HDAC6 Deletion Decreases Pristane-Induced Inflammation and Lupus

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ABSTRACT

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder often occurring in women of childbearing age. SLE is characterized by pathogenic antibody production and inflammation. Histone deacetylase (HDAC) 6 is a class IIb histone deacetylase member. HDAC6 has the ability to catalyze the removal of acetyl groups from lysine residues on non-histone proteins. It has been observed that in lupus mouse models, specific HDAC6 inhibition reduces inflammation. Administration of pristane, a naturally occurring hydrocarbon oil, can result in lupus-like illness and persistent inflammation. In our studies, 0.5 ml of pristane or phosphate buffered saline (PBS) was given intraperitoneally into sex- and age-matched wild type (WT) and HDAC6$^{-/-}$ mice on the C57BL/6 background at 8–12 weeks of age, and mice were euthanized 10 days or 8 months later. The animals were assessed as they aged. Short-term pristane treatment promoted the population of CD11b^{+}Ly6C^{++} inflammatory monocytes and CD11b^{+}Ly6G^{+} neutrophils. Peritoneal recruitment of these inflammatory monocytes and neutrophils in HDAC6$^{-/-}$ mice was significantly decreased compared to the WT mice. Pristane treatment also induced the interferon (IFN) signature genes as determined by RT-qPCR. Furthermore, IFN signature genes were decreased in HDAC6$^{-/-}$ mice compared to the WT mice. In vitro studies in J774 cells revealed that the selective HDAC6 inhibitor (ACY-738) increased acetylation of NF-κB while increasing STAT1-phosphorylation which caused the synthesis of inducible nitric oxide synthase (iNOS) in cells activated by LPS

and IFN-γ. Long-term pristane treatment induced proteinuria in female mice although there were no significant differences between WT and HDAC6$^{-/-}$ animals. HDAC6 deletion significantly inhibited anti-double stranded (ds) DNA IgG level compared with WT mice. Moreover, HDAC6 deletion decreased some lymphocyte populations like T-helper 17 (Th17) cells after pristane treatment while not affecting other cell populations, such as regulatory T cells, total T cells, B cells, and plasma cells. Taken together, these results demonstrate that although HDAC6 inhibition may inhibit some inflammatory pathways, others remain unaffected.

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GENERAL AUDIENCE ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disorder that affects the entire body. It's more common in women of childbearing age. SLE involves the immune system attacking healthy tissues leading to inflammation. One hallmark is the production of autoantibodies. SLE can affect various organs and tissues, causing symptoms like joint pain, skin rashes, and fatigue. Histone Deacetylase 6 (HDAC6) is a specific protein involved in modifying protein function by removing acetyl groups. In lupus, inhibiting HDAC6 has been reported to reduce inflammation. Pristane, a natural oil, can trigger lupus-like symptoms and persistent inflammation. In our studies, we investigated the role of HDAC6 on pristane induced lupus. We used both normal mice (WT) and mice lacking HDAC6 ($HDAC6^{-/-}$). Mice were injected with pristane or a control solution. After 10 days or 8 months, we assessed the mice. We found 10-day pristane treatment increased inflammatory monocytes and neutrophils. $HDAC6^{-/-}$ mice had fewer of these immune cells in their peritoneum. Pristane also activated interferon genes, but this effect was reduced in $HDAC6^{-/-}$ mice. In our studies, a HDAC6 inhibitor increased the acetylation of NF-κB (that would dampen inflammation). Eight-month pristane administration induced proteinuria (protein in urine) in female mice, and this is true for both WT and $HDAC6^{-/-}$ mice. However, HDAC6 deletion decreased autoantibody levels and a pro-inflammatory cell type called Th17. In conclusion, HDAC6 plays a role in lupus-related inflammation.

Targeting HDAC6 might be a potential therapeutic approach for managing lupus symptoms.

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CHAPTER 1

HDAC6 and Systemic Lupus Erythematosus

Introduction

Systemic lupus erythematosus (SLE or lupus) is a systemic autoimmune disorder that often occurs in women of childbearing years [1]. Lupus is characterized by extreme pathogenic autoantibody production and inflammation. Lupus patients have a lot of different immunology abnormalities, which contribute to disease pathology and progression. In spite of the disease heterogeneity, type I interferons (IFN) play an important role as essential pathogenic cytokines in lupus. In lupus, type I IFN signature correlates with disease severity [2; 3]. Type I interferon production can be triggered and driven by extracellular DNA. For instance, when dead or dying cells release their genomic DNA, toll-like receptor 9 (TLR9) identifies it in endosomes and stimulates the production of IFNα in macrophages. Pristane is a natural saturated terpenoid alkane and is a well-known compound that can be used to induce lupus-like disease in mice. In our lupus model, C57BL/6 mice were injected intraperitoneally with single-dose pristane. In accordance with the prior reports, the mortality caused by pristane administration can be as high as 20% in C57BL/6 mice [4]. C57BL/6 mice will develop lupus-like symptoms after single dose of 0.5mL pristane injection intraperitoneally. The symptoms are characterized by excessive autoantibodies and glomerulonephritis [5]. Moreover, several weeks after pristane administration C57BL/6 mice may develop pulmonary hemorrhage [6]. Alveolar hemorrhage could result in early mortality within 4 weeks, which only happens in C57BL/10 and C57BL/6 mice. After pristane injection, macrophages and neutrophils are recruited to the lungs. The hemorrhage typically starts several days later

and reaches a peak approximately 2 weeks after injection. By 4 weeks after pristane injection, alveolar hemorrhage resolves in most mice [5]. After several months, anti-nuclear autoantibodies are increased, resulting in immune complex-mediated glomerulonephritis, similar to lupus-like disease which occurs in humans [7]. Additionally, after eight months of pristane administration, C57BL/6 mice exhibited hypercellularity and moderate expansion of mesangial in the glomeruli [4]. Finally, mice given pristane administration generated high levels of autoantibodies, including anti-dsDNA IgG and anti–Sjögren's-syndrome-related antigen A (anti-SSA) autoantibodies all of which make the pristane mouse model applicable to study lupus.

Histone deacetylase 6 (HDAC6) is a member of class IIb histone deacetylase. HDACs can catalyze the removal of acetyl groups from the lysine residues of histones and other nonhistone proteins. As a result, HDACs can modify many biological functions, like transcription, cell signaling, cell motility, cell survival, inflammation, protein degradation, and angiogenesis [8]. HDACs may have a direct impact on inflammation since it is becoming more and more obvious that they play a basic role in the control of genes that are extensively expressed during inflammation. For example, HDAC6 inhibition encourages Treg-dependent reduction of inflammation and autoimmune [35]. Despite the fact that Sirt1, HDAC9, and HDAC6 all deacetylate Foxp3, the impact of each protein on the transcription factors that control Foxp3 gene expression varies. These results imply that the management of autoimmunity may benefit from the use of a combination strategy of HDAC inhibition. Based on the function and structure studied initially in yeast, HDACs have four classes. Class I HDACs are mainly located in the cell nucleus and exist

in many different types of cells. HDAC1, 2, 3, and 8 are included in class I HDAC. Class I HDACs are primarily responsible for repressing gene transcription by targeting histone proteins. Class IIa and class IIb are two subgroups of class II HDAC. HDAC4, 5, 7, and 9 are included in class IIa HDAC. HDAC6 and 10 are included in class IIb HDAC. Based on domain organization, it is possible for class II HDACs to move between the cytoplasm and the cell nucleus [8]. In previous studies, it has been demonstrated that HDAC6 inhibition leads to the improvement of nephritis in lupus-prone mice. HDAC6 inhibition has been shown to alter NF-κB signaling and decrease the inflammatory response. Taken together these results suggest a therapeutic potential of HDAC6 inhibition to treat lupus disease [9; 10].

The pathogenesis of systemic lupus erythematosus

The inflammatory, chronic autoimmune illness known as systemic lupus erythematosus (SLE) or lupus is marked by a strong production of autoantibodies and the formation of immune complexes. In the United States of America, lupus occurs in around 1.8–7.6 people per 100,000, and 90% of patients are women [11]. The cause of lupus is not fully understood. The underlying mechanisms that cause and maintain disease are sophisticated. It is thought that certain susceptibility genes, epigenetic factors, hormones, and several environmental factors are associated with lupus. An example of the importance of genetic factors is that among monozygotic twins if one develops lupus, the twin will also have a 24% chance of developing disease [31]. Potential environmental risk factors for lupus include smoking, sunlight, vitamin D deficiency, medication, and some infections. These environmental factors can increase the risk of lupus disease. The

mechanism of lupus includes autoantibodies that target one's own tissues and organs initiating an inflammatory immune response. Additionally, individuals with lupus often produce antibodies against double-stranded DNA. The most common autoantibodies seen in lupus are anti-nuclear antibodies, such as anti-dsDNA antibodies. Anti-dsDNA antibodies bind DNA and then form immune complexes that can become deposited in the glomerulus of the kidney triggering an inflammatory response. Continued glomerulonephritis can result in renal insufficiency leading to kidney failure. Lupus can also affect other organs, such as the central nervous system, blood vessels, heart, and lungs. Lupus patients also have a significantly elevated risk of cardiovascular disease, which can also lead to myocardial infarction and heart failure resulting in increased mortality. It is difficult to diagnose lupus and the diagnosis must be based on the combination of experiment tests and patient symptoms. Sadly, there is no known cure for lupus. There are both experimental and symptomatic therapies available to treat lupus including NSAIDs, corticosteroids, methotrexate, and hydroxychloroquine. Recently belimumab a humanized monoclonal antibody that inhibits B-cell activating factor has been approved by the FDA for lupus. Additionally, rituximab an anti-CD20 antibody has also been shown to decrease lupus disease. Although many of the treatments do provide relief they are often given with corticosteroid therapy whose long-term usage has unwanted adverse side effects. Additionally, not all therapies work in lupus patients [32,34]. Lupus patients' life expectancy is lower than normal people, however, eighty to ninety percent of patients can expect a normal life with effective treatment [33].

