HDAC expression and activity is upregulated in diseased lupus-prone mice

Nicole L. Regna a, Miranda D. Vieson a, Alexander M. Gojmerac a, Xin M. Luo a, David L. Caudell b,d, Christopher M. Reilly c,a,*

a Department of Biomedical Sciences & Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, United States
b Wake Forest University Primate Center, Wake Forest School of Medicine, Winston-Salem, NC 27157, United States
c Edward Via College of Osteopathic Medicine, Blacksburg, VA, 24060, United States
d Department of Pathology/Comparative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157, United States

ARTICLE INFO

Article history:
Received 26 August 2015
Received in revised form 1 October 2015
Accepted 5 October 2015
Available online 21 October 2015

Keywords:
SLE
HDAC
B cells
T cells
Glomerular cells

ABSTRACT

Prior studies have shown that pan-HDAC inhibition can decrease disease in lupus mice; however, the mechanisms(s) remain to be elucidated. MRL/Mp-J/Fas−/− (MRL/lpr) mice develop a lupus-like disease characterized by anti-dsDNA production, lymphoproliferation, and immune complex-mediated glomerulonephritis. Early- and late-stage disease (12 and 20 weeks-of-age, respectively) female MRL/lpr mice were compared to age-matched, healthy C57BL/6 mice for HDAC expression and activity in bone marrow (BM) B cells, splenic B and T cells, and glomerular cells. We found that HDAC8 was significantly overexpressed in B cells, splenic T cells and glomerular cells, whereas HDAC9 expression was significantly increased in splenic T cells, BM B cells and glomerular 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. ACY-738 significantly reduced cytoplasmic HDAC activity whereas TsA significantly decreased both nuclear and cytoplasmic HDAC activity. In vitro studies in mesangial cells showed that ACY-738 increased α-tubulin and Hsp90 acetylation resulting in decreased nuclear activation of NF-κB. 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.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. 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 various stages of 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 a 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. Alterations in the balance and functions of T cell subsets in human SLE patients and murine models include: decreased number and function of regulatory T (Treg) cells, elevated Th1 and Th2 cytokines, and increased numbers 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 resulting in altered 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 Ila (HDACs 4, 5, 7, and 9) and class Iib (HDACs 6 and 10). Class Ila 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 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

http://dx.doi.org/10.1016/j.intimp.2015.10.006
1567-5769/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
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 [18]. Knockdown of HDAC5 in mice results in significant heart problems including myocardial hypertrophy and an abnormal cardiac stress response [19]. Germline deletion of HDAC7 in mice has proven to be embryonic lethal due to its role in endothelial cell–cell adhesion [20]. 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 [21]. T cells from both SLE patients and SLE murine models have been demonstrated to overexpress HDAC9 [21]. Furthermore, inhibition of HDAC9 results in increased Treg activation and suppressive function [13]. Due to the significant defects 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 has been demonstrated to deacetylate a number of proteins with specificity for α-tubulin and Hsp90 [22,23]. HDAC6 has increased tissue expression in the kidneys, liver, heart, and pancreas [12]. HDAC6-deficient mice are viable with no significant defects [22]. Mice lacking HDAC6 and those treated with HDAC6 siRNA have hyperacetylated α-tubulin and Hsp90 [15,24]. Hyperacetylation of Hsp90 has been shown to decrease Hsp90 function [22,24,25]. The role of HDAC10 in autoimmune disease remains undetermined.

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 HDAC, SAHA, has been approved by the FDA for the treatment of cutaneous T cell lymphoma [26]. 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].

Due to their suggested role in autoimmune disease, we sought to determine the mRNA expression level of class IIb HDACs (HDAC6 and 10) and one class IIa HDAC (HDAC9) in immune-associated tissues from lupus-prone mice. Next, we investigated whether HDAC activity was increased during SLE disease and if treatment with a selective HDACi would decrease HDAC activity in vitro and the mechanism(s) through which the HDACi exerts its inhibitory effect.

2. Methods
2.1. Mice

Female MRL/Mp-j Fas<sup>−/−</sup> (MRL/lpr) and C57BL/6 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.

