Molecular mechanisms of histone deacetylases in rheumatoid arthritis fibroblast-like synoviocytes
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Chapter 2

THE ACETYL CODE IN RHEUMATOID ARTHRITIS AND OTHER RHEUMATIC DISEASES.

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ABSTRACT

Growing evidence supports the idea that aberrancies in epigenetic processes contribute to the onset and progression of human immune-mediated inflammatory diseases, such as rheumatoid arthritis (RA). Epigenetic regulators of histone tail modifications play a role in chromatin accessibility and transcriptional responses to inflammatory stimuli. Among these, histone deacetylases (HDACs) regulate the acetylation status of histones and non-histone proteins, essential for immune responses. Broad-spectrum HDAC inhibitors (HDACi) are well-known anti-inflammatory agents and reduce disease severity in animal models of arthritis however, selective HDACi remain poorly studied. In this review, we describe emerging findings regarding the aberrant acetyl code in RA and other rheumatic disorders which may help to identify not only novel diagnostic and prognostic clinical biomarkers for RA, but also new targets for epigenetic pharmacological applications.
BACKGROUND

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune and inflammatory disorder characterized by destruction of synovial joints. One main feature of the disease is the infiltration of activated immune cells, including macrophages, T cells, and B cells in the synovial sublining, accompanied by the hyperplastic growth of the synovial intimal lining layer. Resident macrophage and synovial fibroblast-like synoviocytes (FLS) are activated when triggered by immune cell contacts and cytokines, contributing to the perpetuation of the inflammatory status in the synovium, ultimately resulting in the irreversible damage of bone and cartilage.\(^1\) Disease onset in RA appears to result from a complex interaction between a genetically susceptible background and (epigenetic) environmental influences such as diet and smoking.\(^2\) Genome-wide analysis studies (GWAS) have identified multiple genetic risk loci that partially overlap between multiple immune-mediated inflammatory diseases,\(^3\) and somatic mutations have been additionally characterized.\(^4\)

However, genetic predisposition only explains part of disease onset in RA, as the impact of environmental influences has been identified as a crucial combinatory factor that contributes to the risk of developing RA. In order to integrate the gap in our understanding of relationships between environmental and genetic contributors to RA onset and progression, recent studies have made important contributions to identifying the “missing epigenetic link”.\(^5\) Epigenetics, which describes alterations in gene modulation, independent of genomic sequence variations, and filtered through environmental influences, plays a central role in determining gene function and activity that, if altered, eventually confer instability to the immune system. It is not surprising that dysfunction in epigenetic processes, involving DNA methylation, histone modification and non-coding miRNA expression, are associated with the pathogenesis of RA, as well as other autoimmune rheumatic diseases.\(^6\)

An altered epigenetic state has been observed in RA FLS, and a recent report has demonstrated that chronic exposure to tumor necrosis factor (TNF), a major activator of these cells, promotes an “inflammatory cell memory” associated with increased histone acetylation promoting expression of pro-inflammatory chemokine genes.\(^7\) Additionally, an imprinted DNA methylome signature, and the enhanced expression of histone deacetylases (HDACs) and miRNAs have been reported.\(^6\) Together, these observations have led to the suggestion that FLS act as epigenetically imprinted aggressors in the development and progression of RA, rather than simply responders to immune cells infiltrating the synovium.\(^8\) In this review, we describe how aberrant histone modifications and the altered protein acetylome contribute to the inflammatory activation of RA FLS and other cells relevant to RA pathobiology, integrate these findings with current knowledge on related rheumatic diseases and assess the potential of HDACi as a new class of therapeutic agents for the treatment of chronic inflammatory arthritis.
CHAPTER 2

