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Review

Regulation of non-histone proteins by HDAC6 in systemic lupus erythematosus

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ABSTRACT

Therapeutic inhibition of histone deacetylase (HDAC) enzymes has been widely reported for the treatment of many cancers. Recently, increasing evidence of HDACs playing a role in regulating inflammation and immunity has triggered more in-depth investigations on how pharmacologic HDAC inhibitors could be beneficial in treating inflammatory and autoimmune diseases. Initial investigations of HDAC enzymes focused on their ability to regulate gene transcription by removing acetyl groups from lysine residues of core histone proteins. Current research indicates a broad repertoire of non-histone proteins that could also act as substrates for HDAC enzymes, further expanding their regulatory potential in cell processes. There are 18 known HDAC enzymes classified based on structure and function into classes I-IV. As pan-selective HDAC inhibitors have been reported to show adverse side effects, isoform-selective inhibitors are becoming more desirable as pharmacologic agents. In this review, we discuss the current understanding of how HDAC6 contributes to the pathogenesis of systemic lupus erythematosus (SLE), therefore making it a suitable candidate for selective pharmacologic inhibition.

KEYWORDS: HDAC6, SLE, tubulin, β-catenin, HSP90, Smad7, Foxp3, Ku70, treatment

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that affects an estimated 140 per 100,000 individuals in the United States of America [1]. The underlying etiology of SLE is unknown, but multiple influences and predispositions from genetic abnormalities as well as environmental and hormonal factors have been attributed to the development of the disease [2, 3]. Ultimately, patients exhibit abnormalities in immune tolerance, B and T cell signaling and function, innate immune responses, cytokine and chemokine production, apoptosis and subsequent clearance of debris, and autoantibody formation [4, 5]. These abnormalities culminate in progressive, relapsing damage of multiple organs including the kidneys, joints, skin, heart, lungs, blood vessels and brain [6].

Although genome-wide association studies have identified many genes that may play a role in the initiation or progression of SLE [7-9], these studies do not account for potential risks attributed to heritable factors [10], and have failed to identify a unifying switch. This has led researchers to investigate other factors involved in disease pathogenesis. Alterations in gene expression and phenotype which are heritable but do not alter the DNA sequence comprise 'epigenetics' [11]. There is increasing evidence that epigenetics may play a key role in SLE pathogenesis, and epigenetic-targeted therapies may be efficacious [12, 13]. Of particular interest for this review are interactions between DNA and core histone proteins, which are important epigenetic mechanisms regulating the exposure and binding of promoter regions of genes to regulate transcription [14]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) can alter the charge and subsequent binding affinity of core histone proteins through removal or addition of acetyl groups on

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lysine residues and thus alter gene transcription [15-17]. Furthermore, investigations have revealed that HATs and HDACs are also capable of modifying lysine residues on numerous non-histone nuclear and cytosolic proteins [17, 18], which has driven some researchers to alternatively refer to the enzymes as lysine (K) acetyltransferases (KATs) and lysine deacetylases (KDACs).

There are 18 mammalian HDACs which remove acetyl groups from lysine residues in histones and other proteins to control multiple cellular functions including transcription, cell cycle kinetics, cell signaling and cellular transport processes [19]. HDACs are classified into classes I-IV based on structure, homology to yeast HDACs, and function [20, 21]. Class I HDACs (HDAC1, -2, -3, and -8) are nuclear-exclusive enzymes found in a wide range of tissues and cell lines, where they are known for histone modification and repression of transcription [22, 23]. Class II HDACs are further subdivided into class IIa (HDAC4, -5, -7, and -9) and class IIb (HDAC6, and -10) based on domain organization [24], and exhibit selective tissue expression as well as nucleocytoplasmic shuttling, and function through recruitment of distinct cofactors [23]. Class III comprises the sirtuins, which act through a distinct NAD^+-dependent mechanism and are not considered 'classical' HDACs [22]. HDAC11 is the sole member of class IV as phylogenetic analysis revealed very low similarity to HDACs in the other classes [20].