The role of HDAC6 in systemic lupus erythematosus

HDAC6 participates in several essential molecular pathways and exhibits a diverse set of biological functions in different cell types [12; 13]. HDAC6 affects numerous cellular activities in the cytoplasm and many of these are involved in inflammation and the immune response. For instance, HDAC6 affects cell signaling, cell motility, cell survival, and degradation of protein [12; 14]. Various HDACs knockout mice classes cannot survive and have some severe developmental drawbacks. However, mice without HDAC6 are still viable, fertile, develop normally, and have a normal life expectancy. Moreover, HDAC6 knockout mice have normal lymphoid development although the immune response is affected moderately [15]. Previous work on HDAC6 has mainly focused on neurodegenerative diseases and cancer [16; 17; 18; 19; 20; 21; 22; 23; 24]. Recently it has been shown that HDAC6 inhibition could alleviate lupus disease development. It has been documented that lupus-prone mice's glomerular cells, B cells, and T cells all had higher levels of HDAC6 expression and activity [25]. ACY-738 is a highly selective HDAC6 inhibitor [26]. In a previous study, at the initial stage of the lupus disease, the use of a selective HDAC6 inhibitor on NZB/W mice significantly decreased IFN-alpha production. HDAC6 inhibitor treatment also decreases autoreactive B cell responses [36]. Taken together, these results show that selective HDAC6 inhibition improves renal functions and decreases disease development in a lupus-prone mice model at the early stage of the disease. In our studies, we used HDAC6 knockout mice on C57BL/6 background. We injected pristane into the mice to induce lupus in these mice. Additionally, we aimed to clarify the molecular and cellular mechanism of HDAC6 gene

deletion in lupus. Our studies showed how HDAC6 knockout reduces several parameters of lupus disease in the pristane-induced lupus mouse model.

Type I interferons

Type I interferons (IFNs) are polypeptide cytokines released by immune cells when stimulated. These cytokines are involved in T-cell responses, immunoregulation, tumor cell identification, and inflammation. It is not fully understood why type I IFN expression is excessive in lupus. Several pathways regulating the production of type I IFN have been identified. TLR3 recognizes viral dsRNA while TLR4 recognizes LPS. TLR3 and TLR4 are innate immune receptors stimulating the secretion of type I IFN through TIR-domain-containing adapter-inducing interferon-β (TRIF) [27]. Single-stranded RNA is recognized by TLR7/8, and unmethylated CpG DNA is recognized by TLR9. TLR7/8 and TLR9 use myeloid differentiation factor 88 (MyD88) to control the production of type I IFN [28]. It has shown that pristane can induce the production of type I IFN, recruitment of monocytes and neutrophils, and generation of autoantibodies. These activities elicited by pristane solely depended on TLR7 and MyD88 pathways [29;37]. HDAC6 inhibition may protect mice from inflammation via TLR7 and MyD88 pathways. Therefore, we would compare the acetylation level of TLR7 and MyD88 in peritoneal cells from WT and HDAC6$^{-/-}$mice, as well as their mRNA and protein expression levels.

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CHAPTER 2

HDAC6 Deletion Decreases Pristane-Induced Inflammation

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by excessive inflammation and production of pathogenic antibodies. Histone deacetylase 6 (HDAC6) is a class IIb histone deacetylase. It has been reported that selective HDAC6 inhibition decreases inflammation in lupus mouse models. In this study, sex and age-matched wild type (WT) and HDAC6$^{-/-}$ mice were administered 0.5 ml pristane or PBS intraperitoneally (i.p.) at 8-12 weeks of age and were euthanized 10 days later. At sacrifice, body weight and spleen weight were measured, sera were collected, and splenocytes and peritoneal cells were harvested for flow cytometry. We found pristane treatment increased spleen weight in HDAC6$^{-/-}$ mice, but not in WT mice. Flow cytometry results showed that there was no difference in CD4^{+}T cell, CD8^{+} T cells, and total B cell populations in the spleen. Activated T and B cells, as well as plasma cells, were increased by HDAC6 deletion. Pristane administration promoted the population of CD11b^{+}Ly6C^{++} inflammatory monocytes and CD11b^{+}Ly6G^{+} neutrophils. Peritoneal recruitment of these inflammatory monocytes and neutrophils in HDAC6$^{-/-}$ mice was significantly decreased compared to the WT mice. Pristane administration also induced the interferon (IFN) signature genes in peritoneal cells from both WT and HDAC6$^{-/-}$ mice as determined by RT-qPCR. Furthermore, IFN signature genes were not affected in HDAC6$^{-/-}$ mice compared to the WT mice. In vitro studies in J774 cells revealed that the selective HDAC6 inhibitor (ACY-738) increased acetylation of NF-κB while increasing Stat1-phosphorylation which caused the synthesis of inducible nitric oxide synthase (iNOS) in cells activated by LPS and IFN-γ. Taken together, these results demonstrate

that although HDAC6 inhibition may inhibit some inflammatory pathways, others remain unaffected.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease in which body tissues and organs are attacked by its own immune system [1]. Autoantibodies are generated, resulting in the formation of circulating antigen-antibody complexes that become lodged in tiny capillaries and different organ systems, including the kidney and skin [2]. Histone acetylation has been shown to play a critical role in controlling gene expression [3; 4]. The process of acetylation of histone proteins frequently leads to enhanced transcription, whereas deacetylation is linked to gene repression [5]. It has been previously documented by ourselves and others that HDAC inhibitors are effective in the treatment of cancer, hypersensitivity, and autoimmune disease, specifically SLE [6]. However, the use of pan-HDAC inhibitors has shown significant adverse effects limiting the use for chronic conditions [7]. Histone deacetylase (HDAC) 6 is a cytoplasmic class IIb HDAC [8; 9]. HDAC6 modulates protein degradation by deacetylating non-histone proteins, such as heat shock protein (HSP 90), α-tubulin, and NF-κB, and has been shown to play a role in modulation of immune response [9]. It has been demonstrated that overexpression of HDAC6 in macrophages significantly increased the expression of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α via the upregulation of AP-1 and NF-κB signaling pathways [10]. HDAC6 inhibition has been shown to interfere with the NF-κB signaling pathway and decrease inflammation by increasing acetylation of NF-κB resulting in decreased nuclear translocation of NF-κB [11; 12].

One commonly used animal model to study lupus is the pristine-induced disease model. Naturally occurring pristane (2, 6, 10, and 14 tetramethylpentadecane, TMPD) is a

branched alkane that is present in vegetables and mineral oil [13]. When injected into the peritoneum (IP) in mice, pristane induces lupus-like symptoms over time, including the activation of immune cells and the production of autoantibodies against host's own DNA and nuclear antigens [14]. This resembles the autoimmune response observed in human SLE. Interestingly, after pristine administration, in the first two weeks, Severe alveolar hemorrhage occurs in C57BL/6 mice; symptoms include endothelial damage, hemorrhage, and alveolar and perivascular inflammation (small vessel vasculitis, capillaritis) [15]. This leads to a mortality rate as high as 20% in the pristine treated B6 mice [16]. With pristine administration, we aimed to ascertain whether HADC6 deletion would reduce inflammation in our current research. Furthermore, we sought to determine the mechanism by which HADC6 inhibition alters macrophage activation.

Materials and Methods

Mice

The Jackson Lab (Bar Harbor, ME) provided the female WT C57BL/6 mice and the HDAC6 knockout mice on C57BL/6 background. The animals were kept in accordance with the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech College of Veterinary Medicine, in a pathogen-free environment with a regular 12-hour light/dark cycle. All animal procedures were performed in compliance with the IACUC-approved guidelines. WT and HDAC6 knockout mice on the C57BL/6 background were administered a single dose of 0.5 ml pristane (Sigma Aldrich, St. Louis, MO) or PBS intraperitoneally at 8-12 weeks of age. Mice were sacrificed at 10 days after pristane

injection. At sacrifice, body weight and spleen weight were measured, sera were collected, and splenocytes and peritoneal cells were harvested for flow cytometry.

Splenocytes and peritoneal cells isolation

At sacrifice, spleens were collected and minced into small pieces, then transferred on the 70-um cell strainer over a 50 mL conical tube. The spleen pieces were minced and pressed through the strainer with the plunger end of a syringe. The cells were washed through strainer with PBS and then were collected after centrifuge. The cell pellet was resuspended in 2–5 mL of cold 1x RBC Lysis buffer (eBioscience, San Diego, CA) for 5 minutes on ice to lyse the red blood cells. The cell suspension was washed with cold PBS and centrifuged. Splenocytes were collected for flow cytometry analysis. Mouse peritoneal cells were extracted as previously published [17]. In brief, each mouse's abdominal skin was covered in 70% alcohol following euthanasia. A tiny incision was created so that the intact peritoneal wall could be seen. In the peritoneal cavity, 5 mL of ice-cold PBS were injected. Peritoneal fluid was collected after a little massage and centrifuged at 500g for 10 minutes at 4°C. The cells were then resuspended for flow cytometry.

Flow cytometry

Attune NxT (ThermoFisher Scientific, Waltham, MA) or BD FACSAria II (BD Biosciences, San Jose, CA) flow cytometer was used for flow cytometry, peritoneal cells and splenocytes were extracted, blocked with anti-mouse CD16/32 (eBioscience, San Diego, CA), and stained with fluorochrome-conjugated antibodies. Anti-mouse

antibodies used in this study included: Percp/Cy5.5 CD19 (Biolegend 115534), FITC CD3 (Biolegend 100204), APC CD69 (Biolegend 104514), PE CD4 (BD 553730), PE/Cy7 CD138 (Biolegend 142513), Percp/Cy5.5 CD11b (Biolegend 101227), APC/Cy7 Ly6c (Biolegend 128025), and BV421 Ly6G (Biolegend 127627).

RNA isolation and real-time PCR

Using the RNeasy mini kit (Qiagen, Hilden, Germany) as directed by the manufacturer, total RNA was extracted and purified. RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). SYBR Green master mix was used for real-time PCR using an ABI 7500 fast machine (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the endogenous reference gene. The relative mRNA expression levels were computed using the comparative ΔCt technique. Every experiment's samples were conducted in technical triplicates, with each sample consisting of three reaction wells that produced an average threshold cycle value. Primer sequences are listed following. Isg15, forward 5′- GGT GTC CGT GAC TAA CTC CAT -3′, reverse 5′- CTG TAC CAC TAG CAT CAC TGT G -3′; Mx1, forward 5′- GAT CCG ACT TCA CTT CCA GAT GG -3′, reverse 5′- CAT CTC AGT GGT AGT CAA CCC -3′; Irf9, forward 5′- TGT CTG GAA GAC TCG CCT AC -3′, reverse 5′- GCA ACA TCC ATA CGA CCT CTC T -3′; Ifnb, forward 5′- CAG CTC CAA GAA AGG ACG AAC -3′, reverse 5′- GGC AGT GTA ACT CTT CTG CAT -3′; Irf7, forward 5′- TGC TGT TTG GAG ACT GGC TAT -3′, reverse 5′- TCC AAG CTC CCG GCT AAG T -3′; Oas1a, forward 5′- GCC TGA TCC CAG AAT CTA TGC -3′, reverse 5′-

GAG CAA CTC TAG GGC GTA CTG -3′; Cxcl10, forward 5′- CCA AGT GCT GCC GTC ATT TTC -3′, reverse 5′- GGC TCG CAG GGA TGA TTT CAA -3′; Gapdh, forward 5′- AGG TCG GTG TGA ACG GAT TTG -3′, reverse 5′- TGT AGA CCA TGT AGT TGA GGT CA-3′.