HISTONE POST-TRANSLATIONAL MODIFICATIONS

Nucleosomes are the essential building blocks of the chromatin, consisting of short segments of DNA and two copies of core histone proteins (H2A, H2B, H3, and H4). In resting cells, this organization provides a compact chromatin structure, as positive charges of histone proteins counteract negative charges of DNA. Therefore, gene transcription is promoted when histones are modified in a way to create an open, accessible form of chromatin needed for the recruitment of basic transcription machinery. The overall combination of histone post-translational modifications (PTMs) confers histones a specific language, named the “histone code”, which is interpreted by chromatin remodeling proteins and results in the regulation of gene transcription and other biological functions. Therefore, PTMs occurring on histones do not translate into a mere compacting or unwinding of the chromatin. Rather, how a histone mark is “written, read and cancelled” will determine whether gene expression will be ultimately turned on or off. Understanding how the histone code works could lead to therapies that selectively suppress, or induce, the expression of genes deregulated in disease conditions, like cancer and chronic auto-immune diseases. Histone post-translational modifications include a series of approximately 60 covalent biochemical modifications that mostly occur at the N-terminals of histone tails, although some have been observed in the histone globular domain. Here we review the current knowledge on the best-characterized histone PTMs, describe how these are deregulated in RA and in other rheumatic diseases and provide a general summary of these features (Table 1).

Histone acetylation
Acetylation of histones, in particular histone H3 lysine 27 (H3K27) is associated with a relaxed conformation of chromatin that favors transcriptional activity. Additionally, lysine acetylation functions as a recruiter mark for reader enzymes, such as bromodomain (BRD) proteins. Altered histone acetylation has been reported in multiple rheumatic diseases, including global H3 and H4 hypoacetylation in CD4+ T cells of patients with active systemic lupus erythematosus (SLE), with H3 acetylation levels negatively correlating with disease activity scores. In line with this finding, MRL-lpr/lpr mice suffering from an SLE-like condition also exhibit global histone H3 and H4 hypoacetylation. Conversely, H4 was found to be hyperacetylated in systemic sclerosis (SSc) B cells. While studies comparing healthy and disease conditions support the idea that a global aberrant histone acetylation status could occur in pathological conditions, we previously reported that synovial levels of specific H3K18 and global lysine acetylation were not sufficient to discriminate between inflammatory and non-inflammatory forms of arthritis. For this reason, loci-specific analyses of epigenetic markers will likely shed more insight into disease onset and perpetuation.

Recent studies have revealed that an intrinsically higher H4 acetylation around the MMP1 promoter, and H3 acetylation of the IL6 promoter are observed in RA FLS, as compared to OA FLS, and reflect the abundant production of these two inflammatory mediators. Additionally, key activators of FLS, such as IL-1β and TNF, also alter the acetylation status of histones, consequently impacting upon the production of pro-in-
The acetyl code in rheumatoid arthritis

Specifically, IL-1β-mediated induction of cyclooxygenase 2 (COX-2) in OA FLS requires H3 acetylation in the promoter region of this gene, while TNF treatment enhances production of the interferon-responsive gene CXCL10 in RA FLS by favoring both depletion and hyperacetylation of H4 in the CXCL10 promoter. In SLE T lymphocytes, induced levels of H3 acetylation were found in the promoter region of TNFSF7, a gene overexpressed in SLE T cells and triggering immunoglobulin overproduction. Conversely, in SLE monocytes, hyperacetylation of H4 was predominantly reported in the promoter regions of a majority of genes associated with regulation by the IRF1 transcription factor, but only partially overlapped with acetylation changes after IFN-α stimulation. These latter results suggest that changes in histone acetylation may not