In addition to their initial relevance in cancer biology [25], HDAC enzymes are now increasingly being investigated as regulators of inflammation and immunity [22]. As reviewed by Shakespear *et al*., HDACs are documented to play a role in myeloid development, Toll-like receptor (TLR) and interferon (IFN) signaling in innate immune cells, antigen presentation, and development and function of B and T lymphocytes [22]. Subsequently, pharmacologic inhibition of HDACs has been evaluated as a possible treatment modality in a wide spectrum of diseases, including inflammatory and autoimmune diseases [26].

The use of non-selective HDAC inhibitors has been shown to decrease disease in lupus-prone MRL/lpr and NZB/W mice [27-30]. Mechanisms by which HDAC inhibition decreases SLE disease have previously been reviewed by Reilly *et al*. [16]. Some of the highlights include: corrected hypoacetylation states of histones H3 and H4 [31], increased $CD4^+CD25^+Foxp3^+$ T regulatory (Treg) cells [28, 30], reduced Th1- and Th17-inducing cytokines (IL-12 and IL-23) as well as Th1-attracting chemokines [16], and inhibition of germline and post-switch immunoglobulin transcripts in splenic B cells [32]. Most importantly, decreased renal disease (glomerulonephritis and proteinuria) has been consistently reported in studies investigating the use of non-selective HDAC inhibitors to treat lupus in various mouse models [27-30]. However cytotoxicity remains a concern with long-term treatment [29, 33]. Indeed, pan-selective HDAC inhibitors available in the clinic have been associated with abnormalities such as fatigue, nausea, vomiting, diarrhea, thrombocytopenia, neutropenia and cardiac irregularities [34]. Investigation of specific functions for each HDAC isoform in knockout mice reveal that elimination of class I and class IIa HDACs results in embryonic lethal phenotypes or fatal cardiac, vascular, musculoskeletal or neural crest defects [34, 35]. Therefore, it would be desirable to produce HDAC-inhibiting compounds that are time-, cell-, tissue-, and/or isoform-specific to improve safety, while still effectively reducing disease.

HDAC6

HDAC6 is a class IIb HDAC that localizes within the cytoplasm due to inclusion of both a nuclear export signal and a Ser-Glu-containing tetrapeptide domain [21, 36]. Therefore, HDAC6 predominantly contributes to cell functions within the cytoplasm, including cell signaling, activation, survival, motility and protein degradation [37], which can all contribute to inflammation and immunity. HDAC6 knockout mice exhibit a viable phenotype, develop normally, and have no life-limiting defects. Interestingly, lymphocyte development as well as lymphocyte numbers in these mice are normal, and there is a mild decrease in the immune response after antigenic stimulation [38]. Documentation of HDAC6 playing a role in the formation of the immune synapse in T cells [39] and chemotaxis in lymphocytes [40] provides supportive evidence for its immunomodulatory effects. In regards to SLE, we have observed increased expression and activity of HDAC6 within B cells, T cells and glomerular cells of diseased lupus-prone mice [41]. Additionally,

selective HDAC6 inhibition in lupus mice ameloriates disease pathogenesis by decreasing renal histopathology scores, IgG and C3 immune complex deposition, and proteinuria [42]. To further understand how HDAC6 contributes to disease, the remainder of this review discusses the roles of non-histone protein targets of HDAC6 in the context of SLE.

Non-histone substrates of HDAC6

Tubulin

Tubulin heterodimers are the building blocks of microtubules and serve as a target for HDAC6 [37]. Microtubules are vital in maintaining morphology and structure of the cell and many subcellular structures, creating a diverse repertoire of functions that can be regulated by post-transcriptional modifications like acetylation [43]. As reviewed by Li *et al*., multiple studies have linked tubulin acetylation to immune responses [43]; however the role of tubulin acetylation in SLE pathogenesis is yet to be defined.

