Slides were rinsed 3 times with 1X PBS for 5 minutes each. Slides were incubated overnight at 4°C with acetyl-Histone H3 (Lys9) (Alexa Fluor 488,) diluted 1:500 in 1X PBS containing 1% BSA and 0.3% Triton X-100. Slides were rinsed with 1X PBS 3 times, mounted using Vectashield mounting medium, and examined for fluorescence.

2.4.7. Flow cytometric analysis

A single-cell suspension was obtained from the spleens of NZB/W and NZW mice at 38-weeks-of age. Briefly, spleens were dissociated using a wire mesh and the cell suspension was centrifuged for 5 minutes at 300 x *g*. Cells were treated with RBC lysis buffer for 5 minutes at room temperature to lyse erythrocytes, washed 2 times with 1X PBS and then resuspend in flow

cytometry staining buffer. Cells were stained with Allophycocyanin (APC)-conjugated CD3, FITC-conjugated CD4, eFluor450 (eF450)-conjugated CD8a, PerCP-CY5.5-conjugated CD25, and PE-conjugated Foxp3, or Fitc-CD4, APC-ROR-γ, and PE-IL-17, or APC-CD3, Fitc-CD4, and PE-CD8 anti-mouse mAbs (eBioscience, San Diego, CA, USA). Fluorescence was measured using a FACS Aria 1 (BD Biosciences, San Jose, CA) and data was analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

2.4.8. Glomerular Isolation

The cortical tissue was isolated from one kidney of each mouse and pooled by treatment group. The tissue was minced using a surgical blade and then pressed through grading sieves (180 and 150μM mesh). The cells remaining on the 75μM mesh were collected in 1X PBS, force-pressed through a 21-gauge needle, and centrifuged. The pelleted cells were resuspended in 750 U/mL Worthington type I collagenase solution and gently stirred in a water bath at 37°C for 20 minutes. Glomerular cells were pelleted and then resuspended in RNA*later* (QIAGEN, Valencia, CA, USA) and stored at -20°C until RNA isolation.

2.4.9. Isolation of RNA

RNA was isolated using the mirVana miRNA isolation kit according to the manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Briefly, the cells were lysed and mixed with acid-phenol: chloroform for organic extraction. The lysate was centrifuged to separate the organic phases. The upper aqueous phase was removed and mixed with 100% ethanol which was transferred onto a filter cartridge. RNA was eluted from the filter using 95°C elution solution. The eluates were quantified on a spectrophotometer (Nanodrop, Thermo

Scientific, Waltham, MA, USA). An aliquot was taken and diluted to 1 ng/µL for real-time RT-PCR. The eluted RNA was stored at -80°C.

2.4.10. Real-time RT-PCR

IL-10, IL-6 and TGF-β mRNA expression were measured using TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA, USA). The RT master mix was mixed with 10 µL of 1ng/µL RNA template. The negative control received 10 µL of nuclease-free water. RT was performed in an iCycler using the following parameter values: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and held at 4°C. The RT product was stored at -20°C until PCR was performed as described above. The ΔC_T was calculated using the endogenous control GAPDH, and then the $\Delta\Delta C_T$ was determined by calculating the fold change in expression between the NZB/W mice and the NZW controls. All samples were run in triplicate.

2.4.11. ELISA

IL-1β, IL-10, and TGF-β protein 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.

2.4.12. *In vitro* immunoprecipitation and Western blotting

To determine if ITF2357 alters Foxp3 acetylation *in vitro*, 22-week-old female NZB/W mice were euthanized and the spleens were made into single-cell suspensions using wire mesh. At the time of euthanization all mice had a proteinuria of 30-100 mg/dL. Naïve $CD4^+$ T cells

were isolated using magnetic beads and cultured in RPMI media containing 50 μM 2-ME, 1% streptomycin/penicillin, 2 mM HEPES, and 10% FBS at 37°C in a 5% CO_2-humidified incubator. Splenic $CD4^+$ T cells were differentiated into Tregs with anti-CD3 (5μg/ml), anti-CD28 (2μg/ml), recombinant human IL-2 (rhIL-2) (20 U/ml), and TGF-β (2ng/ml). Purified, unstimulated $CD4^+$ T cells were used as a control. Following 72 hours of incubation, Tregs were treated with varying concentrations of ITF2357 for 24 hours and then collected. Foxp3 protein was immunoprecipitated and IgG immunoprecipitation was performed as an isotype control. The Bradford protein assay was used to normalize protein levels. Western blot analysis was performed to determine protein expression of acetylated histones. Briefly, cell lysates were incubated overnight at 4°C with a Foxp3 Ab. A 50% protein G agarose bead slurry was added to the cells and incubated at 4°C for 2 hours. Cells were spun down for 30 seconds at 300 x *g* and rinsed 5 times with cell lysis buffer. The cell pellet was resuspended 1:1 in cell lysis buffer and Laemmli buffer. The samples were heated to 95°C for 5 minutes and then loaded onto a 15% SDS-PAGE gel. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and incubated with antibodies against acetylated histones and β-actin (Cell Signaling, Boston, MA, USA). All experiments were run in triplicate.

2.4.13. Statistical analysis

Statistical analysis was performed using Student's unpaired *t*-test (two-tailed). *P* values less than 0.05 were considered statistically significant.

2.5. Results

2.5.1. HDAC inhibition decreased sera and urinary markers of SLE in NZB/W mice.

Body weight and proteinuria were monitored in NZW and NZB/W mice as they aged. In NZB/W mice treated with DMSO or 5 mg/kg ITF2357, proteinuria levels increased with age. Treatment with 10 mg/kg ITF2357 significantly decreased proteinuria levels as the mice aged (Figure 1 B). Proteinuria remained low in NZW mice regardless of treatment (data not shown). Following euthanization, body weight and spleen weight were measured and the ratio between spleen and body weight was calculated. The spleen: body weight ratio was significantly decreased in NZB/W mice treated with 10mg/kg ITF2357 compared to DMSO-treated NZB/W mice (Figure 1 C). 50% of the DMSO-treated NZB/W mice died before completion of the study. No mice receiving ITF2357 died during the study (Figure 1 A and Table 1).

Table 1. Mouse treatment groups and survival. 20 NZB/W and 15 NZW mice were randomly divided into 3 treatment groups prior to the initiation of treatment. During the study, 5 NZB/W mice in the DMSO treatment group died. No other mice died prior to the termination of the study.

Treatment	Mouse Strain	No. of mice (beginning)	No. of mice (end)
DMSO (control)	NZW	5	5
ITF2357 (5mg/kg)	NZW	5	5
ITF2357 (10mg/kg)	NZW	5	5
DMSO (control)	NZB/W	10	5
ITF2357 (5mg/kg)	NZB/W	5	5
ITF2357 (10mg/kg)	NZB/W	5	5

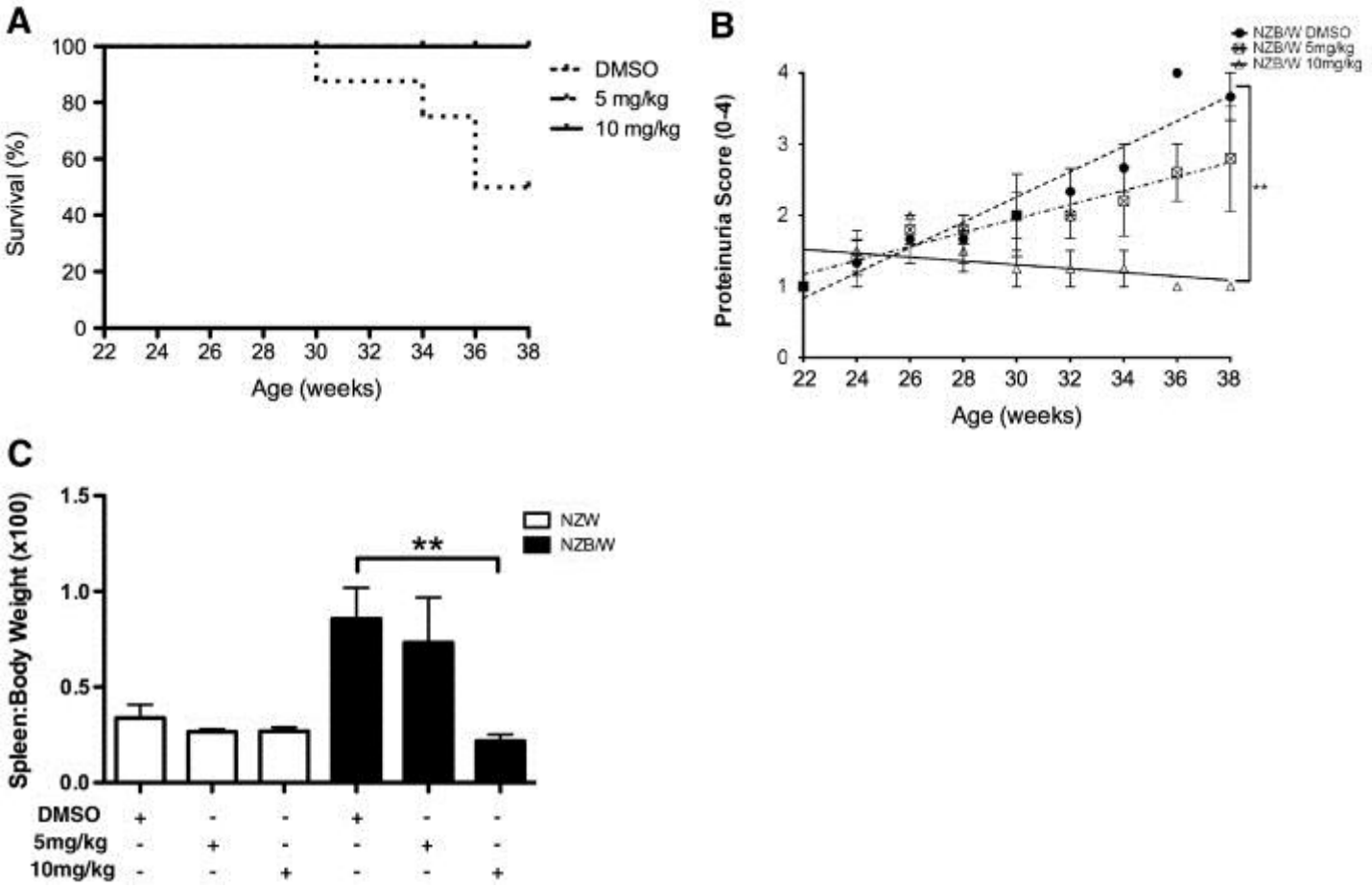
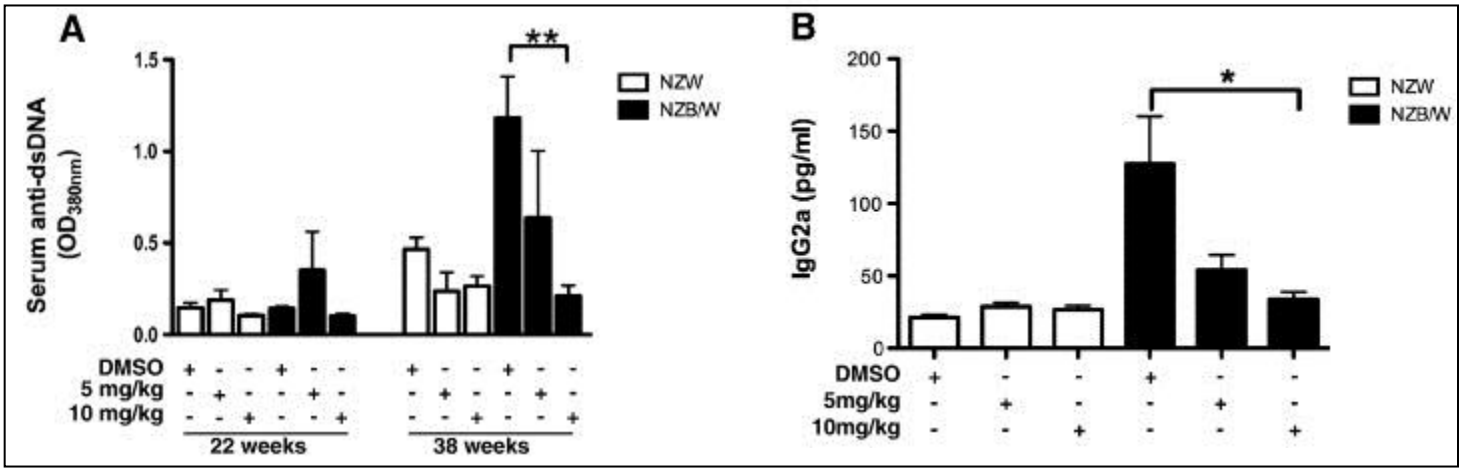


Figure 1. Assessment of disease progression in NZB/W female mice. (A) By 38 weeks-of-age, half of the NZB/W mice receiving the vehicle control alone had died. No NZW (control) mice or mice receiving ITF2357 treatment died before termination of the experiment. (B) Measurement of proteinuria in NZB/W F1 mice receiving intraperitoneal injections of ITF2357 (5 mg/kg in DMSO), (10 mg/kg in DMSO) or vehicle control (DMSO) at 22–38 weeks-of-age. (C) Spleen:body weight ratio was calculated. NZB/W mice treated with ITF2357 (10 mg/kg) had a decreased spleen:body weight ratio at 38 weeks-of-age ($n \geq 5$; $^{**}p < 0.005$).

2.5.2. Treatment with ITF2357 reduced serum anti-double stranded DNA and IgG isotype levels.

To determine if HDAC inhibition alters autoantibody production, serum anti-dsDNA levels were measured in NZW and NZB/W mice every 4 weeks beginning at 22 weeks-of-age through euthanization at 38 weeks-of-age. As the NZB/W mice aged, serum anti-dsDNA levels increased compared to the NZW controls. At 38 weeks-of-age the 10mg/kg ITF2357-treated NZB/W mice had significantly decreased levels of anti-dsDNA compared to the levels in NZB/W mice treated with the vehicle control alone (Figure 2 A). While there was a decrease in anti-dsDNA levels at 38 weeks-of-age from mice that were treated with 5 mg/kg ITF2357, these results were not statistically significant. There were no significant differences in serum anti-dsDNA levels between the groups at 22 weeks-of-age. SLE patients and lupus-prone mice have elevated levels of IgG isotypes, which contribute to disease by

forming immune complexes in the kidneys leading to glomerulonephritis. A correlation between increased levels of IgG2a and glomerulonephritis has been shown by previous research [46]. Our study showed that NZB/W lupus-prone mice have elevated levels of IgG2a and total IgG at 38 weeks-of-age when compared with NZW controls. Treatment with ITF2357 decreased IgG2a and total IgG in a dose-dependent manner. At 10mg/kg, ITF2357 was able to decrease sera levels of IgG2a and total IgG to levels comparable to those in sera from non-diseased NZW mice, however these results were not statistically significant (Figure 2 B-C).



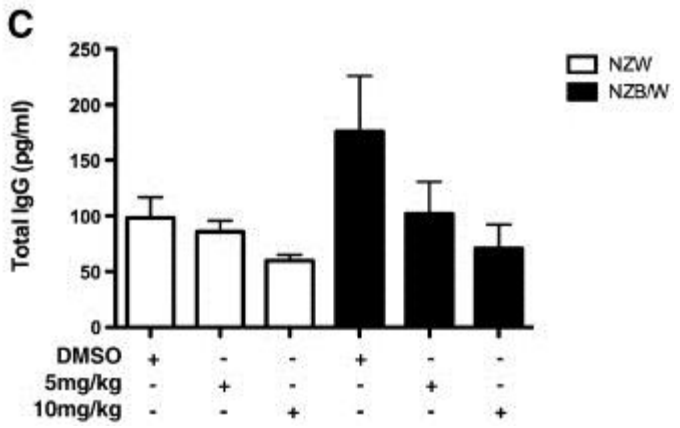


Figure 2 SLE sera biomarkers of disease were decreased in NZB/W mice following HDACi treatment. (A) Measurement of sera anti-dsDNA in NZW and NZB/W mice at 22 weeks-of-age (prior to treatment) and at 38 weeks-of-age (following 16 weeks of treatment). Treatment with ITF2357 (10 mg/kg) significantly decreased anti-dsDNA production in 38-week-old NZB/W mice. (B-C) Sera IgG2a and total IgG levels were decreased in NZB/W mice treated with the HDACi ($n \geq 5$; $^{**}p < 0.005$).

2.5.3. Effects of histone deacetylation on cytokine production.

HDAC inhibitors have been shown to decrease levels of pro-inflammatory cytokines. Therefore we sought to examine the expression of SLE-associated inflammatory cytokines. Cytokine levels were measured in the sera of NZW and NZB/W mice beginning at 22 weeks-of-age every 4 weeks until euthanization at 38 weeks-of-age. TGF-β levels were not significantly different between groups of mice at 22 weeks-of-age (Figure 3 A). As the mice aged, TGF-β decreased in both NZW and NZB/W mice; however, the effect was more marked in the NZB/W mice. Treatment with the HDACi at 10 mg/kg increased TGFβ levels comparable to those in age-matched NZW mice (Figure 3 B). IL-1β sera levels were similar in both NZW and NZB/W

mice at 22 weeks-of-age (Figure 3 C). As the NZB/W mice aged, IL-1β production increased, however, treatment with ITF2357 was able to reverse this effect (Figure 3 D).

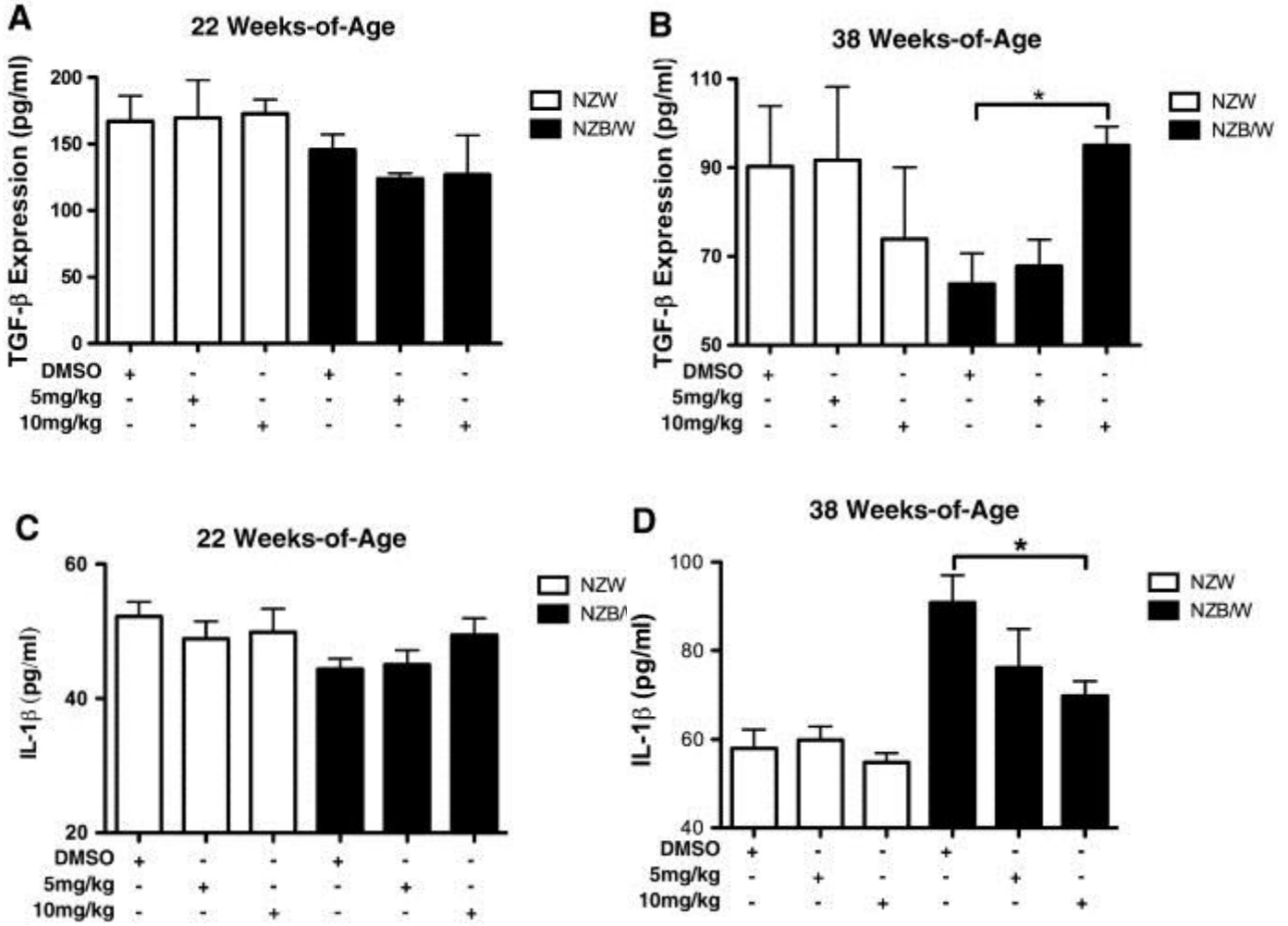


Figure 3. Cytokine production in lupus-prone mice was assessed. (A-D) TGF-β and IL-1β were measured in sera collected from mice prior to treatment (22 weeks-of-age) and following HDACi treatment every 4 weeks until mice were euthanized (38 weeks-of-age). At 22 weeks-of-age there were no significant differences in sera TGF-β or IL-1β (A,C). NZB/W mice treated with vehicle control had decreased levels of TGF-β compared to the NZW mice. The level of sera TGF-β was significantly increased in NZB/W mice treated with ITF2357 (10mg/kg) to levels comparable with NZW control mice at 38 weeks-of-age (B). There was a dose-dependent decrease in sera IL-1β in 38-week-old NZB/W mice treated with ITF2357 ($n \geq 5$; $*p < 0.05$).

2.5.4. Glomerular mRNA expression is altered following HDAC inhibition *in vivo*

Relative glomerular mRNA expression of IL-10, TGF-β, and IL-6 were determined using RT-PCR. HDACi therapy (10mg/kg) was able to decrease IL-10 expression in the glomeruli; however, treatment with 5 mg/kg had no effect on IL-10 expression (Figure 4 A). Treatment with ITF2357 at 5mg/kg resulted in increased TGF-β expression in the glomeruli, however, at the 10 mg/kg dose there was no significant difference in TGF-β expression in NZB/W mice (Figure 4 B). Glomerular IL-6 mRNA expression was significantly decreased in a dose-dependent manner following treatment with ITF2357 (Figure 4 C).

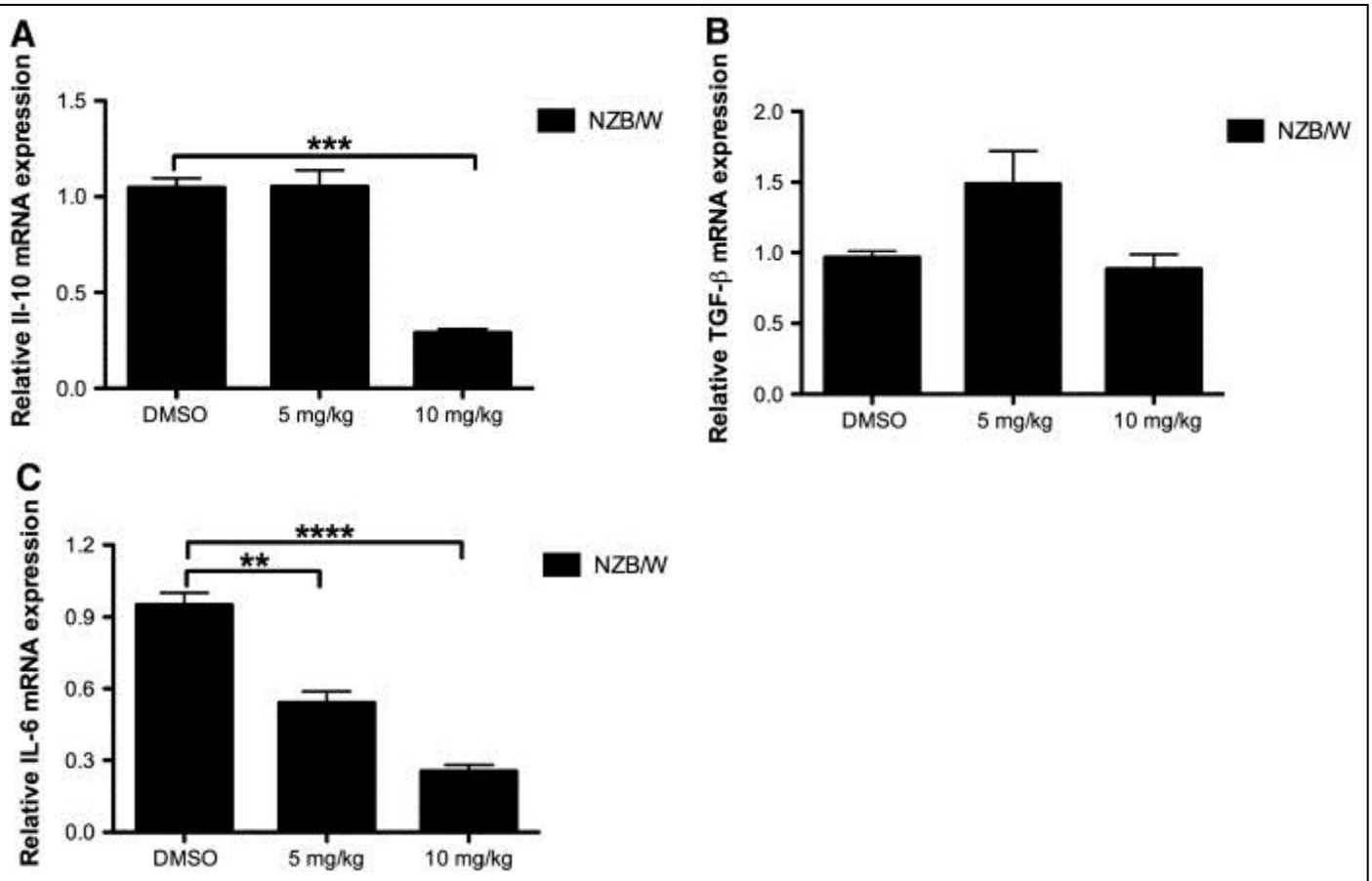
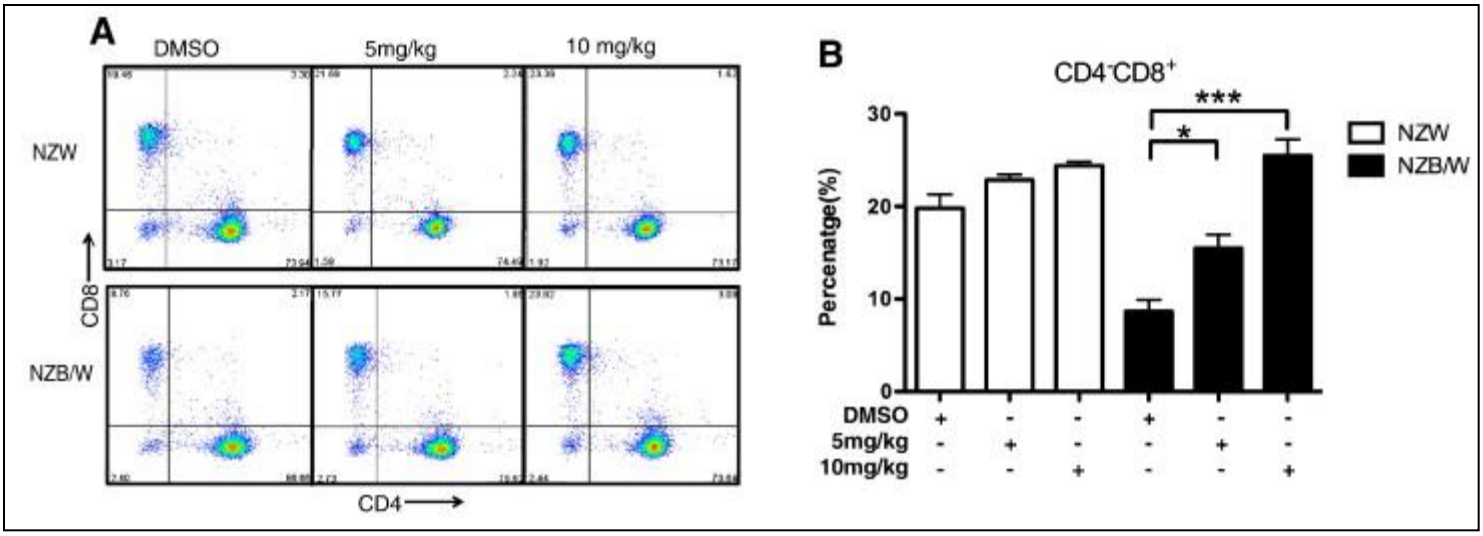


Figure 4. Glomerular mRNA levels were assessed in NZB/W mice. (A) One kidney was removed from each NZW and NZB/W mouse and pooled by group. A glomerular isolation was performed and mRNA levels were quantified using RT-PCR. IL-10 was decreased in the

glomeruli of NZB/W mice injected with ITF2357 (10 mg/kg) compared to those receiving the vehicle control or ITF2357 (5 mg/kg). (B) mRNA levels of TGF-β were increased in NZB/W mice treated with ITF2357 (5mg/kg), but there was no significant difference in NZB/W mice treated with ITF2357 (10mg/kg) when compared to DMSO treated NZB/W mice. (C) Treatment with ITF2357 significantly decreased glomerular IL-6 in a dose-dependent manner ($n \geq 5$; $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.0005$).

2.5.5. Increased differentiation into cytotoxic T cells following HDACi treatment

In order to further characterize the T cell splenic phenotype, levels of $CD4^+$ and $CD8^+$ were assessed. HDAC inhibition by ITF2357 significantly decreased the ratio of CD4:CD8 cells in NZB/W mice by increasing the cytotoxic T cell subset while decreasing the number of Th cells (Figure 5 A-D). NZW mice had a higher percentage of $CD4^-CD8^+$ T cells compared to NZB/W mice. Treatment with ITF2357 significantly increased the percentage of cytotoxic T cells at both the 5 mg/kg and 10 mg/kg dose (Figure 5 A-B). The percentage of Th cells was higher in NZB/W mice compared to NZW mice. Specific class I and II HDAC inhibition resulted in a dose-dependent decrease in Th cells in NZB/W treated mice (Figure 5 A, C). The treatment had no effect on the T cell profile of NZW mice receiving the same treatment (Figure 5A – D).