2.2. 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) lysis 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 RNAlater (Qiagen, Valencia, CA, USA) and stored at −20 °C until RNA isolation or used for cytoplasmic and nuclear extractions.

2.3. Splenocyte isolation

A single-cell suspension was obtained from the spleens of MRL/lpr mice and age-matched 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). naïve T and T<sub>reg</sub> cells were isolated using the appropriate isolation kit according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA, USA). Cells were resuspended in RNAlater (Qiagen, Valencia, CA, USA) and stored at −20 °C until RNA isolation or used for cytoplasmic and nuclear extractions.

2.4. Isolation of glomerular cells

Following euthanization, the glomeruli were removed from MRL/lpr mice and were pooled for glomerular cell isolation as we have previously published [27]. 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 RNAlater (Qiagen, Valencia, CA, USA), and stored at −20 °C until RNA isolation or used for cytoplasmic and nuclear extractions.

2.5. 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.

2.6. Real-time RT-PCR

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

2.7. Nuclear and cytoplasmic extraction

Isolated BM B cells, splenic B and T cells, and glomerular cells were lysed, then cytoplasmic and nuclear protein fractions were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer’s protocol.

2.8. Cell culture

A mouse mesangial cell line (MES 13) transgenic for SV40 was cultured in 75-mm<sup>2</sup> culture flasks at 37 °C in 5% CO<sub>2</sub> 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) for 24 h in order to determine the level of acetylated H3 or α-tubulin. 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 h followed by 24 h of stimulation with LPS/IFN-γ. MES 13 cells were treated with ACY-738 (5 nM) for 2 h 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 NF-κB.

2.9. 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 [28]. The immunoprecipitated protein was normalized using the Bradford protein assay then subjected to the HDAC activity assay.

2.10. 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 min and then loaded onto a 15% SDS-PAGE gel. The HDAC activity colorimetric assay kit (Bio Vision, USA) was used to normalize protein levels. The samples were heated to 96 °C for 5 min 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.

2.11. HDAC activity analysis

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.

2.12. 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% CO₂. Colonies were counted and then collected for Western blot analysis after 8 days of culture.

2.13. 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.

3. Results

3.1. HDAC expression is increased in diseased MRL/lpr 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 [29]. On the other hand, C57BL/6 mice did not exhibit proteinuria, anti-dsDNA or glomerulonephritis regardless of age. In prior studies, we have shown that pan-HDAC inhibitors decrease disease pathogenesis in lupus mice [28]. To explore further the role of HDACs in lupus, we measured mRNA expression levels of HDAC6, 9, and 10 in lupus mice at 12 (early disease) and 20 weeks-of-age (late disease) and compared their expression levels to age-matched non-lupus C57BL/6 mice (Fig. 1). We chose two class Iib members, HDAC6 and 10, because they 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 [30]. We also chose HDAC9, a class Ila HDAC, as it has previously been shown to play an important role in SLE pathogenesis due to its regulation of Treg cells and Hsp90 acetylation [23,31,32]. Relative mRNA expression levels of the class Iib HDACs 6 and 10 and class Ila HDAC9 were determined using real-time RT-PCR. 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, and decreased HDAC10 when compared to age-matched C57BL/6 mice (Fig. 1A). Expression of HDACs 6 and 10 were increased in splenic B cells from diseased MRL/lpr mice; however, there were no significant differences in HDAC9 expression levels between MRL/lpr mice in an early or late disease state (Fig. 1B).

Next we examined the level of HDAC expression in splenic T cells as naive CD4⁺ T cell differentiation has been shown to be dysregulated during SLE [33]. 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 Treg cell function [8,34,35]. In T cells isolated from the spleens of MRL/lpr mice, expression of HDACs 6 and 9 were significantly increased compared to age-matched control mice. Interestingly HDAC10 was significantly decreased in the MRL/lpr mice compared to controls (Fig. 1C).

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 9 expression; however, there was no significant difference in HDAC10 expression compared to age-matched control mice (Fig. 1D).