SLE patients with lupus nephritis almost always exhibit podocyte pathology on renal biopsy [44], the degree of which correlates with proteinuria [45]. Microtubules, and therefore tubulin, are important for the structural integrity and physiology of podocytes [46]. Increased acetylation of tubulin serves as a marker for improved microtubule stability [43]. As previously mentioned, HDAC6 knockout mice exhibit hyperacetylation of tubulin in most tissues [38]. Our laboratory has documented increased alpha-tubulin acetylation in mesangial cells treated with a selective HDAC6 inhibitor *in vitro* [41]. Furthermore, diseased lupus-prone mice had increased HDAC6 expression and activity in glomerular cells, which was reduced after pharmacologic inhibition of HDAC6 [41]. More recently, we have observed decreased lupus nephritis and proteinuria in murine models after pharmacologic HDAC6 inhibition in conjunction with increased acetylation of tubulin in glomerular cells (publication under review). Overall, inhibition of HDAC6 increases acetylation of tubulin and subsequently improves microtubule stability and podocyte structural integrity, which may help alleviate renal damage in SLE.

Another possible mechanism for decreased nephritis observed in lupus-prone mice after selective HDAC6 inhibition may be due to its inhibition of nuclear factor kappa B (NF-κB). NF-κB is a transcription factor that regulates the expression of numerous genes that contribute to the inflammatory response in the kidney [47], and is constitutively activated in many autoimmune diseases including SLE [48]. In addition to increased tubulin acetylation, HDAC6 inhibition also reduced nuclear NF-κB protein in immune-stimulated mesangial cells *in vitro* [41]. Nephrin, a key protein involved in the slit diaphragm, is downregulated during podocyte injury, and when deficient activates NF-κB, which promotes glomerular injury [49]. In human podocytes cultured *in vitro*, promotion of foot process formation and maturation are associated with increased expression of both tubulin and nephrin [50]. The underlying connection between acetylated tubulin and NF-κB in the kidney is uncertain. However, given the current data, it is possible that nephrin in conjunction with tubulin acetylation acts to inhibit NF-κB in glomerular cells.

β-catenin

The function of β-catenin is dependent on its intracellular localization. On the cell membrane, β-catenin plays a role in junctional domains and adherence between epithelial cells. While in the cytoplasm, β-catenin participates in the canonical Wnt/β-catenin signaling cascade resulting in regulation of genes involved in cell proliferation, survival and differentiation [51]. Elevated β-catenin has been documented from kidney biopsies of SLE patients and in the kidney of NZB/W mice with lupus nephritis, suggesting increased Wnt/β-catenin activation [52, 53]. Hyperactivation of the Wnt/β-catenin cascade has been implicated in podocyte dysfunction with subsequent albuminuria as well as in renal interstitial fibrosis [54, 55]. HDAC6 deacetylates β-catenin, which regulates Wnt/β-catenin signaling [37]. When HDAC6 is inhibited, β-catenin nuclear translocation and downstream transcription factor expression are decreased [56, 57]. Therefore, inhibition of HDAC6 may help to diminish hyperactive Wnt/β-catenin signaling in lupus nephritis by increasing the acetylation and nuclear translocation of β-catenin.

Bone marrow transplantation studies in SLE patients and lupus-prone mice revealed abnormalities in mesenchymal stem cells (MSCs), which were further determined to be increased senescence related to

hyperactivation of Wnt/β-catenin signaling. It is thought that this increased senescence in bone marrow MSCs contributes to the failure of syngeneic bone marrow MSC transplantation [58]. The mechanism underlying altered Wnt/β-catentin in bone marrow MSCs in SLE is currently unknown; alteration in the acetylation of β-catenin could be a possibility and warrants further investigation.

Heat shock protein 90

Heat shock protein (HSP) 90 is one of many heat shock proteins, which contribute to housekeeping functions and act as chaperones that play an important role in mediating normal protein folding, prevention of damaging protein aggregation, and transportation of proteins through various cell compartments [59-61]. HSPs are intracellular proteins that may also be released extracellularly, are upregulated in relation to various cell stressors, and contribute to the physiology of inflammation and immune responses [62, 63]. Inhibition of HDAC6 results in hyperacetylation of HSP90, leading to a subsequent loss of HSP90 chaperone activity [64, 65].