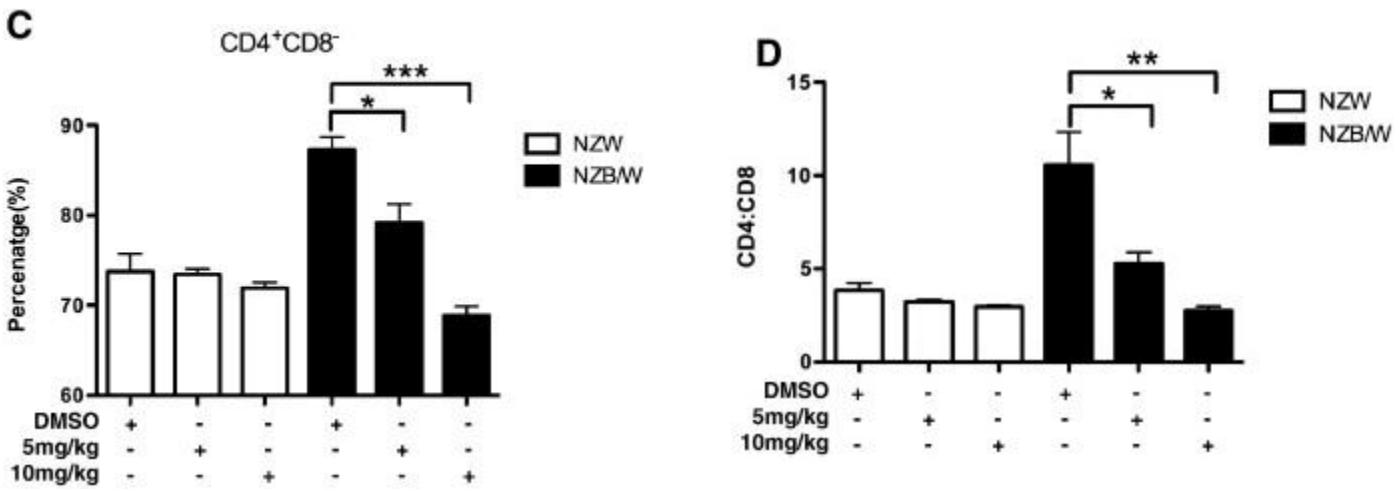


Figure 5. ITF2357 altered helper and cytotoxic T cell profiles (A) Representative images of flow cytometry T cell profiles. (B) The percentage of cytotoxic ($CD4^{-}CD8^{+}$) T cells increased with histone acetylation. (C) HDACi treatment decreased the percentage of Th ($CD4^{+}CD8^{-}$) cells. (D) The ratio of CD4:CD8 T cells decreased significantly in NZB/W mice treated with ITF2357 for 16 weeks ($n \geq 5$; $*p < 0.05$, $**p < 0.005$ $***p<0.0005$).

2.5.6. Inhibition of HDAC increased the number of regulatory T cells

The Treg phenotype was assessed due to its role in the maintenance of self-tolerance and the prevention of autoimmune disease [31]. Following euthanization at 38 weeks-of-age, spleens were removed and single cell suspensions were obtained. Cells were stained for flow cytometric analyses. The percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ T cells (Tregs) was significantly increased in NZB/W mice that had received 10 mg/kg ITF2357 compared to DMSO-treated controls (Figure 6 A- B). NZW mice had no significant change in Treg profiles regardless of treatment (Figure 6 A-D). Treatment with ITF2357 treatment with both the 5 mg/kg and 10 mg/kg resulted in increased percentages of $CD4^{+}CD25^{+}Foxp3^{-}$ and of $CD4^{+}CD25^{-}Foxp3^{+}$ T cells compared to mice treated with the vehicle control alone (Figure 6 A, C – D). Furthermore, treatment with the HDACi significantly increased the percentage of $Foxp3^{-}CD25^{+}$ cells in a dose-dependent manner

in NZB/W mice. However, HDAC inhibition had no effect on the percentage of Foxp3$^-$CD25$^+$ T cells in NZW mice.

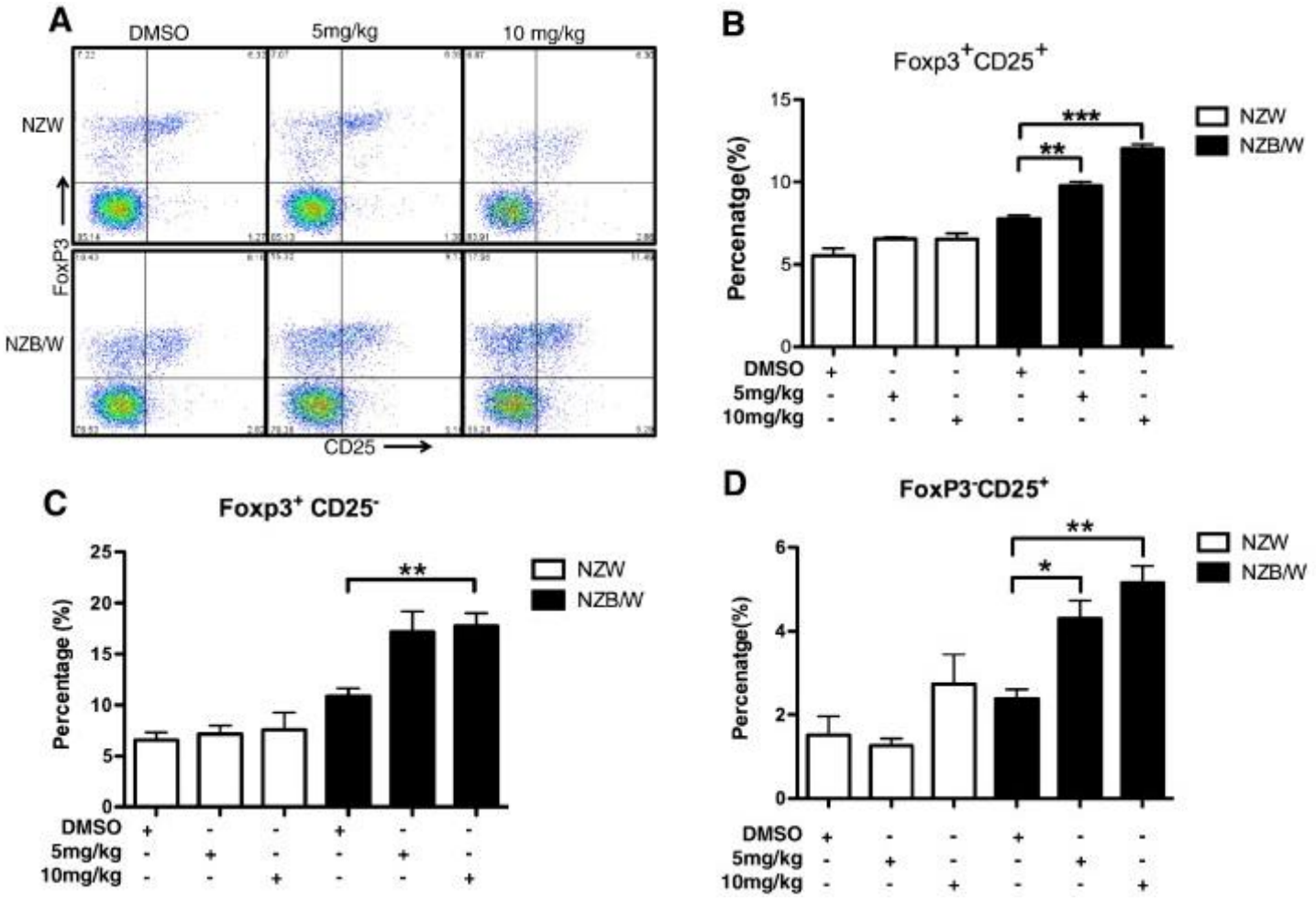


Figure 6. The Treg cell profile was increased following class I and II HDAC inhibition. (A) Representative images of flow cytometry Treg profiles. (B) ITF2357 (10 mg/kg) treatment significantly increased the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells. (C) Percentage of T cells gated on CD4 that were Foxp3$^+$CD25$^-$ in NZW and NZB/W mice treated with ITF2357(5 mg/kg in DMSO), (10 mg/kg in DMSO) or vehicle control (DMSO) for 16 weeks. (D) The percentage of CD25$^+$ T cells is significantly increased following ITF2357 treatment in 38 week old mice ($n \geq 5$; *$p < 0.05$, **$p < 0.005$).

2.5.7. Histone acetylation inhibits Th17 differentiation.

An imbalance between the Treg and Th17 subsets exists throughout the progression of SLE. The Th17 phenotype was assessed in spleens from 38-week-old NZW and NZB/W mice treated with DMSO or 5 mg/kg or 10 mg/kg ITF2357 for 16 weeks. NZB/W mice had decreased levels of $CD4^{+}IL\text{-}17^{+}ROR\text{-}\gamma^{+}$ T cells compared to NZW mice. The percentage of IL-17 producing T cells, measured by flow cytometric analyses, was further reduced in NZB/W mice that were treated with 10mg/kg ITF2357. Treatment with ITF2357 also reduced the percentage of Th17 cell is NZW mice in a dose-dependent manner (Figure 7 A – B).

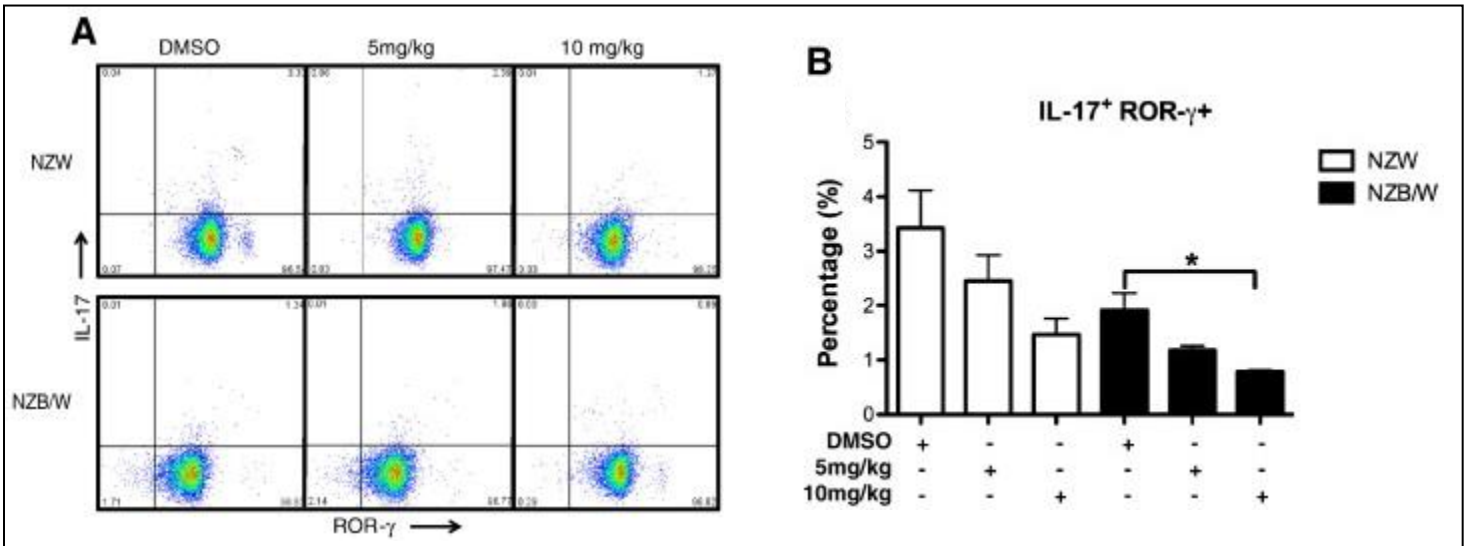
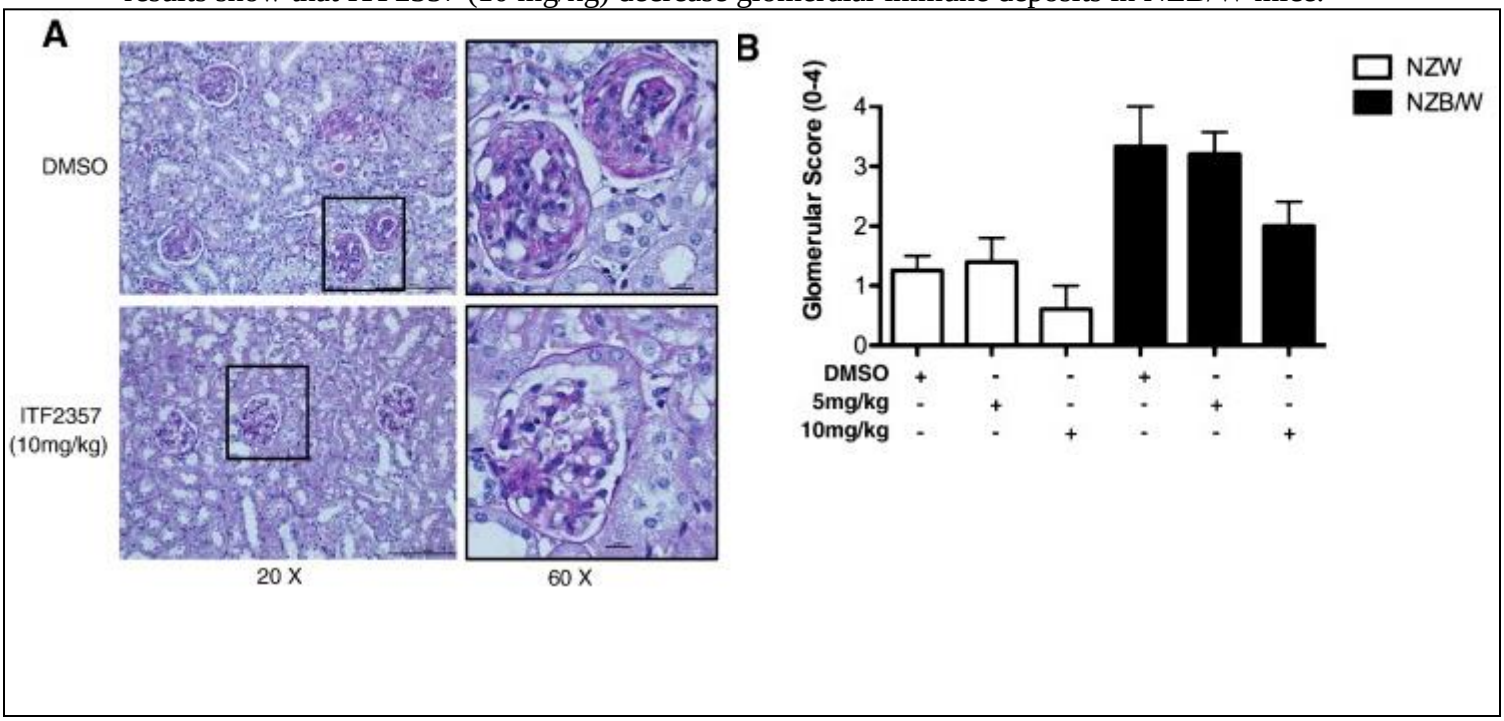


Figure 7. ITF2357 decreased the percentage of Th17 cells. (A) Representative images of flow cytometry Th17 profiles. (B) The percentage of IL-17-producing T cells decreased in mice treated with the HDACi ($n \geq 3$; $^{*}p < 0.05$).

2.5.8. ITF2357 alters renal histopathology and decreases glomerular immune complex deposition.

In order to assess renal disease, kidney sections were embedded in paraffin and stained by PAS. Light microscopy analysis of the kidney sections showed an increase in glomerulonephritis development in NZB/W mice compared to non-diseased NZW mice. DMSO-

treated NZB/W mice had an average glomerular score of 3+ with severe glomerulonephritis. ITF2357 (10 mg/kg) treatment reduced the overall severity of disease by decreasing hypercellularity, crescent formations, and thickening/irregularity of the glomerular basement membrane (Figure 8 A, B). Immune complex and complement deposition within the glomeruli were assessed by immunofluorescent analysis of kidney sections. NZW mice had minimal immune complex deposition regardless of treatment (Figure 8 C-E). Both the number of glomeruli with C3 and IgG deposition as wells as the overall level of deposition within each glomeruli was increased in the NZB/W DMSO-treated mice when compared to NZW controls (Figure 8 F). Following treatment with the HDACi, NZB/W mice had decreased immune complex and complement deposition in the glomeruli (Figure 8 H-J). Taken together these results show that ITF2357 (10 mg/kg) decrease glomerular immune deposits in NZB/W mice.



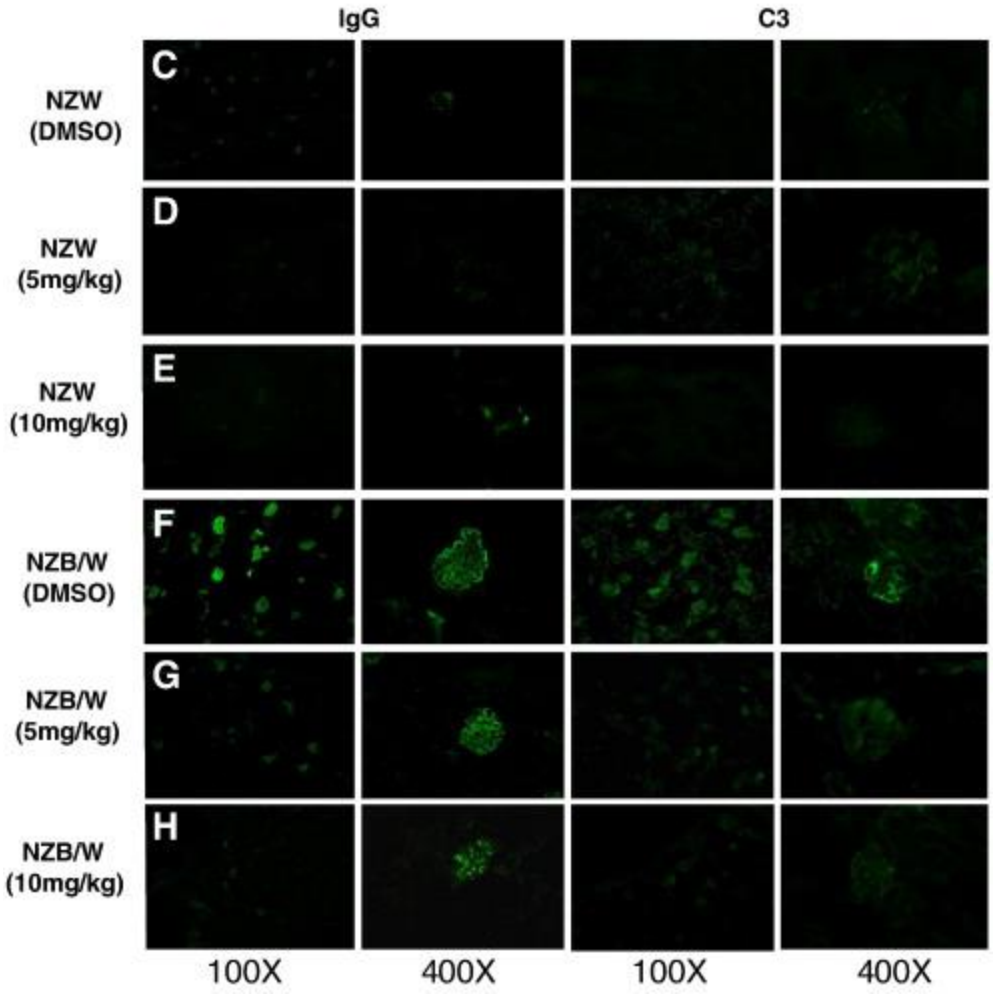


Figure 8. Kidney histopathology and immune complex deposition were assessed. (A-H) Paraffin embedded kidney sections were cut and stained with PAS stain. Sections were assessed for glomerular proliferation, inflammation, number of nuclei per glomerulus, crescent formation, and fibrosis by a pathologist in a blinded manner, and a glomerular score (0-4) was assigned. DMSO-treated NZB/W mice had severe proliferative glomerulonephritis, thickened GBM, and crescent formations. When treated with ITF2357 (10mg/kg) NZB/W mice had improved renal pathology (A-B). 5 μM kidney sections were stained with FITC-conjugated C3 or IgG and assessed for fluorescence intensity. Glomerular deposition of both C3 and IgG was greater in NZB/W (vehicle control) mice compared to NZW mice. ITF2357 treatment was able to decrease immune complex deposition (C-H) ($n \geq 5$; $*p < 0.05$).

2.5.9. Histone acetylation is increased in the kidneys following ITF2357treatment.

Acetylation of the H3 (Lys9) histone is involved with the regulation of nucleosome packaging and structure and is particularly susceptible to post-translational modification. Kidney sections were stained with an Ab for the acetylated H3 histone (FITC) and a nuclear (DAPI) stain and immunofluorescence was assessed. ITF2357 treatment at10 mg/kg increased histone H3 acetylation in both NZW and NZB/W mice (Figure 9). However, treatment at 5 mg/kg ITF2357 was unable to alter H3 histone acetylation (Figure 9 E).

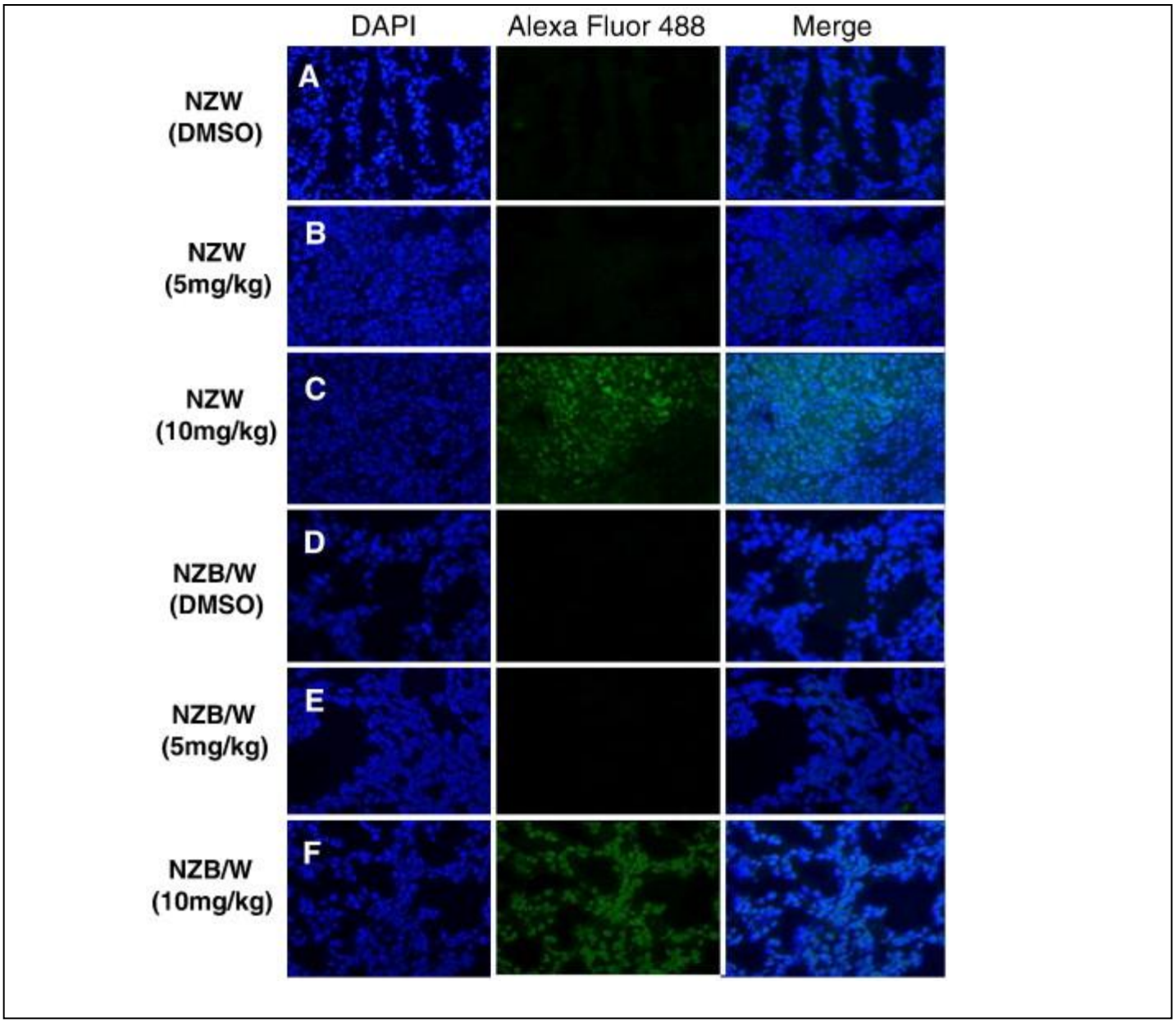


Figure 9. ITF2357 increases histone H3 acetylation in the kidney. (A) Five μM kidney sections from 38-week-old NZW and NZB/W mice treated with DMSO or ITF2357 (5mg/kg or 10mg/kg) were stained for acetyl-histone H3 (lys9). Treatment with ITF2357 (10mg/kg) increased the percentage of FITC (green) stained acetylated H3 histones colocalized with nuclear DAPI (blue) stain in both NZW and NZB/W mice ($n \geq 5$).

2.5.10. *In vitro* treatment with ITF2357 increased Foxp3 acetylation.

Foxp3 acetylation was assessed through immunoprecipitation and Western blot analysis of splenic T cells. A Treg cell phenotype was induced using IL-2, TGF-β and CD3/CD28 co-stimulation. Cells were treated with increasing concentrations of ITF2357 and levels of Foxp3 acetylation were determined by immunoprecipitation and Western blot analysis. Splenic $CD4^+$ T cells were immunoprecipitated with Foxp3 as a control. Induced Tregs were immunoprecipitated with IgG as an isotype control. Treatment with the selective Class I and II HDACi was able to increase acetylation of Foxp3 *in vitro* in a concentration-dependent manner. At a 5 μM concentration the increase in Foxp3 acetylation was significantly increased compared to non-treated controls (Figure 10).

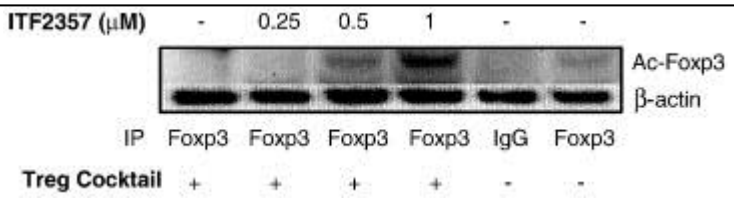


Figure 10. Acetylation of Foxp3 histones. A single-cell suspension was isolated from the spleens of 22-week-old NZB/W mice. Cells were differentiated into Tregs and treated with increasing concentrations of ITF2357 (0.25, 5, and 1 μM). Non-induced splenic cells that did not receive the Treg cocktail (anti-CD3, anti-CD28, rhIL-2, and TGF-β) were used as a control. Foxp3 or IgG, as a control, were immunoprecipitated and Western blot analysis was used to

determine levels of Foxp3 acetylation. Treatment with ITF2357 increased the acetylation of Foxp3 in Treg cells. Experiments were run in triplicate.

2.6. Conclusions

Various HDAC inhibitors have been shown to have therapeutic potential in animal models of multiple autoimmune diseases, including SLE, arthritis, inflammatory bowel disease, and diabetes [41, 42, 47-50]. However, the mechanism through which HDAC inhibitors ameliorate autoimmune disease remains to be elucidated. We sought to determine if ITF2357 would decrease disease in NZB/W mice. NZB/W and NZW mice were treated for 16 weeks beginning at 22 weeks-of-age. Key urinary and sera markers of SLE including proteinuria (Figure 1B) and anti-dsDNA (Figure 2A) were decreased in lupus-prone NZB/W mice treated with ITF2357 throughout the study. Histone acetylation was confirmed through immunofluorescence in the kidney (Figure 9 A-F). DMSO-treated NZB/W mice had a marked increase in immune complex deposition and proliferative glomerulonephritis compared to NZW control mice, which was ameliorated following specific class I and II HDACi therapy (Figure 8). Flow cytometric analysis of splenic T cells showed increased Treg cell numbers (Figure 6), but a decreased Th17 phenotype (Figure 7).

Our results showed that treatment with ITF2357 resulted in increased Foxp3 acetylation in a dose-dependent manner. Recent studies suggest that HDAC inhibitors play a role in the acetylation of non-histone proteins to regulate protein stability, protein-protein interactions, and protein-DNA interactions [33]. Hyperacetylation by HDAC inhibition may prevent ubiquitination and proteasomal degradation, affecting protein stability [51]. One non-histone protein that has been shown to be regulated by HDACs is Foxp3 [35]. Increased Foxp3 acetylation has been demonstrated to prevent polyubiquitination and subsequent proteasomal degradation increasing the stability of the Foxp3 protein and thereby allowing for increased Treg differentiation [35]. Increasing the stability of Foxp3 is important for regulating Treg suppressive

function as well as Treg development [52]. In our study, splenic cells treated with ITF2357 had increased Foxp3 acetylation at the 1 μM concentration (Figure 10). It has been hypothesized that as Foxp3 becomes acetylated, the interaction between transcription factors and the *Foxp3* promoter is increased, thereby increasing Foxp3 expression and Treg cell populations [51].

In the present studies, the Treg ($CD4^+CD25^+Foxp3^+$) phenotype was increased in NZB/W mice treated with ITF2357, a specific class I and II HDACi, compared to DMSO-treated control mice (Figure 7). Stable expression of Foxp3 is necessary for Treg suppression of Th cells and the regulation of autoimmune disease. Research suggests that Tregs may be able to downregulate glomerulonephritis [53, 54]. Our results indicate that the increase in the Treg cell population coincided with decreased glomerulonephritis. In NZB/W mice depleted of $CD4^+CD25^+$ T cells, glomerulonephritis develops in an accelerated manner suggesting that Treg cells are critical for suppression of inflammation in the kidneys [26].

Our study showed that treatment with ITF2357 was able to increase H3 histone acetylation in both NZW and NZB/W mice (Figure 10). Histones can be epigenetically regulated through acetylation or methylation of lysine residues. Acetylation of Lys 9 of the H3 histone has been implicated for its role in transcriptional activation [29]. Previous research has shown that an increase in acetylation of the H3 histone (lys9) correlated with a marked increase in Foxp3 expression [51, 55]. ITF2357 was able to regulate site-specific acetylation of Lys9 in the kidney tissue. We also found that treatment increased the percentage of $Foxp3^+$ Treg cells *in vivo*. These data suggests that by inhibiting class I and II HDACs we were able to increase H3 histone acetylation, further aiding Foxp3 expression.