<table>
<thead>
<tr>
<th>Histone acetylation mark</th>
<th>Localization</th>
<th>Cell/tissue</th>
<th>Disease type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3ac ↑</td>
<td>IL6 promoter (-101/-16 bp)</td>
<td>FLS</td>
<td>RA vs OA</td>
<td>[16]</td>
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<tr>
<td>H4ac ↑</td>
<td>MMP1 promoter (-552/-488; 1524/-1464 bp)</td>
<td>FLS</td>
<td>RA vs OA</td>
<td>[17]</td>
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<tr>
<td>H4ac ↑</td>
<td>CXCL10 promoter (N.A.)</td>
<td>TNF-stimulated FLS</td>
<td>RA</td>
<td>[7]</td>
</tr>
<tr>
<td>H3K18ac ↔</td>
<td>global</td>
<td>Synovial tissue</td>
<td>RA vs OA</td>
<td>[15]</td>
</tr>
<tr>
<td>H3ac↑ H4ac↑</td>
<td>PTGS2 promoter (-266/+12)</td>
<td>IL-1β-stimulated FLS</td>
<td>OA</td>
<td>[18]</td>
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<tr>
<td>H3ac ↓ H4ac ↓</td>
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<td>CD4+ T cells</td>
<td>SLE</td>
<td>[12]</td>
</tr>
<tr>
<td>H3ac↑</td>
<td>TNFSF7 promoter (~533 to ~364 bp)</td>
<td>CD4+ T cells</td>
<td>SLE</td>
<td>[19]</td>
</tr>
<tr>
<td>H4ac↑</td>
<td>179 genes involved in ERK, p38, NFκB, CREB1, and IFNa pathways</td>
<td>Monocytes</td>
<td>SLE</td>
<td>[21]</td>
</tr>
<tr>
<td>H3K9K14ac ↓ H3K23ac ↓ H3K18K23ac ↓ H4K16ac ↓ H4K2K16ac ↓ H4K8K12K16ac ↓ H4K31ac ↓</td>
<td>global</td>
<td>Splenocytes</td>
<td>Murine SLE model</td>
<td>[13]</td>
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<tr>
<td>H4ac ↑ H3ac ↔</td>
<td>global</td>
<td>B cells</td>
<td>SSc</td>
<td>[14]</td>
</tr>
<tr>
<td>H3ac ↓</td>
<td>FLI1 promoter region (-270 to -31bp)</td>
<td>Fibroblasts</td>
<td>SSc</td>
<td>[22]</td>
</tr>
</tbody>
</table>

Table 1. Histone marks associated with RA and other rheumatic disorders

H3=histone 3; H4=histone 4; K=lysine; IL6= interleukin 6; MMP=metalloprotease; CXCL10=C-X-C motif chemokine 10; PTGS2=prostaglandin-endoperoxide synthase 2; TNFSF7=Tumor Necrosis Factor Ligand Superfamily Member 7; ERK= extracellular signal-regulated kinase; p38= mitogen-activated protein kinase 14; NFκB=nuclear factor kappa-light-chain-enhancer of activated B cells; CREB1=CAMP responsive element binding protein 1, IFNα=interferon α; FLI1= Friend Leukemia Virus Integration 1; FLS=fibroblast-like synoviocytes; TNF=tumor necrosis factor; IL-1β=interleukin 1 β; CD4=cluster of differentiation 4; RA=rheumatoid arthritis; OA=osteoarthritis; PsA=psoriatic arthritis; SLE=systemic lupus erythematosus; SSc =systemic sclerosis; N.A.= not available. ↑, ↓, ↔ arrows indicate increased, decreased and comparable histone marks levels, respectively, as compared to healthy individuals or disease controls.
simply reflect the need for chromatin opening following an inflammatory stimulus, but constitute a perpetually deregulated epigenomic mark modulating gene expression in this disease. Finally, the reduced expression of \textit{FLI1}, a suppressor of pathological extracellular matrix deposition, was observed in fibroblasts derived from \(\text{SSc}\) patients, and associated with low levels of H3 and H4 acetylation in \textit{FLI1} promoter region.\textsuperscript{22} Taken together, these observations indicate that therapeutic strategies able to restore the histone acetylation balance could be beneficial to reduce clinical manifestations in rheumatic diseases.

