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**Histology and Histopathology**

*From Cell Biology to Tissue Engineering*

# Treatment with a selective histone deacetylase 6 inhibitor decreases lupus nephritis in NZB/W mice

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**Summary.** To date, there are 18 histone deacetylase (HDAC) enzymes, divided into four classes, which alter protein function by removing acetyl groups from lysine residues. Prior studies report that non-selective HDAC inhibitors decrease disease in lupus mouse models. Concern for adverse side effects of non-selective HDAC inhibition supports investigation of selective-HDAC inhibition. We hypothesized that a selective HDAC-6 inhibitor (HDAC6i) will alleviate disease in a mouse model of lupus by increasing acetylation of alpha-tubulin. Intraperitoneal injections of the selective HDAC6i ACY-1083 (0.3 mg/kg, 1 mg/kg, or 3 mg/kg), vehicle control, or dexamethasone were administered to 21-week-old, female NZB/W mice, 5 days a week, for 13 weeks. Disease progression was evaluated by proteinuria, serum levels of anti-dsDNA antibody, cytokines and immunoglobulins, and post mortem evaluation of nephritis and T cell populations in the spleen. HDAC6i treatment decreased proteinuria, glomerular histopathology, IgG, and C3 scores when compared to vehicle-treated mice. Within glomeruli of HDAC6i-treated mice, there was increased acetylation of alpha-tubulin and decreased NF-κB. Additionally, HDAC6i decreased serum IL-12/IL-23 and Th17 cells in the spleen. Taken together, these results suggest HDAC-6 inhibition may decrease lupus nephritis in NZB/W mice via mechanisms involving acetylation of alpha-tubulin and decreased NF-κB in glomeruli as well as inhibition of Th17 cells.

**Key words:** Lupus, Nephritis, Histone deacetylase inhibitor, Alpha-tubulin

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## Introduction

Urowitz and Gladman (1999) state that understanding the clinical presentation of systemic lupus erythematosus (SLE) now focuses on late stage manifestations that contribute to morbidity and mortality, including chronic renal insufficiency (Urowitz and Gladman, 1999). Lupus nephritis is one of the most costly (Pelletier et al., 2009) and important manifestations of disease in SLE patients, contributing significantly to morbidity and mortality (Houssiau and Lauwerys, 2013). The development of autoantibodies is a mainstay feature of SLE, and contributes to lupus nephritis by cross-reacting with renal antigens, indirectly binding to nuclear material in glomerular basement membranes, or forming immune complexes that circulate and become deposited in kidney glomeruli (Yap and Lai, 2015). Deposition of immune complexes (IC) in the kidneys induces an inflammatory response that disrupts glomerular filtration eventually leading to proteinuria and if not treated can result in end-stage renal disease (Kurts et al., 2013). Over time, changes in the kidneys due to repeated flares of inflammation and damage will ultimately lead to chronic renal failure.

Studies have implicated a potential role for Th17 cells in the pathogenesis of lupus nephritis. Circulating Th17 cells are increased in human SLE patients and correlate with SLE disease activity index scores (Chen et al., 2012; Xing et al., 2012). Additionally, infiltrating T cells within the kidneys of SLE patients exhibit higher expression levels of IL-17, which correlates with multiple disease parameters including glomerular and interstitial disease activity scores, urine protein levels, and elevated blood urea nitrogen levels (Wang et al., 2010). Abnormal IL-17 production in SLE may promote autoimmune disease in at least two ways: (1) increased IL-17 in sites of inflammation (kidney) increases the influx of effector cells therefore amplifying the immune response, and (2) IL-17 can contribute to the excessive activation of the B cell compartment leading to further antibody production (Apostolidis et al., 2011a).

There are at least 18 mammalian histone deacetylase (HDAC) enzymes, which remove acetyl groups from lysine residues in histones and other proteins to control multiple cellular functions including transcription, cell cycle kinetics, cell signaling, and cellular transport processes (Li et al., 2011). HDACs are classified based on structural and functional similarities into classes I-IV, of which classes I and II are most widely studied. Class I HDACs include HDAC1, -2, 3, -6, and -8, and class II HDACs are further subdivided into class IIa (HDAC4, -5, -7, and -9) and class IIb (HDAC6, and -10) (Reilly et al., 2011; Shakespear et al., 2011). In addition to their initial relevance in cancer biology (Marks et al., 2001), HDACs have been reported to play a key role in inflammation and immunity (Shakespear et al., 2011). Subsequently, pharmacologic inhibition of HDACs has been evaluated as a possible treatment modality in a wide spectrum of diseases unrelated to cancer, including inflammatory and autoimmune diseases (Dinarello et al., 2011). In regard to SLE, decreased disease and renal pathology has been reported in various mouse models using pharmacologic inhibition of HDACs or by gene deletion (Mishra et al., 2003; Reilly et al., 2004; 2008; Regna et al., 2014). The majority of HDAC inhibitors are considered "pan-inhibitors" or non-selective inhibitors due to their broad action on multiple enzymes of both class I and II HDACs. Because HDAC I enzymes are ubiquitously distributed throughout the body, pharmacologic inhibition of these enzymes may result in unwanted side effects. Additionally, phenotypes associated with knocking-out class I and class IIa HDACs are often embryonic lethal or have life-limiting developmental abnormalities (Witt et al., 2009). Therefore, selective inhibition of particular HDAC enzymes is desired (Cantley and Haynes, 2013). HDAC6 knock-out mice exhibit a viable phenotype, develop normally, and have no life-limiting defects. More importantly, while lymphocytes in these mice develop normally, there is a mild decrease in the immune response after antigenic stimulation (Zhang et al., 2008). Based on these findings, we have chosen to focus on specific inhibition of HDAC6 for our studies.

The following studies evaluate the ability of a selective HDAC6i (ACY-1083) to decrease lupus nephritis in a murine model of SLE (NZB/W F1 female mice). In association with lupus nephritis, we will also evaluate multiple SLE disease parameters, alpha-tubulin acetylation in the kidneys, and the status of Th17 cells. We hypothesize that treatment of NZB/W F1 female mice with ACY-1083 will decrease lupus nephritis concurrently with decreased Th17 kinetics related to HDAC6i-mediated acetylation of alpha-tubulin.

## Materials and methods

### *Mice*

Female NZB/W F1 and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were used in accordance with the Institutional Animal Care and Use Committee (IACUC) after protocol approval by the IACUC of Virginia Tech University and housed in the animal facility at the Virginia-Maryland College of Veterinary Medicine (Blacksburg, VA, USA). Mice were humanely euthanized with inhalation of isoflurane followed by cervical dislocation and thoracotomy.

### *In vivo treatments and monitoring*

Hydroxy-propyl-methyl cellulose (HPMC, Sigma, St. Louis, MO, USA) was diluted in sterile deionized water at a concentration of 0.05%, autoclaved, then used as the vehicle for all drug solutions. ACY-1083, a histone deacetylase 6 inhibitor (HDAC6i), was courtesy of a generous donation from Acetylon Pharmaceuticals (Boston, MA, USA) for use in all studies. A pharmacokinetic analysis of ACY-1083 was used to determine the optimum doses for the study. Male C67Bl/6 mice were injected with ACY-1083 in 20% hydroxypropyl beta cyclodextrin/0.5% HPMC by intravenous (IV) route at 1 mg/kg or by intraperitoneal (IP) route at 10 mg/kg. Plasma levels of ACY-1083 were determined by LC/MS/MS and quantitated by a standard curve made in matching plasma. Based on these results, ten mice were included in each of 5 treatment groups: (1) vehicle control (HPMC), (2) 0.3 mg/kg ACY-1083, (3) 1 mg/kg ACY-1083, (4) 3 mg/kg ACY-1083, (5) 2 mg/kg dexamethasone (DEX, positive control). Dexamethasone was chosen as the positive control because it can be used in lupus mice without significant side effects (Yuan et al., 2012). Mice were injected IP 5 days/week with a 50uL volume of their respective treatments beginning at 21-weeks-of-age, and treatments continued until euthanasia during late stage clinical disease at 34 weeks-of-age. Proteinuria and body weight were measured bi-weekly before treatment, then weekly after treatment began. Proteinuria was determined 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+=5.

### Measurement of autoantibodies

Sera were collected prior to treatment at 20-weeks-of-age, and then once every 4 weeks until euthanasia. The mice were anesthetized using isoflurane (Piramal Healthcare, Mumbai, Maharashtra, India) and bled from the retro-orbital sinus. The levels of autoantibodies to dsDNA were measured by semi-quantitative ELISA as we have previously described (Regna et al., 2014). Serum from a C57BL/6 mouse and a diseased MRL/*lpr* mouse was used as negative and positive controls, respectively. Values on each plate were normalized to the positive control (set as 1) and a final dilution of 1:3600 was reported.