TGF-β promotes expression of Foxp3, a transcription factor for Treg cells [18, 56]. Our studies showed a decrease in TGF-β in the sera of NZB/W mice receiving vehicle alone (Figure

4B). TGF-β has been demonstrated to play a dual role in SLE pathogenesis [57]. Reduced levels of TGF-β in immune cells can coincide with an increase in TGF-β in target organs leading to autoimmune disease such as lupus [57]. Reduced TGF-β production by immune cells predisposes to autoantibody production, a hallmark of SLE, associated with complement activation, inflammatory cytokine production, and subsequent tissue inflammation and deposition of extracellular matrix [57-59]. Anti-inflammatory cytokines including TGF-β are produced in order to combat inflammation within target organs such as the kidneys inducing the production of extracellular matrix leading to fibrosis [60, 61]. Treatment of NZB/W mice with ITF2357 (10 mg/kg) was able to reverse this effect resulting in an increase in TGF-β in the sera. Furthermore, increased levels of TGF-β in the sera helps naïve $CD4^+$ T cells differentiate into Treg cells which help to regulate autoimmune disease [56].

Anti-dsDNA is produced by autoreactive B cells that are characteristic of patients with SLE. These autoreactive B cells are able to overproduce anti-dsDNA in part due to activation by $CD4^+$ Th cells [5]. Our studies show that NZB/W mice had increased numbers of $CD4^+$ Th cells (Figure 6 C), which correlated with elevated anti-dsDNA sera levels (Figure 2A). However, treatment of NZB/W mice with ITF2357 (10 mg/kg) was able to reduce the percentage of $CD4^+$ T cells as wells as decrease autoantibody production. There was also a marked decrease in the ratio of $CD4^+CD8^+$ cells in NZB/W mice treated with ITF2357 (10 mg/kg) when compared to DMSO-treated control mice (Figure 6 D). $CD4^+$ T cells have increased activation in patients with SLE [61]. Conversely, $CD8^+$ T cells are reduced in number and function resulting in lack of inhibition of autoreactive B cells [13, 62]. Our data suggests that increasing the number of cytotoxic T cells contributes to the decrease in autoantibody production by autoreactive B cells. Splenomegaly, or enlargement of the spleen, is suspected to result from a number of different

factors during SLE including increased lymphocyte proliferation and autoantibody stimulation [63]. Reduced spleen: body weight ratio may be due to reduction in the number of lymphocytes following HDAC inhibition.

HDACi are able to provide a more targeted approach to treatment through inhibition of specific classes of HDACs and have been studied for efficacy in a number of autoimmune diseases [49, 64, 65]. Previous research has demonstrated the ability of HDACi including TSA and SAHA to decrease SLE pathogenesis in NZB/W mice [16, 47]. Our studies have shown the ability of ITF2357 to decrease sera and urinary markers of lupus, increase Treg numbers, while improving renal histopathology. We hypothesize that an increase in Treg cell number and function may reduce autoantibody production resulting in decreased disease activity during SLE. Taken together these data suggest class I and II histone deacetylation plays an important role in the development of lupus and that treatment to inhibit deacetylation can ameliorate SLE disease.

2.7. References

[1] A.A. Aran, C. Putterman, Treatment of lupus nephritis: facing the era of immunotherapy, Panminerva Med, 50 (2008) 235-245.
[2] C.C. Mok, C.S. Lau, Pathogenesis of systemic lupus erythematosus, Journal of clinical pathology, 56 (2003) 481-490.
[3] A. Kuhn, S. Beissert, P.H. Krammer, CD4(+)CD25 (+) regulatory T cells in human lupus erythematosus, Archives of dermatological research, 301 (2009) 71-81.
[4] J.C. Crispin, S.N. Liossis, K. Kis-Toth, L.A. Lieberman, V.C. Kyttaris, Y.T. Juang, G.C. Tsokos, Pathogenesis of human systemic lupus erythematosus: recent advances, Trends in molecular medicine, 16 (2010) 47-57.
[5] J.H. Lee, L.C. Wang, Y.T. Lin, Y.H. Yang, D.T. Lin, B.L. Chiang, Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus, Immunology, 117 (2006) 280-286.
[6] M.J. Ryan, G.R. McLemore, Jr., Hypertension and impaired vascular function in a female mouse model of systemic lupus erythematosus, Am J Physiol Regul Integr Comp Physiol, 292 (2007) R736-742.
[7] D. Perry, A. Sang, Y. Yin, Y.Y. Zheng, L. Morel, Murine models of systemic lupus erythematosus, Journal of biomedicine & biotechnology, 2011 (2011) 271694.
[8] C. Benoist, D. Mathis, A. Satpathy, M. Feuerer, Survey of regulatory T cells (Treg) of the thymus and spleen in males of 33 inbred strains of mice, MPD:25804. Mouse Phenome Database web site, The Jackson Laboratory, (2013).
[9] R. Burnett, G. Ravel, J. Descotes, Clinical and histopathological progression of lesions in lupus-prone (NZB x NZW) F1 mice, Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie, 56 (2004) 37-44.
[10] C.J. Peutz-Kootstra, E. de Heer, P.J. Hoedemaeker, C.K. Abrass, J.A. Bruijn, Lupus nephritis: lessons from experimental animal models, The Journal of laboratory and clinical medicine, 137 (2001) 244-260.
[11] A.N. Theofilopoulos, F.J. Dixon, Murine models of systemic lupus erythematosus, Advances in Immunology, 37 (1985) 269-390.
[12] D.M. Klinman, IgG1 and IgG2a production by autoimmune B cells treated in vitro with IL-4 and IFN-gamma, Journal of immunology, 144 (1990) 2529-2534.

[13] I. Puliaeva, R. Puliaev, C.S. Via, Therapeutic potential of CD8+ cytotoxic T lymphocytes in SLE, Autoimmunity reviews, 8 (2009) 219-223.
[14] J.C. Crispin, V.C. Kyttaris, C. Terhorst, G.C. Tsokos, T cells as therapeutic targets in SLE, Nature reviews. Rheumatology, 6 (2010) 317-325.
[15] J. Zhu, H. Yamane, W.E. Paul, Differentiation of effector CD4 T cell populations (*), Annual review of immunology, 28 (2010) 445-489.
[16] N. Mishra, C.M. Reilly, D.R. Brown, P. Ruiz, G.S. Gilkeson, Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse, Journal of Clinical Investigation, 111 (2003) 539-552.
[17] A. Sabry, H. Sheashaa, A. El-Husseini, K. Mahmoud, K.F. Eldahshan, S.K. George, E. Abdel-Khalek, E.M. El-Shafey, H. Abo-Zenah, Proinflammatory cytokines (TNF-alpha and IL-6) in Egyptian patients with SLE: its correlation with disease activity, Cytokine, 35 (2006) 148-153.
[18] S.A. Apostolidis, L.A. Lieberman, K. Kis-Toth, J.C. Crispin, G.C. Tsokos, The dysregulation of cytokine networks in systemic lupus erythematosus, Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 31 (2011) 769-779.
[19] K. Miyake, M. Akahoshi, H. Nakashima, Th subset balance in lupus nephritis, Journal of biomedicine & biotechnology, 2011 (2011) 980286.
[20] A. Alunno, E. Bartoloni, O. Bistoni, G. Nocentini, S. Ronchetti, S. Caterbi, V. Valentini, C. Riccardi, R. Gerli, Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new, Clinical & developmental immunology, 2012 (2012) 823085.
[21] S. Joshi, L.C. Pantalena, X.K. Liu, S.L. Gaffen, H. Liu, C. Rohowsky-Kochan, K. Ichiyama, A. Yoshimura, L. Steinman, S. Christakos, S. Youssef, 1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A, Molecular and cellular biology, 31 (2011) 3653-3669.
[22] S.A. Apostolidis, J.C. Crispin, G.C. Tsokos, IL-17-producing T cells in lupus nephritis, Lupus, 20 (2011) 120-124.
[23] M.F. Liu, L.H. Lin, C.T. Weng, M.Y. Weng, Decreased CD4+CD25+bright T cells in peripheral blood of patients with primary Sjogren's syndrome, Lupus, 17 (2008) 34-39.

[24] S.M. Wahl, W. Chen, Transforming growth factor-beta-induced regulatory T cells referee inflammatory and autoimmune diseases, Arthritis research & therapy, 7 (2005) 62-68.
[25] J. Wenzel, S. Henze, S. Brahler, T. Bieber, T. Tuting, The expression of human leukocyte antigen-DR and CD25 on circulating T cells in cutaneous lupus erythematosus and correlation with disease activity, Experimental dermatology, 14 (2005) 454-459.
[26] T. Hayashi, K. Hasegawa, C. Adachi, Elimination of CD4(+)CD25(+) T cell accelerates the development of glomerulonephritis during the preactive phase in autoimmune-prone female NZB x NZW F mice, International journal of experimental pathology, 86 (2005) 289-296.
[27] T. Akimova, G. Ge, T. Golovina, T. Mikheeva, L. Wang, J.L. Riley, W.W. Hancock, Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs, Clinical immunology, 136 (2010) 348-363.
[28] U.H. Beier, T. Akimova, Y. Liu, L. Wang, W.W. Hancock, Histone/protein deacetylases control Foxp3 expression and the heat shock response of T-regulatory cells, Current opinion in immunology, 23 (2011) 670-678.
[29] K. Yan, Q. Cao, C.M. Reilly, N.L. Young, B.A. Garcia, N. Mishra, Histone deacetylase 9 deficiency protects against effector T cell-mediated systemic autoimmunity, The Journal of biological chemistry, 286 (2011) 28833-28843.
[30] H. He, N. Lehming, Global effects of histone modifications, Brief Funct Genomic Proteomic, 2 (2003) 234-243.
[31] S. Koarada, Y. Tada, O. Ushiyama, F. Morito, N. Suzuki, A. Ohta, K. Miyake, M. Kimoto, K. Nagasawa, B cells lacking RP105, a novel B cell antigen, in systemic lupus erythematosus, Arthritis & Rheumatism, 42 (1999) 2593-2600.
[32] C.M. Reilly, N. Regna, N. Mishra, HDAC inhibition in lupus models, Molecular medicine, 17 (2011) 417-425.
[33] H.J. Kim, S.C. Bae, Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs, American journal of translational research, 3 (2011) 166-179.
[34] S. Minucci, P.G. Pelicci, Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer, Nature reviews. Cancer, 6 (2006) 38-51.
[35] J. van Loosdregt, Y. Vercoulen, T. Guichelaar, Y.Y. Gent, J.M. Beekman, O. van Beekum, A.B. Brenkman, D.J. Hijnen, T. Mutis, E. Kalkhoven, B.J. Prakken, P.J. Coffer, Regulation of

Treg functionality by acetylation-mediated Foxp3 protein stabilization, Blood, 115 (2010) 965-974.
[36] E.C. Lewis, L. Blaabjerg, J. Storling, S.G. Ronn, P. Mascagni, C.A. Dinarello, T. Mandrup-Poulsen, The oral histone deacetylase inhibitor ITF2357 reduces cytokines and protects islet beta cells in vivo and in vitro, Molecular medicine, 17 (2011) 369-377.
[37] N. Khan, M. Jeffers, S. Kumar, C. Hackett, F. Boldog, N. Khramtsov, X. Qian, E. Mills, S.C. Berghs, N. Carey, P.W. Finn, L.S. Collins, A. Tumber, J.W. Ritchie, P.B. Jensen, H.S. Lichenstein, M. Sehested, Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors, The Biochemical journal, 409 (2008) 581-589.
[38] S. Matalon, B.E. Palmer, M.F. Nold, A. Furlan, A. Kassu, G. Fossati, P. Mascagni, C.A. Dinarello, The histone deacetylase inhibitor ITF2357 decreases surface CXCR4 and CCR5 expression on CD4(+) T-cells and monocytes and is superior to valproic acid for latent HIV-1 expression in vitro, Journal of acquired immune deficiency syndromes, 54 (2010) 1-9.
[39] A. Furlan, V. Monzani, L.L. Reznikov, F. Leoni, G. Fossati, D. Modena, P. Mascagni, C.A. Dinarello, Pharmacokinetics, safety and inducible cytokine responses during a phase 1 trial of the oral histone deacetylase inhibitor ITF2357 (givinostat), Molecular medicine, 17 (2011) 353-362.
[40] E.J. Bodar, A. Simon, J.W. van der Meer, Effects of the histone deacetylase inhibitor ITF2357 in autoinflammatory syndromes, Molecular medicine, 17 (2011) 363-368.
[41] R. Glauben, B. Siegmund, Inhibition of histone deacetylases in inflammatory bowel diseases, Molecular medicine, 17 (2011) 426-433.
[42] A.M. Grabiec, O. Korchynskyi, P.P. Tak, K.A. Reedquist, Histone deacetylase inhibitors suppress rheumatoid arthritis fibroblast-like synoviocyte and macrophage IL-6 production by accelerating mRNA decay, Annals of the rheumatic diseases, 71 (2012) 424-431.
[43] F. Leoni, G. Fossati, E.C. Lewis, J.K. Lee, G. Porro, P. Pagani, D. Modena, M.L. Moras, P. Pozzi, L.L. Reznikov, B. Siegmund, G. Fantuzzi, C.A. Dinarello, P. Mascagni, The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo, Molecular medicine, 11 (2005) 1-15.
[44] J. Tan, S. Cang, Y. Ma, R.L. Petrillo, D. Liu, Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents, Journal of hematology & oncology, 3 (2010) 5.

[45] J. Vojinovic, N. Damjanov, C. D'Urzo, A. Furlan, G. Susic, S. Pasic, N. Iagaru, M. Stefan, C.A. Dinarello, Safety and efficacy of an oral histone deacetylase inhibitor in systemic-onset juvenile idiopathic arthritis, Arthritis and rheumatism, 63 (2011) 1452-1458.
[46] T. Hayashi, K. Hasegawa, Y. Sasaki, T. Mori, C. Adachi, K. Maeda, Systemic administration of interleukin-4 expressing plasmid DNA delays the development of glomerulonephritis and prolongs survival in lupus-prone female NZB × NZW F1 mice, Nephrology Dialysis Transplantation, 22 (2007) 3131-3138.
[47] C.M. Reilly, M. Thomas, R. Gogal, Jr., S. Olgun, A. Santo, R. Sodhi, E.T. Samy, S.L. Peng, G.S. Gilkeson, N. Mishra, The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice, Journal of autoimmunity, 31 (2008) 123-130.
[48] Y. Nasu, K. Nishida, S. Miyazawa, T. Komiyama, Y. Kadota, N. Abe, A. Yoshida, S. Hirohata, A. Ohtsuka, T. Ozaki, Trichostatin A, a histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model, Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society, 16 (2008) 723-732.
[49] D.P. Christensen, M. Dahllof, M. Lundh, D.N. Rasmussen, M.D. Nielsen, N. Billestrup, L.G. Grunnet, T. Mandrup-Poulsen, Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus, Molecular medicine, 17 (2011) 378-390.
[50] X. Zhou, X. Hua, X. Ding, Y. Bian, X. Wang, Trichostatin differentially regulates Th1 and Th2 responses and alleviates rheumatoid arthritis in mice, Journal of clinical immunology, 31 (2011) 395-405.
[51] H. Zhang, Y. Xiao, Z. Zhu, B. Li, M.I. Greene, Immune regulation by histone deacetylases: a focus on the alteration of FOXP3 activity, Immunology and cell biology, 90 (2012) 95-100.
[52] S. Hori, T. Nomura, S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3, Science, 299 (2003) 1057-1061.
[53] H.K. Kang, M.A. Michaels, B.R. Berner, S.K. Datta, Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets, Journal of immunology, 174 (2005) 3247-3255.

[54] H.J. Paust, A. Ostmann, A. Erhardt, J.E. Turner, J. Velden, H.W. Mittrucker, T. Sparwasser, U. Panzer, G. Tiegs, Regulatory T cells control the Th1 immune response in murine crescentic glomerulonephritis, Kidney international, 80 (2011) 154-164.
[55] K.A. Cavassani, W.F.t. Carson, A.P. Moreira, H. Wen, M.A. Schaller, M. Ishii, D.M. Lindell, Y. Dou, N.W. Lukacs, V.G. Keshamouni, C.M. Hogaboam, S.L. Kunkel, The post sepsis-induced expansion and enhanced function of regulatory T cells create an environment to potentiate tumor growth, Blood, 115 (2010) 4403-4411.
[56] X. Zhou, N. Kong, H. Zou, D. Brand, X. Li, Z. Liu, S.G. Zheng, Therapeutic potential of TGF-beta-induced CD4(+) Foxp3(+) regulatory T cells in autoimmune diseases, Autoimmunity, 44 (2011) 43-50.
[57] V. Saxena, D.W. Lienesch, M. Zhou, R. Bommireddy, M. Azhar, T. Doetschman, R.R. Singh, Dual roles of immunoregulatory cytokine TGF-beta in the pathogenesis of autoimmunity-mediated organ damage, Journal of immunology, 180 (2008) 1903-1912.
[58] J.P. Grande, Mechanisms of progression of renal damage in lupus nephritis: pathogenesis of renal scarring, Lupus, 7 (1998) 604-610.
[59] F. Strutz, E.G. Neilson, New insights into mechanisms of fibrosis in immune renal injury, Springer seminars in immunopathology, 24 (2003) 459-476.
[60] W.A. Border, S. Okuda, L.R. Languino, M.B. Sporn, E. Ruoslahti, Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1, Nature, 346 (1990) 371-374.
[61] G.S. Hill, M. Delahousse, D. Nochy, E. Thervet, F. Vrtovsnik, P. Remy, D. Glotz, J. Bariety, Outcome of relapse in lupus nephritis: roles of reversal of renal fibrosis and response of inflammation to therapy, Kidney international, 61 (2002) 2176-2186.
[62] G. Filaci, S. Bacilieri, M. Fravega, M. Monetti, P. Contini, M. Ghio, M. Setti, F. Puppo, F. Indiveri, Impairment of CD8+ T suppressor cell function in patients with active systemic lupus erythematosus, Journal of immunology, 166 (2001) 6452-6457.
[63] C. Mohan, L. Morel, P. Yang, H. Watanabe, B. Croker, G. Gilkeson, E.K. Wakeland, Genetic dissection of lupus pathogenesis: a recipe for nephrophilic autoantibodies, The Journal of clinical investigation, 103 (1999) 1685-1695.
[64] A.M. Grabiec, S. Krausz, W. de Jager, T. Burakowski, D. Groot, M.E. Sanders, B.J. Prakken, W. Maslinski, E. Eldering, P.P. Tak, K.A. Reedquist, Histone deacetylase inhibitors

suppress inflammatory activation of rheumatoid arthritis patient synovial macrophages and tissue, Journal of immunology, 184 (2010) 2718-2728.
[65] C.M. Reilly, M. Thomas, R. Gogal Jr, S. Olgun, A. Santo, R. Sodhi, E.T. Samy, S.L. Peng, G.S. Gilkeson, N. Mishra, The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice, Journal of autoimmunity, 31 (2008) 123-130.

Chapter 3

Specific HDAC6 Inhibition by ACY-738 Reduces SLE Pathogenesis in NZB/W Mice

N. L.Regna[1]
C. B. Chafin[1]
S.E. Hammond[1]
A.G. Puthiyaveetil[2]
D.L. Caudell[3]
C. M. Reilly[1, 4]

[1]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061

[2] Department of Biotechnology, American University of Ras Al Khaimah, United Arab Emirates

[3] Wake Forest University Primate Center and the Department of Pathology/Comparative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157

[4]Edward Via College of Osteopathic Medicine, Blacksburg, VA, 24060

2nd Revision by *Cellular and Molecular Immunology*

3.2. Abstract

Histone deacetylase (HDAC) inhibitors are important modifiers of gene and protein expression. In our studies, we sought to determine if a specific HDAC6 inhibitor (ACY-738) was able to decrease disease in NZB/W lupus-prone mice through regulation of B and T cell differentiation. From 22 – 38 weeks-of-age, NZB/W mice were injected intraperitoneally with 20 mg/kg of ACY-738 (high-dose), 5 mg/kg of ACY-738 (low dose) or DMSO (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 using sera and urinary markers of SLE, immune complex deposition, and renal pathology. Flow cytometric analysis was used to assess thymic and splenic T cell profiles as well as bone marrow, splenic, and peripheral B cell differentiation patterns. Our results showed that HDAC6 inhibition was able to decrease many hallmarks of SLE disease including splenomegaly, immune complex-mediated glomerulonephritis, sera anti-dsDNA levels, and inflammatory cytokine production. ACY-738 treatment decreased the number of double-negative thymic T cells and increased the percentage of splenic T_{reg} cells. Inhibition of HDAC6 altered BM B cell differentiation by increasing 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.

Keywords: B cells, HDAC, Systemic Lupus Erythematosus, T cells

3.3. 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 (T_{reg}) cells, secreting proinflammatory cytokines, and producing autoantibodies.[3] Reduced T_{reg} 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/T_{reg} imbalance has been associated with the development of inflammatory disorders and renal dysfunction[5]. Female New Zealand Black/White (NZB/W) mice mimic human disease in several ways and therefore serve as an acceptable model of SLE. NZB/W mice are generated from the cross of New Zealand Black/BinJ (NZB) and New Zealand White/LacJ (NZW) mice and develop a spontaneous lupus-like disease.[6] Both NZB/W mice and humans with active SLE produce autoantibodies against double-stranded DNA (dsDNA) and histones and develop immune complex-mediated glomerulonephritis.[7]

B cells originate from pluripotent hematopoietic stem cells in the BM. Once the B cell pathway has been selected, B cell development and differentiation occurs in a series of stages, progressing from pro- to pre-, to immature B cells.[8] Pro-B cells ($B220^+CD43^+$) pass through 4 developmental phases: A ($CD24^-BP1^-$), B ($CD24^+BP1^-$), C ($CD24^{lo}BP1^+$), and C′ ($CD24^{hi}BP1^+$), while undergoing heavy chain D-J and V(D)J rearrangement.[9, 10] Following successful IgG heavy chain rearrangement, CD43 expression is downregulated and cells 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^+). [9] Fraction D cells rearrange Ig light chains, begin to express IgM and differentiate

into fraction E or immature B cells.[11] Fraction E cells exit the BM and continue to mature in the spleen. As IgM^+ immature B cells begin to express IgD, they progress into fraction F, or mature B cells.[9]

Hyperactive B cells contribute to SLE, however, the underlying mechanism remains unclear. [2] A number of possible causes have been identified including intrinsic hyper-reactivity resulting in polyclonal B cell activation, defective negative selection, decreased immunoregulatory functions, and altered cytokine production influencing B cell activity.[12] Research in both patients with SLE and murine models has shown abnormalities in B cell development and differentiation.[2, 13] B cell differentiation has been reported to be altered during development in the BM, spleen, and periphery.[13, 14] Due to the ability of B cells to act as antigen-presenting cells (APCS), differentiate into antibody-secreting plasma cells, and secrete immunoregulatory cytokines, agents that modulate B cells may be of potential therapeutic value.[15] Furthermore, a number of B cell depletion therapies have been investigated for their effectiveness in treating SLE. [16]

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.[17, 18] 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 in the treatment of autoimmune diseases including SLE.[19, 20] Traditionally, HDACs were thought to function solely through epigenetic regulation of histone proteins.[21] 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.[21, 22] 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.[23, 24] For this reason, more 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.[25, 26, 27] 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.[28, 29] Treatment with class I and IIb pan-HDAC inhibitors has been shown to increase T_{reg} populations [30, 31], however treatment with selective class I HDAC inhibitors has been shown to have minimal effect on T_{reg} function or numbers.[32] Therefore, it can be reasoned that specific class IIb HDAC inhibition is responsible for T_{reg} function.

The current studies were designed to investigate whether treatment with a specific 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 T_{reg} cells compared to wild type (WT mice).[24, 33] Furthermore HDAC6 is known to be expressed at higher levels in T_{reg} cells, which are downregulated during SLE, than in conventional T cells.[28] HDAC6 inhibition has also been associated with increased DNA-damage leading to a greater apoptotic rate and index. [33] 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-phenylcycloproylamino) pyrimidine-5-carboxamide).

3.4. Materials and Methods

3.4.1. ACY-738 bioavailability

Male C57BL/6 mice were injected intraperitoneally 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 (LC/MS/MS) and calculated using a standard curve. The pharmokinetic (PK) parameters were calculated using WinNonlin software.

3.4.2. Western blot analysis

SH-SY5Y cells were treated with varying concentrations of ACY-738 (1.9 – 500 nM) or vehicle control (DMSO) for 4 hours. Cells were lysed and a histone extraction kit was used according to the manufacturer's protocol (Epigentek, Farmingdale, NY, USA). Western blot analysis was used to determine levels α-tubulin acetylation (Sigma Aldrich, St. Louis, MO, USA) and β-actin (Cell Signaling Technology, Boston, MA, USA) in the cytoplasmic fraction. The histone extract fractions were western blotted for acetylated histone H3 (K9) (Cell Signaling Technology, Boston, MA, USA) and histone H4 (Millipore, Billerica, MA, USA).

3.4.3. 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).

3.4.4. *In vivo* treatment

Mice were injected intraperitoneally 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 euthanization 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.

3.4.5. Measurement of autoantibodies

Sera were collected prior to initiation of treatment at 22 weeks-of-age and every 4 weeks until euthanization. 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 hours and then centrifuged for 15 min at 10,000x*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 Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A final dilution of 1:800 was reported.

3.4.6. Isolation of B cells from the BM

BM cells were flushed in PBS with 1% BSA from the femurs of NZB/W mice following euthanization. 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 B22 and Flourescein Isothiocyanate (FITC)-conjugated anti-mouse CD43 mAbs to identify pro-B cell ($B220^+$ $CD43^+$) and pre-B cell ($B220^+$ $CD43^-$) populations. Pro-B cell populations were further stained with Phycoerythirn (PE)-conjugated anti-mouse BP1 and PECy5 or Peridinin-chlorophyll proteins (PerCP)-conjugated anti-mouse CD24 mAbs to identify fractions A ($B220^+$ $CD43^+$ $CD24^-$ $BP1^-$), B ($B220^+$ $CD43^+$ $CD24^+$ $BP1^-$), C ($B220^+$ $CD43^+$ $CD24^{lo}$ $BP1^+$), and C' ($B220^+$ $CD43^+$ $CD24^{hi}$ $BP1^+$). Pre-B cells fractions were further stained with PE-conjugated anti-mouse IgD and PECy5-conjugated anti-mouse IgM mAbs to identify fractions D ($B220^+$ $CD43^-$ IgM^- IgD^-), E ($B220^+$ $CD43^-$ IgM^+ IgD^-) and F ($B220^+$ $CD43^-$ IgM^+ IgD^+). Fractions were measured by flow cytometric analysis. All antibodies were purchased from eBioscience (San Diego, CA, USA). Flow cytometry was performed at that 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).

3.4.7. Isolation of splenic B cells

Following euthanization, 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 stagesusing flow cytometry: T1 (IgM^+ $CD23^-$ $AA4.1^+$ $CD21^-$), T2 (IgM^{hi} $CD23^+$ $AA4.1^+$ $CD21^+$), T3 (IgM^{lo} $CD23^+$ $AA4.1^+$ $CD21^+$), F_0 (IgM^+ $CD23^+$ $AA4.1^-$ $CD21^-$), MZ (IgM^+ $CD23^-$ $AA4.1^-$ $CD21^+$) or B1 (IgM^+ $CD23^-$ $AA4.1^-$ $CD21^-$). Flow cytometry data was analyzed using FlowJo.

3.4.8. Isolation of peripheral B cells

Blood was collected prior to euthanization using retro-orbital bleeding. RBCs from the peripheral blood were lysed and single cell suspensions were labeled using FITC-conjugated B220 and PerCep710 conjugated IgM anti-mouse mAbs (eBioscience, San Diego, CA, USA). Mature B cells were identified as IgM^+B220^+ cells. Flow cytometry data was analyzed using FlowJo.

3.4.9. Pathology

At the time of euthanization, the kidneys were removed and cut in half. One half of the kidney from each mouse was fixed in formalin, embedded in paraffin, sectioned, and stained with Periodic acid-Schiff (PAS). Kidney sections were scored (0 – 4) for glomerular proliferation, inflammation, crescent formation, necrosis, and by a pathologist, in a blinded manner.

3.4.10. 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.

3.4.11. 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.

3.4.12. 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 RNA*later* (QIAGEN, Valencia, CA, USA), and stored at -20°C until RNA isolation.

3.4.13. 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 diluted to 1 ng/µL for real-time RT-PCR. The eluted RNA was stored at -80°C.

3.4.14. Real-time RT-PCR

IL-6, *IL-10* and *TGF-β* mRNA expression were measured using TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA, USA) . The $\Delta\Delta C_T$ was calculated using the endogenous control GAPDH, and then the ΔC_T 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.

3.4.15. 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.

3.4.16. 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.

3.5. Results

3.5.1. 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 (Figure 1 A – B). There were no significant differences in the percentages of pro- or pre-B cells between diseased and pre-diseased mice (Figure 1 B).

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 ($B220^{+}$ $CD43^{+}$ $CD24^{lo}$ $BP1^{+}$), and C' ($B220^{+}$ $CD43^{+}$ $CD24^{hi}$ $BP1^{+}$) (Figure 1 C – D). Diseased NZB/W mice had significantly fewer cells in fractions C and C′ when compared to pre-diseased mice (Figure 1 D). Pre-B cells were further divided into fractions D ($B220^{+}$ $CD43^{-}$ IgM^{-} IgD^{-}), E ($B220^{+}$ $CD43^{-}$ IgM^{+} IgD^{-}) and F ($B220^{+}$ $CD43^{-}$ IgM^{+} IgD^{+}) (Figure 1 E – 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 (Figure 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 of defective B cells leading to an increased number of autoreactive B cells leaving the BM.