**WRITERS, READERS AND ERASERS OF THE HISTONE CODE**

Chromatin-remodeling proteins that add, recognize and remove covalent chemical modifications on histone proteins are referred to as “writers, readers and erasers”, respectively.\textsuperscript{6} In this review, we specifically assess proteins that regulate the acetylation of histones and other proteins. Other enzymes affecting diverse post-translational modifications are known, and described elsewhere.\textsuperscript{11} Histone acetyltransferases (HAT) mediate the transfer of acetyl groups from acetyl coenzyme A (acetyl-CoA) to the \(-\epsilon\)-amino groups of lysine residues in histones and non-histone proteins. Multiple HATs proteins are known, such as the p300 and CREB-binding protein (p300/CBP), the Gcn5-related N-acetyltransferases (GNATs), the MYST-domain lysine acetyltransferases (MYSTs) and the Histone Acetyltransferase 1 (HAT1 or KAT1), differing in their roles and cellular localization. However, they share similar catalytic domains in the proximity of the acetyl-CoA binding site.\textsuperscript{23} Histone deacetylases (HDACs) counterbalance HAT activity by deacetylating histones, as well as non-histone proteins. Eighteen human HDACs enzymes have been identified, divided into four classes based on their phylogenetic comparison with yeast: class I Rpd3-like proteins (HDACs 1-3 and 8); class II Hda1-like proteins, subdivided into class IIA (HDAC4-5, 7 and 9) and class IIB (HDAC6 and 10) HDACs; class III Sir2-like sirtuins (SIRT1-7) and class IV (HDAC11). While class I, II and IV HDACs enzymatic activity requires zinc to deacetylate acetyl lysine substrates, sirtuins require nicotinamide adenine dinucleotide (NAD+) as a cofactor. Additionally, tissue distribution and cellular localization of different HDACs classes varies: class I HDACs are ubiquitously expressed in tissues and are almost exclusively present in cell nuclei; class II HDACs display a specific tissue distribution, and shuttle between the nucleus and cytoplasm to serve as tracking proteins for other HDACs and co-repressor complexes.\textsuperscript{6}

The global level of histone acetylation results from a balance between opposing writing and erasing activities of HATs and HDACs. However, the interpretation of these acetylation marks ultimately determines their functional outcome. Bromodomains (BRDs) are protein domains that specifically recognize \(-\epsilon\)-amino groups of lysine acetylation motifs. Initially found associated with nuclear, but not cytoplasmic HATs, BRDs were thought to have a prominent role not only in the recognition of acetylated histones, but also in chromatin structure and transcriptional activation. To date, approximately 60 human BRD family members have been identified, including the bromodomain and extraterminal (BET) domain subfamily BRD2, BRD3, BRD4, and BRDT.\textsuperscript{24}
The acetyl code in rheumatoid arthritis

The class I HDAC unbalance in rheumatic disorders

When the physiological equilibrium between HATs, HDACs and reader proteins is altered, deregulation of gene transcription can result in pathological conditions, such as cancer and chronic inflammation. In RA, a global increase of HDAC activity was reported in peripheral blood mononuclear cell (PBMCs), which was not affected after 12-week conventional anti-TNF therapy. On the contrary, HAT activity levels did not differ between PBMCs of RA patients and healthy individuals. This study suggested that an unstable HAT/HDAC balance in RA may be due to an overloaded HDAC activity. Conversely, elevated HAT p300 levels were found in SSc skin biopsies and SSc fibroblasts, supporting the idea that an increased HAT activity may play a prominent role in the HAT/HDAC disequilibrium of other rheumatologic diseases.

Subsequent studies have aimed to unravel a potential link between the inflammatory status in RA and the expression or activity of distinct HDAC classes. We have reported that, upon IL-1β and TNF stimulation, the activity of specific class I and IIb, but not IIa, HDACs was increased in RA FLS. In addition, class I HDAC member expression was found to be elevated in RA FLS and synovial tissue, correlating with the expression of inflammatory mediators, such as TNF and MMP1. Class I HDAC1 has consistently been found to be upregulated in RA FLS, synovial tissue and PBMCs and, most recently, in SLE PBMCs. HDAC1 knockdown in RA FLS predominantly affected cell proliferation and migration, while impairment of HDAC1/2 had little influence on the regulation of immune-related genes. Nevertheless, mice lacking HDAC1 display decreased TNF and increased IL10 expression, in experimental arthritis experiments, associated with reduced paw inflammation, cartilage loss and bone damage in the CIA model, suggesting cell-specific inflammatory functions of HDAC1. With regard to the class I HDAC2, limited studies of genetically manipulated mice makes the interpretation of the role of this enzyme in RA difficult. Currently available reports have relied upon the use of inhibitors targeting both HDAC1 and HDAC2, two proteins that are highly homologous with redundant developmental functions in mice. Silencing gene expression of HDAC1 or HDAC2 individually led to a modest regulation of RA FLS proliferation and apoptosis, while knockdown of both family members reinforced pro-apoptotic effects. HDAC2 was additionally found to be overexpressed in chondrocytes from OA patients, and shown to induce osteoclastogenesis and bone resorption, two phenomena also associated with RA pathology.