Elevated levels of HSP90 in peripheral blood mononuclear and lymphoid cells of SLE patients [66-68] and within the spleen of lupus-prone MRL/lpr mice [69, 70] is attributed to increased IL-6 and enhanced expression of the hsp90β gene [66, 67]. Further studies in IL-6 transgenic mice support the notion that elevated IL-6 results in higher HSP90 levels and also correlates with the production of anti-HSP90 autoantibodies [70]. Anti-HSP90 autoantibodies are primarily of the IgG isotype [71] and SLE patients with elevated levels of these autoantibodies are more likely to have low levels of C3 and renal disease [72]. Autoantibodies to HSP90 have been detected in glomerular and mesangial deposits in SLE patients with glomerulonephritis [73], implying a pathogenic nature of these autoantibodies.

In lupus-prone MRL/lpr mice, treatment with the HSP90 inhibitor ganetespib decreased proteinuria, total number of IgG-positive glomeruli and glomerular pathology scores [74]. Within mesangial cells, expression of nitric oxide (NO), IL-6 and IL-12 in response to inflammatory stimuli was decreased after inhibition of HSP90 [75]. The decrease in these inflammatory cytokines is likely related to a reduction in the expression of inhibitor of κB (IκB) kinase and decreased nuclear factor-κB (NF-κB) translocation to the nucleus, as we have observed in J774 macrophages [76]. Further, HSP90 inhibition in macrophages also prevents HSP90 chaperoning of newly synthesized cytokines [77]. While HSP90 inhibition results in decreased inflammatory cytokine expression, we found that there were no differences in IgG or C3 deposition in glomeruli or glomerular pathology scores in MRL/lpr mice despite reductions in proteinuria following treatment with 17-DMAG when compared to controls [75]. However, pharmacologic inhibition of HDAC6 results in increased HSP90 acetylation and decreased nuclear translocation of NF-κB in immune stimulated mesangial cells [41], as well as decreased glomerular pathology scores and deposition of IgG and C3 in NZB/W mice [42].

Abnormalities in T cell signaling, phenotype, activation, and function all play a role in the pathogenesis of SLE [78]. HSP90 plays a role in the activation of T cells by stabilizing lymphocyte-specific protein tyrosine kinase (Lck) [79] and by being an essential regulator for the expression of LAT (linker for activation of T cells) [80]. In our laboratory, inhibition of HSP90 in MRL/lpr mice decreased the number of double negative T cells and increased $CD8^+$ T cells within the spleen, culminating in a reduced CD4/CD8 ratio [75]. Further, reductions in $CD4^+$ T cells in the lymph nodes and spleen after inhibition of HSP90 homologue gp96 have been documented in mice with lupus-like disease [81]. In both of these studies, alterations in T cell populations occurred in conjunction with ameliorated lupus-like disease in mice [76, 81], suggesting therapeutic potential of HSP90 inhibition in T cell-mediated diseases. As HSP90 is a substrate of HDAC6 [64, 65], HDAC6 inhibition also carries the potential to exert similar results in treating autoimmune diseases like SLE. In fact, selective HDAC6 inhibition with ACY-738 in NZB/W mice decreased double negative T cells in the thymus in addition to increasing Treg cells in the spleen [42]. Additionally, in our current studies, we have found that selective HDAC6 inhibition in NZB/W mice decreased the number of Th17 cells in the spleen (unpublished data). Whether these alterations in T cell subtypes are attributed to modulation of HSP90 or other HDAC6 substrates warrants further investigation.

Plasmacytoid dendritic cells (pDCs) are the primary secretors of type I interferons [82] in response to engagement of TLRs 7 and 9 by nucleic acids [83]. Increased stimulation of pDCs subsequently increases the secretion of interferon (IFN)-α and is implicated in the maintenance and progression of disease in SLE [84]. Recently, HSP90 has been shown to be crucial in TLR 7/9-mediated INF-α production by pDCs through associating with and delivering TLR7/9 from the endoplasmic reticulum to early endosomes and mediating self-nucleic acid recognition in SLE [85].

Smad7

Transforming growth factor-beta (TGF-β) is an important mediator of fibrosis in multiple chronic kidney diseases, including lupus nephritis [86]. Interestingly, reduced levels of TGF-β in immune cells coincides with increased levels in target organs [87], including kidneys in SLE patients with lupus nephritis [88]. These imbalances predispose to autoantibody production and contribute to tissue inflammation and extracellular matrix production.