In vitro experiments

Acquired from ATCC (TIB-67), J774A.1 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml of penicillin and streptomycin, and maintained at 37 °C in an incubator with 5% CO2 humidification. When cells were 80% confluent, cells were treated with the selective HDAC6 inhibitor ACY-738 (Adooq Bioscience, Irvine, CA), and stimulated with LPS (Sigma Aldrich, St. Louis, MO) and IFN-γ (R&D Systems, Minneapolis, MN) for 24 hours.

Western blot and antibodies

BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) was used to measure the protein concentrations after total protein was extracted and lysed in RIPA buffer containing 1% protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein in the lysates were mixed with SDS loading buffer and boiled for 5 minutes. The protein samples were separated by electrophoresis on 4-12% SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred to polyvinylidenedifluoride membranes. The membrane was then blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% bovine serum albumin (TBST buffer).

The membrane was then probed with primary and secondary antibodies. The blot was scanned on an Odyssey Clx Imager (LI-COR Biosciences, Lincoln, NE). All antibodies were purchased from Cell Signaling Technology (Danvers, MA). The primary antibodies dilution ratio is 1:1000. The western blot antibodies and catalog number are as follows. NF-κB p65 (8242S), Acetyl-NF-κB p65 (Lys310) (12629S), iNOS (13120S), Histone H3 (4499T), Acetyl-Histone H3 (Lys9) (9649T), Acetyl-α-Tubulin (Lys40) (5335T), α-Tubulin (3873T), Phospho-Stat1 (Tyr701) (9167T), Stat1 (14994T), Phospho-NF-κB p65 (Ser536) (3033T), Anti-mouse IgG (H^+L) (5257P), and Anti-rabbit IgG (H^+L) (5151P). Using ImageJ software and densitometric analysis, the amounts of proteins were quantified and normalized to housekeeping protein.

Statistical analysis

The one-way ANOVA for multiple comparisons or the student's t-test for single comparisons were used to evaluate statistical differences between sample groups. Data are shown as mean ± SEM. Statistical significance was determined by P value (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Results

HDAC6 gene deletion did not alter the body weight in pristine treated animals but increased spleen and spleen to body weight ratio.

WT and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 12 weeks of age. After 10 days of pristine injection, the animals were

euthanized and we measured body and spleen to body weight (Figure 1). Although body weights were not significantly altered in any group, spleen weights were significantly increased in the HDAC6$^{-/-}$ pristine treated mice compared to the controls. Additionally, spleen to body weight ratio was significantly increased in the HDAC6$^{-/-}$ pristine treated mice compared to the controls.

HDAC6 gene deletion inhibited peritoneal recruitment of inflammatory monocytes and neutrophils after pristane administration.

At sacrifice, cells were collected from the peritoneum and assessed by flow cytometry. Activated monocytes were identified as CD11b^{+}Ly6c^{++} and neutrophil cells were identified as CD11b^{+}Ly6G^{+} (Figure 2). In both the WT and HDAC6$^{-/-}$ mice pristine administration increased the numbers of activated neutrophils and monocytes. In the HDAC6$^{-/-}$ pristine treated animals there was significantly less recruitment of activated monocytes and neutrophils compared to the WT mice.

Pristine administration increased the percentage of activated T and B cells in HDAC6$^{-/-}$ animals.

Ten days after pristine administration, the spleens were removed and splenocytes were isolated. Flow cytometry was used to assess cell activation (Figure 3). There was no significant difference on CD4^{+} T or CD8^{+} T cells in any of the treatment groups of controls. However, the early activation marker in immune cells and implicated in T cell differentiation (CD69^{+}) was significantly increased after pristine administration in the HDAC6$^{-/-}$ animals. Furthermore, CD69^{+} expression was significantly increased in the

HDAC6$^{-/-}$ pristine treated mice compared to the pristine WT animals. In B cells, CD69 was increased in both the WT and the HDAC6$^{-/-}$ pristine treated animals. Furthermore, the percentages of plasmablast (CD19^{+}CD138^{+}) as well as plasma cells (CD19^{-}CD138^{+}) were significantly increased in the HDAC6$^{-/-}$ mice treated with pristine.

HDAC6 knockout didn't affect interferon (IFN) signature genes expression induced by pristane administration.

After 10 days of pristine treatment, a portion of the peritoneal cells were collected and mRNA was isolated. We used real-time qPCR to assess several IFN signature genes (Figure 4). We examined expression of the type I IFN signature genes Ifnb, Mx1, Oas1a, Irf7, Irf9, Cxcl10, and Isg15 and normalized the expression to the housekeeping gene GAPDH. The IFN signature was significantly increased for all mRNA levels examined in mice that were treated with pristine in both the WT and the HDAC6$^{-/-}$ animals with the exception of IFN-β which was decreased. Furthermore, there was no difference in the non-treated peritoneal cells from WT mice and HDAC6$^{-/-}$ animals.

Stimulated J774 macrophages with treated with the HDAC6 inhibitor increased acetylation of NF-κB and iNOS expression.

To investigate further the role of HDAC6 inhibition on macrophage function, we use J774 cells line. Initially, experiments were conducted to determine the concertation of the selective HDAC6 inhibitor ACY-738 that would increase alpha tubulin acetylation without increasing histone acetylation (Figure 5). We found that at 1 μM, α-tubulin was acetylated but not histone H3. When J774 cells were stimulated with a cocktail of

LPS/IFN-γ, iNOS expression was increased. It has previously been reported that in macrophages stimulated with LPS treated with a HDAC6 inhibitor lead to increased NF-κB acetylation and this decreased iNOS expression [18]. We found that 1 μM concertation of ACY-738 induced a-tubulin significantly and increased NF-κB acetylation without affecting Histone H3 acetylation. Surprisingly, when the macrophages were treated with ACY-738 and stimulated with LPS/IFN-γ we measured an increase in iNOS expression. While acetylation of NF-κB increased with ACY-738, phosphorylation of NF-κB was not affected by ACY-738. Furthermore, p-STAT-1 was significantly increased by ACY-738 after LPS/IFN-γ stimulation.

Discussion

Histone deacetylase 6 (HDAC6) dysregulation plays a significant role in the development and progression of various human diseases [19; 20]. HDAC6 is an enzyme responsible for removing acetyl groups from various cytosolic proteins and transcription factors that influence cell signaling and gene expression [8; 21]. Dysregulation of HDAC6 has been linked to several pathological conditions, including cancer, neurodegenerative disorders, autoimmune diseases, and cardiovascular ailments. In cancer, overexpression of HDAC6 can promote uncontrolled cell growth and inhibit cell death, contributing to tumorigenesis and tumor progression [22; 23; 24]. In neurodegenerative diseases such as Parkinson's and Alzheimer's, altered HDAC6 activity has been associated with abnormal protein aggregates and impaired cellular clearance mechanisms, contributing to neuronal damage and degeneration [25; 26; 27; 28; 29; 30]. In inflammation, and in particular SLE, we have previously reported that selective HDAC6 inhibition can decrease disease in lupus

mice [6; 31; 32; 33]. In our studies, we aimed to determine whether HDAC6 gene deletion would inhibit inflammation in the pristine lupus mouse model. The C57BL/6 pristine lupus mouse model does not develop lupus-like disease until several months after initial injection [14]. However, these mice often develop severe lung involvement that may result in up to 20% mortality by 2 weeks after injection [16]. In our present studies, we aimed to determine if HDAC6 deletion would decrease early inflammation in this mouse lupus model.

We found that gene deletion of HDAC6 significantly decreased inflammatory monocytes and activated neutrophils accumulated in the peritoneum 10 days after pristine injection compared to WT mice. This was particularly interesting as HDAC6 inhibition has been shown to decrease neutrophil activation in Pseudomonas aeruginosa-induced inflammation and infection in cystic fibrosis [34]. Moreover, studies by Yan and coworkers found differing effects of HADC6 gene deletion in the LPS induced model of acute peritonitis [35]. They found that neutrophil activation was largely unaffected while macrophage activation was enhanced in the WT animals whereas it was blunted in the $HDAC6^{-/-}$ animals. This apparent dichotomy could be due to the different mechanisms by which the immune system is activated.

LPS binds to the extracellular domain of TLR4 located on the surface of immune cells including dendritic cells, macrophages, and monocytes. Binding of LPS to TLR4 then forms a complex with its co-receptor MD-2 (myeloid differentiation protein 2), which is necessary for efficient LPS recognition [36; 37]. The TLR4-MD-2 complex triggers a

conformational change in TLR4, leading to the recruitment of adapter proteins, particularly MyD88 (myeloid differentiation primary response 88) and TRIF (TIR domain-containing adapter-inducing interferon-β) [38]. The MyD88-dependent pathway is the primary and rapid signaling cascade. It leads to the activation of IRAK (interleukin-1 receptor-associated kinase) family members, followed by the activation of TRAF6 (tumor necrosis factor receptor-associated factor 6) [39]. This cascade results in the activation of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [40]. Activation of NF-κB then translocates to the nucleus, where it induces the transcription of pro-inflammatory genes, including those encoding cytokines such as IL-1β (interleukin-1 beta), TNF-α (tumor necrosis factor-alpha), and IL-6 (interleukin-6) [41].

In contrast, the intracellular mechanisms of pristane-induced inflammation are complex and involve the activation of immune cells and various signaling pathways. While the exact mechanisms may vary depending on the specific context and cell types involved, pristane is believed to act through both Pattern Recognition Receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), on the surface or within the cytoplasm of immune cells [42]. TLR activation, in particular, can trigger intracellular signaling cascades, including NF-κB and MAPK (mitogen-activated protein kinase) pathways which are central to the regulation of inflammatory gene expression [43; 44; 45; 46; 47; 48].

Additionally, pristane-induced inflammation has been associated with the activation of inflammasomes and multiprotein complexes that regulate the processing and release of pro-inflammatory cytokines, especially IL-1β [49]. Furthermore, the NLRP3 (NOD-like receptor family, pyrin domain-containing inflammasome) has been implicated in pristane-induced inflammation [50]. Through these mechanisms, pristane has been linked to the development of autoimmune diseases and can lead to the production of autoantibodies [51].