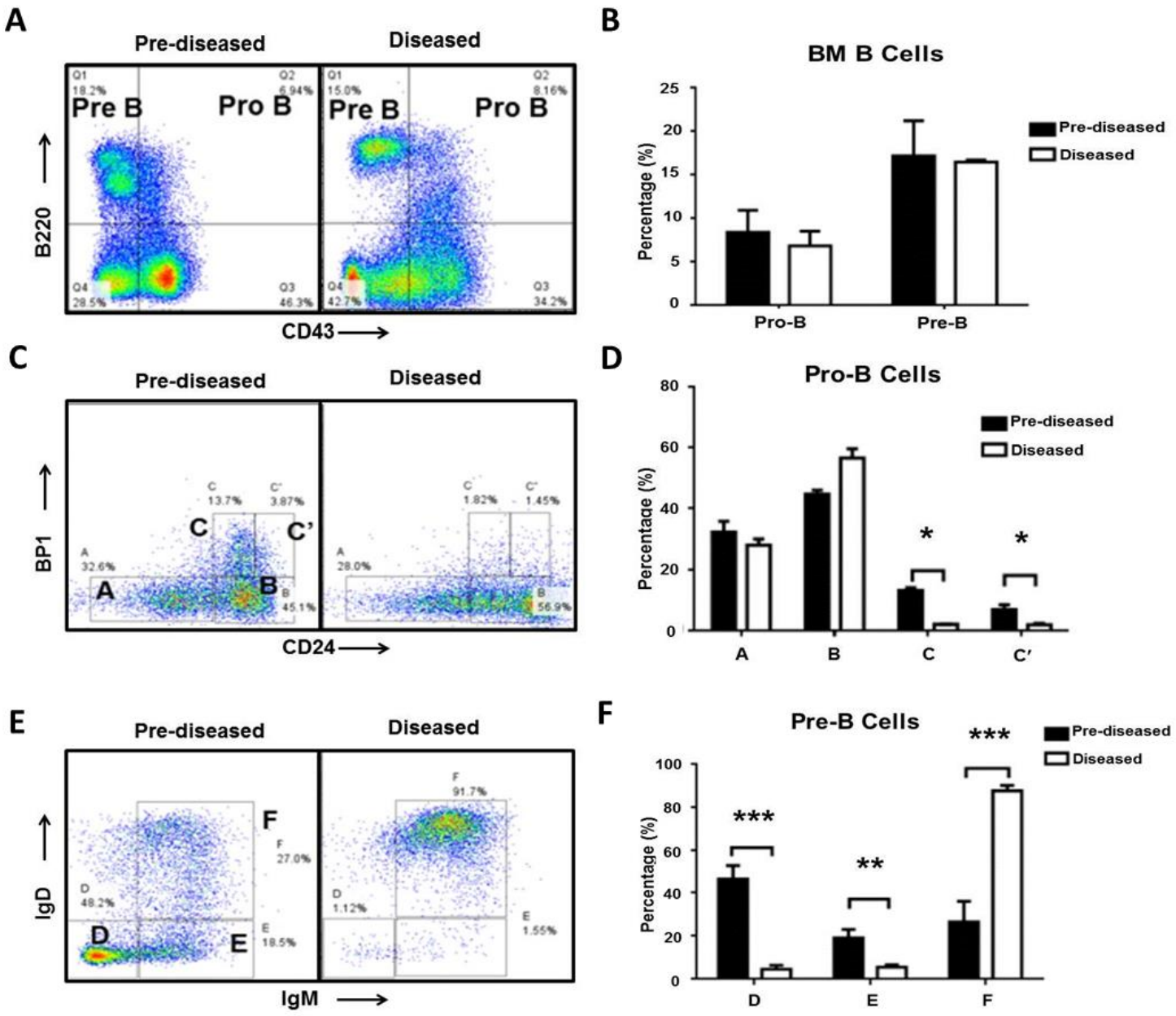


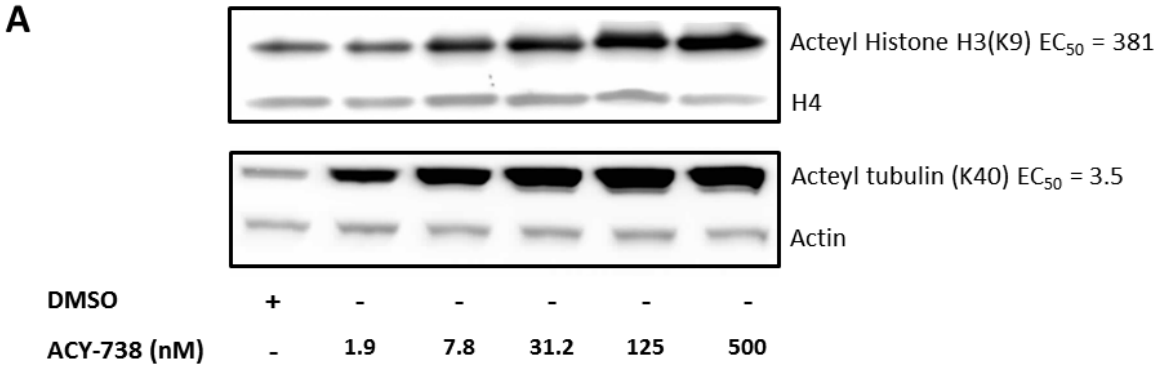
Figure 1 Evaluation of pro- and pre-B cells in NZB/W mice. BM cells were harvested from pre-diseased and diseased NZB/W mice and labeled with fluorescently tagged antibodies specific for pro- and pre-B cells. (A) Representative images of B cells labeled with CD43 and B220. (B) There were no significant 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 ($CD24^{lo}BP1^{+}$) and C′ ($CD24^{hi}BP1^{+}$).

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 ($n = 3$; *$p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$).

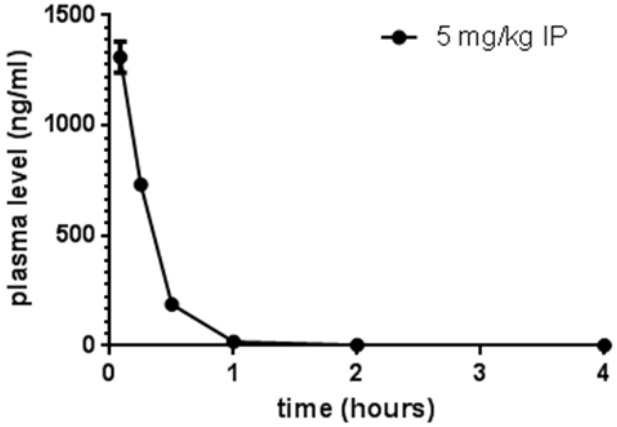
3.5.2. Selectivity and bioavailability of ACY-738 were determined.

We chose to use a selective HDAC6 inhibitor to treat NZB/W mice due to previous reports of enhanced T_{reg} cell activity following HDAC6 inhibition[27]. The compound ACY-738 induced the acetylation of α-tubulin (a measure of HDAC6 inhibition) in cultured SH-SY5Y cells at a concentration of 1.9 nM after four hours of treatment (Figure 2A). We treated cells with varying concentrations of ACY-738 and examined the acetylation status of α-tubulin (K40) and histone H3 (K9). We determined that ACY-738 has a 100-fold greater selectivity for increasing acetylation of the HDAC6 associated protein, α-tubulin (EC_{50} = 3.5 nM), over class I HDAC associated protein, histone H3 (EC_{50} = 381 nM). These results indicate that ACY-738 selectively targets HDAC6 at low nanomolar concentrations. To determine the dose that would achieve a plasma concentration of approximately 2 nM for up to four hours. 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 four hours after the dose average 5.6 nM (figure 2B).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.



B

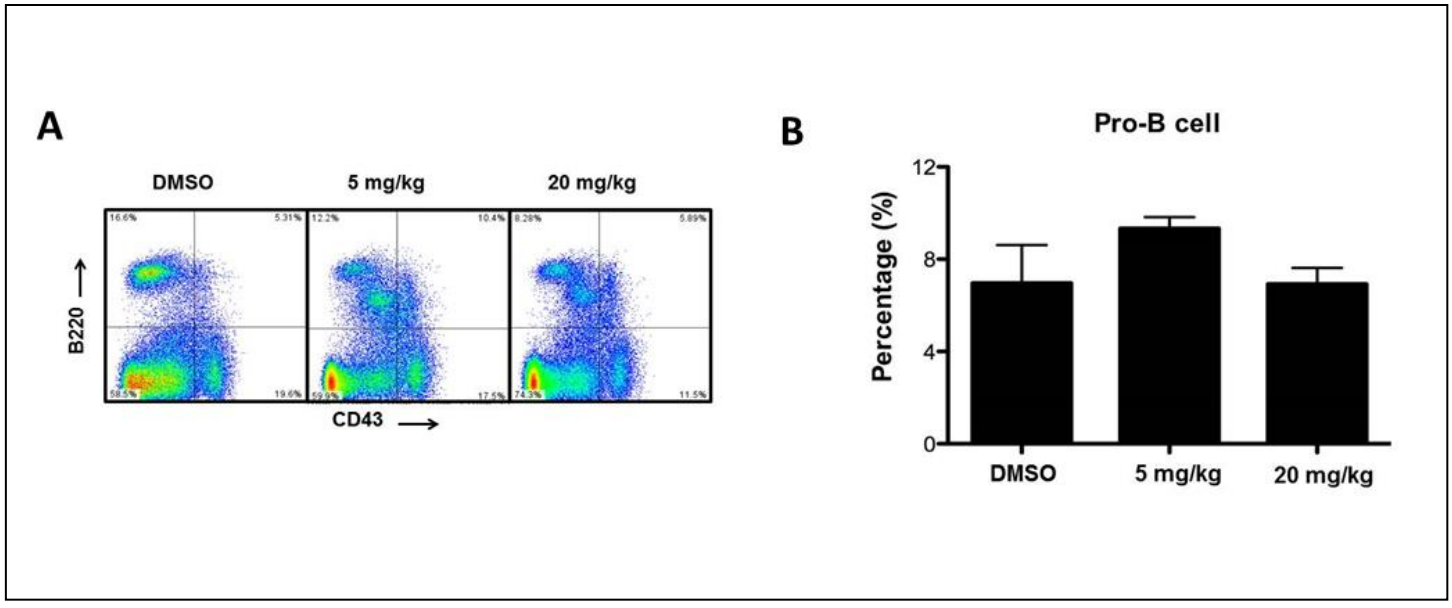


PK parameters	Unit	Value
T_{max}	hr	0.0830
C_{max}	ng/mL	1310
Terminal $t_{1/2}$	hr	3.10
AUC_{last}	hr*ng/mL	412
AUC_{INF}	hr*ng/mL	416

Figure 2. ACY-738 acetylation status and PK parameters. (A) Western blot analysis of α-tubulin and histone H3 acetylation status. ACY-738 treatment was 100-fold more selective in increasing α-tubulin acetylation tubulin (EC_{50} = 3.5 nM) than histone H3 acetylation tubulin (EC_{50} = 381 nM). (B) PK data following i.p. injection of 5 mg/kg ACY-738. ACY-738 reached a maximum plasma concentration of 1310 ng/mL at 0.830 hours following treatment.

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

We tested whether inhibiting 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 pro-B cells was not significantly altered following HDAC6 inhibition (Figure 3 A – 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 stages A and C (Figure 3 C – D). The pre-B cell population was significantly decreased in a dose-dependent manner following HDAC6 inhibition in NZB/W mice (Figure 3 A, E). The developmental pre-B cell stages were also significantly altered. At both the high and low 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 (Figure 3F – G). HDAC6 inhibition is able to reduce the number of cells that survive BM differentiation resulting in fewer B cells continuing development in the periphery.



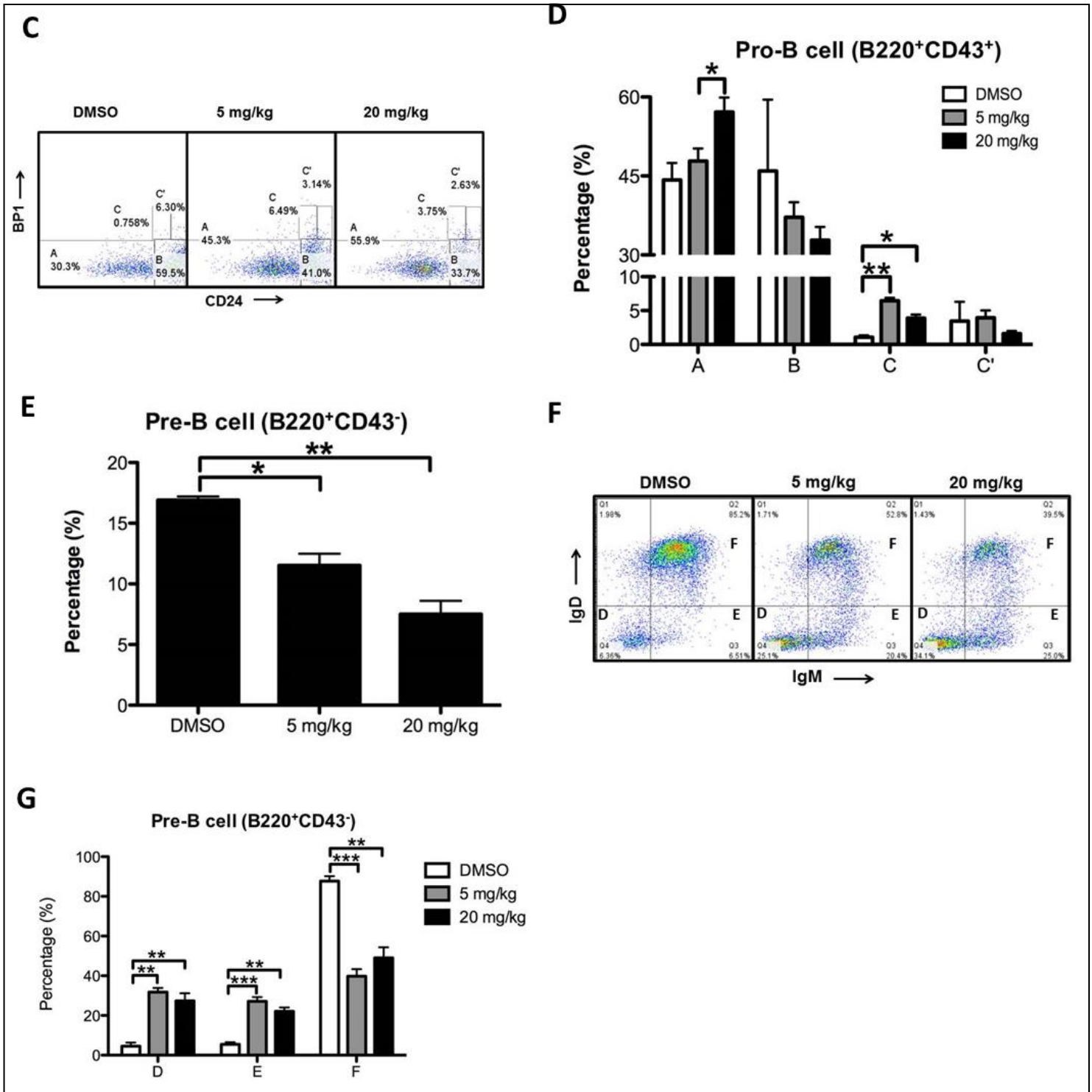


Figure 3 HDAC6 inhibition altered pro- and pre-B cell populations in the BM. Harvested BM cells were stained with B220 and CD43. (A) Representative flow cytometry image of pro-B cell ($B220^{+}CD43^{+}$) and pre-B cell ($B220^{+}CD43^{-}$) populations. (B) There were no significant differences in the percentages of pro-B cells amongst treatment groups. (C) Representative flow

diagram of pro-B cell fractions: A, B, C, and C′. (D) Treatment with ACY-738 significantly increased the percentage of pro-B cells in fractions A and C. (E) The percentage of pre-B cells was significantly decreased in a dose-dependent manner following HDAC6i treatment. (F) Representative flow cytometry image of pre-B cell fractions D, E, and F. (G) At 38 weeks-of-age, the percentage of pre-B cells in fractions D and E was significantly increased, while the percentage of cells in fraction F was significantly decreased at both the low and high doses of ACY-738 ($n \geq 3$; *$p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$).

3.5.4. 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. [34] 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, F_0, B1, and MZ. However, treatment with ACY-738 did not significantly affect these populations of B cells at either the low or high dose (Figure 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 [14, 35]. A single-cell suspension was obtained from the blood of 38-week-old NZB/W mice and strained for IgM and B220. Treatment had no effect on the percentage of peripheral B cells (IgM^+B220^+) in 38-week-old NZB/W mice (Figure 4 B – C).

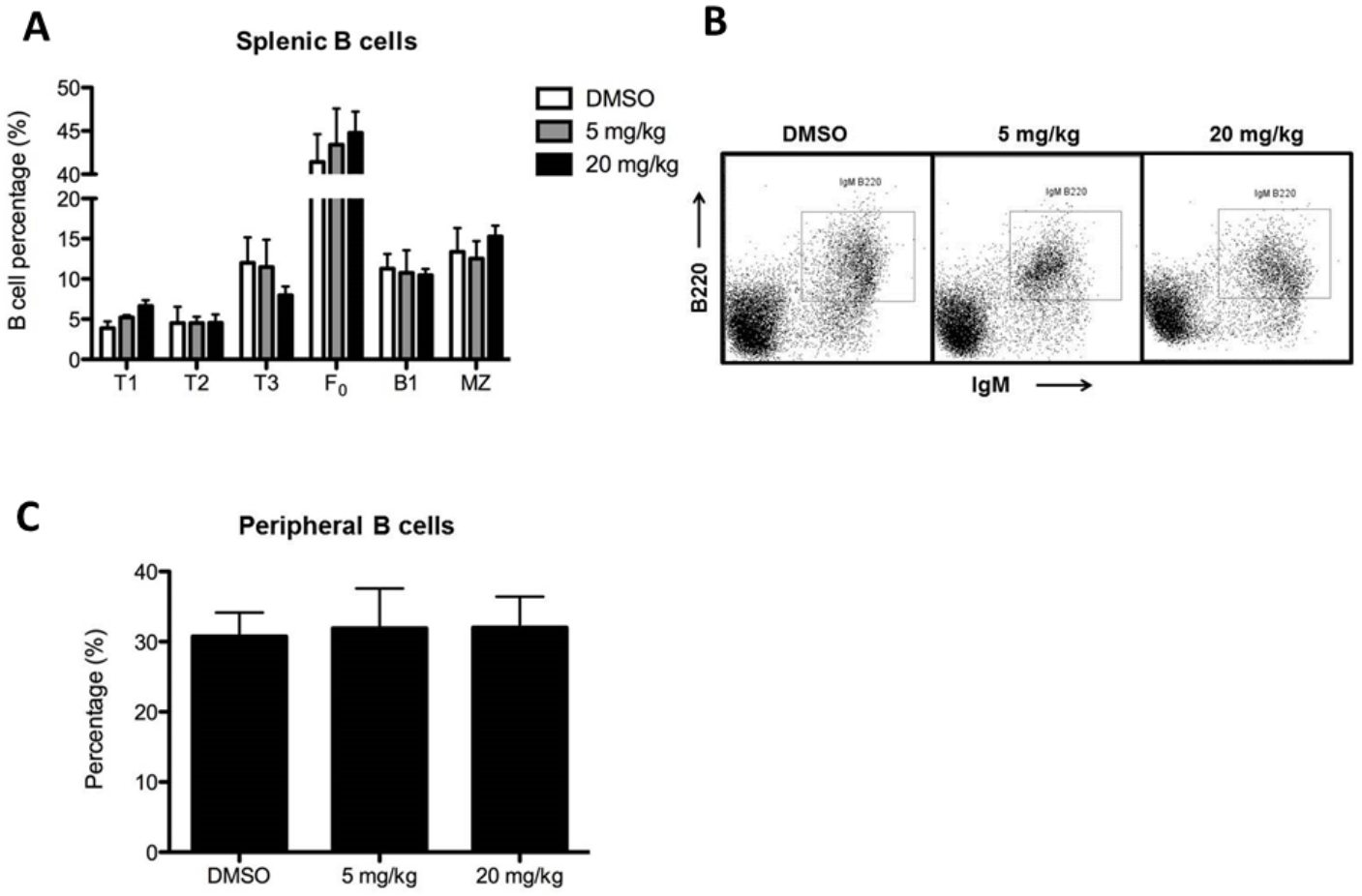


Figure 4 ACY-738 had no effect on splenic or peripheral B cells. (A) Single cell suspension of splenocytes were divided into developmental stages T1 ($IgM^{+}CD23^{-}AA4.1^{+}CD21^{-}$), T2 ($IgM^{hi}CD23^{+}AA4.1^{+}CD21^{+}$), T3 ($IgM^{lo}CD23^{+}AA4.1^{-}CD21^{+}$), F_0 ($IgM^{+}CD23^{+}AA4.1^{-}CD21^{-}$), MZ ($IgM^{+}CD23^{-}AA4.1^{-}CD21^{+}$), and B1 ($IgM^{+}CD23^{-}AA4.1^{-}CD21^{-}$). There were no significant differences following HDAC6 inhibition in any of the splenic B cell developmental (B) Representative flow cytometry diagram of peripheral B cells ($IgM^{+}B220^{+}$). (C) ACY-738 had no effect on the percentage of peripheral B cells at either the low or high dose HDAC6i ($n \geq 3$).

3.5.5. *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.[36, 37, 38] 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 (Figure 4 B, 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 (Figure 5 B, C).

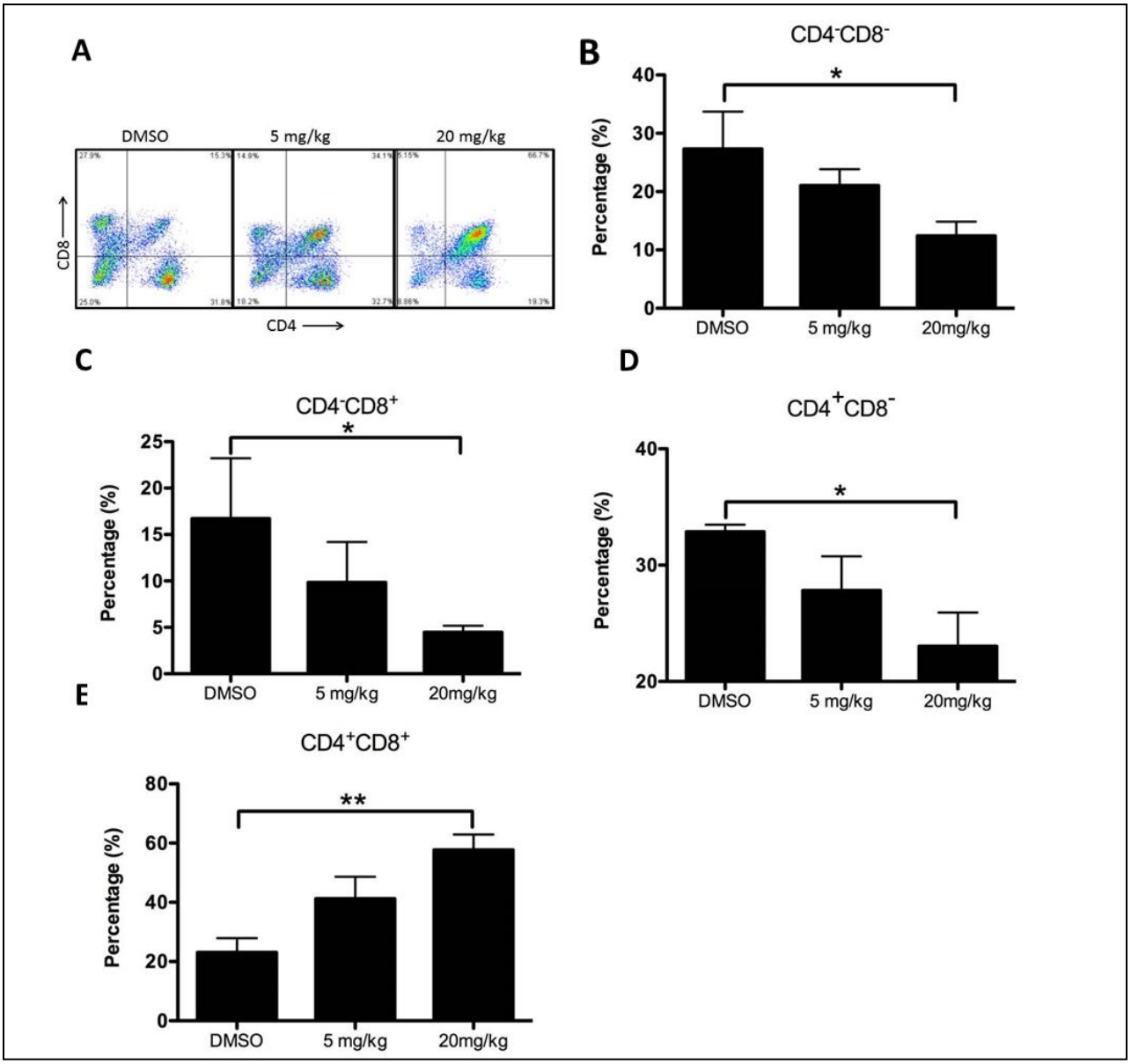


Figure 5. The percentage of DN thymic T cells is reduced following HDAC6 inhibition. (A) Representative flow cytometry images of thymocytes gated on CD3 and labeled with CD4 and CD8. (B) ACY-738 treatment decreased the percentage of DN T cells in a dose-dependent manner. (C) There was a reduction in the percentage of $CD3^+CD4^+CD8^-$ and (D) $CD3^+CD4^-CD8^+$ T cells following HDAC6 inhibition. (E) Double positive ($CD3^+CD4^+CD8^+$) thymic T cell numbers were increased in a dose-dependent manner following 16 weeks of treatment with ACY-738 ($n \geq 3$; *$p < 0.05$, ** $p < 0.005$).

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

The T_{reg} phenotype was assessed due to its role in the maintenance of self-tolerance and the prevention of autoimmune disease.[39] During active SLE the overall number and function of T_{reg} cells is reduced.[40] 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 both doses compared to mice treated with vehicle control alone (Figure 6 A – B).

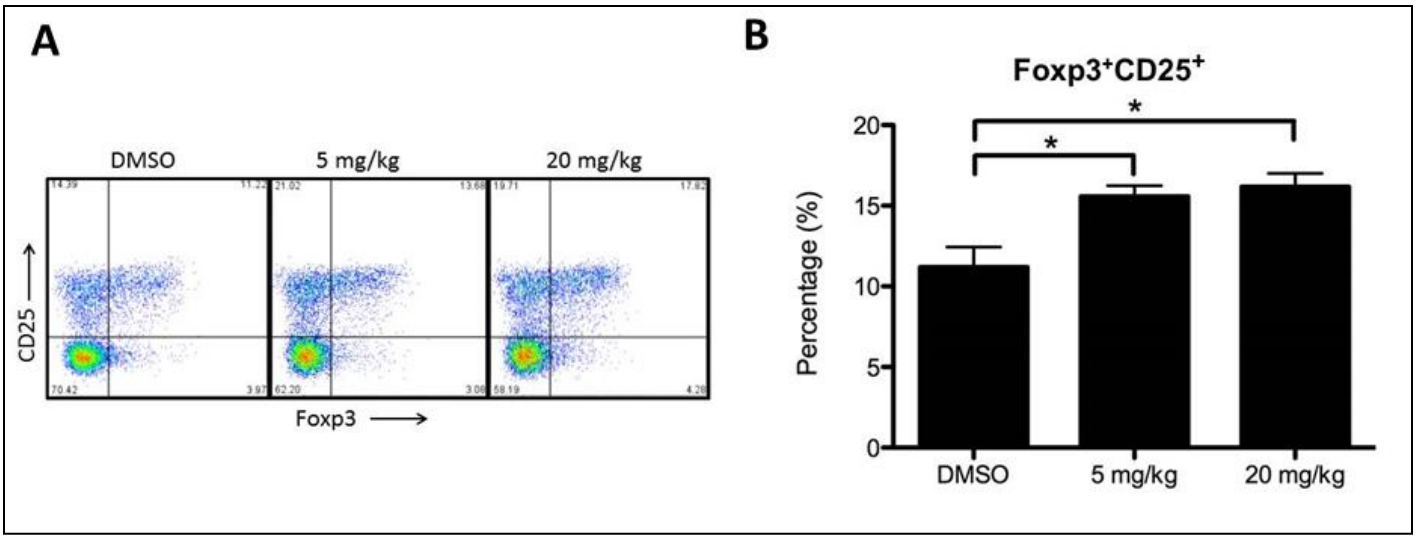


Figure 6. Specific HDAC6 inhibition increased the T_{reg} phenotype in NZB/W mice. (A) Representative flow diagram of splenocytes gated on CD4 and labeled with Foxp3 and CD25. (B) Treatment with ACY-738 significantly increased the percentage of T_{reg} cells ($CD4^+Foxp3^+CD25^+$) ($n \geq 5$; $*p < 0.05$).

3.5.7. 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 high or low 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 (Figure 7 A). 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 (Figure 7 B). 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 F1 mice (Figure 7 C). Because SLE is characterized by enlargement of the spleen, splenomegaly was assessed in mice following euthanization 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 concentration dependent manner (Figure 7 D – E).

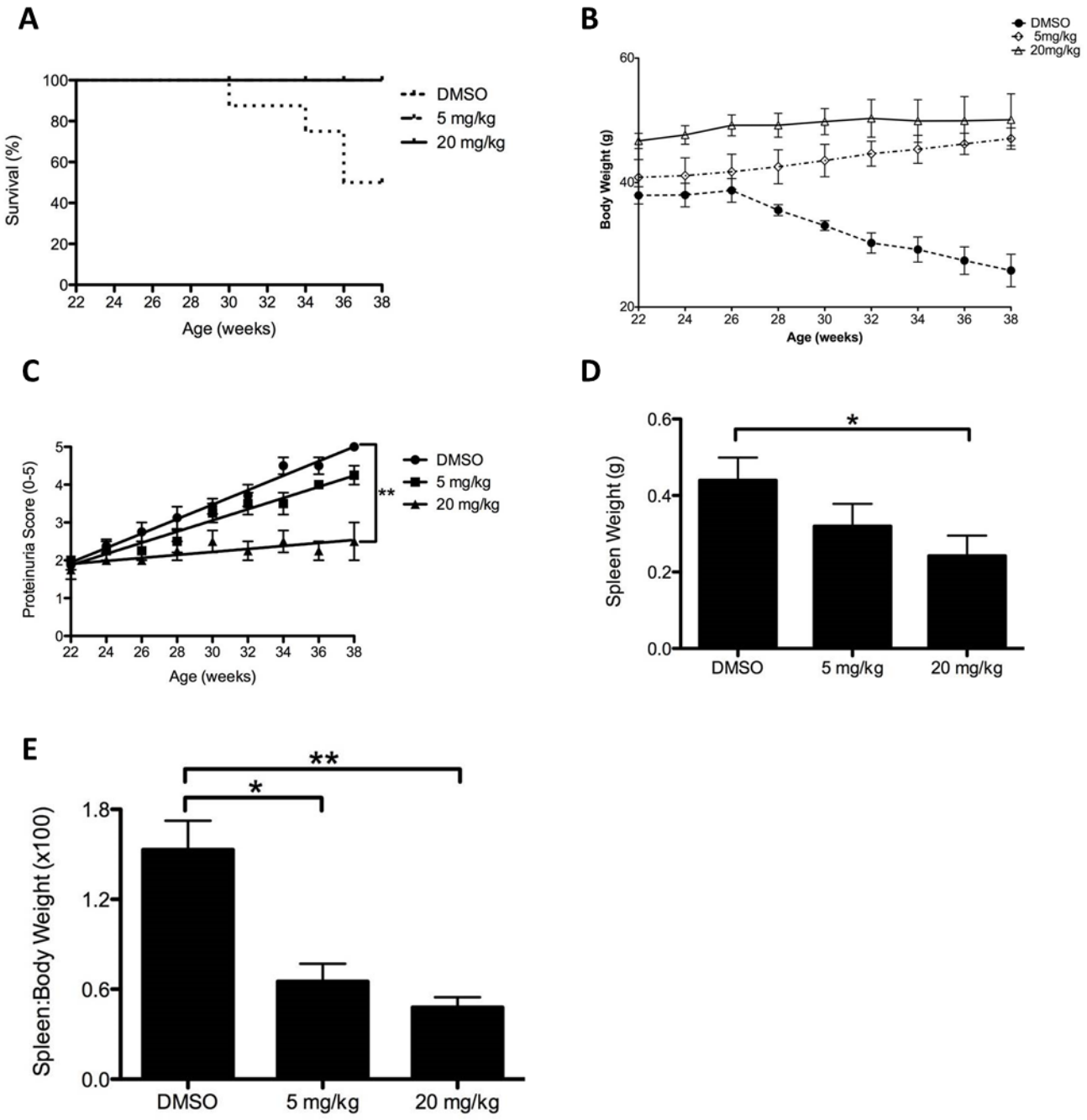


Figure 7 Assessment of survival rate and disease progression in NZB/W mice. (A) By 38 weeks-of-age, half of the NZB/W mice receiving the vehicle control alone had died. No mice receiving the HDAC6i died before termination of the study. (B) Body weight increased as NZB/W mice aged, until mice reached 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 – 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 ($n \geq 5$; *$p < 0.05$, ** $p < 0.005$).