### Measurement of serum cytokines and immunoglobulin isoforms

Total IgG, $IgG_{2a}$, IL-12/IL-23, and TGF-β protein levels were measured in the sera by quantitative ELISA according to manufacturer's protocol (eBioscience, San Diego, CA, USA). Plates were read on a microplate spectrophotometer at 450 nm.

### Splenocyte isolation and flow cytometric analysis

Spleens were aseptically removed from the mice after euthanasia and dissociated into single cell suspensions and red blood cells were lysed with ACK lysis buffer (Lonza, Alpharetta, GA, USA) according to manufacturer's protocol as we have previously described (Regna et al., 2015b). For evaluation of T cell populations, two sets of splenocytes were washed with cold flow cytometry buffer followed by staining of cell surface antigens by either CD4-FITC and CD25-PerCP-Cy5.5 (Treg) or CD4-FITC only (Th17). Next, both sets of T cells (Treg and Th17) were fixed and permeabilized with Foxp3/Transcription factor Fix/Perm buffer (eBiosciences, San Diego, CA, USA) followed by intracellular antigen staining with Foxp3-PE (Treg) or RORγ-APC and IL-17-PE (Th17). Both sets of T cells were sorted by in a FACS Aria 1 flow cytometer (BD Biosciences, San Jose, CA, USA) then analyzed by FlowJo Software (Tree Star, Ashland, OR, USA).

### Renal histopathology

At the time of euthanasia, both kidneys were removed. One kidney was fixed in 10% neutral buffered formalin for 24 hours, then routinely processed, embedded in paraffin, sectioned at 4-5 μm, 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 (D.L. Caudell) in a blinded manner.

### Renal IgG and C3 accumulation

One kidney was placed in OCT media and snap-frozen in a slurry containing dry ice and 2-methylbutane (Fisher Scientific, Hampton, NH, USA). The frozen kidney was cryosectioned at 4 μm thickness and mounted on charged, glass, microscope slides. Mounted frozen sections were fixed in acetone for 10 min, then washed 3 times with PBS for 5 min each. Next, the sections were incubated with goat anti-mouse IgG conjugated to FITC (1:100, Sigma, St. Louis, MO, USA) or goat anti-mouse C3 conjugated to FITC (1:100, Cederalane, Burlington, NC, USA) antibodies in a humid chamber for 1 h. Slides were mounted using Vectashield mounting media (Vector Labs, Burlingame, CA, USA) and examined by an Olympus IX73 fluorescent microscope (Olympus America Inc., Center Valley, PA, USA). Deposition of IgG and C3 within glomeruli was scored (0-3) by a board-certified veterinary anatomic pathologist (M.D. Vieson).

### Acetylated alpha-tubulin, Histone 3, and NF-κB immunofluorescence in glomerular cells

The frozen kidney was further sectioned at 5-7 μm thickness, fixed in 4% formaldehyde for 15 min, then washed 3 times with PBS for 5 min each. Next, the sections were blocked with normal goat serum for 1 hour then incubated with rabbit anti-mouse acetylated-alpha-tubulin (K40) or rabbit anti-mouse nuclear factor – kappa B (NF-κB) (Cell Signaling Technologies, Danvers, MA, USA) overnight at 4°C. Sections were washed with PBS 3 times for 5 min each, then incubated with either goat anti-rabbit IgG conjugated to R-Phycoerythrin (PE) or goat anti-rabbit IgG conjugated to FITC (Sigma, St. Louis, MO, USA) for 2 hours. Sections stained for acetylated alpha-tubulin were washed thrice with PBS, then incubated with rabbit anti-mouse acetylated-histone H3 (Lys9) conjugated with Alexa-Fluor488 (Cell Signaling Technologies, Danvers, MA, USA) overnight at 4°C. Sections were washed thrice with PBS, then mounted with Vectashield mounting media with DAPI (Vector Labs, Burlingame, CA, USA) and examined by a Zeiss LSM800 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

### Cytoplasmic and nuclear NF-κB in mesangial cells, in vitro

SV40-Mes13 mesangial cells (ATCC, Manassas, VA, USA) were cultured in complete media composed of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 Medium (75/25) with 14 mM HEPES (Corning Cellgro, Manassas, VA, USA) supplemented with 5% fetal bovine serum (HyClone, Logan, UT, USA) and 1% Penicillin/Streptomycin (Corning Cellgro, Manassas, VA, USA). Cells were incubated in a humidified, 5% $CO_2$ incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and treated with ACY-1083 for 2 hours

before being stimulated by 1 ug/mL LPS (Sigma, St. Louis, MO, USA) and 100 ng/mL IFN-γ (Cedarlane Laboratories Limited, Burlington, NC, USA) or DI water for 24 hours. HPMC is used as the vehicle control, similar to *in vivo* studies.

Cytoplasmic and nuclear protein fractions were isolated utilizing NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Protein lysates were quantitated and normalized by Bradford assay, then subjected to electrophoresis in 7.5% Criterion TGX Precast gels (Bio-Rad, Hercules, CA, USA) followed by protein transfer to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were thoroughly washed, blocked with TBS with 5% non-fat milk, then incubated with primary antibodies against NF-ϰB and beta-actin (Cell Signaling Technologies, Danvers, MA, USA) overnight. Membranes were then thoroughly washed and incubated with horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit IgG (secondary) antibodies (Cell Signaling Technologies, Danvers, MA, USA) followed by thorough washing then addition of Amersham ECL Detection Reagents (GE Healthcare Life Sciences, Logan, UT, USA). Membranes were then exposed and images acquired with a Kodak 4000MM imaging station (Carestream, Rochester, NY, USA).

### *Statistics*

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). Linear regression analysis was performed to analyze differences in urine protein levels, body weight, dsDNA autoantibodies, IgG isotypes, and serum cytokine levels over time (age). Differences between the slopes and intercepts of different treatment groups were determined by analysis of covariance (ANCOVA). Differences among groups with more than two conditions were analyzed using one-way ANOVA followed by further analysis using Tukey's multiple comparison tests. Differences between two means were assessed with unpaired two-tailed t-tests. A p-value $<0.05$ was considered statistically significant.

## Results

### *ACY-1083 inhibits HDAC6, selectively*

ACY-1083 was generated as a result of a drug discovery effort at Acetylon Pharmaceuticals to produce a highly selective inhibitor of HDAC6 (Fig. 1A). The compound was tested against recombinant HDACs in a biochemical assay as previously described (Bradner et al., 2010). ACY-1083 inhibits HDAC6 with a potency of 4 nM and HDAC1 (the next most affected target) with a potency of 961 nM (Fig. 1B). ACY-1083 does not potently inhibit HDAC8 or any class IIa or Class III HDAC (not shown). In a pharmacokinetic study ACY-1083 has a plasma half-life of 3.8 hours when injected intraperitonealy (IP). Dose levels for the *in vivo* experiments were chosen to achieve a plasma level high enough to inhibit HDAC6 but not high enough to inhibit HDAC1.

### *ACY-1083 treatment had no effects on mouse survival and body weight, and decreased proteinuria and splenic weight*

Body weight was monitored every other week before the initiation of treatment, then weekly until euthanasia at 34 weeks-of-age. There were no significant changes in body weights between the study groups over time.

Proteinuria was monitored weekly in all treatment groups as they aged (Fig. 2A). The urine protein score increased over time in HPMC-treated NZB/W F1 mice as expected. Treatment with ACY-1083 (0.03 mg/kg) did no significantly decrease proteinuria compared to the HPMC controls. However, 1 mg/kg and 3 mg/kg doses significantly decreased proteinuria compared to the HPMC-treated animals ($p<0.01$ and $p<0.001$, respectively).

Spleen weight was evaluated in all treatment groups after euthanasia at 34 weeks-of-age. A mild dose-response of decreasing splenic weight associated with increasing doses of ACY-1083 is noted, however it was not statistically significant (Fig. 2B). Spleens from the DEX-treated mice were significantly smaller than the

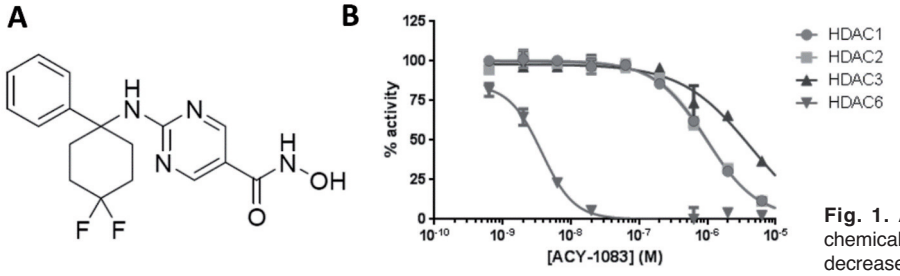


**Fig. 1.** ACY-1083 is selective for HDAC6. **A.** Basic chemical structure of ACY-1083. **B.** ACY-1083 selectively decreased HDAC6 activity with a potency of 4 nM.

other treatment groups ($p<0.001$).

