The Role of Histone Deacetylase 6 Inhibition on Systemic Lupus Erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a chronic multifactorial inflammatory autoimmune disease with heterogeneous clinical manifestations. Among different manifestations, lupus nephritis (LN) remains a major cause of morbidity and mortality. There are few FDA approved treatments for LN. In general, they are non-selective and lead to global immunosuppression with significant side effects including an increased risk of infection. In the past 60 years, only one new drug, belimumab was approved for lupus disease with modest efficacy in clinic and not approved for patients suffering for nephritis. Therefore, it is urgent to develop new treatments to replace or reduce the use of current ones.

Histone deacetylase 6 (HDAC6) plays a variety of biologic functions in a number of important molecular pathways in diverse immune cells. Both innate and adaptive immune cells contribute to pathogenesis of lupus. Among those cells, B cells play a central role in pathogenesis of lupus nephritis in an anti-body dependent manner through differentiation into plasma cells (PCs). As a result, HDAC6 inhibitors represent an entirely new class of agents that could have potent effects in SLE. Importantly, the available toxicity profile suggests that HDAC6 inhibitors could be advanced into SLE safely.

We have demonstrated previously that histone deacetylase (HDAC6) expression is increased in animal models of systemic lupus erythematosus (SLE) and that inhibition of HDAC6 decreased disease. ACY-738 is a hydroxamic acid HDAC6 inhibitor that is highly selective for HDAC6. In our current studies, we tested if an orally selective HDAC6 inhibitor, ACY-738, would decrease disease pathogenesis in a lupus mouse model with established early disease. Moreover, we sought to delineate the cellular and molecular mechanism(s) of action of a selective HDAC6 inhibitor in SLE. In order to define the mechanism by which HDAC6 inhibition decreases disease pathogenesis in NZB/W mice by using RNAseq to evaluate the transcriptomic signatures of splenocytes from treated and untreated mice coupled with applied computational cellular and pathway analysis. In addition, we sought to bridge between the transcriptomic data obtained from the HDAC6 treated mice and human gene expression information to determine the relevance to this target in possibly controlling human lupus.

We treated 20-week-old (early-disease) NZB/W F1 female mice with two different doses of the selective HDAC6 inhibitor (ACY-738) for 4~5 weeks. As the mice aged, we determined autoantibody production and cytokine levels by ELISA, and renal function by measuring proteinuria. At the termination of the study, we performed a comprehensive analysis on B cells, T cells, and innate immune cells using flow cytometry and examined renal tissue for immune-mediated pathogenesis using immunohistochemistry and immunofluorescence. We then used RNAseq to determine the genomic signatures of splenocytes from treated and untreated mice and applied computational cellular and pathway analysis to reveal multiple signaling events associated with B cell activation and differentiation in SLE that were modulated by HDAC6 inhibition.

Our results showed a reduced germinal center B cell response, decreased T follicular helper cells and diminished interferon (IFN)-γ production from T helper cells in splenic tissue. Additionally, we found the IFN-α-producing ability of plasmacytoid dendritic cells was decreased along with immunoglobulin isotype switching and the generation of pathogenic autoantibodies. Renal tissue showed decreased immunoglobulin deposition and reduced inflammation as judged by glomerular and interstitial inflammation.

The molecular pathways by which B cells become pathogenic PC secreting autoantibodies in SLE are incompletely characterized. RNA sequence data showed that PC development was abrogated and germinal center (GC) formation was greatly reduced. When the HDAC6 inhibitor-treated lupus mouse gene signatures were compared to human lupus patient gene signatures, the results showed numerous immune and inflammatory pathways increased in active human lupus were significantly decreased in the HDAC6 inhibitor treated animals. Pathway analysis suggested alterations in cellular metabolism might contribute to the normalization of lupus mouse spleen genomic signatures, and this was confirmed by direct measurement of the impact of the HDAC6 inhibitor on metabolic activities of murine spleen cells.

Taken together, these studies show selective HDAC6 inhibition decreased several parameters of disease pathogenesis in lupus-prone mice. The decrease was in part due to inhibition of B cell development and response. RNA sequence data analysis show HDAC6 inhibition decreases B cell activation signaling pathways and reduces PC differentiation in SLE and suggests that a critical event might be modulation of cellular metabolism.

The Role of Histone Deacetylase 6 Inhibition on Systemic Lupus Erythematosus

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General Audience Abstract

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease by which immune cells mistakenly attacks healthy self-cells in different organs. Kidney inflammation occurs in nearly 50% of patients with lupus resulting in kidney damage leading to end stage renal disease. Lupus nephritis (LN) is major cause of morbidity and mortality associated with SLE. Current treatments for LN consist primarily of immunosuppressants that block the immune response and leave the patients with unwanted side effects including an increased risk of infection. To circumvent the unwanted side effects, we explored a novel mechanism to target the immune response. My project was to determine whether histone deacetylase 6 (HDAC6) inhibition would suppress the autoimmune inflammatory response in lupus. We found that inhibition of HDAC6 was effective at attenuating early LN, probably by down-regulating innate immune response, which suppressed subsequent adaptive immune responses downstream. HDAC6 inhibition affected the innate immune response by inhibiting type I interferon production by plasmacytoid dendritic cells. HDAC6 inhibition affected the cell mediated immune response by decreasing T helper cell and B cell activation. To determine the mechanism by which HDAC6 inhibits immune cells activation, we used RNAseq to reveal HDAC6 inhibition on multiple signaling events associated with the induction of lupus disease. These results suggest that HDAC6 could be a potential therapeutic target in the early stage of LN.

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Chapter 1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the body's immune system produces autoantibodies against normal healthy tissue or against cellular components to form immune complexes that are deposited in various tissues inducing inflammation leading to tissue damage (1). Autoantibodies are produced by activated autoreactive B cells through antibody class switching and somatic hypermutation, finally differentiation into antibody secreted plasma cells with help from activated autoreactive T cells (2). In early disease, several studies have shown a critical role for the pro-inflammatory cytokine IFN-alpha (3-6). Additionally, studies using lupus-prone mice have demonstrated that the ablation of plasmacytoid dendritic cells (pDCs) which are the major source of IFN-alpha prevented lupus nephritis (LN) progression, reduced activation of autoreactive T and B cells, decreased autoantibodies in the circulation and renal deposition (7, 8). These studies highlight the critical involvement of innate immune activation in the initiation of SLE. LN occurs in approximately 50% SLE patients and is a major cause of morbidity and mortality in this disease (9). Although much is known about the mechanisms regulating T cell: B cell collaboration and PC generation in SLE, many details of the intracellular event regulating this process have not yet been delineated.

Post-translational modification (PTM) of proteins is an important means to regulate protein: protein interactions and downstream cellular functions. Among the various PTMs of proteins, acetylation plays a major role. Acetylation/deacetylation events are reversible PTM on lysine residues of histone and non-histone proteins and are essential for specific protein: protein

interactions and in the nucleus for gene regulation. These reactions are typically catalyzed by enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. HDACs are classified into four subclasses: three Zn2+-dependent classes (I, II, and IV), and one NAD+-dependent class III. Class II is subdivided into class IIa and class IIb. HDAC6 belongs to HDAC class IIb and is largely cytoplasmic in location. It is associated with non-histone substrates, including α-tubulin, heat shock protein 90 (HSP90), and cortactin and others and has been shown to modulate immune cell function in various ways, including modifying BCL6 function and B cell maturation (10-14).

In our previous studies, we have demonstrated that inhibition of HDAC6, a class IIb HDAC, resulted in the amelioration of LN in lupus-prone mice associated with several altered immune responses, with minimal toxicity, suggesting therapeutic potential to treat LN (15-17). SLE, is a chronic inflammatory disease that is generally divided into the early autoimmune initiating stage and the later autoimmune effector stage (18-20). It is still not well understood whether the effects of HDAC6 inhibition to altered autoimmune responses are at the priming stage or effector stage.

In the following chapters, I have included literature reviews to summarize current research progresses related to the roles of HDACs, in particular HDAC6 in the development of SLE. This is followed by an original research study showing that HDAC6 inhibition was able to downregulate the autoimmune responses in early disease and inhibit the initiation of LN. Finally, based on this research study, a possible treatment strategy with HDAC6 inhibitor administration is proposed and discussed as a further research direction in the future.

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Chapter 2. The Impact of Protein Acetylation/Deacetylation on Systemic Lupus Erythematosus (Literature Review)

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Abstract

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease in which the body's immune system mistakenly attacks healthy cells. Although the exact cause of SLE has not been identified, it is clear that both genetics and environmental factors trigger the disease. Identical twins have a 24% chance of getting lupus disease if the other one is affected. Internal factors such as female gender and sex hormones, the major histocompatibility complex (MHC) locus and other genetic polymorphisms have been shown to affect SLE, as well as external, environmental influences such as sunlight exposure, smoking, vitamin D deficiency, and certain infections. Several studies have reported and proposed multiple associations between the alteration of the epigenome and the pathogenesis of autoimmune disease. Epigenetic factors contributing to SLE include microRNAs, DNA methylation status, and the acetylation/deacetylation of histone proteins. Additionally, the acetylation of non-histone proteins can also influence cellular function. A better understanding of non-genomic factors that regulate SLE will provide insight into the mechanisms that initiate and facilitate disease and also contribute to the development of novel therapeutics that can specifically target pathogenic molecular pathways.

Introduction

Systemic lupus erythematosus (SLE) is a pathophysiologically complex systemic autoimmune disease affecting multiple organs [1-3]. Epigenetic regulation refers to the change of the epigenomic pattern that in turn alters gene expression specifically related to modified DNA sequences [4]. Epigenetic abnormalities in cancer research has been widely studied for decades. Because disease pathogenesis between autoimmune diseases and cancer share some similarities, epigenetic contributions have been proposed in the regulation of autoimmune disease [5]. Epigenetic regulation may involve microRNAs, acetylation and methylation of histone proteins and DNA methylation—all of which have been linked to the initiation, onset, progression and perpetuation of SLE [6-8]. Attenuation of lupus-like disease in murine models via treatment with histone modifiers has been reported in many studies. In regard to histone acetylation/deacetylation, different isoforms of histone deacetylase enzymes may be predominantly nuclear, cytoplasmic or shuttle between the nucleus and cytoplasm. Our studies have demonstrated that histone deacetylases (HDACs) are significantly upregulated in lymphocytes in MRL/lpr lupus prone mice [9, 10]. Other studies have also found elevated histone acetylation in innate immune cells, such as monocytes [11-13]. Moreover, studies have shown that global alteration of DNA methylation is pathogenic in lymphocytes and innate immune cells; specifically, hypomethylation has been highly correlated with disease activities in SLE patients [14]. In this review, we will briefly summarize the role of DNA methylation in SLE and highlight immunopathogenic contributions of acetylation and deacetylation to lupus.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease that involves genetic predisposition, epigenetic modification and environmental factors that lead to alteration in both the innate and adaptive immune responses, including abnormalities in apoptotic cell clearance, cytokine production, and dendritic cell, B-cell and T-cell activation [1, 15, 16]. Lupus is highly associated with deficiency of apoptotic clearance [17]. While the initial triggers vary, the excessive accumulation of apoptotic debris, in particular microparticles containing nuclear material, can activate antigen presenting cells including dendritic cells and B cells, which initiate the cellular interactions that lead to the generation of antinuclear antibodies through interactions with autoreactive T cells [17, 18]. T cells not only activate B-cell responses but also infiltrate target tissues and cause damage. Type I and type II interferons (TFN I/II), tumor necrosis factors (TNF), B-lymphocyte stimulators (BLys), interleukin 6, interleukin 17, interleukin 18, interleukin 21, and many other cytokines are involved in autoimmune priming and induce inflammatory mediated tissue injury in patients with lupus [19-21].

One of the most severe manifestations of SLE is lupus nephritis (LN). LN remains a major cause for morbidity and mortality in SLE patients [3, 22-28]. Glomerulonephritis (GN) is the most common form of LN, which is frequently accompanied by tubulointerstitial and/or vascular lesions [29]. In the kidney, immune complexes containing anti-DNA and anti-nucleosome antibodies contribute to lupus nephritis and initially deposit in the endothelial and mesangial areas, and then in the basement membrane and epithelial areas [30]. Importantly, these immune complexes initiate a further influx of inflammatory cells by activating the complement cascade.

Genetic variations suggest a predisposition to SLE development [31]. Both single gene deficiency, such as complement C1q and C4, and the effect of a large number of genetic variations including single-nucleotide polymorphisms (SNPs) within noncoding regions of immune response–related genes are associated with SLE [31-33]. However, these findings can only account for about 15% of the heritability of SLE, suggesting that factors other than genetic variations may have additional effects on SLE development [34]. UV light exposure is a well-documented environmental trigger of SLE [2, 29]. However, paradoxically, avoidance of sunlight to reduce UV light exposure can result in a vitamin D deficiency, which is negatively associated with SLE severity [35]. Additionally, viral infections may trigger SLE by self-antigen mimicry and induction of inflammation [36-38]. As the disease ratio of female to male is about 9 to 1, hormones and the X chromosome are believed to play a role in the increased prevalence of SLE among women [39, 40]. Epigenetic changes, including DNA methylation, histone modifications (acetylation and methylation), and acetylation of non-histone proteins, influence DNA accessibility to transcription factors, as well as translocation and addition of proteins resulting in altered gene expression. These epigenetic changes have emerged as possible mechanisms that initiate or contribute to the pathology of SLE.

Methylation in SLE

Methylation of C bases in CG pairs, known as DNA methylation, is characterized by the addition of a methyl group to the carbon residue in a gene promoter region. Histones are a group of conserved proteins associated with the DNA strand that regulate DNA stability, replication, and transcription through different histone modifications, including methylation and acetylation. The modification of DNA and histones may act as epigenetic regulators of gene expression. DNA methylation promotes the repression of gene transcription through prevention of transcription

factors binding to the chromatin structure [41]. Histone modifications can be very dynamic based on the activation and differentiation status of the cell (Figure 1) [42, 43].

The induction of this lupus-like syndrome depends on the level of methylation of genome DNA from the apoptotic cells. Studies have reported that perturbation of DNA methylation results in the activation of the apoptotic pathway. Aberrant clearance of apoptotic DNA plays a critical role in the development of SLE. It has also been shown that the injection of extracted DNA from stimulation-induced apoptotic lymphocytes into a mouse model induced a lupus-like disease, characterized by the production of anti-dsDNA antibodies and lupus nephritis [44-46]. Upon re-methylation of apoptotic DNA, the ability to induce anti-dsDNA antibody was dampened. In contrast, an increase in DNA demethylation boosts its ability to stimulate an autoimmune response [47]. Toll-like receptor 9 (TLR9), which recognizes hypomethylated DNA to induce an inflammatory response in innate immune cells, drives an enhanced type I interferon response that is likely to contribute to the DNA methylation effect on SLE development [48].

Studies have used DNA methylation inhibitors to induce lupus-like diseases in murine models and found impaired DNA methylation in SLE patients, both of which support the relationship between DNA hypomethylation and lupus disease [49-51]. CD11a is an integrin involved in cellular adhesion and co-stimulation and provides a critical initial interaction between T cells and antigen-presenting cells, stabilizing the immune synapse. CD11a is overexpressed in lupus via the regulation of DNA methylation. Lu et al. show that overexpression of CD11a from CD4+ T cells in patients with active lupus is positively correlated with SLE disease activity index

(SLEDAI) score. They further demonstrated that demethylation of the promoter region of CD11a is higher in active lupus patients compared with inactive patients and normal controls [52].

Enhancer of Zeste homologue2 (EZH2) can act as an epigenetic regulator of gene expression, either through trimethylation of lysine 37 in histone 3 (H3K27me3) or direct control of DNA methylation by recruiting DNA methyltransferase [53, 54]. Unlike other histone methylation, which relaxes chromatin and turns on gene transcription, trimethylation of H3K27 causes the chromatin to compact and repress gene transcription [55-57]. In lupus patients, H3K27me3 is enriched in the hematopoietic progenitor kinase 1 (HPK1) promotor region [58, 59]. As HPK1 acts as a negative regulator of T cell-mediated IFNγ and IgG production, it may explain part of the lupus pathophysiology. Additionally, EZH2 is highly expressed in naïve CD4+ T cells in lupus patients compared to healthy controls and positively correlates with lupus disease activity. Increased EZH2 expression also induces overexpression of junctional adhesion molecule A (JAM-A) through DNA hypomethylation. In this regard, upregulated JAM-A promotes T cell adhesion and T cell survival, increasing the ability for T cells to migrate to inflamed tissues in lupus [60].

CD40L (CD154) and CD70 are two molecules that are expressed on activated T cells and involved in co-stimulation during T cell: B cell interaction. Both molecules have been shown to be upregulated on T cells in patients with active SLE compared to lupus patients with inactive disease or healthy individuals. Furthermore, hypomethylation of these molecules at the promoter regions of T cells has been demonstrated to promote disease [61-63]. Recently, a study by Ulff-Muller and coworkers reported that hypermethylated DNA in B cells contributed to SLE, which

contrasts with DNA hypomethylation in T cells [64]. In addition to methylation of DNA, acetylation of histone and non-histone proteins has been reported to play a role in the development of lupus and has been of particular interest in our research studies [65].

Acetylation in SLE

The acetylation and deacetylation of amino-acid residues within histone tails has been shown to be a main factor influencing chromatin structure and modulating gene transcription, both positively and negatively [66, 67]. Histone acetylation regulates gene transcription in different ways. First, acetylation of lysine residues within histone tails neutralizes the positive charge of histone proteins, loosening chromatin structure. This increases the accessibility of transcription factors to the promoter regions of their target genes [68]. Second, acetylated histones also function as binding sites for other proteins that act as transcriptional co-activators. In contrast, histone deacetylation promotes transcriptional suppression via chromatin compaction [69]. Third, direct acetylation and deacetylation of transcription factors and proteins, other than histones, have been shown to have both positive or negative regulating roles on gene expression (Figure 2) [70].

Histone acetylation is controlled by the opposite actions of two large families of enzymes—the histone acetyltransferases (HATs) and histone deacetylases (HDACs).

The HDAC superfamily encodes 11 proteins with a highly conserved deacetylase domain (Table 1). These proteins can be classified into four families (class I, IIa, IIb and IV), which differ in function, cellular and subcellular localization, and expression patterns. The class I HDAC family consists of HDAC1, 2, 3 and 8 [71-73]. They are expressed ubiquitously, localized

predominantly to the nucleus, and display high enzymatic activity toward histone substrates. HDAC1, HDAC2 and HDAC3 are nearly identically in repressive complexes [74], whereas HDAC8 seems to work alone without binding to repressive complexes [72].

HDAC4, 5, 7 and 9 belong to the class IIa HDAC family. They have conserved binding sites for the transcription factor myocyte enhancer factor 2 (MEF2) and the chaperone protein 14-3-3. These HDACs can translocate between the nucleus and the cytoplasm after phosphorylation by kinases and thereafter binding to the chaperone protein 14-3-3 [75-78]. The regulated phosphorylation of class IIa HDACs functions as a linker between extracellular signals and transcriptional changes in the nucleus, and plays key roles in different tissues during development and disease. Different from other HDACs, class IIa HDACs show relatively restricted expression patterns, with HDAC4 expressed in the brain, HDAC5 and HDAC9 enriched in muscle, the heart and brain, and HDAC7 mostly found in the thymus and endothelial cells [75, 79-81].

The Class IIb family consists of HDAC6 and HDAC10. HDAC6 mainly resides in the cytoplasm, and the function of HDAC6 is not well understood [82-84]. The direct targets of HDAC6 include the cytoskeletal proteins α-tubulin and cortactin, transmembrane proteins such as the interferon receptor IFNαR, and chaperones [85-89]. HDAC11 is the only class IV HDAC discovered to date, but its function is not well delineated [90, 91].

The roles of HDACs in lupus have been reported by us and others, and HDAC inhibitors are indicated to be a possible new treatment for lupus. There are pan-HDACs and selective HDAC inhibitors. Pan-HDAC inhibition causes a global interference of epigenetic programming which

could result in both therapeutic as well as adverse effects. In contrast, selective HDAC inhibition has less toxicity based on their limited substrates, location, and specific enzyme activities.

In T cells of SLE patients, CD40L and IL-10 are overexpressed, whereas IFN-gamma is downregulated [92]. After treatment with trichostatin A (TSA), a reversible pan inhibitor of HDAC, the expression levels of CD40L, IL-10 and IFN-gamma were corrected in patient T cells, suggesting the involvement of HDACs in abnormal T cell activation in SLE patients through at least regulating CD40L and IL-10 expression, which are both critically involved in T cell: B cell interaction and B cell activation. TSA also suppressed IL-2 production by activated T cells by downregulating T cell receptor zeta chain signaling [93]. Treatment of either TSA or another HDAC inhibitor, suberonylanilide hydroxamic acid (SAHA) targeting HDAC I in MRL/lpr lupus-prone mice showed reduced proteinuria, glomerulonephritis, spleen weight, and mesangial cell inflammation, associated with an increased accumulation of acetylated histones H3 and H4 in total cellular chromatin [94, 95]. Recently Hu et al. reported reduced H3 and H4 acetylation and decreased IFN-gamma expression in mouse splenocytes of SLE patient peripheral blood mononuclear cell (PBMC) [96]. Conversely, Garcia et al. reported hypoacetylation in splenocytes of MRL/lpr lupus-prone mice compared to MRL/MpJ control mice [97]. Besides the effects of HDACs on T cell activation, it has been reported that HDAC1 is recruited to the IgH enhancer region, and TSA treatment of B cells reduced the production of anti-DNA autoantibodies directly, highlighting the influence of HDACs on B cells in lupus mice [98]. In New Zealand Black/White (NZB/W) F1 female mice, TSA administration resulted in an increase in regulatory T cells and a decrease of CD69+ activated T helper cells which correlated with reduced lupus nephritis [99]. Another pan-HDAC inhibitor, panobinostat targeting class I, II and

IV HDACs, decreased the percentage of autoreactive plasma cells and reduced the production of autoantibodies in MRL/Lpr mice in pre-disease stage [100].

Hu et al. reported differential functions and expression patterns of different HDACs in MRL/lpr mice. They demonstrated increased SIRT1 expression but decreased HDAC7 expression in MRL/lpr mice compared to MRL control mice [101]. SIRT1 siRNA treatment reduced tubulointerstitial scores but had no effect on proteinuria and serum autoantibody levels, which was different from the effects of the pan HDAC inhibitor TSA. HDAC3 and 11 have been reported to be decreased in monocytes from SLE patients compared to healthy individuals, suggesting their possible roles in suppressing autoimmune responses [102]. In addition, the transcription factor RFX1 which recruits the co-repressor HDAC1 is reduced in T cells of SLE patients. This results in the overexpression of CD11a and CD70 on T cells of SLE patients, suggesting the immunosuppressive effect of HDAC1 and suggests the immune repressive effect of TSA may work on HDACs other than HDAC1 [103]. HDAC9 deficiency in MRL/lpr mice has been shown to reduce lupus symptoms and increase survival rates compared to HDAC9 intact MRL/lpr mice [104]. In the study, effector T cells in the HDAC9-/- mice switched from a Th1 and Tfh into a Th2 phenotype with increased acetylation of histone proteins globally at the IL-4 gene locus, suggesting HDAC9 inhibition may benefit SLE patients. However, TSA is a pan HDAC inhibitor. Another study, using a selective class I and II HDAC inhibitor (ITF2357) demonstrated reduced disease in NZB/W F1 mice, suggesting that class I and II HDAC are involved in lupus pathogenesis [105]. In addition to regulating protein-translating genes, HDACs have also been reported to regulate microRNAs that suppress B cell responses [106]. We have recently reported that HDAC6 inhibition upregulated microRNA targeting AID and Blimp-1,

which are critical factors in B cell responses, resulting in reduced lupus disease in MRL/lpr mice. Furthermore, we found, reduced germinal center B cells, T follicular cells and IFN-gamma secreting cells suggesting HDAC6 inhibition contributed to the downregulation of adaptive immune response in lupus nephritis [107]. In our studies, we have shown that selective HDAC6 inhibition decreases IFN-alpha production in initiation stage of the disease. Furthermore, we found that administration of the selective HDAC6 inhibitor ACY-738 *in vitro* led to decreased IFN-alpha production in a dose-dependent manner [107]. HDACs also promote neutrophil extracellular traps (NETs) which act as an important source of self-antigens [108]. Our previous studies have shown MRL/lpr mice have increased levels of HDAC6 and HDAC9 compared to non-autoimmune B6 mice. However, B6 animals showed increased expression of HDAC10 compared to MLR/lpr lupus prone mice in splenic B and T cells [9]. As HDAC6 is primarily localized to the cytosol, the selective HDAC6i, ACY-738, blocks HDAC activity in the cytoplasm while not affecting nuclear histones [109]. We found that HDAC6i treatment of B cells increased NFkB acetylation, prevented translocation to the nucleus, and suppressed B cell development at the pre-B cell stage [110]. Further treatment with ACY-738 in NZB/W F1 mice reduced lupus nephritis, sera anti-dsDNA level associated with increased splenic Tregs, and decreased Th17 cells [110, 111]. These studies suggest that HDAC6 is involved in lupus development by various mechanisms. Transcription factor Fli-1 regulates G-CSF production to control neutrophil infiltration into the kidneys, causing kidney inflammation in lupus [112-114]. Deacetylation at aa380 decreases Fli-1 driven activation of the G-CSF promoter to decrease inflammatory cytokine secretion in lupus prone mice [115]. In human studies, Th17 cells play a pivotal role in the contribution to the pathogenesis of SLE via secreting the IL-17 inflammatory cytokine. Increasing HDAC3 acetylation led to overexpression of IL-17A through

downregulating expression of transcription factor RFX1, which suggests HDAC3 acts as a nuclear epigenetic regulator in SLE patients [116].

Metabolism and Epigenetic Crosstalk in Lupus

The methylation/demethylation of DNA and histones and acetylation/deacetylation of histone and nonhistone proteins alter gene expression and immune cell function in lupus. However, these epigenetic reactions are reversible and can be affected by the availability of substrates from metabolic pathways. Advancements in the field of immunometabolism have suggested that aberrant metabolic pathways may also play a critical role in the pathogenesis of lupus disease [117].

Lupus patients have been reported to exhibit a depletion of intracellular glutathione, a vital cellular antioxidant [118]. Glutathione depletion has been reported to increase the target of the rapamycin (mTOR) signaling pathway [119, 120]. The mTOR signaling pathway acts as a central regulator in cell metabolism, growth, proliferation and survival by mainly controlling energy utilization and protein synthesis, and this pathway exists in almost all immune cells [121, 122]. Activation of mTOR occurs in T cells and other cell types in lupus and is responsible for multiple pathogenic processes [123-127]. Recent studies have suggested that the addition of N-acetyl L-cysteine (NAC), which helps to replenish intracellular glutathione, decreases lupus through blocking mTOR [128].

The kynurenine pathway is a metabolic pathway leading to the production of nicotinamide adenine dinucleotide (NAD+) from the degradation of the essential amino acid tryptophan. Disruption in the pathway is associated with certain genetic disorders [129-131]. In lupus

patients, it was reported that NAC significantly reduced kynurenine, which also decreased mTOR signalling. These results suggest that reversal of glutathione depletion by the amino acid precursor or inhibition of the kynurenine pathway may reduce the activation of mTOR in SLE [119, 132, 133]. Rapamycin, a specific mTOR inhibitor, can effectively decrease lupus disease in both lupus-prone mice and patients [124]. Rapamycin was shown to promote demethylation of genes at the 5-position of cytosine (5mC) in the mTOR pathway in naïve CD4+ T cells. This inhibited the differentiation of Th1 and Th17 cells that can both contribute to lupus development [134].

The activation of mTOR promotes glycolysis and lipogenesis to generate acetyl-CoA, which the substrate histone acetylation relies on to provide an acetyl group and complete the reaction. Indeed, one of the targets of mTOR is the acetylation of histone proteins. However, the consequence of this is not clear. In one study, it was shown that acetylation of histone H3 at lysine 56, H3K56ac, was directly inhibited by rapamycin [135, 136]. However, H3K56ac was activated by mTOR and promoted mTOR-dependent growth, suggesting positive feedback between mTOR activation and histone acetylation [137]. Furthermore, histone deacetylation is mediated by the nicotinamide adenine dinucleotide (NAD) dependent sirtuin deacetylase. The bio-generation of NAD from the kynurenine pathway is upregulated in lupus. Additionally, it has been shown that the kynurenine pathway also promotes mTOR activation which, as discussed above, promotes histone acetylation [131, 138-140]. Therefore, an overall balance and availability of metabolic substrates may determine opening or closing of gene transcriptions by affecting the level of histone acetylation or deacetylation, respectively.

Summary

Aberrant epigenome gene regulation and modification plays a crucial role in the pathogenesis of SLE. Comprehensive understanding of how epigenetic modification and acetylation/deacetylation of non-histone proteins corrects or promotes autoimmune disease will enable us to gain further insight into the pathogenic mechanisms of autoimmune disorders. By understanding how acetylation/deacetylation and methylation/demethylation modulate gene expression and cell signaling, we will be able to more effectively target the signaling cascades and gene expressions that initiate and promote aberrant cell function in SLE.

Figures

Figure 1.

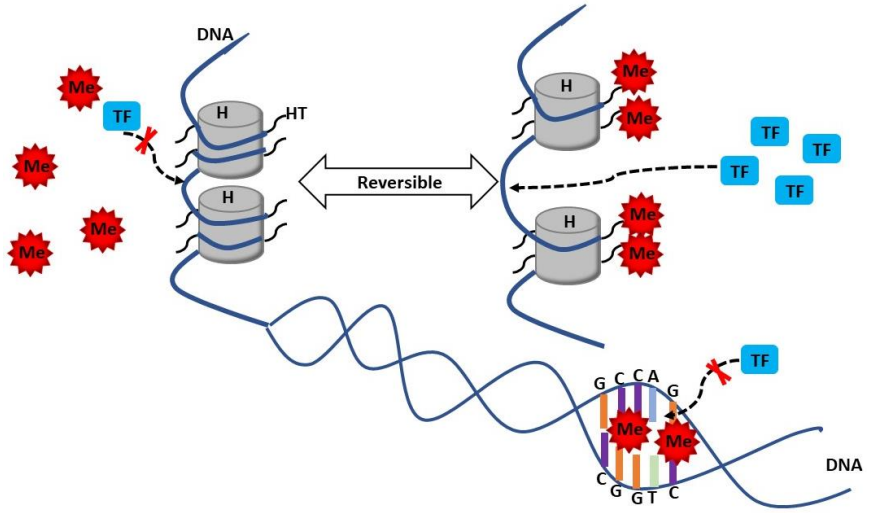

Figure 1. Histone and DNA methylation and demethylation. Histone methylation adds methyl group to the residue of histone tails and increases gene transcription by uncoiling DNA from histone and opening more DNA binding sites to transcriptional factors. DNA methylation happens between paired CG groups in DNA sequences. It prohibits the transcriptional factors binding to DNA and represses the gene transcription. H: Histone; HT: Histone tails; Me: Methylation; TF: Transcriptional factor.

Figure 2.

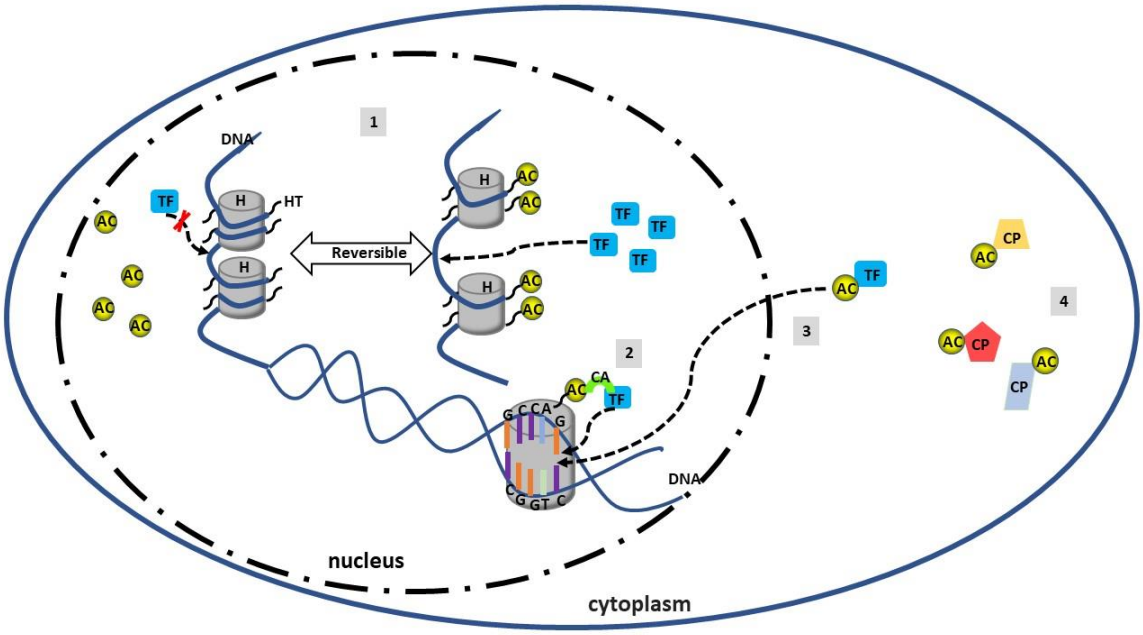

Figure 2. Histone and nonhistone protein modified by acetylation and deacetylation. Acetylation and deacetylation refer to removal or addition of acetyl group from targeted protein or DNA. (**1**) Histone acetylation and deacetylation is a dynamic and reversible reaction that alters the structure of histones and affect the gene transcription by loosening or compacting the DNA. (**2**) Acetylated histones can act as a binding site for other proteins which are co-activators of transcriptional factors. (**3**) Nucleus translocation and DNA binding affinity of transcriptional factors in the cytoplasm can be modified by acetylation or deacetylation. (**4**) Acetylation and deacetylation can regulate other nonhistone proteins in the cytoplasm and alter their function in cellular activities. H: Histone; HT: Histone tails; AC: Acetylation; TF: Transcriptional factor; CA: Co-activator; CP: Cytoplasm protein.

Tables

Table 1. Summary of HDAC Classifications.

Histone deacetyla-ses (HDAC) Classifica-tion	Enzyma-tic Activity	Mechanism of Action	Location	Substrates	HDAC Inhibitor	Autoimmu-nity and systemic lupus erythemat-osus (SLE) Involvement
Class I.						
HDAC1	Enhanced when incorporated into complexes	1 class I catalytic domain	Nucleus	p53, RB, MyoD, NF-kB, DNMTI, DNMT3a, MBD2, Sp1, BRCA1, MeCP2, ATM, Smad7 [61, 141]	Valproic acid, phenylbutyrate, MS-275, Romidepsin, Suberoylanilide Hydroxamic Acid [142]	Overexpression of HDAC1 increases the activity of the 3'-IgH enhancers. HDAC1 is recruited to the IgH enhancer region, and TSA treatment of B cells reduced the production of anti-DNA autoantibodies.
HDAC2	Enhanced when incorporated into complexes	1 class I catalytic domain	Nucleus	RB, NF-kB, BRCA1, DNMTI [143]	Valproic Acid, phenylbutyrate, Suberoylanilide Hydroxamic Acid, MS-275, Romidepsin [142, 144, 145]	Critical for transcriptional regulation, cell cycle progression and developmental processes.
HDAC3	Enhanced when	1 class I catalytic	Nucleus/Cytoplasm	RB, NF-kB,	Valproic Acid,	HDAC3 gene

	incorporated into complexes	domain		Smad7, Stat3, SRY [143]	Suberoylanilide Hydroxamic Acid, MS-275 [142, 146]	expression is decreased in SLE monocytes, involved in macrophage polarization.
HDAC8	Fully active in isolation	1 class I catalytic domain	Nucleus	Not Reported	Suberoylanilide Hydroxamic Acid, Resveratrol, APHA, Curcumin [142, 147]	Downregulate the expression of pro-inflammatory cytokines (TNF-alpha, TGF-beta, IL-1beta, and IL-6).
Class IIa.						
HDAC4	Weak enzymatic activity in isolation	1 class II catalytic domain	Nucleus/Cytoplasm	GCMa, GATA-1, HP-1 [141, 148, 149]	Not reported	Role in pro-inflammatory gene expression.
HDAC5	Weak enzymatic activity in isolation	1 class II catalytic domain	Nucleus/Cytoplasm	GCMa, Smad7, HP-1 [141, 150]	TSA [142]	HDAC5 mRNA expression is enhanced in inflammatory states.
HDAC7	Weak enzymatic activity in isolation	1 class II catalytic domain	Nucleus/Cytoplasm	PLAG1, PLAG2 [141, 151]	Not reported	Promotes inflammatory responses in macrophages, regulates TLR responses in macrophages, regulates LPS signaling.
HDAC9	Weak enzymatic activity in isolation	1 class II catalytic domain	Nucleus/Cytoplasm	Not Reported	Suberoylanilide Hydroxamic Acid, MS-	Regulates Foxp3-dependent suppression.