3.5.8. 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.[41] Diseased NZB/W mice have elevated production of anti-dsDNA as well as increased production of IgM and IgG isotypes (specifically IgG2a) [42]. To determine the effect of specific HDAC6 inhibition on disease in NZB/W mice, serum anti-dsDNA 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 (Figure 8 A – 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 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 the vehicle control-treated mice ($p < 0.05$). Following treatment with 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 (Figure 8 A). The HDAC6i treatment significantly decreased levels of IgG2a and total IgG (Figure 8 B – C).

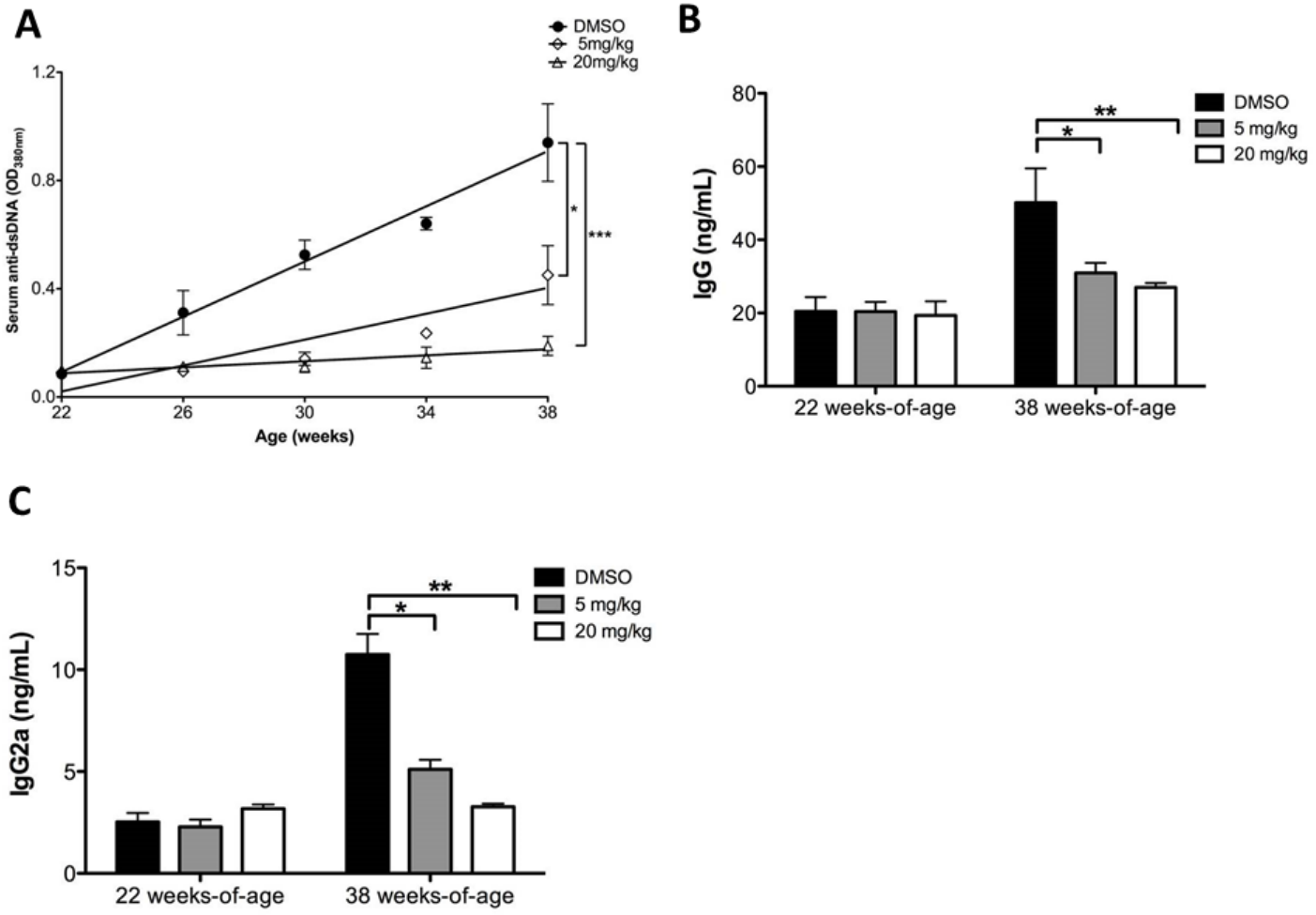


Figure 8 Evaluation of SLE sera biomarkers of disease in NZB/W mice following ACY-738 therapy. (A) Measurement of sera anti-dsDNA in NZB/W mice at 22 weeks-of-age (prior to treatment) and 38 weeks-of-age (following 16 weeks of treatment). There were no significant differences in anti-dsDNA levels prior to the initiation of treatment. Anti-dsDNA increased in the NZB/W mice as they aged; however, HDAC6 inhibition was able to decrease production in

38-week-old mice. (B) IgG2a levels were measured prior to initiation of treatment and prior to euthanization at 38 weeks-of-age. Anti-dsDNA levels increased over time, but ACY-738 treatment significantly decreased IgG2a in a dose-dependent manner. (C) At 38 weeks-of-age, total IgG was slightly decreased in mice that received the HDAC6i ($n \geq 5$; *$p < 0.05$, ** $p < 0.005$).

3.5.9. 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.[43, 44] Conversely, lymphocyte production of TGF-β has been shown to be decreased during active SLE.[45] Beginning at 22 weeks-of-age, cytokine levels were measured in the sera every 4 weeks until euthanization at 38 weeks-of-age (Figure 9 A – B). As the NZB/W mice aged, sera levels of TGF-β decreased whereas levels of IL-1β increased (Figure 9 A – B). HDAC6 inhibition attenuated the reduction of TGF-β production as the mice aged in a dose-dependent manner (Figure 9 A). Treatment with both the low and high dose of ACY-738 attenuated sera IL-1β production as the NZB/W mice aged (Figure 9 B).

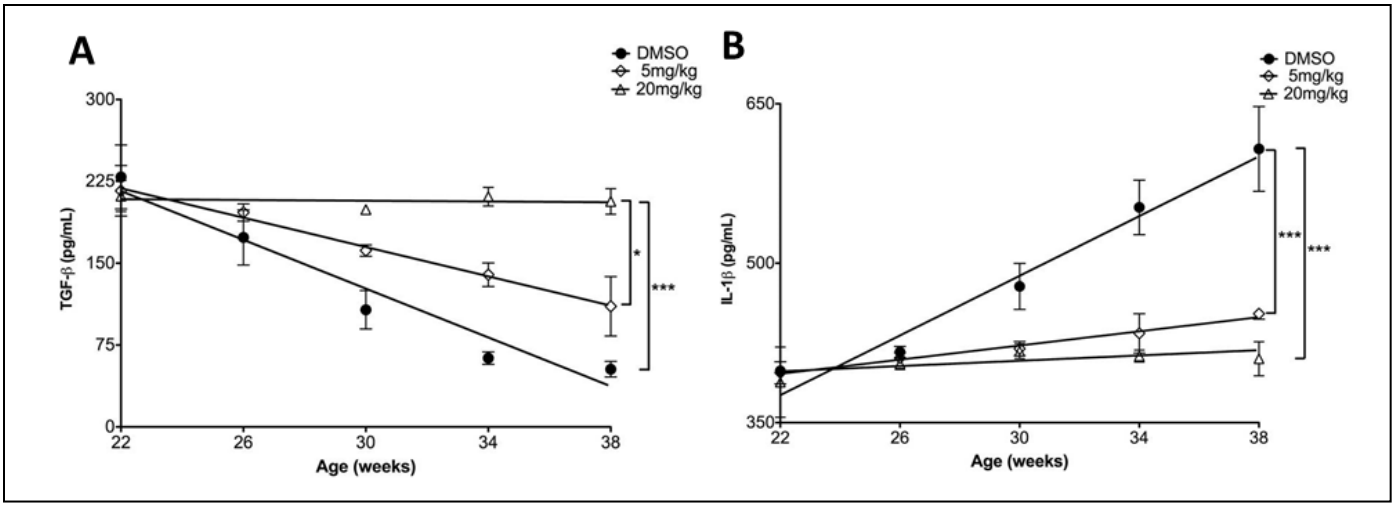


Figure 9 Cytokine production in lupus-prone mice was assessed. (A – B) TGF-β and IL-1β were measured in sera collected from mice prior to treatment (22 week-of-age) and following HDAC6i treatment for 16 weeks. At 22 weeks-of-age there were no significant differences in cytokine levels amongst the three groups. (A) Following treatment, TGF-β levels had significantly decreased in vehicle control-treated mice, however, treatment with ACY-738 was able to reverse this trend in a dose-dependent manner. (B) Levels of IL-1β were elevated in 38-week-old NZB/W mice. HDAC6 inhibition significantly decreased sera IL-1β ($n \geq 3$; $*p < 0.05$, $*** p < 0.0005$).

3.5.10. 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.[46] Altered mRNA glomerular expression can lead to fibrosis and irreversible glomerular damage.[47] 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.[48] 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.[49] 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.[50]

Following euthanization, 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 (Figure 10). Furthermore, glomerular *TGF-β* mRNA was decreased following inhibition of ACY-738 in a dose-dependent manner (Figure 10). Taken together these results suggest that HDAC6 inhibition by ACY-738 reduces production of SLE-associated cytokine mRNA.

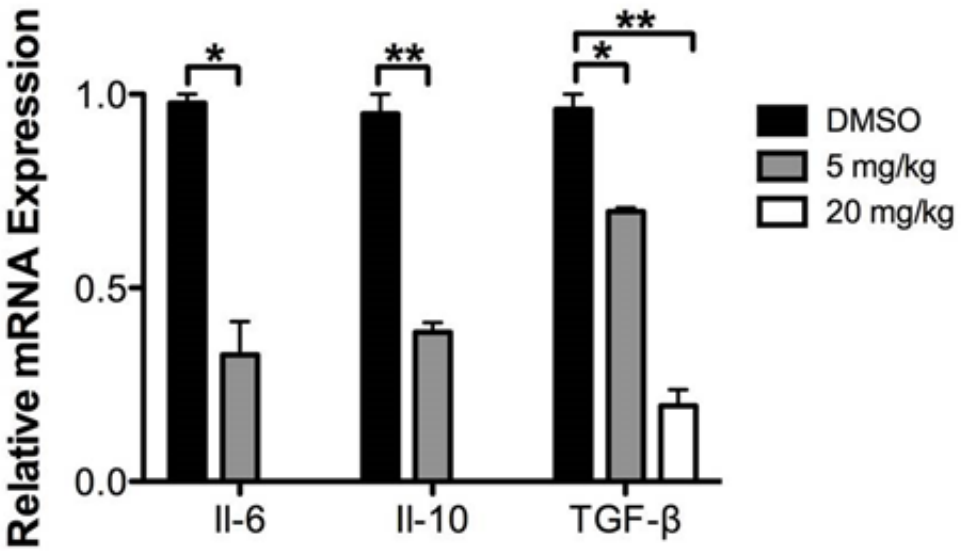


Figure 10 Glomerular mRNA levels were assessed in 38-week-old mice. Relative glomerular levels of *IL-6, IL-10,* and *TGF-β* mRNA were determined using real time RT-PCR. Treatment with ACY-738 decreased *IL-6, IL-10,* and *TGF-β* were all decreased in a dose-dependent manner. Following treatment with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

3.5.11. 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 (Figure 11 A). 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 (Figure 11 B). 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 (Figure 11 C – D).

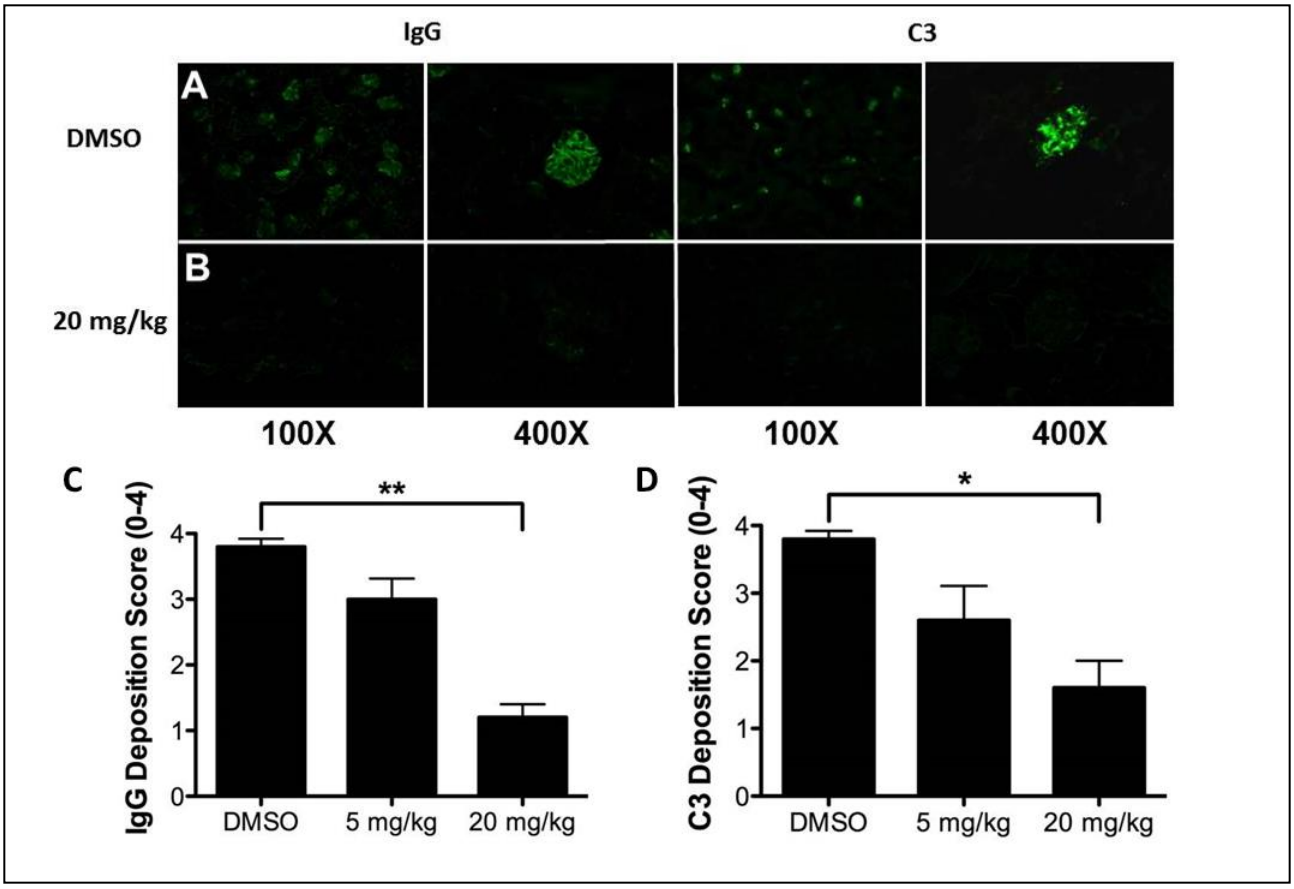


Figure 11 HDAC6 inhibition decreased glomerular immune complex deposition. 5 μM kidney sections were stained with FITC-conjugated C3 or IgG and assessed for fluorescence intensity. (A – B) Representative images of glomerular deposition of both C3 and IgG in NZB/W mice treated with the vehicle control 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 ($n \geq 5$; *$p < 0.05$, ** $p < 0.005$).

3.5.12. HADAC6 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 (Figure 12 A). Treatment with 5 mg/kg ACY-738 did not significantly 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 (Figure 12 B). 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 (Figure 12 C).

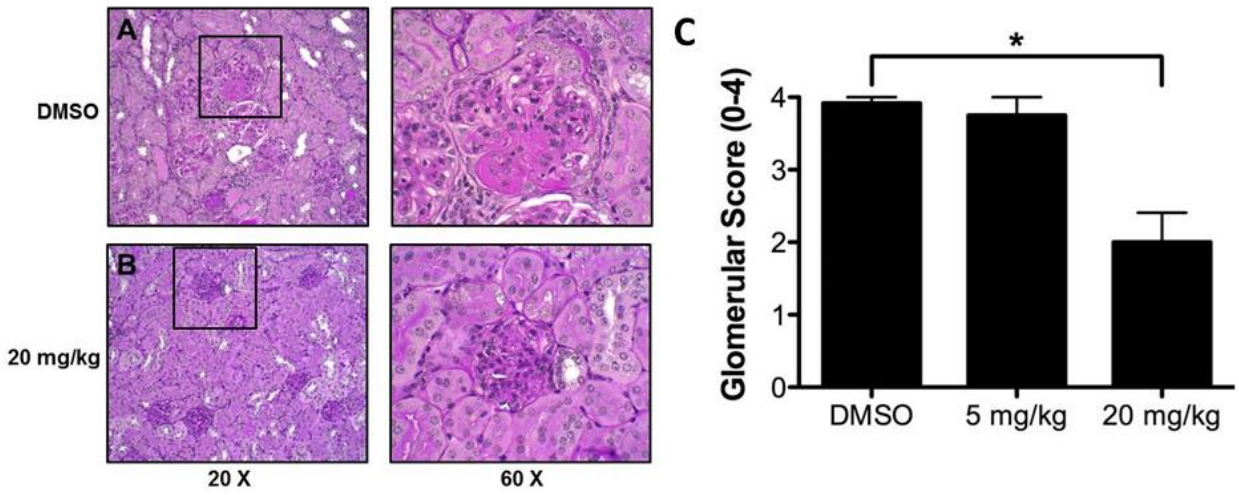


Figure 12 SLE-associated renal pathology was decreased following ACY-738 therapy. Paraffin-embedded kidneys were sectioned and then stained with PAS. (A) Representative image of severe glomerulonephritis (thickened GBM and crescent formations) from an NZB/W mouse treated with the vehicle control alone. (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 ($n \geq 5$; $*p < 0.05$).

3.6. Discussion

Treatment with ACY-738 was able to decrease SLE disease by correcting aberrant B and T cell differentiation. We showed that BM differentiation of B cells was altered in diseased NZB/W mice with increased survival of differentiating B cells resulting in an accumulation of cells in the late pre-B cell fraction F. 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 the aberrant B cell differentiation in NZB/W mice by increasing the percentages of cells in late pro-B cell and early pre-B cell fractions. Furthermore, HDAC6 inhibition decreased the accumulation of cells in late pre-B fraction F, reversing the increased survival of BM B cells characteristic of SLE. Treatment with ACY-738 was also able to alter T cell differentiation in NZB/W mice. The percentages of T_{reg} cells were increased, while the percentages of thymic DN T cells were decreased following HDAC6i therapy for 16 weeks. 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.

Our results suggest that there is 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. Diseased NZB/W mice had decreased numbers of pro-B cells in the late C and C′ fractions and in the early pre-B cell fractions D and E when compared to pre-diseased NZB/W mice. 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 properly undergo B cell differentiation possibly due to

altered regulation of B cell checkpoints, resulting in failure to remove defective B cells. 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.[8] In healthy individuals, approximately 55-75% of the repertoire produced by Ig gene rearrangement in the BM is autoreactive.[51] These autoreactive B cells are removed at two checkpoints.[12] The majority of autoreactive B cells are removed in the BM while the cells are still immature.[52] 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.[51] 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.[52] Our results show that the greatest difference in B cell development in NZB/W mice occurs during BM differentiation at the pre-B/pro-B stages indicating a possible apoptotic defect in NZB/W mice.

ACY-738 is a selective HDAC6 inhibitor with minimal reactivity against other class II HDACs and 100-fold less selectivity against class I HDACs.[53] Jochems et al. showed a dose-dependent enzymatic selective inhibition of recombinant HDCA6 over HDACS 1, 2, and 3 following treatment with ACY-738.[53] Class I HDAC inhibition is known to significantly reduce the total number of peripheral lymphocytes.[54] A complete blood count was performed along with a blood smear prior to euthanization of the mice treated with the low and high dose of ACY-738. 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 theses doses of ACY-738. Inhibition of HDAC6 using ACY-738 was able to correct the abnormal percentages of late pro-B/early pre-B cells associated with diseased NZB/W mice. While ACY-738 treatment had no effect on the total numbers of pro-B cells, there was a

significant decrease in the percentage of total pre-B cells. HDAC6 inhibition increased the percentage of early-stage pro-B cells in fraction A and the number of cells in the early pre-B cell developmental stages D and E. Furthermore treatment with ACY-738 decreased the percentage of late-stage pre-B cells in fraction F. These results indicate HDAC6 inhibition decreased the percentage of cells that developed into immature B cells coupled with a shift from late-stage subsets to early-stage pro- and pre-B cell subsets. These data indicate that HDAC6 inhibition is able to increase the removal of B cells during BM differentiation. During V(D)J and class switch recombination in the BM, DNA double strand breaks occur resulting in apoptosis of autoreactive B cells.[8] Previous studies implicate HDAC6 in the regulation of apoptosis through increased DNA damage.[33] Our results showed the HDAC6 inhibition had the greatest effect on the pro- and pre B cell populations, which could indicate that ACY-738 functions by correcting the apoptotic defect that is present in SLE.

The effect of HDAC6 inhibition on developmental splenic stages and peripheral B cell populations was also investigated. In our studies we found that ACY-738 did not affect the percentages of B cells in the periphery or the distribution of B cells in the splenic developmental stages. After immature B cells leave the BM, they continue to develop in secondary lymphoid organs including the spleen.[8] As immature B cells develop into mature B cells they become antigen-specific.[8] B cells that escape negative selection during BM differentiation may mature in the spleen to become marginal zone or follicular cells.[55] $IgM^{+}IgD^{+}$ B cells leave the BM as transitional cells and enter the spleen where they mature into follicular B cells or marginal-zone B cells.[8, 55] ACY-738 did not alter the percentages of B cells in transitional, follicular or marginal zone stages. These results suggest that ACY-738 is acting during early B cell development in the BM and not on peripheral of splenic B cells.

Anti-dsDNA is produced by autoreactive B cells that escape negative and positive selection during B cell development.[3, 12] 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.[56] 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 doesn't affect numbers of peripheral B cells, it does alter mature plasma cell production of autoantibodies. This further indicates the ability of HDAC6 inhibition to remove autoreactive B cells that produce 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. Depletion of T_{reg} cells in mice leads to overproduction of Abs however, upon transfer of T_{reg} cells back into mice, Ab production is significantly reduced.[57] 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.*[27] 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.[40] Studies have reported that T_{reg} cell numbers are reduced during active SLE, but numbers are increased following treatment and clinical improvement.[40] It has been demonstrated that pan-HDAC inhibitors, but not class I specific HDAC inhibitors, are able to increase populations of T_{reg} cells.[24] Taken together these data suggest that the T_{reg} profile is most likely regulated by a class IIb HDAC, such as HDAC6.

Naïve $CD4^+$ T cells differentiate into T_{reg} cells following TGF-β stimulation, which promotes Foxp3 expression.[58] 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 HDAC6i 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.[49] 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.[59] 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.[49, 59] Our results showed the ability of HDAC6 inhibition to reverse these trends by increasing the sera levels of TGF-β, while decreasing *TGF-β* in the glomeruli of the kidneys. This indicates that HDAC6 inhibition may increase T_{reg} populations through altered TGF-β production.

During, SLE there is an imbalance between the production of Th1 and Th2 cytokines.[60] We investigated whether HDAC6i 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.[61] 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 pathogenicity of a number of autoimmune diseases including SLE.[62] In lupus-prone mice that are deficient in IL-1β, sera levels of autoantibodies are decreased and the manifestation of disease is much milder.[63] Our results indicate that ACY-738 is able to decrease IL-1β in a dose-dependent manner that 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 percentage 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 pre-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. Future studies are currently defining the mechanism through which ACY-738 regulates abnormal B cell development observed in SLE patients.

References

1. Crispin JC, Liossis SN, Kis-Toth K, Lieberman LA, Kyttaris VC, Juang YT, *et al.* Pathogenesis of human systemic lupus erythematosus: recent advances. *Trends in molecular medicine* 2010, **16**(2)**:** 47-57.

2. Grammer AC, Lipsky PE. B cell abnormalities in systemic lupus erythematosus. *Arthritis research & therapy* 2003, **5 Suppl 4:** S22-27.

3. Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. *Journal of clinical pathology* 2003, **56**(7)**:** 481-490.

4. Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C, *et al.* Regulatory T cells in patients with systemic lupus erythematosus. *Journal of autoimmunity* 2006, **27**(2)**:** 110-118.

5. Alunno A, Bartoloni E, Bistoni O, Nocentini G, Ronchetti S, Caterbi S, *et al.* Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new. *Clinical & developmental immunology* 2012, **2012:** 823085.

6. Burnett R, Ravel G, Descotes J. Clinical and histopathological progression of lesions in lupus-prone (NZB x NZW) F1 mice. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie* 2004, **56**(1-2)**:** 37-44.

7. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Advances in Immunology* 1985, **37:** 269-390.

8. Hardy RR, Hayakawa K. B cell development pathways. *Annual review of immunology* 2001, **19:** 595-621.

9. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *The Journal of experimental medicine* 1991, **173**(5)**:** 1213-1225.

10. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, *et al.* Ordered rearrangement of immunoglobulin heavy chain variable region segments. *The EMBO journal* 1984, **3**(6)**:** 1209-1219.

11. Ehlich A, Schaal S, Gu H, Kitamura D, Muller W, Rajewsky K. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell* 1993, **72**(5)**:** 695-704.

12. Dorner T, Giesecke C, Lipsky PE. Mechanisms of B cell autoimmunity in SLE. *Arthritis research & therapy* 2011, **13**(5)**:** 243.

13. Anolik J, Sanz I. B cells in human and murine systemic lupus erythematosus. *Current opinion in rheumatology* 2004, **16**(5)**:** 505-512.

14. Korganow AS, Knapp AM, Nehme-Schuster H, Soulas-Sprauel P, Poindron V, Pasquali JL, *et al.* Peripheral B cell abnormalities in patients with systemic lupus erythematosus in quiescent phase: decreased memory B cells and membrane CD19 expression. *Journal of autoimmunity* 2010, **34**(4)**:** 426-434.

15. Jacob N, Stohl W. Autoantibody-dependent and autoantibody-independent roles for B cells in systemic lupus erythematosus: past, present, and future. *Autoimmunity* 2010, **43**(1)**:** 84-97.

16. Cambridge G, Isenberg DA, Edwards JC, Leandro MJ, Migone TS, Teodorescu M, *et al.* B cell depletion therapy in systemic lupus erythematosus: relationships among serum B lymphocyte stimulator levels, autoantibody profile and clinical response. *Annals of the rheumatic diseases* 2008, **67**(7)**:** 1011-1016.

17. Chen C, Rowell EA, Thomas RM, Hancock WW, Wells AD. Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *The Journal of biological chemistry* 2006, **281**(48)**:** 36828-36834.

18. Geiman TM, Robertson KD. Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together? *Journal of cellular biochemistry* 2002, **87**(2)**:** 117-125.

19. Reilly CM, Regna N, Mishra N. HDAC inhibition in lupus models. *Molecular medicine* 2011, **17**(5-6)**:** 417-425.

20. Dinarello CA, Fossati G, Mascagni P. Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer. *Molecular medicine* 2011, **17**(5-6)**:** 333-352.

21. Kim HJ, Bae SC. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *American journal of translational research* 2011, **3**(2)**:** 166-179.

22. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. *Gene* 2005, **363:** 15-23.

23. Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W, *et al.* Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2003, **9**(10 Pt 1)**:** 3578-3588.

24. de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H, *et al.* Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. *Molecular and cellular biology* 2011, **31**(10)**:** 2066-2078.

25. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood* 2009, **114**(18)**:** 3727-3735.

26. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O, *et al.* Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood* 2010, **115**(5)**:** 965-974.

27. Beier UH, Wang L, Han R, Akimova T, Liu Y, Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. *Science signaling* 2012, **5**(229)**:** ra45.

28. Hancock WW. Rationale for HDAC inhibitor therapy in autoimmunity and transplantation. *Handbook of experimental pharmacology* 2011, **206:** 103-123.

29. Zhang Y, Kwon S, Yamaguchi T, Cubizolles F, Rousseaux S, Kneissel M, *et al.* Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. *Molecular and cellular biology* 2008, **28**(5)**:** 1688-1701.

30. Reilly CM, Thomas M, Gogal Jr R, Olgun S, Santo A, Sodhi R, *et al.* The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice. *Journal of autoimmunity* 2008, **31**(2)**:** 123-130.

31. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood* 2008, **112**(6)**:** 2340-2352.

32. Akimova T, Ge G, Golovina T, Mikheeva T, Wang L, Riley JL, *et al.* Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs. *Clinical immunology*, **136**(3)**:** 348-363.

33. Wang L, Xiang S, Williams KA, Dong H, Bai W, Nicosia SV, *et al.* Depletion of HDAC6 enhances cisplatin-induced DNA damage and apoptosis in non-small cell lung cancer cells. *PloS one* 2012, **7**(9)**:** e44265.

34. Grimaldi CM, Michael DJ, Diamond B. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *Journal of immunology* 2001, **167**(4)**:** 1886-1890.

35. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, *et al.* Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *Journal of immunology* 2000, **165**(10)**:** 5970-5979.

36. Tsokos GC, Balow JE. Phenotypes of T lymphocytes in systemic lupus erythematosus: decreased cytotoxic/suppressor subpopulation is associated with deficient allogeneic cytotoxic responses rather than with concanavalin A-induced suppressor cells. *Clinical immunology and immunopathology* 1983, **26**(2)**:** 267-276.

37. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, *et al.* Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *Journal of immunology* 2008, **181**(12)**:** 8761-8766.

38. Hammond DM, Nagarkatti PS, Gote LR, Seth A, Hassuneh MR, Nagarkatti M. Double-negative T cells from MRL-lpr/lpr mice mediate cytolytic activity when triggered through adhesion molecules and constitutively express perforin gene. *The Journal of experimental medicine* 1993, **178**(6)**:** 2225-2230.

39. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, *et al.* Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological reviews* 2006, **212:** 8-27.

40. Horwitz DA. Regulatory T cells in systemic lupus erythematosus: past, present and future. *Arthritis research & therapy* 2008, **10**(6)**:** 227.

41. Vyse TJ, Drake CG, Rozzo SJ, Roper E, Izui S, Kotzin BL. Genetic linkage of IgG autoantibody production in relation to lupus nephritis in New Zealand hybrid mice. *The Journal of clinical investigation* 1996, **98**(8)**:** 1762-1772.

42. Ehlers M, Fukuyama H, McGaha TL, Aderem A, Ravetch JV. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *The Journal of experimental medicine* 2006, **203**(3)**:** 553-561.

43. Handwerger BS, Rus V, da Silva L, Via CS. The role of cytokines in the immunopathogenesis of lupus. *Springer seminars in immunopathology* 1994, **16**(2-3)**:** 153-180.

44. Dean GS, Tyrrell-Price J, Crawley E, Isenberg DA. Cytokines and systemic lupus erythematosus. *Annals of the rheumatic diseases* 2000, **59**(4)**:** 243-251.

45. Ohtsuka K, Gray JD, Stimmler MM, Toro B, Horwitz DA. Decreased production of TGF-beta by lymphocytes from patients with systemic lupus erythematosus. *Journal of immunology* 1998, **160**(5)**:** 2539-2545.

46. Dixon FJ. Murine SLE models and autoimmune disease. *Hospital practice* 1982, **17**(3)**:** 63-73.

47. McMurray RW, Hoffman RW, Nelson W, Walker SE. Cytokine mRNA expression in the B/W mouse model of systemic lupus erythematosus--analyses of strain, gender, and age effects. *Clinical immunology and immunopathology* 1997, **84**(3)**:** 260-268.

48. Ishida H, Muchamuel T, Sakaguchi S, Andrade S, Menon S, Howard M. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice. *The Journal of experimental medicine* 1994, **179**(1)**:** 305-310.

49. Saxena V, Lienesch DW, Zhou M, Bommireddy R, Azhar M, Doetschman T, *et al.* Dual roles of immunoregulatory cytokine TGF-beta in the pathogenesis of autoimmunity-mediated organ damage. *Journal of immunology* 2008, **180**(3)**:** 1903-1912.

50. Aringer M, Smolen JS. Cytokine expression in lupus kidneys. *Lupus* 2005, **14**(1)**:** 13-18.

51. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, *et al.* Defective B cell tolerance checkpoints in systemic lupus erythematosus. *The Journal of experimental medicine* 2005, **201**(5)**:** 703-711.

52. Lu L, Chaudhury P, Osmond DG. Regulation of cell survival during B lymphopoiesis: apoptosis and Bcl-2/Bax content of precursor B cells in bone marrow of mice with altered expression of IL-7 and recombinase-activating gene-2. *Journal of immunology* 1999, **162**(4)**:** 1931-1940.

53. Jochems J, Boulden J, Lee BG, Blendy JA, Jarpe M, Mazitschek R, *et al.* Antidepressant-Like Properties of Novel HDAC6 Selective Inhibitors with Improved Brain Bioavailability. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 2013.

54. Dowdell KC, Pesnicak L, Hoffmann V, Steadman K, Remaley AT, Cohen JI, *et al.* Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, diminishes lymphoproliferation in the Fas -deficient MRL/lpr(-/-) murine model of autoimmune lymphoproliferative syndrome (ALPS). *Experimental hematology* 2009, **37**(4)**:** 487-494.

55. Lian ZX, Kita H, Okada T, Hsu T, Shultz LD, Dorshkind K, *et al.* Increased frequency of pre-pro B cells in the bone marrow of New Zealand Black (NZB) mice: implications for a developmental block in B cell differentiation. *Developmental immunology* 2002, **9**(1)**:** 35-45.

56. Hanrotel-Saliou C, Segalen I, Le Meur Y, Youinou P, Renaudineau Y. Glomerular antibodies in lupus nephritis. *Clinical reviews in allergy & immunology* 2011, **40**(3)**:** 151-158.

57. Hayashi T, Hasegawa K, Adachi C. Elimination of CD4(+)CD25(+) T cell accelerates the development of glomerulonephritis during the preactive phase in autoimmune-prone female NZB x NZW F mice. *International journal of experimental pathology* 2005, **86**(5)**:** 289-296.

58. Zhou L, Lopes JE, Chong MM, Ivanov, II, Min R, Victora GD, *et al.* TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 2008, **453**(7192)**:** 236-240.

59. Grande JP. Mechanisms of progression of renal damage in lupus nephritis: pathogenesis of renal scarring. *Lupus* 1998, **7**(9)**:** 604-610.

60. Arora V, Verma J, Marwah V, Kumar A, Anand D, Das N. Cytokine imbalance in systemic lupus erythematosus: a study on northern Indian subjects. *Lupus* 2012, **21**(6)**:** 596-603.

61. Maeda K, Mehta H, Drevets DA, Coggeshall KM. IL-6 increases B-cell IgG production in a feed-forward proinflammatory mechanism to skew hematopoiesis and elevate myeloid production. *Blood* 2010, **115**(23)**:** 4699-4706.

62. Maczynska I, Millo B, Ratajczak-Stefanska V, Maleszka R, Szych Z, Kurpisz M, *et al.* Proinflammatory cytokine (IL-1beta, IL-6, IL-12, IL-18 and TNF-alpha) levels in sera of patients with subacute cutaneous lupus erythematosus (SCLE). *Immunology letters* 2006, **102**(1)**:** 79-82.

63. Voronov E, Dayan M, Zinger H, Gayvoronsky L, Lin JP, Iwakura Y, *et al.* IL-1 beta-deficient mice are resistant to induction of experimental SLE. *European cytokine network* 2006, **17**(2)**:** 109-116.

Chapter 4

Overexpression of HDAC6 in lupus-prone mice can be inhibited by ACY-738 *in vitro*

N. L.Regna[1]
S.E. Hammond[1]
David L. Caudell[2]
C. M. Reilly[1, 3]

[1]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061

[2]Wake Forest University Primate Center and the Department of Pathology/Comparative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157

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

Submitted to *Immunology & Cell Biology*; Under review

4.2. Abstract

Prior studies have shown that pan-HDAC inhibition can decrease disease in lupus mice; however, the mechanism(s) remain(s) to be elucidated. MRL/MpJ-*Fas*^{lpr} (MRL/lpr) mice develop a lupus-like disease characterized by anti-dsDNA production, lymphoproliferation, and immune complex-mediated glomerulonephritis. Early and late disease (12 and 20 weeks-of-age) female MRL/lpr mice were compared to age-matched, healthy C57BL/6 mice for HDAC expression and activity in bone marrow B cells, splenic B and T cells, and glomerular cells. We found that *HDAC6* was significantly overexpressed in B cells and glomerular cells, whereas *HDAC9* expression was significantly increased in splenic T cells. Due to the overexpression of *HDAC6*, we tested whether treatment with a selective HDAC6 inhibitor (ACY-738) or a pan-HDAC inhibitor (TsA) would decrease HDAC activity. Treatment with ACY-738 (5 nM) significantly reduced cytoplasmic HDAC activity whereas treatment with TsA was able to significantly decrease both nuclear and cytoplasmic HDAC activity. *In vitro* studies in mesangial cells showed that ACY-738 is specific for HDAC6 through α-tubulin acetylation and resulted in decreased Hsp90 and a decrease in nuclear activation of NFκB. Treatment of pre-B cells with ACY-738 decreased the Bcl-2:Bax ratio leading to a pro-apoptotic environment. These results suggest that increased *HDAC6* expression and activity contribute to SLE pathogenesis, and isoform-selective HDAC inhibitors may prove beneficial in the treatment of SLE by acetylating key signaling and transcription factors in inflammation and cell activation.

4.3. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the activation of autoreactive T and B cells [1-3]. During SLE, pathogenic autoantibodies directed against nuclear components, including nucleic acids and histones, are produced contributing to multiorgan inflammation and tissue damage [4]. Studies have shown a number of abnormalities in B cell development in SLE patients including pre-immune B cell maturation, negative selection, receptor editing, and somatic hypermutation [5]. Furthermore, when autoreactive B cells encounter self-antigen (Ag), they form immune complexes (ICs) which become lodged in glomerular capillaries resulting in glomerulonephritis [6]. Aberrant T cell development is also thought to contribute to disease in SLE patients. The number and function of regulatory T (T_{reg}) cells is decreased in both SLE patients and murine models. Furthermore, both humans and mice with SLE have elevated Th1 and Th2 cytokines as well as an increase in the number of Il-17-producing Th17 cells, which contribute to inflammation [7, 8].

Histone deacetylases (HDACs) catalyze the removal of acetyl groups on both histone and non-histone proteins thereby altering protein stability and function [9, 10]. HDACs have been shown to play a role in the regulation of a number of proteins involved with cell cycle, motility, immunity, inflammation, and apoptosis [11]. HDACs can be grouped into four classes: class I, II, III, and IV. Class I HDACs (HDACs 1, 2, 3, and 8) have ubiquitous tissue expression and are involved with cell survival and proliferation. HDACS 1, 2, and 3 are found solely in the nucleus; however, HDAC8 can be found in either the nucleus or the cytoplasm[12]. Class II HDACs can be further subdivided into class IIa (HDACs 4, 5, 7, and 9) and class IIb (HDACs 6 and 10). Class IIa HDACs are able to shuttle back and forth between the nucleus and the cytoplasm, whereas class IIb HDACs are primarily cytosolic [9]. HDAC11 is the sole member of the HDAC

class IV and can be found in either the nucleus or the cytoplasm, but has a tendency to co-localize with HDAC6 in the cytoplasm[12]. Studies suggest that class II HDACs may be preferable targets for treating chronic disease without detrimental side effects [13-15]. While inhibiting class I HDACs has shown some efficacy in the treatment of autoimmune disease, they have also been associated with cytotoxicity following long-term treatment [16, 17]. For these reasons, we have focused this study on the expression of class II HDACs and the role they may play in SLE pathogenesis.

Class IIa HDACs (4, 5, 7, and 9) are highly expressed in the heart, brain, and smooth muscle [12]. Mice lacking HDAC4 are viable, but have significant defects including chondrocyte hypertrophy and premature ectopic ossification. Knockdown of HDAC5 in mice results in significant heart problems including myocardial hypertrophy and an abnormal cardiac stress response. Germline deletion of HDAC7 in mice has proven to be embryonic lethal due to its role in endothelial cell-cell adhesion. HDAC9 overexpression is believed to contribute to SLE pathogenesis. HDAC9 deficient MRL/lpr mice have decreased levels of autoantibody production, inflammatory cytokine production, and glomerulonephritis coupled with prolonged survival. T cells from both SLE patients and SLE murine models have been demonstrated to overexpress HDAC9 [18]. Furthermore, inhibition of HDAC9 results in increased T_{reg} activation and suppressive function [13]. Due to the adverse effects associated with class IIa HDAC knockout mice, inhibition of these HDACs for the treatment of disease may produce a number of adverse side effects.

Class IIb HDACs (HDAC6 and HDAC10) are found primarily in the cytoplasm. HDAC6 is a class IIb HDAC and has been demonstrated to deacetylate a number of proteins with specificity for α-tubulin and Hsp90 [19, 20]. HDAC6 has increased tissue expression in the

kidneys, liver, heart, and pancreas [12]. HDAC6-deficient mice are viable with no significant defects [12]. Mice lacking HDAC6 and those treated with HDAC6 siRNA have hyperacetylated α-tubulin and Hsp90. Hyperacetylation of Hsp90 has been shown to decrease Hsp90 function [19]. The role of HDAC10 in autoimmune disease remains largely undetermined [12].

Due to the ubiquitous nature of class I HDACs, inhibition not only alters autoimmune associated pathways, but also disrupts normal physiological functions [12]. For this reason, pan-HDAC inhibitors and class I selective HDAC inhibitors tend to be cytotoxic, and therefore the development of isoform-selective HDAC inhibitors for the treatment of chronic diseases including SLE is warranted. The class I and II HDACi, SAHA, has been approved by the FDA for the treatment of cutaneous T cell lymphoma [21]. However, we have previously published that while long-term treatment with SAHA can reduce disease in lupus-prone mice, its long-term use may have deleterious effects [16].

For these studies, we sought to determine the level of mRNA expression in immune-associated tissues from lupus-prone mice of class IIb HDACs along with the class IIa HDAC, HDAC9, due to their suggested role in autoimmune disease. Next, we investigated whether HDAC activity was increased during SLE disease and if treatment with a selective HDAC6i would to decrease HDAC activity *in vitro* and the mechanism(s) through which the HDAC6i exerts it inhibitory effect.

4.4. Materials and Methods

4.4.1 Mice

Female MRL/MpJ-*Fas*^{lpr} (MRL/lpr) and C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in the animal facility at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM, Blacksburg, VA, USA). All mice were used in accordance with the Institutional Animal Care and Use Committee of Virginia Tech.

4.4.2 Proteinuria

Proteinuria was measured by a 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.

4.4.3 Measurement of autoantibodies

Sera were collected prior to euthanization at 12 and 20 weeks-of-age. The mice were anesthetized using isoflurane (Piramal Healthcare, Mumbai, Maharashtra, India) and cardiac puncture was used to collect blood. Blood was allowed to clot for 2 hours and then centrifuged for 15 min at 10,000x*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 Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A final dilution of 1:400 was reported.

4.4.4. Isolation of BM B cells

BM cells were harvested from the tibias and femurs of MRL/lpr mice and age-matched C57BL/6 mice following euthanization. Briefly, BM cells were flushed in PBS with 1%BSA followed by RBC lysis by ammonium chloride potassium (ACK) lysing solution. B cells were isolated using the Dynal Mouse B Cell Negative Isolation Kit according to the manufacturer's protocol (Invitrogen, Life Technologies, Grand Island, NY, USA). Cells were resuspended in RNA*later* (Quiagen, Valencia, CA, USA) and stored at -20°C until RNA isolation or used for cytoplasmic and nuclear extractions.

4.4.5. Splenocyte isolation

A single-cell suspension was obtained from the spleens of MRL/lpr mice and age-matches C57BL/6 mice following euthanization. Briefly, the spleen was removed from each mouse and dissociated across a sterile wire mesh in a petri dish containing ice-cold PBS with 1% BSA. RBCs were lysed using RBC lysis buffer and cells were pelleted and washed with PBS. B cells were isolated using the Dynal Mouse B Cell Negative Isolation Kit according to the manufacturer's protocol (Invitrogen, Life Technologies). T cells were isolated using the T_{reg} isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA). Cells were resuspended in RNA*later* (QIAGEN) and stored at -20°C until RNA isolation or used for cytoplasmic and nuclear extractions.

4.4.6. Isolation of glomerular cells

Following euthanization, the glomeruli were removed from MRL/lpr mice and were pooled for mesangial cell isolation. This procedure was repeated three separate times for each group. Briefly, the cortical tissue was isolated from one kidney of each mouse and pooled by

group. Next, cortical tissue was pressed through grading sieves (180, 150, and 75 µm mesh) and resuspended in 750 U/mL Worthington type I collagenase at 37°C for 20 min. Glomerular cells were pelleted, resuspended in RNA*later* (QIAGEN), and stored at -20°C until RNA isolation or used for cytoplasmic and nuclear extractions.

4.4.7. 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 diluted to 1 ng/µL for real-time RT-PCR. The eluted RNA was stored at -80°C.

4.4.8. Real-time RT-PCR

HDAC6, *HDAC9*, and *HDAC10* mRNA expression were measured using TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA, USA). The ΔC_T was calculated using the endogenous control GAPDH, and then the $\Delta\Delta C_T$ was determined by calculating the fold change in expression between MRL/lpr mice and age-matched control mice. All samples were run in triplicate.

4.4.9. Nuclear and cytoplasmic extraction

Isolated BM B cells, splenic B and T cells, and glomerular cells were lysed and separate cytoplasmic and nuclear protein fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's protocol.

4.4.10. Cell Culture

A mouse mesangial cell line (MES 13) transgenic for SV40 was cultured in 75-mm^2 culture flasks at 37°C in 5% CO_2 in DMEM and Ham's F12 medium with 14 mM HEPES (3:1), supplemented with 10% FBS and 1% streptomycin-penicillin solution (Cellgro, Manassas, VA, USA). LPS (1 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and IFN-γ (100 ng/mL) (Cedarlane Laboratories Limited, Burlington, NC, USA) were used to induce immune stimulation in mesangial cells. MES 13 cells were treated with varying concentrations of ACY-738 (1, 2, 5, 10 or 100 nM) in order to determine the level of acetylated H3 or α-tubulin for 24 hours. In order to determine the level of Hsp90 acetylation and iNOS protein, MES 13 cells were treated with varying concentrations of ACY-738 (0.5, 1, or 5 nM) for 2 hours followed by 24 hours of stimulation with LPS/IFN-γ. MES 13 cells were treated with ACY-738 (5 nM) for 2 hours and stimulated with LPS/IFN-γ for 15, 30, or 60 mins for nuclear extraction in order to determine the effect of HDAC6 inhibition on NFκB.

4.4.11 Immunoprecipitation

To determine the level of HDAC6 activity in BM, splenic, and glomerular cells from MRL/lpr and C57BL/6 mice, HDAC6 protein was immunoprecipitated as previously published [22]. The immunoprecipitated protein was then subjected to the HDAC activity assay. To determine the effect of HDAC6 inhibition on Hsp90 acetylation in mesangial cells, Hsp90 protein was immunoprecipitated and then subjected to Western blot analysis for acetylated lysines (Cell Signaling).

4.4.12. Western blot analysis

In order to determine the specificity of ACY-738 for HDAC6, α-tubulin and histone H3 acetylation was determined using Western blot analysis. Briefly, cells were lysed and the Bradford protein assay was used to normalize protein levels. The cell pellet was resuspended 1:1 in cell lysis buffer and Laemmli buffer. The samples were heated to 95°C for 5 minutes and then loaded onto a 15% SDS-PAGE gel. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and incubated with antibodies against acetylated lysines, acetylated α-tubulin, acetylated histone H3, histone H4, iNOS, NF-κB, or β-actin (Cell Signaling, Boston, MA, USA). All experiments were run in triplicate.

4.4.13. HDAC activity assay

The HDAC activity colorimetric assay kit (Bio Vision, USA) was used according to the manufacturer's protocol to determine the level of HDAC activity in freshly isolated B, T, and glomerular cells. Briefly, nuclear and cytoplasmic cell lysates were incubated with the HDAC colorimetric substrate followed by treatment with the lysine developer. The plate was read at 405 nm on a Spectramax 340PC microplate spectrophotometer.

4.4.14 Pre-B colony formation assay

A single-cell suspension of BM cells was obtained from 8-week-old MRL/lpr mice as described earlier. Isolated BM cells were mixed with Methocult M3630 medium (StemCell Technologies, Vancouver, Canada) in a 1:10 (v/v) ratio according to the manufacturer's protocol. Cells were plated in triplicate in pre-tested culture dishes and incubated in a humidified incubator

at 37°C and 5% CO_2. Colonies were counted and then collected for Western blot analysis after 8 days of culture.

4.4.15. Statistical analysis

Statistical analysis was performed using Student's unpaired *t*-test (two-tailed). When three or more independent groups were compared a one-way ANOVA was used followed by Tukey's post-test. *P* values less than 0.05 were considered statistically significant.

4.5. Results

4.5.1 MRL/lpr mice had significant disease activity compared to control mice.

Female MRL/lpr mice develop an autoimmune inflammatory disease that reflects lupus pathologies including proteinuria, autoantibody production, and glomerulonephritis by 18 weeks-of-age [23]. MRL/lpr mice and age-matched controls were evaluated for SLE markers of disease including splenomegaly, proteinuria, autoantibody production, and glomerulonephritis. Spleen weight and spleen:body weight were significantly higher in the 20-week-old MRL/lpr mice compared to age-matched controls and the 12-week-old MRL/lpr mice (Figure 1 A – B). When compared to age-matched control mice, the 20-week-old MRL/lpr mice had significantly higher proteinuria (Figure 1 C). The sera were analyzed for autoantibody production using an ELISA for anti-dsDNA. We found that the 12 and 20-week-old MRL/lpr mice had significantly higher sera anti-dsDNA production compared to age-matched C57BL/6 mice (Figure 1 D – E).

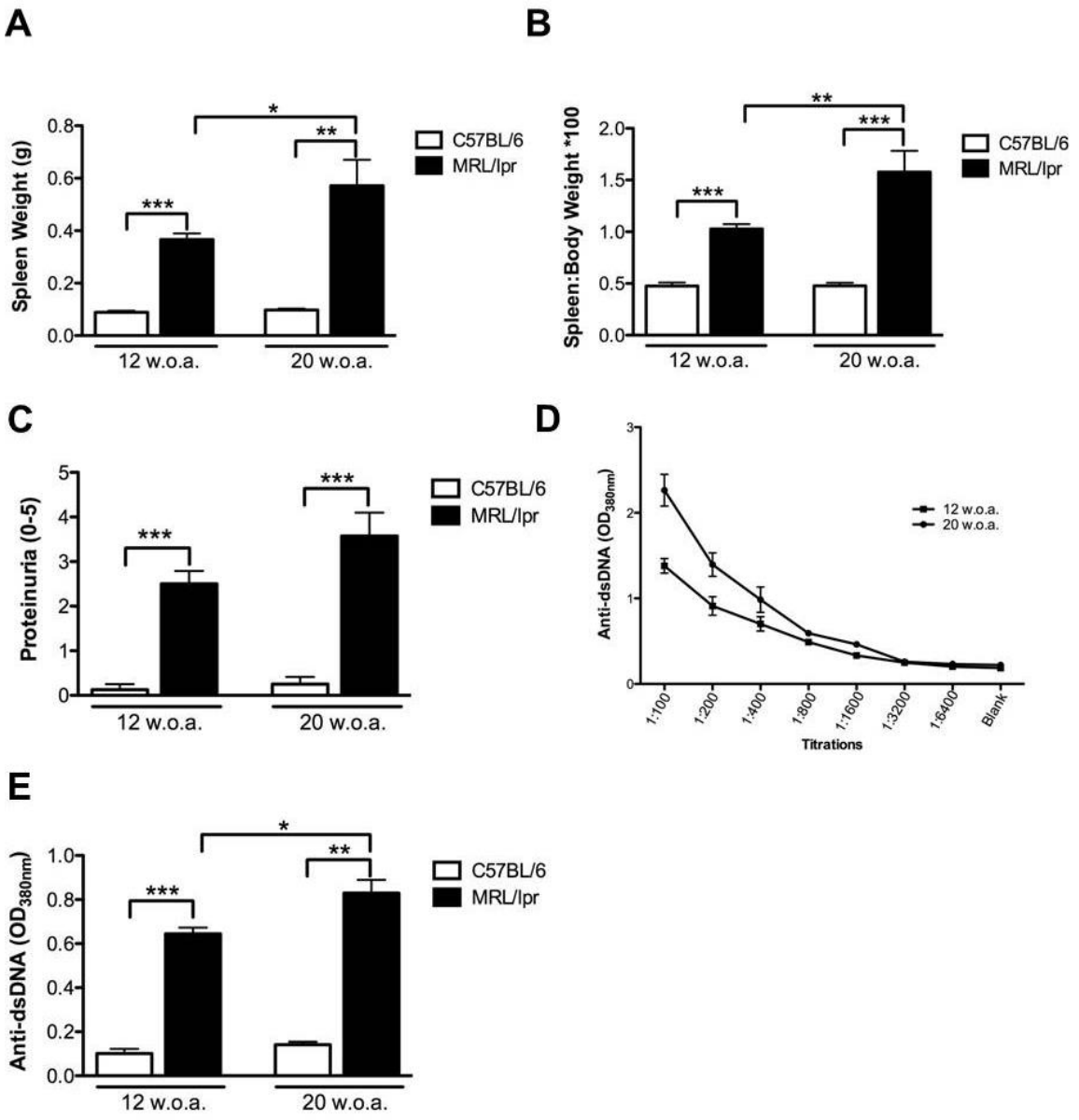


Figure 1 Identification of disease progression in MRL/lpr mice. Hallmarks of SLE disease were evaluated at 12 or 20 weeks-of-age prior to euthanization in MRL/lpr mice and age-matched control mice. (A) Proteinuria was significantly higher in the lupus prone-mice at 20 weeks-of-age compared to control mice. (B) Following euthanization average spleen weight was determined by group. (C) Spleen:body weight ratio was calculated and multiplied by 100. MRL/lpr mice had decreased spleen:body weight ratio. (D-E) Anti-dsDNA was significantly higher in MRL/lpr mice at 18 weeks-of-age ($n \geq 6$; *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$).

4.5.2 ACY-738 selectively increases α-tubulin acetylation levels *in vitro*.

Following HDAC6 inhibition, α-tubulin becomes acetylated [24]. Histone H3 becomes hyperacetylated following inhibition of class I HDACs [25]. In order to determine the concentration at which ACY-738 selectively inhibits HDAC6 without inhibiting class I HDACs, Western blot analysis was performed to determine α-tubulin and histone H3 acetylation levels. Mesangial cells were cultured and treated with increasing concentrations of ACY-738 for 24 hrs. We found that ACY-738 at 5 nM was able to significantly increase the level of α-tubulin acetylation while having little to no effect on acetylation on histone H3 (Figure 2).

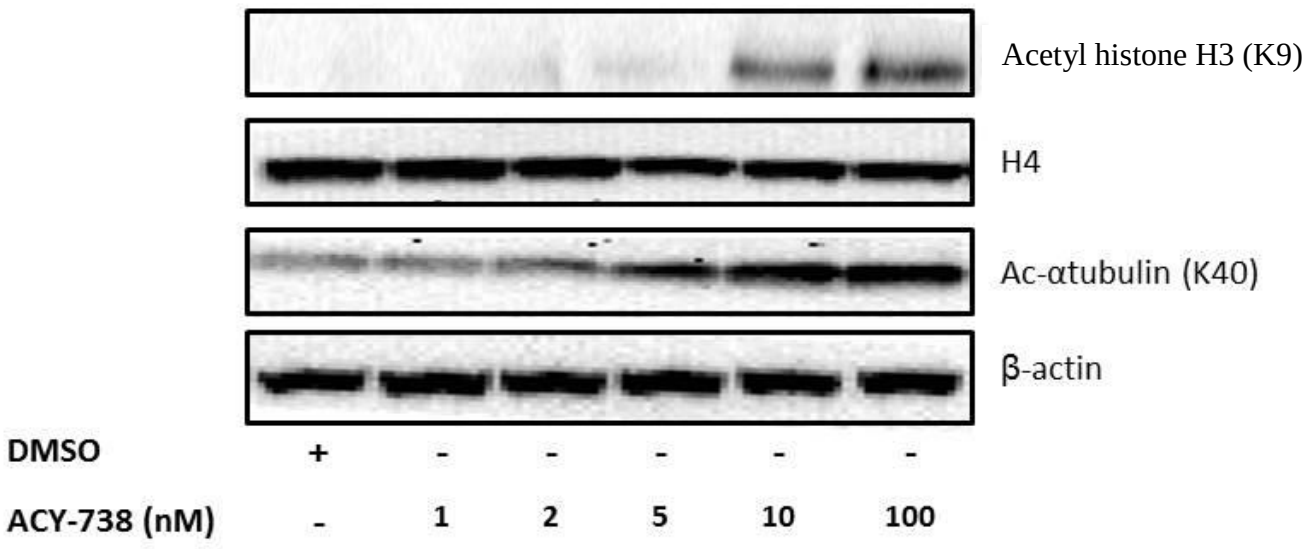


Figure 2 ACY-738 treatment increases α-tubulin acetylation. Treatment of mesangial cells with a selective HDAC6i increased α-tubulin acetylation in a concentration dependent manner. At the 5 nM concentration, ACY-738 treatment for 24 hrs was able to increase α-tubulin acetylation without increasing the acetylation of the H3 histone. All experiments were run in triplicate.

4.5.3. HDAC expression is altered in diseased MRL/lpr mice.

Class IIb HDACs may serve as potential therapeutic targets for the treatment of SLE, due to their regulation of Hsp90 [12, 15]. Increased Hsp90 levels during SLE have been associated with an increase in IL-6, B cell activation and autoantibody production [26]. HDAC9, a class IIa HDAC, may also play an important role in SLE pathogenesis due to its regulation of T_{reg} cells and Hsp90 acetylation [20, 27, 28]. Relative mRNA expression levels of the class IIb HDACs *6, 9,* and *10* were determined using real time RT-PCR (Figure 3). B cells isolated from the BM of 12- and 20-week-old MRL/lpr mice had significantly higher mRNA expression of HDACs *6* and *9* when compared to age-matched C57BL/6 mice. However, there were no significant differences in the expression of *HDAC10* between lupus-prone mice and control mice (Figure 3 A). Expression of HDACs *6, 9,* and *10* were increased in splenic B cells from diseased MRL/lpr mice; however, there were no significant differences between HDAC expression levels between MRL/lpr mice in an early or late disease state (Figure 3 B).

Next, we examined the level of HDAC expression in splenic T cells as naïve $CD4^+$ T cell differentiation has been shown to be dysregulated during SLE [29]. SLE patients and murine models have an imbalance between the Th1 and Th2 phenotypes as well as an increase in the number of Th17 cells and a decrease in T_{reg} cell function [7, 30, 31]. In T cells isolated from the spleens of MRL/lpr mice, expression of HDACs *6* and *9* were all significantly increased compared to age-matched control mice. Furthermore, the greatest difference in mRNA expression was determined to be with *HDAC9* (Figure 3 C).

The glomerulus is a capillary tuft at the beginning of the nephron that functions to filter blood. Proper glomerular filtration is maintained by the glomerular basement membrane through the interaction of podocytes, endothelial cells, and mesangial cells. During SLE circulating ICs

become lodged in the glomeruli resulting in inflammation and if unresolved can result in renal failure. Therefore, we also examined HDAC expression in glomerular cells. We found that glomerular cells from MRL/lpr mice had significantly higher *HDAC6* and *HDAC9*; however, there was no significant difference in *HDAC10* expression compared to age-matched control mice (Figure 3 D).