### *Treatment with ACY-1083 had no effect on autoantibody levels and maintained lower levels of total IgG and $IgG_{2a}$ in the serum*

Serum was collected every 4 weeks beginning at 16 weeks-of-age and at the time of euthanization at 34 weeks-of-age for measurement of anti-dsDNA antibody levels. Similar to the vehicle control (HPMC) group ($p<0.001$), serum anti-dsDNA antibody levels increased as the mice aged in each treatment group ($p<0.001$). Mice treated with DEX had significantly lower anti-dsDNA antibodies levels ($p<0.001$) at 28, 32, and 34 weeks-of-age (Fig. 3A) compared to those treated with HPMC.

Levels of total IgG and $IgG_{2a}$ were evaluated in the sera of mice beginning at 20 weeks-of-age, then every 4 weeks, and again at the time of euthanasia at 34 weeks-of-age. There was no significant increase or decrease in the levels of total IgG or $IgG_{2a}$ over time in all treatment groups evaluated. After 13 weeks of treatment, serum levels of total IgG and $IgG_{2a}$ were lower in mice treated with ACY-1083 or DEX when compared to the HPMC group (Fig. 3B,C), which was statistically significant for $IgG_{2a}$ levels in mice treated with 1 or 3 mg/kg ACY-1083 or DEX ($p<0.05$).

### *ACY-1083 treatment decreased Th17 cells and Treg cells in the spleen*

Following euthanasia at 34 weeks-of-age, spleens were removed and single cell suspensions were obtained. Splenocytes were roughly quantitated by hemocytometer then evaluated by flow cytometric analysis for percentages of T cell subsets. There was a similar number of total splenocytes isolated from mice treated with HPMC ($M=177.3x10^6$, $SEM=32.07x10^6$) or ACY-1083 at 0.3 mg/kg ($M-142.8x10^6$, $SEM=19.45x10^6$), 1 mg/kg ($M=114.6x10^6$, $SEM=18.87x10^6$), or 3 mg/kg ($M=126.0x10^6$, $SEM=24.09x10^6$) doses. However, dexamethasone treatment significantly decreased

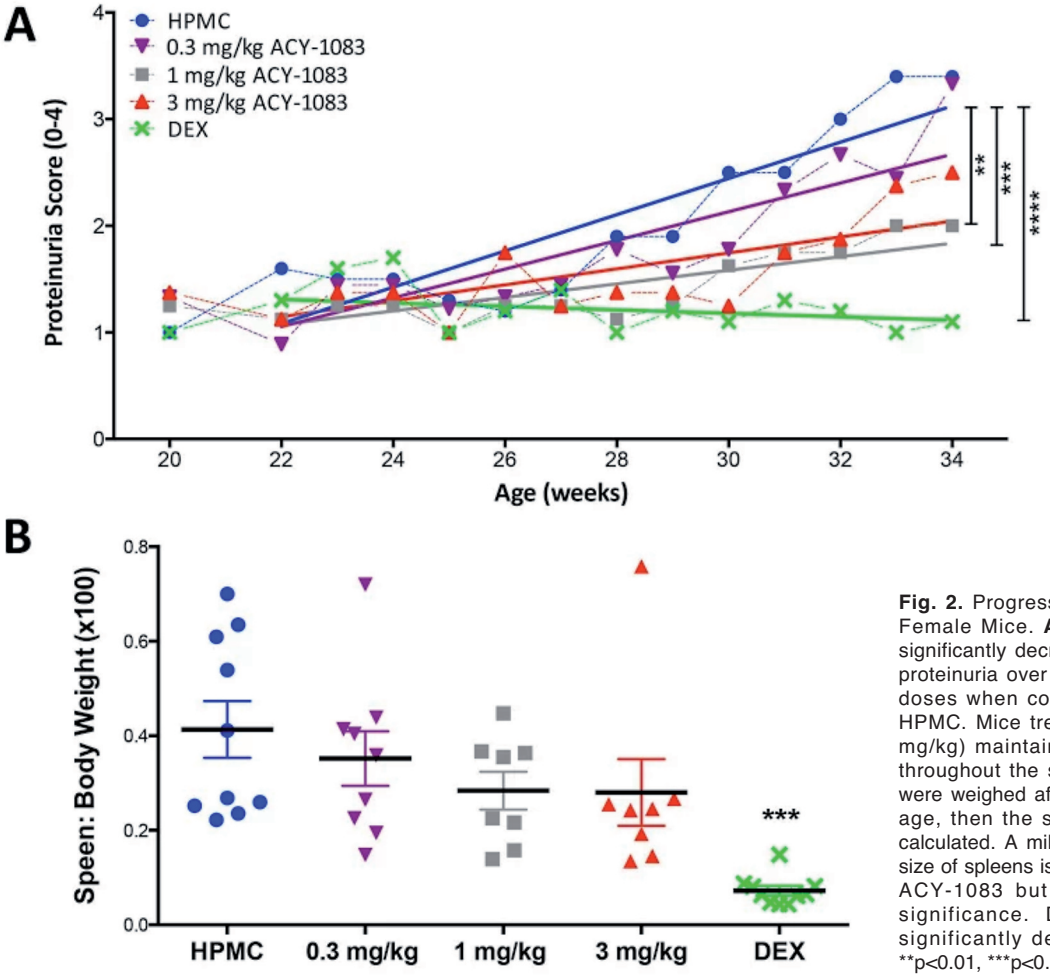


**Fig. 2.** Progression of Disease in NZB/W F1 Female Mice. **A.** Treatment with ACY-1083 significantly decreased the rate of elevation in proteinuria over time at 1 mg/kg and 3 mg/kg doses when compared to mice treated with HPMC. Mice treated with dexamethasone (2 mg/kg) maintained low urine protein scores throughout the study (34 weeks). **B.** Spleens were weighed after euthanasia at 34 weeks-of-age, then the spleen:body weight ratio was calculated. A mild dose-dependent decrease in size of spleens is noted with increasing doses of ACY-1083 but does not reach statistical significance. Dexamethasone (2 mg/kg) significantly decreased spleen size. ($n\geq8$; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

the total number of splenocytes (M=4.88x$10^6$, SEM=0.52x$10^6$) compared to HPMC ($p<0.001$).

There is a mild dose-dependent decrease in the percentage of $CD4^+IL\text{-}17^+ROR\gamma^+$ (Th17) cells in the spleen with increasing doses of ACY-1083 (Fig. 4A,B), which was statistically significant at the 3 mg/kg dose ($p<0.05$). Mice treated with DEX had significantly lower percentages of Th17 cells in the spleen ($p<0.01$) compared to vehicle control (HPMC) mice.