					275 [142]	Increase in Treg cells—decrease in suppressive activity. HDAC9 inhibition may benefit SLE patients as shown in MRL/Lpr mice.
Class IIb.						
HDAC6	Acts on structural proteins	2 class II catalytic domains with 1215 amino acids. SE14 repeats. BUZ is ZnF domain	Mainly cytoplasmic	Smad7, α-Tubulin, Hsp90 [143, 152]	M344 [111, 141, 142]	HDAC6 is overexpressed in SLE—causes an increased B cell development and response. Inhibition causes reduced germinal center B cells, T follicular cells and IFN-gamma secreting cells.
HDAC10	Not measurable	2 class II catalytic domains	Nucleus/Cytoplasm	Not reported	Not reported	Overexpressed in B cells from the spleen.
Class IV.						
HDAC11	Regulates immune activation and immune tolerance	1 class IV catalytic domain	Nucleus	Not reported	Not reported	Gene expression is decreased in SLE monocytes, negative

	transcription al regulator

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Chapter 3. Selective HDAC6 inhibition decreases early stage of lupus nephritis by downregulating both innate and adaptive immune responses

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Abstract

We have previously demonstrated that HDAC6 expression is increased in animal models of systemic lupus erythematosus (SLE) and that inhibition of HDAC6 decreased disease. In our current studies, we tested if an orally active selective HDAC6 inhibitor would decrease disease pathogenesis in a lupus mouse model with established early disease. Additionally, we sought to delineate the cellular and molecular mechanism(s) of action of a selective HDAC6 inhibitor in SLE. We treated 20-week-old (early-disease) NZB/W F1 female mice with two different doses of the selective HDAC6 inhibitor (ACY-738) for five weeks. As the mice aged, we determined autoantibody production and cytokine levels by ELISA, and renal function by measuring proteinuria. At the termination of the study, we performed a comprehensive analysis on B cells, T cells, and innate immune cells using flow cytometry and examined renal tissue for immune-mediated pathogenesis using immunohistochemistry and immunofluorescence. Our results showed a reduced germinal center B cell response, decreased T follicular helper cells and diminished IFN-α production from T helper cells in splenic tissue. Additionally, we found the IFN-alpha-producing ability of plasmacytoid dendritic cells was decreased along with immunoglobulin isotype switching and the generation of pathogenic autoantibodies. Renal tissue showed decreased immunoglobulin deposition and reduced inflammation as judged by glomerular and interstitial inflammation. Taken together, these studies show selective HDAC6 inhibition decreased several parameters of disease pathogenesis in lupus-prone mice. The decrease was in part due to inhibition of B cell development and response.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the body's immune system produces autoantibodies against normal healthy tissue or against cellular components to form immune complexes that are deposited in various tissues inducing inflammation leading to tissue damage (1). Autoantibodies are produced by activated autoreactive B cells through antibody class switching and somatic hypermutation with help from activated autoreactive T cells (2). In early disease, several studies have shown a critical role for the pro-inflammatory cytokine IFN-alpha (3-6). Additionally, studies using lupus-prone mice have demonstrated that the ablation of plasmacytoid dendritic cells (pDCs) which are the major source of IFN-alpha prevented lupus nephritis (LN) progression, reduced activation of autoreactive T and B cells, decreased autoantibodies in the circulation and renal deposition (7, 8). These studies highlight the critical involvement of innate immune activation in the initiation of SLE. LN occurs in approximately 50% SLE patients and is a major cause of morbidity and mortality in this disease (9). Recently, several studies have provided evidence that epigenetic factors play a crucial role in the initiation and progression of the development of SLE, including LN (10).

Epigenetic factors include miRNA, methylation of DNA and acetylation of histones as well as non-histone proteins. There exists a balance of histone acetylaces (HATs) and histone deacetylases (HDACs) catalyzing the addition or removal of acetyl groups on proteins which alter their stability and function (11). There are 18 mammalian HDACs, which remove acetyl groups from lysine residues on histones and other proteins that can lead to modifications in multiple cellular functions including transcription, cell cycle kinetics, cell signaling, and cellular

transport processes (12). HDACs are classified based on structure and function homology originally studied in yeast. Currently, HDAC's fall into four different classes. Class I HDACs (HDAC1, -2, -3, and -8) reside primarily in the nucleus and are distributed in a wide range of cells and tissues where they are predominantly responsible for targeting histones proteins to repression gene transcription. Class II HDACs are subdivided into class IIa (HDAC4, -5, -7, and -9) and class IIb (HDAC6, and -10) based on domain organization and exhibit selective tissue expression and may shuttle between the cytosol and the nucleus (12). Class III comprises the sirtuins, which act through a distinct NAD^+-dependent mechanism and are not considered "classical" HDACs (13). HDAC11 is the sole member of class IV as phylogenetic analysis revealed very low similarity to HDACs in the other classes (14).

In our previous studies, we have demonstrated that inhibition of HDAC6, a class IIb HDAC, resulted in the amelioration of LN in lupus-prone mice associated with several altered immune responses, with minimal toxicity, suggesting therapeutic potential to treat LN (15-17). SLE, is a chronic inflammatory disease that is generally divided into the early autoimmune initiating stage and the later autoimmune effector stage (18-20). It is still not well understood whether the effects of HDAC6 inhibition to altered autoimmune responses are at the priming stage or effector stage. Here, using lupus-prone mice, we demonstrated that HDAC6 inhibition was able to downregulate the autoimmune responses in early disease and inhibit the initiation of LN.

Materials and methods

Mice and ACY-738 treatment

Female NZBWF1/J (NZB/W) and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and maintained under specific pathogen-free conditions at Virginia Tech College of Veterinary Medicine. For ACY-738 treatment, NZB/W mice were given a diet mixed with two different doses of the HDAC6 inhibitor, ACY-738 (50 mg/kg diet or 200 mg/kg diet), which was purchased from ENVIGO (Huntingdon, UK). All procedures strictly followed the requirements of the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech.

Leukocyte isolation and flow cytometric analysis

Bone marrow leukocytes. Bones from both hind limbs of each mouse were cracked gently in a mortar containing phosphate buffered saline (PBS) by using a pestle. Bone marrow was released by gentle stirring after the addition of C10 medium (RPMI 1640, 10% FBS, 1 mM sodium pyruvate, 1% 100× MEM non-essential amino acids, 10 mM HEPES, 55 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, all from Life Technologies, Grand Island, NY). The suspension was cleared by passing through a 70-μm sterile cell strainer and layered on the top of Ficoll-Paque Plus (GE Healthcare, Pittsburg, PA). After centrifugation at 1,363×*g* without break for 30 min at room temperature, mononuclear cells in the buffy coat layer were collected. In studies with pure pDCs, they were then sorted as $DAPI^{-}CD11c^{+}CD11b^{-}PDCA1^{+}B220^{+}$ cells.

Spleen leukocytes

Spleens were collected, smashed and then washed through 70-μm cell strainers with C10 medium. Red blood cells were lysed with RBC lysis buffer (eBioscience, San Diego, CA). Cell suspensions were filtered through 70-μm cell strainers to get rid of debris clumps.

Ex vivo stimulation of splenocytes

Splenocytes ($1x10^6$ per well) were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 ug/ml ionomycin (Sigma-Aldrich) and 1x Brefeldin A (BFA) (eBioscience) in 200 μl C10 and cultured in the 96-well flat bottom plate at 37°C with 5% CO_2 for 5 hours. Then splenocytes were collected for flow cytometry.

Flow cytometric analysis

For surface marker staining, cells were blocked with anti-mouse CD16/32 (eBioscience), stained with fluorochrome-conjugated antibodies, and analyzed with BD FACSAria II (BD Biosciences, San Jose, CA) or Attune NxT (ThermoFisher Scientific, Waltham, MA) flow cytometer. For intracellular marker staining, after surface staining, cells were fixed and permeabilized with Fix/Perm buffer (eBioscience) before staining intracellular markers. Anti-mouse antibodies used in this study include: CD11b, CD11c, Siglec-H, B220, Ly6a, MHCII, CD19, CD138, IgD, GL7, IgM, CD43, CD3, CD4, Foxp3, PD-1 (Biolegend), CCR5, IL-10, IFN-gamma (BD Biosciences). Flow cytometry data were analyzed with FlowJo.

Ex vivo HDAC6 inhibition

For the drug inhibition experiment, bone marrow mononuclear cells (BMMCs) from C57BL/6 mice were isolated and seeded in a 96-well flat bottom plate at a density of $2x10^6$ cells/ml in 200 μl C10 per well. The cells were then incubated with various concentrations of ACY-738 overnight and stimulated with ODN1585 CpG (5 μM final) for an additional 6 hours. Supernatants were then harvested and stored in a -80 °C freezer.

For the small-interfering RNA (siRNA) interference experiment, plasmacytoid dendritic cells (pDCs) were enriched from BMMCs using negative selection with a MACS kit (MACS Catalog #130-092-786) and seeded in a 24-well flat-bottom plate at a density of $1.5x10^6$ cells/ml in 400 μl C10 per well. siRNA complexes were prepared in HiPerfect reagent (QIAGEN, Hilden, Germany) according to the manufactures' instructions, and added onto the cells at 100 μl per well drop-wise, followed by 1-day incubation at 37°C with 5% CO_2. Then pDCs were stimulated with ODN1585 CpG (5 μM final) for another 6 hours. Supernatants were harvested and stored at -80 °C.

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected directly from the heart, transferred into microcentrifuge tubes and incubated for 30 min at room temperature, and then centrifuged at 1500×*g* for 5 min. Serum was collected and stored at -80°C. Detection of anti-double-stranded DNA (dsDNA) IgG was described previously (21). A similar procedure was used to detect anti-dsDNA IgG2a. Total IgG and IgG2a

concentrations were determined with mouse IgG ELISA kits according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, Texas).

Immunofluorescence

Kidneys were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek, Torrance, CA) and rapidly frozen in a freezing bath of dry ice and 2-methylbutane. Frozen OCT samples were cryosectioned and unstained slides were stored at -80°C. Frozen slides were warmed to room temperature and let dry for 30 min, followed by fixation in -20°C cold acetone at room temperature for 10 min. After washing in PBS, slides were blocked with PBS containing 1% bovine serum albumin (BSA) and anti-mouse CD16/32 for 20 min at room temperature. Slides were then incubated with fluorochrome-conjugated antibody mixture for 1 h at room temperature in a dark humid box. Slides were mounted with Prolong Gold containing DAPI (Life Technologies, Carlsbad, CA). The following anti-mouse antibodies were used in immunohistochemical analysis: IgG-PE, IgG 2a-PE (eBioscience, Santa Clara, CA), anti-C3-FITC(Cedarlanelabs, Burlington, Canada). Slides stained with antibodies were read and pictured with EVOS® FL microscope (Advanced Microscopy Group, Grand Island, NY) and a 40× objective. Six randomly picked glomeruli from each sample were pictured and then analyzed by using ImageJ software to calculate the deposition of IgG, IgG2a and C3.

Proteinuria score and kidney histopathology

Proteinuria was measured weekly with Uristix 4 (SIEMENS, city, state). A scale of 0-4 was used that corresponded to negative-trace, 30 mg/dL, 100 mg/dL, 300 mg/dL and ≥2000 mg/dL total

protein, respectively. For 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 4-5μm, and stained with Periodic acid-Schiff (PAS). Kidney sections were assigned a glomerular pathology score (0-4) based on a cumulative evaluation of glomerular proliferation, inflammation, crescent formation, necrosis, and fibrosis by a board-certified veterinary anatomic pathologist (M.D. Vieson) in a blinded manner. Briefly, the score ranges from 0, normal glomeruli, to 4, which includes glomeruli characterized by marked mesangial thickening, hypercellularity, segmental necrosis, crescents, and hyalinized end-stage glomeruli.

Statistical analysis

For the comparison of two groups, unpaired student's *t*-test was used. For the comparison of more than two groups, one-way ANOVA and Tukey's post-test were used. Results were considered statistically significant when $P<0.05$. In some experiments, linear regression analysis and Grubbs' test for identification of outliers were used. All analyses were performed with Prism Graphpad software.

Results

Inhibition of HDAC6 prevents LN progression

We have previously reported on the pharmacokinetics of the selective HDAC6 inhibitor ACY-783 (16). To study the effects of HDAC6 inhibition on the initiation of LN, we treated 20-week-old NZB/W F1 female (NZB/W) mice the selective HDAC6 inhibitor, ACY-738. Two different concentrations were mixed in the food to evaluate a dose-dependent effect for this compound. During the treatment, there were no statistically significant changes in food consumption or body weight (data not shown). Weekly monitoring of proteinuria showed that starting from 23 weeks of age, NZB/W mice treated with both doses of ACY-738 had significantly lower proteinuria scores compared to those without treatment (Fig. 1A). Linear regression analysis showed a significant increase in proteinuria in the untreated group. While proteinuria in 50 mg/kg ACY-738-treated group remained relatively unchanged, there is a statistically significant decrease in proteinuria noted in 200 mg/kg ACY-738-treated group. At the termination of the treatment (25 weeks-of-age), kidney sections were evaluated for glomerulonephritis (Figure 1B). Given our scoring parameters, the 50 mg/kg ACY-738-treated group was significantly lower than that of untreated group (Fig. 1B). The 200 mg/kg treated group also had a trend of lower score though it didn't reach but was close to statistical significance compared to controls ($p=0.059$). Taken together, these results suggest HDAC6 inhibition administered in the early stage of LN can help prevent the progression of LN.

HDAC6 inhibition reduces IFN-alpha production from pDCs

To study the mechanism by which selective HDAC6 inhibition prevents the initiation of LN, we focused on the possible changes of the innate immune responses, particularly pDCs as they have

been reported to play a key role in the initiation LN through the production of IFN-alpha (7, 8). We found that the administration of ACY-738, lead to a statistically significant dose-dependent increase of the percentage of MHC-II$^+$ pDCs (particularly MHC-II$^+$Ly6a$^+$ pDCs, gated according to the isotype control) and a significant decrease of the percentage of MHC-II$^-$Ly6a$^-$ pDCs in the bone marrow compared to the untreated group (Fig. S1A, Fig. 2A and B). Furthermore, the absolute number of bone marrow pDCs did not change (Fig. S1B). To further evaluate the effect of HDAC6 inhibition on pDC IFN-alpha production, we sorted pDCs from the bone marrow, stimulated with CpG, cultured at 37°C for 6 hours, and then collected supernatant to detect the IFN-alpha production of pDCs. We found that *ex vivo* IFN-alpha producing ability of bone marrow pDCs from the 50 mg/kg ACY-738-treated group was significantly lower than that of untreated group (Fig. 2C). The pDCs from the 200 mg/kg ACY-738-treated group was also decreased although it did not reach statistical significance (Fig. 2C). These results suggest that HDAC6 inhibition may inhibit differentiation of bone marrow MHC-II$^-$Ly6a$^-$ pDCs which are the cells thought to produce the large amounts of IFN-alpha in the initial stage of LN (22, 23). Consistent with the reduced IFN-alpha production of pDCs in the bone marrow of ACY-738-treated groups, through RNA sequencing of total splenocytes, we found a set of IFN-alpha regulated genes significantly different between 0 mg/kg and 200 mg/kg ACY-738-treated groups as shown in Table 1 and Table 2. Compared to INTERFEROME database(24), we found that the expression of splenic IFN-alpha-downregulated genes (Table 1) and –upregulated genes (Table 2) were significantly higher and lower in 200mg/kg ACY-738-treated group respectively, suggesting the lower IFN-alpha level in the spleen of HDAC6 inhibition group. To evaluate further the influence of selective HDAC6 inhibition on the function of pDCs, we cultured total bone marrow cells or enriched bone marrow pDCs from C57BL/6 mice in medium containing

various concentrations of ACY-738. In parallel experiments we used HDAC6 siRNAs to specifically knockdown HDAC6. We found that in a concentration-dependent manner, ACY-738 at 1 μM significantly suppressed CpG-induced IFN-alpha production from pDCs without significant cytotoxicity (Figure 3A). Furthermore, using siRNA to directly knockdown HDAC6 in pDCs we found a significant decrease of IFN-alpha production upon CpG stimulation without affecting the survival of pDCs (Figure 3B). Taken together, our results suggest that HDAC6 inhibition can suppress IFN-alpha production from pDCs, which may in turn prevent the initiation of LN.

HDAC6 inhibitor dampens adaptive immunity critical for LN development

As IFN-alpha is a broad activator of the immune system (25) and studies have shown the promoting effects of IFN-alpha on the activation of B cells and T cells in lupus-prone mice (26), we sought to determine if changes of B cell and T cell responses occurred following HDAC6 inhibition during the initiation stages of LN in NZB/W mice. Consistent with the reduced IFN-alpha responses, the absolute number of plasma cells ($CD138^{+}B220^{-}$) and both the percentage and absolute number of plasmablasts ($CD138^{low}B220^{+}$) in the bone marrow of ACY-738-treated groups were significantly lower than those of the untreated group (Fig. 4A, C and D). In addition, the spleen weight and the percentage of germinal center (GC) B cells ($GL7^{+}IgD^{-}$) in the spleen of ACY-738-treated groups were significantly decreased as well (Fig. S2A, Fig. 4B and E). Importantly, the HDAC6 inhibitor-mediated reduction of spleen weight and B cell responses were dose-dependent, with more apparent effects in the 200 mg/kg ACY-738 group compared to the 50 mg/kg ACY-738 group. GC B cells are responsible for the generation of pathogenic IgGs, particularly IgG2a isotype (27) with high affinity to self-antigens in NZB/W mice. This response

of GC B cells requires help from corresponding T helper cells (28). In our studies, we sought to compare T helper cell responses in the spleen between ACY-738-treated and untreated mice. We found the percentage of T follicular helper cells ($PD1^+CXCR5^+$) were significantly lower in the two ACY-738-treated groups compared to the untreated group (Fig. 5A). Next, we purified splenic T cells, placed them in culture, then stimulated the cultured cells with PMA, ionomycin and BFA for 5 hours and determined cytokine production using intracellular staining. We found that the percentages of T helper cells secreting either IL-10 or IFN-gamma, both pathogenic in NZB/W mice (29, 30), were significantly reduced in two ACY-738-treated groups compared to the untreated group (Fig. 5B and C), while the percentage of IL-4 producing T helper cells was not changed (Fig. S2B). As $CD25^+Foxp3^+$ Tregs didn't show significant difference among the three groups (data not shown), the increased IL-10 producing T helper cells should be the pathogenic population. In addition, the percentage of IFN-gamma producing $CD8^+$ T cells was reduced in the spleen by ACY-738 treatment (Fig. S2C), indicating a general decrease of the Th1 cytokine response. These results suggest that HDAC6 inhibition downregulated B cell responses, as well as the T cell responses that promote B cell responses at the initiation stage of LN in NZB/W mice.

Pathogenic autoantibodies decrease with HDAC6 inhibition

A direct inducer of LN is the generation and deposition of pathogenic autoantibodies in the glomerular regions of the kidney. In LN, the activation of pDCs, B cells and T cells all result in the generation of pathogenic autoantibodies. To determine if HDAC6 inhibition would decrease pathogenic Ig production, we measured IgG antibody levels including autoreactive IgGs with or without HDAC6 inhibition. The results showed that the levels of total IgG and IgG2a in the

serum of 50 mg/kg ACY-738-treated group were significantly lower than those of the untreated group (Fig. S3A). The levels of anti-dsDNA IgG and IgG2a in the serum of both 50 mg/kg and 200 mg/kg ACY-738-treated groups had a trend to decrease compared to the untreated group but did not reach statistical significance (Fig. S3B). On the contrary, the deposition of both IgG and IgG2a was significantly reduced in two ACY-738-treated groups compared to the untreated group (Fig. 6A and B). Moreover, C3 deposition was also significantly reduced in the 200 mg/kg ACY-738 group (Fig. 6C), suggesting downregulation of the complement system upon HDAC6 inhibition. Together, these results suggest that the pathogenic autoantibody responses in the kidney of NZB/W mice were suppressed by HDAC6 inhibition.

Discussion

HDAC inhibitors are epigenetic regulators that have been used effectively to treat hematological malignancies (31). Despite the different classes of HDAC inhibitors that have been used successfully as anticancer agents, their use in immunology has not been widely reported. As recently published by Grammer and Lipsky HDAC inhibition in lupus will effect pathways and not work solely through a signal enzyme or gene (32). They reported the QUADrATIC study showed 447 significant positive connections to the HDAC signature in SLE and predicted that the HDAC inhibitors vorinostat and valproic acid would have positive effects in altering the lupus gene signature. As both vorinostat and valproic acid are considered non-selective HDAC inhibitors affecting HDAC1, 2, 3 and 6, our studies are aimed to determine the efficacy of selectively inhibiting HDAC6. Our previous work using NZB/W mice demonstrated that HDAC6 inhibition has beneficial effects in lupus nephritis (15, 16). However, there are two major differences between our current study and previous studies of HDAC6 inhibition in NZB/W mice. First, in our previous studies, we examined the changes of established lupus nephritis symptoms at the late stage influenced by continuous HDAC6 inhibition starting from the early pre-disease stage until the endpoint of the late stage. Although in these studies we demonstrated the beneficial effects of the HDAC6 inhibitor on lupus nephritis, it was not known whether HDAC6 inhibition disrupted the priming of autoreactive B cell responses at the early stage or just suppressed the induced inflammation in the kidney at the later effector stage. To further answer this question, our current study focused on the effects of HDAC6 inhibition to the priming of autoimmune responses at the early stage. And we found that the beneficial effects of HDAC6 inhibition started early before the onset of lupus nephritis symptoms. Second, the

immune mechanisms we investigated behind HDAC6 inhibition are different between our previous studies and current study. In previous studies, we mainly focused on the changes of B cell and T cell development from precursors to immature naïve cells to mature naïve cells and the changes of different subpopulations of B cells in the spleen. However, in the current study, we moved forward to mainly examine not only the adaptive immune factors but also the innate immune factors critical for the priming of autoimmune responses. In our current studies, we show that an orally active selective HDAC6 inhibitor can decrease disease in a murine model of SLE with early established disease. Inhibition of HDAC6 in early disease led to a reduction of bone marrow plasmablasts as well as plasma cells which are considered to be the producers of pathogenic antibodies in SLE. Additionally, we saw a dose-dependent decrease in splenic GC B cells which was not observed in previous studies. And a decrease in IgG deposition and glomerular pathology socres in the kidney was consistent with the effect of long-term HDAC6 inhibition until late stage of LN in previous studies. These results suggest that HDAC6 inhibition to decrease LN starts early at the priming stage of humoral autoimmune responses. Moreover, the short term HDAC6 inhibition ended at the early stage of LN resulted in reduced IFN-gamma producing Th1 cells and T follicular helper cells. However, there was no change of Th17 cells which have been shown to reduce with long term HDAC6 inhibition until the late stage of LN in previous studies. These different influences of HDAC6 inhibition on different effector T cell subtypes at different stages of LN may reflect the stepwise pathogenic functions of various T cells with Th1 and T follicular helper cells critical at the early stage to induce pathogenic autoIgG2 antibodies and Th17 cells important at the late stage to promote the inflammation in the nephritic kidney.

There have been other studies published on the use of non-selective (pan- HDAC inhibitor) including the compound romidepsin which was found to reduce B-cell survival, leaving proliferation and differentiation intact. Interestingly, different pan-HDAC inhibitors have shown various results in regard to targeting B cells and/or T cells. This was evident in the studies comparing the pan-HDAC inhibitor panobinostat and vorinostat which altered B cell function in multiple myeloma and T cell function in cutaneous T cell lymphoma respectively(33, 34). Also of interest, the action of panobinostat on B cells occurred at low concentrations (in the nanomolar range), whereas at elevated concentrations effects on T cell function were observed (35). In our prior studies we previously showed the pan-HDAC inhibitor vorinostat (also known as suberanilohydroxamic acid) could decrease disease in lupus mice through targeting T cells, although it did have some toxicity at elevated concentrations when administered long-term to lupus mice (36, 37).

In lupus there has been many different ways to approach treating disease pathogenesis, including targeting B cells, T cells, plasma cells or cells of the innate immune system. In support of specifically target B cells in SLE, the B-cell-depleting antibody rituximab (anti-CD20) has been used therapeutically to dampen the antibody response and reduce autoimmunity (38). However, clinical trials were somewhat disappointing, possibly because rituximab was not very effective at targeting plasma cells that are CD20-negative. The studies using panobinostat showed efficacy in targeting all B cells including plasma cells (35), which may be a better therapeutic target. Although these results are promising, the rationale for more selective HDAC inhibition remains paramount as we seek to understand their precise mechanism. In our studies, we saw a decrease in plasma cell differentiation along with a decrease in IFN-alpha production suggestion that

HDAC6i inhibition may be an effective treatment in the early stages of SLE. We did not observe any alterations in weight with the treatment between the treated and untreated groups. As we continue to investigate the therapeutic efficacy of long term HDAC6 inhibition in SLE, it will be imperative that, by decreasing renal disease, we increase the life span of the animals with this therapy. Future studies are currently being planned. In addition, whether HDAC6 inhibition will be a first line defense to treat lupus nephritis still needs further animal model, pre-clinical and even clinical studies. For current clinical relevance, it is indeed more important to investigate the effects of the later intervention by HDAC6 inhibition starting after disease onset, which we haven't done yet but is worthy to do in future studies. However, with the improvement of early disease biomarker studies, one of the future directions in lupus treatment should be early diagnosis and early prevention of symptoms onset. From our previous and current studies, HDAC6 inhibition has the ability to suppress autoimmune responses early and sustains this ability to ameliorate the symptoms at later stage of lupus nephritis, which provides a potential early intervention strategy for lupus nephritis along with the development of early diagnosis in the future.

Since non-selective blockade of class I and class II HDACs does not allow elucidation of their mechanism in renal damage, more studies using selective HDACs are warranted. HDAC6 is a class IIb HDAC that localizes within the cytoplasm due to its inclusion of both a nuclear export signal and Ser-Glu-containing tetrapeptide domain (39, 40). HDAC6 affects multiple cellular functions in the cytoplasm including cell signaling, activation, survival, motility, and protein degradation (41) , which can all contribute to inflammation and immunity. One rationale for targeting HDAC6 is that knockout mice exhibit a viable phenotype, develop normally, and have

no life-limiting defects. This is not necessarily true for all HDACs as several other HDAC knock out murine models show either embryonic lethality or severe developmental defects. Interestingly, lymphocyte development and numbers in these mice are normal, although there is a mild decrease in the immune response after antigenic stimulation (42). In regards to SLE, we have previously observed increased expression and activity of HDAC6 within B cells, T cells, and glomerular cells of diseased lupus-prone mice (17).

We have previously found that ACY-783 decreased the activation of NF-□B in kidney mesangial cells although there may be additional targets in the renal tissue that are affected by HDAC6 inhibition (15). In our current studies, we found that that the selective HDAC6 inhibitor ACY-738 dose dependently decreased the percentage of BM pDCs. It did not affect the viability of the cultured BM pDCs up to 1 uM but did significantly decrease the production of IFN-α in the nanomolar range. This indicates that ACY-738 could exert therapeutic effects with no apparent toxicity. The ability of HDAC6 inhinitor to inhibit IFN-α production was confirmed using siRNA to HDAC6. These results are significant as several studies have shown that IFN-α facilitates the maturation of myeloid DC and contribute to T-cell activation and follicular T-helper cell differentiation (43-45). IFN-α also has the ability to directly stimulate CD4 T cells and enhance antigen-specific B-cell activation (46, 47). Of note, in our studies, IFN-□ has been shown to increase TLR7 expression in B cells, and promotes B-cell proliferation and differentiation into early plasmablasts which we were able to abrogate through HDAC6 inhibition. It has also been reported by Mathian and coworkers that IFN-α is critical for GC formation in NZB/W mice and for inducing pathogenic IgG2a autoantibodies (48). In our studies we noted that with ACY-738, both GC formation as well as IgG2a antibody formation

were decreased. We are not sure in this study whether HDAC6 inhibition has direct effects on autoreactive B cell and T cell responses or only indirect effects on them through downregulating IFNa. As one of our previous studies showed that B cells and T cells in another lupus model, MRL/lpr mice, expressed HDAC6, even at significantly higher level compared to those in non-lupus mice(17), we speculate that HDAC6 inhibition should have both direct and indirect effects on the alteration of B cell and T cell responses. To demonstrate this hypothesis, we will create NZB/W mice with pDC-, T cell- and B cell-specific HDAC6 depletion, respectively in future studies.

In regard to the decrease in glomerulonenephritis seen in our studies, there is emerging evidence that HDACs may play a role in renal fibrogenesis (49). The mechanisms by which HDACs mediates renal damage remain elusive, but may be associated with regulating the expression of inflammatory and profibrotic genes and activation of cell signaling pathways that mediate renal fibrosis (50). In another model of renal damage, Pang et al. (2009) demonstrated that treatment with trichostatin A (TSA), a pan-HDAC inhibitor that can block both class I and class II HDACs, can attenuate renal fibrosis in a murine model of unilateral ureteral obstruction (51). In our prior studies we found TSA to exert protective effects in both the NZB/W and MRL/lpr animal models (36, 52). In a recent paper by Manson et al. (2014) they demonstrated that TSA treatment induced BMP-7 expression which is important as an antifibrotic molecule (53). Furthermore, Marumo et al. and Liu et al. showed that HDAC inhibition alleviates renal fibrosis through suppression of inflammatory responses in the injured kidney (50, 54).

In summary, we found that we could inhibit HDAC6 using an orally active compound and that this leads to decreased IFN-α production and the inhibition of BM plasmablasts as well as plasma cells. Additionally, the decrease in IFN-α resulted in reduced GC formation and decreased pathogenic IgG2a antibody production. These coupled with improved renal scores and decreased proteinuria suggest that HDAC6 inhibition may be therapeutically beneficial for the treatment of SLE.

Figures

Figure 1.

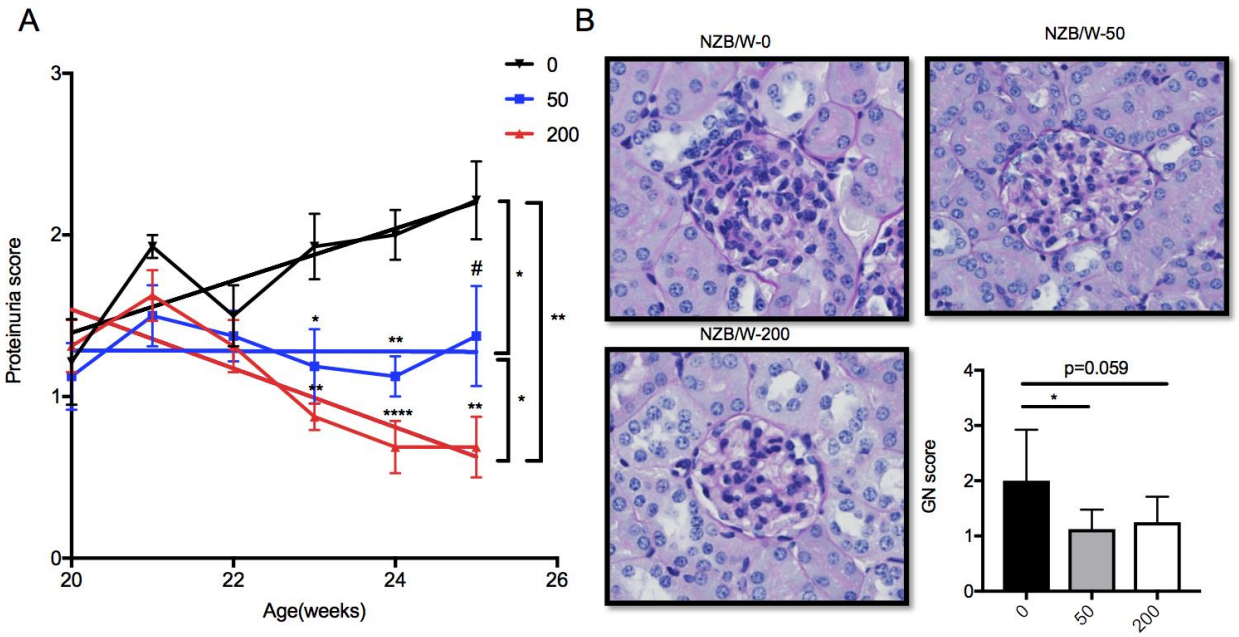

Figure 1. Inhibition of HDAC6 prevents LN progression. (A) NZB/W mice showing proteinuria as they age. The tendency of proteinuria changes in three groups are indicated by the slope of lines representatively. 0=control; 50 (mg/kg ACY-738); 200 (mg/kg ACY-738). (B) Representative PAS image of the glomerulus from NZB/W mice in three different group are shown along with pathology score for glomerular lymphoproliferative mononuclear cell infiltration. Data are shown as mean + standard error of the mean (SEM), n=8 mice in each group, # P=0.057, * P<0.05, ** P<0.01, *** P<0.001, one-way ANOVA.

Figure 2.

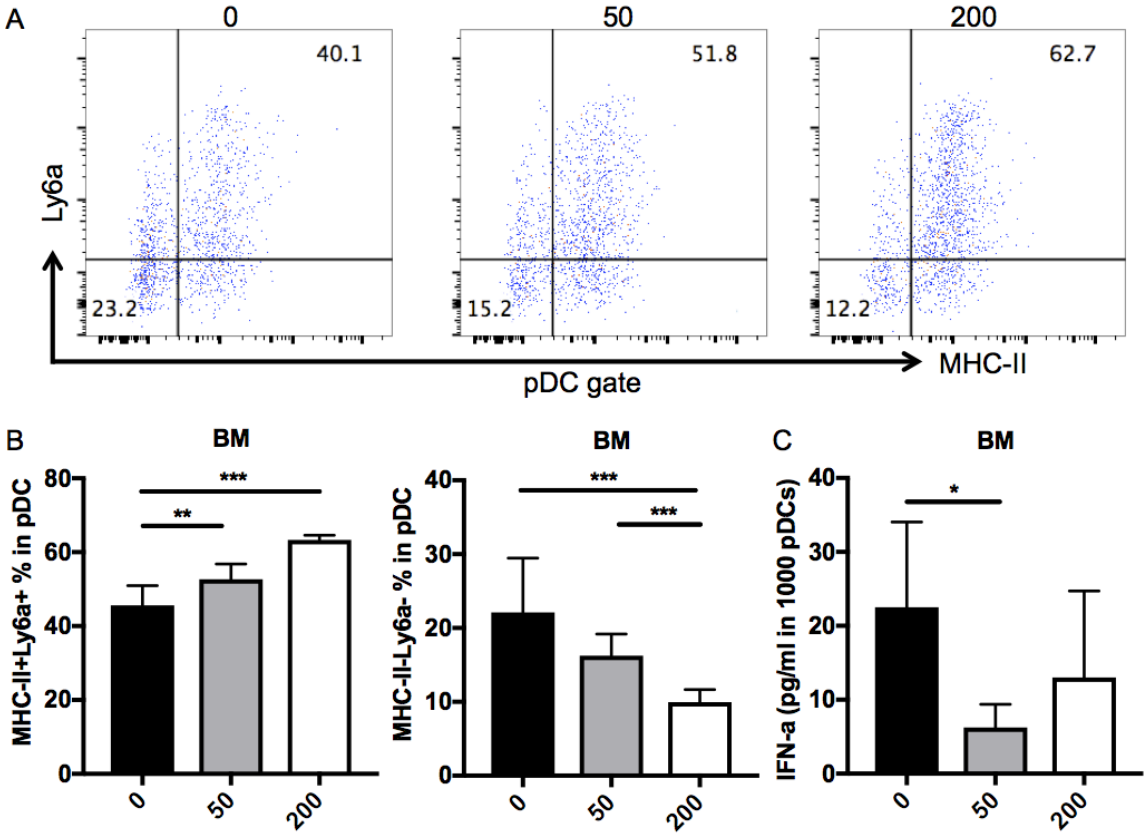

Figure 2. In vivo study of HDAC6 inhibition reduces IFN-alpha production from pDCs. (A) Representative flow cytometry plots of subtypes of pDCs in bone marrow are shown. (B) The percentage of pDCs with low ability ($MHCII^+$ $Ly6a^+$ pDCs) and high ability ($MHCII^-$ $Ly6a^-$ pDCs) of secreting IFN-α. (C) IFN-α production of sorted pDCs from bone marrow upon CpG stimulation for 12 h. * P<0.05, ** P<0.01, *** P<0.001, one-way ANOVA. n≥4.

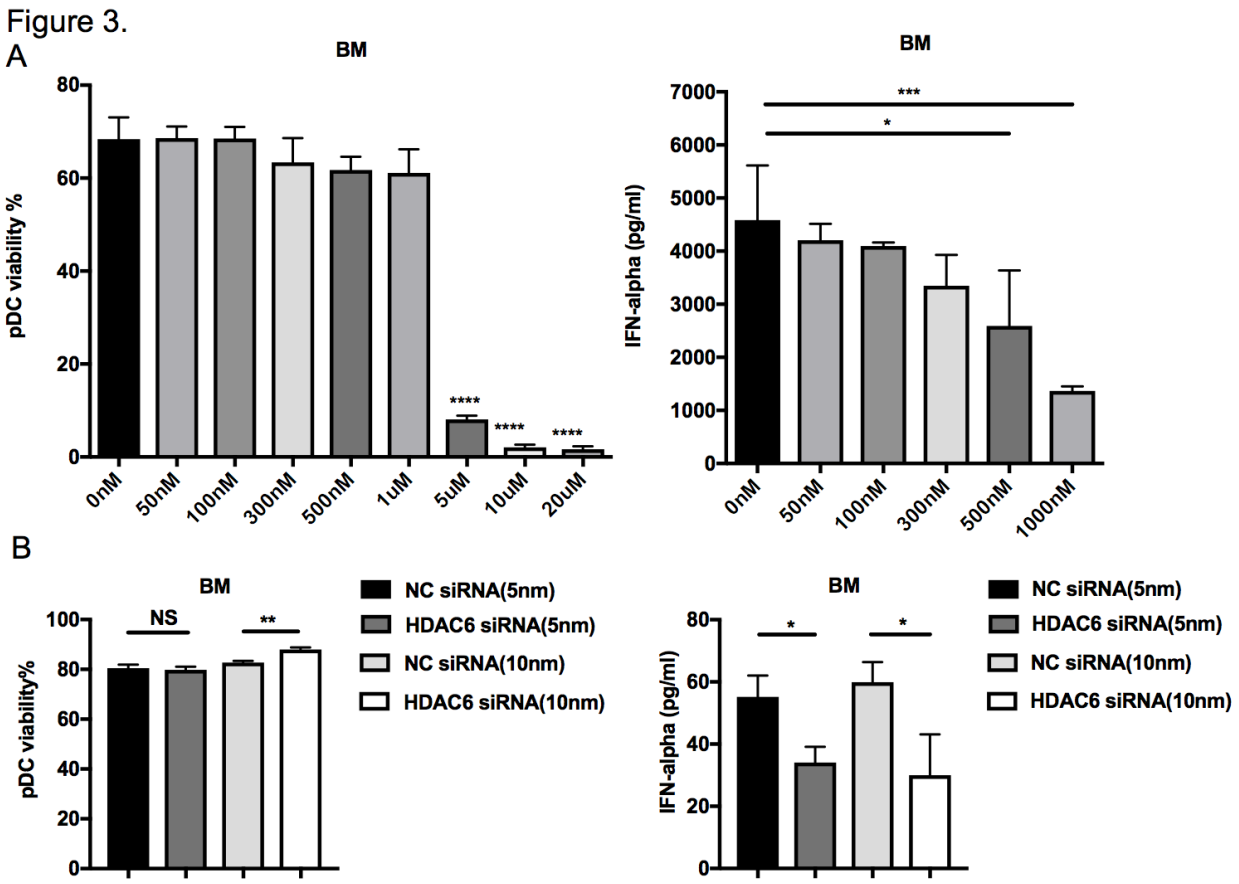

Figure 3. Ex vivo study of HDAC6 inhibition reduces IFN-alpha production from pDCs.