We have reported that class I HDAC3 silencing in RA FLS suppresses IFN-β expression and, in turn, abrogates signal transducer and activator of transcription 1 (STAT1) activation. A similar finding was described in murine HDAC3-/- macrophages, which, upon triggering by LPS, were unable to activate half of their inflammatory gene response due to a defect in interferon signaling. Interestingly, this study also indicated that loss of HDAC3 in LPS-induced macrophages was associated with a reduction in acetylation in the IFNB1 promoter region, suggesting that a similar mechanism of action could occur in RA FLS lacking HDAC3 activity. Additionally, a whole-genome microarray study revealed that gene expression changes in murine HDAC3-/- macrophages closely resembled the gene expression profile of wild-type macrophages stimulated with IL-4, indicating that HDAC3 deletion may affect cell polarization, and drive macrophages to an alternatively activated, anti-inflammatory phenotype.
Class II HDACs and sirtuins: anti-inflammatory friends or targetable foes?

Unlike class I HDACs, class II HDACs have been less extensively characterized in RA. Multiple studies have reported anti-inflammatory functions of some of these family members, or other non-immune functions restricted to specific cell types. We have found that RA synovial HDAC5 expression negatively correlated with synovial expression of IL6 and with clinical parameters of RA disease activity. Additionally, RA FLS exposure to multiple inflammatory cytokines, such as IL-1β, TNF, and different TLR ligands, induced a robust and time-dependent decrease in HDAC5 production. Interestingly, lower HDAC5 expression exacerbated the production of IL-1β-driven and type I interferon-dependent chemokines in RA FLS, and promoted angiogenesis in endothelial cells. These studies suggest that, upon the activation of an inflammatory response, HDAC5 is “turned off”, possibly via changes in expression and/or nuclear export, to release its inhibitory function on transcriptional complexes that include the pro-inflammatory class I HDAC3.

Expression of another class II HDAC, HDAC4, is reduced in the cartilage of OA patients, and its overexpression decreases the production of Runx2, MMPs and pro-inflammatory genes associated with cartilage degradation, while increasing the production of type II collagen. Despite contrary findings reported in a different study, most recently the HDAC4 gene locus was found to be predominantly hypomethylated in the subchondral bone of OA patients. This epigenetic alteration could possibly explain the reduced expression levels observed in the above-mentioned study, and support the idea of a protective role of HDAC4 in preserving cartilage integrity. Similarly, HDAC7 was shown to suppress Runx2 activity and osteoblast differentiation, and to prevent chondrocyte survival and proliferation.

HDAC9 has been found to be increased in the T cell compartment of animal models of SLE, and its depletion was beneficial for disease activity rates. These data, combined with previous evidence showing HDAC9 as a suppressor of Foxp3 in Tregs, provide additional indications that HDAC9 may act as an epigenetic regulator in T cell-mediated systemic autoimmunity. While HDAC6 also plays a role in T cell tolerance and in macrophage activation, its depletion and inhibition in RA FLS did not affect IL-1β-mediated inflammatory gene expression responses, underlining possible cell type-specific functions of HDACs.

Sirtuins, class III HDACs, are known to play a role in cellular stress, inflammation, as well as cell metabolism. Studies of sirtuins in RA, however, are limited and varying results have led to a lack of consensus regarding their potential contributions to this disease. SIRT1 was found to be overexpressed in RA synovial tissue, FLS, and monocytes, but reduced in total RA PBMCs. In one recent study myeloid-specific SIRT1 knockout mice displayed reduced disease severity after collagen-induced arthritis (CIA), possibly due to critical regulation of dendritic cell maturation. In an independent study, mice transgenically overexpressing SIRT1 displayed an impaired differentiation of monocytes into macrophages, suppressed NF-κB transcriptional activity and reduced expression of pro-inflammatory cytokines. In SSc, SIRT1 has been proposed as a key regulator of fibrosis, as its expression is suppressed in SSc skin fibroblasts and further reduced by hypoxic conditions. However different reports had led to opposing suggestions either favor activation or inhibition of SIRT1 to attenuate fibrotic responses. SIRT2 KO was also investigated in arthritis models, and contributed to worsening of the disease and aggra-
vated inflammation.\textsuperscript{52} Finally, SIRT6 activity in chondrocytes was suggested to play a role in the maintenance of chondrocyte survival and cartilage homeostasis.\textsuperscript{46}