Treatment with ACY-1083, at all doses investigated

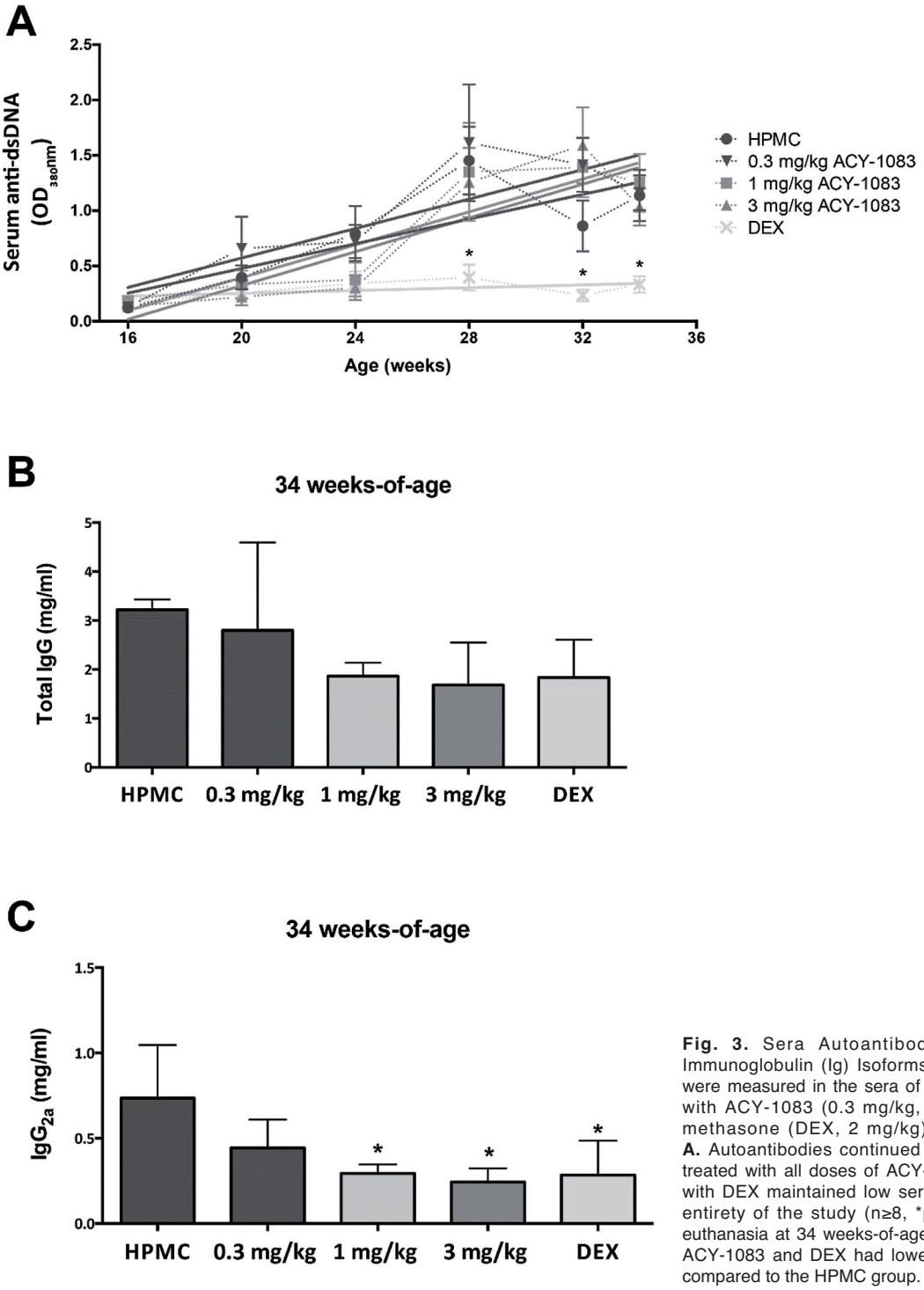


**Fig. 3.** Sera Autoantibodies Targeting dsDNA and Immunoglobulin (Ig) Isoforms. Anti-dsDNA and Ig isoforms were measured in the sera of NZB/W F1 female mice treated with ACY-1083 (0.3 mg/kg, 1 mg/kg, or 3 mg/kg), dexamethasone (DEX, 2 mg/kg) or vehicle control (HPMC). **A.** Autoantibodies continued to increase over time in mice treated with all doses of ACY-1083 and HPMC. Mice treated with DEX maintained low serum autoantibody levels for the entirety of the study ($n\geq8$, $^*p<0.05$). **B, C.** By the time of euthanasia at 34 weeks-of-age, mice treated with all doses of ACY-1083 and DEX had lower levels of total IgG and $IgG_{2a}$ compared to the HPMC group. (n=4, $^*p<0.05$)

mildly decreased the number of $CD4^+CD25^+Foxp3^+$ (Treg) cells in the spleen (Fig. 4C,D) in comparison to the vehicle control group, which reached statistical significance in the 3 mg/kg group ($p<0.05$). Furthermore, DEX treated animals showed a significantly decreased the percentage of Treg cells in

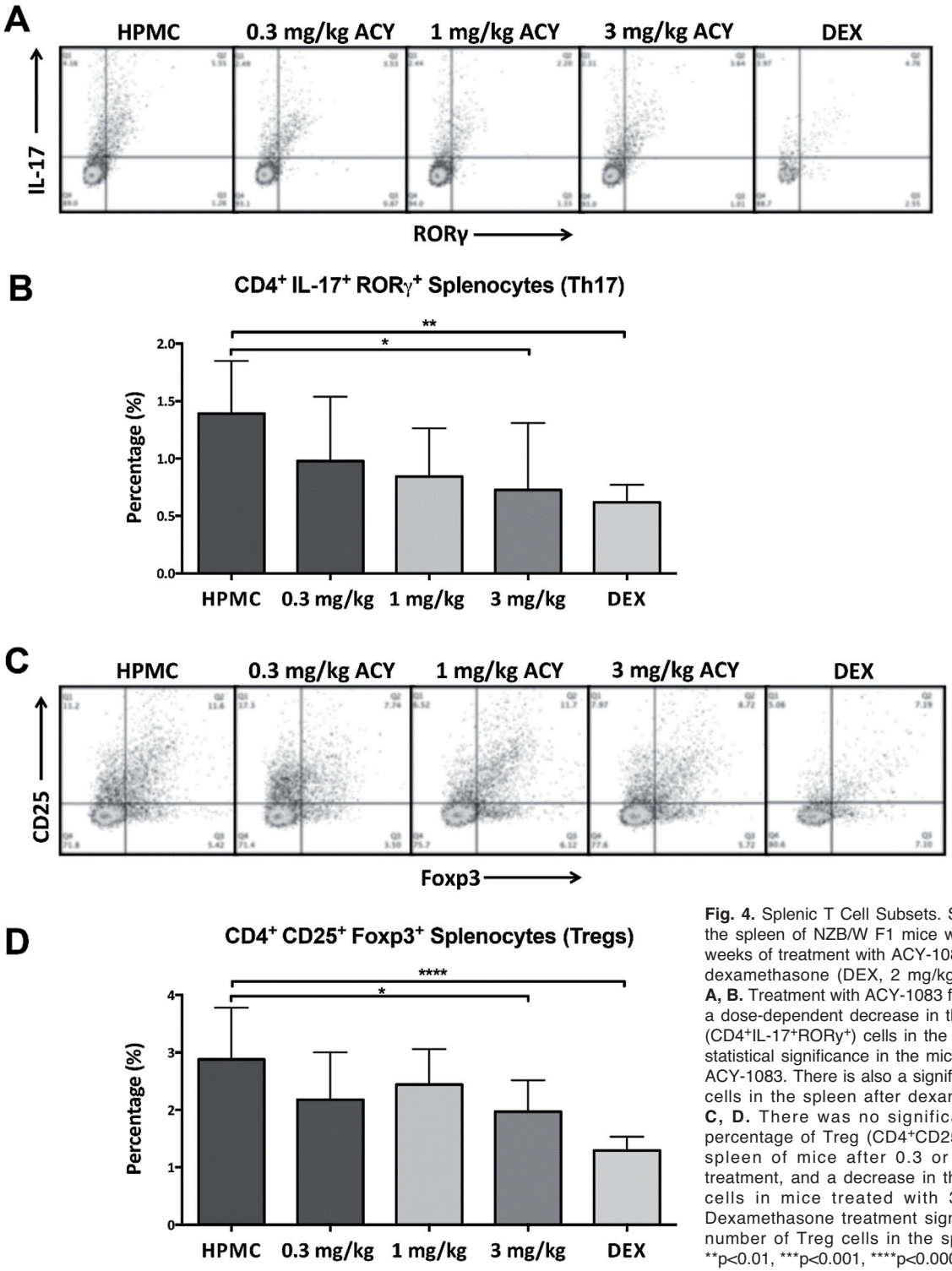


**Fig. 4.** Splenic T Cell Subsets. Subsets of T cells from the spleen of NZB/W F1 mice were assessed after 13 weeks of treatment with ACY-1083 (0.3, 1, or 3 mg/kg), dexamethasone (DEX, 2 mg/kg), or vehicle (HPMC). **A, B.** Treatment with ACY-1083 for 13 weeks resulted in a dose-dependent decrease in the percentage of Th17 ($CD4^+IL\text{-}17^+RORγ^+$) cells in the spleen, which reached statistical significance in the mice treated with 3 mg/kg ACY-1083. There is also a significant depletion of Th17 cells in the spleen after dexamethasone treatment. **C, D.** There was no significant alteration in the percentage of Treg ($CD4^+CD25^+Foxp3^+$) cells in the spleen of mice after 0.3 or 1 mg/kg ACY-1083 treatment, and a decrease in the percentage of Treg cells in mice treated with 3 mg/kg ACY-1083. Dexamethasone treatment significantly depleted the number of Treg cells in the spleen. ($n\geq8$; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

the spleen (p<0.0001).