(A) The viability of sorted pDC from bone marrow after treatment with different concentrations of the selective HDAC6 inhibitor (ACY-738) for 24 hr. (B) IFN-α production of treated pDCs with HDAC6 inhibitor from bone marrow upon CpG stimulation for 24 hr. (C) The viability of sorted pDC from bone marrow after treatment with two different concentration of negative control siRNA and HDAC6 siRNA respectively for 24h. (D) IFN-α production of treated pDCs with siRNA from bone marrow upon CpG stimulation for 24 h. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, one-way ANOVA. n=3.

Figure 4.

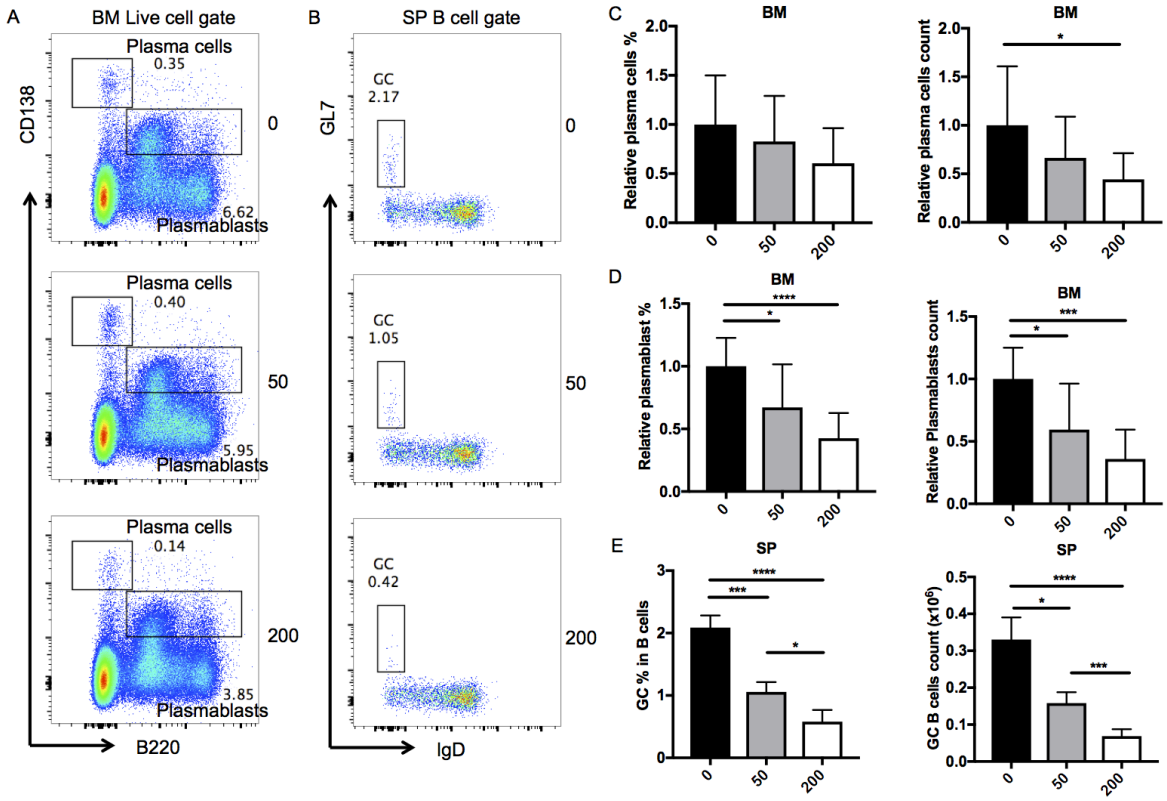

Figure 4. HDAC6 inhibition decreases B cell responses. (A) Representative flow cytometry plots for plasma cells and plasmablasts in the bone marrow. (C and D) Graphical representation of the relative percentage and absolute cell count of plasma cells and plasmablasts in the bone marrow. (B) Representative flow cytometry plots of B cells of germinal center in the spleen. (E) Graphical representation of relative percentage and absolute cell numbers of B cells of germinal center in the spleen. Representative flow cytometry plots are shown. * $P<0.05$, *** $P<0.001$, one-way ANOVA. $n\geq4$.

Figure 5.

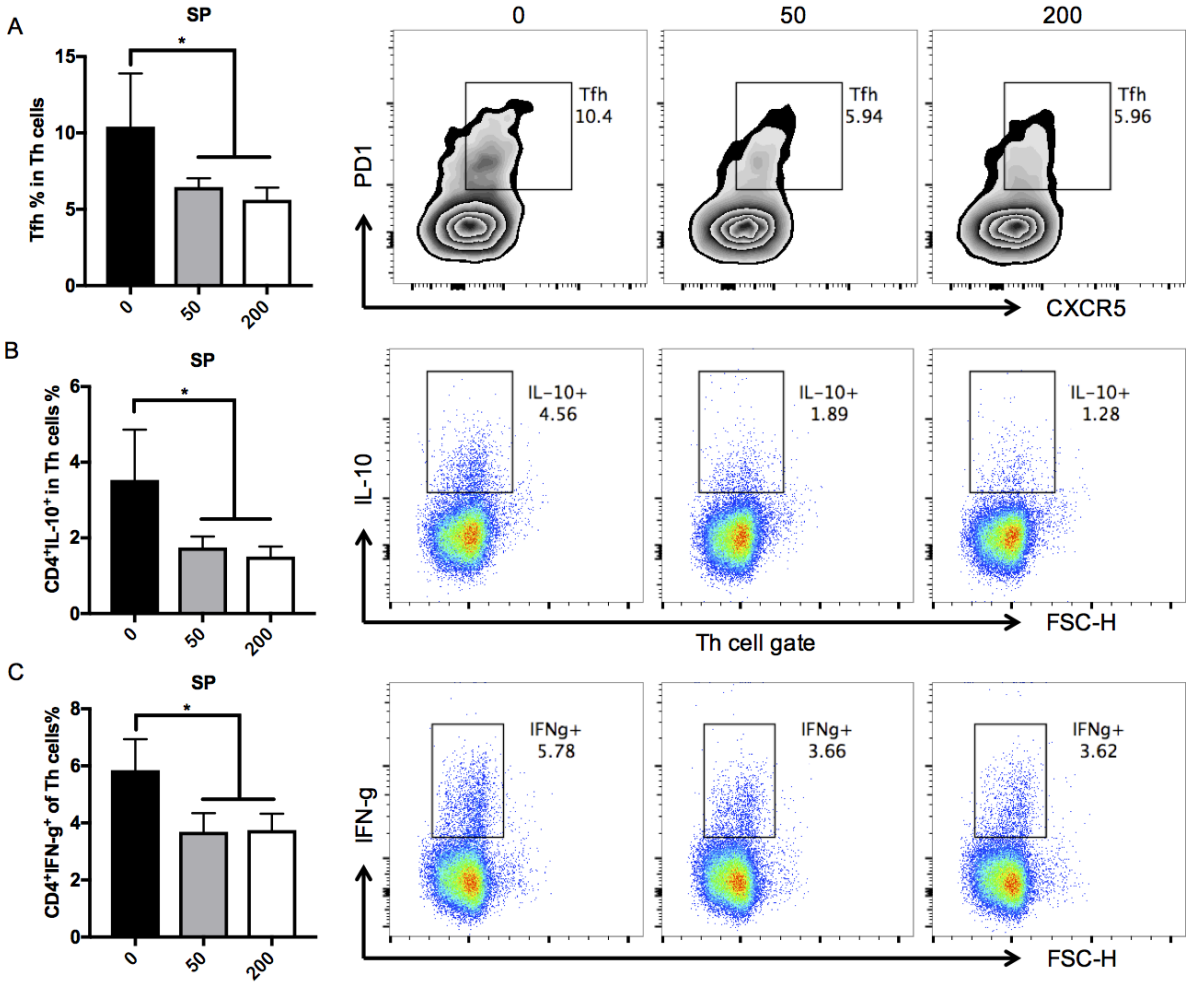

Figure 5. HDAC6 inhibition reduces pathogenic T cell responses. (A) Graphical representation and histogram of the percentage of follicular T cells in total T helper cells in the spleen in NZB/W mice treated with different doses of ACY-783. (B) Graphical representation and histogram of the percentage of IL-10 secreting cells in total $CD4^+$ T helper cells in the spleen of NZB/W mice treated with different doses of ACY-783. (C) Graphical representation and histogram showing percentage of interferon-gamma secreting cells in total $CD4^+$ T helper cells in the spleen of NZB/W mice treated with different doses of ACY-783. * $P<0.05$, ** $P<0.01$, one-way ANOVA. Representative images are shown. n=4.

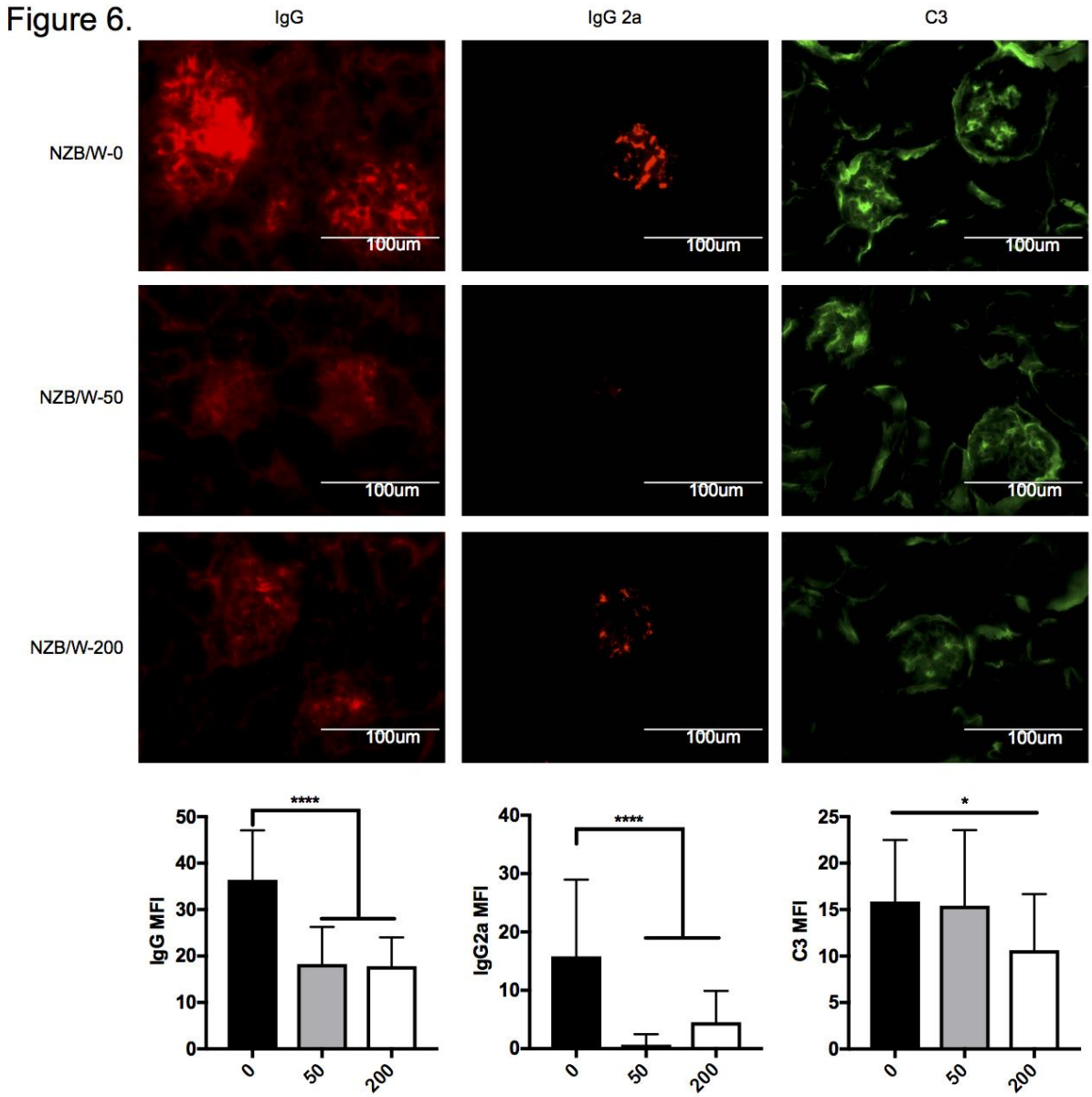

Figure 6. Pathogenic autoantibodies decrease with HDAC6 inhibition. (Top) Representative images of fluorescent staining for IgG, IgG2a and C3 in the glomerulus of NZB/W mice treated with various doses of ACY-738. (Bottom) graphical representation of mean fluorescent intensity (MFI) of IgG, IgG2a and C3, * $P<0.05$, ** $P<0.01$, one-way ANOVA. n=24.

Tables

Table 1. IFN-alpha downregulated genes from RNA sequencing of total splenocytes

IFN-alpha downregulated genes		
gene	log2_fold_change (ACY 738: 0 mg/ 200 mg)	p value
1100001G20Rik	-0.79508	9.00E-04
7SK	-1.05718	5.00E-05
Ankrd13b	-0.743131	0.00195
Aqp9	-0.893753	0.00015
Art4	-0.685621	0.0029
Atp7b	-0.951345	5.00E-05
Car3	-2.26838	0.00075
Ccl17	-1.70116	5.00E-04
Ccl24	-1.11797	5.00E-05
Ctrb1	-1.68403	5.00E-05
Ear6	-1.11203	0.00025
Elovl7	-0.752606	0.0033
Epdr1	-1.09842	5.00E-05
Gstk1	-1.21378	5.00E-05
Gzma	-0.614498	5.00E-05
Klf1	-1.41685	5.00E-05
Krt18	-1.88806	5.00E-05
Lphn1	-0.714992	7.00E-04
Mgl2	-1.55538	5.00E-05
Mgst2	-0.745639	0.00265
Myc	-0.468861	7.00E-04
Pdzk1ip1	-1.06259	1.00E-04
Plekha5	-0.576601	9.00E-04
Prg2	-0.713751	1.00E-04
Retnlg	-0.602896	0.00085
S100a8	-0.920403	5.00E-05
S100a9	-0.942064	5.00E-05
Sbf2	-0.790673	3.00E-04
Sord	-0.687697	0.00015
Sphk1	-1.4623	5.00E-05
Tcf15	-2.48992	5.00E-05
Trnp1	-1.32613	0.00015
Wdr60	-0.824671	0.00315

Table 2. IFN-alpha upregulated genes from RNA sequencing of total splenocytes

IFN-alpha upregulated genes		
gene	log2_fold_change (ACY 738: 0 mg/ 200 mg)	p value
Ablim1	0.470431	0.00075
Apobec1	0.431714	0.0031
B4galt5	0.522463	0.00025
Bcl6	0.620676	5.00E-05
Cd244	0.581184	0.0026
Cd83	0.722924	5.00E-05
Ceacam1	0.833845	5.00E-05
Csrp3	1.46752	5.00E-05
Cybb	0.597421	5.00E-05
Dusp16	0.788321	5.00E-05
Ect2	0.708839	5.00E-05
Ehd4	0.557537	5.00E-05
Gm12250	0.650353	0.00115
Gm16589	0.800247	5.00E-05
Gng11	1.22316	5.00E-05
Igkv12-98	1.37593	5.00E-05
Igkv3-10	0.738	5.00E-05
Igkv5-48	0.693764	5.00E-05
Il1b	0.642912	0.00015
Kif11	0.530139	0.00065
Lag3	1.1179	5.00E-05
Lair1	1.04921	5.00E-05
Man2a1	0.603639	5.00E-05
Manf	0.689241	0.00115
Ms4a6d	0.818086	5.00E-05
Msr1	0.822638	0.00335
Nuf2	0.544983	0.00255
Nupr1	1.44185	5.00E-05
Parp14	0.609205	5.00E-05
Pdia3	0.519802	0.00015
Phf6	0.532949	0.00015
Pik3ap1	0.538156	5.00E-05
Serpine1	1.33431	5.00E-05
Sh3bp2	0.604478	1.00E-04
Tpx2	0.498344	0.0015

Supplementary Figures

Figure S1.

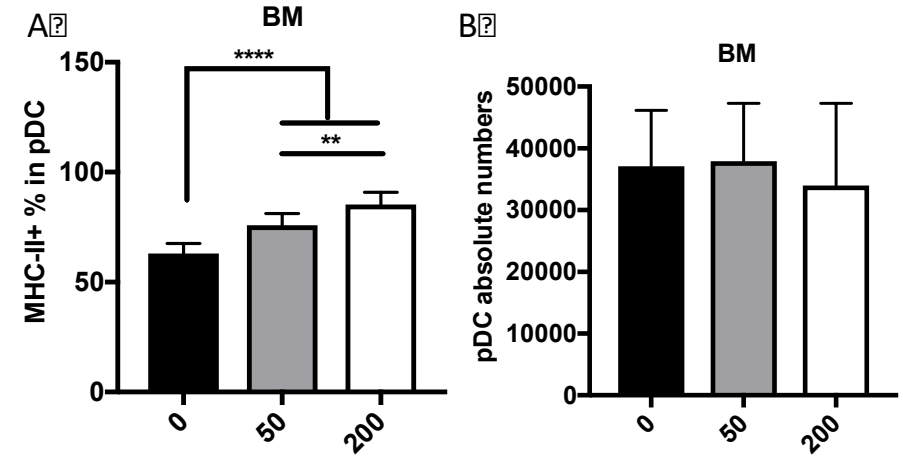

Figure S1. (A)The percentage of activated pDCs ($MHCII^+$ pDCs) in the bone marrow. (B) The absolute cell counts of pDCs in the bone marrow. * P<0.05, ** P<0.01, one-way ANOVA. n=8

Figure S2.

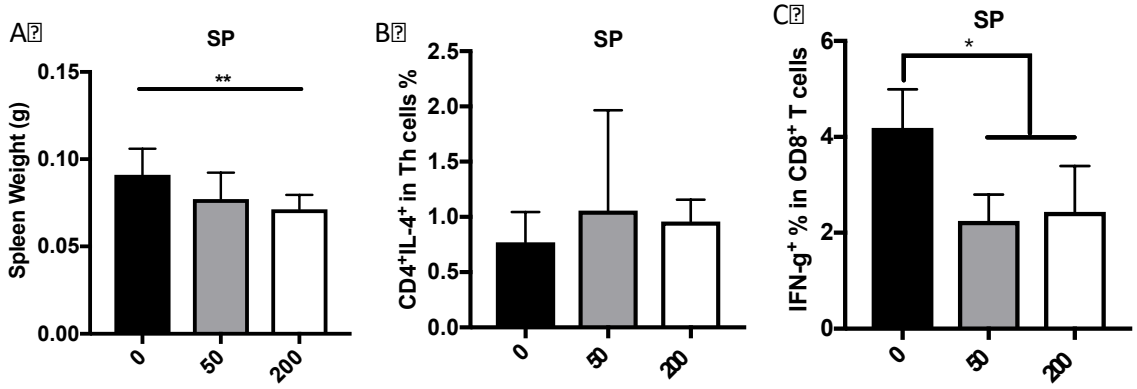

Figure S2. (A) Spleen weight. (B) The percentage of IL-4 secreting cells in total $CD4^+$ T helper cells in the spleen. (C) The percentage of IFN-gamma secreting cells in total $CD8^+$ T helper cells in the spleen. * P<0.05, ** P<0.01, one-way ANOVA. n≥4.

Figure S3.

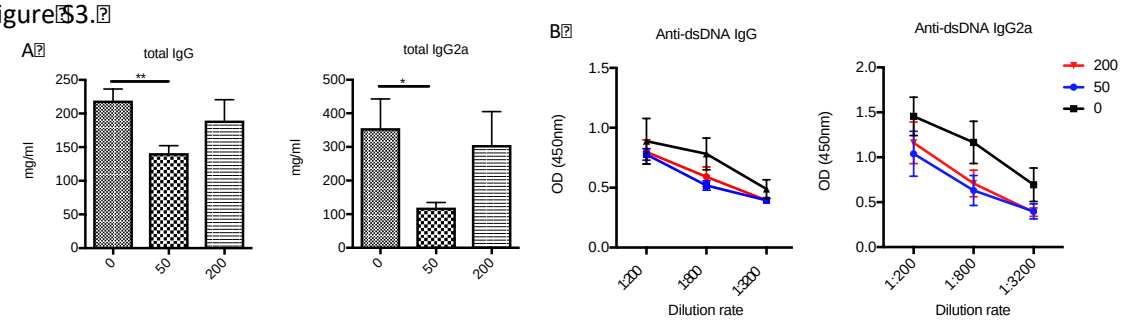

Figure S3. (A) The amount of total IgG and IgG2a in the serum. (B) The OD (450nm) value of anti-dsDNA IgG and anti-dsDNA IgG2a in the serum. * P<0.05, ** P<0.01, one-way ANOVA. n=4.

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Chapter 4. Selective Histone Deacetylase 6 Inhibition Normalize B Cell Activation and Germinal Center Formation in a Model of Systemic Lupus Erythematosus

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Abstract

Autoantibody production by plasma cells (PCs) plays a pivotal role in the pathogenesis of systemic lupus erythematosus (SLE). The molecular pathways by which B cells become pathogenic PC secreting autoantibodies in SLE are incompletely characterized. Histone deactylase 6 (HDAC6) is a unique cytoplasmic HDAC that modifies the interaction of a number of tubulin- associated proteins; inhibition of HDAC6 has been shown to be beneficial in murine models of SLE, but the downstream pathways accounting for the therapeutic benefit have not been clearly delineated. In the current study, we sought to determine whether selective HDAC6 inhibition would abrogate abnormal B cell activation in SLE. We treated NZB/W lupus mice with the selective HDAC6 inhibitor, ACY-738, for four weeks beginning at 20 weeks-of age. After only 4 weeks of treatment, manifestation of lupus nephritis (LN) were greatly reduced in these animals. We then used RNAseq to determine the genomic signatures of splenocytes from treated and untreated mice and applied computational cellular and pathway analysis to reveal multiple signaling events associated with B cell activation and differentiation in SLE that were modulated by HDAC6 inhibition. PC development was abrogated and germinal center (GC) formation was greatly reduced. When the HDAC6 inhibitor-treated lupus mouse gene signatures were compared to human lupus patient gene signatures, the results showed numerous immune and inflammatory pathways increased in active human lupus were significantly decreased in the HDAC6 inhibitor treated animals. Pathway analysis suggested alterations in cellular metabolism might contribute to the normalization of lupus mouse spleen genomic signatures, and this was confirmed by direct measurement of the impact of the HDAC6 inhibitor on metabolic activities of murine spleen cells. Taken together, these studies show HDAC6 inhibition decreases B cell

activation signaling pathways and reduces PC differentiation in SLE and suggest that a critical event might be modulation of cellular metabolism.

Introduction

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease characterized by the production of pathogenic antibodies with the formation of immune complexes that can be deposited in various tissues. Plasma cells (PCs) are differentiated B cells responsible for the production of antibodies that provide defense from invading foreign pathogens. After activation, B cells either (a) form short-lived extrafollicular plasmablasts that are critical for early protective immunity or (b) enter specialized regions of secondary lymphoid tissue that facilitate T cell: B cell collaboration, either germinal centers (GCs) or extra-follicular foci and undergo extensive proliferation eventually becoming PC that produce high avidity antibody via somatic hypermutation. In lupus, PCs differentiated from active B cells produce autoantibodies such as anti-dsDNA and anti-RNA–binding proteins, which bind self-antigens forming immune complex that deposit in blood vessels and renal glomeruli leading to vasculitis and nephritis. Although much is known about the mechanisms regulating T cell: B cell collaboration and PC generation in SLE, many details of the intracellular event regulating this process have not yet been delineated.

Post-translational modification (PTM) of proteins is an important means to regulate protein: protein interactions and downstream cellular functions. Among the various PTMs of proteins, acetylation plays a major role. Acetylation/deacetylation events are reversible PTM on lysine residues of histone and non-histone proteins and are essential for specific protein: protein interactions and in the nucleus for gene regulation. These reactions are typically catalyzed by enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. HDACs are classified into four subclasses: three Zn^{2+}-dependent classes (I, II, and IV), and

one NAD^+-dependent class III. Class II is subdivided into class IIa and class IIb. HDAC6 belongs to HDAC class IIb and is largely cytoplasmic in location. It is associated with non-histone substrates, including α-tubulin, heat shock protein 90 (HSP90), and cortactin and others and has been shown to modulate immune cell function in various ways, including modifying BCL6 function and B cell maturation [1].

We have previously shown that the selective HDAC6 inhibitor ACY-738 given to pre-disease lupus-prone NZB/W mice prevented the onset of lupus nephritis (LN). In our current studies, we treated NZB/W mice for only four weeks after disease onset and sought to determine mechanisms by which this cytoplasmic HDAC inhibitor might alter the cellular functions involved in lupus pathogenesis and especially the maintenance of GC and PC generation. To accomplish this, we assessed changes in the mRNA transcriptome mediated by selective HDAC6 inhibition using RNA-Sequencing (RNA-seq) analysis of whole splenocytes. We found that HDAC6 inhibition in NZB/W mice led to global changes in gene expression. Phenotypically, we found that decreased glomerulonephritis was coupled with reduced IgG and C3 deposition and decreased GC and PC populations. Furthermore, we observed reduced B cell activation following HDAC6 inhibitor treatment. Underlying this was a change in cellular metabolism. Taken together, these data indicate that targeting autoreactive B cells through increased acetylation may limit cell activation and differentiation in lupus, thereby provide therapeutic benefit.

Materials and Method

Mice and ACY-738 treatment

Female New Zealand Black/White F1 (NZB/WF1/J) (NZB/ W) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). For ACY-738 treatment, NZB/W mice were given a diet mixed with or without 200mg/kg ACY-738, which was purchased from Envigo (form 8640, Huntingdon, UK). Treatment started at 20 weeks-of-age when the animals began to show signs of mild proteinuria (30mg/dl by dipstick analysis). All animals were allowed food and water *ad libitum.* Treatment was continued for four weeks at which time, animals were euthanized.

Immunofluorescence

At the termination of the experiment, the spleens and kidneys were removed. One portion of the spleen and the kidney was embedded in Tissue-TekVR optimal cutting temperature compound (O.C.T.TM) (Sakura Finetek, Torrance, CA, USA) and frozen rapidly in a freezing bath of dry ice and 2-methylbutane. Frozen OCT samples were cryosectioned into 5 and 10 μm sections respectively. Frozen slides were warmed to room temperature and allowed to dry for 30 min, followed by fixation in cold acetone at room temperature for 10 min. After washing in PBS, slides were blocked with PBS containing 1% bovine serum albumin (BSA) and anti-mouse CD16/32 for 20 min at room temperature. Slides were then incubated with a fluorochrome-conjugated antibody mixture for 1 h at room temperature in a dark humid box. Slides were mounted with Prolong Gold containing DAPI (Life Technologies, Carlsbad, CA, USA). The following anti-mouse antibodies were used in immunohistochemical analysis: anti-IgG-phycoerythrin (PE) (eBioscience, Santa Clara, CA, USA) and anti-C3-fluorescein isothiocyanate (FITC) (Cedarlanelabs, Burlington, Canada), anti-IgD-phycoerythrin (PE) (eBioscience, Santa

Clara, CA, USA), anti-CD3-APC (Biolegend, San Diego, CA, USA), Peanut Agglutinin (PNA)-fluorescein isothiocyanate (FITC) (Burlingame, CA,USA), anti-CD138- phycoerythrin (PE) (eBioscience, Santa Clara, CA, USA) and anti-IgM-V450 (BD bioscience, Franklin Lakes, NJ). Slides stained with antibodies were read and visualized with an EVOSVR FL microscope (Advanced Microscopy Group, Grand Island, NY, USA) and a x40 and x20 objective for kidney and for spleen, respectively. Six randomly selected glomeruli from each sample were pictured and then analyzed by using ImageJ software (National Institutes of Health, Rockville, MD, USA) to calculate the deposition of IgG and C3. For spleens, a total of 20 spots were imaged for each group of 4 mice, with five random spots imaged from each mouse, from which representative figures were selected.

mRNA-isolation and sequencing

Total RNA was isolated from whole splenocytes using the miRNeasy Mini Kit (Qiagen,Germantown, MD, USA) per manufacturer's instructions. To remove residual amounts of DNA contamination in isolated RNA, on-column DNase digestion with RNase-Free DNase was performed. The RNA concentration was quantified using a NanoDrop 2000. Total RNA was sent to Beckman Coulter (Danvers, MA, USA) for 2x100bp paired-end Illumina RNA sequencing with an average of 40 million reads per sample. Sequencing data (FASTQ files) was trimmed for both adaptor sequences and quality using a combination of ea-utils and Btrim [2, 3]. Sequencing reads were then aligned to the genome (Ensembl.org 38.74) using Bowtie2/Tophat2 [4, 5] and counted via HTSeq [6].

Gene set variation analysis (GSVA)

The open source GSVA (V1.25.0) software package for R/Bioconductor [7] was used as a non-parametric, unsupervised method for estimating the variation of pre-defined gene sets in patient and control samples of microarray expression data sets. Raw RNAseq counts transformed into log2 expression values for pre-defined gene sets were used as the inputs for GSVA. Enrichment scores (GSVA scores) were calculated non-parametrically using a Kolmogorov Smirnoff (KS)-like random walk statistic; a negative value for a particular sample and gene set indicated that the gene set has a lower expression than the same gene set in a sample with a positive value. The enrichment scores (ES) were the largest positive and negative random walk deviations from zero, respectively, for a particular sample and gene set. The positive and negative ES for a particular gene set depend on the expression levels of the genes that form the pre-defined gene set. The increased transcripts for SLE plasma cells (PC) were taken from Lugar et al [8] to determine the enrichment of PC. Tfh cells were determined by expression of *Bcl6*[9], *Pdcd1*, *Icos*, *Ascl2* [10] and *Tnfsf4* [11]. Markers of germinal centers were determined by expression of *Gcsam[12]*, *Nuggc* [13], *Rgs13* [14], *Klhl6* [15], *Aicda* [16], *Bcl6* [9] and *Irf4* [17].

I-Scope analysis

I-scope is a tool used to identify immune infiltrates in gene expression datasets. I-scope was created through an iterative search of more than 17,000 genes identified in more than 50 microarray datasets. From this search, 1226 candidate genes were identified and researched for restriction in hematopoietic cells as determined by the HPA, GTEx and FANTOM5 datasets (www.proteinatlas.org)[18]; 926 genes met the criteria for being

mainly restricted to hematopoietic lineages (brain, reproductive organs exclusions). These genes were researched for immune cell specific expression in 30 hematopoietic sub-categories: T cells, regulatory T cells, activated T cells, anergic cells, CD4 T cells, CD8 T cells, gamma- delta T cells, NK/NKT cells, T & B cells, B cells, activated B cells, T &B & monocytes, monocytes & B cells, MHC Class II expressing cells, monocyte dendritic cells, dendritic cells, plasmacytoid dendritic cells, Langerhans cells, myeloid cells, plasma cells, erythrocytes, neutrophils, low density granulocytes, granulocytes, platelets, and all hematopoietic. Transcripts are entered into I-scope and the number of transcripts in each category are calculated and represent the specific immune cell populations in each dataset.

Pathway analyses

Ingenuity Pathway Analysis (IPA) software (Qiagen, Venlo, Netherlands) was used to calculate Z scores based on increased and decreased transcript levels in HDAC6 inhibitor samples compared with transcript levels in controls) [19]. Z scores ≥ 2 or ≤ -2 and overlap p values $\leq .05$ were considered significant. IPA scores were used to determine whether pathways were up-regulated or repressed based on whether transcripts were increased or decreased relative to controls in the entry dataset.

Gene ontology (GO) biological pathway (BP) analysis

Increased and decreased transcripts were annotated with GO BP terms separately and overlap p values were determined. Pathways were considered enriched or reduced if they had associated *p* values < 0.01.

Biologically informed gene glustering (BIG-C)

BIG-C is a custom functional clustering tool developed to annotate the biological meaning of large lists of genes. Separately, increased and decreased genes are sorted into 52 categories based on their most likely biological function and/or cellular localization based on information from multiple online tools and databases including UniProtKB/Swiss-Prot, GO Terms, MGI database, KEGG pathways, NCBI PubMed, and the Interactome. Each gene is placed into only one category based on its most likely function to eliminate the redundancy in enrichment sometimes found in GO BP annotation [20, 21].

Comparison of HDAC6 inhibitor- treated NZB/W RNA seq to human SLE tissue microarray data

The comparison analysis feature of IPA [19] was used to compare the Z scores between processed microarray data from DE analysis of four human SLE tissue experiments and the DE analysis of the HDAC6 inhibitor treated versus untreated NZB/W mice. Raw data from lupus tissue datasets were obtained from the GEO repository: GSE36700 for lupus synovium (4 OA, 4 SLE patients), GSE72535 for discoid lupus skin (8 healthy control (HC), 9 DLE), GSE32591 for LN dissected glomerulus WHO class 3 or 4 (32 HC, 22 SLE) and GSE32591 for lupus nephritis dissected tubulointerstitium from WHO CLASS 3 or 4 LN(32 HC, 22 SLE). Differential Gene Expression (DE) was carried out for each dataset of SLE tissue samples and controls. GCRMA normalized expression values were variance corrected using local empirical Bayesian shrinkage before calculation of DE using the ebayes function in the open source BioConductor LIMMA package (https://www.bioconductor.org/packages/release/bioc/html/limma.html) [22]. Resulting

p-values were adjusted for multiple hypothesis testing and filtered to retain DE probes with an FDR < 0.05 [23].

Metabolic enzyme function studies

Citrate synthase (CS) catalyzes the formation of citrate and coenzyme A (CoASH) from acetyl-CoA and oxaloacetate. CoASH reduces DTNB and CS activity was determined from the reduction of DTMB over time [24]. Briefly, at sacrifice splenocytes from ACY-738 treated and control mice were lysed ($1x10^6$ cells/200 µl) in a buffer containing 0.1% Triton X-100, 1 mM EDTA, 50 mM Tris, pH 7.4, and Protease Inhibitor Cocktail (Nacalai Tesque). The CS assay was carried out using 20 µl of the lysates in 96-well plates. CS activity was measured by adding 80 µl of the reaction solution containing 0.1 mM DTNB, 0.3 mM acetyl-CoA, 1 mM oxaloacetate, and 50 mM Tris at pH 7.4 to each well. Absorbance was measured on a spectrophotometer (BioTek Synergy 2, Winooski Vermont, USA.) at 405nm at 37C every 12 seconds for 5 minutes. Total protein concentration of the lysates was quantified by a Bio- Rad Protein Assay, and CS activity was normalized to the total protein concentration. CS activity was calculated as the rate of increase of absorbance with time. All samples were run in triplicate. Maximum activity was calculated and reported as µmol/mg/min.

For the determination of β-hydroxyacyl-CoA dehydrogenase activity, the oxidation of NADH to NAD was measured [24, 25]. In this procedure, splenocytes were added to 190µl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. Following a 2-minute background reading, 15µl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 5 minutes at 37C. Maximum activity was calculated and reported as µmol/mg/min.

Cytochrome c oxidase, which transfers electrons between complex III and IV of the electron transport chain, was assayed based on the oxidation of ferrocytochrome c to ferricytochrome c by cytochrome c oxidase. Horse heart cytochrome c (Sigma Aldrich, 2mg/ml) was dissolved in a 10mM potassium phosphate buffer containing 10mg/ml of sodium dithionite. 10ul of splenocyte extracts were added to 290ul of the reduced cytochrome c test solution. The rate of cytochrome C oxidation was measured spectrophotometrically as a reduction in absorbance at 550 nm every 10 seconds for 5 minutes at 37C. Maximum cytochrome c oxidase activity was expressed relative to protein content and reported as μmol/mg/min.

Fatty acid and glucose oxidation studies

Splenocytes were isolated from spleens from eight-week-old NZB/W female mice. T cells and B cells were enriched from splenocytes using negative selection with a magnetic-activated cell sorting kit (Miltenyi Biotec, Auburn CA). Cells were seeded in a 24-well flat- bottomed plate at a density of $1x10^6$ cells/ml in 1ml RPMI-1640 (HyClone, South Logan, UT) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (HyClone), $5{\cdot}5 \times 10^{-2}$ mM 2-mercaptoethanol (Gibco BRL Life Technologies, Paisley, UK) and 10% heat-inactivated bovine calf serum (HyClone) per well. For T cells stimulation, plates were pre-coated with anti-CD3 (Invitrogen) and T cells were stimulated with anti-CD28 (Invitrogen) with or without the addition of 4uM ACY-738 or DMSO (control) followed by 24h incubation at 37^0C with 5% CO_2. B cells were cultured with Lipopolysaccharide (LPS: *Escherichia coli* serotype 0111:B4; Sigma-Aldrich, St Louis, MO) (50 ug/ml) and treated with ACY-738 (4uM) or DMSO (control) for 24 hr after which the cells were collected and metabolism analysis performed. Substrate metabolism was assessed as previously described [24]. Briefly, fatty acid

oxidation was measured using radiolabeled fatty acid ([1-^{14}C]-palmitic acid, American Radiolabeled Chemicals, St. Louis, MO.) to quantify $^{14}CO_2$ production from the oxidation of isolated B and T cells. Cells were incubated in 0.5μCi/mL of [1-^{14}C]-palmitic acid for 1 hour after which the media was acidified with 200 μL 45% perchloric acid for 1 hour to liberate $^{14}CO_2$. The $^{14}CO_2$ was trapped in a tube containing 1 M NaOH, which was then placed into a scintillation vial with 5 mL scintillation fluid. The vial's ^{14}C concentrations were measured on a 4500 Beckman Coulter scintillation counter. Glucose oxidation was assessed in the same manner as fatty acid oxidation with the exception that [U-^{14}C] glucose was substituted for [1-^{14}C]-palmitic acid. Oxidation values were normalized to total protein content as assessed via a commercially available bicinchroninic acid (bca) procedure (Thermo Fisher Scientific, Waltham, MA) and expressed as nM/mg protein/hr.