In regard to T and B cell activation, pristane has been shown to activate B cells and promote the production of antibodies. It induces the formation of long-lived antibody-secreting cells in the peritoneal cavity of mice. We found that there was an increase in B cell activation with pristine treatment and the increase in B cell activation was greater in the HDAC6$^{-/-}$ animals compared to the WT mice. Furthermore, that was an increase in the plasmablast cells in the pristine treated HDAC6$^{-/-}$ animals compared to the WT pristine treated mince. Similarly, pristane has been reported to activate certain subsets of T cells [52]. It can influence the differentiation and activation of CD4^{+} T helper cells [53]. We found increased activation markers of T cells along with increased B cell differentiation to plasma cells in HDAC6$^{-/-}$ mice treated with pristine. It has been reported that pristine increases autoantibody production and nephritis through TLR7 or IFN receptor mediated type I interferon (IFN-I) production [54]. Following HDAC6 suppression, it was discovered that activated CD4^{+} T-cells expressed less of the apoptotic signaling receptor FAS, whereas CD8^{+} T-cells showed no changes in this regard [57]. Together with these findings that link HDAC6 to the regulation of T-cell survival, surface

marker expression was changed in both $CD8^+$ and $CD4^+$ T-cells. Specifically, stimulated T-cells treated with an isotype-selective HDAC6 inhibitor showed increased expression of the activation molecule CD69 [57]. Another group found the peritoneal wash contained considerably more $CD69^+$-activated CD4 and CD8 T cells when the HDAC6 inhibitor ACY1215 was used [58].

Surprisingly, we found HDAC6 inhibition did not suppress iNOS expression in LPS/ IFN-γ stimulated J774 cells. Yang and co-workers have previously reported that macrophages from $HDAC6^{-/-}$ knock mice have decreased iNOS production with LPS stimulation [55]. As LPS acts predominantly through NF-κB to inhibit iNOS, IFN-γ pathway acting through STAT1 to induce iNOS expression can act independent of NF-κB. IFN-γ stimulates the generation of nitric oxide (NO) and prevents the activation of the NLRP3 inflammasome, which primes macrophages for antimicrobial activities and can therefore enhance pro-inflammatory signaling. Furthermore, the contribution by p38 to the induction of iNOS and apoptosis is independent of NF-κB nuclear translocation [56]. A difference in our findings compared to that of Yang and coworkers could be due to the duration of pretreatment of the HDAC6 inhibitor. In our studies, we added ACY-738 concurrent with LPS/IFN-γ for 24 hours. In their studies, they pretreated the cells for 3 hours prior to stimulation.

In summary, we discovered that in the early inflammatory response to pristane, HDAC6 deletion inhibited the recruitment of inflammatory monocytes and neutrophils in the peritoneum. The HDAC6 inhibitor was found to raise NF-κB acetylation, decrease NF-

κB phosphorylation and total NF-κB protein level in in vitro investigations using J774 cells.

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Declaration of interest

The authors declare no conflict of interest.

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Figures

Figure 1

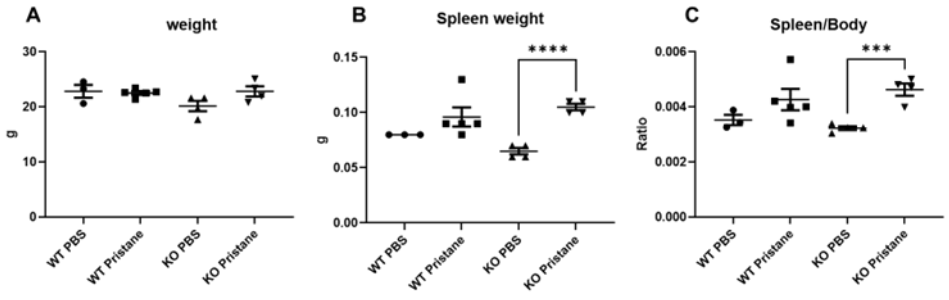

Figure 1. Pristane treatment increased spleen weight in HDAC6$^{-/-}$ mice, but not in WT mice. WT and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 8-12 weeks of age and were euthanized 10 days later. (A, B) Body weight and spleen weight were measured. (C) The ratio of spleen weight to body weight. Mice number (WT PBS n=3; WT Pristane n=5; KO PBS n=4; KO Pristane n=4). The one-way ANOVA for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Figure 2

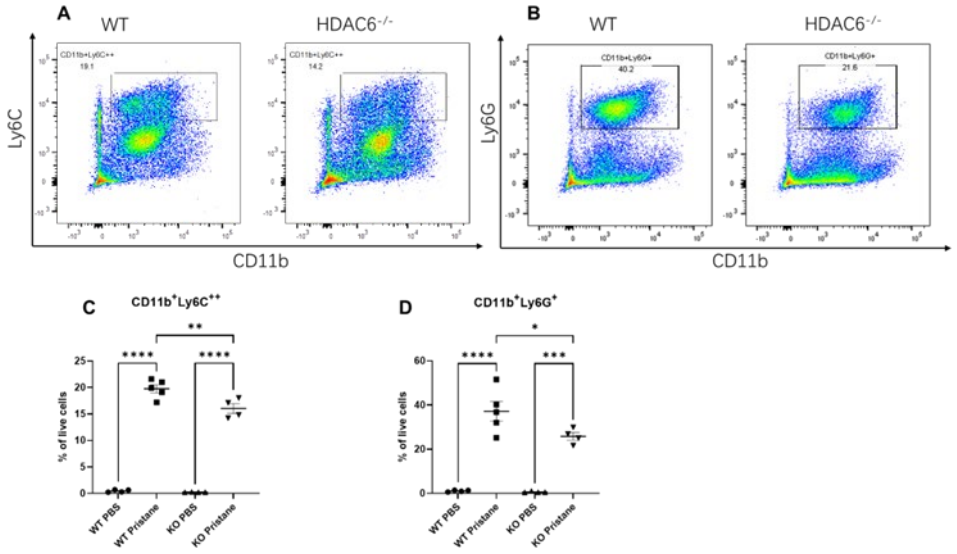

Figure 2. HDAC6 knockout inhibited peritoneal recruitment of inflammatory monocytes and neutrophils after pristane treatment. WT and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 8-12 weeks of age and were euthanized 10 days later. Peritoneal cells were isolated and stained with CD11b, Ly6C, and Ly6G antibodies. (A, B) Representative flow cytometry picture of inflammatory monocytes (CD11b^{+}Ly6C^{++}) and neutrophils (CD11b^{+}Ly6G^{+}). (C, D) The percent of inflammatory monocytes (CD11b^{+}Ly6C^{++}) and neutrophils (CD11b^{+}Ly6G^{+}) to the live cells. Mice number (WT PBS n=4; WT Pristane n=5; KO PBS n=4; KO Pristane n=4). The one-way ANOVA for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Figure 3

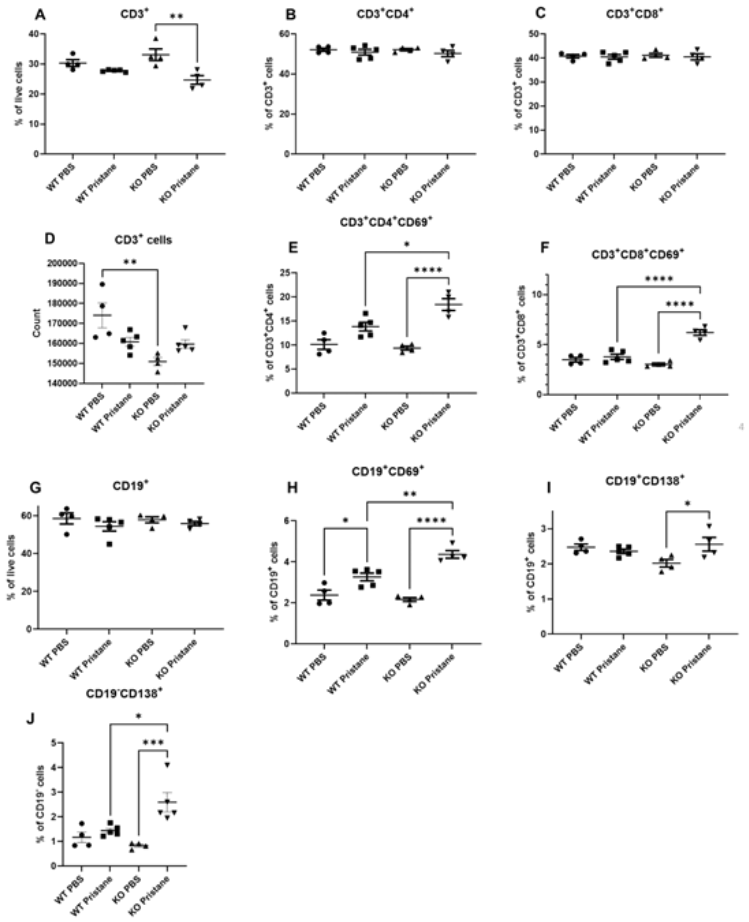

Figure 3. HDAC6 knockout increased expression of CD69 on T cells and B cells. WT and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 8-12 weeks-of-age and were euthanized 10 days later. Splenocytes were isolated and stained with CD3, CD4, CD8, CD19, CD69, and CD138 antibodies. (A) The percent of CD3$^+$ T cells to the live cells. (B-J) The percent of CD3$^+$CD4$^+$ T helper cells, CD3$^+$CD8$^+$ cytotoxic T cell, CD69$^+$ T cells, CD19$^+$ B cells, CD69$^+$cells, CD138$^+$ plasma cells and the total number of CD3$^+$ T cells. Mice number (WT PBS n=4; WT Pristane

n=5; KO PBS n=4; KO Pristane n=5). The one-way ANOVA for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Figure 4