### *Treatment of NZB/W F1 female mice with ACY-1083 decreased serum IL-12/IL-23 p40 levels in a dose-dependent manner and decreased serum TGF-β*

Serum was collected from mice every 4 weeks starting at 20 weeks-of-age and again at the time of euthanasia (34 weeks-of-age) for evaluation of cytokines involved in polarization of T cell subsets. Over time, serum TGF-β levels decreased in mice treated with ACY-1083 at either the 0.3 mg/kg (p=0.02) or 3 mg/kg (p=0.005) doses (Fig. 5A). TGF-β levels were maintained in mice treated with 1 mg/kg ACY-1083, vehicle control, or dexamethasone. After 13 weeks of treatment (Fig. 5B), TGF-β decreased in the serum of mice treated with ACY-1083 at 0.3, 1 or 3 mg/kg doses (not significant), and significantly increased in mice treated with dexamethasone (p<0.05).

Serum IL-12/IL-23 p40 levels progressively increased over time (Fig. 5C) in mice treated with vehicle control (p=0.01) and 0.3 mg/kg ACY-1083 (p=0.03). Mice treated with 1 mg/kg or 3 mg/kg ACY-1083 also experienced increasing serum levels of IL-12/IL-23 p40 over time (p<0.02). However, the rate of increase was reduced in both treatment groups, which reached statistical significance in the 3 mg/kg group (p=0.03). In contrast, IL-12/IL-23 p40 levels decreased over time in mice treated with dexamethasone (p<0.001). At the time of euthanasia (Fig. 5D), there was a dose-dependent decrease in serum IL-12/IL-23 p40 levels in mice treated with ACY-1083, although not statistically significant. Dexamethasone treatment, however, significantly reduced IL-12/-IL23 p40 levels in the serum of NZB/W F1 mice by the end of the study (p<0.05).

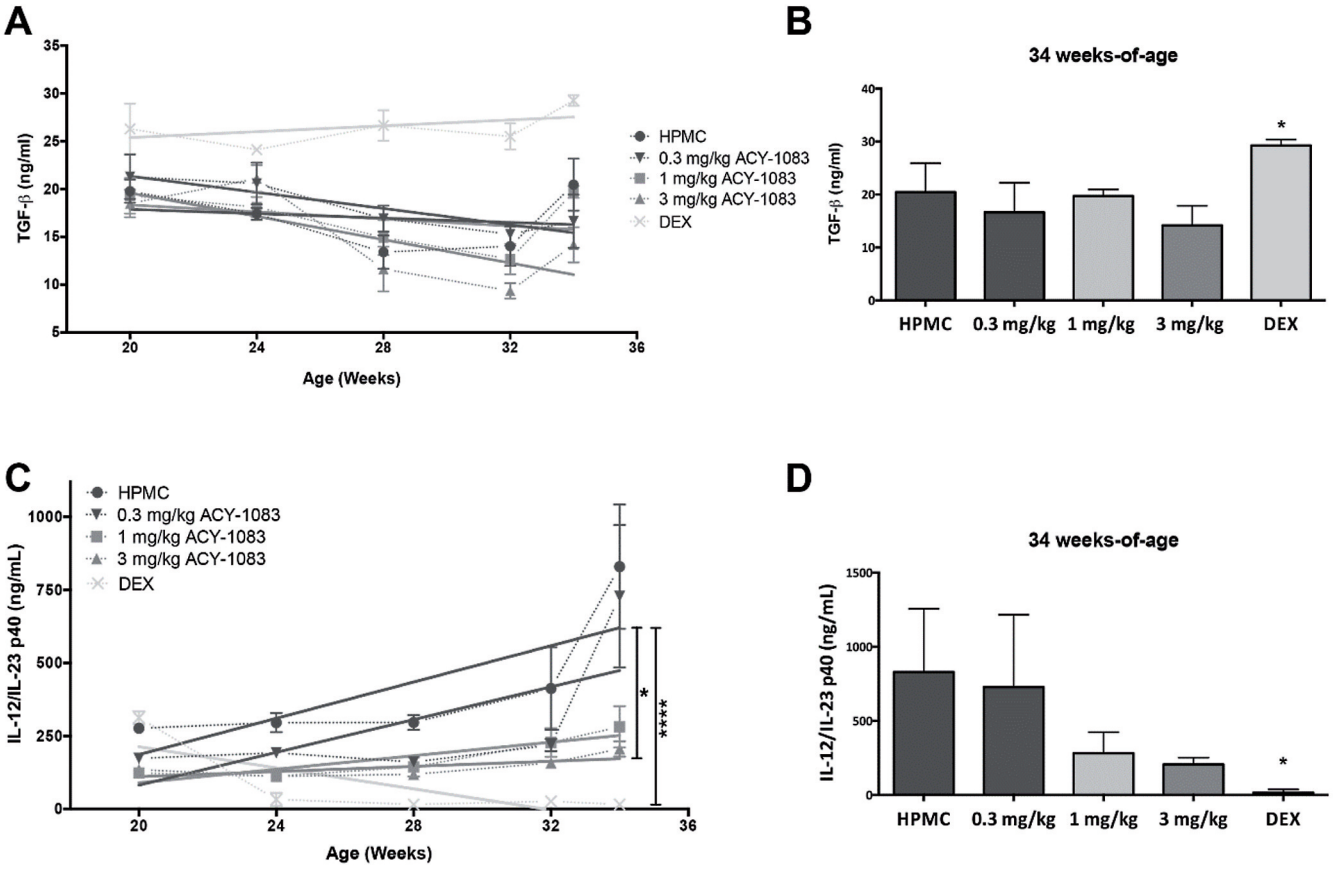


**Fig. 5.** Serum Levels of TGF-β and IL-12/IL-23. Serum cytokine levels were evaluated every 4 weeks from NZB/W F1 female mice treated with ACY-1083 (0.3, 1, or 3 mg/kg), vehicle (HPMC), or dexamethasone (DEX, 2 mg/kg). **A.** Serum TGF-β levels decreased over time in mice treated with 0.3, 1, or 3 mg/kg ACY-1083 and remained unchanged in mice treated with vehicle (HPMC) and dexamethasone (2 mg/kg). **B.** At the time of euthanasia (34 weeks-of-age), serum TGF-β levels were lower in mice treated with ACY-1083 (not statistically significant), and significantly increased in mice treated with dexamethasone (2 mg/kg). **C.** Serum IL-12/IL-23 p40 increased over time in mice treated with vehicle control and 0.3 mg/kg ACY-1083. Serum levels were maintained in mice treated with 1 mg/kg or 3 mg/kg ACY-1083, and serum levels decreased in mice treated with dexamathasone (2 mg/kg). **D.** By the end of the study (34 weeks-of-age), there is a dose-dependent decrease in serum levels of IL-12/IL-23 p40 in mice treated with ACY-1083, and a significant decrease mice treated with dexamethasone (2 mg/kg). (n≥3, *p<0.05, ****p<0.0001)