Code and data availability

R bioconductor packages limma and Gene set variation analysis (GSVA) are open source code available at www.bioconductor .org.

Statistics

Data was analyzed by student t test with GraphPad Prism software. Statistically significant differences are followed by *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; ****P ≤ 0.0001.

Study approval

The animal experiments strictly followed the requirement of the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech, USA and maintained under specific pathogen-free conditions at Virginia Tech College of Veterinary Medicine. All of operations of animals are in compliance with the Guide for the Care and Use of Laboratory Animals.

Results

ACY-738 is selective for HDAC6 inhibition

ACY-738 is a hydroxamic acid HDAC6 inhibitor that is highly selective for HDAC6. In preliminary experiments, we found that ACY-738 inhibits HDAC6 with a potency of 1.5 nM and HDAC1 (the next most affected target) with a potency of 93 nM (Figure 1A, 1B). In a cell-based assay using HCT-116 cells, we found ACY-738 induced tubulin acetylation (a marker of HDAC6 inhibition) at 800 nM, whereas acetylation of H3K9 (a marker of Class 1 HDAC inhibition) at that concentration was minimal suggesting that the inhibition was primarily cytosolic. As previously reported, 100 mg/kg/day of ACY-738 in rodent chow achieves an estimated plasma concentration of 100 nM [26]. In our studies, the mean plasma concentration of ACY-738 at different time intervals was 212 nM as determined by mass spectrometry (LC/MS, Agilux, Worcester, MA, USA).

Inhibition of HDAC6 improves established LN

To simulate the therapeutic paradigm in human lupus, we treated 20-week-old NZB/W F1 female (NZB/W) mice with established LN with the selective HDAC6 inhibitor ACY-738. After only 4 weeks, ACY-738-treated mice exhibited significantly less renal pathology than the untreated group (Figure 2A). Moreover, the deposition of IgG and C3 in glomeruli, which contribute to the progression of renal inflammation, was significantly decreased in the ACY-738 treated group compared to the untreated control group (Figure. 2B).

Suppression of B cell responses by HDAC6 inhibitor

To investigate the mechanisms of HDAC6 inhibition on autoimmune responses, we analyzed changes in splenic composition by carrying out bulk RNA sequencing on total splenocytes from ACY-738-treated and untreated NZB/W mice (Figure 3). Analysis of global gene expression changes by hierarchical clustering showed that 3911 transcripts were differentially expressed between the treated and untreated samples. Among these, 1922 genes were up-regulated, and 1989 genes were down-regulated in the ACY-738-treated group compared to the control group. To determine whether HDAC6 inhibition led to changes in cell populations in the spleen of treated mice, we employed the I-Scope clustering program that permitted identification of immune and inflammatory cell types based on gene expression. Control experiments were performed to demonstrate the specificity and lack of cross reactivity of I-scope (Supplementary Figure 5). We found that HDAC6 inhibition led to a profound decrease in transcripts associated with plasma cells, B cells and inflammatory myeloid cells (Figure 4A, Supplementary Data 1,2) as well as more modest decreases in other immune/inflammatory cells. Next, gene set variation analysis (GSVA) was carried out to determine whether there was enrichment in transcripts identifying these populations. Indeed, we found that plasma cell, Tfh cell, and GC signatures were all decreased following 4 weeks of HDAC6 inhibitor treatment, as compared to the untreated control (Figure 4B). To further validate the impact of the HDAC6 inhibitor on germinal center B cell response, we alternatively assessed the changes of spleens and Peyer's patches from C57BL/6J HDAC6 -/- mice compared to C57BL/6J mice by flow cytometry (Supplementary Figure 2). We documented a reduction of T follicular helper cells (Tfh) in spleens and Peyer's patches of HDAC6 knockout mice compared to wild type C57BL/6J mice. Different from lupus-prone mice, the lack of HDAC6 in mice of B6 background showed no reduction of splenic spontaneous germinal centers in steady state, suggesting differences in the

molecular pathways leading to spontaneous germinal center formation versus those forming in spleens of lupus-prone mice. To confirm our RNA sequencing results, we carried out immunohistofluorescence (IHF) microscopy of splenic sections to evaluate the presence of plasma cells and GCs (Figure 4C and 4D). Consistent with the RNA sequencing results, both $CD138^+$ PC (Figure 4C) and PNA^+ GC (Figure 4C, 4D and supplementary Figure 1) were dramatically reduced in the ACY-738-treated group, suggesting that HDAC6 treatment suppressed GC activity and subsequent PC generation and/or survival.

HDAC6 inhibition reduces B cell signaling in NZB/NZW F1 mice

To evaluate whether HDAC6 inhibition specifically might inhibit B cell signaling, we employed IPA canonical pathway analysis to assess the pattern of change in differential gene expression in HDAC6-treated mice (Figure 5). HDAC6 inhibition was found to reduce transcripts involved in both the BCR and the TLR dependent PI3K signaling pathway in B cells, as well as decreasing transcription factors, NF-κB, ELK1, c-JUN and ATF, which control cell growth, differentiation, and homeostasis of many cells including B cells. To validate the role of HDAC6 in regulation of B cell activation signaling suggested by analysis of RNAseq data, we have carried out *in vitro* stimulation experiments with HDAC6-/- mice and NZB/W mice. As seen in the figures (Supplementary Figure 3 and Figure 4), reduced activation of B cells was noted in B cells from C57BL/6J/HDAC6-/- mice as well as ACY-738 treated NZB/W mice (Supplementary Figure 3 and Figure 4).

HDAC6 inhibition alters gene transcripts associated with inflammation and cellular metabolism

To investigate further the specific pathways by which HDAC6 inhibition decreased the molecular basis of lupus, several additional analyses were carried out (Figure 6). IPA was used to determine the biological pathways significantly affected by HDAC6 inhibitor treatment (Figure 6A.) There were only five significant signaling pathways increased by HDAC6 inhibitor treatment ($p \leq 0.05$; $Z \geq 2$) We found that HDAC6 inhibition led to an increase in glutathione metabolism and the gamma-glutamyl cycle, which may be related to the activation of the mercapturic acid pathway for the detoxification of foreign compounds. With regard to pathways down-regulated with HDAC6 inhibition, there were 59 pathways with Z scores ≤ -2 and p values ≤ 0.05; pathways associated with immune signaling, B cell signaling, myeloid inflammatory pathways and phagocytosis—all pathways previously demonstrated to be important in the pathogenesis of human SLE [27-29]. Next, GO biological pathway enrichment analysis was carried out separately on increased and decreased transcripts and categories with significant overlap p values were determined (Figure 6B). GO biological pathway analysis confirmed the increase in biochemical processes associated with drug metabolism shown by IPA, but processes related to cilium assembly were most highly enriched. The most decreased GO categories were related to the immune and inflammatory response, B cell receptor signaling, cell division, ER stress and unfolded protein responses, NF-κB signaling and phagocytosis. Furthermore, a decrease in the interferon gene signature as well as pattern recognition receptors such as TLRs was also observed. These results illustrate that the IPA pathways and the GO biological pathway analysis showed similar changes in transcription and signaling profiles.

Next, the enrichment of transcripts increased or decreased in HDAC6 treated NZB/NZW mice were assessed using the BIG-C clustering algorithm and chi square analysis to evaluate significant enrichment of BIG-C categories (Figure 6C). In agreement with published data from human patients treated with HDAC inhibitors, we observed a significant metabolic shift as evidenced by the increase in biochemical markers in the cytoplasm, including enzymes associated with fatty acid synthesis, mitochondrial and peroxisome activity [30] (Supplementary Data 7). Furthermore, the observed decrease in transcripts associated with the unfolded protein response, golgi, ER, and cell cycle transcripts associated with recently generated plasma cells support an overall reduction in plasma cells (Supplementary Data 8). Thus, the three analytical methodologies demonstrated that HDAC6 inhibitor treatment led to increased transcripts associated with biochemical pathways and cytoskeletal events and decreased transcripts associated with plasma cells and immune networks.

HDAC6 inhibition alters cellular metabolism

For immune cells to become activated, metabolic processes increase to support activation, proliferation, and differentiation. Although pathways associated with the mitochondria and cellular biochemistry were affected by HDAC6 inhibition, it was unclear whether a specific type of metabolism was predominating after treatment. Increased transcripts related to cellular energy production demonstrated nine genes associated with glycolysis (*Fbp1* (negative regulator), *Ier3* (negative regulator), *G6pc3*, *Pfkm*, *Aldoc*, *Dhktd1*, *Prkaa2*, *Khk*, *Eno2*), 12 genes involved in oxidative phosphorylation (*Taz, Atp5s, Slc25a23, Cox4l2, Cox6b2, Ndufb3, mt-Nd2, mt-Nd4, mt-Cytb*, *Nipsap2*, *Coq7* and *Nubpl*), seven fatty acid beta-oxidation genes (*Acsbg1*, *Slc27a6*, *Slc27a1*, *Ivd*, *Pex5*, *Pex7*, *Hadh*, *Decr1*, *Echdc2*, *Acad11*) and four genes associated with the

TCA cycle (*Pdk2* (negative regulator), *Idh2, Sdhaf4, Dhtkd1*). Among decreased transcripts, there were nine genes associated with glycolysis (*Pgk1, Pgam1, Pfkfb3, Hk2, Pfkp* (expressed in platelets and fibroblasts), *Zbtb7a, Nupr1, Hif1a, Tpil1*), seven with oxidative phosphorylation (*Coa5, Nupr1, Pgk1, Atp7a, Bid, Vcp, Pde12*), two with fatty acid beta oxidation (*Abcd1, Abcd2*), and four with the TCA cycle (*Glud1, Idh1, Pdha1, Pdpr*). To determine whether the altered transcripts induced by HDAC inhibition led to altered metabolic pathways in lupus mice, we examined the enzyme activity of proteins involved in electron transport chain function, the tricarboxylic acid cycle, and fatty acid beta oxidation in the spleens of lupus mice treated with the HDAC6 inhibitor ACY-728 for a 4-week period (Figure 7). We observed a significant decline in citrate synthase enzyme function in response to HDAC inhibition ($p = 0.043$). The activity of citrate synthase is a biochemical marker of mitochondrial density and oxidative capacity [31, 32]. The activities of beta hydroxyacyl CoA dehydrogenase (βHAD), a key regulatory enzyme in the beta oxidation of fatty acids to acetyl CoA was unchanged with HDAC6 inhibition whereas cytochrome c oxidase, important in the function of mitochondrial electron transport chain function, was decreased but not statistically significant ($p = 0.053$).

To investigate further the role of ACY-738 on the metabolic function of B and T cells, we performed *in vitro* experiments on cells isolated from NZB/W female lupus mice. Purified B cells and T cells were stimulated with LPS or anti CD3/CD28 for 24 hours with or without 4 uM of ACY-738 (Figure 8). We have previously reported that at this concentration of ACY-738 is effective at inhibiting inflammatory mediator production and activation in immune cells without toxicity [33]. Glucose is a major source for energy and biosynthesis in activated T and B cells [34, 35]. In the cell, glucose undergoes a 10-step reaction to generate pyruvate which is either

reduced into lactate by lactate dehydrogenase in the cytosol, or transported into the mitochondria via the mitochondrial pyruvate carrier complex where it is converted into acetyl-CoA by the pyruvate dehydrogenase complex, a process that is tightly regulated by the pyruvate dehydrogenase kinase (*Pdk1*), which can phosphorylate pyruvate dehydrogenase complex and inhibit its activity. When B cells were treated with ACY-738, CO_2 produced from oxidation of glucose was significantly decreased ($p = 0.044$). In T cells there was a reduction in CO_2 after treatment, but it was not significant ($p = 0.16$) (Figure 8.) Next, we investigated the amount of CO_2 production from fatty acids (palmitate) with and without ACY-738. Similarly, we found that ACY-738 did not decrease CO_2 production from fatty acids in stimulated B cell and T cells significantly (P=0.09, B cells and P=0.06, T cells).

HDAC6 inhibition in mice decreases pathogenic signaling pathways that are up-regulated in active human SLE

In order to demonstrate the relevance of our findings regarding HDAC6 inhibitor-mediated suppression of molecular pathways in lupus mice, we compared the downregulated pathways to those found to be up-regulated in active human lupus. Specifically, we compared the pathways down-regulated by HDAC6 inhibition in NZB/W mice to pathways up-regulated in human lupus affected organs, including skin, synovium and kidney (Figure 9). Our results showed that the molecular pathways decreased by the HDAC6 inhibitor in NZB/W mice are also highly up-regulated in human SLE affected tissues. For example, ACY-738 treatment of NZB/W mice significantly decreased a total of 59 IPA canonical pathways ($Z \leq -2$, $p \leq .05$). Of these pathways, 38 (64%) had significant positive Z scores ($Z > 2$) for at least two of three human SLE affected tissues. For the remaining 21 IPA canonical pathways decreased by ACY-738, positive Z scores

less than 2 were found for most of the human SLE affected tissues. ACY-738 treatment of mice increased a total of 5 canonical pathways, and none were significantly decreased in human SLE although glutathione mediated detoxification (Z = 3.5 in HDAC6 inhibitor treated mice) had negative Z scores for human lupus skin (-1.8) and lupus nephritis (-1.6). The striking overlap in canonical pathways affected by HDAC6 inhibition and the aberrant pathways in human SLE affected tissues confirms the relevance of the murine lupus results in predicting potential benefit in human lupus.

Discussion

In the current studies, we sought to define the mechanism by which HDAC6 inhibition decreases disease pathogenesis in NZB/W mice by using RNAseq to evaluate the transcriptomic signatures of splenocytes from treated and untreated mice coupled with applied computational cellular and pathway analysis. In addition, we sought to bridge between the transcriptomic data obtained from the HDAC6 treated mice and human gene expression information to determine the relevance to this target in possibly controlling human lupus. We found that PC development was abrogated and GC formation was greatly reduced in HDAC6 inhibitor-treted NZB/W mice. When we compared the HDAC6 inhibitor treated lupus mouse gene signatures to human lupus patient gene signatures, the results showed numerous immune and inflammatory pathways increased in active human lupus affected tissue were significantly decreased in the HDAC6 inhibitor treated animals. Pathway analysis suggested alterations in cellular metabolism might contribute to the normalization of lupus mouse spleen genomic signatures, and this was confirmed by direct measurement of the impact of the HDAC6 inhibitor on metabolic activities of murine spleen cells. Taken together, our studies suggest that HDAC6 may decrease germinal center activity and B cell activation and reduces several signaling pathways required for PC differentiation in the context of LN. Moreover, the molecular pathways suppressed by the HDAC6 inhibitor were frequently overexpressed in human lupus tissue. Of importance, our data also suggests that HDAC6 inhibition corrects aberrant cellular metabolism observed in lupus. There are numerous signaling pathways, metabolic events, and transcription factors that regulate the differentiation of B cells into PC [36-40]. Our rationale for continued investigations to define the molecular events in lupus immunopathogenesis mediated by HDAC6 is related to the uncertainty of the non-redundant roles of HDAC6 in immune function in general and lupus in

particular. HDAC6 knock out mice (HDAC6$^{-/-}$) have grossly normal immune cell development. However, HDAC6$^{-/-}$ mice show a four-fold decrease in antibody production in response to immunization with a T cell-dependent antigen. Furthermore, responses to RNA but not DNA viruses are reduced in HDAC6-deficient mice [41, 42]. HDAC6 is a unique member of the HDAC family that largely resides within the cytoplasm and regulates the acetylation status of a number of cytoplasmic proteins. These include proteins involved in the tubulin cytoskeleton as well as the proteasome. HDAC6 inhibition, therefore, has the potential to alter a variety of cellular functions [43, 44]. Inhibition of HDAC6 has also beneficial effects treating multiple myeloma, an expansion of malignant PCs that secrete abnormal antibodies [45, 46]. In lupus, HDAC6 may act to regulate both innate and adaptive immune responses [47-51]. HDAC6 acts as a coactivator for interferon-beta (IFN-β) induction, and HDAC6 inhibition prevents IFN-β expression [52]. Indeed, we found that the IFN signature is decreased. β-catenin also serves a target of HDAC6; deacetylation of β-catenin facilitates it translocation to the nucleus to serve as a co-activator for IRF3-mediated transcription, a possible mechanism for its impact on IFN- β production [53]. In B cells, HDAC6 inhibition leads to the acetylation of NF□B which prevents its nuclear translocation [54, 55]. Alpha tubulin regulates the cellular cytoskeleton and is acetylated by HDAC6 inhibitors. Increased acetylation of alpha tubulin may inhibit the B-T cell interaction by preventing B cell migration and germinal center formation. Indeed, Tfh-B cell collaboration requires interaction of CD40L and IL-4 with CD40 and IL-4L, respectively. We have previously shown that HDAC6 inhibition resulted in a decreased Tfh population and reduced CD40 and IL-4L activities in B cells [33]. The current studies confirm that HDAC6 inhibition decreased the Tfh population in lupus mice. Additionally, regulation of B cell activation involves tyrosine kinase regulation. P85/P110-PI3K belongs to class IA PI3K

mediated signals which regulate B cell commitment and differentiation. PI3K signaling pathways can be activated in a Toll like receptor (TLC)-dependent or B cell receptor (BCR)-dependent manner. Following treatment with ACY-738, we found decreased PI3K transcripts which are important for B cell inflammatory signaling [56]. Bruton's tyrosine kinase (Btk) is also an important component of BCR signaling. Of note, increased Btk expression has been observed in human autoimmune disease [57]. Prior studies have shown Bkt activation controls the entry of peripheral naïve B cells into the follicle, survival and maturation of B cells, and plasma cell differentiation [58]. Recent studies have demonstrated that inhibition of Btk reduces autoantibody production and pathogenesis [59, 60]. Btk inhibition reduces B cell activation, differentiation of PC and autoantibody class-switching [57]. In our current studies, we found that Btk expression and signaling cascade was suppressed by HDAC6 inhibition, the suppression of Btk may have occurred through inhibition of PI3K signaling. In summary, the HDAC6 inhibitor suppresses expression of a number of pathways that are essential for B cell activation and differentiation of PC. Whether the therapeutic effect in SLE is based on inhibition of one pathway or many activation pathways required for germinal center formation and PC differentiation and survival remains to be completely defined.

HDAC6 inhibitor treatment was also demonstrated to have an effect on cellular metabolism. This was shown *in vivo* in treated mice and *in vitro* with cultured lymphocytes. In regard to cellular metabolism, we observed a significant metabolic shift as evidenced by the increase in gene expression profiles of biochemical markers in the cytoplasm, including mitochondrial enzymes associated with fatty acid oxidation and peroxisome activity which others have reported with HDAC6i [61, 62]. Despite an increase in mRNA content of mitochondrial enzymes, we observed

a significant decline in citrate synthase enzyme function in response to HDAC6 inhibition. The activity of citrate synthase is a biochemical marker of mitochondrial density and oxidative capacity [31, 32]. Perhaps the increased gene expression signature is compensatory to a reduced enzyme activity. Indeed, it has been shown previously that mitochondrial metabolism, including citrate synthase activity, is downregulated in response to HDAC6 inhibition [63]. This is an important finding as O_2 consumption was found to be increased in SLE patents relative to control subjects [30]. Furthermore, the electron transport chain complex I has been identified as the main source of oxidative stress in SLE [64]. B cell differentiation to PC requires a terminal increase in oxidative phosphorylation in order to generate antibodies [65]. The activities of beta hydroxyacyl coA dehydrogenase (βHAD), a key regulatory enzyme in the beta oxidation of fatty acids to acetyl CoA was unchanged with HDAC6 inhibition whereas cytochrome c oxidase, important in the function of the mitochondrial electron transport chain function was decreased but not significantly. Metabolic control of mitochondrial ROS production and glucose utilization have long been recognized as regulators of cellular activation within T cells [66]. In particular, glucose utilization via the pentose phosphate pathway (PPP) and output of NADPH have been reported to regulate the mitochondrial transmembrane potential during T cell activation and chronic activation of $CD4^+$ T cells from lupus-prone mice and SLE patients occurs with high levels of oxygen consumption [67]. Indeed, in other immune-mediated inflammatory diseases, it has been reported that there is increased activation of the citric acid cycle is associated with disease [68]. Taken together, these studies suggest that HDAC6 inhibition may decrease lupus disease by regulating immunologic as well as metabolic function.

To investigate further whether HDAC6 inhibition directly decreased cellular metabolism or whether the changes noted in treated animals were secondary to quieting of the immune

response, we stimulated NZB/W B and T cells *in vitro* and with and without the HDAC6 inhibitor ACY-738 and found that glucose metabolism was significantly decreased in B cells and that fatty acid oxidation was also reduced with HDAC6 inhibition. Combining our gene expression studies along with our *in vitro* metabolic studies suggest that glucose metabolism is critical for immune cell activation and inflammatory cytokine production. A recent study of human $CD4^+$ T cells showed upregulation in metabolism, including pyruvate oxidation and TCA cycle utilization [69], resulting in cell polarization and production of IFN-γ production. Our *in vitro* results suggest that ACY-738 may limit cell metabolism and decrease the spontaneous activation of lupus T and B cells.

In summary, we have shown that selective HDAC6 inhibition corrects abnormal B cell activation and differentiation in NZB/W mice that display early onset disease. The correction in B cell differentiation and activation correlated with less severe renal disease. Specifically, HDAC6 inhibition decreased several signaling pathways that are critical for B cells differentiation to PC. In addition to HDAC6 inhibiting B cell and T cell activation, several metabolic and enzymes pathways that are known to be increased in active lupus were also ameliorated. This was demonstrated *in vivo* and *in vitro*. Finally, when RNA profiles from the NZB/W mice were compared to humans with lupus, our results demonstrate that the many of genes upregulated in lupus patients were decreased lupus mice with HDAC6 inhibition. Taken together, these studies suggest that selective HDAC6 inhibition may be a potential therapeutic for the treatment of lupus nephrites.

Figures

Figure 1.

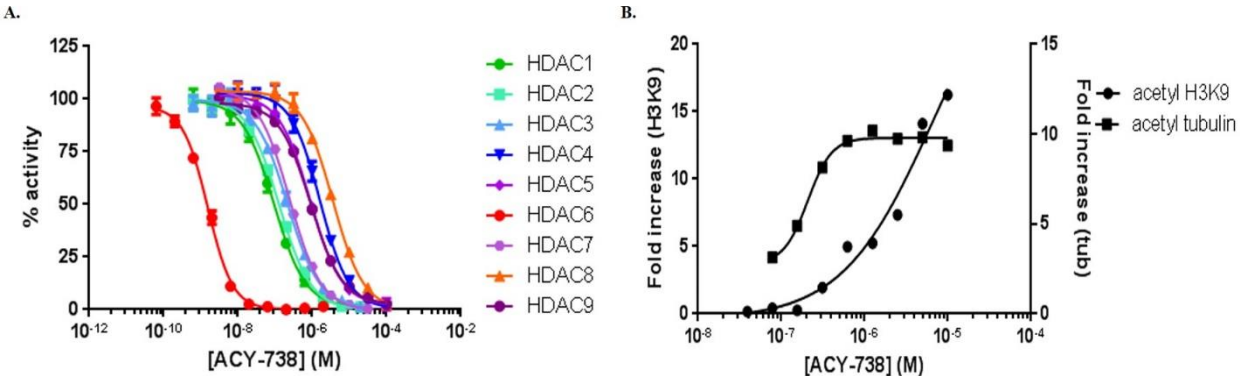


Figure 1. ACY-738 is a selective inhibitor or HDAC6. ACY-738 inhibits HDAC6 with a potency of 1.5 nM and HDAC1 (the next most affected target) with a potency of 93 nM (left). In a cell based assay in HCT-116 cells ACY-738 Induces tubulin acetylation (a marker of HDAC6 inhibition) at 800 nM, whereas histone acetylation (a marker of Class 1 HDAC inhibition) at that concentration is minimal (right).

Figure 2.

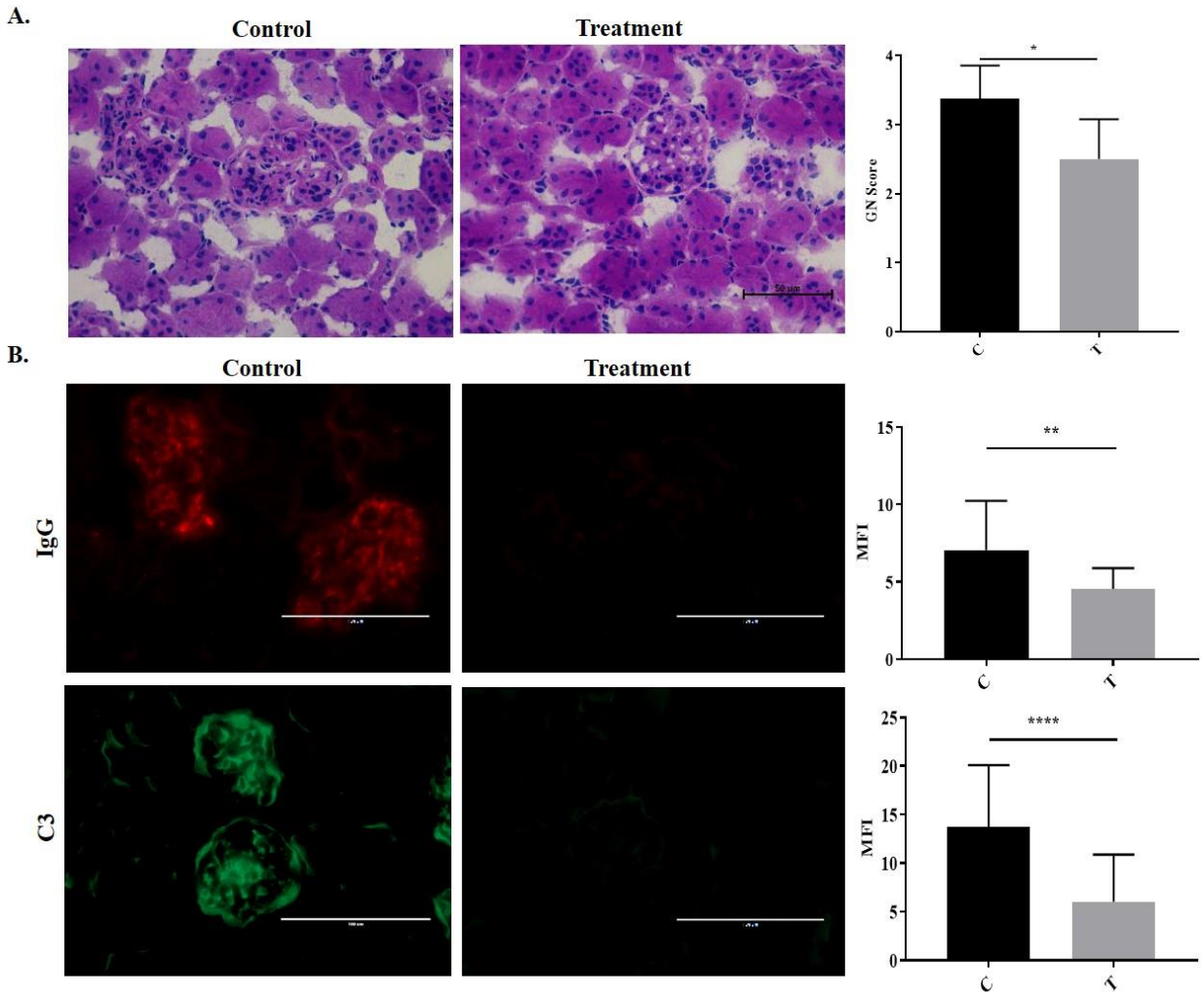


Figure 2. Inhibition of histone deacetylase HDAC6 reduced Ig and C deposition in NZB/W lupus nephritis. (A) Representative Hematoxylin and Eosin (H&E) staining image of kidney glomerular region along with pathology score which reflects the severity of membranoproliferative changes and distribution. (B) Representative immunohistological staining of kidney section for IgG and C3. Graphic analysis of mean fluorescent intensity (MFI) of IgG and C3 is also provided. Data are shown as mean ± standard error of the mean (s.e.m) n=4 mice for each group; T-test; *P<0.05, **P<0.01, ****P<0.0001.

Figure 3.

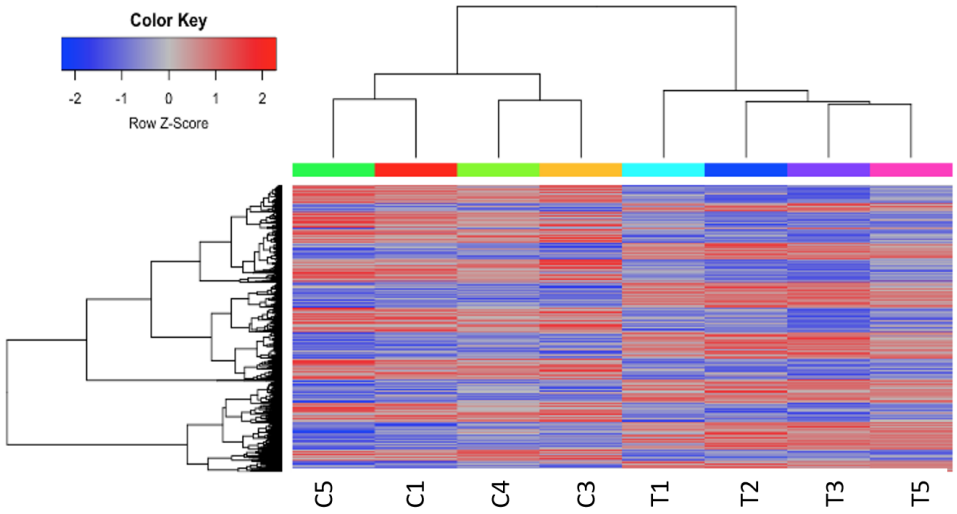


Figure 3. HDAC6i treatment of NZB/NZW F1 mice induced global gene expression changes in whole splenocytes. Hierarchical clustering of 3911 transcripts (1922up, 1989 down) that differed significantly (FDR < 0.1) between control (C1, C3, C4 and C5) and treated mice (T1, T2, T3 and T5).

Figure 4.

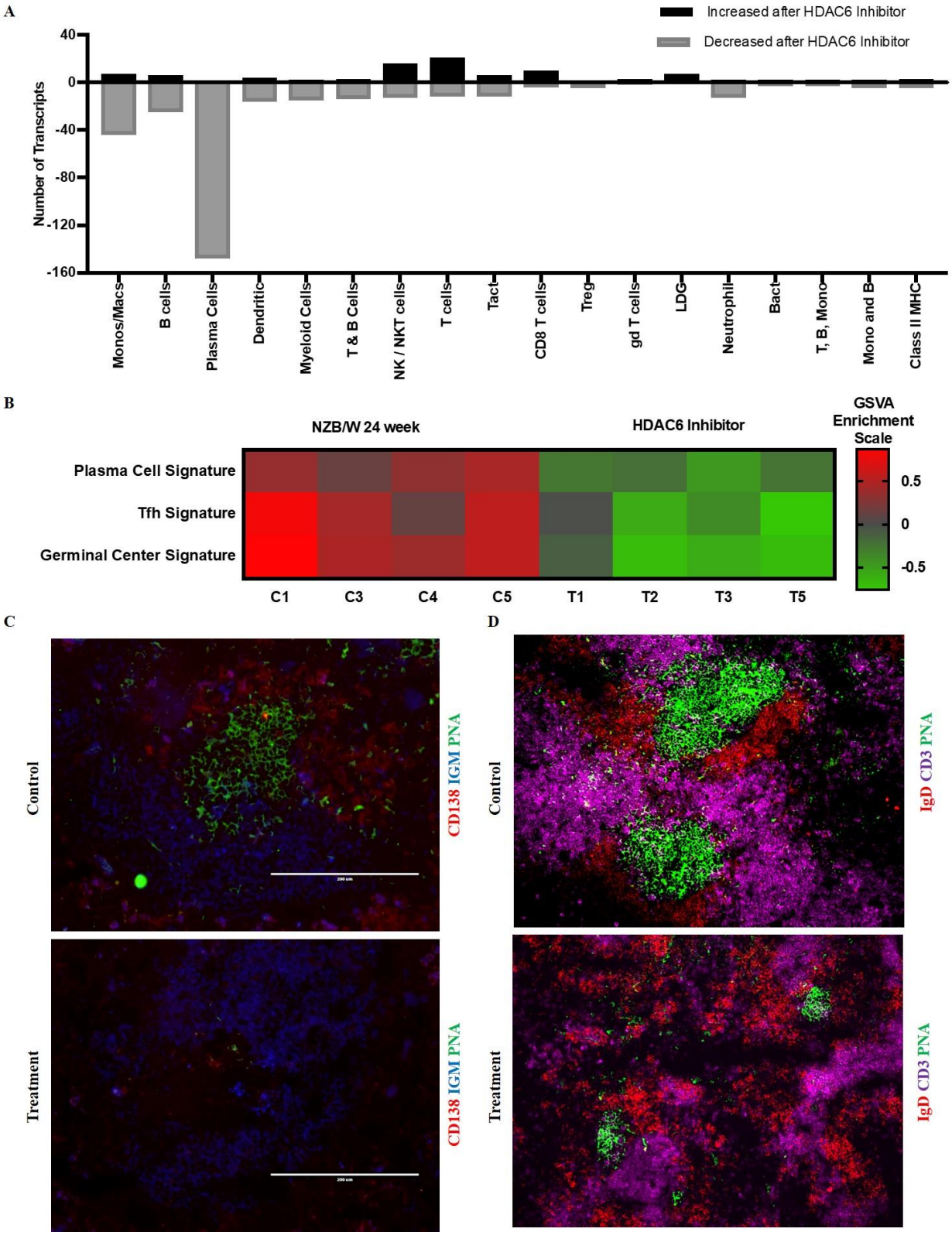

A
Increased after HDAC6 Inhibitor
Decreased after HDAC6 Inhibitor
Number of Transcripts
40
0
-40
-80
-120
-160
Monos/Macs
B cells
Plasma Cells
Dendritic
Myeloid Cells
T & B Cells
NK / NKT cells
T cells
Tact
CD8 T cells
Treg
gd T cells
LDG
Neutrophil
Bact
T, B, Mono
Mono and B
Class II MHC
B
NZB/W 24 week
HDAC6 Inhibitor
GSVA Enrichment Scale
0.5
0
-0.5
Plasma Cell Signature
Tfh Signature
Germinal Center Signature
C1
C3
C4
C5
T1
T2
T3
T5
C
Control
Treatment
CD138 IGM PNA
D
Control
Treatment
IgD CD3 PNA

Figure 4. HDAC6i treatment results in significantly decreased GC activity and PC formation. (A) I-Scope hematopoietic cell enrichment demonstrated that HDAC6 inhibition decreased PC, B cells and inflammatory myeloid cells. The number of transcripts corresponding to each cell type increased or decreased after HDAC6 inhibitor treatment are shown. Gene symbols for transcripts for PC, B cells and inflammatory myeloid cell are in Supplementary Data 1 (increased transcripts) and Supplementary Data 2 (decreased transcripts). (B) GSVA was carried out to determine the enrichment of PC, Tfh cells and GC in each HDAC6 inhibitor treated and control NZB/NZW mouse (Methods lists genes used for GSVA enrichment modules). (C) Representative splenic section stained with anti-CD138, anti-IgM and PNA. (D) Representative splenic section stained for T cells, follicular B cells and GC with anti- CD3, anti-IgD and PNA.

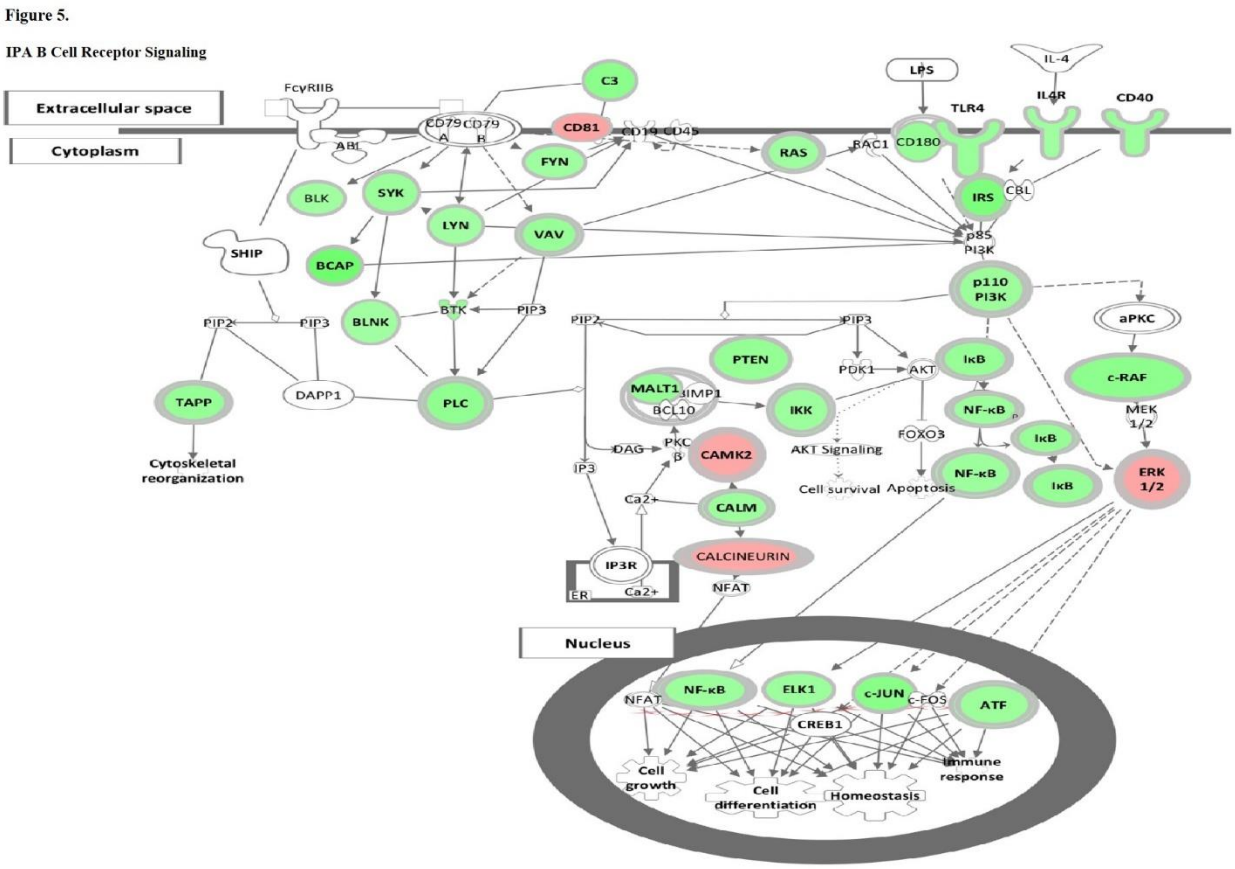


Figure 5. HDAC6 inhibition repressed B cell signaling pathways in NZB/NZW mice. The IPA Canonical Signaling Pathway "B Cell Receptor Signaling" had a Z score of -3.1. Transcripts differentially expressed between HDAC6 inhibitor treated and untreated NZB/NZW mice were overlaid on genes in the IPA pathway. Decreased transcripts are shown in green, while increased transcripts are shown in pink.