Smad7 is an inhibitory molecule involved in the TGF-β signaling cascade and acts by promoting ubiquitination and degradation of receptor complexes [89]. Additionally, Smad7 contributes to the suppression of renal inflammation by inducing IκB and therefore inhibiting NF-κB-driven inflammatory responses [90]. Gene therapy to increase the expression of Smad7 in kidneys has been documented to decrease inflammation and histologic damage as well as ameliorate chronic kidney diseases, including autoimmune crescentic glomerulonephritis in mice [91, 92]. Smad7 synthesis is increased in podocytes, but not mesangial cells, treated *in vitro* with TGF-β and in NZB/W mice with immune-mediated glomerular injury [93]. While overexpression of Smad7 inhibits profibrotic Smad3-dependent TGF-β signaling in podocytes, it may alternatively shift TGF-β signaling activities towards apoptotic responses [93]. TGF-β also enhances transcription of Smad7 in peripheral blood mononuclear cells (PBMCs). However, in one study, PBMCs from 50% of lupus patients failed to transcribe Smad7 in response to TGF-β [94]. Whether this resistance is also found in renal cells of SLE patients, and how this resistance in PBMCs plays a role in the propagation of lupus nephritis is uncertain. HDACs have been found to interact with and deacetylate Smad7 resulting in its decreased stability [95]. Our laboratory has previously documented increased expression and activity of HDAC6 in glomerular cells of diseased MRL/lpr mice [41], which may be contributing to the progression of lupus nephritis due to the decreased stability of Smad7. While currently unproven, it is possible that this mechanism is partly responsible for the decreased lupus nephritis we have documented in NZB/W mice treated with HDAC6 inhibitors [42].

Interstitial inflammation and scarring in lupus nephritis is more reliable in identifying SLE patients that are at the greatest risk of developing renal failure [96]. TGF-β is a crucial cytokine that triggers myofibroblastic differentiation, which contributes to chronic fibrotic diseases [97]. Immunohistochemical studies using alpha-smooth muscle actin (α-SMA) show myofibroblastic differentiation in the interstitium of diseased kidneys [98], and in experimental renal scarring experiments alpha-SMA-positive interstitial cells increased over time as tubulointerstitial fibrosis progressed [99]. In one study, silencing of HDAC6 by RNA interference impaired TGF-β-induced α-SMA expression in fibroblasts [100]. Therefore, HDAC6 inhibition carries the potential to suppress the progression of renal fibrosis by blocking α-SMA expression.

Forkhead box P3 (Foxp3)

An important down-stream molecule in the TGF-β cascade is Foxp3, a transcription factor for regulatory T cells (Tregs); TGF-β promotes expression of Foxp3 and differentiation of Tregs from naïve $CD4^+$ T cells [101]. Complete loss of Foxp3 protein results in a lack of Tregs, and Foxp3-deficient (*scurfy*) mice develop a severe and fatal autoimmune disease [102, 103]. Tregs comprise approximately 2% of the $CD4^+$ T cell population in humans [104] and function to maintain immune tolerance to self-antigens and to suppress excessive and deleterious immune responses [105]. Reduced numbers and function of circulating Tregs have been reported in human SLE patients [106-109], as well as resistance of lupus effector cells to Treg-cell suppression [110]. In regards to lupus-prone mice, Treg cell frequencies are reduced in NZB/W mice before disease onset, while frequencies are mainly

reduced in diseased MRL/lpr mice and continue to decline as disease progresses [111]. Importantly, Treg cells suppress inflammation in the kidney, as depletion of $CD4^+CD25^+$ (Treg) cells in NZB/W mice results in accelerated development of lupus glomerulonephritis [112].

Treatment with non-selective HDAC inhibitors or a selective HDAC6 inhibitor has been shown to increase splenic Treg cell percentages in conjunction with decreased disease parameters in lupus-prone mice [28, 30, 42]. Our laboratory has also recently documented increased HDAC6 expression and activity in splenic $CD4^+CD25^+$ cells from diseased MRL/lpr mice [41]. Two studies have reported decreased suppressive functions of Tregs from diseased MRL/lpr mice [113, 114], which may be related to this elevated HDAC6. Tregs from HDAC6 knockout mice express more Foxp3 and exhibit enhanced suppressive function *in vitro* and *in vivo* [115]. Furthermore, there is more acetylated Foxp3 in Tregs in the absence of HDAC6, implying that HDAC6 deacetylates Foxp3 [116]. Collectively, HDAC6 inhibition results in Foxp3 acetylation, which increases Foxp3 stability and leads to increased Treg cell differentiation [117], development and function [118].