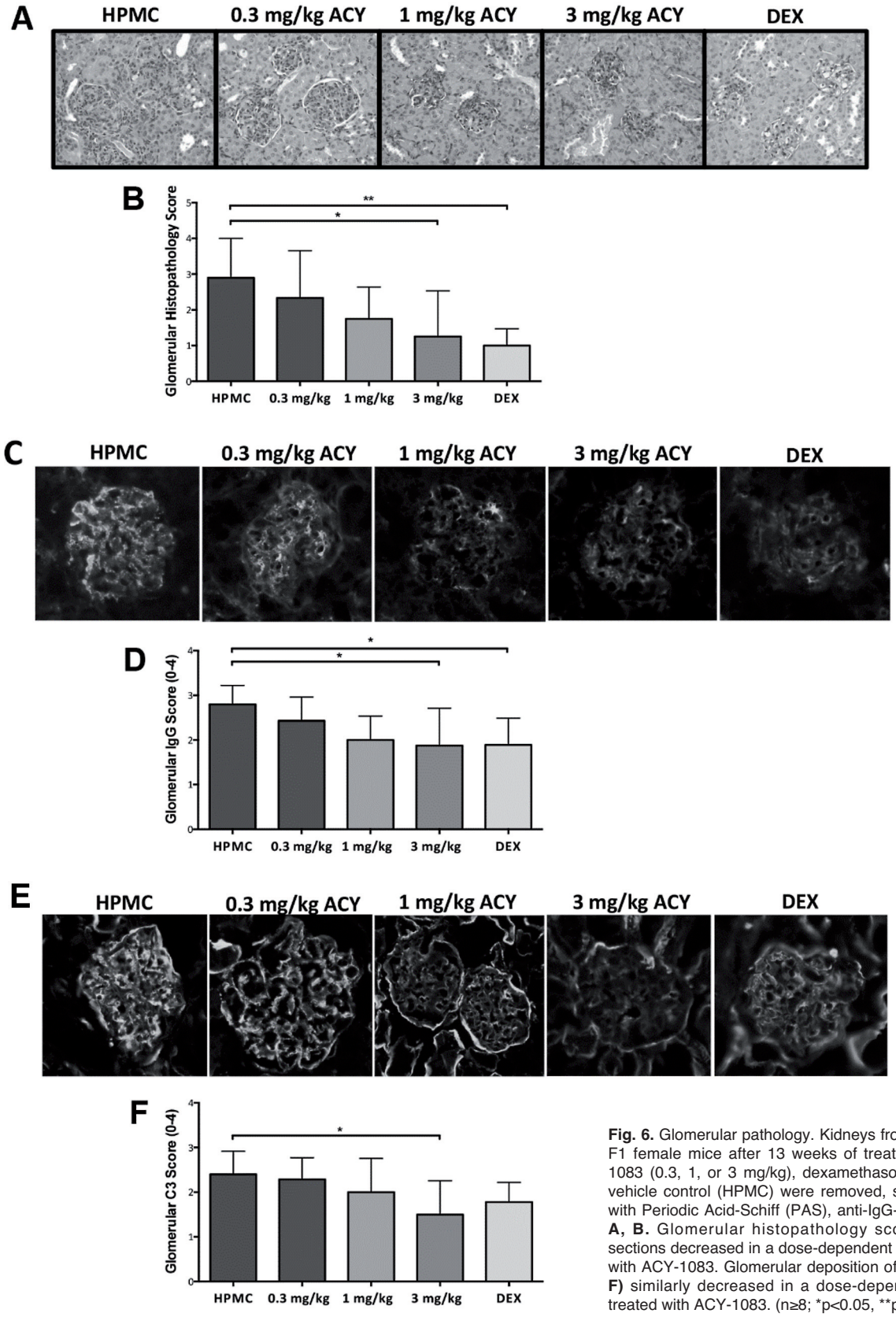


**Fig. 6.** Glomerular pathology. Kidneys from 34-week-old NZB/W F1 female mice after 13 weeks of treatment with either ACY-1083 (0.3, 1, or 3 mg/kg), dexamethasone (DEX, 2 mg/kg), or vehicle control (HPMC) were removed, sectioned, then stained with Periodic Acid-Schiff (PAS), anti-IgG-FITC, or anti-C3-FITC. **A, B.** Glomerular histopathology scores on PAS-stained sections decreased in a dose-dependent manner in mice treated with ACY-1083. Glomerular deposition of IgG **(C, D)** and C3 **(E, F)** similarly decreased in a dose-dependent manner in mice treated with ACY-1083. (n≥8; *p<0.05, **p<0.01)

### *Treatment with ACY-1083 decreased lupus nephritis as well as glomerular IgG and C3 deposition in a dose-dependent manner*

After euthanasia, kidneys were evaluated by histopathology and immunofluorescent microscopy for severity of lupus nephritis and accumulation of immune complexes within the glomerulus. Evaluation of PAS-stained sections showed a dose-dependent decrease in the glomerular histopathology score with increasing doses of ACY-1083 (Fig. 6A,B), which reached statistical significance in the 3 mg/kg ACY-1083 group ($p<0.05$). Mice treated with dexamethasone (DEX, 2 mg/kg) had the lowest glomerular histopathology scores ($p<0.01$) compared to the other treatment groups. Frozen kidney sections stained with fluorescent-tagged antibodies revealed dose-dependent decreases in the deposition of IgG (Fig. 6C,D) and C3 (Fig. 6E,F) within the glomeruli of ACY-1083-treated. Deposition of IgG and C3 were significantly decreased in mice treated with 3 mg/kg ACY-1083 ($p<0.05$) when compared to mice treated with the vehicle. Dexamethasone treatment also decreased glomerular deposition of IgG ($p<0.05$) and C3 (not statistically significant).

### *Treatment with ACY-1083 increased alpha-tubulin acetylation and decreased NF-κB in glomerular cells*

After 17 weeks of treatment, mice were euthanized and kidneys were collected and snap-frozen for immunofluorescent investigation of alpha-tubulin (α-Tub) and histone H3 (H3) acetylation at lysine residues

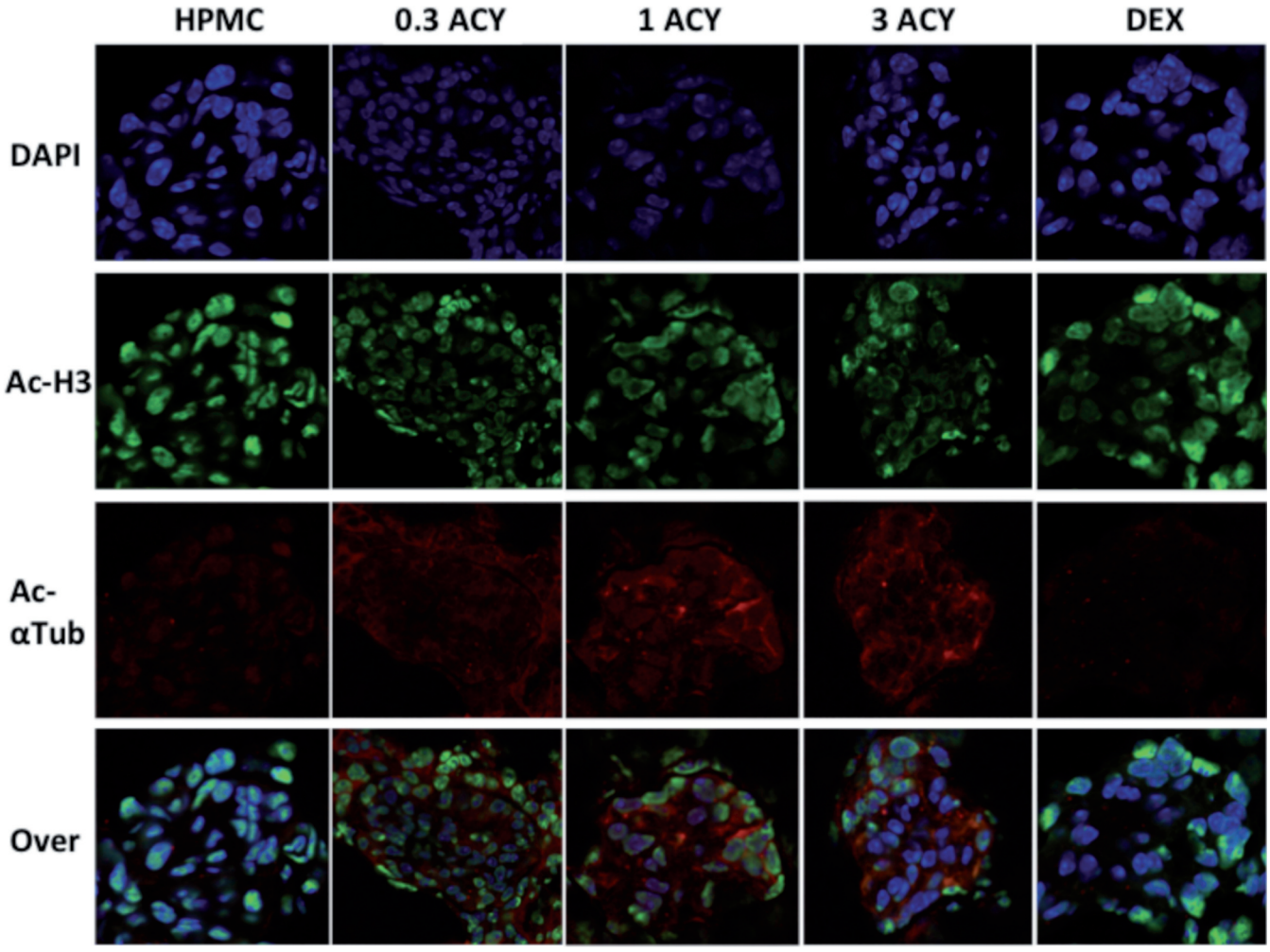


**Fig. 7.** Alpha-Tubulin and Histone 3 Acetylation in Glomerular Cells. Kidneys from NZB/W F1 female mice treated with ACY-1083 (0.3, 1, or 3 mg/kg), dexamethasone (DEX, 2 mg/kg), or vehicle (HPMC) for 13 weeks were removed, snap-frozen, sections, then evaluated for acetylation of histone 3 (H3) and alpha-tubulin (α-Tub) by immunofluorescence. Treatment with ACY-1083 increased the acetylation of α-Tub (R-Phycoerythrin) within glomerular cells. In comparison, there was no increase in α-Tub acetylation after treatments with HPMC or DEX. No differences are noted in H3 acetylation (AlexaFluor488) between all treatment groups (n=5).