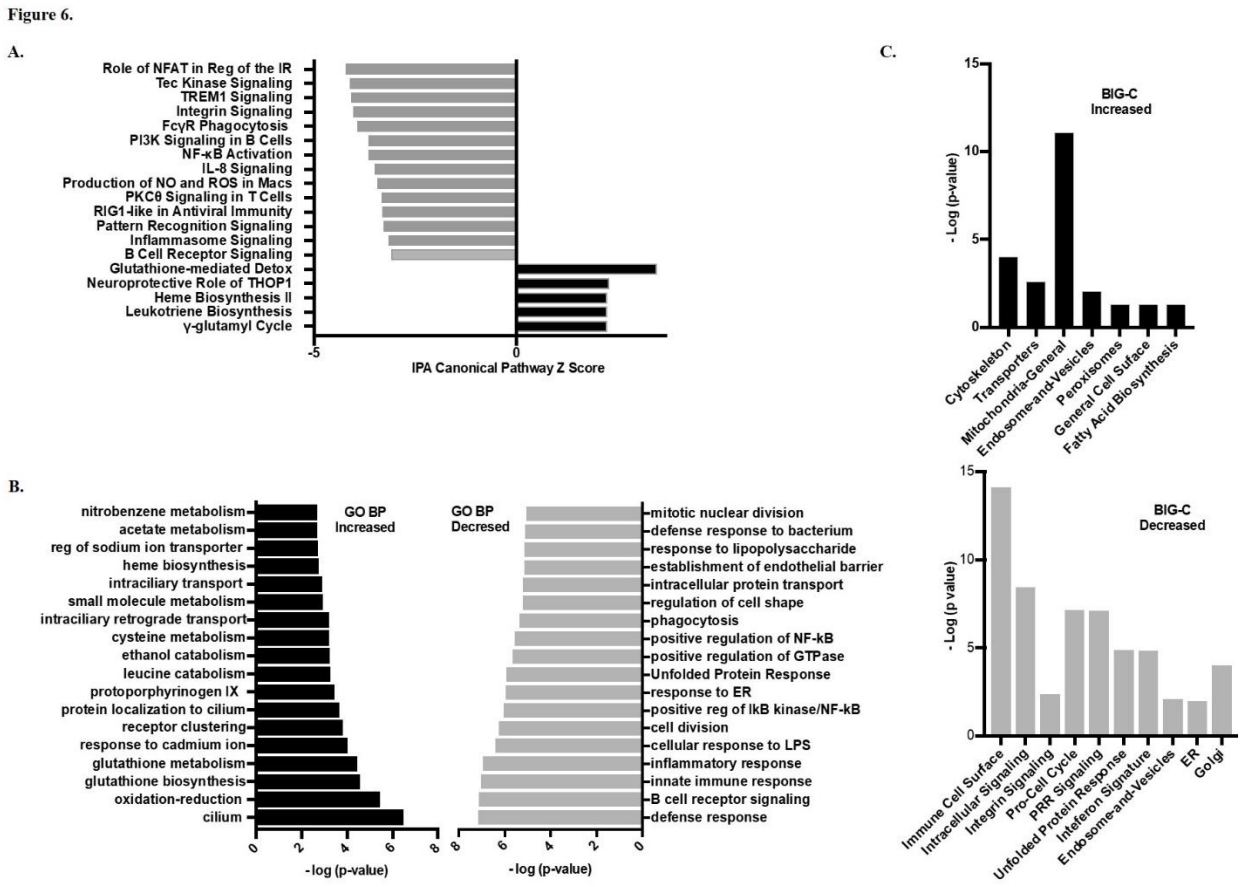


Figure 6. Inhibition of HDAC6 altered transcripts associated with cellular metabolism.

(A) Ingenuity pathway analysis (IPA) was performed on the differentially expressed transcripts between HDAC6 inhibitor treated and untreated NZB/NZW mice. The most significant signaling pathways increased or decreased by Z score analysis with an overlap p value ≤ 0.05 are shown. The full list of significant increased and decreased pathways and the genes used to determine significance are in Supplementary Data 3 (increased) and Supplementary Data 4 (decreased). (B) GO biological pathway enrichment analysis of the top most increased and decreased pathways by lowest overlap p value significance. A full list of GO biological pathways enriched ($p < .01$) are in Supplementary Data 5 (increased) and Supplementary Data 6 (decreased). (C) BIG-C pathway enrichment was performed using increased (left) or decreased (right) transcripts from the DE analysis of HDAC6 inhibitor treated NZB/NZW mice compared

to NZB/NZW mice. The -log (p value) is shown for the enriched categories. Gene symbols corresponding to each category are listed in Supplementary Data 7 (increased) and Supplementary Data 8 (decreased).

Figure 7.

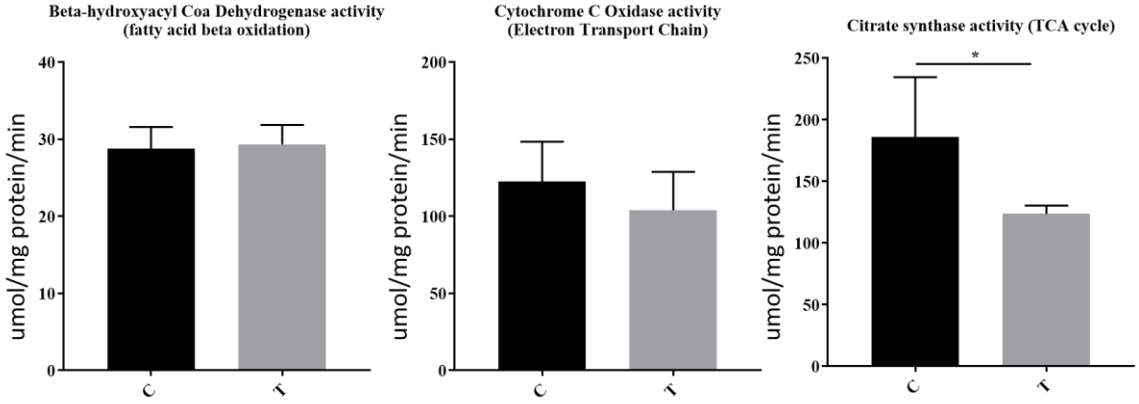


Figure 7. HDAC6 inhibition decreased citrate synthase activity and cytochrome c oxidase activity in NZB/W mice. Four weeks of treatment of NZB/W mice with the HDAC6 inhibitor ACY-738 lead to a significant decrease in the rate limiting enzyme of the TCA cycle ($p = .043$), and a decrease in cytochrome C oxidase activity ($p=0.053$) while having no effect on beta hydroxyacyl coa dehydrogenase in splenocytes (n=5).

Figure 8

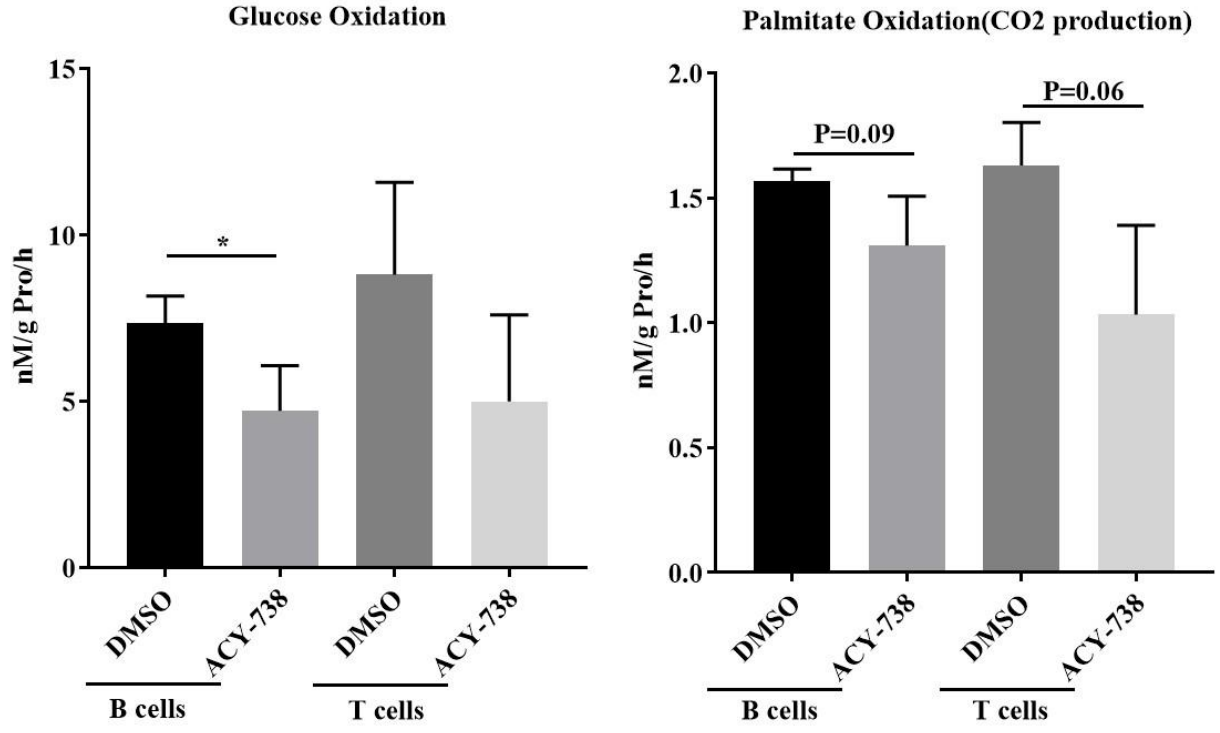


Figure 8. HDAC6 inhibition decreases glucose and fatty acid oxidation in T and B cells from NZB/W mice. T cells and B cells from 12-week old NZB/W female were purified and stimulated with anti CD3/CD28 or LPS respectively for 24 hours with or without the addition of 4 uM ACY-738 (DMSO only was used as control). After 24 hours of culture, CO_2 production from the oxidation of glucose and palmitate were determined from three separate experiments in triplicate (n=3).

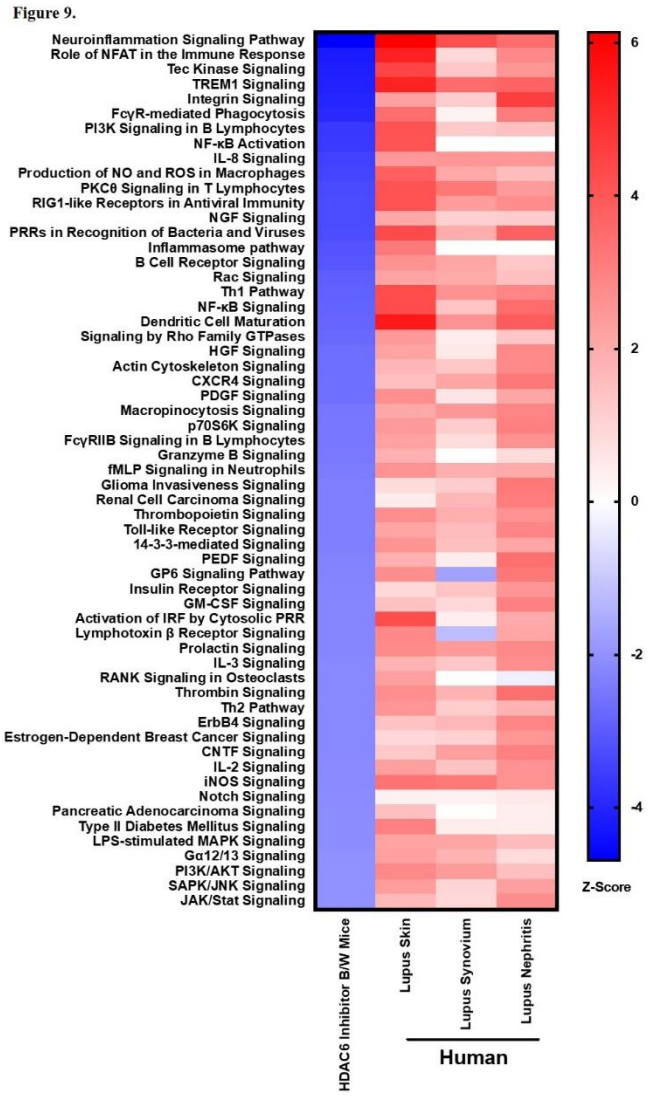
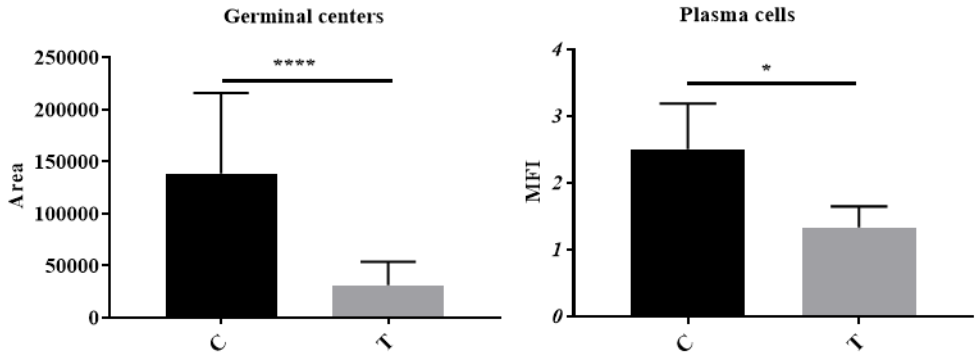


Figure 9. HDAC6 inhibition decreases lupus gene signature pathways in NZB/W mice that are increased in active human SLE. IPA canonical signaling pathways increased in human SLE microarray tissue datasets were compared to signaling pathways in NZB/W mice decreased by the HDAC6 inhibitor. Z scores greater or less than 2 are considered significant.

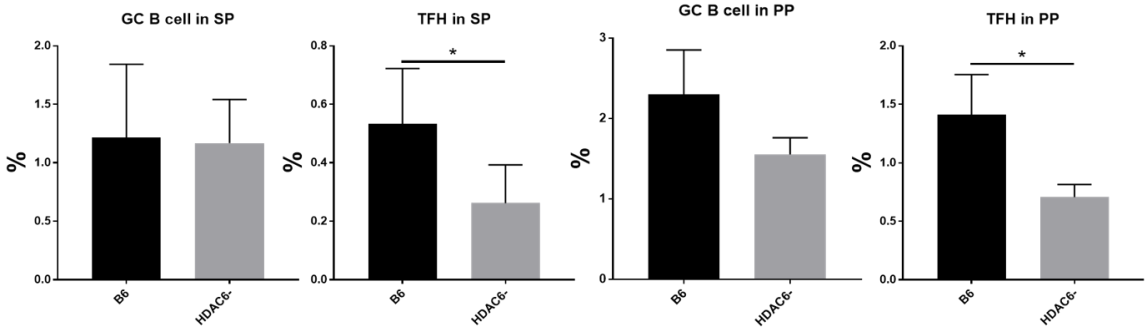
Supplementary Figures

Supplementary Figure 1.



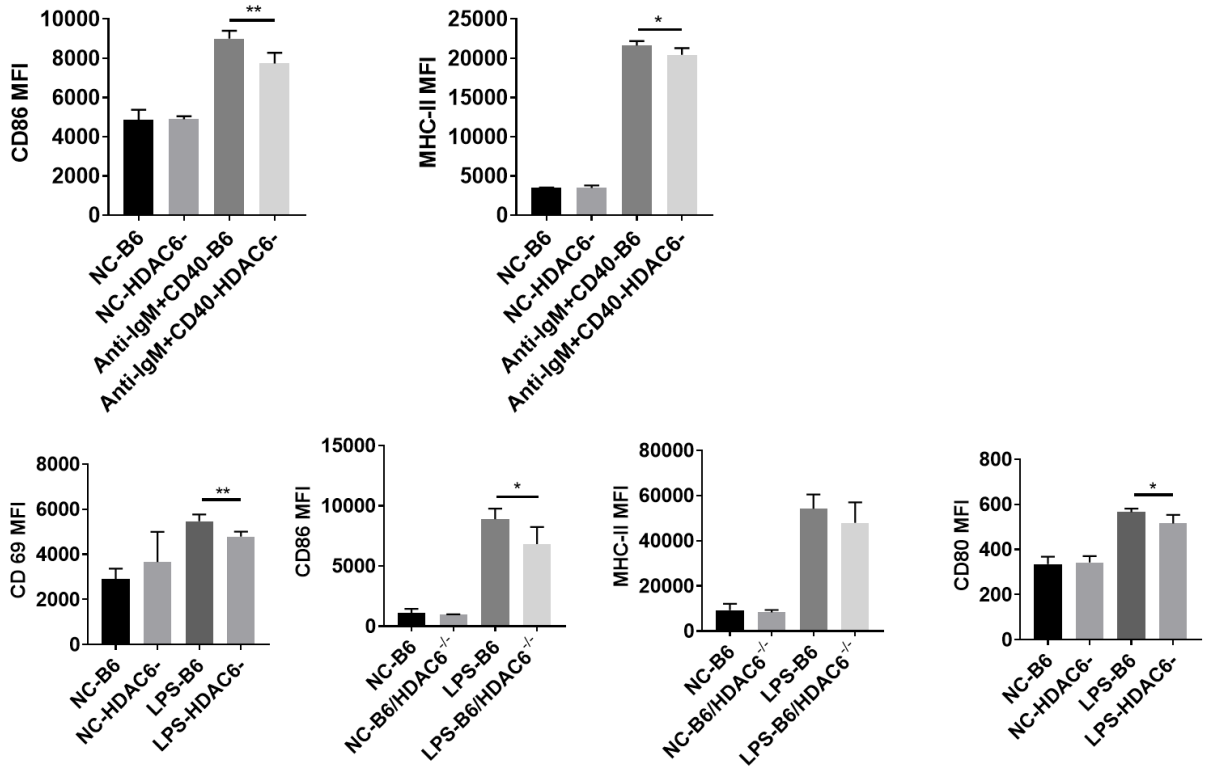
Supplementary Figure 1. Quantified germinal center formation in NZB/W female mice at 24 weeks-of age-treated with (T) or without (C) ACY-738 for four weeks. We randomly picked 5 germinal centers from each spleen sample and analyzed by using Image J software to calculate the size of the germinal center. N=20, * P<0.05, **** P<0.0001.

Supplementary Figure 2.



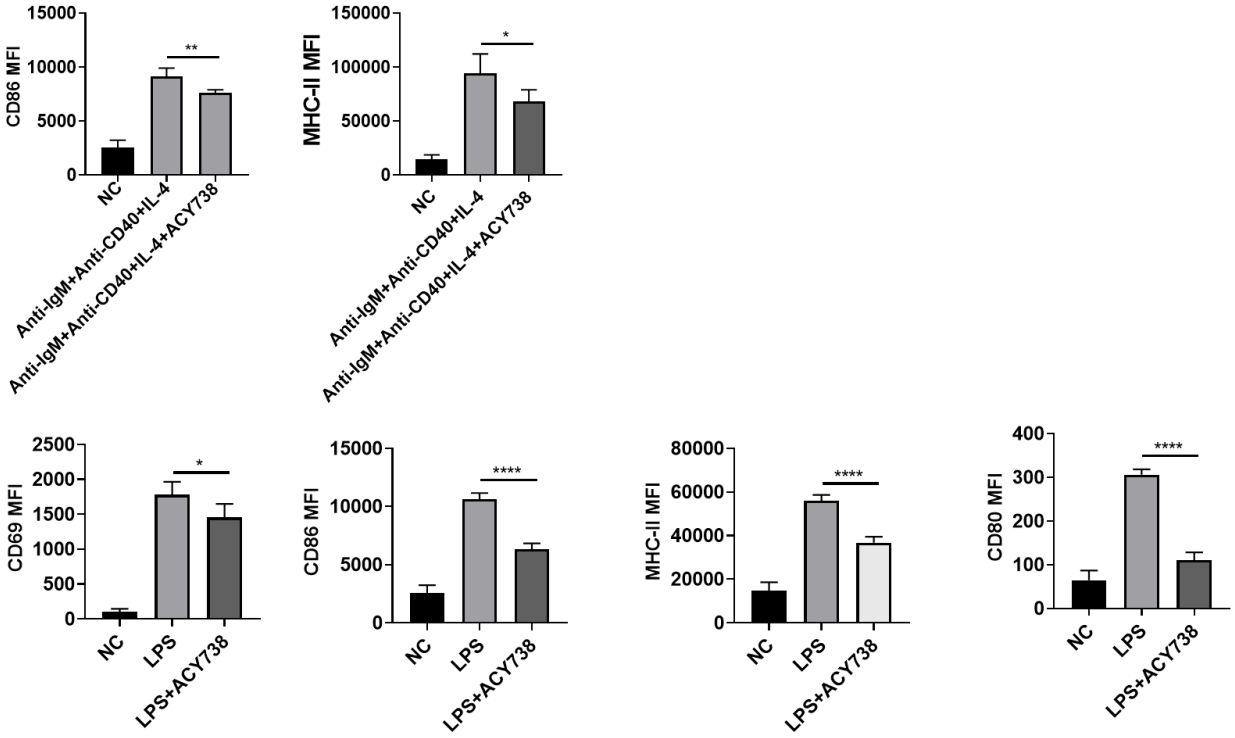
Supplementary Figure 2: Flow cytometry of GC B cells and TFH assessed by Flow cytometry in C57/B6 mice and C57B6HDAC6-/- mice. For spleen, n=5, for Peyer's patch, n=3. Germinal center B cells are gated by CD19+, GL7+, IgD-. * P<0.05.

Supplementary Figure 3.



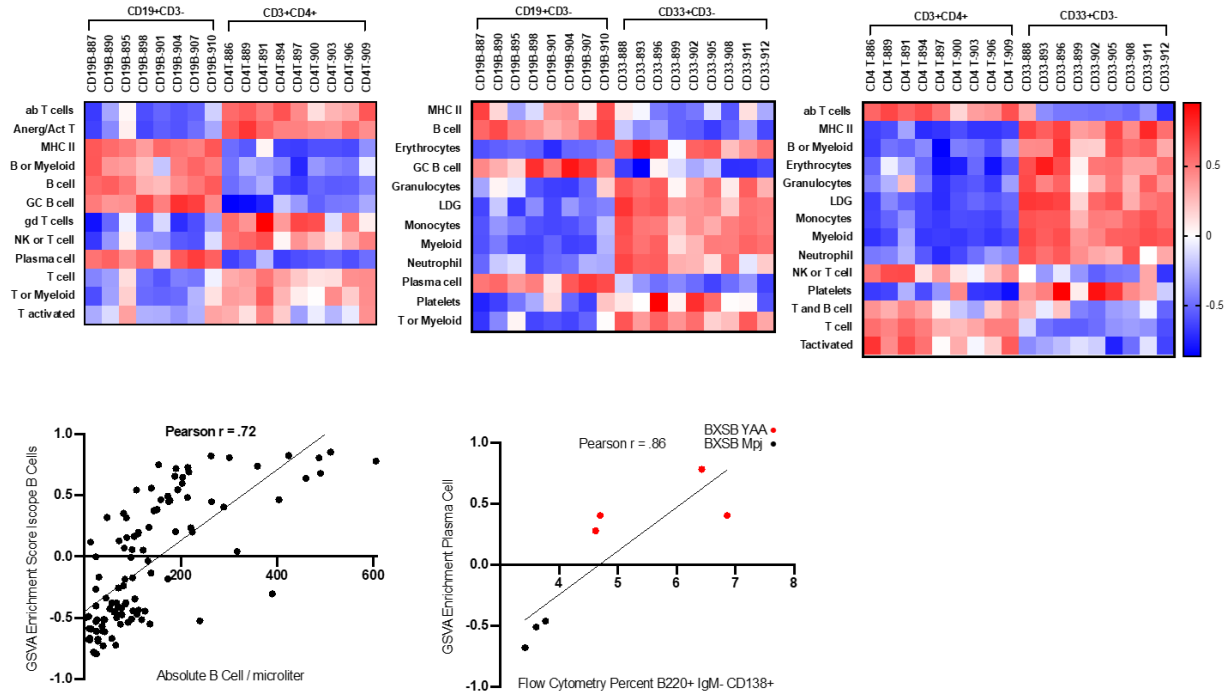
Supplementary Figure 3: Flow cytometry of sorted B cells from C57BL/6J mice and C57BL/6J/HDAC6-/- mice stimulated with LPS or anti-IgM, anti-CD40 for 24 hours. The results showed reduced expression of activation markers of B cells CD86 and MHCII in C57BL/6J/HDAC6-/- mice compared to C57BL/6J mice with stimulation of anti-IgM and anti-CD40. In addition, MFI of CD69, CD86, and CD80 are downregulated in C57BL/6J/HDAC6-/- mice with stimulation of LPS. N=5. * $P<0.05$, ** $P<0.01$.

Supplementary Figure 4.



Supplementary Figure 4: Flow cytometry of sorted B cells from NZB/W mice stimulated with LPS or anti-IgM, anti-CD40 and then treated with ACY738 for 24 hours. The results showed reduced expression of activation markers of B cells CD86 and MHCII in ACY-738 treated B cells with stimulation of anti-IgM and anti-CD40. In addition, MFI of CD69, CD86, MHC-II and CD80 are significantly downregulated in ACY-738 treated B cells with stimulation of LPS. N=5. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Supplementary Figure 5



Supplementary Figure 5. (A) Control experiments demonstrate the specificity and lack of cross reactivity of I-scope. Experiments were performed on the DE analysis of healthy control purified CD3+CD4+ T cells, CD19+CD3- B and Plasma Cells, and CD33+CD3- Myeloid cells from microarray dataset GSE10325. The genes in each I-scope category (29 categories in total; hematopoietic general was not used) were used as modules for gene set variation analysis to determine the specificity of each module and cross-reactivity to other cell types. For each comparison, only categories with at least three genes above the Interquartile Range threshold were considered for statistical analysis. Significance of GSVA enrichment scores was determined using Sidak's multiple comparisons test. Adjusted p values below .05 were considered significant. (B) Demonstration of strong relationship of human B cell / microliter counts to GSVA enrichment scores for the I-scope B cell category on 105 human subjects from microarray dataset GSE88884. Demonstration of the strong relationship of mouse flow

cytometry values for plasma cells (B220+IgM-CD138+) and the GSVA enrichment scores using the I-scope plasma cell module on BXSB Yaa and BXSB MPJ mice.

Supplementary Tables

Supplementary Data 1

Gene Symbols from Iscope Categories Increased by HDAC6 Inhibitor Treatment

B cells	Dendritic	Monocytes / Macrophages	Myeloid	Plasma Cells
6	4	7	1	0
Cd1d1	*Il15*	*Ier3*	*Clec4b2*	
Gng7	*Cd1d1*	*Ccl17*		
Vpreb1	*Cd209a*	*Mfge8*		
Ly6d	*Osm*	*Clec9a*		
Fam129c		*Il15*		
Cd37		*Mgl2*		
		Lgals9		

Supplementary Data 2
Gene Symbols from Iscope Categories Decreased by HDAC6 Inhibitor Treatment

B cells	Monocytes / Macrophages	Dendritic	Myeloid	Plasma Cells	More plasma cells	More Plasma Cells
25	**44**	**16**	**15**	**148**		
Tnfrsf8	*Tlr8*	*Cd300e*	*Ms4a4a*	*Jchain*	*Ighv1-54*	*Igkv10-94*
Slamf1	*Tnfaip3*	*Vsig4*	*Clec7a*	*Hmmr*	*Ighv1-56*	*Igkv10-95*
Havcr1	*Tnip3*	*Hmmr*	*Pik3ap1*	*Hvcn1*	*Ighv1-58*	*Igkv12-41*
Tlr7	*Ace*	*Igsf6*	*Btk*	*Cd38*	*Ighv1-61*	*Igkv12-46*
Irf4	*Ms4a4a*	*Adamdec1*	*Fgr*	*Slamf7*	*Ighv1-62-3*	*Igkv13-85*
Tlr9	*Clec4e*	*Il18bp*	*Bach1*	*Hyou1*	*Ighv1-63*	*Igkv15-103*
Rgs13	*Cd300e*	*Il12b*	*Treml4*	*Fkbp11*	*Ighv1-*	*Igkv17-*

					66	*121*
Aicda	*Clec4a3*	*Il27*	*Ms4a6c*	*Mzb1*	*Ighv1-7*	*Igkv17-127*
Sh2b2	*Tnfrsf1b*	*Themis2*	*Itgax*	*Ighg1*	*Ighv1-73*	*Igkv2-109*
Samsn1	*Vsig4*	*Cd180*	*Apoc1*	*Igkc*	*Ighv1-75*	*Igkv2-116*
Pou2af1	*Lilra5*	*Slamf1*	*Slpi*	*Tnfrsf17*	*Ighv1-76*	*Igkv2-137*
Btk	*Ms4a2*	*Il21r*	*Gm15931*	*Ighd*	*Ighv1-77*	*Igkv3-1*
Klhl6	*Tgm2*	*Cd83*	*Lilrb4a*	*Stil*	*Ighv1-78*	*Igkv3-11*
Pkn1	*Msr1*	*Cnr2*	*Cd300lb*	*Parpbp*	*Ighv1-83*	*Igkv3-12*
Blk	*Hmmr*	*Ulbp1*	*Cd300ld*	*Ighd1-1*	*Ighv1-84*	*Igkv3-2*
Blnk	*Pilra*	*Fcgr1*		*Ighd2-7*	*Ighv10-1*	*Igkv3-4*
Gcsam	*Igsf6*			*Ighe*	*Ighv13-2*	*Igkv3-9*
Tnfsf8	*Siglec1*			*Ighg3*	*Ighv14-2*	*Igkv4-51*
Tnfrsf13c	*Clec4d*			*Ighj1*	*Ighv14-3*	*Igkv4-57-1*
Cd22	*Fpr2*			*Ighj2*	*Ighv14-4*	*Igkv4-58*
Nuggc	*Csf1r*			*Ighj3*	*Ighv2-2*	*Igkv4-61*
Elf1	*Clec7a*			*Ighv1-12*	*Ighv2-3*	*Igkv4-63*
Snap23	*Adgre1*			*Ighv1-14*	*Ighv2-4*	*Igkv4-69*
Ncf1	*Smpdl3b*			*Ighv1-18*	*Ighv2-6*	*Igkv4-70*
H2-Ob	*Adamdec1*			*Ighv1-19*	*Ighv2-6-8*	*Igkv4-73*
	Cd5l			*Ighv1-2*	*Ighv2-7*	*Igkv4-74*
	Cfb			*Ighv1-20*	*Ighv2-9*	*Igkv4-77*
	Il18bp			*Ighv1-21*	*Ighv2-9-1*	*Igkv4-86*
	Il10			*Ighv1-21-1*	*Ighv3-3*	*Igkv5-37*

	Il12b			*Ighv1-22*	*Ighv3-4*	*Igkv5-39*
	Ctla2b			*Ighv1-25*	*Ighv3-6*	*Igkv5-43*
	Il27			*Ighv1-26*	*Ighv5-12*	*Igkv5-45*
	C6			*Ighv1-28*	*Ighv5-15*	*Igkv6-14*
	Serping1			*Ighv1-30*	*Ighv5-16*	*Igkv6-23*
	Cybb			*Ighv1-31*	*Ighv5-17*	*Igkv6-29*
	Cxcl10			*Ighv1-33*	*Ighv5-2*	*Igkv6-32*
	Slc11a1			*Ighv1-36*	*Ighv5-4*	*Igkv8-21*
	Lmnb1			*Ighv1-37*	*Ighv5-6*	*Igkv8-24*
	Hvcn1			*Ighv1-39*	*Ighv5-9*	*Igkv8-28*
	Mpeg1			*Ighv1-4*	*Ighv5-9-1*	*Igkv8-30*
	Clec4n			*Ighv1-42*	*Ighv6-5*	*Igkv9-120*
	Csf2rb2			*Ighv1-43*	*Ighv6-6*	*Igkv9-123*
	Lyz1			*Ighv1-47*	*Ighv7-1*	*Igkv9-124*
	Fcgr1			*Ighv1-5*	*Ighv7-2*	*Igkv9-129*
	Fcgr3			*Ighv8-8*	*Ighv7-3*	*Iglc4*
				Igip	*Ighv7-4*	*Igll1*
				Igkj2	*Ighv8-11*	*Igkj5*
				Igkj3	*Ighv8-2*	*Igkv1-117*
					Ighv8-4	*Igkv1-122*
					Ighv8-5	*Igkv1-99*

Supplementary Data 3

Ratio	z-score	Molecules
0.387	3.464	*GSTZ1,GSTM2,MGST2,GSTM5,GSTM3,GGH,GSTM4,Gsta4,GSTO2,MGST3, GSTP1,GSTK1*
0.218	2.294	*PRSS50,HLA-A, PRSS12,CTSG,PRSS33,SERPINA3,PRSS41,PRKAG1,C1R,PRSS16,PRSS36,LONP1,Prss30,ACE,GZMA,GZMK,ENDOU,KLK8,TPSAB1/TPSB2,HPN,APP,PREP,PRKAR2B,PRTN3,MAPT,ST14*
0.556	2.236	*UROD,PPOX,UROS,ALAD,HMBS*
0.385	2.236	*MGST2,GGT5,GGT1,MGST3,GGT7*
0.571	2.121	*CHAC1,GGT5,GCLM,GGT1,GSS,CHAC2,GGACT,GGT7*

Supplementary Data 4

Ingenuity (IPA) Canonical Pathways with Negative Z scores	-log(p-value)	Ratio	z-score	Molecules
Neuroinflammation Signaling Pathway	3.88	0.228	-4.695	*TRAF3,TGFBR1,AGER,TICAM2,TGFBR3,TLR8,NCSTN,MAPK13,CX3CR1,CXCL10,TGFBR2,IKBKB,IKBKG,PIK3CG,MAPK3,CYBB, PLA2G4F,PLA2G12A,FGFR1,NFKB2,MAPK12,TLR9,IRF7,PTPN11,GAB1,CD40,PLCG2, MAPT,SYK,GAD1,SLC6A1,PIK3R6,CFLAR, CX3CL1,ICAM1,HLA-A,PIK3R5,MFGE8,FZD1,NFKB1,SLC6A13, GRINA,PLA2G4E,JUN,TLR7,CASP8,NOS2,PPP3CA,NLRP3,PIK3C2A,GRB2,IL10,MYD88,MAPK6,ACVR1,IL1R1,IRAK3,CSF1R,XIAP, APP,PLA2G4A,TLR4,RIPK1,IL12B,H2-Eb2,HLA-DOB,Tlr13,TIRAP,IRAK4,PSEN1,BIRC2*
Role of NFAT in Regulation of the Immune Response	3.52	0.247	-4.217	*BLNK,RAF1,FYN,Calm1 (includes others),HLA-A,NFKBIE,GNB5,PIK3R5,CSNK1A1,FCER1A,KRAS,GNA14,NFKB1,FCGR1A,GNG7,IKBKB,IKBKG,GNG11,JUN,PIK3CG,MAPK3,XPO1,GNA13,FCGR3A/FCGR3B,PPP3CA,CALML5,PIK3C2A,FCGR2A,GRB2,FGFR1,CSNK1G3,NFKB2,GNAZ,TLR9,BTK,GAB1,PTPN11,H2-Eb2,PLCG2,SYK,LYN,PIK3R6,MS4A2,HLA-DOB,MEF2C,GNAL*