**Misreading the acetyl code in RA**

Amongst bromodomain (BRD) proteins, which recognize and interpret protein acetylation, BRD2, BRD3 and BRD4 have been reported to play a functional role in controlling inflammation. Even though the expression levels of these proteins is not different between synovial tissues derived from RA and OA patients,\textsuperscript{53} single nucleotide polymorphisms (SNPs) were found in the BRD2 locus of RA patients and associated with antibody positivity for citrullinated alpha-enolase peptide 1 (CEP-1) and cyclic citrullinated peptides (CCP). A single SNP was also identified for BRD1, and linked to early-phase radiological joint destruction in RA patients.\textsuperscript{54} Overall, these data suggest that the altered activity and expression of histone-modifying enzymes, and the presence of polymorphisms associated with histone readers could be associated with RA pathogenesis.

**BEYOND HISTONES: ACETYLATION OF NON-HISTONE PROTEINS**

Accumulating evidence suggests that the majority of HDACs are able to deacetylate non-histone targets. During the course of evolution, HDACs evolved earlier than histones, suggesting that non-histone proteins represent their primary substrates. Thus, the term lysine deacetylases (KDACs) would be more accurate, as it refers to the real biological activity of these molecules, rather than to their historical targets. In the last decade, approximately 3600 lysine acetylation sites in 1750 proteins were identified by high throughput mass spectrometry and in part implicated in a variety of cellular and immunological processes.\textsuperscript{55}

In RA, PBMCs revealed an altered acetylome, with one of the predominantly acetylated proteins identified as α-enolase (ENO1), a protein previously associated with the induced pro-inflammatory responses of RA monocytes and macrophages, as well as protection against apoptosis in RA FLS.\textsuperscript{56, 57} The acetylation of ENO1 was found to be required for protein activity, and facilitated lymphocyte activation.\textsuperscript{58} Deregulated signaling pathways in RA, including nuclear factor-kappa B (NF-κB), mitogen activated protein kinases (MAPKs), forkhead box (FOX) proteins and Janus tyrosine kinase (JAK)/STAT pathway, contribute to inflammatory gene expression and cellular survival in the synovium, and have been reported to be modulated by reversible acetylation.\textsuperscript{5} SIRT2-mediated deacetylation of the NF-κB p65 subunit, and SIRT1-dependent deacetylation of FoxO3, were both shown to be beneficial in the reduction of MMP, cytokine, and inflammatory gene expression in CIA, while limiting chemokine and pro-angiogenic factor gene expression in RA FLS.\textsuperscript{52, 59} Although direct evidence of acetylation of MAPK and JAK/STAT signaling molecules in RA has not been demonstrated, evidence from available literature suggests that HDAC1, -2, and -3 bind to and acetylate MAPK phosphatase 1 (MKP-1), while genetic silencing or inhibition of these HDACs impairs STAT1 transcriptional activity.\textsuperscript{60, 61} Taken together, these findings suggest a critical role for acetylation in the activation of classical inflammatory responses.
In RA and in other forms of inflammatory arthritis, cartilage degradation is susceptible to reactive oxygen species (ROS) production, which is counterbalanced by the antioxidant action of mitochondrial enzyme superoxide dismutase 2 (SOD2). SOD2 acetylation was associated with the impaired activity of the protein, and found increased in human OA cartilage. Interestingly, SIRT3 administration restored physiological SOD2 activity in vitro, while its deletion caused progressive knee destruction in vivo, supporting a crucial role for this mitochondrial deacetylase in the prevention of oxidative stress and cartilage degradation. The tumor suppressor p53, a protein also sensitive to oxidative stress, whose inactivated form was already shown to contribute to inefficient apoptotic responses of stromal cells in the inflamed joint, was found hyperacetylated in RA FLS following TNF treatment. While originally it was speculated that this modification would not affect p53 transcriptional activity, it is now known that acetylation of p53 promotes protein stability and activation and that SIRT1-mediated deacetylation facilitates its degradation. In conclusion, studies aiming to unravel the abnormalities in the RA acetylome, and their regulation and recognition by writers and readers proteins, may help to identify specific epigenetic drugs useful in the treatment of RA.