40 and 9, respectively (Fig. 7), and NF-ϰB (Fig. 8A). In all treatment groups, staining for acetylated-H3 exhibited diffuse nuclear reactivity in all the glomerular cells, and there were no significant changes in either the staining pattern or amount of positive-staining nuclei between treatment groups. There was no specific

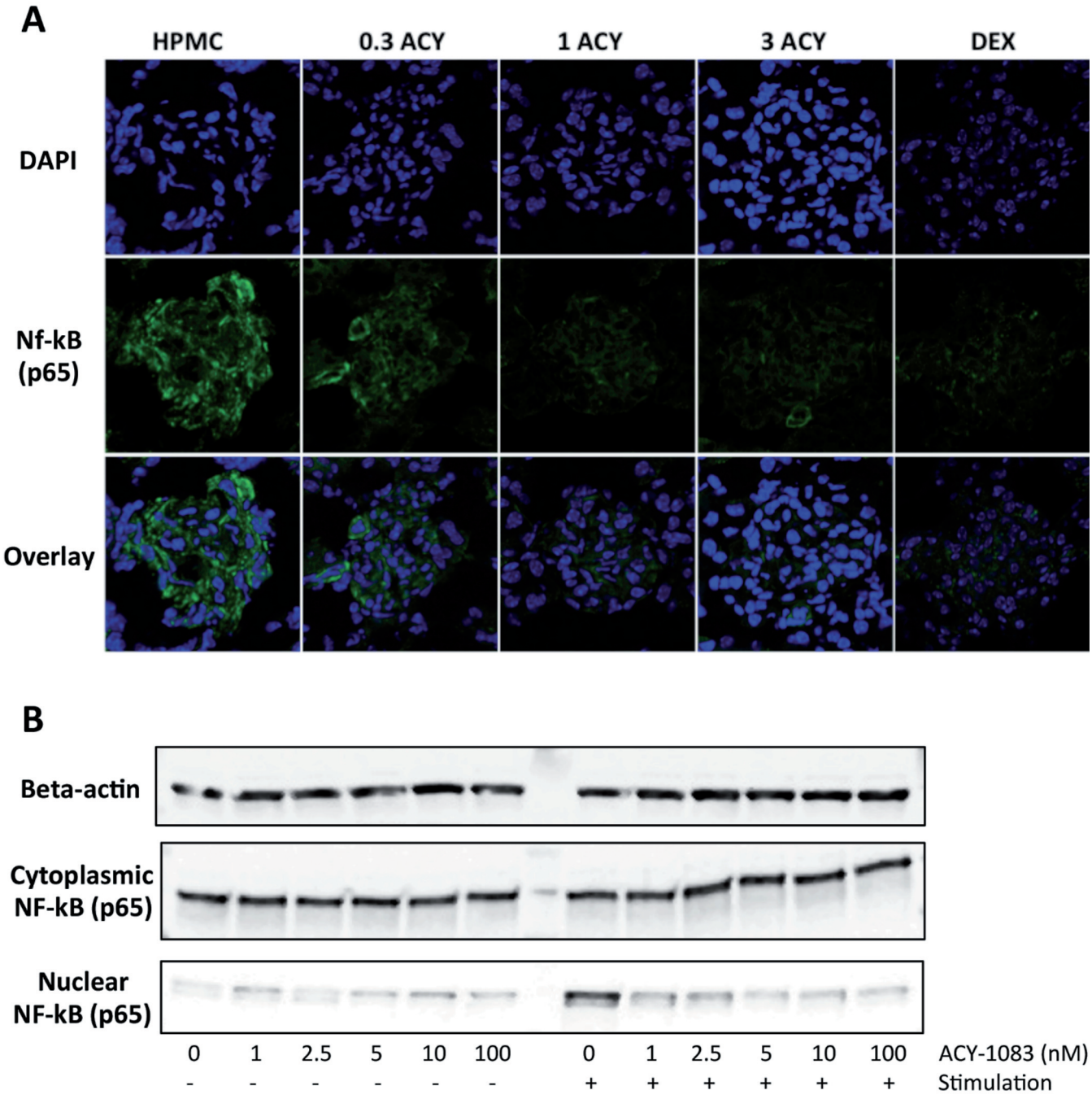


**Fig. 8.** NF-κB in glomerular and mesangial cells. **A.** Snap-frozen sections of kidneys from NZB/W mice treated with ACY-1083 (0.3, 1, or 3 mg/kg), dexamethasone (DEX, 2 mg/kg), or vehicle (HPMC) were immunofluorescently evaluated for NF-κB (p65). Glomerular cells from HPMC-treated mice exhibited the strongest and most abundant NF-κB staining, which decreased in a dose-dependent manner in mice treated with ACY-1083. There is limited NF-κB staining in glomerular cells from DEX-treated mice. (n=3) **B.** Mesangial (SV40/Mes13) cells were treated with varying concentrations of ACY-1083 for 2 hours, then stimulated with 1 ug/mL LPS and 100 ng/mL IFN-γ or DI water for 24 hours. Cytoplasmic and nuclear protein fractions were extracted and evaluated by western blot for NF-κB. Treatment with ACY-1083 decreased nuclear NF-κB after LPS/INF-γ stimulation in a concentration-dependent manner.

positive staining for acetylated α-Tub within the glomeruli of mice treated with HPMC, DEX, or 0.3 mg/kg ACY-1083. Minimal, punctate, multifocal cytoplasmic staining is noted in glomerular cells in mice treated with 1 mg/kg ACY-1083, and more numerous and prominent cytoplasmic reactivity is noted in mice treated with 3 mg/kg ACY-1083. Glomerular cells exhibit diffuse, granular, cytoplasmic immunoreactivity to NF-κB, which decreases in a dose-dependent manner with increasing doses of ACY-1083. There is minimal staining in glomerular cells of mice treated with DEX.

### *ACY-1083 decreased nuclear NF-κB protein in mesangial cells, in vitro*

An SV40/Mes13 murine mesangial cell line was utilized to determine how mesangial cells contribute to the changes noted *in vivo*. The cells were cultured with varying concentrations of ACY-1083 (0, 1, 2.5, 5, 10, or 100 nM) then stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ or DI water for 24 hours. Nuclear and cytoplasmic protein fractions were then extracted and evaluated by western blot to investigate NF-kB levels (Fig. 8B). The levels of NF-κB within cytoplasmic protein fractions remained unchanged in all treatment groups. However, nuclear NF-κB increased in mesangial cells stimulated with LPS/IFN-γ and decreased in a concentration-dependent manner in LPS/IFN-γ-stimulated cells treated with ACY-1083.

## Discussion

Histone deacetylases (HDACs) are increasingly being investigated for their roles in modulating inflammation and immunity (Shakespear et al., 2011), and therefore becoming therapeutic targets candidates for various inflammatory and autoimmune diseases (Dinarello et al., 2011). Due to the potential of adverse side effects associated with non-selective HDAC inhibition (Witt et al., 2009), we sought to determine the effects of specific HDAC6 inhibition on SLE disease in NZB/W F1 female mice. Glomerular cells from lupus-prone mice exhibit increased expression and activity of HDAC6, particularly in the cytoplasm, which is reduced when treated with a selective HDAC6i (ACY-738) (Regna et al., 2015a,b). One of the major functions of HDAC6, a cytoplasmic, class IIb HDAC, is controlling the acetylation status of alpha-tubulin (Zhang et al., 2003). Overexpression of HDAC6 results in hypoacetylation of tubulin, while inhibition of HDAC6 results in hyperacetylation (Zhang et al., 2003). In the current study, ACY-1083 increased acetylation of alpha-tubulin and did not alter the acetylation status of histone 3 within glomerular cells of NZB/W F1 mice confirming specific inhibition of HDAC6. Further, there was a concurrent decrease in SLE disease parameters after treatment with ACY-1083, including decreased proteinuria, lupus nephritis, and splenic weight. These data suggest that the acetylation status of alpha-tubulin plays a role in the progression of SLE disease in NZB/W F1 female mice.

To determine the contribution of glomerular changes from mesangial cells, SV40/Mes13 mesangial cells were cultured with ACY-1083 then stimulated with LPS and IFN-γ. Treatment with ACY-1083 increased acetylated alpha-tubulin and concurrently decreased nuclear NF-κB protein in mesangial cells. Another HDAC6-selective inhibitor, ACY-738, has also been shown to increase alpha-tubulin acetylation and decrease nuclear NF-κB in mesangial cells with a similar treatment and stimulation protocol (Regna et al., 2015a). NF-κB is constitutively activated in many autoimmune diseases, including SLE (Brown et al., 2008), and contributes to the inflammatory response in the kidney by regulating the expression of numerous important contributory genes (Sanz et al., 2010). The underlying connection between acetylated tubulin and NF-κB in the kidney is uncertain. One theory is that acetylated tubulin acts in concert with nephrin, a key protein involved in the slit diaphragm, to inhibit NF-κB in glomerular cells (Vieson and Reilly, 2015). Other mechanisms related to HDAC inhibition that may contribute to decreased NF-κB in glomeruli, independently of alpha-tubulin acetylation, include inhibition of heat shock protein (Hsp) 90 (Kovacs et al., 2005; Kekatpure et al., 2009; Shimp et al., 2012; Regna et al., 2015a,b), and modulation of Smad7 stability (Simonsson et al., 2005; Wang et al., 2005; Vieson and Reilly, 2015).