Ku70

Ku70 is a component of DNA repair machinery responsible for non-homologous end joining (NHEJ) of double strand breaks [119] and is also a substrate for HDAC6 [37]. HDAC6 deacetylates ku70, which plays a role in apoptosis through regulating cytoplasmic ku70 interactions with pro-apoptotic protein Bax or anti-apoptotic protein FLIP. In both mechanisms, inhibition of HDAC6 leads to increased apoptosis [120-122]. Abnormalities in apoptosis and clearance of apoptotic cells have been implicated in the etiopathogenesis of SLE. Defective apoptosis may contribute to the breakdown of tolerance by allowing autoreactive T and B lymphocytes to survive, allowing exposure of autoantigens to the immune system, and contributing to cell damage as an effector mechanism [123]. Studies have shown abnormalities in early checkpoints regulating B cell development and removal of autoreactive B cells within the bone marrow in SLE [124]. We have also recently identified alterations in the proportions of B cells in various stages of development and differentiation in the bone marrow of diseased NZB/W mice, suggestive of a possible apoptotic defect [42]. Furthermore, HDAC6 inhibition applied to pre-B cells *in vitro* increased Bax protein, which was associated with decreased cell growth [41]. Lastly, a recent genomic admixture mapping and molecular modeling study discovered an intronic single nucleotide polymorphism (SNP) that disrupts the activity of ku70/80 binding at a newly discovered SLE susceptibility locus [125]. This abnormality could contribute to autoantibody production and interaction [125] since the ku70/80 complex mediates the predominant pathway of NHEJ during immuno-globulin class switch recombination [126]. Based on these results, further studies are warranted to establish a possible link among ku70, Bax protein, and HDAC6 inhibition in the regulation of bone marrow B cell development in SLE.

CONCLUSION

Treatment for SLE has relied on the administration of nonsteroidal anti-inflammatory drugs, anti-malarial agents, glucocorticoids and immunosuppressants (cyclophosphamide, methotrexate, mycophenolate mofetil, etc.) [2, 127]. These treatment regimens are often intensive, associated with side effects, and still carry the potential for relapse and progression of disease flares [128]. Continued research of the molecular mechanisms involved in SLE pathogenesis has led to the development of biological agents, like monoclonal antibodies, that target B cells, T cells, cytokines and components of the complement system [129]. However, there have been several disappointments in clinical trials involving these approaches [129]. Within the past 50 years, belimumab has been the only therapy approved by the US Food and Drug Administration for non-renal SLE [130]. Lupus nephritis is one of the most important manifestations of disease in SLE, contributing significantly to morbidity and mortality [131]. While recent prognostic studies have documented improvement in survival rates in SLE patients [132], the incidence of end-stage renal disease attributed to lupus nephritis has not changed [133] and several questions remain unanswered. Therefore, the investigation for effective and safe treatments is still paramount in SLE research.

The multifactorial etiology and involvement of multiple branches of immunity and inflammation

in SLE creates a difficult disease to effectively and safely treat and manage. The biggest challenge in developing a compound for treatment is finding a balance between its specificity for SLE-associated aberrations and minimizing unwanted and deleterious side effects. As this review highlights, HDAC6 carries the potential to play a role in multiple target areas involved in SLE pathogenesis by controlling the acetylation status of its many substrates. Furthermore, when HDAC6 is knocked out, mice exhibit a viable phenotype with few alterations in the immune response [38], suggesting that inhibition of HDAC6 carries a certain level of safety. In addition to the potential of being a safe and efficacious treatment modality for SLE, HDAC6 inhibition has unveiled additional molecular pathways and abnormalities that will enhance our knowledge of SLE pathogenesis as they are investigated further.

CONFLICT OF INTEREST STATEMENT

The authors of this review have no conflicts of interest.

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