Tec Kinase Signaling	2.98	0.241	-4.116	*FYN,GNB5,PIK3R5,FCER1A,GNA14,NFKB1,GNG7,BLK,PAK1,ITGA3,GNG11,RHOB,PIK3CG,HCK,GNA13,ACTG2,FGR,PRKCA,VAV2,STAT6,PIK3C2A,GRB2,FGFR1,NFKB2,STAT3,GNAZ,MAPK12,TLR9,BTK,TLR4,RHOQ,GAB1,PTPN11,PRKCD,PLCG2,LYN,PIK3R6,MS4A2,VAV1,STAT2,GNAL*
TREM1 Signaling	3.82	0.32	-4.082	*ICAM1,NLRP3,IL10,GRB2,MYD88,CIITA,LAT2,TLR8,CD83,STAT3,NFKB2,NFKB1,TLR9,NLRC5,TLR4,NOD2,MPO,CD40,MAPK3,NLRC3,PLCG2,TLR7,Tlr13,ITGAX*
Integrin Signaling	2.68	0.224	-4.025	*RAF1,RAPGEF1,MAP3K11,KRAS,PTEN,ITGB3,ITGAE,ITGA3,PAK1,RHOB,MAPK3,PIK3CG,GRB7,ITGAV,ACTG2,CAPN5,ACTR2,CRKL,FGFR1,BCAR3,GSN,TLR9,TTN,RAP1A,RAC3,RHOQ,GAB1,PTPN11,PLCG2,PIK3R6,ACTN4,TSPAN6,FYN,ARPC1B,PIK3R5,CRK,ITGB8,BCAR1,ACTR3,PPP1R12A,PFN4,PIK3C2A,GRB2,ACTN2,ITGAL,GIT1,ITGB2,LIMS1,ITGAX*
FcγR-mediated Phagocytosis in Macs and Monos	3.05	0.28	-3.922	*FYN,ARPC1B,CRK,FCGR1A,PTEN,PAK1,ACTR3,MAPK3,EZR,PIK3CG,HCK,ACTG2,FCGR3A/FCGR3B,FGR,PRKCA,VAV2,ACTR2,RPS6KB1,FCGR2A,RAC3,PLD4,NCF1,SYK,PRKCD,LYN,VAV1*
PI3K Signaling in B Lymphocytes	5.04	0.3	-3.667	*BLNK,CD81,FYN,RAF1,Calm1 (includes others),PDIA3,NFKBIE,ATF6,KRAS,NFKB1,PTEN,BLK,PLCD3,IKBKB,IKBKG,JUN,PIK3CG,MAPK3,PPP3CA,CAMK2B,VAV2,IL4R,CALML5,C3,NFKB2,MALT1,BTK,TLR4,CD40,CD180,PLCG2,SYK,SH2B2,LYN,VAV1,PIK3AP1,PLEKHA2,ELK1,CAMK2G*
NF-κB Activation	3.96	0.31	-3.657	*RAF1,NFKBIE,PIK3R5,KRAS,NFKB1,ITGB3,IKBKB,ITGA3,IKBKG,MAPK3,PIK3CG,ITGAV,PRKCA,MAP3K14,PIK3C2A,GRB2,FGFR1,NFKB2,TLR9,ITGAL,ITGB2,RIPK1,GAB1,PTPN11,PRKCD,PIK3R6,EIF2AK2*
IL-8 Signaling	2.69	0.228	-3.507	*RAF1,ICAM1,GNB5,PIK3R5,EGF,VEGFB,KRAS,NFKB1,IQGAP1,CCND1,GNG7,ITGB3,IKBKB,IKBKG,GNG11,JUN,RHOB,PIK3CG,MAPK3,CYBB,ITGAV,GNA13,LASP1,PRKCA,RPS6KB1,PIK3C2A,GRB2,FGFR1,VEGFC,IRAK3,MAPK12,TLR9,RAC3,PLD4,ITGB2,MPO,CCND2,RHOQ,ARAF,GAB1,PTPN11,PRKCD,PIK3R6,IRAK4,ITGAX*

Production of NO and ROS in Macrophages	3.65	0.247	-3.429	*MAP3K15,MAP3K11,APOF,NFKBIE,PIK3R5,MAPK13,MAP3K5,NFKB1,IKBKB,LYZ,IKBKG,JUN,RHOB,PPP1R12A,PPM1J,PIK3CG,MAPK3,HOXA10,CYBB,NOS2,TNFRSF1B,PRKCA,MAP3K14,MAP2K7,PTPN6,APOM,PIK3C2A,GRB2,PPP2R5D,FGFR1,NFKB2,PCYOX1,MAPK12,TLR9,RAP1A,MAP3K12,TLR4,NCF1,MPO,RHOQ,GAB1,PTPN11,PRKCD,PLCG2,PIK3R6,MAP3K8,IRF8,MAP3K3*
PKCθ Signaling in T Lymphocytes	4.27	0.27	-3.333	*MAP3K15,FYN,CACNA2D2,MAP3K11,HLA-A,NFKBIE,CACNA1H,PIK3R5,KRAS,MAP3K5,NFKB1,CACNA1F,IKBKB,IKBKG,JUN,PIK3CG,MAPK3,CACNG8,PPP3CA,CAMK2B,VAV2,MAP3K14,CACNB1,PIK3C2A,GRB2,FGFR1,CACNA1C,NFKB2,MALT1,TLR9,RAC3,CACNA1A,MAP3K12,GAB1,PTPN11,H2-Eb2,PLCG2,PIK3R6,HLA-DOB,VAV1,MAP3K8,MAP3K3,CAMK2G*
Role of RIG1-like Receptors in Antiviral Innate Immunity	1.61	0.273	-3.317	*IFIH1,TANK,IKBKB,TRAF3,IRF7,IKBKG,RIPK1,NFKBIE,DDX58,NFKB2,CASP8,NFKB1*
NGF Signaling	3.5	0.273	-3.307	*MAP3K15,RAF1,MAP3K11,PIK3R5,CRK,KRAS,MAP3K5,NFKB1,IKBKB,IKBKG,PIK3CG,MAPK3,RPS6KA2,SMPD3,MAP3K14,RPS6KB1,PIK3C2A,GRB2,FGFR1,NFKB2,TLR9,MAPK12,RAP1A,MAP3K12,RPS6KA6,PTPN11,GAB1,PRKCD,PLCG2,PIK3R6,MAP3K8,ELK1,MAP3K3*
PRRs in Recognition of Bacteria and Viruses	4.1	0.277	-3.286	*PIK3R5,TLR8,EIF2S1,NFKB1,IFIH1,PIK3CG,MAPK3,TLR7,OSM,CLEC6A,PRKCA,OAS1,NLRP3,C3,C5AR1,OAS2,PIK3C2A,IL10,MYD88,GRB2,FGFR1,NFKB2,MAPK12,TLR9,OAS3,TLR4,CLEC7A,NOD2,IRF7,GAB1,PTPN11,IL12B,PRKCD,PLCG2,SYK,DDX58,PIK3R6,EIF2AK2*
Inflammasome pathway	3.62	0.5	-3.162	*TLR4,NOD2,NLRP3,MYD88,CTSB,NEK7,NFKB2,CASP8,PANX1,NFKB1*
B Cell Receptor Signaling	6.88	0.297	-3.101	*RAF1,MAP3K15,MAP3K11,KRAS,MAPK13,BCL6,PTEN,IKBKB,IKBKG,PIK3CG,MAPK3,MAP3K14,RPS6KB1,PTPN6,FGFR1,MALT1,NFKB2,MAPK12,TLR9,RAP1A,MAP3K12,GAB1,PTPN11,INPP5F,PLCG2,SYK,PIK3R6,VAV1,PIK3AP1,INPP5K,CAMK2G,MAP2K6,BLNK,Calm1 (includes others),NFKBIE,PIK3R5,IGHG1,MAP3K5,N*

				FKB1,OCRL,JUN,CD22,PPP3CA,CAMK2B, VAV2,MAP2K7,CALML5,PIK3C2A,FCGR2A ,GRB2,BTK,INPP5J,LYN,MEF2C,MAP3K8,E LK1,MAP3K3
Rac Signaling	3.79	0.282	-2.959	*ABI2,RAF1,MAP3K11,ARPC1B,PIK3R5,KR AS,PIP5K1B,IQGAP1,NFKB1,ITGA3,PAK1, ACTR3,JUN,CYFIP2,PIK3CG,MAPK3,CYBB ,ACTR2,RPS6KB1,TIAM1,MAP2K7,PIK3C2 A,GRB2,FGFR1,NFKB2,TLR9,GAB1,PTPN1 1,CYFIP1,PIP5K1C,CD44,PIK3R6,ELK1*
Th1 Pathway	3.22	0.259	-2.921	*MAP2K6,SOCS3,CD40LG,ICAM1,HLA-A,KLRD1,PIK3R5,NCSTN,CD8A,NFKB1,NF IL3,PIK3CG,KLRC1,DLL1,TNFSF11,NOTC H3,PIK3C2A,IL10,GRB2,FGFR1,IL27,STAT 3,TLR9,ITGB2,CD40,GAB1,PTPN11,IL12B, H2-Eb2,ICOS,PIK3R6,HLA-DOB,VAV1,NOTCH1,PSEN1*
NF-κB Signaling	6.63	0.298	-2.885	*RAF1,CSNK2A1,TRAF3,CD40LG,TGFBR1,T GFBR3,TLR8,KRAS,TNFRSF17,IL1R2,TGFB R2,IKBKB,TNIP1,IKBKG,PIK3CG,LTBR,TA B1,MAP3K14,FGFR1,NFKB2,MALT1,TLR9, ARAF,GAB1,CD40,PTPN11,PLCG2,PIK3R6, FGFRL1,MAP2K6,NFKBIE,PIK3R5,TNFAIP 3,EGF,NFKB1,TANK,TLR7,CASP8,TNFRSF 1B,TNFSF11,MAP2K7,PIK3C2A,MYD88,GR B2,IRAK3,IL1R1,TLR4,RIPK1,MAP3K8,BTR C,EIF2AK2,TIRAP,MAP3K3,IRAK4*
Dendritic Cell Maturation	3.65	0.247	-2.832	*CD40LG,ICAM1,PDIA3,HLA-A,LEPR,NFKBIE,PIK3R5,CD83,MAPK13,IG HG1,NFKB1,FCGR1A,PLCD3,IKBKB,CD1D ,IKBKG,IL1RL2,PIK3CG,MAPK3,LTBR,COL 11A2,TNFRSF1B,FCGR3A/FCGR3B,TAB1, MAP3K14,PIK3C2A,IL10,FCGR2A,MYD88, GRB2,FGFR1,IL15,NFKB2,MAPK12,TLR9,T LR4,Cd1d2,CD40,GAB1,PTPN11,IL12B,H2-Eb2,PLCG2,PIK3R6,HLA-DOB,STAT2,IRF8,COL3A1*
Signaling by Rho Family GTPases	3.63	0.234	-2.774	*RAF1,MAP3K11,CDH22,CDH24,GNB5,CD C42EP2,PIP5K1B,PAK1,ITGA3,RHOB,PIK3 CG,EZR,MAPK3,CYBB,GNA13,ACTG2,ACT R2,FGFR1,ARHGEF15,SEPT7,NFKB2,GNA Z,MAPK12,TLR9,PKN1,MAP3K12,RHOQ,C DH5,GAB1,PTPN11,PIP5K1C,CYFIP1,PIK3 R6,ARHGEF18,ARHGEF9,GNAL,ARPC1B,S*

				EPT3,PIK3R5,CDH23,SEPT11,GNA14,NFKB1,IQGAP1,GNG7,CDH11,ACTR3,GNG11,JUN,PPP1R12A,ARHGEF3,MAP2K7,PIK3C2A,SEPT4,GRB2,MYLPF,SEPT1,ELK1,MSN
HGF Signaling	5.6	0.322	-2.667	*CDKN2A,MAP3K15,RAF1,RAPGEF1,MAP3K11,PIK3R5,KRAS,MAP3K5,CCND1,PAK1,ITGA3,JUN,HGF,PIK3CG,MAPK3,PRKCA,MAP3K14,MAP2K7,PIK3C2A,GRB2,CRKL,FGFR1,STAT3,MAPK12,TLR9,RAP1A,ELF1,MAP3K12,GAB1,PTPN11,PRKCD,PLCG2,CDKN1A,PIK3R6,MAP3K8,ELK1,MAP3K3*
Actin Cytoskeleton Signaling	2.14	0.211	-2.655	*ABI2,RAF1,FGD3,ARPC1B,PDGFA,PIK3R5,EGF,KRAS,CRK,PIP5K1B,IQGAP1,BCAR1,FGF13,PAK1,ITGA3,ACTR3,CYFIP2,PPP1R12A,FLNA,PIK3CG,EZR,MAPK3,GNA13,ACTG2,MATK,VAV2,ACTR2,TIAM1,PIK3C2A,GRB2,MYLPF,CRKL,FGFR1,ACTN2,GSN,TLR9,TTN,RAC3,GIT1,TIAM2,GAB1,PTPN11,CYFIP1,PIP5K1C,PIK3R6,VAV1,ACTN4,MSN*
PDGF Signaling	4.11	0.311	-2.646	*RAF1,CSNK2A1,PDGFA,PIK3R5,CRK,KRAS,OCRL,MYC,JUN,MAPK3,PIK3CG,SPHK1,PRKCA,PIK3C2A,GRB2,FGFR1,CRKL,STAT3,TLR9,GAB1,PTPN11,INPP5J,INPP5F,PLCG2,PIK3R6,EIF2AK2,INPP5K,ELK1*
CXCR4 Signaling	1.33	0.2	-2.646	*RAF1,GNB5,PIK3R5,CRK,KRAS,GNA14,BCAR1,GNG7,PAK1,GNG11,JUN,RHOB,PIK3CG,MAPK3,GNA13,PRKCA,PIK3C2A,GRB2,MYLPF,FGFR1,ADCY6,GNAZ,TLR9,MAPK12,RHOQ,GAB1,PTPN11,PRKCD,LYN,PIK3R6,ELK1,ELMO2,GNAL*
Macropinocytosis Signaling	4.11	0.321	-2.524	*PDGFA,PIK3R5,EGF,KRAS,ITGB8,ITGB3,PAK1,RAB5A,HGF,PIK3CG,PRKCA,PIK3C2A,GRB2,FGFR1,TLR9,RAB34,CSF1R,ITGB2,ABI1,PTPN11,GAB1,CSF1,PLCG2,PRKCD,PIK3R6,ACTN4*
p70S6K Signaling	1.71	0.22	-2.502	*RAF1,YWHAH,PDIA3,PIK3R5,KRAS,PLCD3,MAPK3,PIK3CG,PPM1J,EEF2K,PRKCA,RPS6KB1,IL4R,YWHAG,PIK3C2A,GRB2,PPP2R5D,FGFR1,TLR9,BTK,PTPN11,GAB1,MAPT,SYK,PLCG2,PRKCD,LYN,PIK3R6,AGTR1*
FcγRIIB Signaling in B	3.03	0.291	-2.5	*BLNK,CACNB1,CACNA2D2,PIK3C2A,GRB2,FGFR1,CACNA1H,PIK3R5,CACNA1C,KRA*

Lymphocytes				*S,MAPK12,TLR9,CACNA1A,CACNA1F,BTK, PTPN11,GAB1,SYK,PIK3CG,PLCG2,LYN,PI K3R6,CACNG8*
Granzyme B Signaling	1.64	0.375	-2.449	*CASP9,APAF1,BID,CASP8,LMNB1,PARP1*
fMLP Signaling in Neutrophils	3.02	0.26	-2.414	*RAF1,Calm1 (includes others),ARPC1B,NFKBIE,GNB5,PIK3R5,KR AS,NFKB1,GNG7,IKBKG,ACTR3,GNG11,PI K3CG,MAPK3,CYBB,PPP3CA,PRKCA,ACT R2,CALML5,PIK3C2A,GRB2,FGFR1,FPR2, NFKB2,TLR9,FPR1,NCF1,GAB1,PTPN11,P RKCD,PIK3R6,ELK1*
Toll-like Receptor Signaling	3.29	0.303	-2.357	*MAP2K6,MAP3K14,TICAM2,MYD88,TLR8, TNFAIP3,MAPK13,NFKB2,IRAK3,NFKB1,M APK12,TLR9,TLR4,IKBKB,IKBKG,JUN,IL12 B,TLR7,EIF2AK2,TIRAP,ELK1,TAB1,IRAK4*
Thrombopoietin Signaling	2.24	0.277	-2.357	*RAF1,PIK3C2A,GRB2,FGFR1,PIK3R5,KRA S,STAT3,TLR9,MYC,JUN,PTPN11,GAB1,MA PK3,PRKCD,PLCG2,PIK3CG,PIK3R6,PRK CA*
Glioma Invasiveness Signaling	1.89	0.257	-2.357	*TIMP3,PIK3C2A,GRB2,FGFR1,HMMR,PIK 3R5,KRAS,TLR9,ITGB3,RHOQ,RHOB,PTPN 11,GAB1,MAPK3,PIK3CG,PIK3R6,ITGAV,C D44*
Renal Cell Carcinoma Signaling	1.73	0.241	-2.357	*RAPGEF1,RAF1,PIK3C2A,GRB2,FGFR1,PI K3R5,CRK,KRAS,HIF1A,TLR9,RAP1A,PAK1 ,JUN,PTPN11,GAB1,CUL2,MAPK3,HGF,PI K3CG,PIK3R6*
14-3-3-mediated Signaling	2.27	0.237	-2.353	*RAF1,YWHAH,PDIA3,TP73,PIK3R5,KRAS, MAP3K5,PLCD3,JUN,MAPK3,PIK3CG,TUB B4A,PRKCA,TUBB1,TUBB3,YWHAG,PIK3C 2A,GRB2,FGFR1,TUBA4A,TLR9,MAPK12,G AB1,TUBB6,PTPN11,PRKCD,MAPT,PLCG2 ,PIK3R6,CDKN1B,ELK1*
PEDF Signaling	2.78	0.276	-2.294	*RAF1,PIK3C2A,GRB2,NFKBIE,FGFR1,SER PINF1,PIK3R5,KRAS,MAPK13,NFKB2,NFK B1,MAPK12,TLR9,HNF1B,IKBKB,IKBKG,P TPN11,GAB1,MAPK3,PIK3CG,PIK3R6,CFL AR,CASP8,ELK1*
GP6 Signaling Pathway	4.33	0.284	-2.271	*FYN,Calm1 (includes others),COL4A5,PIK3R5,COL4A2,Col17a1,C ol6a4,ITGB3,COL5A1,LAMC1,PIK3CG,LAM A1,COL11A2,KLF12,COL27A1,PRKCA,COL 5A2,CALML5,COL4A1,PIK3C2A,GRB2,FGF R1,LAMC3,COL20A1,TLR9,BTK,COL13A1, GAB1,PTPN11,PRKCD,PLCG2,SYK,LYN,PI*

				K3R6,ADAM10,COL4A4,COL7A1,COL3A1
Insulin Receptor Signaling	2.57	0.241	-2.263	*RAF1,SOCS3,FYN,RAPGEF1,SGK1,PIK3R5,CRK,KRAS,STXBP4,OCRL,PRKAG1,PTEN,SCNN1A,PPP1R12A,PIK3CG,MAPK3,PTPN1,RPS6KB1,PIK3C2A,GRB2,CRKL,FGFR1,ACLY,TLR9,GRB10,RHOQ,PRKAR2B,GAB1,PTPN11,INPP5J,INPP5F,SH2B2,PIK3R6,INPP5K*
Lymphotoxin β Receptor Signaling	3.28	0.313	-2.236	*MAP3K14,TRAF3,PIK3C2A,GRB2,FGFR1,APAF1,PIK3R5,NFKB2,NFKB1,TLR9,DIABLO,IKBKB,IKBKG,CASP9,PTPN11,GAB1,MAPK3,PIK3CG,PIK3R6,LTBR,BIRC2*
Activation of IRF by Cytosolic Pattern Recognition Receptors	3.23	0.317	-2.236	*TRAF3,IL10,NFKBIE,ZBP1,IRF9,NFKB2,ADAR,NFKB1,MAPK12,ISG15,IFIH1,TANK,IKBKB,IRF7,IKBKG,RIPK1,JUN,CD40,DDX58,STAT2*
GM-CSF Signaling	2.74	0.288	-2.236	*RAF1,PIK3C2A,GRB2,FGFR1,PIK3R5,KRAS,STAT3,TLR9,CCND1,CSF2RB,PTPN11,GAB1,MAPK3,PIK3CG,LYN,HCK,PIK3R6,ELK1,PPP3CA,CAMK2G,CAMK2B*
Prolactin Signaling	2.04	0.253	-2.236	*FYN,SOCS3,RAF1,PIK3C2A,GRB2,FGFR1,PIK3R5,KRAS,CEBPB,STAT3,TLR9,MYC,JUN,PTPN11,GAB1,MAPK3,PRKCD,PLCG2,PIK3CG,PIK3R6,PRKCA*
IL-3 Signaling	3.49	0.301	-2.2	*RAPGEF1,RAF1,PIK3R5,KRAS,PAK1,JUN,MAPK3,PIK3CG,PPP3CA,PRKCA,STAT6,PTPN6,PIK3C2A,IL3RA,GRB2,FGFR1,CRKL,STAT3,TLR9,CSF2RB,PTPN11,GAB1,PRKCD,PIK3R6,ELK1*
RANK Signaling in Osteoclasts	6.56	0.353	-2.197	*MAP2K6,MAP3K15,RAF1,Calm1 (includes others),MAP3K11,NFKBIE,PIK3R5,MAPK13,MAP3K5,NFKB1,IKBKB,IKBKG,JUN,PIK3CG,MAPK3,PPP3CA,MAP3K14,CALML5,MAP2K7,TNFSF11,PIK3C2A,GRB2,FGFR1,NFKB2,GSN,TLR9,MAPK12,XIAP,MAP3K12,GAB1,PTPN11,PIK3R6,MAP3K8,ELK1,MAP3K3,BIRC2*
Thrombin Signaling	1.95	0.211	-2.197	*RAF1,GATA1,PDIA3,GNB5,PIK3R5,EGF,KRAS,MAPK13,GNA14,NFKB1,GNG7,PLCD3,IKBKB,GNG11,RHOB,PPP1R12A,PIK3CG,MAPK3,GNA13,ARHGEF3,PRKCA,CAMK2B,*

				RPS6KB1,PIK3C2A,GRB2,MYLPF,ARHGEF15,FGFR1,ADCY6,NFKB2,GNAZ,MAPK12,TLR9,RHOQ,GAB1,PTPN11,PRKCD,PLCG2,PIK3R6,ELK1,ARHGEF9,GNAL,CAMK2G
Th2 Pathway	2.93	0.247	-2.191	*SOCS3,TNFSF4,ICAM1,TNFRSF4,TGFBR1,HLA-A,TGFBR3,PIK3R5,NCSTN,NFKB1,TGFBR2,JUN,PIK3CG,CCR8,STAT6,IL4R,DLL1,NOTCH3,PIK3C2A,IL10,GRB2,FGFR1,ACVR1,TLR9,ITGB2,CD40,GAB1,PTPN11,IL12B,H2-Eb2,ICOS,PIK3R6,CXCR6,HLA-DOB,VAV1,NOTCH1,PSEN1*
ErbB4 Signaling	2.09	0.264	-2.183	*RAF1,ADAM17,NRG2,PIK3C2A,GRB2,FGFR1,PIK3R5,NCSTN,KRAS,TLR9,PTPN11,GAB1,MAPK3,PRKCD,PLCG2,PIK3CG,PIK3R6,PSEN1,PRKCA*
CNTF Signaling	1.96	0.266	-2.183	*RPS6KB1,RAF1,PIK3C2A,GRB2,FGFR1,PIK3R5,KRAS,STAT3,TLR9,LIFR,RPS6KA6,PTPN11,GAB1,MAPK3,PIK3CG,PIK3R6,RPS6KA2*
Estrogen-Dependent Breast Cancer Signaling	1.61	0.238	-2.183	*PIK3C2A,GRB2,FGFR1,PIK3R5,KRAS,NFKB2,NFKB1,TLR9,CCND1,Akr1b7,JUN,PTPN11,GAB1,MAPK3,PIK3CG,PIK3R6,ELK1,ESR1,HSD17B14*
iNOS Signaling	3.3	0.356	-2.138	*CALML5,Calm1 (includes others),MYD88,NFKBIE,MAPK13,IRAK3,NFKB2,NFKB1,MAPK12,IKBKB,TLR4,IKBKG,JUN,NOS2,TAB1,IRAK4*
IL-2 Signaling	1.62	0.25	-2.138	*RAF1,CSNK2A1,PIK3C2A,GRB2,FGFR1,PIK3R5,KRAS,TLR9,JUN,PTPN11,GAB1,MAPK3,PIK3CG,SYK,PIK3R6,ELK1*
Notch Signaling	1.32	0.263	-2.121	*FURIN,DLL1,ADAM17,NOTCH3,DTX3,NCSTN,HES1,NOTCH1,PSEN1,RFNG*
Pancreatic Adenocarcinoma Signaling	5.55	0.317	-2.117	*CDKN2A,RAF1,TGFBR1,PA2G4,PIK3R5,EGF,VEGFB,KRAS,E2F3,NFKB1,CDKN2B,CCND1,RAD51,TGFBR2,CASP9,PIK3CG,MAPK3,RALGDS,E2F2,TFDP1,PIK3C2A,GRB2,FGFR1,VEGFC,MDM2,NFKB2,STAT3,TLR9,MAPK12,PLD4,PTPN11,GAB1,E2F7,CDKN1A,PIK3R6,CDKN1B,ELK1,NOTCH1*
LPS-stimulated MAPK Signaling	4.4	0.322	-2.117	*MAP2K6,RAF1,NFKBIE,PIK3R5,KRAS,MAPK13,MAP3K5,NFKB1,IKBKB,IKBKG,PAK1,JUN,MAPK3,PIK3CG,PRKCA,MAP3K14,PIK3C2A,GRB2,FGFR1,NFKB2,TLR9,MAPK12,TLR4,PTPN11,GAB1,PRKCD,PIK3R6,ELK*

				1
Type II Diabetes Mellitus Signaling	3.3	0.253	-2.117	*SOCS3,CACNA2D2,PRKAB2,NFKBIE,CACNA1H,PIK3R5,MAP3K5,NFKB1,PRKAG1,CACNA1F,IKBKB,IKBKG,ACSBG1,PIK3CG,MAPK3,PRKAA2,CACNG8,TNFRSF1B,SMPD3,PRKCA,MAP3K14,CACNB1,MAP2K7,PIK3C2A,GRB2,FGFR1,CACNA1C,CEBPB,NFKB2,TLR9,MAPK12,CACNA1A,GAB1,PTPN11,PRKCD,SH2B2,PIK3R6,SLC27A6,SLC27A1*
Gα12/13 Signaling	3.22	0.259	-2.058	*RAF1,NFKBIE,CDH22,TBXA2R,CDH24,PIK3R5,CDH23,KRAS,MAP3K5,NFKB1,CDH11,IKBKB,IKBKG,JUN,PIK3CG,MAPK3,GNA13,VAV2,MAP2K7,PIK3C2A,GRB2,MYLPF,FGFR1,NFKB2,TLR9,MAPK12,BTK,LPAR6,CDH5,GAB1,PTPN11,PIK3R6,MEF2C,VAV1,ELK1*
SAPK/JNK Signaling	3.69	0.288	-2.043	*MAP3K11,PIK3R5,CRK,KRAS,MAP3K5,GNG7,GNG11,JUN,GADD45A,PIK3CG,GNA13,TAB1,MAP2K7,PIK3C2A,GRB2,FGFR1,CRKL,MAPK12,TLR9,MAPK8IP1,RAC3,DAXX,MAP3K12,RIPK1,GAB1,PTPN11,PIK3R6,DUSP4,ELK1,MAP3K3*
PI3K/AKT Signaling	2.59	0.248	-2.043	*RAF1,YWHAH,NFKBIE,KRAS,MAP3K5,NFKB1,CCND1,OCRL,PTEN,IKBKB,IKBKG,ITGA3,HSP90B1,PPM1J,MAPK3,PIK3CG,RPS6KB1,YWHAG,GRB2,PPP2R5D,MDM2,NFKB2,MAPK8IP1,GAB1,INPP5J,INPP5F,LIMS1,CDKN1A,MAP3K8,CDKN1B,INPP5K*
JAK/Stat Signaling	3.09	0.289	-2.041	*STAT6,SOCS3,RAF1,PTPN6,PIK3C2A,GRB2,FGFR1,PIK3R5,KRAS,CEBPB,STAT3,NFKB2,NFKB1,TLR9,PIAS3,JUN,PTPN11,GAB1,MAPK3,PIK3CG,CDKN1A,PTPN1,PIK3R6,STAT2*

Supplementary Data 5
Go Biological Pathway Terms by p value for the transcripts increased by HDAC6 inhibitor treatment

GO Term	**Term**	**pvalue**	**Annotated**	**Significant**	**Expected**	**pvalue**	**Neg Log (p-value)**	**Term**	**GOtype**	**responsons**

										e
GO:0042384	cilium assembly	3.25E-07	153	35	12.5	3.25E-07	6.49E+00	cilium assembly	BP	UP
GO:0055114	oxidation-reduction process	3.53E-06	946	117	77.27	3.53E-06	5.45E+00	oxidation-reduction process	BP	UP
GO:0006750	glutathione biosynthetic process	2.65E-05	13	7	1.06	2.65E-05	4.58E+00	glutathione biosynthetic process	BP	UP
GO:0006749	glutathione metabolic process	3.62E-05	46	18	3.76	3.62E-05	4.44E+00	glutathione metabolic process	BP	UP
GO:0046686	response to cadmium ion	9.23E-05	36	11	2.94	9.23E-05	4.03E+00	response to cadmium ion	BP	UP
GO:0043113	receptor clustering	0.000151492	44	12	3.59	0.000151492	3.82E+00	receptor clustering	BP	UP
GO:0061512	protein localization to cilium	0.000219761	22	8	1.8	0.000219761	3.66E+00	protein localization to cilium	BP	UP
GO:0006782	protoporphyrinogen IX biosynthetic process	0.000344241	9	5	0.74	0.000344241	3.46E+00	protoporphyrinogen IX biosynthetic process	BP	UP
GO:0006552	leucine catabolic process	0.00054405	3	3	0.25	0.00054405	3.26E+00	leucine catabolic process	BP	UP
GO:0006068	ethanol catabolic process	0.000581648	6	4	0.49	0.000581648	3.24E+00	ethanol catabolic process	BP	UP

GO:0006534	cysteine metabolic process	0.000642137	10	5	0.82	0.000642137	3.19E+00	cysteine metabolic process	BP	UP
GO:0035721	intraciliary retrograde transport	0.000642137	10	5	0.82	0.000642137	3.19E+00	intraciliary retrograde transport	BP	UP
GO:0044281	small molecule metabolic process	0.001175761	164	195	134.28	0.001175761	2.93E+00	small molecule metabolic process	BP	UP
GO:0035735	intraciliary transport involved in cilium morphogenesis	0.001269198	7	4	0.57	0.001269198	2.90E+00	intraciliary transport involved in cilium morphogenesis	BP	UP
GO:0006783	heme biosynthetic process	0.001736561	21	10	1.72	0.001736561	2.76E+00	heme biosynthetic process	BP	UP
GO:2000649	regulation of sodium ion transmembrane transporter activity	0.001966232	36	9	2.94	0.001966232	2.71E+00	regulation of sodium ion transmembrane transporter activity	BP	UP
GO:0006083	acetate metabolic process	0.002043103	4	3	0.33	0.002043103	2.69E+00	acetate metabolic process	BP	UP
GO:0018916	nitrobenzene metabol	0.002043103	4	3	0.33	0.002043103	2.69E+00	nitrobenzene metabolic process	BP	UP

	ic process									
GO:0045724	positive regulation of cilium assembly	0.002043103	4	3	0.33	0.002043103	2.69E+00	positive regulation of cilium assembly	BP	UP
GO:0006677	glycosylceramide metabolic process	0.002663323	13	5	1.06	0.002663323	2.57E+00	glycosylceramide metabolic process	BP	UP
GO:0042219	cellular modified amino acid catabolic process	0.002663323	13	5	1.06	0.002663323	2.57E+00	cellular modified amino acid catabolic process	BP	UP
GO:0046689	response to mercury ion	0.002663323	13	5	1.06	0.002663323	2.57E+00	response to mercury ion	BP	UP
GO:0043001	Golgi to plasma membrane protein transport	0.003465353	32	8	2.61	0.003465353	2.46E+00	Golgi to plasma membrane protein transport	BP	UP
GO:1901017	negative regulation of potassium ion transmembrane transporter activity	0.003866549	14	5	1.14	0.003866549	2.41E+00	negative regulation of potassium ion transmembrane transporter activity	BP	UP
GO:	dicarbo	0.0044	7	14	6.45	0.004489	2.35E+00	dicarboxylic	B	U

0043648	xylic acid metabolic process	89029	9			029		acid metabolic process	P	P
GO:0060632	regulation of microtubule-based movement	0.004770823	15	7	1.23	0.004770823	2.32E+00	regulation of microtubule-based movement	BP	UP
GO:0048069	eye pigmentation	0.004796711	5	3	0.41	0.004796711	2.32E+00	eye pigmentation	BP	UP
GO:0071918	urea transmembrane transport	0.004796711	5	3	0.41	0.004796711	2.32E+00	urea transmembrane transport	BP	UP
GO:0046688	response to copper ion	0.004952292	27	7	2.21	0.004952292	2.31E+00	response to copper ion	BP	UP
GO:0006833	water transport	0.005413787	15	5	1.23	0.005413787	2.27E+00	water transport	BP	UP
GO:0006979	response to oxidative stress	0.005470968	353	43	28.83	0.005470968	2.26E+00	response to oxidative stress	BP	UP
GO:0050808	synapse organization	0.006053111	189	26	15.44	0.006053111	2.22E+00	synapse organization	BP	UP
GO:0051260	protein homooligomerization	0.006310122	247	32	20.17	0.006310122	2.20E+00	protein homooligomerization	BP	UP
GO:0002223	stimulatory C-type lectin receptor signalin	0.006667992	2	2	0.16	0.006667992	2.18E+00	stimulatory C-type lectin receptor signaling pathway	BP	UP

	g pathway									
GO:0007181	transforming growth factor beta receptor complex assembly	0.006667992	2	2	0.16	0.006667992	2.18E+00	transforming growth factor beta receptor complex assembly	BP	UP
GO:0018283	iron incorporation into metallo-sulfur cluster	0.006667992	2	2	0.16	0.006667992	2.18E+00	iron incorporation into metallo-sulfur cluster	BP	UP
GO:0038162	erythropoietin-mediated signaling pathway	0.006667992	2	2	0.16	0.006667992	2.18E+00	erythropoietin-mediated signaling pathway	BP	UP
GO:0046502	uroporphyrinogen III metabolic process	0.006667992	2	2	0.16	0.006667992	2.18E+00	uroporphyrinogen III metabolic process	BP	UP
GO:0050747	positive regulation of lipoprotein metabolic process	0.006667992	2	2	0.16	0.006667992	2.18E+00	positive regulation of lipoprotein metabolic process	BP	UP
GO:0071284	cellular response to	0.006667992	2	2	0.16	0.006667992	2.18E+00	cellular response to lead ion	BP	UP

	lead ion									
GO:1902855	regulation of nonmotile primary cilium assembly	0.006667992	2	2	0.16	0.006667992	2.18E+00	regulation of nonmotile primary cilium assembly	BP	UP
GO:0042073	intraciliary transport	0.006874301	37	14	3.02	0.006874301	2.16E+00	intraciliary transport	BP	UP
GO:0050771	negative regulation of axonogenesis	0.007449195	36	8	2.94	0.007449195	2.13E+00	negative regulation of axonogenesis	BP	UP
GO:0001501	skeletal system development	0.008300412	454	52	37.08	0.008300412	2.08E+00	skeletal system development	BP	UP
GO:0032482	Rab protein signal transduction	0.008636413	528	59	43.13	0.008636413	2.06E+00	Rab protein signal transduction	BP	UP
GO:0006691	leukotriene metabolic process	0.008839224	23	6	1.88	0.008839224	2.05E+00	leukotriene metabolic process	BP	UP
GO:0035162	embryonic hemopoiesis	0.008839224	23	6	1.88	0.008839224	2.05E+00	embryonic hemopoiesis	BP	UP
GO:0042373	vitamin K metabolic process	0.009011774	6	3	0.49	0.009011774	2.05E+00	vitamin K metabolic process	BP	UP
GO:0045213	neurotransmitter recepto	0.009011774	6	3	0.49	0.009011774	2.05E+00	neurotransmitter receptor metabolic	BP	UP

	r metabolic process							process		
GO:0018342	protein prenylation	0.009171769	11	4	0.9	0.009171769	2.04E+00	protein prenylation	BP	UP
GO:0042178	xenobiotic catabolic process	0.009171769	11	4	0.9	0.009171769	2.04E+00	xenobiotic catabolic process	BP	UP
GO:0071243	cellular response to arsenic-containing substance	0.009171769	11	4	0.9	0.009171769	2.04E+00	cellular response to arsenic-containing substance	BP	UP

Supplementary Date 6
Go Biological Pathway Terms by P value for transcripts decreased by HDAC6 inhibitor treatment

GO Term	Annotated	Significant	Expected	pvalue	Neg Log (p-value)	Term	GOtype	response
GO:0042832	24	12	1.91	6.86E-08	7.16E+00	defense response to protozoan	BP	DOWN
GO:0050853	38	17	3.03	7.40E-08	7.13E+00	B cell receptor signaling pathway	BP	DOWN
GO:0045087	471	99	37.52	9.05E-08	7.04E+00	innate immune response	BP	DOWN
GO:0006954	502	100	39.99	1.18E-07	6.93E+00	inflammatory response	BP	DOWN
GO:0071222	142	35	11.31	3.88E-07	6.41E+00	cellular response to lipopolysaccharide	BP	DO

								W N
GO:0051301	568	86	45.24	5.65E-07	6.25E+00	cell division	BP	D O W N
GO:0043123	159	32	12.66	8.91E-07	6.05E+00	positive regulation of I-kappaB kinase/NF-kappaB signaling	BP	D O W N
GO:0034976	128	40	10.2	1.09E-06	5.96E+00	response to endoplasmic reticulum stress	BP	D O W N
GO:0030968	51	16	4.06	1.20E-06	5.92E+00	endoplasmic reticulum unfolded protein response	BP	D O W N
GO:0043547	424	62	33.77	2.29E-06	5.64E+00	positive regulation of GTPase activity	BP	D O W N
GO:0051092	100	23	7.97	2.85E-06	5.55E+00	positive regulation of NF-kappaB transcription factor activity	BP	D O W N
GO:0006909	139	34	11.07	4.54E-06	5.34E+00	phagocytosis	BP	D O W N
GO:0008360	119	25	9.48	6.11E-06	5.21E+00	regulation of cell shape	BP	D O W N
GO:0006886	780	115	62.13	6.62E-06	5.18E+00	intracellular protein transport	BP	D O W N
GO:0061028	29	11	2.31	7.12E-06	5.15E+00	establishment of endothelial barrier	BP	D O W N
GO:0032496	287	63	22.86	7.31E-06	5.14E+00	response to lipopolysaccharide	BP	D O W N