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

Specific HDAC isoforms can be found alone in either the nucleus or cytoplasm, or in both the nucleus and the cytoplasm. The class Iib HDACs (HDAC6 and HDAC10) are found primarily 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. An 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 (Fig. 2). In the BM, we found a significant elevation in the cytosolic levels of HDAC activity in MRL/lpr 20 week-old mice compared to controls, while the nuclear fraction showed elevated HDAC activity, which reached...
cells, CD4+ CD25+ cells had greater nuclear HDAC activity; however, age-matched healthy control mice. Similar to the HDAC activity in B, T, and glomerular cells from MRL/lpr mice, we sought to determine whether there was a significant difference in HDAC activity when compared to age-matched controls at 20 weeks-of-age, with nuclear HDAC activity showing the greatest differences (Fig. 2B).

Targeting of different HDAC isoforms, including HDAC6 and HDAC9, has been demonstrated to increase Treg cell function through a number of different mechanisms [13,31]. We isolated CD4+ CD25− cells and CD4+ CD25+ T cells from the spleens of MRL/lpr and C57BL/6 mice and evaluated the level of HDAC activity to determine associations between spleen disease and Treg HDAC activity. We found that CD4+ CD25+ cells had more HDAC activity than CD4+ CD25− T cells regardless of mouse strain or age (Fig. 2C). Additionally, CD4+ CD25+ cell HDAC activity was significantly greater in the MRL/lpr mice compared to age-matched healthy control mice. Similar to the HDAC activity in B cells, CD4+ CD25+ cells had greater nuclear HDAC activity; however, we also found an increase in the cytoplasmic HDAC activity. 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 (Fig. 2D).

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

Because our results show elevated HDAC expression 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 HDAC activity in these cells. In order to determine the level of HDAC6 activity, HDAC6 protein was immunoprecipitated from nuclear and cytoplasmic fractions isolated from B, T, and glomerular cells, then 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 (Fig. 3). HDAC6 activity was significantly higher in the cytoplasm in MRL/lpr mice compared to C57BL/6 mice.

3.4. HDAC6 inhibition increases α-tubulin acetylation and decreases cytoplasmic HDAC activity in B, T, and glomerular cells

Following HDAC6 inhibition, α-tubulin becomes acetylated [36]. Furthermore, Histone H3 becomes hyperacetylated following inhibition of class I HDACs [37]. In order to determine the concentration at which ACY-738 selectively inhibits HDAC6 without inhibiting class I HDACs, mesangial cells were cultured and treated with increasing concentrations of ACY-738 for 24 h, followed by Western blot analysis to determine α-tubulin and histone H3 acetylation levels. 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 of histone H3 (Fig. 4A).

In order to assess the ability of a selective HDAC6i (ACY-738) to inhibit nuclear and cytoplasmic HDAC activity, isolated BM B cells, splenic B and T cells, and glomerular cells were treated with either Trichostatin A (TsA, a class I and II HDACi) or ACY-738. As previously discussed, a 5 nM concentration of class I and II HDACs at this concentration [38]. Not surprisingly, we found that ACY-738 (5 nM) had a greater inhibitory effect on the cytoplasmic fractions compared to nuclear fractions from isolated B, T, and glomerular cells. The ability of ACY-738 to inhibit HDAC activity was not dependent upon the age or strain of mouse or on the cell type. TsA (1 μM) was able to inhibit HDAC activity in both cytoplasmic and nuclear fractions regardless of mouse strain or age (Fig. 4B–E).
3.5. In vitro inhibition of HDAC6 in mesangial cells results in inhibition of Hsp90 and a decrease in NF-κB 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 [25,39]. 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 [30]. Mesangial cells are the primary resident immunoregulatory cells found in the glomerulus and act to maintain proper glomerular filtration [40]. Our study showed that treatment of mesangial cells with ACY-738 resulted in a slight, but not significant, increase in Hsp90 acetylation and decrease of non-acetylated Hsp90 (Fig. 5A). HDAC6 inhibition also resulted in a decrease in iNOS protein (Fig. 5A and B), which previous studies have shown to be upregulated in the kidneys of SLE patients and mice with active disease [41,42].

During SLE, NF-κB regulates both B and T cell development as well as T cell activation. NF-κB controls the immune response through regulation of inflammatory cytokine gene expression. During SLE, increased nuclear translocation of NF-κB leads to increased iNOS and IL-6 production and a pro-inflammatory environment in the mesangium [43]. Our results showed that nuclear levels increased in a time-dependent manner from 0 to 60 mins; however, treatment with ACY-738 reduced nuclear levels of NF-κB in cultured mesangial cells (Fig. 5C).