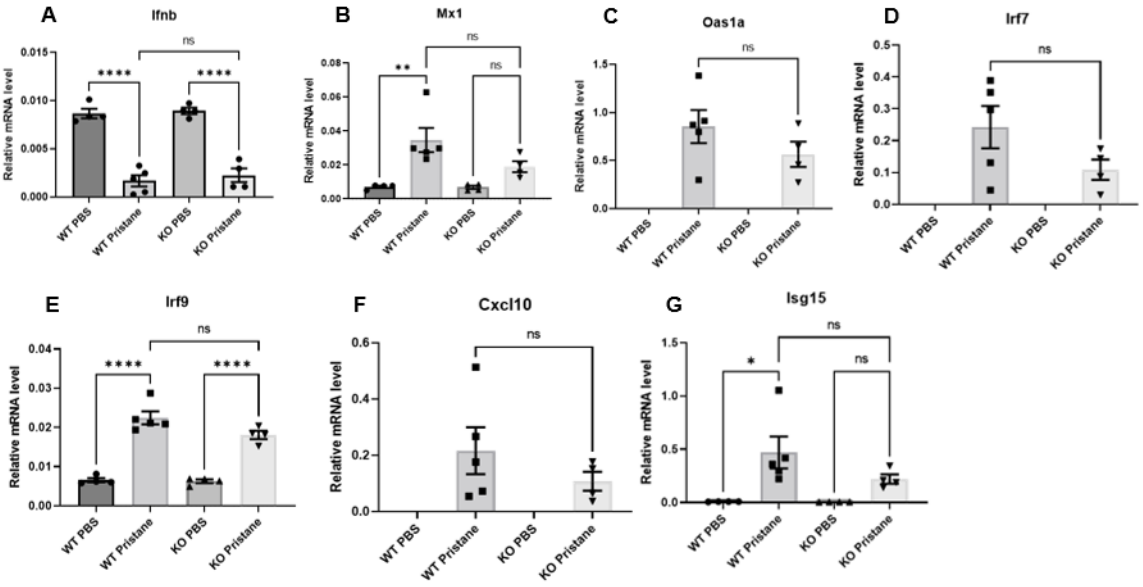

Figure 4. HDAC6 knockout didn't affect interferon (IFN) signature genes expression induced by pristane treatment. WT and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 8-12 weeks of age and were euthanized 10 days later. Peritoneal cells were isolated and RNA was extracted. (A-G) Ifnb, Mx1, Oas1a, Irf7, Irf9, Cxcl10, Isg15 mRNA levels were determined by real time PCR and normalized to the GAPDH levels. Mice number (WT PBS n=4; WT Pristane n=5; KO PBS n=4; KO Pristane n=4). The one-way ANOVA for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Figure 5

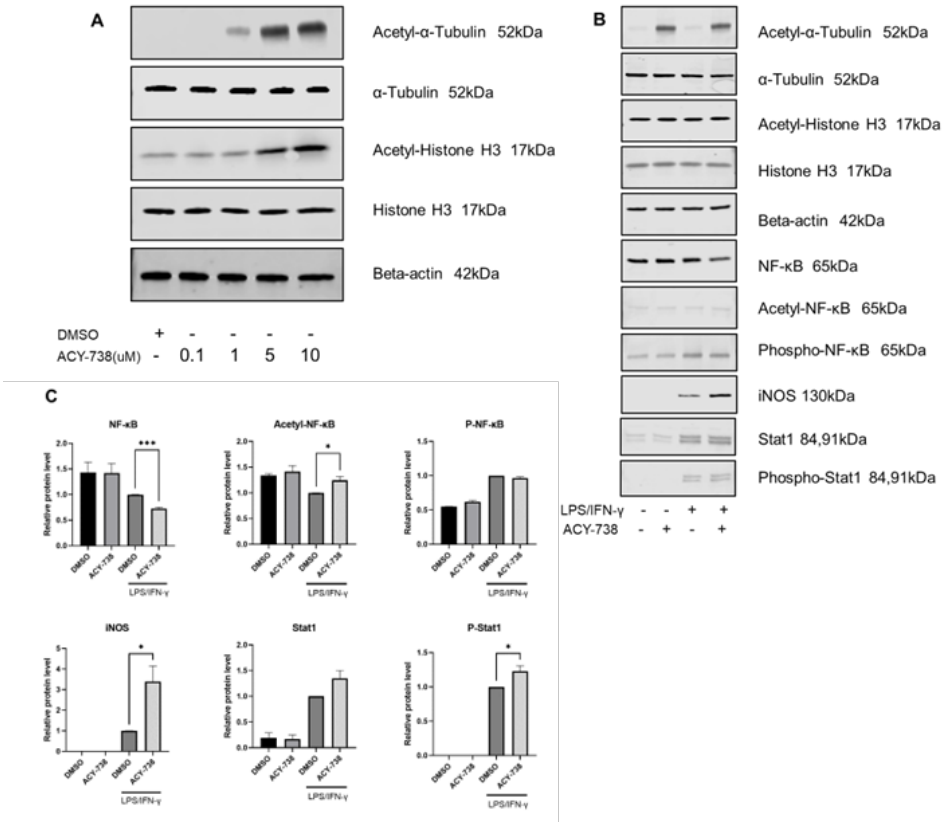

Figure 5. HDAC6 inhibitor ACY-738 inhibited the NF-κB signaling *in vitro*. J774A mouse macrophage cells were treated with different concentrations of ACY-738 for 24 hours. (A) Western blot analysis showed that 1 μM ACY-738 was able to significantly increase the level of α-tubulin acetylation while not affect the acetylation of histone H3. (B) J774A mouse macrophage cells were treated with 1 μM ACY-738 as well as LPS (1 μg/mL) and IFN-γ (100 ng/mL) for 24 hours. Western blot analysis showed that with LPS/ IFN-γ stimulation, 1 μM ACY-738 was able to decrease the protein level of NF-κB. (C) Protein quantification of NF-κB, Acetyl- NF-κB, Phospho- NF-κB, iNOS, Stat1, and Phospho-Stat1 by using ImageJ software. The experiments were repeated three times independently.

CHAPTER 3

HDAC6 Deletion Decreases Pristane-Induced Lupus

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Abstract

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder often occurring in women of childbearing age. SLE is characterized by pathogenic antibody production and inflammation. Histone deacetylase 6 (HDAC6) is a class IIb histone deacetylase member. The removal of acetyl groups from lysine residues on nonhistone proteins can be catalyzed by HDAC6. It has been observed that in lupus mouse models, specific HDAC6 inhibition reduces inflammation. Pristane, a naturally occurring hydrocarbon oil, can result in lupus-like illness and persistent inflammation. In this study, sex and age-matched wild type (WT) and HDAC6$^{-/-}$ mice on the C57BL/6 background were injected with 0.5 ml pristane or PBS intraperitoneally at 8-12 weeks of age and were euthanized 8 months later. The animals were assessed as they aged. At sacrifice, body weight, spleen weight, and kidney weight were measured, sera and urine were collected, and splenocytes were harvested for flow cytometry. We found pristane treatment didn't alter the spleen, kidney, and body weight. Pristane treatment induced proteinuria in female mice with no significant differences between WT and KO animals. HDAC6 deletion significantly inhibited pristane-induced anti-dsDNA IgG compared with WT mice. Moreover, HDAC6 deletion decreased T helper 17 (Th17) cells after pristane treatment. In summary, HDAC6 deletion alleviated some aspects of pristane-induced lupus by decreasing anti-dsDNA IgG and Th17 cells.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune illness in which the body's immune system attacks its own organs and tissues [1]. Autoantibodies are generated in SLE patients, and these antibodies bind to circulating antigens and lodge in small blood vessels in different organ systems, including the skin and kidney [2]. It has been demonstrated that histone acetylation is essential for regulating gene expression [3; 4]. Histone protein acetylation frequently causes an increase in transcription, whereas deacetylation is linked to the repression of genes [5]. HDAC inhibitors are effective in treating autoimmune diseases, including SLE, allergies, and cancer [6; 7; 8]. Conversely, the use of pan-HDAC inhibitors has been shown to induce serious adverse effects, making their use for long-term illnesses limited [9]. Histone deacetylase (HDAC) 6 is a cytoplasmic class IIb HDAC [10; 11]. HDAC6 controls protein degradation; deacetylation of non-histone proteins such as heat shock protein (HSP90), α-tubulin, and NF-κB has been demonstrated to alter the immune response [11]. It has been demonstrated that overexpression of HDAC6 significantly increases the expression of pro-inflammatory cytokines in macrophages, including TNF-α, IL-6, and IL-1β, by upregulating the AP-1 and NF-κB signaling pathways [12]. Conversely, HDAC6 inhibition disrupts the NF-κB signaling pathway and reduces inflammation by inducing NF-κB's acetylation, which in turn inhibits nuclear translocation [13; 14].

The pristane induced lupus mouse model has been utilized to study SLE. In mice, pristane injections into the peritoneum cause lupus-like symptoms over time, such as immune cell activation and the development of autoantibodies against the host's DNA

and nuclear antigens similar to the autoimmune reaction seen in SLE in humans [15]. Pristane is present in trace amounts in a wide variety of plants, marine life, and mineral oils. Interestingly, C57BL/6 (B6) mice have substantial alveolar hemorrhage in the first two weeks upon pristine treatment. This bleeding is accompanied by endothelial damage, hemorrhage, and alveolar and perivascular inflammation including small artery vasculitis [16]. This results in a 20 percent mortality rate in the B6 mice that are pristine stimulated [17]. The goal of our current studies was to ascertain whether HADC6 gene deletion would lessen the lupus disease in the pristine induced model.

Material and Methods

Mice

The Jackson Lab (Bar Harbor, ME) provided the female WT C57BL/6 mice and the HDAC6 knockout mice on C57BL/6 background. The animals were kept in accordance with the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech College of Veterinary Medicine, in a pathogen-free environment with a regular 12-hour light/dark cycle. All animal procedures were performed in compliance with the IACUC-approved guidelines. WT and HDAC6 knockout mice on the C57BL/6 background were administered a single dose of 0.5 ml pristane (Sigma Aldrich, St. Louis, MO) or PBS intraperitoneally at 8-12 weeks of age. Mice were sacrificed at 8 months after pristane injection. At sacrifice, body weight, spleen weight, and kidney weight were measured, serum and urine were collected, and splenocytes cells were harvested for flow cytometry. Once the whole blood had been collected, it was allowed to clot by keeping it undisturbed at room temperature for fifteen to thirty minutes. Next, the blood was centrifuged at

1,000–2,000 x g for 10 minutes. The serum was transferred into a sterile tube after centrifugation. Urine were collected into sterile tubes by gently pressing on the mouse bladder to expel urine.

Anti-dsDNA IgG, total IgG, creatinine, and albumin detection

Anti-dsDNA IgG in the sera was detected by using Mouse Anti-dsDNA ELISA Kit (Signosis EA-5201). Total IgG in the sera was detected by using IgG (Total) Mouse Uncoated ELISA Kit with Plates (Invitrogen 88-50400-22). Creatinine in the urine was detected by using Creatinine (urinary) Colorimetric Assay Kit (Cayman Chemical 500701). Albumin in the urine was detected by using Mouse Albumin ELISA Kit (Bethyl Laboratories E99-134). Every step of the process was carried out as directed by the manufacturer's protocols.