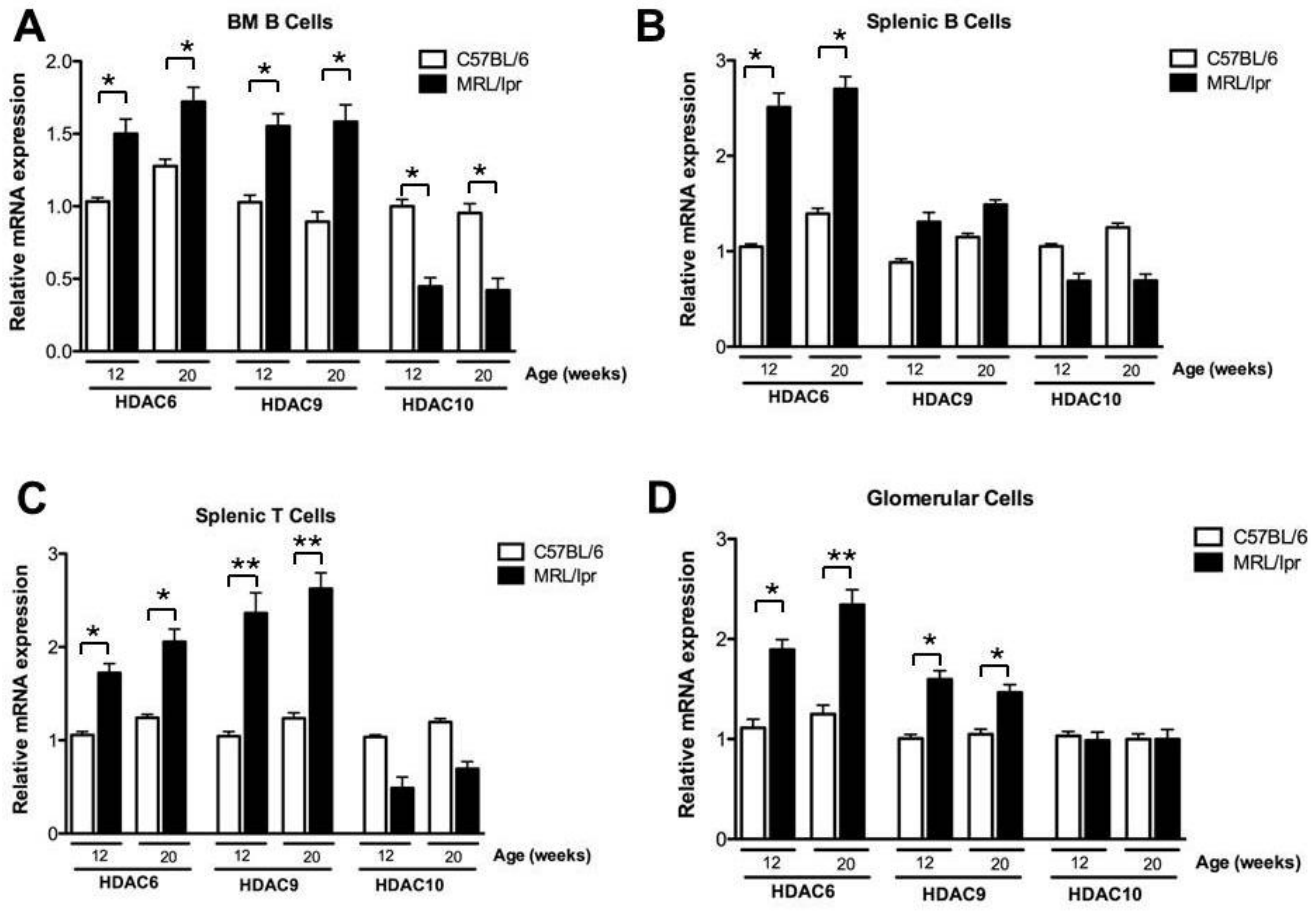


Figure 3 HDAC6, 9, and 10 expression in immune cells from 12- and 20-week-old lupus and non-lupus mice. Real-time RT-PCR was used to determine expression of HDACs 6, 9, and 10 in B cells, T cells, and glomerular cells from lupus and control mice. (A) BM B cells from MRL/lpr mice had significantly higher expression of HDAC6 and HDAC9 mRNA compare to age-matched, healthy C57BL/6 mice; however, there were no significant differences in the

expression of HDAC10. (B) HDAC6 and HDAC10 were significantly higher expressed in B cells isolated from the spleens of MRL/lpr mice. (C-D) Similarly to the BM B cells, splenic T cells and glomerular from the lupus-prone mice had increased expression of HDACs 6 and 9, but no significant differences in the expression of HDAC10 were noted ($n \geq 6$; *$p < 0.05$, ** $p < 0.01$).

4.5.4. Nuclear and cytoplasmic HDAC activity is increased in diseased lupus-prone mice.

Specific HDAC isoforms can be found in the nucleus alone, the cytoplasm alone, or in both the nucleus and the cytoplasm. The class IIb HDACs (HDAC6 and HDAC10) are found only in the cytoplasm, whereas HDAC9 shuttles between the nucleus and the cytoplasm based on HDAC activity. We sought to determine whether or not there was an increase in HDAC activity in immune-associated tissues as well as whether activity was primarily cytoplasmic or nuclear. A HDAC activity assay was used in order to determine the level of HDAC activity in B, T, and glomerular cells. We found that HDAC activity was significantly greater in BM B cells, splenic B and T cells, and glomerular cells isolated from MRL/lpr mice compared to age-matched C57BL/6 mice (Figure 4). We found no significant difference in the cytosolic levels of HDAC activity, while the nuclear fraction showed significantly elevated HDAC activity in both early and late diseased mice (Figure 4 A). B cells from the spleen of both early- and late-diseased MRL/lpr mice had significantly increased levels of both cytoplasmic and nuclear HDAC activity when compared to age-matched controls, with nuclear HDAC activity showing the greatest differences (Figure 4B).

Targeting of different HDAC isoforms, including HDAC6 and HDAC9, has been demonstrated to increase T_{reg} cell function through a number of different mechanisms [13, 27] .

We examined the level of HDAC activity in T_{reg} cells and $CD4^+CD25^-$ T cells from diseased lupus mice in order to determine association between SLE disease and T_{reg} HDAC activity. ($CD4^+CD25^+$) cells and $CD4^+CD25^-$ T cells were isolated from the spleens of MRL/lpr and C57BL/6 mice. Our study showed that T_{reg} cells had more HDAC activity than $CD4^+CD25^-$ T cells regardless of mouse strain or age. However, the T_{reg} cell HDAC activity was significantly greater in the MRL/lpr mice compared to age-matched healthy control mice. Similarly to B cell HDAC activity, T_{reg} cell cells had greater nuclear HDAC activity; however, we also found an increase in the cytoplasmic T_{reg} HDAC activity (Figure 4 C). Furthermore, significantly elevated HDAC activity was found in glomerular cells from MRL/lpr mice in cytoplasmic and nuclear fractions of early- and late-disease animals compared to age-matched controls (Figure D).

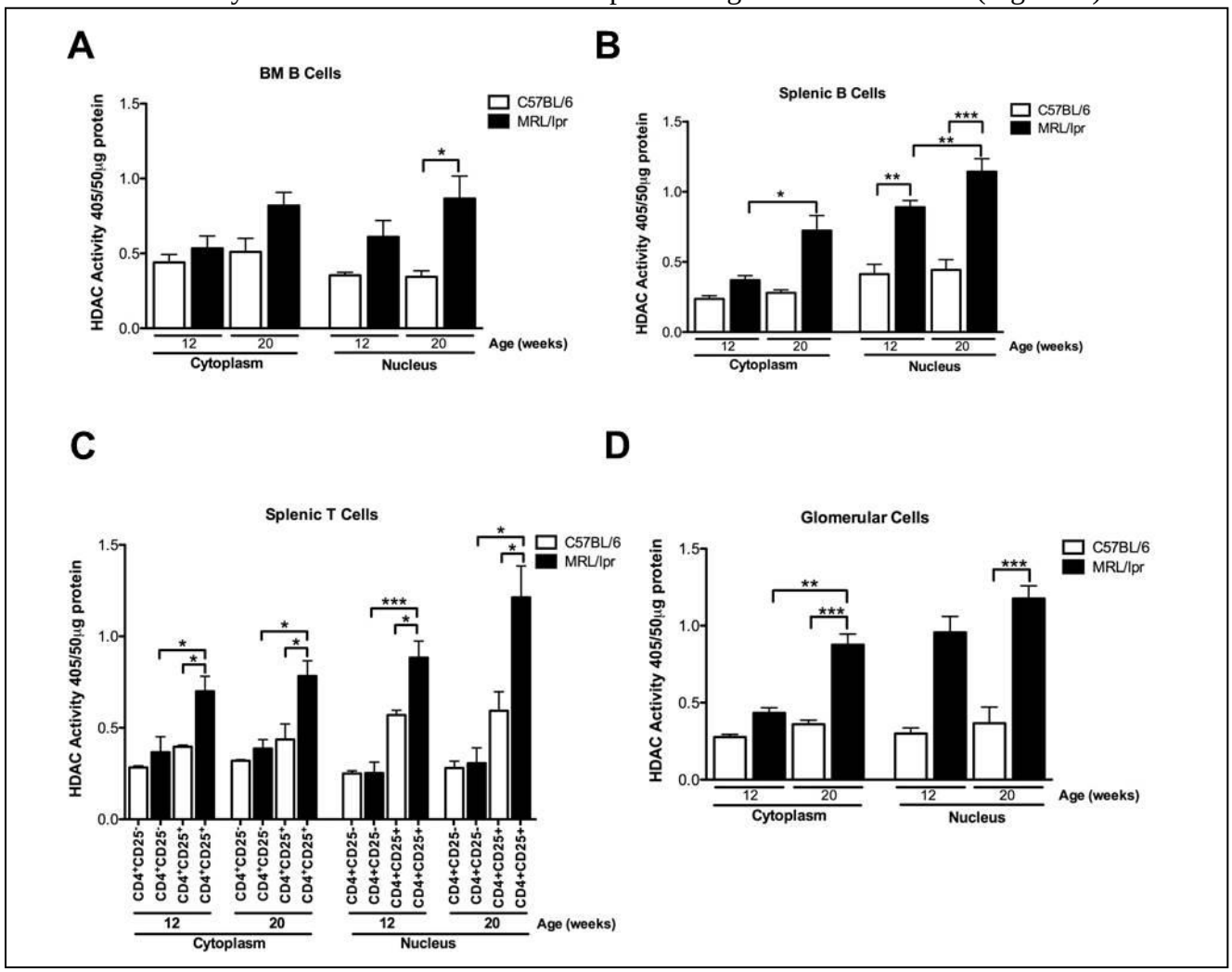


Figure 4 HDAC activity is increased in B, T, and glomerular cells in 12- and 20-week-old lupus mice compared to non-lupus controls. An HDAC activity assay was used to assess HDAC activity in B cells, T cells, and glomerular cells from lupus and control mice. (A) In B cells isolated from the BM of 20-week-old MRL/lpr mice, there was a significant increase in nuclear cytoplasmic activity when compared to C57BL/6 mice; however, there was no significant difference in the level of HDAC activity at 12 weeks-of-age. BM B cells from MRL/lpr mice did not have significantly more cytoplasmic HDAC activity. (B) The level of both cytoplasmic and nuclear HDAC activity was significantly increased in splenic B cells from lupus-prone mice when compared to healthy, age-matched controls. (C) T_{reg} cells isolated from the spleens of MRL/lpr mice had significantly higher nuclear and cytoplasmic HDAC activity compared to age-matched controls. T_{reg} HDAC activity did not continue to further increase ad disease progressed in MRL/lpr mice. (D) Both cytoplasmic and nuclear HDAC activity was increased in glomerular cells from SLE mice. As the mice aged from 12 to 20 weeks-of-age and diseased progressed, HDAC activity continued to increase in glomerular cells from MRL/lpr mice ($n \geq 6$; *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.5.5. HDAC6 activity is increased in diseased MRL/lpr mice.

Due to our results showing that HDAC6 expression was elevated in BM B cells, splenic T cells, and glomerular cells from MRL/lpr mice, we sought to determine whether there was a specific increase in HDAC6 activity in these mice. In order to determine the level of HDAC6 activity, HDAC6 protein was immunoprecipitated from isolated B, T, and glomerular cells and nuclear and cytoplasmic fractions and subjected to the HDAC activity assay. We found that HDAC6 activity was primarily cytoplasmic; however, some HDAC6 nuclear activity was present

in isolated B, T, and glomerular cells (Figure 5). HDAC6 activity was significantly higher in MRL/lpr mice compared to C57BL/6 mice. Furthermore, B, T, and glomerular cells isolated from 20-week-old MRL/lpr mice had significantly higher HDAC6 activity than 12-week-old MRL/lpr mice indicating an association between HDAC6 activity and disease progression (Figure 5).

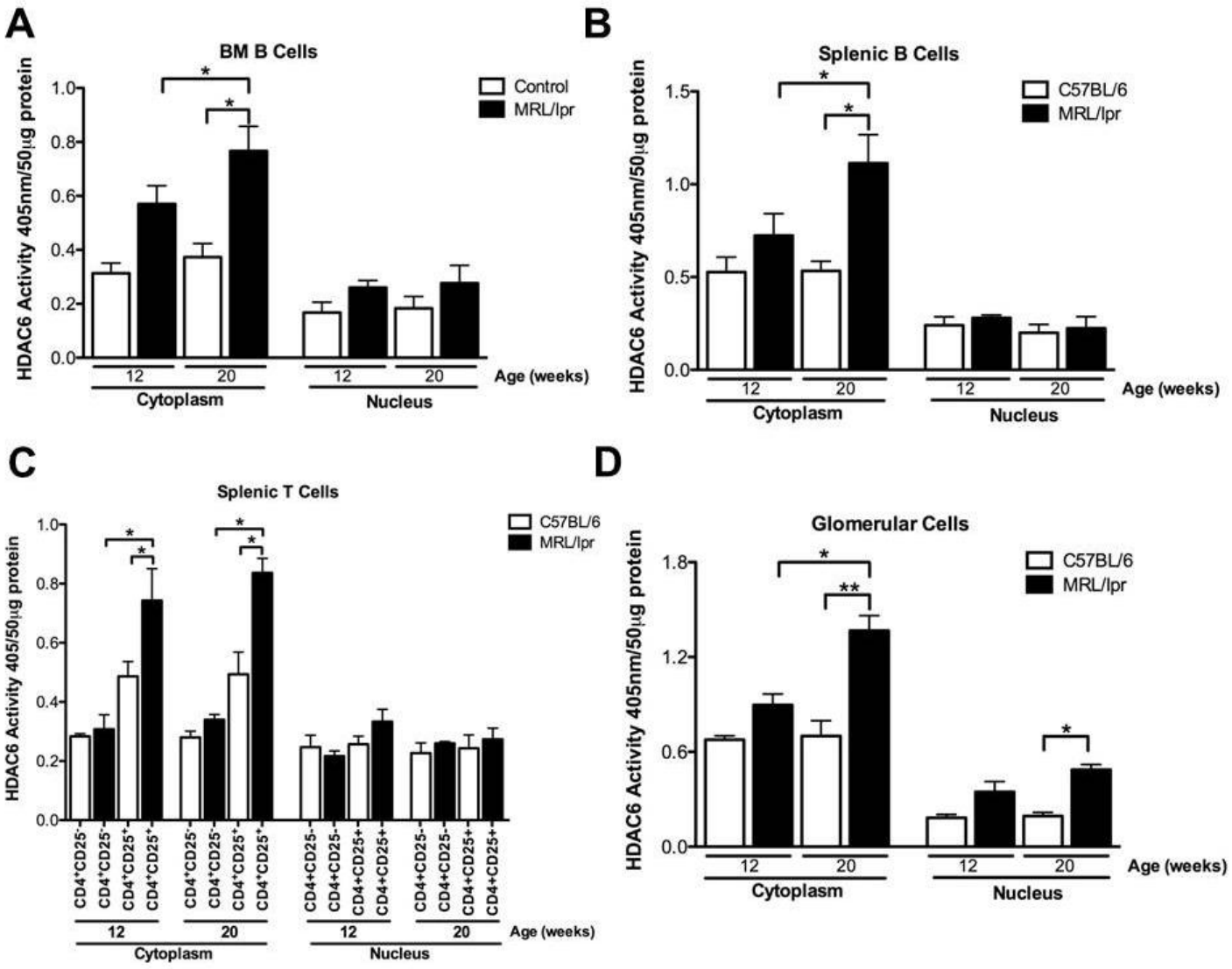


Figure 5 HDAC6 activity is increased in splenic B cells and glomerular cells in 12- and 20-week-olf MRL/lpr mice compared to C57BL/6 controls. HDAC6 was immunoprecipitated

from isolated B, T, and glomerular cells and subjected to an HDAC activity assay. (A) There were no significant differences in HDAC6 activity in B cells isolated from the BM of MRL/lpr and C57BL/6 mice. (B) Splenic B cells had significantly higher cytoplasmic HDAC6 activity from lupus-prone mice at 20 weeks-of-age. There were no significant differences in the cytoplasmic HDAC6 activity at 12 weeks-of-age or in the nuclear HDAC activity. (C) T_{reg} cells (D) Cytoplasmic HDAC6 activity was increased in glomerular cells isolated from MRL/lpr mice regardless of age. HDAC6 activity continued to increase in glomerular cells as disease progressed in the lupus-prone mice ($n \geq 6$; *$p < 0.05$, ** $p < 0.01$).

4.5.6 HDAC6 inhibition primarily decreases cytoplasmic HDAC activity in B, T, and glomerular cells.

In order to assess the ability of a selective HDAC6i to inhibit nuclear and cytoplasmic HDAC activity; isolated BM B cells, splenic B and T cells, and glomerular cells were treated with a class I and II HDACi, Trichostatin A(TsA), or an HDAC6i, ACY-738. A concentration of 5 nM was selected for use in these experiments, due to our previous study indicating that at 5 nM, ACY-738 is able to increase α-tubulin acetylation without increasing acetylation of histone H3 indicating selectivity for HDAC6. We selected a 1 μM concentration of TsA for use in these studies based off of previously published work indicating that TsA (1 μM) was able to inhibit class I and II HDACs at this concentration [32]. Not surprisingly, we found that ACY-738 (5 nM) had a greater inhibitory effect on the cytoplasmic fractions from isolated B, T, and glomerular cells. A 1 μM concentration of TsA was selected due to previous studies indicating its ability to decrease class I and II HDACs and decrease HDAC activity at this concentration [33, 34]. TsA (1 μM) was able to inhibit HDAC activity in both cytoplasmic and nuclear

fractions regardless of mouse strain or age. The ability of ACY-738 to inhibit HDAC activity was not dependent upon the age or strain of mouse or on the cell type (Figure 6).

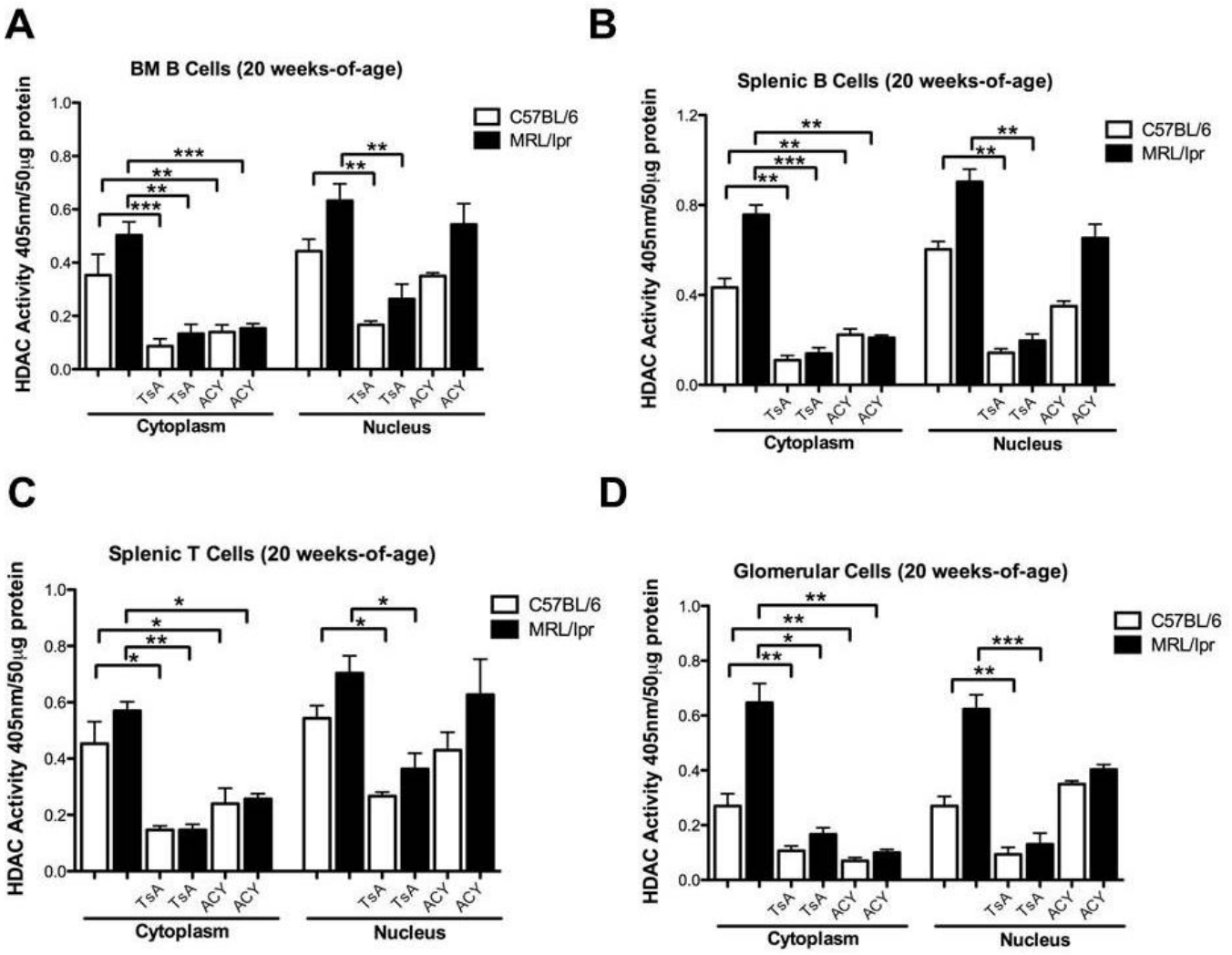


Figure 6 Cytoplasmic HDAC activity is decreased with selective HDAC6 inhibition while cytoplasmic and nuclear activity is decreased following pan-HDACi therapy. Isolated cells were treated with class I and II HDACi, TsA, or the HDAC6 selective inhibitor, ACY-738. (A-D) ACY-738 (5 nM) was able to decrease cytoplasmic HDAC activity in BM B cells, splenic B and T cells, and glomerular cells. Treatment with TsA (1 μM) was able to significantly decrease both nuclear and cytoplasmic HDAC activity in both MRL/lpr and C57BL/6 mice ($n \geq 6$; *$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

4.5.7 *In vitro* inhibition of HDAC6 in mesangial cells results in inhibition of Hsp90 and a decrease in NFκB nuclear translocation.

Hyperacetylation of Hsp90 results in a decrease in Hsp90 function by limiting its chaperone activity. HSp90 activity is required for the proper folding of a number of client proteins, including IKK, a key component of the IκB signaling pathway and NF-κB nuclear translocation. Lack of Hsp90 activity results in misfolded IKK and its subsequent degradation, thereby, preventing NF-κB nuclear translocation [35, 36]. Sera levels of Hsp90 have been proven to be elevated in SLE patients and approximately 50% of patients generate anti-Hsp90 autoantibodies. Some SLE patients have glomerular deposits of Hsp90 and elevated Hsp90 levels have been correlated to IL-6 production and severity of glomerulonephritis [26]. Mesangial cells are the primary resident immunoregulatory cells found in the glomerulus and act to maintain proper glomerular filtration [37]. Our study showed that treatment of mesangial cells with ACY-738 resulted in an increase in Hsp90 acetylation (Figure 7A). HDAC6 inhibition also resulted in a decrease in iNOS protein (Figure 7B), which previous studies have shown is upregulated in the kidneys from SLE patients and mice with active disease [38, 39].

During SLE, NFκB regulates both B and T cell development as well as T cell activation. NFκB controls the immune response through regulation of inflammatory cytokine gene expression. During SLE, increased nuclear translocation of NFκB leads to increased iNOS and IL-6 production and a pro-inflammatory environment in the mesangium [40]. Our results showed that nuclear levels increased in a time-dependent manner from 0 - 60 mins; however, treatment with ACY-738 reduced nuclear levels of NFκB in cultured mesangial cells (Figure 7 C).

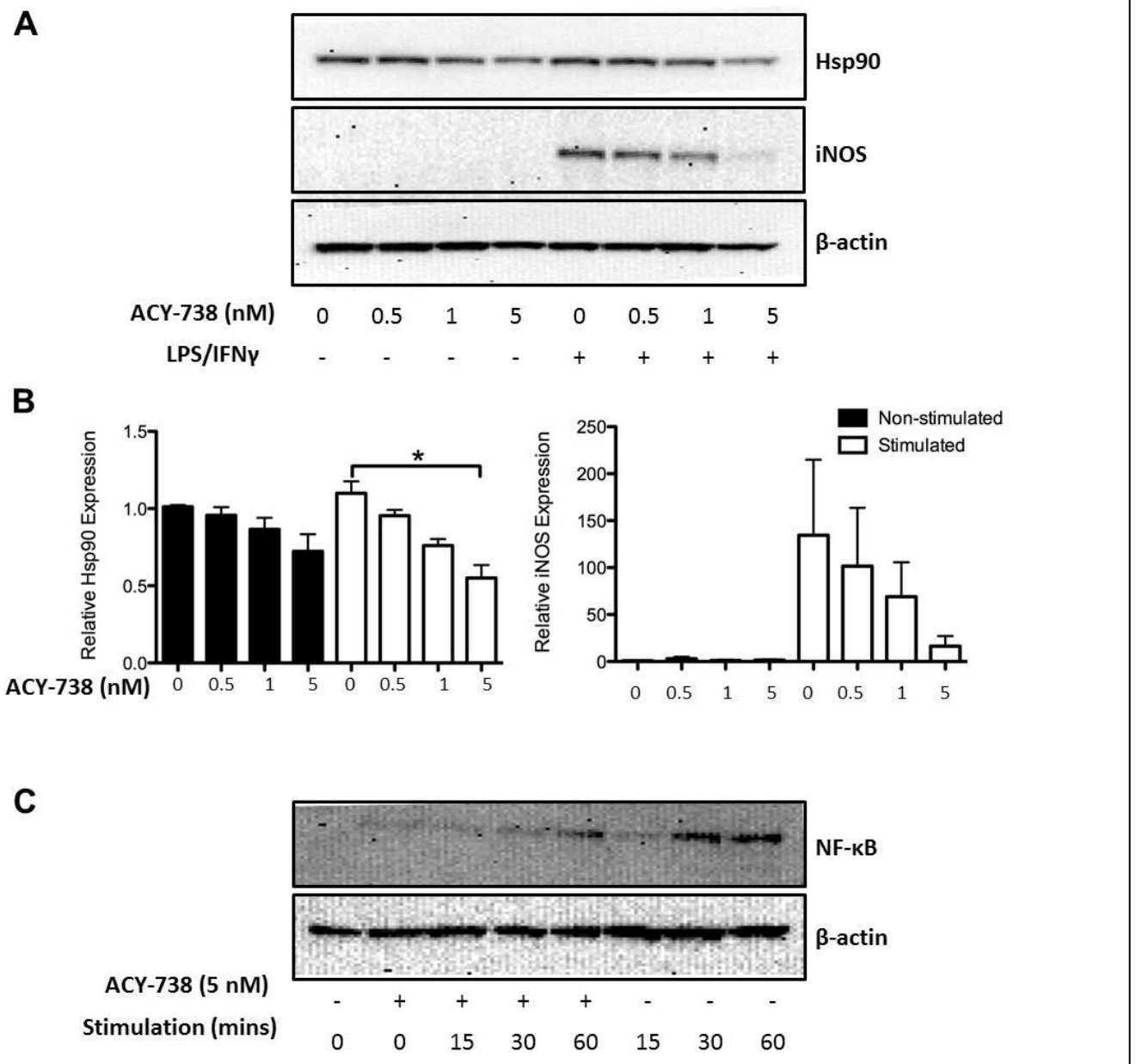


Figure 7 HDAC6 inhibition alters mesangial cell protein expression *in vitro.* (A-B)Western blot analysis showing a decrease in iNOS and Hsp90 protein levels in mesangial cells stimulated with LPS/IFN-γ for 24 hours following treatment with ACY-738. (C) ACY-738 (5 nM) treatment was also able to decrease the nuclear levels for NF-κB following LPS/IFN-γ-stimulation of mesangial cells in a time dependent manner Nuclear levels of NF-κB. All experiments were run in triplicate ($n \geq 3$; $^*p < 0.05$).

4.5.8. *In vitro* inhibition of HDAC6 in BM pre-B cells results in a pro-apoptotic environment.

During lymphopoiesis, B cells in the BM pass through two main stages: pro-B and pre-B. Previous research has shown that B cell development during the pre-B phase is dysregulated in lupus-prone mice [41]. The pre-B cell dysregulation correlated to SLE disease activity and was corrected following treatment with the selective HDAC6i, ACY-738. Pre-B cells were cultured using the Methocult assay and CFUs were counted. Treatment of pre-B cells with ACY-738 reduced the average number of CFUs formed after 7 days of culture (Figure 8A). Cells were collected and subjected to Western blot analysis for the apoptosis associated Bax and Bcl-2 proteins. Treatment of ACY-738 increased the level of Bax, without altering Bcl-2 expression (Figure 8B). Bax expression is known to promote apoptosis, while Bcl-2 is a known inhibitor of apoptosis.