Increased serum immunoglobulin titers are some of the more reliable indicators of SLE activity in human SLE patients. Immunoglobulins of the IgG isotype directed against dsDNA, C1q, and nucleosomes (autoantibodies) are commonly analyzed titers because they correlate significantly with disease manifestations of SLE, particularly lupus nephritis (Manson et al., 2009; Mok et al., 2010; Yang et al., 2012). The NZB/W F1 mouse model shares many of these clinical manifestations, including markedly elevated serum levels of IgG antibodies that bind dsDNA and histones (Andrews et al., 1978; Theofilopoulos and Dixon, 1985). Deposition of these autoantibodies, particularly IgG anti-dsDNA and $IgG_{2a}$ in glomeruli is one of the major contributors to the pathogenesis of lupus nephritis in both human patients (Rothfield and Stollar, 1967) and in lupus mouse models (Andrews et al., 1978; Ebling and Hahn, 1980). Importantly, $IgG_{2a}$ is considered one of the more pathogenic subclasses of immunoglobulin due to its unique ability to bind and activate the Fcγ receptor FcγIV (Nimmerjahn et al., 2005). We have previously shown that treatment with a class I and II HDAC inhibitor (ITF2357) significantly decreased anti-dsDNA antibodies, as well as total IgG and $IgG_{2a}$ in the serum of NZB/W mice (Regna et al., 2014). In our current study, inhibition of HDAC6 with ACY-1083 had no significant effect on the serum levels of anti-dsDNA antibodies in NZB/W F1 mice. In contrast, serum levels of total IgG and $IgG_{2a}$ did not significantly increase over time, and were lower in mice treated with ACY-1083.

The development of autoantibodies is a mainstay feature of SLE, and contributes to lupus nephritis by cross-reacting with renal antigens, indirectly binding to nuclear material in glomerular basement membranes, or forming immune complexes that will circulate and become entrapped within glomeruli (Yap and Lai, 2015). However, there is a discordance between serum levels of anti-dsDNA antibodies and lupus nephritis (Deshmukh et al., 2006), which has been reported in humans (Alba et al., 2003) and various lupus-prone mouse strains (Kong et al., 2003; Singh et al., 2003). Similarly, we saw a decrease in lupus nephritis and glomerular IgG deposition with increasing doses of ACY-1083 despite increasing levels of anti-dsDNA in the serum of NZB/W F1 female mice. This discrepancy may be due to the differential ability of different subclasses of these antibodies to deposit in the kidney and incite an inflammatory response (Bagavant et al., 2002). Therefore, determining the subclass, cross-reactivity with different cellular antigens, and the ability to deposit in the kidneys is more important in determining the pathogenic potential of anti-dsDNA antibodies than the overall serum titer, alone (Deshmukh et al., 2006).

Elevated IL-17 production has been reported to be instrumental in the inflammatory cascade in SLE patients (Martin et al., 2014) and also plays a role in the pathogenesis of lupus in various mouse models including NZB/W F1 mice (Hou et al., 2012). Elevated Th17 cells have been detected in the peripheral blood of human patients with lupus nephritis and correlated with disease activity index scores (Chen et al., 2012; Xing et al., 2012). Our laboratory has previously shown decreased splenic Th17 cells with concomitant reduction in SLE disease parameters in MRL/lpr mice after treatment with the non-selective HDACi, ITF2357 (Regna et al., 2014). In the current studies, we observed a similar dose-dependent decrease in the number of Th17 cells in the spleen. This decrease mirrors the dose-dependent decrease in lupus nephritis pathology in ACY-1083-treated mice suggesting that HDAC6 inhibition decreases lupus nephritis in NZB/W F1 mice by decreasing Th17 cells in the spleen.

In addition to the decreases in splenic Th17 cells and lupus nephritis, pharmacologic HDAC 6 inhibition in NZB/W F1 mice resulted in a dose-dependent decrease in serum IL-12/IL-23. IL-23 is a heterodimer that shares a common p40 subunit with IL-12 as well as some similar signal-transduction components (Lankford and Frucht, 2003). However, IL-23 is different from IL-12 because it is paramount in the production of Th17 cells and the production of pro-inflammatory cytokines IL-22 and IL-17 (Chen et al., 2007). In human SLE patients, serum IL-23 levels and the number of Th17 cells are significantly elevated compared to controlled subjects suggesting that the IL-23/IL-17 axis is important in the inflammatory status in SLE (Wong et al., 2008). Additionally, glomerular IL-17 and IL-23 expression levels positively correlate with SLE activity and renal histopathology scores in lupus nephritis patients (Chen et al., 2012). Treatment of dendritic cells isolated from the peripheral blood of humans with the non-specific HDAC inhibitors trichostatin A or suberoylanilide hydroxamic acid (SAHA) reduced IL-23 and IL-12 production after stimulation with LPS and INF-γ (Bosisio et al., 2008).

Multiple reports have shown decreased numbers and function of CD4+ regulatory T (Treg) cells in patients with active SLE (Lyssuk et al., 2007; Valencia et al., 2007; Suen et al., 2009). Also, restoring or increasing these Treg populations has decreased the severity of disease in SLE patients (Barath et al., 2007) and in NZB/W mice (Reilly et al., 2008). In particular, we have previously reported that HDAC inhibitors (Trichostatin A and ITF2357) increased regulatory T cell populations, which correlated with decreased disease in NZB/W mice (Reilly et al., 2008; Regna et al., 2014). In the current study, we did not see an increase in the Treg population despite reduction in disease severity in NZB/W mice. Instead, CD4+CD25+Foxp3+ Tregs were slightly decreased in mice treated with the HDAC6 inhibitor. Similarly, treatment with the HDAC inhibitor Trichostatin has also been reported to decrease the number of Tregs in C57BL6 mice (Liu et al., 2010). This decrease in Treg cells may be due to a global suppression of T cell development and HDAC6 inhibition regulates several other transcription factors necessary for T cell development. Along with the decrease in splenic Tregs, we also saw a decrease in serum TGF-β levels. TGF-β promotes expression of Foxp3 (Apostolidis et al., 2011b; Zhou et al., 2011) and naïve $CD4^+$ T cell differentiation into Treg cells (Zhou et al., 2011). Based on our findings, reduction of disease in NZB/W F1 female mice after treatment with the selective HDAC6 inhibitor ACY-1083 may be attributed to alterations in the number and function of cell populations other than regulatory T cells.

### *Conclusions*

Lupus nephritis is a major contributor to the morbidity and mortality in SLE patients (Houssiau and Lauwerys, 2013) for which a specific, safe, and effective treatment regimen is not currently available. Pharmacologic inhibition of HDACs offers these desired treatment characteristics, and has consistently decreased renal disease in lupus-prone mice (Mishra et al., 2003; Reilly et al., 2004, 2008; Regna et al., 2014). Concerns for adverse side effects associated with non-selective HDAC inhibition (Witt et al., 2009; Cantley and Haynes, 2013) directed these studies towards specifically targeting HDAC6. Similar to non-specific HDAC inhibition, these studies show that selective inhibition of HDAC6 decreases SLE disease in lupus-prone mice by decreasing lupus nephritis. Within the glomeruli, HDAC6i increased acetylation of alpha-tubulin and decreased nuclear NF-κB. A connection between alpha-tubulin and NF-κB has not been clearly elucidated and renders further investigation. In comparison to other

studies in our laboratory utilizing HDAC inhibitors (Mishra et al., 2003; Reilly et al., 2004, 2008; Regna et al., 2014), the current study shows no significant alterations in the amount of Treg cells and a decrease in Th17 cells. These data suggest that ACY-1083 may decrease disease by mechanisms targeting predominantly Th17 cells, instead of Tregs. Not only do Th17 cells play a role in the pathogenesis in SLE (Martin et al., 2014), they are also implicated in multiple inflammatory and autoimmune diseases (Bedoya et al., 2013). Therefore, further studies are warranted to further investigate the role ACY-1083 plays, as a specific HDAC6 inhibitor, in Th17 cell pathobiology.

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