Splenocytes isolation

At sacrifice, spleens were collected and minced into small pieces, then were transferred on the 70-um cell strainer over a 50 mL conical tube. The spleen pieces were minced and pressed through the strainer with the plunger end of a syringe. The cells were washed through strainer with PBS and then were collected after centrifuge. The cell pellet was resuspended in 2–5 mL of cold 1x RBC Lysis buffer (eBioscience, San Diego, CA) for 5 minutes on ice to lyse the red blood cells. The cell suspension was washed with cold PBS and centrifuged. Splenocytes were collected for flow cytometry analysis.

Kidney histopathology

Within moments of extraction, kidneys were fixed in formalin. In the Histopathology Laboratory at Virginia Tech College of Veterinary Medicine, fixed tissues were paraffin-embedded, sectioned, and stained for Periodic Acid-Schiff (PAS). A board-certified veterinary anatomic pathologist blindly evaluated each kidney segment for glomerular proliferation, inflammation, crescent development, necrosis, and fibrosis before assigning a glomerular pathology score (0–4). In summary, glomeruli with hypercellularity, crescents, significant mesangial thickening, segmental necrosis, and hyalinized end-stage glomeruli fall within the score range of 0 (normal glomeruli) to 4.

Flow cytometry

Attune NxT (ThermoFisher Scientific, Waltham, MA) or BD FACSAria II (BD Biosciences, San Jose, CA) flow cytometer was used for flow analysis. Peritoneal cells and splenocytes were extracted, blocked with anti-mouse CD16/32 (eBioscience, San Diego, CA), and stained with fluorochrome-conjugated antibodies. Anti-mouse antibodies used in this study included: APC CD3 (Biolegend 100236), PE/Cy7 CD4 (Biolegend 116016), PE/Dazzle594 CD8 (Biolegend 100762), APC/Cy7 PD-1 (Biolegend 135223), Percp/Cy5.5 CXCR5 (Biolegend 145507), Pacific Blue CD25 (Biolegend 102021), PE Foxp3 (Biolegend 126403), Percp/Cy5.5 CD44 (MBL FP10377002), APC/Cy7 CD62L (Biolegend 104428), PE RORγT (Invitrogen 12-6981-82), PE B220 (Biolegend 103207), APC/Cy7 CD138 (Biolegend 142529), Pacific Blue CD19 (Biolegend 115526), PE/Cy7 CD38 (Biolegend 102717), Percp/Cy5.5 GL7 (Biolegend 144609).

Statistical analyses

The student's t-test for single comparisons was used to evaluate statistical differences between sample groups. Data are shown as mean ± SEM. Statistical significance was determined by P value (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Results

HDAC6 gene deletion did not alter the body weight in pristine treated mice.

WT and HDAC6$^{-/-}$ mice on the C57BL/6 background were injected with 0.5 ml pristane or PBS at 12 weeks of age. Two weeks following injection, C57BL/6 mice frequently experience a severe pulmonary hemorrhage that can result in up to 20% mortality [17]. We also observed this phenomenon in our studies (Table 1). The remaining mice that recovered from the initial pulmonary hemorrhage were evaluated and utilized for the experiments. After 8 months of pristine injection, the animals were euthanized and we measured body, spleen, and kidney weight as well as the ratio of spleen and kidney weight to body weight (Figure 1). The results showed that body weights, spleen weights, kidney weights, and the ratio were not significantly altered in any group.

HDAC6 gene deletion decreased proteinuria and anti-dsDNA IgG level after pristane treatment.

At sacrifice, urine and sera were collected. We measured creatinine and albumin levels (proteinuria) in the urine as well as anti-dsDNA IgG level in the sera (Figure 2). Pristane treatment resulted in a decrease in creatinine in the KO female group compared to the

WT female mice (Figure 2A). Pristane treatment induced proteinuria in WT female mice to a greater extent than that in the HDAC6$^{-/-}$female mice although it did not reach statistical significance (Figure 2B). When the albumin/creatinine ratios were compared there was no significant difference between any of the groups (Figure 2C). In the pristine treated HDAC6$^{-/-}$ female mice there were significantly fewer levels of anti-dsDNA IgG antibody levels compared to the WT female mice (Figure 2D). The were no significant difference in total IgG levels in any treatment group (Figure 2E). The anti-dsDNA IgG to total IgG ratio was significantly decreased in HDAC6$^{-/-}$ female mice compared with WT female mice after pristane treatment.

HDAC6 deletion decreased Th17 cells after pristane treatment.

Eight months after pristine treatment, the spleens were removed and splenocytes were isolated. Flow cytometry was used to assess cell activation (Figure 3). When the flow cytometry results were analyzed for B cells (Figure 3A), plasma cells (Figure 3B), germinal center cells (Figure 3C), T cells (Figure 3D), cytotoxic T cells (Figure 3F), memory T cells (Figure 3G), T follicular helper cells (Figure 3I), and regulatory T cells (Figure 3J), there was no significant difference between pristane treated WT female mice and pristane treated HDAC6$^{-/-}$ female mice. Pristane treatment significantly increased the T cells (Figure 3D) and the plasma cells (Figure 3B) in WT female mice. There was a significant increase of helper T cells in pristane treated HDAC6$^{-/-}$ female mice compared with pristane treated WT female mice (Figure 3E). Furthermore, the percentages of Th17 cells ($CD3^{+}CD4^{+}ROR\gamma t^{+}$) were significantly decreased in the HDAC6$^{-/-}$ female mice treated with pristane compared with WT female mice treated with pristane (Figure 3H).

Discussion

Dysregulation of HDAC6 has been reported to play a role in a variety of human diseases [18; 19]. The role of HDAC6 is to remove the acetyl groups from a variety of transcription factors and cytosolic proteins that affect gene expression and cell signaling [10; 20]. Numerous pathological problems, including cancer, neurological diseases, autoimmune diseases, and cardiovascular maladies, have been associated with dysregulation of HDAC6. Overexpression of HDAC6 in cancer can suppress cell death and encourage unchecked cell proliferation, which can aid in the development and progression of tumors [21; 22; 23]. Changes in HDAC6 activity have been linked to aberrant protein aggregates and compromised cellular clearance systems in neurodegenerative illnesses like Parkinson's and Alzheimer's, which exacerbate neuronal damage and degeneration [24; 25; 26; 27; 28; 29]. We have previously shown that selective HDAC6 inhibition can reduce disease in various SLE animal models [6; 30; 31; 32]. In the present work, we investigated whether deletion of the HDAC6 gene would reduce inflammation in the mice model of pristine induced lupus. It takes several months for the C57BL/6 mice to exhibit signs of lupus-like illness following its first pristane injection [15]. In our studies, we investigated whether HDAC6 deletion might reduce lupus characteristics in this mouse model.

We found that gene deletion of HDAC6 significantly decreased anti-dsDNA IgG level 8 months after pristine injection compared to female WT mice. Pristane treatment induced proteinuria in WT female mice to a greater extent than that in the HDAC6$^{-/-}$female mice

although there was no significant difference. We also measured the renal function of pristane treated mice. However, there was no significant difference between PBS group and pristane group for histopathological scores (Supplemental data). Daniel et al. found that mice given pristane exhibited higher levels of anti-dsDNA autoantibody compared to mice given PBS [33]. They also found that PBS and pristane mice did not significantly differ in their albuminuria. Taken together, urinary albumin level is not often monitored in pristane treated C57BL/6 mice.

Pristane has been demonstrated to stimulate B cells and encourage the generation of antibodies with reference to T and B cell activation. It causes the peritoneal cavity of mice to become filled with long-lived cells that secrete antibodies. We found that there was an increase in plasma cells activation with pristine treatment. Similarly, pristane has been shown to activate specific T cell subsets [34]. It can influence the differentiation and activation of $CD4^+$ T helper cells [35]. We found increased activation markers of T cells in WT female mice treated with pristine. According to reports, pristine stimulates the formation of autoantibodies and nephritis by means of TLR7 or IFN receptor-mediated type I interferon (IFN-I) production [36]. Th17 cells are a subset of CD4 T cells that secrete interleukin-17 (IL-17), a cytokine that promotes inflammation [37]. Th17 cells are involved in host defense and are a component of the adaptive immune system. However, Th17 cells are also strongly linked to autoimmune disorders and have the potential to cause significant illness [38]. We found the percentages of Th17 cells were significantly decreased in the female $HDAC6^{-/-}$ mice treated with pristine compared with female WT mice. Regulatory T (Treg) cells play an important role in SLE. Dysfunction of Treg cells

leads to effector T cells overactivation, which in turn fuels autoimmune inflammation and accelerates the course of SLE [39]. In this study, there was no significant difference in the percent of Treg cells between PBS treated mice and pristane treated mice.

In summary, we discovered that in pristane treated female C57BL/6 mice, HDAC6 deletion inhibited the anti-dsDNA level significantly and also decreased the percentages of Th17 cells significantly. HDAC6 deletion protected female C57BL/6 mice from pristane induced lupus.

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Declaration of interest

The authors declare no conflict of interest.

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Supplementary Material

Additional files are provided.