PHARMACOLOGICAL INHIBITION OF HISTONE ACETYLATION READERS AND ERASERS: THERAPEUTIC APPLICATIONS

Based on the growing number of observations that the epigenome is deregulated in RA and in other rheumatic diseases, novel compounds were developed in the last years with the aim to mitigate the abnormal activity of epigenetic proteins, and to restore physiological gene expression patterns. In the context of histone modifying enzymes, the number of HDAC inhibitors (HDACi) currently used at different stages of discovery and clinical trials outnumber those of other epigenomic targets. While this can be attributed in part to their extensive use in cancer research and treatment, studies in the last decade have also highlighted that HDACi display immunomodulatory functions at concentrations far lower than those used in cancer treatment.

Different modulators of HDAC and BET activity have demonstrated therapeutic potential in in vitro and in vivo models of rheumatic diseases, summarized in Figure 1. Treatment of biopsy explants, macrophages, FLS and PBMCs of RA patients with “first” generation HDACi, such as trichostatin A (TSA), resulted in the suppression of cytokines that play a crucial role in RA and SLE pathology. However, given the broad effects of first generation inhibitors, and the need for long-term safe and efficacious treatment, synthetic HDACi with optimized pharmacokinetic properties were developed. The so-called “second” generation HDACi include compounds as SAHA (vorinostat), FK228 (romidepsin), LBH-589 (panobinostat), all approved by the FDA for the treatment of hematological malignancies, and additional ones such as ITF2357 (givinostat), valproic acid and MS-275 (entinostat), which are currently in phase II and III clinical trials for different types of cancer and haematological disorders.

We previously reported that ITF2357 reduces expression of a broad spectrum of pro-inflammatory cytokines and chemokines, MMPs and anti-apoptotic genes in IL-1β-exposed
The acetyl code in rheumatoid arthritis

Figure 1. Timeline: History of the therapeutic use of HDAC and BET inhibitors in rheumatic disease models

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>Selective HDAC MS-275 displays better therapeutic effects in CIA model, compared to MTX and pan-HDAC SAHA</td>
</tr>
<tr>
<td>2010</td>
<td>The pan-HDAC TSA, PheBut, FK228 and SAHA reduce cytokine expression, synovial hyperplasia and bone destruction in AA, AMA and CIA models of RA</td>
</tr>
<tr>
<td>2011</td>
<td>Clinical efficacy of the orally active pan-HDAC ITF2357 in phase I trial in SOJIA patients</td>
</tr>
<tr>
<td>2012</td>
<td>Pan-HDAC ITF2357 displays inflammatory-suppressive properties in RA FLS</td>
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<tr>
<td>2013</td>
<td>Selective HDAC MI-192 reduces cytokines expression in RA but not healthy PBMCs, unlike pan-HDAC TSA</td>
</tr>
<tr>
<td>2013</td>
<td>Anti-inflammatory effects of BET inhibitor Q1 first assessed in CIA model of arthritis</td>
</tr>
<tr>
<td>2013</td>
<td>ITF2357 first tested in SLE-prone NZB/W marine model</td>
</tr>
<tr>
<td>2014</td>
<td>BET inhibitor JQ1 reduces SLE pathogenesis in MRL-lpr/lpr mice</td>
</tr>
<tr>
<td>2014</td>
<td>Resveratrol reduces fibrosis progression in vitro and in vivo models of SSC</td>
</tr>
<tr>
<td>2015</td>
<td>BET inhibitors JQ1 and BET151 display anti-inflammatory properties in RA FLS</td>
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<tr>
<td>2015</td>
<td>Butyrate and ITF-8 used to suppress cytokines production in in vitro acute gout model</td>
</tr>
<tr>
<td>2016</td>
<td>Selective HDAC3/6i recapitulates the anti-inflammatory effects of ITF2357 in RA FLS</td>
</tr>
<tr>
<td>2016</td>
<td>Selective HDAC ACY-738 limits SLE development in NZB/W mice</td>
</tr>
</tbody>
</table>