GO:0050830	58	16	4.62	7.81E-06	5.11E+00	defense response to Gram-positive bacterium	BP	DOWN
GO:0007067	358	59	28.52	9.11E-06	5.04E+00	mitotic nuclear division	BP	DOWN
GO:0007568	261	42	20.79	9.39E-06	5.03E+00	aging	BP	DOWN
GO:0035335	93	21	7.41	1.03E-05	4.99E+00	peptidyl-tyrosine dephosphorylation	BP	DOWN
GO:0006898	203	35	16.17	1.12E-05	4.95E+00	receptor-mediated endocytosis	BP	DOWN
GO:0002755	16	8	1.27	1.15E-05	4.94E+00	MyD88-dependent toll-like receptor signaling pathway	BP	DOWN
GO:0007179	135	29	10.75	2.01E-05	4.70E+00	transforming growth factor beta receptor signaling pathway	BP	DOWN
GO:0071260	69	17	5.5	2.11E-05	4.67E+00	cellular response to mechanical stimulus	BP	DOWN
GO:0018279	13	7	1.04	2.24E-05	4.65E+00	protein N-linked glycosylation via asparagine	BP	DOWN
GO:0034097	575	89	45.8	2.54E-05	4.60E+00	response to cytokine	BP	DOWN
GO:0006888	70	17	5.58	2.59E-05	4.59E+00	ER to Golgi vesicle-mediated transport	BP	DOWN
GO:0032733	23	9	1.83	3.69E-05	4.43E+00	positive regulation of interleukin-10	BP	DO

						production		W N
GO:003 0433	46	13	3.66	4.16E-05	4.38E+00	ER-associated ubiquitin-dependent protein catabolic process	BP	D O W N
GO:004 8008	46	13	3.66	4.16E-05	4.38E+00	platelet-derived growth factor receptor signaling pathway	BP	D O W N
GO:003 2695	14	7	1.12	4.18E-05	4.38E+00	negative regulation of interleukin-12 production	BP	D O W N
GO:003 5556	2309	318	183.92	5.35E-05	4.27E+00	intracellular signal transduction	BP	D O W N
GO:000 2532	35	11	2.79	5.50E-05	4.26E+00	production of molecular mediator involved in inflammatory response	BP	D O W N
GO:004 3407	62	20	4.94	6.99E-05	4.16E+00	negative regulation of MAP kinase activity	BP	D O W N
GO:003 4122	22	10	1.75	8.33E-05	4.08E+00	negative regulation of toll-like receptor signaling pathway	BP	D O W N
GO:000 0188	20	8	1.59	8.40E-05	4.08E+00	inactivation of MAPK activity	BP	D O W N
GO:003 0890	37	11	2.95	9.71E-05	4.01E+00	positive regulation of B cell proliferation	BP	D O W N
GO:005 1241	900	125	71.69	0.0001010 1	4.00E+00	NA	BP	D O W N
GO:000 9967	1128	163	89.85	0.0001069 4	3.97E+00	positive regulation of signal transduction	BP	D O W N

GO:0032956	253	38	20.15	0.0001123	3.95E+00	regulation of actin cytoskeleton organization	BP	DOWN
GO:0008285	546	79	43.49	0.00011325	3.95E+00	negative regulation of cell proliferation	BP	DOWN
GO:0051607	174	29	13.86	0.00011444	3.94E+00	defense response to virus	BP	DOWN
GO:0043406	169	32	13.46	0.00011589	3.94E+00	positive regulation of MAP kinase activity	BP	DOWN
GO:0001525	388	60	30.91	0.00012039	3.92E+00	angiogenesis	BP	DOWN
GO:0050728	92	22	7.33	0.00014073	3.85E+00	negative regulation of inflammatory response	BP	DOWN
GO:0000186	45	12	3.58	0.00015097	3.82E+00	activation of MAPKK activity	BP	DOWN
GO:0045078	12	6	0.96	0.00015357	3.81E+00	positive regulation of interferon-gamma biosynthetic process	BP	DOWN
GO:0050777	103	20	8.2	0.00015929	3.80E+00	negative regulation of immune response	BP	DOWN
GO:0045944	952	107	75.83	0.0001688	3.77E+00	positive regulation of transcription from RNA polymerase II promoter	BP	DOWN
GO:0002677	5	4	0.4	0.00018784	3.73E+00	negative regulation of chronic inflammatory response	BP	DOWN
GO:0050732	36	13	2.87	0.00018793	3.73E+00	negative regulation of peptidyl-tyrosine	BP	DO

						phosphorylation		W N
GO:0030889	17	7	1.35	0.00019117	3.72E+00	negative regulation of B cell proliferation	BP	D O W N
GO:0006919	74	16	5.89	0.00019471	3.71E+00	activation of cysteine-type endopeptidase activity involved in apoptotic process	BP	D O W N
GO:0046777	209	32	16.65	0.00026487	3.58E+00	protein autophosphorylation	BP	D O W N
GO:0016446	13	6	1.04	0.00026598	3.58E+00	somatic hypermutation of immunoglobulin genes	BP	D O W N
GO:0001782	33	12	2.63	0.00028817	3.54E+00	B cell homeostasis	BP	D O W N
GO:0032727	13	8	1.04	0.00030339	3.52E+00	positive regulation of interferon-alpha production	BP	D O W N
GO:0002634	9	5	0.72	0.00030573	3.51E+00	regulation of germinal center formation	BP	D O W N
GO:1901701	735	122	58.54	0.00033021	3.48E+00	NA	BP	D O W N
GO:0010506	230	34	18.32	0.00033947	3.47E+00	regulation of autophagy	BP	D O W N
GO:0032088	63	14	5.02	0.00035625	3.45E+00	negative regulation of NF-kappaB transcription factor activity	BP	D O W N
GO:0007229	84	19	6.69	0.00036158	3.44E+00	integrin-mediated signaling pathway	BP	D O W N

GO:2000377	134	23	10.67	0.00036273	3.44E+00	regulation of reactive oxygen species metabolic process	BP	DOWN
GO:0001783	24	8	1.91	0.0003675	3.43E+00	B cell apoptotic process	BP	DOWN
GO:0007030	79	16	6.29	0.00042764	3.37E+00	Golgi organization	BP	DOWN
GO:0010212	140	27	11.15	0.00043262	3.36E+00	response to ionizing radiation	BP	DOWN
GO:0046325	14	6	1.12	0.00043413	3.36E+00	negative regulation of glucose import	BP	DOWN
GO:0031396	155	30	12.35	0.00045383	3.34E+00	regulation of protein ubiquitination	BP	DOWN
GO:0045071	37	10	2.95	0.00047276	3.33E+00	negative regulation of viral genome replication	BP	DOWN
GO:0001788	3	3	0.24	0.00050451	3.30E+00	antibody-dependent cellular cytotoxicity	BP	DOWN
GO:0010835	3	3	0.24	0.00050451	3.30E+00	regulation of protein ADP-ribosylation	BP	DOWN
GO:0051088	3	3	0.24	0.00050451	3.30E+00	PMA-inducible membrane protein ectodomain proteolysis	BP	DOWN
GO:0002467	15	9	1.19	0.0005225	3.28E+00	germinal center formation	BP	DOWN
GO:0001798	6	4	0.48	0.00052778	3.28E+00	positive regulation of type IIa hypersensitivity	BP	DO

								W N
GO:0060696	6	4	0.48	0.00052778	3.28E+00	regulation of phospholipid catabolic process	BP	D O W N
GO:0016064	97	25	7.73	0.0005592 1	3.25E+00	immunoglobulin mediated immune response	BP	D O W N
GO:0006977	10	5	0.8	0.0005713 2	3.24E+00	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	BP	D O W N
GO:0033623	10	5	0.8	0.0005713 2	3.24E+00	regulation of integrin activation	BP	D O W N
GO:0048012	10	5	0.8	0.0005713 2	3.24E+00	hepatocyte growth factor receptor signaling pathway	BP	D O W N
GO:0006302	147	24	11.71	0.0005824 3	3.23E+00	double-strand break repair	BP	D O W N
GO:0045766	105	19	8.36	0.0005834 2	3.23E+00	positive regulation of angiogenesis	BP	D O W N
GO:0042093	38	10	3.03	0.0005962 8	3.22E+00	T-helper cell differentiation	BP	D O W N
GO:0006950	2860	427	227.81	0.0006061	3.22E+00	response to stress	BP	D O W N
GO:0010832	15	6	1.19	0.0006749 3	3.17E+00	negative regulation of myotube differentiation	BP	D O W N
GO:0032703	15	6	1.19	0.0006749 3	3.17E+00	negative regulation of interleukin-2 production	BP	D O W

								N
GO:0000281	26	8	2.07	0.00067615	3.17E+00	mitotic cytokinesis	BP	DOWN
GO:0002639	33	9	2.63	0.00084016	3.08E+00	positive regulation of immunoglobulin production	BP	DOWN
GO:0032715	33	9	2.63	0.00084016	3.08E+00	negative regulation of interleukin-6 production	BP	DOWN
GO:0007184	21	7	1.67	0.00086076	3.07E+00	SMAD protein import into nucleus	BP	DOWN
GO:0050871	64	19	5.1	0.00086586	3.06E+00	positive regulation of B cell activation	BP	DOWN
GO:0032735	27	8	2.15	0.00089435	3.05E+00	positive regulation of interleukin-12 production	BP	DOWN
GO:0050856	27	8	2.15	0.00089435	3.05E+00	regulation of T cell receptor signaling pathway	BP	DOWN
GO:0043542	134	22	10.67	0.0008955	3.05E+00	endothelial cell migration	BP	DOWN
GO:0010770	143	23	11.39	0.00092642	3.03E+00	positive regulation of cell morphogenesis involved in differentiation	BP	DOWN
GO:0016310	1852	270	147.52	0.00092869	3.03E+00	phosphorylation	BP	DOWN
GO:0002456	97	22	7.73	0.00096009	3.02E+00	T cell mediated immunity	BP	DOWN
GO:003	21	10	1.67	0.0009674	3.01E+00	mitotic G1 DNA	BP	D

1571				1		damage checkpoint		DOWN
GO:0002313	11	5	0.88	0.0009788	3.01E+00	mature B cell differentiation involved in immune response	BP	DOWN
GO:0006614	11	5	0.88	0.0009788	3.01E+00	SRP-dependent cotranslational protein targeting to membrane	BP	DOWN
GO:0007035	11	5	0.88	0.0009788	3.01E+00	vacuolar acidification	BP	DOWN
GO:0007076	11	5	0.88	0.0009788	3.01E+00	mitotic chromosome condensation	BP	DOWN
GO:0060670	11	5	0.88	0.0009788	3.01E+00	branching involved in labyrinthine layer morphogenesis	BP	DOWN
GO:0048569	16	6	1.27	0.0010074	3.00E+00	post-embryonic organ development	BP	DOWN
GO:0044728	62	13	4.94	0.0010248	2.99E+00	NA	BP	DOWN
GO:0070301	62	13	4.94	0.0010248	2.99E+00	cellular response to hydrogen peroxide	BP	DOWN
GO:0046649	542	105	43.17	0.0010259	2.99E+00	lymphocyte activation	BP	DOWN
GO:0050663	134	26	10.67	0.0011218	2.95E+00	cytokine secretion	BP	DOWN
GO:0043124	48	11	3.82	0.0011385	2.94E+00	negative regulation of I-kappaB kinase/NF-kappaB signaling	BP	DOW

								N
GO:0050766	48	11	3.82	0.00113855	2.94E+00	positive regulation of phagocytosis	BP	DOWN
GO:0031936	7	4	0.56	0.00115364	2.94E+00	negative regulation of chromatin silencing	BP	DOWN
GO:0034162	7	4	0.56	0.00115364	2.94E+00	toll-like receptor 9 signaling pathway	BP	DOWN
GO:0045348	7	4	0.56	0.00115364	2.94E+00	positive regulation of MHC class II biosynthetic process	BP	DOWN
GO:0045359	7	4	0.56	0.00115364	2.94E+00	positive regulation of interferon-beta biosynthetic process	BP	DOWN
GO:0045714	7	4	0.56	0.00115364	2.94E+00	regulation of low-density lipoprotein particle receptor biosynthetic process	BP	DOWN
GO:0033003	35	12	2.79	0.00115971	2.94E+00	regulation of mast cell activation	BP	DOWN
GO:0030334	561	88	44.69	0.00117996	2.93E+00	regulation of cell migration	BP	DOWN
GO:0051347	421	85	33.53	0.00126496	2.90E+00	positive regulation of transferase activity	BP	DOWN
GO:0071897	86	20	6.85	0.00132381	2.88E+00	DNA biosynthetic process	BP	DOWN
GO:0033628	35	9	2.79	0.00133078	2.88E+00	regulation of cell adhesion mediated by integrin	BP	DOWN
GO:190	925	116	73.68	0.0013728	2.86E+00	NA	BP	D

2580				8				O W N
GO:0002708	105	25	8.36	0.0013871	2.86E+00	NA	BP	D O W N
GO:0006281	388	57	30.91	0.00138974	2.86E+00	DNA repair	BP	D O W N
GO:0051983	64	13	5.1	0.0013964	2.85E+00	regulation of chromosome segregation	BP	D O W N
GO:0010564	414	62	32.98	0.00141281	2.85E+00	regulation of cell cycle process	BP	D O W N
GO:0051591	96	17	7.65	0.00142156	2.85E+00	response to cAMP	BP	D O W N
GO:0045910	17	6	1.35	0.00145268	2.84E+00	negative regulation of DNA recombination	BP	D O W N
GO:0060251	17	6	1.35	0.00145268	2.84E+00	NA	BP	D O W N
GO:1902236	17	6	1.35	0.00145268	2.84E+00	negative regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway	BP	D O W N
GO:0031331	186	32	14.82	0.0015012	2.82E+00	NA	BP	D O W N
GO:0001774	12	5	0.96	0.00156824	2.80E+00	microglial cell activation	BP	D O W N
GO:0006266	12	5	0.96	0.00156824	2.80E+00	DNA ligation	BP	D O

								W N
GO:0061299	12	5	0.96	0.00156824	2.80E+00	retina vasculature morphogenesis in camera-type eye	BP	D O W N
GO:0010543	23	7	1.83	0.0015758	2.80E+00	regulation of platelet activation	BP	D O W N
GO:0030335	322	47	25.65	0.00160968	2.79E+00	positive regulation of cell migration	BP	D O W N
GO:0006611	50	11	3.98	0.00162488	2.79E+00	protein export from nucleus	BP	D O W N
GO:0048013	36	9	2.87	0.00165063	2.78E+00	ephrin receptor signaling pathway	BP	D O W N
GO:0065008	2841	321	226.29	0.00166301	2.78E+00	NA	BP	D O W N
GO:0034113	43	10	3.43	0.00167834	2.78E+00	heterotypic cell-cell adhesion	BP	D O W N
GO:0030198	195	28	15.53	0.00169552	2.77E+00	extracellular matrix organization	BP	D O W N
GO:0032269	747	112	59.5	0.0017104	2.77E+00	negative regulation of cellular protein metabolic process	BP	D O W N
GO:0006260	239	38	19.04	0.00171239	2.77E+00	DNA replication	BP	D O W N
GO:0050727	216	47	17.2	0.00173628	2.76E+00	regulation of inflammatory response	BP	D O W N

GO:0001932	939	160	74.79	0.00177501	2.75E+00	regulation of protein phosphorylation	BP	DOWN
GO:0016197	196	28	15.61	0.00183136	2.74E+00	endosomal transport	BP	DOWN
GO:0051640	365	49	29.07	0.00185375	2.73E+00	organelle localization	BP	DOWN
GO:0032648	37	12	2.95	0.0018772	2.73E+00	regulation of interferon-beta production	BP	DOWN
GO:0001922	4	3	0.32	0.00189769	2.72E+00	B-1 B cell homeostasis	BP	DOWN
GO:0010694	4	3	0.32	0.00189769	2.72E+00	positive regulation of alkaline phosphatase activity	BP	DOWN
GO:0034154	4	3	0.32	0.00189769	2.72E+00	toll-like receptor 7 signaling pathway	BP	DOWN
GO:0038094	4	3	0.32	0.00189769	2.72E+00	Fc-gamma receptor signaling pathway	BP	DOWN
GO:0038145	4	3	0.32	0.00189769	2.72E+00	macrophage colony-stimulating factor signaling pathway	BP	DOWN
GO:0045356	4	3	0.32	0.00189769	2.72E+00	positive regulation of interferon-alpha biosynthetic process	BP	DOWN
GO:0045719	4	3	0.32	0.00189769	2.72E+00	negative regulation of glycogen biosynthetic process	BP	DOWN
GO:1900225	4	3	0.32	0.00189769	2.72E+00	regulation of NLRP3 inflammasome complex	BP	DO

						assembly		W N
GO:200 0617	4	3	0.32	0.0018976 9	2.72E+00	positive regulation of histone H3-K9 acetylation	BP	D O W N
GO:000 7155	1252	171	99.72	0.0019004 8	2.72E+00	cell adhesion	BP	D O W N
GO:004 6627	30	8	2.39	0.0019024 1	2.72E+00	negative regulation of insulin receptor signaling pathway	BP	D O W N
GO:006 0706	30	8	2.39	0.0019024 1	2.72E+00	cell differentiation involved in embryonic placenta development	BP	D O W N
GO:005 1129	501	66	39.91	0.0019730 5	2.70E+00	NA	BP	D O W N
GO:000 8347	37	9	2.95	0.0020292 3	2.69E+00	glial cell migration	BP	D O W N
GO:003 2757	37	9	2.95	0.0020292 3	2.69E+00	positive regulation of interleukin-8 production	BP	D O W N
GO:200 0278	37	9	2.95	0.0020292 3	2.69E+00	regulation of DNA biosynthetic process	BP	D O W N
GO:003 4248	18	6	1.43	0.0020333 4	2.69E+00	NA	BP	D O W N
GO:007 1407	353	44	28.12	0.0020511 1	2.69E+00	cellular response to organic cyclic compound	BP	D O W N
GO:005 0731	145	26	11.55	0.0020542 8	2.69E+00	positive regulation of peptidyl-tyrosine phosphorylation	BP	D O W N

GO:0002828	24	7	1.91	0.00207333	2.68E+00	regulation of type 2 immune response	BP	DOWN
GO:0030866	24	7	1.91	0.00207333	2.68E+00	cortical actin cytoskeleton organization	BP	DOWN
GO:0060445	24	7	1.91	0.00207333	2.68E+00	branching involved in salivary gland morphogenesis	BP	DOWN
GO:0031349	209	49	16.65	0.0021490 8	2.67E+00	NA	BP	DOWN
GO:0034138	8	4	0.64	0.0021618 3	2.67E+00	toll-like receptor 3 signaling pathway	BP	DOWN
GO:0043373	8	4	0.64	0.0021618 3	2.67E+00	NA	BP	DOWN
GO:0048102	8	4	0.64	0.0021618 3	2.67E+00	autophagic cell death	BP	DOWN
GO:2000059	8	4	0.64	0.0021618 3	2.67E+00	negative regulation of protein ubiquitination involved in ubiquitin-dependent protein catabolic process	BP	DOWN
GO:0032874	120	23	9.56	0.0022793 4	2.64E+00	positive regulation of stress-activated MAPK cascade	BP	DOWN
GO:0042542	107	23	8.52	0.0023280 3	2.63E+00	response to hydrogen peroxide	BP	DOWN
GO:0001820	13	5	1.04	0.0023821 5	2.62E+00	serotonin secretion	BP	DOWN
GO:004	13	5	1.04	0.0023821	2.62E+00	positive regulation of	BP	D

3306				5		mast cell degranulation		O W N
GO:0051336	1087	146	86.58	0.00243345	2.61E+00	NA	BP	D O W N
GO:0070507	118	19	9.4	0.00244364	2.61E+00	regulation of microtubule cytoskeleton organization	BP	D O W N
GO:0048754	172	25	13.7	0.00246353	2.61E+00	branching morphogenesis of an epithelial tube	BP	D O W N
GO:0060603	38	9	3.03	0.00247394	2.61E+00	mammary gland duct morphogenesis	BP	D O W N
GO:0018107	68	13	5.42	0.00247749	2.61E+00	peptidyl-threonine phosphorylation	BP	D O W N
GO:0033157	297	38	23.66	0.00253342	2.60E+00	regulation of intracellular protein transport	BP	D O W N
GO:0009968	943	135	75.11	0.00260412	2.58E+00	negative regulation of signal transduction	BP	D O W N
GO:0006897	495	91	39.43	0.00267622	2.57E+00	endocytosis	BP	D O W N
GO:0002548	25	7	1.99	0.00268403	2.57E+00	monocyte chemotaxis	BP	D O W N
GO:0045577	25	7	1.99	0.00268403	2.57E+00	regulation of B cell differentiation	BP	D O W N
GO:0045672	25	7	1.99	0.00268403	2.57E+00	positive regulation of osteoclast differentiation	BP	D O W

								N
GO:0042176	326	54	25.97	0.00277021	2.56E+00	regulation of protein catabolic process	BP	DOWN
GO:0044130	19	6	1.51	0.00277346	2.56E+00	negative regulation of growth of symbiont in host	BP	DOWN
GO:0061099	19	6	1.51	0.00277346	2.56E+00	negative regulation of protein tyrosine kinase activity	BP	DOWN
GO:0030330	56	15	4.46	0.00281528	2.55E+00	DNA damage response, signal transduction by p53 class mediator	BP	DOWN
GO:0033036	2273	288	181.05	0.00289214	2.54E+00	NA	BP	DOWN
GO:0070059	56	15	4.46	0.00293585	2.53E+00	intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	BP	DOWN
GO:0008284	779	91	62.05	0.00297031	2.53E+00	positive regulation of cell proliferation	BP	DOWN
GO:0032722	39	9	3.11	0.00299253	2.52E+00	positive regulation of chemokine production	BP	DOWN
GO:0050868	81	18	6.45	0.00303523	2.52E+00	negative regulation of T cell activation	BP	DOWN
GO:0046822	184	26	14.66	0.00303843	2.52E+00	regulation of nucleocytoplasmic transport	BP	DOWN
GO:0030100	180	33	14.34	0.0030981	2.51E+00	regulation of endocytosis	BP	DOWN

GO:0002474	54	11	4.3	0.00310544	2.51E+00	antigen processing and presentation of peptide antigen via MHC class I	BP	DOWN
GO:0045454	62	12	4.94	0.00321669	2.49E+00	cell redox homeostasis	BP	DOWN
GO:0071375	206	33	16.41	0.003219	2.49E+00	cellular response to peptide hormone stimulus	BP	DOWN
GO:0002690	70	13	5.58	0.00323242	2.49E+00	positive regulation of leukocyte chemotaxis	BP	DOWN
GO:0042267	44	12	3.5	0.00342212	2.47E+00	natural killer cell mediated cytotoxicity	BP	DOWN
GO:0033198	26	7	2.07	0.00342378	2.47E+00	response to ATP	BP	DOWN
GO:0002685	127	26	10.12	0.00345444	2.46E+00	regulation of leukocyte migration	BP	DOWN
GO:0034123	14	5	1.12	0.00346439	2.46E+00	positive regulation of toll-like receptor signaling pathway	BP	DOWN
GO:0070234	14	5	1.12	0.00346439	2.46E+00	positive regulation of T cell apoptotic process	BP	DOWN
GO:0071800	14	5	1.12	0.00346439	2.46E+00	podosome assembly	BP	DOWN
GO:0042771	40	9	3.19	0.00359317	2.44E+00	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	BP	DOWN
GO:004	3216	381	256.16	0.0035989	2.44E+00	NA	BP	D

4765				4				O W N
GO:0032436	55	11	4.38	0.00360791	2.44E+00	positive regulation of proteasomal ubiquitin-dependent protein catabolic process	BP	D O W N
GO:0032760	55	11	4.38	0.00360791	2.44E+00	positive regulation of tumor necrosis factor production	BP	D O W N
GO:0032147	201	38	16.01	0.00361756	2.44E+00	activation of protein kinase activity	BP	D O W N
GO:0010470	33	8	2.63	0.00364408	2.44E+00	regulation of gastrulation	BP	D O W N
GO:0050829	33	8	2.63	0.00364408	2.44E+00	defense response to Gram-negative bacterium	BP	D O W N
GO:0002517	9	4	0.72	0.0036467	2.44E+00	T cell tolerance induction	BP	D O W N
GO:0002645	9	4	0.72	0.0036467	2.44E+00	positive regulation of tolerance induction	BP	D O W N
GO:0000086	71	13	5.66	0.00367457	2.43E+00	G2/M transition of mitotic cell cycle	BP	D O W N
GO:0010629	1271	142	101.24	0.00368538	2.43E+00	negative regulation of gene expression	BP	D O W N
GO:0030593	63	12	5.02	0.00369151	2.43E+00	neutrophil chemotaxis	BP	D O W N
GO:0035994	20	6	1.59	0.00369813	2.43E+00	response to muscle stretch	BP	D O W

								N
GO:0051043	20	6	1.59	0.00369813	2.43E+00	NA	BP	DOWN
GO:0071822	1352	167	107.69	0.00375019	2.43E+00	NA	BP	DOWN
GO:0007010	967	132	77.02	0.0037941	2.42E+00	cytoskeleton organization	BP	DOWN
GO:0043065	488	69	38.87	0.00379943	2.42E+00	positive regulation of apoptotic process	BP	DOWN
GO:0031334	159	23	12.66	0.00380848	2.42E+00	positive regulation of protein complex assembly	BP	DOWN
GO:0050680	114	18	9.08	0.00388563	2.41E+00	negative regulation of epithelial cell proliferation	BP	DOWN
GO:0007584	178	25	14.18	0.00391231	2.41E+00	response to nutrient	BP	DOWN
GO:0043524	141	21	11.23	0.00392118	2.41E+00	negative regulation of neuron apoptotic process	BP	DOWN
GO:0070374	123	19	9.8	0.00393245	2.41E+00	positive regulation of ERK1 and ERK2 cascade	BP	DOWN
GO:0071363	410	72	32.66	0.00398004	2.40E+00	cellular response to growth factor stimulus	BP	DOWN
GO:0022411	366	44	29.15	0.00405373	2.39E+00	NA	BP	DOWN
GO:000	160	23	12.74	0.0041212	2.38E+00	cellular response to	BP	D

9267				3		starvation		O W N
GO:0034644	56	11	4.46	0.00417333	2.38E+00	cellular response to UV	BP	D O W N
GO:2001236	157	26	12.51	0.0041837	2.38E+00	regulation of extrinsic apoptotic signaling pathway	BP	D O W N
GO:0045581	27	7	2.15	0.00430908	2.37E+00	negative regulation of T cell differentiation	BP	D O W N
GO:0034502	34	8	2.71	0.00443833	2.35E+00	protein localization to chromosome	BP	D O W N
GO:0035924	34	8	2.71	0.00443833	2.35E+00	cellular response to vascular endothelial growth factor stimulus	BP	D O W N
GO:0038061	34	8	2.71	0.00443833	2.35E+00	NIK/NF-kappaB signaling	BP	D O W N
GO:0001812	5	3	0.4	0.00446246	2.35E+00	positive regulation of type I hypersensitivity	BP	D O W N
GO:0002513	5	3	0.4	0.00446246	2.35E+00	tolerance induction to self antigen	BP	D O W N
GO:0022614	5	3	0.4	0.00446246	2.35E+00	membrane to membrane docking	BP	D O W N
GO:0023035	5	3	0.4	0.00446246	2.35E+00	CD40 signaling pathway	BP	D O W N
GO:0032211	5	3	0.4	0.00446246	2.35E+00	negative regulation of telomere maintenance via telomerase	BP	D O W

								N
GO:0034638	5	3	0.4	0.00446246	2.35E+00	phosphatidylcholine catabolic process	BP	DOWN
GO:0045345	5	3	0.4	0.00446246	2.35E+00	positive regulation of MHC class I biosynthetic process	BP	DOWN
GO:0050859	5	3	0.4	0.00446246	2.35E+00	negative regulation of B cell receptor signaling pathway	BP	DOWN
GO:0051256	5	3	0.4	0.00446246	2.35E+00	mitotic spindle midzone assembly	BP	DOWN
GO:0060058	5	3	0.4	0.00446246	2.35E+00	positive regulation of apoptotic process involved in mammary gland involution	BP	DOWN
GO:1901026	5	3	0.4	0.00446246	2.35E+00	ripoptosome assembly involved in necroptotic process	BP	DOWN
GO:1902563	5	3	0.4	0.00446246	2.35E+00	NA	BP	DOWN
GO:0010811	98	16	7.81	0.00449913	2.35E+00	positive regulation of cell-substrate adhesion	BP	DOWN
GO:0007169	457	74	36.4	0.00451911	2.34E+00	transmembrane receptor protein tyrosine kinase signaling pathway	BP	DOWN
GO:0002824	107	24	8.52	0.00459527	2.34E+00	NA	BP	DOWN
GO:0070372	188	31	14.97	0.00459828	2.34E+00	regulation of ERK1 and ERK2 cascade	BP	DOWN
GO:004	356	72	28.36	0.0046479	2.33E+00	positive regulation of	BP	D

5860				8		protein kinase activity		O W N
GO:0006801	49	10	3.9	0.00465093	2.33E+00	superoxide metabolic process	BP	D O W N
GO:0007041	49	10	3.9	0.00465093	2.33E+00	lysosomal transport	BP	D O W N
GO:0070527	49	10	3.9	0.00465093	2.33E+00	platelet aggregation	BP	D O W N
GO:0019221	289	42	23.02	0.00470758	2.33E+00	cytokine-mediated signaling pathway	BP	D O W N
GO:0032091	65	12	5.18	0.00480999	2.32E+00	negative regulation of protein binding	BP	D O W N
GO:0007160	162	23	12.9	0.0048113	2.32E+00	cell-matrix adhesion	BP	D O W N
GO:0042098	162	23	12.9	0.0048113	2.32E+00	T cell proliferation	BP	D O W N
GO:0030099	322	52	25.65	0.00481538	2.32E+00	myeloid cell differentiation	BP	D O W N
GO:0048011	21	6	1.67	0.00483307	2.32E+00	neurotrophin TRK receptor signaling pathway	BP	D O W N
GO:0030220	15	5	1.19	0.00485912	2.31E+00	platelet formation	BP	D O W N
GO:0045780	15	5	1.19	0.00485912	2.31E+00	positive regulation of bone resorption	BP	D O W

								N
GO:0045892	1041	111	82.92	0.0048796 3	2.31E+00	negative regulation of transcription, DNA-templated	BP	DOWN
GO:0042326	361	68	28.75	0.0048885	2.31E+00	negative regulation of phosphorylation	BP	DOWN
GO:0046637	42	9	3.35	0.0050749 9	2.29E+00	NA	BP	DOWN
GO:0032868	203	32	16.17	0.0051958 6	2.28E+00	response to insulin	BP	DOWN
GO:0010976	182	25	14.5	0.0052307 3	2.28E+00	positive regulation of neuron projection development	BP	DOWN
GO:0015031	1439	192	114.62	0.0052700 6	2.28E+00	protein transport	BP	DOWN
GO:0046677	51	13	4.06	0.0052865 1	2.28E+00	response to antibiotic	BP	DOWN
GO:0006875	374	46	29.79	0.0053312 4	2.27E+00	cellular metal ion homeostasis	BP	DOWN
GO:0071417	371	56	29.55	0.0053355 7	2.27E+00	cellular response to organonitrogen compound	BP	DOWN
GO:0007257	28	7	2.23	0.0053569 2	2.27E+00	activation of JUN kinase activity	BP	DOWN
GO:0032459	28	7	2.23	0.0053569 2	2.27E+00	regulation of protein oligomerization	BP	DOWN
GO:006	28	7	2.23	0.0053569	2.27E+00	regulation of dendritic	BP	D

1001				2		spine morphogenesis		O W N
GO:0098751	28	7	2.23	0.00535692	2.27E+00	NA	BP	D O W N
GO:0009888	1742	204	138.75	0.00535797	2.27E+00	tissue development	BP	D O W N
GO:0046834	35	8	2.79	0.00535909	2.27E+00	lipid phosphorylation	BP	D O W N
GO:0046635	50	10	3.98	0.00540745	2.27E+00	positive regulation of alpha-beta T cell activation	BP	D O W N
GO:0072503	313	40	24.93	0.00542744	2.27E+00	NA	BP	D O W N
GO:0050864	102	33	8.12	0.00543061	2.27E+00	regulation of B cell activation	BP	D O W N
GO:0006906	100	16	7.97	0.00550061	2.26E+00	vesicle fusion	BP	D O W N
GO:0007098	58	11	4.62	0.00551463	2.26E+00	centrosome cycle	BP	D O W N
GO:0045931	109	17	8.68	0.00559453	2.25E+00	positive regulation of mitotic cell cycle	BP	D O W N
GO:0002726	10	4	0.8	0.00569696	2.24E+00	positive regulation of T cell cytokine production	BP	D O W N
GO:0006268	10	4	0.8	0.00569696	2.24E+00	DNA unwinding involved in DNA replication	BP	D O W

								N
GO:0006465	10	4	0.8	0.00569696	2.24E+00	signal peptide processing	BP	DOWN
GO:0019471	10	4	0.8	0.00569696	2.24E+00	4-hydroxyproline metabolic process	BP	DOWN
GO:0032740	10	4	0.8	0.00569696	2.24E+00	positive regulation of interleukin-17 production	BP	DOWN
GO:0036010	10	4	0.8	0.00569696	2.24E+00	protein localization to endosome	BP	DOWN
GO:0060768	10	4	0.8	0.00569696	2.24E+00	regulation of epithelial cell proliferation involved in prostate gland development	BP	DOWN
GO:0048522	4067	503	323.95	0.0057003	2.24E+00	NA	BP	DOWN
GO:0010038	272	34	21.67	0.00592298	2.23E+00	response to metal ion	BP	DOWN
GO:0006955	988	195	78.7	0.0059234	2.23E+00	immune response	BP	DOWN
GO:0031663	45	11	3.58	0.00593102	2.23E+00	lipopolysaccharide-mediated signaling pathway	BP	DOWN
GO:0007595	43	9	3.43	0.0059741	2.22E+00	lactation	BP	DOWN
GO:0097305	348	47	27.72	0.00600019	2.22E+00	response to alcohol	BP	DOWN
GO:000	57	17	4.54	0.0060550	2.22E+00	B cell activation	BP	D

2312				8		involved in immune response		DOWN
GO:0007249	224	47	17.84	0.0060668	2.22E+00	I-kappaB kinase/NF-kappaB signaling	BP	DOWN
GO:0034142	22	6	1.75	0.00620435	2.21E+00	toll-like receptor 4 signaling pathway	BP	DOWN
GO:0044843	147	32	11.71	0.00621804	2.21E+00	NA	BP	DOWN
GO:0042981	1264	165	100.68	0.00624224	2.20E+00	regulation of apoptotic process	BP	DOWN
GO:0051272	338	52	26.92	0.0062483	2.20E+00	positive regulation of cellular component movement	BP	DOWN
GO:0007043	59	11	4.7	0.00630179	2.20E+00	cell-cell junction assembly	BP	DOWN
GO:0014065	84	14	6.69	0.00631068	2.20E+00	phosphatidylinositol 3-kinase signaling	BP	DOWN
GO:0001970	2	2	0.16	0.00634097	2.20E+00	positive regulation of activation of membrane attack complex	BP	DOWN
GO:0002760	2	2	0.16	0.00634097	2.20E+00	positive regulation of antimicrobial humoral response	BP	DOWN
GO:0031938	2	2	0.16	0.00634097	2.20E+00	regulation of chromatin silencing at telomere	BP	DOWN
GO:0032661	2	2	0.16	0.00634097	2.20E+00	NA	BP	DOW

								N
GO:0032804	2	2	0.16	0.00634097	2.20E+00	negative regulation of low-density lipoprotein particle receptor catabolic process	BP	DOWN
GO:0033277	2	2	0.16	0.00634097	2.20E+00	abortive mitotic cell cycle	BP	DOWN
GO:0034136	2	2	0.16	0.00634097	2.20E+00	negative regulation of toll-like receptor 2 signaling pathway	BP	DOWN
GO:0035026	2	2	0.16	0.00634097	2.20E+00	leading edge cell differentiation	BP	DOWN
GO:0043316	2	2	0.16	0.00634097	2.20E+00	cytotoxic T cell degranulation	BP	DOWN
GO:0043686	2	2	0.16	0.00634097	2.20E+00	co-translational protein modification	BP	DOWN
GO:0044338	2	2	0.16	0.00634097	2.20E+00	canonical Wnt signaling pathway involved in mesenchymal stem cell differentiation	BP	DOWN
GO:0044339	2	2	0.16	0.00634097	2.20E+00	canonical Wnt signaling pathway involved in osteoblast differentiation	BP	DOWN
GO:0045368	2	2	0.16	0.00634097	2.20E+00	positive regulation of interleukin-13 biosynthetic process	BP	DOWN
GO:0045401	2	2	0.16	0.00634097	2.20E+00	positive regulation of interleukin-3 biosynthetic process	BP	DOWN
GO:0045425	2	2	0.16	0.00634097	2.20E+00	positive regulation of granulocyte macrophage colony-stimulating factor biosynthetic process	BP	DOWN