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Figures

Figure 1

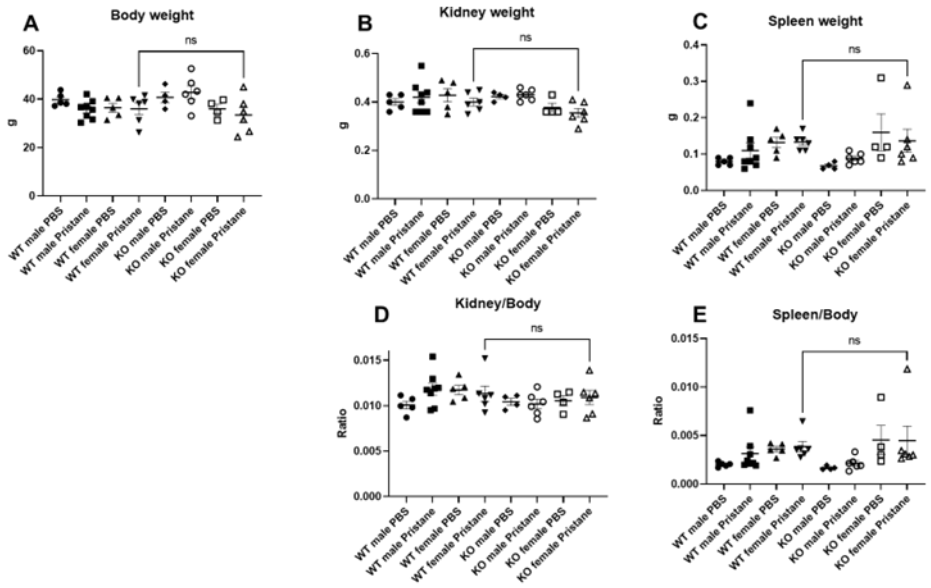

Figure 1. HDAC6 knockout did not alter the body weight, kidney weight, and spleen weight after pristane treatment. Wild type and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 8-12 weeks of age and were euthanized 8 months later. (A-C) Body weight, kidney weight, and spleen weight were measured. (D) The ratio of kidney weight to body weight. (E) The ratio of spleen weight to body weight. Mice number (WT male PBS n=5; WT male Pristane n=8; WT female PBS n=5; WT female Pristane n=6;KO male PBS n=4; KO male Pristane n=6; KO male PBS n=4; KO male Pristane n=6). The student's t-test for single comparisons was used to evaluate statistical differences between sample groups. (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Figure 2

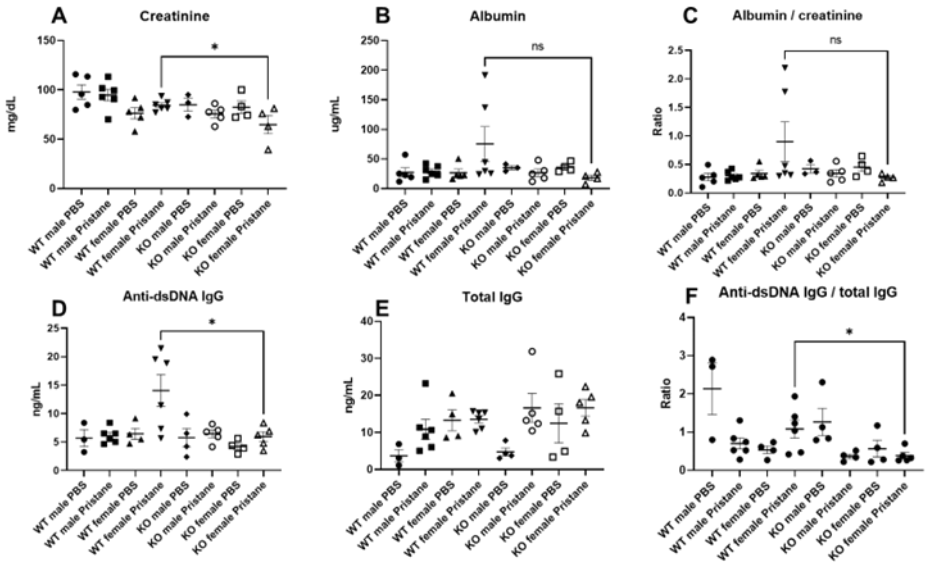

Figure 2. HDAC6 knockout reduced anti-dsDNA IgG level after pristane treatment. Wild type and HDAC6$^{-/-}$ mice on the C57BL/6 background were administered 0.5 ml pristane or PBS at 8-12 weeks of age and were euthanized 8 months later. Urine and serum were collected. Creatinine and albumin levels in the urine were detected by using kits. Anti-dsDNA IgG and total IgG levels in the serum were detected by using ELISA kits. (A) The creatinine level in the urine. (B) The albumin level in the urine. (C) The ratio of albumin to creatine. (D) The anti-dsDNA IgG level in the serum. (E) The total IgG level in the surum. (F) The ratio of anti-dsDNA IgG to total IgG. Mice number (WT male PBS n=5; WT male Pristane n=8; WT female PBS n=5; WT female Pristane n=6;KO male PBS n=4; KO male Pristane n=6; KO male PBS n=4; KO male Pristane n=6). The student's t-test for single comparisons was used to evaluate statistical differences between sample groups. (*P < 0.05, **P < 0.01, ***P < 0.001, ****p<0.0001; NS, not significant).

Figure 3

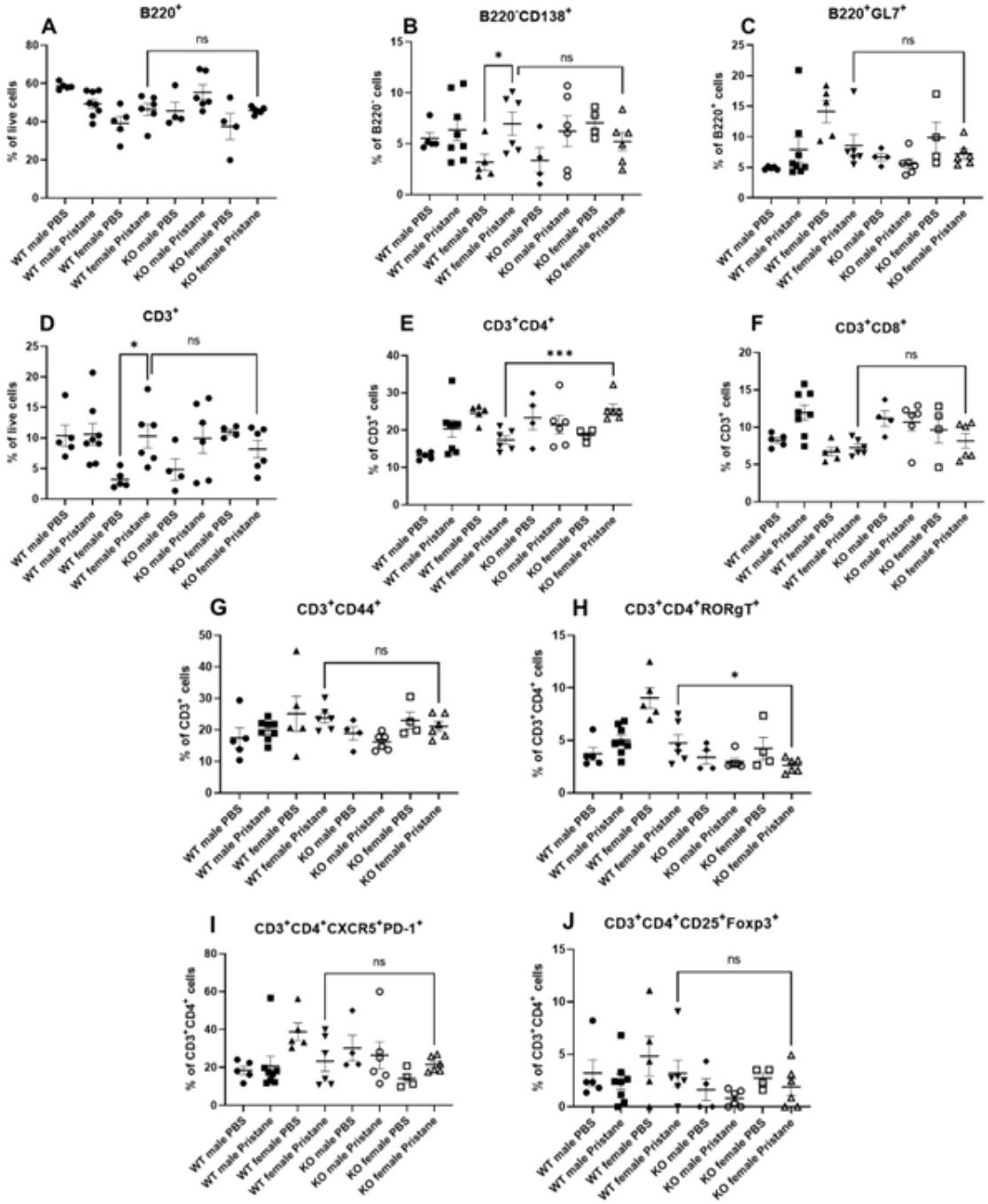

Figure 3. HDAC6 knockout altered some lymphocyte populations after pristane treatment. Wild type and HDAC6$^{-/-}$ mice on the C57BL/6 background were

administered 0.5 ml pristane or PBS at 8-12 weeks of age and were euthanized 8 months later. Splenocytes were isolated and stained with different antibodies. (A) The percent of $B220^+$ B cells in the live cells. (B) The percent of plasma cells in $B220^-$ cells. (C) The percent of germinal center cells in $B220^+$ cells. (D) The percent of $CD3^+$ T cells in live cells. (E) The percent of $CD4^+$ T cells in $CD3^+$ T cells. (F) The percent of $CD8^+$ T cells in $CD3^+$ T cells. (G) The percent of memory T cells in $CD3^+$ T cells. (H) The percent of Th17 cells in $CD3^+CD4^+$ T cells. (I) The percent of T follicular helper cells (Tfh) in $CD3^+CD4^+$ T cells. (J) The percent of regulatory T cells in $CD3^+CD4^+$ T cells. Mice number (WT male PBS n=5; WT male Pristane n=8; WT female PBS n=5; WT female Pristane n=6; KO male PBS n=4; KO male Pristane n=6; KO male PBS n=4; KO male Pristane n=6). The student's t-test for single comparisons was used to evaluate statistical differences between sample groups. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$p<0.0001$; NS, not significant).

Supplemental Figures

Supplemental Figure 1

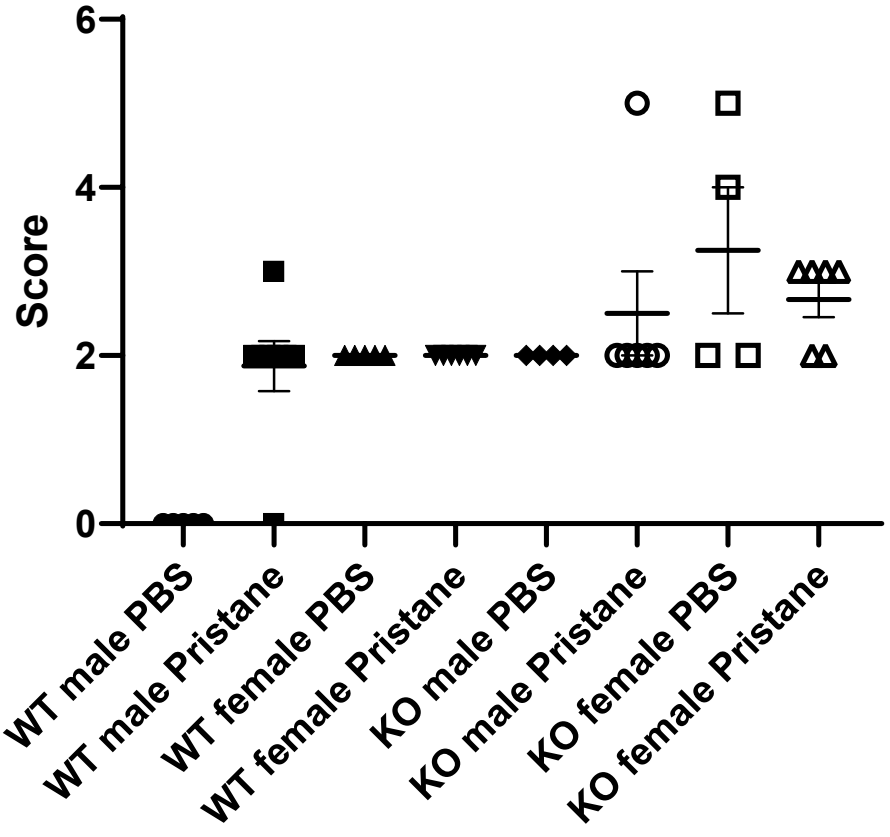

Figure S1. Histopathological scores were quantified by mesangial expansion and membrane thickening.