3.6. 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 [44]. 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 (Fig. 6A). Cells were collected and subjected to Western blot analysis for evaluation of apoptosis-associated Bax and Bcl-2 proteins. Treatment of ACY-738 increased the level of Bax, without altering Bcl-2 expression (Fig. 6B). Bax expression is known to promote apoptosis, while Bcl-2 is a known inhibitor of apoptosis.
4. Discussion

We found that the expression and activity of class II HDACs (6 and 9) were upregulated in early- and late-diseased MRL/lpr mice compared to age-matched C57BL/6 control mice. On the other hand, HDAC10 was decreased in MRL/lpr mice compared to age-matched C57BL/6 animals in BM B cells and splenic T cells, but not in glomerular cells. 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 (1 μM) decreased both nuclear and cytoplasmic HDAC activity. Inhibition of HDAC6 in mesangial cells led to an increase in acetylated Hsp90 as well as a decrease in nuclear levels of NF-κB. 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 NF-κB 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 [45]. The role of HDAC10 in the cytoplasm remains to be determined; however, it has been shown to be a transcriptional modulator in the nucleus [46]. HDAC10 expression was downregulated in BM B and T cells in MRL/lpr mice compared to controls. However, HDAC10 expression was not altered with respect to the increased disease activity suggesting 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 [21]. HDAC9 has been implicated in the regulation and the suppressive function of Treg cells [31]. Our study showed that HDAC9 was significantly overexpressed in splenic T cells in MRL/lpr mice compared to C57BL/6 mice. 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 CD4+ CD25+ cells, which have decreased immunosuppressive function in SLE patients and murine models of disease [47]. We found CD4+ CD25+ 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 Treg 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 [48]. Overexpression of HDAC9 by splenic T cells may contribute to the decrease in Treg cell number and function associated with MRL/lpr mice [49]. In our studies, HDAC activity in CD4+ CD25− 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 regards to regulation of these cells. It is important to note we used CD4+ CD25− as a surrogate marker for Treg cells. In order for these cells to be phenotyped as Tregs, Foxp3 must be expressed as reports...
have described that activated T cells may also contain CD25+ in older MRL/lpr mice [50].

HDAC6 is a class IIb HDAC with a structure similar 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 [25,30]. 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 Tregs [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 was able to increase α-tubulin acetylation without significantly increasing the acetylation of the H3 histone. (B–E) Isolated cells were treated with class I and II HDACi, TsA, or the HDAC6 selective inhibitor, ACY-738 for 24 h. 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 ≥ 6; *p < 0.05, **p < 0.01, ***p < 0.001).
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 NF-κB in immune-stimulated mesangial cells. When NF-κB is activated, it is translocated into the nucleus allowing for gene regulation [53]. We also found that HDAC6 inhibition decreased Hsp90 in a concentration-dependent manner in cultured mesangial cells. Increased Hsp90 acetylation results in inhibition of Hsp90 chaperone function and proteosomal degradation of Hsp90 client proteins [24]. 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 [30]. Hsp90 plays a key role in the induction of iNOS and is required for transcriptional factor binding to iNOS promoters. NF-κB 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 [54].

We have found that pre-B cell development is dysregulated in lupus-prone mice (unpublished data). 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 [55]. 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 [56]. 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 [57–59]. Studies have suggested that treatment of SLE with HDAC inhibitors may be able to correct aberrant B and T cell development [60]. SLE is a chronic autoimmune disease requiring long-term treatment [61]. 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 [16,62]. Our results suggest that overexpression of HDAC6 cells is preferred [16,62]. Our results suggest that overexpression of HDAC6 inhibits NF-κB 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.

References


[41] V. Parietti, F. Monneaux, M. Decossas, S. Muller, Function of CD4+,CD25+ Treg cells from MRL/lpr mice were functionally more active in vitro but did not prevent spontaneous as well as adriamycin-induced nephropathy in vivo, Rheumatology 51 (2012) 1357–1367.