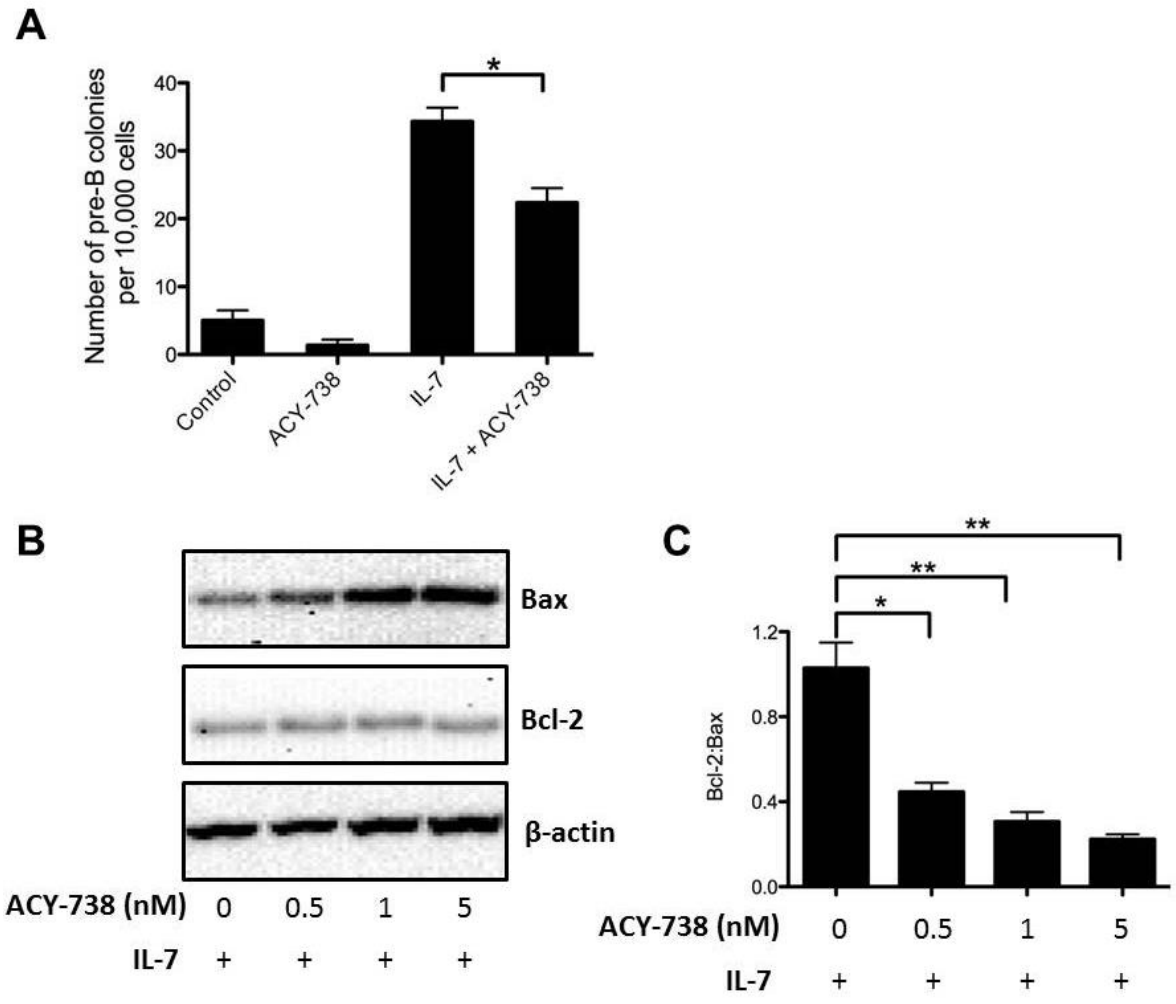


Figure 8 Apoptotic markers are increased in pre-B cells following treatment with ACY-738. Pre-B cells were cultured using a Methocult assay in the presence or absence of IL-7 and ACY-738. (A) ACY-738 inhibited the growth of pre-B cells *in vitro* when treated with IL-7. (B) Cultured pre-B cells subjected to Western blot analysis were found to have increased Bax protein. (C) The Bcl-2:Bax ratio was determined using densitometric analysis. Treatment with ACY-738 decreased the Bcl-2:Bax ratio in cultured pre-B cells. All experiments were run in triplicate ($n \geq 6$; *$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

4.6. Discussion

We found that expression of class II HDACs (6 and 9) as well as HDAC activity was upregulated in diseased MRL/lpr mice compared to healthy control mice. Conversely, we found that HDAC10 had a significant decrease in expression in BM B cells from MRL/lpr mice, but no significant differences in splenic or glomerular cells when compared to non-lupus controls. As disease progresses in MRL/lpr mice, expression of HDACs (*6* and *9*) and HDAC activity continued to increase in the immunoregulatory cells examined. However, there was no significant difference in the level of *HDAC10* mRNA expression between lupus and non-lupus mice except in BM B cells where it was significantly decreased. Furthermore, both nuclear and cytoplasmic HDAC activity was increased in BM B cells, splenic B and T cells, and glomerular cells. HDAC6 activity was found to be primarily cytoplasmic and most significantly increased in 20-week-old MRL/lpr mice. Treatment with the specific HDAC6i, ACY-738 (5 nM), was found to primarily decrease cytoplasmic HDAC activity, whereas treatment with TsA (1μM) decreased both nuclear and cytoplasmic HDAC activity. Inhibition of HDAC6 in mesangial cells led to a decrease in Hsp90 as well as a decrease in nuclear levels of NFκB. ACY-738 was able to inhibit pre-B cell proliferation through increased Bax expression *in vitro.* Taken together these results suggest that HDAC6 inhibition is able to decrease cytoplasmic HDAC activity through regulation of NFκB and Hsp90 in glomerular cells and through regulation of the Bcl-2:Bax ratio in pre-B cells from the BM.

To date there have been relatively few studies on the role of HDAC10 and its expression during disease. HDAC10 is a class IIb HDAC that resides in both the nucleus and the cytoplasm [42]. The role of HDAC10 in the cytoplasm remains to be determined; however, it has been shown to be a transcriptional modulator in the nucleus [43]. We found that expression of

HDAC10 was not significantly different splenic B and T cells and glomerular cells from MRL/lpr mice compared to age-matched controls. Interestingly, we found that *HDAC10* expression was downregulated in BM B cells, which warrants further investigation. However, *HDAC10* expression was not altered with increased disease activity. These data suggest that HDAC10 may not be a major factor in SLE disease.

We have previously published that HDAC9 deficient MRL/lpr mice have decreased disease activity suggesting a role for HDAC9 in SLE pathogenesis [18]. HDAC9 has been implicated in the regulation and the suppressive function of T_{reg} cells [27]. Our studies showed that *HDAC9* was most significantly overexpressed in splenic T cells. Due to the aberrant T cell development associated with SLE, we examined HDAC activity in splenic T cells. Specifically, we compared the HDAC activity of $CD4^+CD25^-$ T cells and T_{reg} cells, which have decreased immunosuppressive function in SLE patients and murine models of disease [44]. We found T_{reg} cells had increased HDAC activity regardless of the strain or age of the mouse when compared to $CD4^+CD25^-$ T cells. HDAC9 is thought to inhibit Foxp3 function and that the nuclear export of HDAC9 is required for optimal T_{reg} suppressive function [14]. Once bound to Foxp3, HDAC9 decreases acetylation levels of Foxp3 resulting in increased polyubiquitination, proteasomal degradation, and therefore decreased Foxp3 stability and function [45]. Overexpression of *HDAC9* by splenic T cells may contribute to the decrease in T_{reg} cell number and function associated with MRL/lpr mice [46]. HDAC activity in T_{reg} cells from lupus-prone mice was found to be both nuclear and cytoplasmic, suggesting a role for HDACs that are both nuclear and cytoplasmic in nature in the regulation of T_{reg} cells.

HDAC6 is a class IIb HDAC with a structure to HDAC9; however, HDAC6 primarily resides in the cytoplasm. Substrates of HDAC6 include α-tubulin and Hsp90, which become

deacetylated when HDAC6 catalyzes the removal of acetyl groups [26, 36]. We investigated the level of *HDAC6* expression in immune cells isolated from early- and late-disease lupus-prone mice due to the role of HDAC6 in cell development and regulation of gene transcription. Studies with HDAC6 KO mice have shown a role for HDAC6 in T cell survival and activation specifically as a negative regulator of T_{regs} [15]. We found that *HDAC6* was overexpressed in BM B cells, splenic B and T cells, and glomerular cells from diseased MRL/lpr mice. Furthermore, when HDAC6 was isolated from these immune cells, we found that HDAC6 activity was increased in both B and T cells from diseased lupus mice. These results suggest that HDAC6 may play a role in the dysregulation of both B and T cell development during SLE.

Since HDAC6 was overexpressed in all of the immune cells isolated from MRL/lpr mice, we tested whether a specific HDAC6i would be able to decrease HDAC activity *in vitro.* At a 5 nM concentration, ACY-738 significantly increased α-tubulin acetylation without significantly altering acetylation of histone H3. Furthermore, B, T, and glomerular cells treated with ACY-738 had significantly decreased cytoplasmic HDAC activity while having no significant effect on nuclear HDAC activity. These results suggest that ACY-738 is selective for HDAC6 over class I HDACs at a 5 nM concentration.

Glomerulonephritis is the leading cause of morbidity and mortality in SLE patients [47]. We found that HDAC6 was overexpressed in glomerular cells from diseased MRL/lpr mice. Furthermore, isolated glomerular cells from MRL/lpr mice had a cytoplasmic increase in HDAC activity. Since HDAC6 is found in the cytoplasm, these results suggest that HDAC6 overexpression may contribute to SLE renal pathology. *In vitro* immune stimulation of mesangial cells by LPS/IFN-γ led to increased activation of NF-κB in a time-dependent manner. HDAC6 inhibition by ACY-738 (5 nM) was found to decrease nuclear translocation of NFκB in

immune-stimulated mesangial cells. When NF-κB is activated, it is translocated into the nucleus allowing for gene regulation [48]. We also found that HDAC6 inhibition decreased Hsp90 in a concentration-dependent manner in cultured mesangial cells. Decreased Hsp90 production results in inhibition of Hsp90 chaperone function and proteosomal degradation of Hsp90 client proteins [49]. Furthermore, Hsp90 plays a role in activation of lymphocytes and antigen presentation and has been demonstrated to be elevated in the serum from SLE patients. During SLE, elevated Hsp90 levels have been correlated to IL-6 and autoantibody production suggesting that Hsp90 may contribute to disease progression [26]. Hsp90 plays a key role in the induction of iNOS and is required for transcriptional factor binding to iNOS promoters [50]. NFκB is a known inducer of iNOS and when Hsp90 is inhibited there is a subsequent reduction in the ability of NF-κB to induce iNOS expression [50].

We have found that pre-B cell development is dysregulated in lupus-prone mice [41]. When pre-B cells were cultured from a single-cell suspension isolated from the BM, treatment with ACY-738 inhibited the formation of pre-B cell CFUs. Furthermore, treatment with this selective HDAC6i increased the pro-apoptotic protein, Bax, without altering expression of the apoptosis inhibitor, Bcl-2. During SLE the ratio of Bcl-2:Bax has been shown to be increased in lymphocytes suggesting a role of decreased apoptosis in SLE pathogenesis [51]. The elevated ratio of Bcl-2:Bax in lymphocytes from SLE patients is believed to contribute to the survival of autoreactive B cells that produce pathogenic autoantibodies [52]. The ability of HDAC6 inhibition to decrease the ratio of Bcl-2:Bax in pre-B cells may help to increase the removal of autoreactive B cells during active SLE.

Altered histone acetylation has recently been implicated in the pathogenesis of a number of diseases including cancer, cardiac hypertrophy, diabetes, and multiple sclerosis [53-55].

Studies have suggested that treatment of SLE with HDAC inhibitors may be able to correct aberrant B and T cell development. SLE is a chronic autoimmune disease requiring long-term treatment [56]. Use of pan-HDAC inhibitors may not be optimal in SLE as unwanted side effects may arise with long-term treatment, therefore the development of isoform-selective HDAC inhibitors for the treatment of lupus to specifically target abnormal immune cells is preferred [16, 57]. Our results suggest that overexpression of HDAC mRNA and HDAC activity in B, T, and glomerular cells contributes to disease in MRL/lpr lupus-prone mice. We found that HDAC6 inhibition by ACY-738 is able to decrease Hsp90 expression while inhibiting NFκB nuclear translocation. Taken together these data suggest that inhibition of specific HDAC isoforms may be beneficial in the treatment of SLE and that further studies are warranted to determine their efficacy.

4.8. References

[1] Crispin JC, Liossis SN, Kis-Toth K, Lieberman LA, Kyttaris VC, Juang YT *et al.* Pathogenesis of human systemic lupus erythematosus: recent advances. Trends in molecular medicine, 2010;16**:**47-57.

[2] Grammer AC, Lipsky PE. B cell abnormalities in systemic lupus erythematosus. Arthritis research & therapy, 2003;5 Suppl 4**:**S22-7.

[3] Davidson A, Diamond B. Autoimmune diseases. The New England journal of medicine, 2001;345**:**340-50.

[4] Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. Journal of clinical pathology, 2003;56**:**481-90.

[5] Dorner T, Giesecke C, Lipsky PE. Mechanisms of B cell autoimmunity in SLE. Arthritis research & therapy, 2011;13**:**243.

[6] Agnello V, Koffler D, Kunkel HG. Immune complex systems in the nephritis of systemic lupus erythematosus. Kidney international, 1973;3**:**90-9.

[7] Miyake K, Akahoshi M, Nakashima H. Th subset balance in lupus nephritis. Journal of biomedicine & biotechnology, 2011;2011**:**980286.

[8] Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C *et al.* Regulatory T cells in patients with systemic lupus erythematosus. Journal of autoimmunity, 2006;27**:**110-8.

[9] Reilly CM, Regna N, Mishra N. HDAC inhibition in lupus models. Molecular medicine, 2011;17**:**417-25.

[10] Dinarello CA, Fossati G, Mascagni P. Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer. Molecular medicine, 2011;17**:**333-52.

[11] Lemoine M, Younes A. Histone deacetylase inhibitors in the treatment of lymphoma. Discovery medicine, 2010;10**:**462-70.

[12] Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: What are the cancer relevant targets? Cancer letters, 2009;277**:**8-21.

[13] de Zoeten EF, Wang L, Sai H, Dillmann WH, Hancock WW. Inhibition of HDAC9 increases T regulatory cell function and prevents colitis in mice. Gastroenterology, 2010;138**:**583-94.

[14] Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM *et al.* Deacetylase inhibition promotes the generation and function of regulatory T cells. Nature medicine, 2007;13**:**1299-307.

[15] de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H *et al.* Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. Molecular and cellular biology, 2011;31**:**2066-78.

[16] Reilly CM, Mishra N, Miller JM, Joshi D, Ruiz P, Richon VM *et al.* Modulation of renal disease in MRL/lpr mice by suberoylanilide hydroxamic acid. Journal of immunology, 2004;173**:**4171-8.

[17] Wagner JM, Hackanson B, Lubbert M, Jung M. Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. Clinical epigenetics, 2010;1**:**117-36.

[18] Yan K, Cao Q, Reilly CM, Young NL, Garcia BA, Mishra N. Histone deacetylase 9 deficiency protects against effector T cell-mediated systemic autoimmunity. The Journal of biological chemistry, 2011;286**:**28833-43.

[19] Zhang Y, Kwon S, Yamaguchi T, Cubizolles F, Rousseaux S, Kneissel M *et al.* Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. Molecular and cellular biology, 2008;28**:**1688-701.

[20] Hancock WW. Rationale for HDAC inhibitor therapy in autoimmunity and transplantation. Handbook of experimental pharmacology, 2011;206**:**103-23.

[21] Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. The oncologist, 2007;12**:**1247-52.

[22] Regna NL, Chafin CB, Hammond SE, Puthiyaveetil AG, Caudell DL, Reilly CM. Class I and II histone deacetylase inhibition by ITF2357 reduces SLE pathogenesis in vivo. Clinical immunology, 2014;151**:**29-42.

[23] Perry D, Sang A, Yin Y, Zheng YY, Morel L. Murine models of systemic lupus erythematosus. Journal of biomedicine & biotechnology, 2011;2011**:**271694.

[24] Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A *et al.* HDAC6 is a microtubule-associated deacetylase. Nature, 2002;417**:**455-8.

[25] Drogaris P, Villeneuve V, Pomies C, Lee EH, Bourdeau V, Bonneil E *et al.* Histone deacetylase inhibitors globally enhance h3/h4 tail acetylation without affecting h3 lysine 56 acetylation. Scientific reports, 2012;2**:**220.

[26] Shukla HD, Pitha PM. Role of hsp90 in systemic lupus erythematosus and its clinical relevance. Autoimmune diseases, 2012;2012**:**728605.

[27] Beier UH, Wang L, Han R, Akimova T, Liu Y, Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. Science signaling, 2012;5**:**ra45.

[28] Beier UH, Akimova T, Liu Y, Wang L, Hancock WW. Histone/protein deacetylases control Foxp3 expression and the heat shock response of T-regulatory cells. Current opinion in immunology, 2011;23**:**670-8.

[29] Crispin JC, Kyttaris VC, Terhorst C, Tsokos GC. T cells as therapeutic targets in SLE. Nature reviews Rheumatology, 2010;6**:**317-25.

[30] Alunno A, Bartoloni E, Bistoni O, Nocentini G, Ronchetti S, Caterbi S *et al.* Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new. Clinical & developmental immunology, 2012;2012**:**823085.

[31] Chan RW, Lai FM, Li EK, Tam LS, Chow KM, Li PK *et al.* Imbalance of Th1/Th2 transcription factors in patients with lupus nephritis. Rheumatology, 2006;45**:**951-7.

[32] Noh H, Oh EY, Seo JY, Yu MR, Kim YO, Ha H *et al.* Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-beta1-induced renal injury. American journal of physiology Renal physiology, 2009;297**:**F729-39.

[33] Li D. Selective degradation of the IkappaB kinase (IKK) by autophagy. Cell research, 2006;16**:**855-6.

[34] Kovacs JJ, Murphy PJ, Gaillard S, Zhao X, Wu JT, Nicchitta CV *et al.* HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Molecular cell, 2005;18**:**601-7.

[35] Schlondorff D. Roles of the mesangium in glomerular function. Kidney international, 1996;49**:**1583-5.

[36] Bollain-y-Goytia JJ, Ramirez-Sandoval R, Daza L, Esparza E, Barbosa O, Ramirez D *et al.* Widespread expression of inducible NOS and citrulline in lupus nephritis tissues.

Inflammation research : official journal of the European Histamine Research Society [et al], 2009;58:61-6.

[37] Reilly CM, Farrelly LW, Viti D, Redmond ST, Hutchison F, Ruiz P *et al.* Modulation of renal disease in MRL/lpr mice by pharmacologic inhibition of inducible nitric oxide synthase. Kidney international, 2002;61:839-46.

[38] Peairs A, Radjavi A, Davis S, Li L, Ahmed A, Giri S *et al.* Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. Clin Exp Immunol, 2009;156:542-51.

[39] Palanichamy A, Bauer JW, Yalavarthi S, Meednu N, Barnard J, Owen T *et al.* Neutrophil-mediated IFN activation in the bone marrow alters B cell development in human and murine systemic lupus erythematosus. Journal of immunology, 2014;192:906-18.

[40] Guardiola AR, Yao TP. Molecular cloning and characterization of a novel histone deacetylase HDAC10. The Journal of biological chemistry, 2002;277:3350-6.

[41] Kao HY, Lee CH, Komarov A, Han CC, Evans RM. Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. The Journal of biological chemistry, 2002;277:187-93.

[42] Horwitz DA. Regulatory T cells in systemic lupus erythematosus: past, present and future. Arthritis research & therapy, 2008;10:227.

[43] van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O *et al.* Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood, 2010;115:965-74.

[44] Parietti V, Monneaux F, Decossas M, Muller S. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. Arthritis and rheumatism, 2008;58:1751-61.

[45] Yung S, Chan TM. Autoantibodies and resident renal cells in the pathogenesis of lupus nephritis: getting to know the unknown. Clinical & developmental immunology, 2012;2012:139365.

[46] Zubair A, Frieri M. NF-kappaB and systemic lupus erythematosus: examining the link. Journal of nephrology, 2013;26:953-9.

[47] Rao R, Fiskus W, Yang Y, Lee P, Joshi R, Fernandez P *et al.* HDAC6 inhibition enhances 17-AAG--mediated abrogation of hsp90 chaperone function in human leukemia cells. Blood, 2008;112**:**1886-93.

[48] Luo S, Wang T, Qin H, Lei H, Xia Y. Obligatory role of heat shock protein 90 in iNOS induction. American journal of physiology Cell physiology, 2011;301**:**C227-33.

[49] Cui JH, Qiao Q, Guo Y, Zhang YQ, Cheng H, He FR *et al.* Increased apoptosis and expression of FasL, Bax and caspase-3 in human lupus nephritis class II and IV. Journal of nephrology, 2012;25**:**255-61.

[50] Graninger WB, Steiner CW, Graninger MT, Aringer M, Smolen JS. Cytokine regulation of apoptosis and Bcl-2 expression in lymphocytes of patients with systemic lupus erythematosus. Cell death and differentiation, 2000;7**:**966-72.

[51] Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. Trends in immunology, 2011;32**:**335-43.

[52] Christensen DP, Dahllof M, Lundh M, Rasmussen DN, Nielsen MD, Billestrup N *et al.* Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus. Molecular medicine, 2011;17**:**378-90.

[53] Buglio D, Mamidipudi V, Khaskhely NM, Brady H, Heise C, Besterman J *et al.* The class-I HDAC inhibitor MGCD0103 induces apoptosis in Hodgkin lymphoma cell lines and synergizes with proteasome inhibitors by an HDAC6-independent mechanism. British journal of haematology, 2010;151**:**387-96.

[54] Doria A, Iaccarino L, Ghirardello A, Zampieri S, Arienti S, Sarzi-Puttini P *et al.* Long-term prognosis and causes of death in systemic lupus erythematosus. The American journal of medicine, 2006;119**:**700-6.

[55] Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. Molecular cancer research : MCR, 2007;5**:**981-9.

Chapter 5

Future Directions

N.L. Regna[1]
C. M. Reilly[1, 2]

[1]Department of Biomedical Sciences & Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061
[2]Edward Via College of Osteopathic Medicine, Blacksburg, VA, 24060

5.2. Proposed experiments

We have recently shown altered differentiation of B cell Hardy fractions in diseased New Zealand Black/White (NZB/W) mice. Furthermore, preliminary experiments demonstrate the ability of histone deacetylase (HDAC) 6 inhibition to correct aberrant BM B cell differentiation in lupus-prone mice to patterns similar to healthy mice. We have also shown that HDAC6 is overexpressed in B, T, and glomerular cells in diseased lupus mice, suggesting a role for HDAC6 in SLE pathogenesis. Future experiments will aim to elucidate the mechanism(s) through which HDAC6 inhibition alters development progression of BM B cells.

Our current studies have shown that inhibition of HDAC6 leads to hyperacetylation of α-tubulin, a major constituent of microtubules [1-3]. Future experiments will aim to determine the effect of α-tubulin acetylation on the developmental progression of BM B cells from pre-diseased NZB/W mice. We will seek to define the role of α-tubulin acetylation on microtubule structure and stabilization in B cells and determine whether HDAC6 inhibition can alter B cell differentiation independent of α-tubulin acetylation. Microtubules, which consist of α-and β-tubulin, play an important role in cell structure and shape [4, 5]. 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. B cells not only require contact with the stroma, but are also stimulated by stromal cell production of growth factors including IL-7 and stem cell factor (SCF) [6]. Preliminary experiments have shown that diseased NZB/W mice have decreased numbers of late pro-B/early pre-B cells and an accumulation of mature fraction F pre- B cells. The differential development may be explained by alteration in stroma-B cell interactions. Acetylation of α-tubulin has been demonstrated to increase the stability of microtubules leading to altered stromal cell contact [7].

As B cells undergo development, the status of their Ig gene rearrangements change [8]. Due to preliminary experiments indicating altered distribution of B cells in developmental Hardy fractions along with reversal following HDAC6 inhibition, we believe that α-tubulin acetylation may affect expression of genes involved with various stages of B cell development. For B cells plated in direct contact with stromal cells the % adhesion will be determined in order to evaluate whether α-tubulin hyperacetylation alters the ability of B cells to bind to and interact with stromal cells. Cytokine production will be measured from the media of cells in direct contact with the stroma, and cells separated by the transwell diffusion chamber. The presence of IL-7 and SCF, required growth factors for B cell development, will be determined using enzyme linked immunosorbent assay (ELISA) to evaluate the effect of HDAC6 inhibition as well as stromal cell contact on cytokine production. B cells will be collected following 4 days of treatment and sorted into Hardy developmental fractions in order to determine the effect of direct stromal cell contact. α-tubulin will be mutated at its acetylation site, lysine 40 , to alanine in order to prevent acetylation of α-tubulin in order to determine whether α-tubulin acetylation is needed for the alterations in BM B cell differentiation following HDAC6 inhibition. These studies will show whether or not HDAC6 inhibition alters B cell development through altered stromal cell contact and whether or not any changes in B cell development are dependent upon α-tubulin acetylation.

Apoptosis is required for the development and proper functioning of the immune system. During B cell lymphopoiesis, apoptosis is critical for the deletion of B cells with nonfunctional and self-reactive antigen receptors [8]. B lymphocytes differentiate from HSCs within the bone marrow where their development is regulated by both positive and negative selection [9]. Gene rearrangement to produce a vast B cell repertoire is imprecise and requires regulation [10]. In

healthy individuals when autoreactive B cells are generated they are signaled for removal during B cell development in the bone marrow [11]. During SLE, these B cells are able to bypass apoptosis and continue to develop and differentiate into mature autoantibody producing B cells [12]. Preliminary experiments have shown that there is an accumulation of immature B cells in the BM of diseased NZB/W mice. Treatment with an HDAC6i was able to significantly decrease the percentage of cells in this developmental subset. The accumulation of immature B cells may indicate a failure in apoptotic mechanisms to remove autoreactive B cells during development in SLE. This apoptotic failure may be due to dysregulation of apoptotic regulators including BCL-2 family proteins and p53. P53 has been demonstrated to be non-functional in humans with active SLE [13].

We will seek to characterize the effect of HDAC6 inhibition on pro- and pre-B cell apoptosis in the BM. Our preliminary data indicate altered BM B cell differentiation in diseased NZB/W mice resulting in an abnormal distribution of B cells in developmental stages. Furthermore, our data indicate that treatment with an HDAC6i is able to reverse the abnormal percentage of cells in the pro- and pre-B cell developmental Hardy fractions. Inhibition of HDAC6 may be able to affect BM differentiation patterns by altering apoptosis of specific B cell Hardy subsets. The rates at which precursor B cells undergo apoptosis at successive stages of differentiation in normal, healthy mouse BM has been well defined by Osmond et al [14]. We plan to determine if treatment with an HDAC6i is able to alter these apoptotic trends in B cells from the BM of lupus-prone mice. Our studies will define the apoptotic index and rate of BM Hardy fractions following HDAC6 inhibition.

We also plan to examine the effect of HDAC6 inhibition on pro- and anti-apoptotic gene and protein expression. If we determine the apoptosis to be significantly altered in the BM by HDAC6 inhibition we will seek to determine whether apoptosis in the BM is caspase dependent. We will look at Bax and Bcl-2 in order to determine the Bax/Bcl-2 ratio. The Bax protein is a member of the bcl-2 family that promotes apoptosis. The ratio of Bcl-2 to Bax determines the susceptibility of a cell to apoptosis. Conversely, the Bcl-2 protein is a known inhibitor of apoptosis [15]. The Bcl-2to Bax ratio is significantly higher during active SLE, which may support the survival of autoreactive B cells [16]. We will also seek to determine the expression of activated caspases, particularly, caspase-3. Activation of caspase-3 is the critical step in determining whether or not a cell will undergo apoptosis [17]. When caspase-3 becomes activated it initiates a chain reaction and at this point it becomes impossible to reverse commitment to apoptosis [18]. Caspase activation is known to be dysregulated during SLE [19]. These studies would determine whether HDAC6 inhibition is able to alter B cell development in the BM through altered apoptosis.

5.3. References

[1] de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H *et al.* Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. Molecular and cellular biology, 2011;31**:**2066-78.

[2] Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A *et al.* HDAC6 is a microtubule-associated deacetylase. Nature, 2002;417**:**455-8.

[3] Piperno G, LeDizet M, Chang XJ. Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. The Journal of cell biology, 1987;104**:**289-302.

[4] Zhang Y, Li N, Caron C, Matthias G, Hess D, Khochbin S *et al.* HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. The EMBO journal, 2003;22**:**1168-79.

[5] Nogales E. Structural insight into microtubule function. Annual review of biophysics and biomolecular structure, 2001;30**:**397-420.

[6] Funk PE, Varas A, Witte PL. Activity of stem cell factor and IL-7 in combination on normal bone marrow B lineage cells. Journal of immunology, 1993;150**:**748-52.

[7] Takemura R, Okabe S, Umeyama T, Kanai Y, Cowan NJ, Hirokawa N. Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. Journal of cell science, 1992;103 (Pt 4)**:**953-64.

[8] Lu L, Osmond DG. Apoptosis and its modulation during B lymphopoiesis in mouse bone marrow. Immunological reviews, 2000;175**:**158-74.

[9] Carsetti R. The development of B cells in the bone marrow is controlled by the balance between cell-autonomous mechanisms and signals from the microenvironment. The Journal of experimental medicine, 2000;191**:**5-8.

[10] Melchers F, ten Boekel E, Seidl T, Kong XC, Yamagami T, Onishi K *et al.* Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. Immunological reviews, 2000;175**:**33-46.

[11] MacLennan IC. Autoimmunity. Deletion of autoreactive B cells. Current biology : CB, 1995;5**:**103-6.

[12] Dorner T, Giesecke C, Lipsky PE. Mechanisms of B cell autoimmunity in SLE. Arthritis research & therapy, 2011;13**:**243.

[13] Herkel J, Mimran A, Erez N, Kam N, Lohse AW, Marker-Hermann E *et al.* Autoimmunity to the p53 protein is a feature of systemic lupus erythematosus (SLE) related to anti-DNA antibodies. Journal of autoimmunity, 2001;17**:**63-9.

[14] Osmond DG. B cell development in the bone marrow. Seminars in immunology, 1990;2**:**173-80.

[15] Reed JC, Zha H, Aime-Sempe C, Takayama S, Wang HG. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Advances in experimental medicine and biology, 1996;406**:**99-112.

[16] Graninger WB, Steiner CW, Graninger MT, Aringer M, Smolen JS. Cytokine regulation of apoptosis and Bcl-2 expression in lymphocytes of patients with systemic lupus erythematosus. Cell death and differentiation, 2000;7**:**966-72.

[17] Krishnan S, Kiang JG, Fisher CU, Nambiar MP, Nguyen HT, Kyttaris VC *et al.* Increased caspase-3 expression and activity contribute to reduced CD3zeta expression in systemic lupus erythematosus T cells. Journal of immunology, 2005;175**:**3417-23.

[18] Cui JH, Qiao Q, Guo Y, Zhang YQ, Cheng H, He FR *et al.* Increased apoptosis and expression of FasL, Bax and caspase-3 in human lupus nephritis class II and IV. Journal of nephrology, 2012;25**:**255-61.

[19] Santo L, Hideshima T, Kung AL, Tseng JC, Tamang D, Yang M *et al.* Preclinical activity, pharmacodynamic, and pharmacokinetic properties of a selective HDAC6 inhibitor, ACY-1215, in combination with bortezomib in multiple myeloma. Blood, 2012;119**:**2579-89.