Supplemental Table 1

Group	Mice number before pristane treatment	Mice number after pristane treatment
WT male PBS	5	5
WT male Pristane	15	9
WT female PBS	5	5
WT female Pristane	8	6
HDAC6$^{-/-}$ male PBS	4	4
HDAC6$^{-/-}$ male Pristane	10	4
HDAC6$^{-/-}$ female PBS	4	4
HDAC6$^{-/-}$ female Pristane	15	10

Table S1. The mice number before and after pristane treatment.

CHAPTER 4

Discussion and Future Directions

In our studies, we found HDAC6 deletion decreased pristane-induced $CD11b^{+}Ly6C^{++}$ inflammatory monocytes and $CD11b^{+}Ly6G^{+}$ neutrophils, inhibited NF-κB signaling, decreased anti-dsDNA IgG level, and reduced the percentage of Th17 cells. However, there are still many unknown issues that need to be addressed.

Future studies should include an investigation of the mRNA expression level, protein expression level, and acetylation level of TLR7 and MyD88 to determine if differences exist in WT and $HDAC6^{-/-}$ mice. HDAC6 knockout may affect other proteins and their acetylation status. In the type I IFN pathway, single-stranded RNA and DNA trigger TLR7/9 dependent pathway signaling through adaptor MyD88 and signaling effector mediators like TRAF3, TRAF6, IRAK-1, and IRAK-4. Finally, downstream transcriptional events start and induce the IFN genes expression through the transcriptional factor IRF7. Future inquiries could be expanded to investigate if this pathway is altered in HDAC6 deficient mice. Proteome analysis is also warranted to determine how HDAC6 deletion influences protein expression. We could utilize mass spectrometry (MS) technology to identify the acetylome of the peritoneal cells in HDAC6 knockout mice. As HDAC6 can remove acetyl groups from lysine residues of the protein, it would be expected that HDAC6 knockout mice have increased acetylation levels of its substrates. For these studies, we could collect the protein of the peritoneal cells in HDAC6 knockout mice, and then digest the protein by using trypsin. In order to enrich

the acetylated peptides, anti-acetyllysine beads would be incubated with the digested peptides. Acetylated peptides would then be measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and acetylated peptides relative abundance would be determined. Utilizing MaxQuant analysis [1], we would identify increased acetylation of proteins in the peritoneal cells in HDAC6 knockout mice to compare with WT mice. Increased acetylation of proteins would be confirmed by immunoprecipitation with acetyl beads. To elucidate the functional implications of the modified acetylation level, we would conduct pathway analysis on the acetyl-proteome by utilizing the PANTHER analysis [2] and KEGG pathway analysis. To validate whether proteins with increased acetylation level actually interacts with HDAC6, we would study the interactome of HDAC6 by utilizing immunoprecipitation (IP) and MS. We would immunoprecipitate HDAC6 and check the interactome by using LC-MS/MS. The protein interaction would then be confirmed by co-immunoprecipitation (co-IP). Combining the acetylome and interactome of HDAC6, we would hopefully identify potential target proteins of HDAC6. The pathway analysis should give us insight into the function of these target proteins, which should participate in immune system regulation.

In C57BL/6 mice, pristane injection induces pulmonary hemorrhage within the first two weeks following injection. This results in up to 20 percent of the animals having to be euthanized. Therefore, differences in immune cells between HDAC6 knockout and WT mice in long-term pristane treatment studies become harder to assess as the mortality in early inflammatory period may tend to skew the results in the surviving animals. As we observed, only two of the WT mice developed proteinuria and three female WT mice had

a higher level of anti-dsDNA IgG after pristane administration. In order to fully understand the immune cells profiles and immune cell gene expression difference between HDAC6 knockout mice and WT mice, single cell RNA sequencing (scRNA-seq) could be utilized to identify different cell types and precise gene expression related to lupus diseases [3; 4; 5; 6; 7; 8; 9; 10; 11]. Traditionally, flow cytometry could be utilized to identify the composition of immune cells and bulk RNA sequencing was used to detect gene expression in lupus. Nevertheless, flow cytometry is imperfect because it utilizes a limited set of cell markers while bulk RNA sequencing could not detect gene expression differences of specific type cells [12]. scRNA-seq of mouse splenocytes is an unbiased comprehensive systematic approach to simultaneously identify and characterize the population and composition along with transcriptional profile of immune cells in the spleen [13]. Moreover, scRNA-seq can identify and annotate transcriptomic signatures of specific type cells as well as genetic variants in lupus. The transcriptomic signatures of immune cells help us identify novel pathogenic factors and pathways [14]. For these experiments, we could isolate the splenocytes of HDAC6 knockout mice and wild type mice after pristane administration, then use Chromium Next GEM Single Cell 3' Reagent Kits v3.1. We will lyse the cell, prepare the library, reverse transcribe the barcoded RNA, amplify the cDNA, add the sample index and Illumina adapters, send the library for sequencing, generate the reads, and perform data analysis by utilizing R and other bioinformatic packages. After analyzing the results of scRNA-seq, we could characterize distinct cell subsets in the spleen, like B cells, T cells, monocytes, macrophages, plasmacytoid dendritic cells (pDC), and natural killer (NK) cells. Among these cells, we could identify pathological populations that contributed to lupus disease. We could also

identify the heterogeneity within an immune cell population and discover cell networks that affect each other cells through interaction and communication intercellularly. To fully elucidate the molecular mechanism and chromatin accessibility landscape behind pristane-induced lupus, we could perform the single cell assay for transposase accessible chromatin sequencing (scATAC-seq) [15; 16; 17]. This technology can delineate the immune cells active regulatory DNA landscape in pristane-induced lupus mice at the single-cell level. This method can help us explore the transcription factors (TFs) that potentially contribute to inflammation responses to pristane. By using scATAC-seq, we could identify the cell type-specific gene regulation networks and characterize cell type-specific TF motifs. In brief, we will collect peripheral blood mononuclear cells (PBMCs) from HDAC6 knockout mice and WT mice after pristane administration, and Chromium Next GEM Single Cell ATAC v2 kit will be used following the manufacturer's guide. Main steps of data processing include processing barcode, aligning genome, making duplicate, calling perks, calling cells, clustering and projecting to t-SNE, annotating peak, analyzing TF motifs, and analyzing differentially accessible peaks. After analyzing the data, we would be able to identify the distinct cell types and cell type-specific TF motifs, then compare the open chromatin patterns between HDAC6 knockout and WT mice, and finally identify the key TFs in HDAC6 knockout mice in response to pristane treatment.

It has been reported that HDAC6 is a key regulator of cytoskeletal dynamics. It alters two important cytoskeletal substrates: cortactin, a protein related to filamentous actin (F-actin) that is necessary for actin-related protein(Arp) 2/3-mediated actin network assembly, and α-tubulin [18; 19]. Microtubule-dependent cell motility is regulated by HDAC6-mediated

deacetylation. In mammalian cells, overexpression of HDAC6 causes tubulin hypoacetylation and facilitates chemotactic cell migration [20]. On the other hand, different pharmacological or genetic approaches that limit HDAC6 function cause hyperacetylation of tubulin and microtubule, increase the buildup of focal adhesions, and impair fibroblast mobility [21; 22]. Whereas cortactin is hypoacetylated when HDAC6 is overexpressed, it is hyperacetylated when HDAC6 activity is inhibited, which also hinders cortactin's translocation to the cell periphery, inhibits its interaction with F-actin, and hampers cell motility [19]. Thus, through deacetylating α-tubulin and cortactin, HDAC6 controls cell motility. Our studies show that HDAC6 deletion decreased pristane-induced recruitment of $CD11b^{+}Ly6C^{++}$ inflammatory monocytes and $CD11b^{+}Ly6G^{+}$ neutrophils. To study further the role of HDAC6 inhibition on myeloid cell function, we would examine the migration ability of macrophages and neutrophils. We would separate the inflammatory monocytes and neutrophils in the peritoneal cavities of WT and $HDAC6^{-/-}$ mice after pristane 10-day treatment. Then we would culture these cells and measure the migration ability by transwell assays. To investigate the possibility that macrophage movement requires deacetylase activity of HDAC6, HDAC6-specific inhibitors could be added to the cultures including tubacin and ACY-738. Then with inflammatory stimulation, monocytes and neutrophils migration ability could be assessed. Because HDAC6 has been connected to cytoskeletal regulation and phagocytosis necessitates the dynamic remodeling of the cytoskeleton, we could also investigate the function of HDAC6 in the control of cytoskeletal remodeling during phagocytosis. For these studies, we could use bone marrow-derived macrophages from WT and $HDAC6^{-/-}$ mice and treat them with *E. coli*, and assess the phagocytotic ability of the cells using

flow cytometric analysis or immunofluorescence labeling. HDAC6's primary substrate, cortactin, binds F-actin to promote actin filament formation and branching, which are essential for phagocytosis and cell motility [23]. We would investigate the formation of filopodial protrusion and cortactin translocation in macrophages of WT and $HDAC6^{-/-}$ mice by using immunostaining. Macrophages would be immunostained with antibodies against cortactin and F-actin to check the formation of filopodial protrusion and cortactin translocation.

A potent technique for producing conditional gene knockout mice and understanding gene function *in vivo* is the Cre/loxP system. Conditional HDAC6 knock-out mice are useful tools for studying the roles of different cell types and tissues in disease models and during development. To study the role of HDAC6 in the B cells, T cells, and Th17 cells, we would generate the $CD19^{cre}HDAC6^{fl/fl}$ mice, $Lck^{cre}HDAC6^{fl/fl}$ mice, and $Il17a^{cre}HDAC6^{fl/fl}$ mice. And then we would analyze the several parameters of pristane-induced lupus to fully understand the role of HDAC6 in different cell types.

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