GO:0046110	2	2	0.16	0.00634097	2.20E+00	NA	BP	DOWN
GO:0051563	2	2	0.16	0.00634097	2.20E+00	smooth endoplasmic reticulum calcium ion homeostasis	BP	DOWN
GO:0060101	2	2	0.16	0.00634097	2.20E+00	negative regulation of phagocytosis, engulfment	BP	DOWN
GO:0071226	2	2	0.16	0.00634097	2.20E+00	cellular response to molecule of fungal origin	BP	DOWN
GO:0072573	2	2	0.16	0.00634097	2.20E+00	tolerance induction to lipopolysaccharide	BP	DOWN
GO:0072719	2	2	0.16	0.00634097	2.20E+00	cellular response to cisplatin	BP	DOWN
GO:1900248	2	2	0.16	0.00634097	2.20E+00	negative regulation of cytoplasmic translational elongation	BP	DOWN
GO:1902525	2	2	0.16	0.00634097	2.20E+00	regulation of protein monoubiquitination	BP	DOWN
GO:2000417	2	2	0.16	0.00634097	2.20E+00	negative regulation of eosinophil migration	BP	DOWN
GO:0002446	27	9	2.15	0.00655691	2.18E+00	neutrophil mediated immunity	BP	DOWN
GO:0010165	29	7	2.31	0.00658463	2.18E+00	response to X-ray	BP	DOWN
GO:0033598	29	7	2.31	0.00658463	2.18E+00	mammary gland epithelial cell	BP	DO

						proliferation		W N
GO:0034698	29	7	2.31	0.00658463	2.18E+00	response to gonadotropin	BP	D O W N
GO:0045736	29	7	2.31	0.00658463	2.18E+00	negative regulation of cyclin-dependent protein serine/threonine kinase activity	BP	D O W N
GO:1903035	136	31	10.83	0.00659934	2.18E+00	NA	BP	D O W N
GO:0034349	16	5	1.27	0.00660988	2.18E+00	glial cell apoptotic process	BP	D O W N
GO:0071236	16	5	1.27	0.00660988	2.18E+00	cellular response to antibiotic	BP	D O W N
GO:0051054	129	19	10.28	0.0066389	2.18E+00	positive regulation of DNA metabolic process	BP	D O W N
GO:0002429	114	33	9.08	0.00667897	2.18E+00	immune response-activating cell surface receptor signaling pathway	BP	D O W N
GO:0061572	111	17	8.84	0.00672558	2.17E+00	actin filament bundle organization	BP	D O W N
GO:0032649	80	18	6.37	0.00681367	2.17E+00	regulation of interferon-gamma production	BP	D O W N
GO:2000106	82	17	6.53	0.00685106	2.16E+00	NA	BP	D O W N
GO:0051239	2276	304	181.29	0.00686063	2.16E+00	NA	BP	D O W N

GO:0001568	539	81	42.93	0.00708409	2.15E+00	blood vessel development	BP	DOWN
GO:0001816	521	100	41.5	0.00709576	2.15E+00	cytokine production	BP	DOWN
GO:0006974	616	96	49.07	0.00715579	2.15E+00	cellular response to DNA damage stimulus	BP	DOWN
GO:0010498	336	52	26.76	0.00738309	2.13E+00	proteasomal protein catabolic process	BP	DOWN
GO:0051302	236	30	18.8	0.0073911	2.13E+00	regulation of cell division	BP	DOWN
GO:0050715	77	13	6.13	0.00746101	2.13E+00	positive regulation of cytokine secretion	BP	DOWN
GO:0002697	274	57	21.82	0.0074629	2.13E+00	NA	BP	DOWN
GO:0055074	309	39	24.61	0.00747165	2.13E+00	calcium ion homeostasis	BP	DOWN
GO:0051235	280	38	22.3	0.00757297	2.12E+00	NA	BP	DOWN
GO:0050710	37	8	2.95	0.00762952	2.12E+00	negative regulation of cytokine secretion	BP	DOWN
GO:0061098	37	8	2.95	0.00762952	2.12E+00	positive regulation of protein tyrosine kinase activity	BP	DOWN
GO:0042325	1182	191	94.15	0.0077278	2.11E+00	regulation of phosphorylation	BP	DO

								W N
GO:0043112	130	23	10.35	0.00774883	2.11E+00	receptor metabolic process	BP	D O W N
GO:0008608	23	6	1.83	0.00783795	2.11E+00	attachment of spindle microtubules to kinetochore	BP	D O W N
GO:0030511	23	6	1.83	0.00783795	2.11E+00	positive regulation of transforming growth factor beta receptor signaling pathway	BP	D O W N
GO:0030279	69	12	5.5	0.00784991	2.11E+00	negative regulation of ossification	BP	D O W N
GO:00512601	247	31	19.67	0.00785195	2.11E+00	protein homooligomerization	BP	D O W N
GO:0035850	30	7	2.39	0.00800965	2.10E+00	NA	BP	D O W N
GO:0042269	30	7	2.39	0.00800965	2.10E+00	regulation of natural killer cell mediated cytotoxicity	BP	D O W N
GO:0033043	987	133	78.62	0.00810548	2.09E+00	regulation of organelle organization	BP	D O W N
GO:0043086	687	95	54.72	0.00824881	2.08E+00	negative regulation of catalytic activity	BP	D O W N
GO:0061515	53	10	4.22	0.00826343	2.08E+00	NA	BP	D O W N
GO:0002366	187	44	14.9	0.00828483	2.08E+00	leukocyte activation involved in immune response	BP	D O W N

GO:0045351	13	7	1.04	0.00834792	2.08E+00	type I interferon biosynthetic process	BP	DOWN
GO:2000257	13	6	1.04	0.00835998	2.08E+00	NA	BP	DOWN
GO:0032494	11	4	0.88	0.00839305	2.08E+00	response to peptidoglycan	BP	DOWN
GO:0035589	11	4	0.88	0.00839305	2.08E+00	G-protein coupled purinergic nucleotide receptor signaling pathway	BP	DOWN
GO:0043312	11	4	0.88	0.00839305	2.08E+00	neutrophil degranulation	BP	DOWN
GO:0045651	11	4	0.88	0.00839305	2.08E+00	positive regulation of macrophage differentiation	BP	DOWN
GO:0002756	6	3	0.48	0.00839713	2.08E+00	MyD88-independent toll-like receptor signaling pathway	BP	DOWN
GO:0014010	6	3	0.48	0.00839713	2.08E+00	Schwann cell proliferation	BP	DOWN
GO:0022417	6	3	0.48	0.00839713	2.08E+00	protein maturation by protein folding	BP	DOWN
GO:0051133	6	3	0.48	0.00839713	2.08E+00	NA	BP	DOWN
GO:0090073	6	3	0.48	0.00839713	2.08E+00	positive regulation of protein homodimerization activity	BP	DOWN
GO:2001046	6	3	0.48	0.00839713	2.08E+00	positive regulation of integrin-mediated	BP	DO

						signaling pathway		W N
GO:0072001	289	39	23.02	0.0084278	2.07E+00	renal system development	BP	D O W N
GO:0032944	178	36	14.18	0.00864345	2.06E+00	NA	BP	D O W N
GO:1902582	1132	155	90.17	0.00872434	2.06E+00	NA	BP	D O W N
GO:0050482	17	5	1.35	0.00875871	2.06E+00	arachidonic acid secretion	BP	D O W N
GO:0060337	17	5	1.35	0.00875871	2.06E+00	type I interferon signaling pathway	BP	D O W N
GO:0070570	17	5	1.35	0.00875871	2.06E+00	regulation of neuron projection regeneration	BP	D O W N
GO:0060759	70	12	5.58	0.00880525	2.06E+00	NA	BP	D O W N
GO:0071456	105	16	8.36	0.0088089	2.06E+00	cellular response to hypoxia	BP	D O W N
GO:0009892	2268	280	180.65	0.00892973	2.05E+00	negative regulation of metabolic process	BP	D O W N
GO:0002704	38	8	3.03	0.00900434	2.05E+00	NA	BP	D O W N
GO:0043551	38	8	3.03	0.00900434	2.05E+00	regulation of phosphatidylinositol 3-kinase activity	BP	D O W N

GO:0035303	137	22	10.91	0.00901987	2.04E+00	regulation of dephosphorylation	BP	DOWN
GO:0034341	62	11	4.94	0.00919995	2.04E+00	response to interferon-gamma	BP	DOWN
GO:0042991	79	13	6.29	0.00924944	2.03E+00	transcription factor import into nucleus	BP	DOWN
GO:0042110	383	65	30.51	0.00926742	2.03E+00	T cell activation	BP	DOWN
GO:0045088	175	43	13.94	0.00936539	2.03E+00	regulation of innate immune response	BP	DOWN
GO:0036473	54	10	4.3	0.0094357	2.03E+00	NA	BP	DOWN
GO:1901215	165	27	13.14	0.00948681	2.02E+00	negative regulation of neuron death	BP	DOWN
GO:2000134	45	16	3.58	0.00952825	2.02E+00	negative regulation of G1/S transition of mitotic cell cycle	BP	DOWN
GO:0034329	130	23	10.35	0.00956741	2.02E+00	cell junction assembly	BP	DOWN
GO:0051093	865	97	68.9	0.00958491	2.02E+00	NA	BP	DOWN
GO:0051247	1086	170	86.5	0.00963704	2.02E+00	positive regulation of protein metabolic process	BP	DOWN
GO:0015804	31	7	2.47	0.00964944	2.02E+00	neutral amino acid transport	BP	DO

								W N
GO:0051091	189	37	15.05	0.00971286	2.01E+00	positive regulation of sequence-specific DNA binding transcription factor activity	BP	D O W N
GO:0030336	174	25	13.86	0.00973076	2.01E+00	negative regulation of cell migration	BP	D O W N
GO:0045191	24	6	1.91	0.00975949	2.01E+00	regulation of isotype switching	BP	D O W N
GO:1902042	24	6	1.91	0.00975949	2.01E+00	negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	BP	D O W N
GO:0045785	301	47	23.98	0.00976571	2.01E+00	positive regulation of cell adhesion	BP	D O W N
GO:0032355	134	19	10.67	0.00991023	2.00E+00	response to estradiol	BP	D O W N
GO:2001020	134	19	10.67	0.00991023	2.00E+00	regulation of response to DNA damage stimulus	BP	D O W N
GO:0050778	398	95	31.7	0.00994119	2.00E+00	positive regulation of immune response	BP	D O W N
GO:0043066	793	97	63.16	0.00995229	2.00E+00	negative regulation of apoptotic process	BP	D O W N

Supplementary Data 7
Gene Symbols for Enriched BIG-C Categories Increased by the HDAC6 inhibitor

Cytoskeleton	**Transporters**	**Endosome and Vesicles**	**Mitochondria**	**Fatty Acid Biosynthesis**	**Peroxisomes**	**ROS Protection**	**Cell Surface**
Sgce	*Slc6a1*	*Syt3*	*Gpat2*	*Echdc2*	*Gstk1*	*Prdx2*	*Prnp*

Mapt	*Slc51a*	*Syngr1*	*Gls2*	*Acss2*	*Nudt12*	*Txnrd2*	*Tspan6*
Krt18	*Kcnk12*	*Rab3b*	*Amt*	*Hadh*	*Hacl1*	*Gstp1*	*Ptpru*
Lrch2	*Slc27a6*	*Snx31*	*Cyp11a1*	*Mecr*	*Pex11a*	*Prdx6*	*Pcdh7*
Nphp1	*Slc12a5*	*Clstn1*	*Nme4*	*Decr1*	*Paox*		*Folr1*
Pfn4	*Atp4a*	*Syt5*	*Dhtkd1*	*Pcx*	*Pex6*		*Cdh22*
Spata7	*Slc6a13*	*Sytl1*	*Me3*		*Pex11b*		*Dlk1*
Dync2li1	*Kcnab3*	*Unc13b*	*Nmnat3*		*Pex7*		*Adora3*
Kif17	*Slc22a18*	*Pacsin3*	*Maoa*		*Pxmp4*		*Mc1r*
Spire2	*Kcnh3*	*Spag8*	*Clybl*		*Pex5*		*Tmem205*
Ang	*Kcnh2*	*Scg5*	*Tmlhe*				*Sema6c*
Snph	*Slc22a17*	*Prss16*	*Slc25a23*				*Adgrd1*
Krt86	*Slc7a4*	*Fam109b*	*Tdrkh*				*Ms4a3*
Mapre3	*Aqp9*	*Vamp5*	*Ldhd*				*Smo*
Dlg4	*Abcg2*	*Rab17*	*Hmgcs2*				*Adgrg7*
Homer2	*Fxyd1*	*Rab38*	*Fahd1*				*Ppfia3*
Eda	*Slc44a5*	*Ocrl*	*Bphl*				*Ramp3*
Myo1d	*Kcnd1*	*Slc9a9*	*Chchd6*				*Cd248*
Actn2	*Cacna1c*	*Snx22*	*Aldh5a1*				*Gpr27*
Plekhg4	*Tfr2*	*Dennd6b*	*Mthfd2l*				*Muc13*
Ptpdc1	*Cacng8*	*Arl4c*	*Acad10*				*Lrfn1*
Kif7	*Aqp11*	*Tmem9*	*Pyroxd2*				*Cadm3*
Tnni1	*Aqp1*	*Tmem163*	*Slc25a35*				*Plxna2*
Ank3	*Trpm1*	*Sytl3*	*Hint2*				*Pth1r*
Tubb3	*Kcng2*	*Stx2*	*Bckdhb*				*Epor*
Ift81	*Slc2a10*	*Appl2*	*Lipt1*				*Cysltr1*
Gpr4	*Abcb4*	*Rab27b*	*Coq4*				*Adgrl1*
Dock6	*Kcnh7*	*Stxbp1*	*Nipsnap1*				*Tspan17*
Ttc8	*Clcn1*	*Abca5*	*Cyp27a1*				*Unc5a*
Ttc12	*Slc27a1*	*Mamdc4*	*Mccc2*				*Adam33*
Wdr35	*Clcn2*	*Rab23*	*Aldh4a1*				*Chrne*
Epb41l4b	*Slc38a5*	*Als2cl*	*Pccb*				*Lsr*
Ehbp1	*Kcne3*	*Scrn2*	*Iba57*				*Enpp5*
Spag4	*Slc39a8*	*Sft2d3*	*Ppox*				*Lhfpl2*
Odf2l	*Kcnip3*	*Ap4b1*	*Glrx5*				*Tspan33*
Mylpf	*Akap7*	*Lamtor2*	*Amacr*				*Adgrg1*

Ift43	*Kcnc3*	*Rab24*	*Ethe1*				*Mmp17*
Nefh	*Kctd14*	*Hap1*	*Acp6*				*Baiap2l2*
Wdr60	*Slc41a3*	*Flot2*	*Lyrm1*				*Cldn10*
Ttll1	*Aqp3*	*Dennd1a*	*Sfxn2*				*Cdh24*
Dennd2a	*Tesc*	*Rab3d*	*Dguok*				*Arvcf*
Wdr19	*Slc16a7*	*Fcho1*	*Agk*				*Tmem41a*
Vill	*Slc14a1*	*Rabep2*	*Sfxn4*				*Sema6b*
Kptn	*Slc9a5*	*Vps16*	*Mcee*				*Ptger3*
Pls3	*Kcnj8*	*Ap1b1*	*Immp2l*				*Cd34*
Nek3	*Cacna1a*	*Ap1g2*	*Clpb*				*Neo1*
Bbs2	*Cnga1*		*Ivd*				*Eng*
Kifc2	*Spns3*		*Mtfr1l*				*F11r*
Bbs1	*Slc29a2*		*Naxe*				*Palm*
Dmd	*Slc43a1*		*Adck5*				*Fgfr1*
Arl6	*Slc4a8*		*Sfxn5*				*Mfap3l*
Dzip1	*Slc16a5*		*Pcca*				*Gpr82*
Fuz	*Slc29a4*		*Coq7*				*Hrh1*
Fnbp1l	*Cbarp*		*Ppa2*				*Gprc5c*
Rpgr	*Bspry*		*Akap1*				*Agrn*
Bbs9	*Slco2b1*		*Mccc1*				*Art3*
Tubb4a	*Stom*		*Acadsb*				*Amot*
Kif9	*Cacna2d2*		*Ccdc58*				*Plscr3*
Ccdc14	*Cacnb1*		*Slc25a24*				*Plpp2*
Palld	*Slc29a1*		*Fxn*				*Sdc2*
Bbs4	*Ano10*		*Suox*				*Spa17*
Nek8	*Slc39a4*		*Acad11*				*Gpc4*
Krt10	*Slc2a9*		*Slc25a39*				*Plxdc2*
Arhgap18	*Slco3a1*		*Pstk*				*Tmem17*
Ift74	*Kctd12*		*Acad8*				*Tacstd2*
Hook2	*Atp9a*		*Fpgs*				*Il1rl2*
Dctn6	*Slc50a1*		*Coq2*				*Anxa9*
Tubg2	*Slc19a1*		*Timm10b*				*Sema4f*
Eml2	*Kctd2*		*Tk2*				*Efna1*
Ccdc114	*Kctd13*		*Taz*				*Cpne2*
Gsn	*Slc39a3*		*Mipep*				*Fgfrl1*

Cnn3	*Ttyh3*		*Dhodh*				*Ephb6*
Matk	*Slc6a20a*		*Adck1*				*Adgra2*
Katnal1	*Ank*		*Abcb8*				*Reck*
Cep72	*Cbarp*		*2310061I04 Rik*				*Magi2*
Mks1	*Gm44509*		*1700021F05 Rik*				*Grik5*
Klc4							*Gp5*
Pick1							*Tbxa2r*
Kifap3							*Ramp1*
Cep57l1							*Plxna3*
Sgcb							*Tgfb3*
Klhdc1							*Mpp7*
Ift122							*Tmem273*
Lrrc45							*Tlcd2*
Vmac							*Gpr19*
Fntb							*Ly6d*
Sfi1							*Plscr4*
Cep41							*Prr7*
Tube1							*Adgr5*
Spata6							*Tmem107*
Cep19							*Ptprs*
Mob3b							*Smim13*
Cep131							*Krtcap3*
Fhl3							*Ano8*
Tuba4a							*Gphn*
Ccdc61							*Tjp3*
Ick							*Lpar6*
Ift27							*Smim19*
Ip6k2							*Adora2a*
Marveld1							*Amigo2*
Ankra2							*Ncstn*
Pdlim1							*Tmem8a*
Tpm1							*Cercam*

Tubb4a							

Supplementary Data 8
Gene Symbols for Enriched BIG-C Categories Decreased by the HDAC6 inhibitor

Cell Surface	Intracellular Signaling	PRR Signaling	IFN gene Signature (IGS)	Pro Cell Cycle	Unfolded Protein & Stress	Endosome and Vesicles	Endoplasmic Reticulum	Golgi	Integrin Signaling
Ms4a4b (includes others)	*Dusp4*	*Tlr13*	*Mx1/Mx2*	*Cep55*	*Chac1*	*Dnm3*	*Edem1*	*Fam20c*	*Gna13*
Ctla2a/Ctla2b	*Gnaz*	*Oasl2*	*Ifi44*	*Ncapg*	*Bhlha15*	*Zfyve9*	*Edem3*	*Mest*	*Lamc1*
Igkv5-39	*Dusp14*	*Oas2*	*Rsad2*	*Nek2*	*Xbp1*	*Itsn2*	*Edem2*	*Slc9a7*	*Itgav*
Ighv1-37	*Csrp3*	*Tlr8*	*Rtp4*	*Cep85l*	*Edem1*	*Ehd4*	*Erp44*	*Atp7a*	*Plcg2*
Ighv5-16	*Gng11*	*Tlr13*	*Eif2ak2*	*Cdca2*	*Creb3l2*	*Dab2*	*Erlec1*	*Cgnl1*	*Spock2*
Cd300ld	*Rgs4*	*Oas3*	*Ifitm3*	*Prc1*	*Derl3*	*Smap1*	*Creld2*	*B3gnt9*	*Raf1*
Ace	*Evc*	*Tlr7*	*Sp110*	*Ndc80*	*Sel1l*	*Capza1*	*Ddn*	*Plagl1*	*Col17a1*
Ighv1-31	*Pik3ap1*	*Zbp1*	*Gbp2*	*Foxm1*	*Hspa5*	*Washc4*	*Ryr3*	*Chst2*	*Actg2*
Igkv12-41	*Rgs13*	*Dusp16*	*Sp100*	*Ect2*	*Edem3*	*Eea1*	*Hsp90b1*	*Fgd4*	*Lamc3*
Ighv1-33	*Aicda*	*Irf7*	*Cmpk2*	*Clspn*	*Dnajc3*	*Arfgap3*	*Sec24d*	*B3galt1*	*Bcar1*
Ighv1-84	*Gzmk*	*Oasl2*	*Ifit1*	*Ttk*	*Insig1*	*Snx2*	*Kcnrg*	*Chst3*	*Itgb8*
Ighv1-20	*Fkbp11*	*Tirap*	*Ifit3b*	*Esco2*	*Ube2j1*	*Arpc1b*	*Tram2*	*Glcci1*	*Lama1*
Cx3cr1	*Sik2*	*Isg15*		*Bub1*	*Edem2*	*Arap1*	*Txndc11*	*Ica1l*	*Col5a2*
Ighv5-9	*Foxp3*	*Irf8*		*Dsn1*	*Dnajb9*	*Cyth4*	*Txndc5*	*Fndc3b*	*Hspg2*

Ighv1-42	*Rgs16*	*Nlrc3*		*Top1*	*Erp44*	*Rab5a*	*Sdf2l1*	*Xylt1*	*Fbn2*
Ighv1-39	*Nos2*	*Irf4*		*Fen1*	*Hsph1*	*Vps26a*	*Hyou1*	*Chst1*	*Eln*
Crybg3	*Acod1*	*Nlrc5*		*Kntc1*	*Rpn1*	*Arfgap2*	*Sec24a*	*Fut8*	*Col4a1*
Ighv1-26	*Zc3h12d*	*Tlr9*		*Ncaph*	*Herpud1*	*Git1*	*Prr11*	*Man2a1*	*Itgae*
Ighv5-4	*Dusp3*	*Irak4*		*Aurkb*	*Man1b1*	*Rab35*	*Hspa13*	*Slc39a7*	*Vcan*
Ms4a4a	*Tnip3*	*Trim14*		*Ska3*	*Vmp1*	*Vps26b*	*Dnajb11*	*Rab43*	*Col4a2*
Tgm3	*Sh3bgrl2*	*Lrrfip1*		*Cdc20*	*Ubxn4*		*Manf*	*Manea*	*Col3a1*
Ighv5-12	*Amotl1*	*Ticam2*		*Cenpe*	*Pdia4*		*Sec61a1*	*Atp8b2*	*Col13a1*
Ighv5-6	*Sh2b2*	*Irf9*		*Kif11*	*Tmem214*		*Pdia6*	*Gcnt2*	*Spon1*
Ighv2-9-1	*Spry1*	*Ddx58*		*Cdca5*	*Calr*		*Atp2a2*	*Parp9*	*Ecm1*
Ighv2-9	*Nfil3*	*Nlrp3*		*Top2a*	*Atf6*		*Lrrc59*	*Cyb5d1*	
Ackr4	*Cmip*	*Tnfaip3*		*Cdc42bpb*	*Erlec1*		*Mtdh*	*Rab39b*	
Ighv1-5	*Ikzf3*	*Tlr4*		*Bub1b*	*Canx*		*Tor3a*	*Bhlhe40*	
Ighv5-9-1	*Tiam1*	*Casp4*		*Uhrf1*	*Sec63*		*Ssr4*	*B4galt5*	
Igkv3-2	*Parp14*	*Ifih1*		*Incenp*	*Vcp*		*Pdia3*	*Qpctl*	
Ighv3-4	*Prdm1*	*Nod2*		*Spdl1*	*St13*		*Slc35e1*	*Itpripl2*	
Ighv5-15	*Bcl6*	*Zc3hav1*		*E2f2*	*Nploc4*		*Slc35b1*	*Uso1*	
Clec4e	*Styx*	*Traf3*		*Cdc45*	*Ero1l*		*Atp10d*	*Mgat2*	
Ighv1-36	*Themis2*	*Arl16*		*Nusap1*	*Ero1lb*		*Plod2*	*Cog5*	
Ighv1-21	*Trib1*	*Rnf41*		*Dbf4*			*Ergic1*	*Serinc5*	
Ighv1-	*Grk3*	*Irak3*		*Sgo1*			*Sec23*	*Tpst1*	

66							*a*		
Ighj3	*Stard8*	*Myd88*		*Mis18bp1*			*Surf4*	*Sec14l1*	
Efnb2	*Ppp1r12a*	*Trim35*		*Espl1*			*Sec11c*	*Slc30a7*	
Ccr6	*Fgr*	*Tank*		*Cenpl*			*Mlec*	*Tmed5*	
Ighd2-7	*Rps6ka2*	*Ifi213*		*Ccnd2*			*Rrbp1*	*Pask*	
Gcsam	*Irak1bp1*			*Cep57*			*Erap1*	*Gcnt1*	
Ighv1-19	*Dck*			*Lats2*			*Lclat1*	*Pdxdc1*	
Igkv4-51	*Ikzf2*			*E2f3*			*Slc33a1*	*Psen1*	
Ighv8-4	*Shcbp1*			*Mcm6*			*Ssr3*	*Rnf157*	
Ighv1-73	*Lat2*			*Cep68*			*Ttc9c*	*G2e3*	
Lepr	*Mzb1*			*Cdk14*			*Ergic2*	*Ddhd1*	
Ighv1-7	*Prkab2*			*Rfc1*			*Stt3a*	*B3gnt5*	
Gjb2	*Socs3*			*Ccnd1*			*Sqle*	*Man1a2*	
Ighv1-28	*Ccdc88c*			*Cdc25b*			*Alg2*	*Syngr2*	
Igkv5-45	*Dusp5*			*Mcm5*			*Elovl5*	*St6galnac4*	
Ighv1-61	*Rasgrp3*			*Mis12*			*Clptm1l*	*Gsap*	
Ighj1	*Prkcd*			*Helb*			*Ssr1*	*Arcn1*	
Ighv1-18	*Batf*			*Mcmbp*			*Gdap2*	*St8sia4*	
Ighg3	*Map3k8*			*Cenpj*			*Sec23b*	*Tmf1*	
Igkv1-99	*Crkl*			*Cdc27*			*Spcs3*	*Bicd2*	
Igkv17-121	*Phlpp1*			*Plk3*			*Trim59*	*Gga2*	
Ighv8-11	*Ptpn1*			*Nek7*			*Mia2*	*Pde4dip*	
Ighv1-58	*Stat3*			*Pold1*			*Srp72*	*Slc38a10*	
Ptgir	*Samsn*			*Nde1*			*Soat1*	*Cnst*	

	1								
Ighv7-3	Pou2af1			Mcm4			Yipf5	Alkbh5	
Ighv1-47	Pptc7			Ccng1			Kctd20	Copb2	
Ighv1-75	Rel			Ccnb1-ps			Dnajc14	Rab30	
Ighv1-12	Nfam1			Cenpc1			Sec23ip	C1galt1	
S1pr2	Mob3a			C330027C09Rik			Stim2	Fam20b	
Cd300e	Pten						Aldh3a2	Chpf2	
Ighv1-63	Pla2g7						Ankrd13c	Tm9sf3	
Ighv1-83	Malt1						Ero1l	Slc30a5	
Ulbp1	Rnf19b						Ero1lb	Gorasp2	
Ighv2-2	Prkca						Deaf1	Fut11	
Igkv4-57-1	Bach1						Cyp51	Osbpl9	
Ighv1-25	Nfkbie						Rab1	Atp2c1	
Cd300lb	Btk							Copb1	
Igkv2-116	Mef2c							Vps54	
Ighv6-6	Ptpn11							Gosr2	
Igkv9-124	Casp8ap2							Copa	
Pcdhgc3	Nfkbiz							Stip1	
Ighv1-22	Klhl6							Copg1	
Ighv1-4	Lpxn							Tmed10	
Ighv1-21-1	Stk40							Arl1	
Drd4	Map3k5							Calu	
Ighv7-	Stat2							Pcsk7	

2									
Igkv10-94	*Map3k3*							*Gdi2*	
Igkv8-24	*Ppp4r1*							*Furin*	
Ighv2-4	*Ibtk*							*Gpr107*	
Thbd	*Stap1*							*Gga1*	
Igkv9-123	*Tnip2*							*Man1a*	
Ighv8-5	*Syk*								
Klra2	*Pkn1*								
Ighv2-7	*Ywhah*								
Sgpp2	*Rps6kb1*								
Clec4a3	*Spred1*								
Ighv2-6-8	*Map3k14*								
Igkv15-103	*Stat6*								
Itgae	*Dusp6*								
Igkv4-63	*Bcar3*								
S1pr5	*Grb2*								
Ighv3-3	*Ikbkb*								
Emp2	*Crk*								
Igkv5-37	*Camkk2*								
Lag3	*Nfkb2*								
Vasn	*Elk1*								
Igkv3-1	*Rnf31*								
Igkj5	*Nfkb1*								
Ighv14-4	*Sh3bp5*								
Cdhr5	*Pip5k1c*								
Igkv10-95	*Btbd10*								

Igkv13-85	*Ppp4r2*								
Tnfrsf1b	*Mapk1ip1l*								
Ighv2-3	*Kras*								
Ighj2	*Tnfaip8*								
Igkv8-30	*Hck*								
Lair1	*Abi1*								
Bmp8a	*Fyn*								
Igkv17-127	*Prkx*								
Fcer1a	*Ptpn12*								
Ighv1-62-3	*Grk2*								
Ptpro	*Gimap4*								
Igkv3-4	*Pld4*								
Ighv8-8	*Sash3*								
Vsig4	*Stt3b*								
Ighv2-6	*Ikbkg*								
Igkv9-129	*Pxk*								
Ighv14-3	*Ptpn6*								
Igkv4-69	*Csnk1g3*								
Ighv6-5	*Ywhag*								
Igkv8-21	*Plaa*								
Igkv5-43	*Tesk1*								
Igkv4-86	*Blk*								
Ighe	*Mknk1*								
Ceacam1	*Blnk*								

Igkv6-32	*Ppp6r1*								
Ighg1	*Vav1*								
Nt5e	*Sav1*								
Ighv10-1	*Lyn*								
Igkv3-11	*Csnk1a1*								
Ighv3-6	*Ppp2r5c*								
Astl	*Sh2d3c*								
Ighv1-14	*Ppm1f*								
Igkv4-73	*Stk24*								
Igkv4-74	*Tnip1*								
Igkv2-137	*Ppp2r5d*								
Igkv4-70	*Map3k11*								
Ighv5-17	*Glyr1*								
Mreg	*1700019D03Rik*								
Epha4	*Gm10031*								
Ighv1-54	*H13*								
Igkv6-14	*Calm1 (includes others)*								
Gpr35									
Igkv6-29									
Igkv6-23									
Ighd1-1									
Gja4									
Ighv5-									

2									
Igkv3-9									
Lrp8									
Ighv1-77									
Igkv3-12									
Lilra5									
Nrcam									
Igkv12-46									
Tigit									
Adgrg6									
Ighv1-78									
Ighv1-76									
Ighv13-2									
Ighv7-1									
Igkv4-77									
Ighv14-2									
Ccr8									
Ighv1-2									
Igkj3									
Efnb1									
Ighv1-56									
Slamf7									
Muc20									
Ighv8-2									
Il1r2									
Ighv1-30									
Ighv1-43									

Treml4									
Ptprg									
Ms4a2									
Cd244									
Nrp1									
Igsf11									
Igkv2-109									
Igkv4-61									
Tnfrsf4									
Tgm2									
Cd83									
Igkv8-28									
Plxnb2									
Lifr									
Clec1b									
Igkv4-58									
Tnfsf8									
Crybg3									
Slamf1									
Notch1									
Cdh11									
Msr1									
Sdc1									
Trabd2b									
Pdcd1									
Ighv7-4									
Adam19									
Gngt2									
Cdh5									
Hmmr									

Jchain									
Igkv9-120									
Havcr1									
Ctla4									
Pilra									
Kazn									
Colec12									
Fut4									
Susd2									
C5ar1									
Igkj2									
Igkv1-122									
P2ry12									
Il13ra1									
Igkv1-117									
Ptger2									
Gp9									
Igsf6									
Siglec1									
Cldn7									
Paqr5									
Clec4d									
Fpr1									
Tgfbr1									
Fpr2									
Il21r									
Notch3									
Itgal									
Igkc									
Ptch1									
Alcam									
Plxnd1									
Ptprk									

Ldlrad3									
Sema4d									
Gpm6b									
Csf1r									
Ptpre									
Ms4a6c									
Cd300a									
Scimp									
Icos									
P2ry14									
Epcam									
Tnfrsf17									
Ptafr									
Acvr1									
Entpd1									
Clec7a									
Ltbr									
Jmjd4									
Adam9									
Dcbld1									
Itgax									
Rftn1									
Jaml									
Cd180									
Enpp1									
P2ry6									
Ptger4									
Plekha2									
Itgb3									
Myo1g									

Icam1									
Ldlr									
Cmklr 1									
Cd40									
Adam 17									
Tnfrsf 13c									
Pitpnb									
Adgre 1									
Cnr2									
Ighd									
Cerk									
Pon2									
Sdc3									
Cd22									
Susd6									
Cpne3									
Slamf 6									
Il17ra									
Sell									
Adam 10									
Cd38									
Pqlc3									
Tmem 65									
Tgfbr2									
Itgb2									
Sppl2 a									
Ms4a6 b									
Cd44									
Tspan 14									
Igkv9-120									
Igkv9-123									

Igkv9-124									
Igkv9-129									
Iglc4									
Igll1									
Icam4									
Fcgr4									
Klra2									
VASN									
Clec4n									
Csf2rb2									
Fcgr1									
Fcgr3									
Il4ra									
Gm15931									
Lilrb4a									
Siglece									
Sirpb1c									
Ms4a6d									
Ttc7									
Igip									

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Chapter 5. Discussion and Future Directions

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease with heterogenous clinical manifestations. Twenty-five gene loci in the genome are involved in the susceptibility of the disease. Some of these gene loci suggest that plasmacytoid dendritic cell (pDC) signaling through toll like receptors (TLRs) and Type I interferon (IFNs) may be involved in lupus progression (1-5). Further studies have demonstrated that IFN-alpha is a central mediator in the pathogenesis of SLE (6-9). Moreover, pDCs as major producers of type I IFNs have also been reported to secrete IFN-alpha upon self-antigen stimulation and play a key role in the initiation of lupus nephritis (LN) (9, 10). Histone deacetylase (HDAC)6 belongs to class IIb of the HDAC family (11-13). Besides deacetylase-dependent enzymatic activity on tubulin (14), heat shock protein (HSP)90 and cortactin (15, 16), HDAC6 can also interact with proteins in cellular processes such as protein acetylation and ubiquitination (17-19). The versatile functions of HDAC6 suggest that it participate in cell signaling, activation, survival, motility, and degradation of misfolded proteins, which can all contribute to inflammation and autoimmunity (20).

In our study, with the treatment of NZB/W mice with an HDAC6 inhibitor in the initial stage of the disease, we found a significant decrease in the production of IFN-alpha along with the reduction of MHC-II^{-} Ly6a^{-} pDCs and increase of MHC-II^{+} Ly6a^{+} pDCs. These changes are associated with reduced autoreactive B cell responses, decreased B-cell help to pathogenic T cells, and improved renal functions. This suggests that HDAC6 inhibition may be a new therapeutic strategy for SLE which works by dampening the IFN-alpha response of pDCs. Therefore, for future investigations, we hypothesize that HDAC6 inhibition acetylates proteins in

IFN-alpha producing signaling pathway in pDCs and impairs the production of IFN-alpha in lupus-susceptible mice, thus ameliorating lupus disease.

Moreover, as we demonstrated the decrease of Th1 and Tfh cells in the spleen of NZB/W mice treated with ACY-738 at the early stage of lupus development, it is highly possible that HDAC6 is involved in the differentiation of naïve $CD4^+$ T cells into Th1 and Tfh cell subpopulations. We can study the *in vitro* Th cell differentiation with the addition of ACY-738 to directly study the influence of HDAC6 inhibition on T cells.

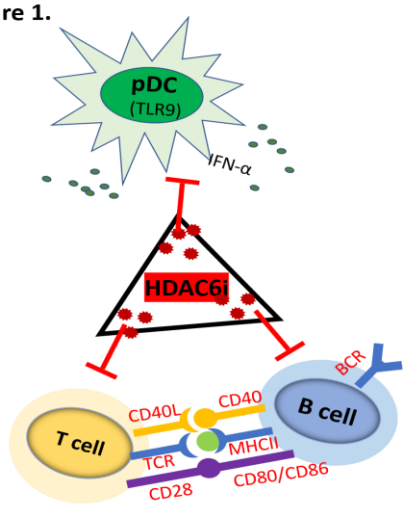
In addition, our data of decreased germinal center B cell and plasma cell populations are consistent with RNA-seq analysis on the transcriptomic signatures of splenocytes, where we also found decreased germinal center activities and B-cell activation signaling pathways. Further studies on the metabolism of splenocytes suggest that HDAC6 inhibition-induced alterations of cellular metabolism might contribute to the normalization of the autoimmune genomic signatures associated with murine lupus. However, as the RNA-seq data were generated from bulk splenocytes with global inhibition of HDAC6, we did not specifically study the effect of HDAC6 inhibition in single-cell populations. In the future, we can generate B cell- or T cell-specific HDAC6 conditional knockout mice on lupus background to further study the mechanism of how HDAC6 inhibition alters cell signaling pathways and affects cellular functions.

Finally, we compared the gene signatures between the HDAC6 inhibitor-treated lupus mice and human lupus patients that indicated numerous immune and inflammatory pathways increased in both human and mouse. However, to make HDAC6 inhibitors as a possible new treatment

method in the clinic, the effects and safety of HDAC6 inhibitors should be tested in an *in vivo* model with the characteristics of the human immune system before clinical trials can be carried out. The generation of NSG humanized lupus mouse model can be one attractive solution for this issue. By transferring peripheral blood mononuclear cells of SLE patients into immunocompromised NSG mice, a humanized lupus mouse model can be established. Different HDAC6 inhibitors will be tested in this model to confirm what have been found in lupus-prone mouse models and to examine the possible toxicity of these drugs on the human immune system.

There are still many questions waiting to be answered before the actual application of HDAC6 inhibitors as a treatment for SLE. For example, what are the molecular targets of HDAC6 or HDAC6 inhibitors in different immune cells? Will the inhibition of HDAC6 affect normal immune responses against infections and/or tumor elimination? What is the optimal dose of HDAC6 inhibitors that will also prevent possible side effects? How to specifically deliver HDAC6 inhibitors to targeted immune cell populations without affecting other cells in order to limit side effects? These questions require further investigations in both mouse and humanized lupus models.

Summary Figure 1.



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