HDAC=histone deacetylase; BET=bromodomain and extra-terminal motif; TSA=trichostatin A; PheBut=phenylbutyrate; SAHA=suberoylanilide hydroxamic acid; MTX=methotrexate; AA=adjuvant arthritis; AMA=autoantibody-mediated arthritis; CIA=collagen-induced arthritis; SLE=systemic lupus erythematosus; FLS=fibroblast-like synoviocytes; PBMCs=peripheral blood mononuclear cells.
posed RA FLS, partially via inhibition of type I IFN production, or through destabilization of cytokine mRNA decay. Additionally, when used in CIA and adjuvant-induced arthritis (AIA) murine models, ITF2357 reduced bone destruction and joint swelling, and was efficacious both in therapeutic and prophylactic regimens, while SAHA and FK228 also displayed protective properties against murine rheumatic models in vivo. Interestingly, while low dose of SAHA limited disease activity scores in a rat CIA model of arthritis, higher concentration exacerbated arthritic symptoms, hinting that reduced dosage ranges could provide a better therapeutic window and possibly limit off-target effects. Given the remarkable anti-arthritic properties in pre-clinical studies, ITF2357 reached an open-label phase I clinical trial and was orally administered to children suffering from systemic onset juvenile idiopathic arthritis (SOJIA), showing excellent safety, and a remarkable improvement of the affected joints. Although, with the exclusion of SOJIA, second generation HDACi have not yet entered clinical trials for the treatment of rheumatic diseases, evidence from studies in vitro and in vivo support the idea that these might be considered in the future.

Inhibitors of histone acetylation readers, such as bromodomain proteins, were first discovered as potent anti-inflammatory agents in bacteria-induced septic shock. Later, they were proposed as therapeutic agents in different models of chronic inflammation. One BET bromodomain inhibitor (BETi), JQ1, blocked Th17 differentiation in vivo, protected mice from the development of autoimmune encephalitis and collagen-induced arthritis, and was also beneficial in a murine lupus model. In RA FLS, an inhibitor similar to JQ1, I-BET151, was shown to suppress the expression of chemokines and pro-inflammatory factors, consistent with parallel experiments in which BRD2,3 and 4 expression was silenced. Comparable findings in RA FLS were also confirmed with the use of JQ1, underlining the shared anti-inflammatory mechanism of action of these BETi. Despite extensive research, many questions remain unanswered regarding the specific role of histone enzymes in RA pathogenesis. The “next” generation inhibitors for histone erasers and readers should ideally consist of small molecules with enhanced target specificity, lower potential cytotoxicity, and increase efficacy in disease treatment. While specific bromodomain inhibitors are still in development, selective HDACi have started to emerge in the last years.

ISOFORM-SELECTIVE VERSUS PAN-SELECTIVE HDAC INHIBITORS

While the use of pan-HDAC inhibitors (pan-HDACi) has demonstrated anti-inflammatory effects both in vitro and in vivo, the contribution of specific HDACs to inflammatory processes is not completely unraveled. In order to gain more insight to this question, and to limit the possible side-effects which might be associated with the use of pan-HDACi, small molecules with improved HDAC selectivity have been developed in the last years. The reported values for the inhibitory activity of pan- or selective HDACi are summarized in Table 2. Class I HDAC expression or activity were most commonly associated with RA disease activity, and elevated in the synovial compartment. Growing interest in the understanding
of the individual contributions of these HDACs members in RA pathology has therefore fostered the development of class I specific HDAC inhibitors. Initial studies demonstrated that MS-275, an HDAC inhibitor with preferential effects against class I HDACs, displayed better anti-rheumatic properties in prophylactic CIA models, as compared to the pan-specific HDAC inhibitor SAHA and methotrexate (MTX). In line with these findings, recent pre-clinical studies of MPT0G009, an HDAC inhibitor with some selectivity for class I and class IIb HDACs, showed that this novel inhibitor displayed more pronounced anti-arthritic effects and pharmacokinetics in vivo, compared to SAHA. Butyrate, a natural HDAC inhibitor targeting class I HDACs and derived by the processing of dietary fibers, was recently shown to reduce urate crystal-induced cytokine expression in an in vitro model of acute gout, while phenylbutyrate was previously reported to favor anti-inflammatory responses in AIA arthritis model. The current limitation in the use of butyrate and other short-chain fatty acids, such as valproic acid, remains the low inhibitory effectiveness of these compounds, as millimolar concentrations are normally needed to insure cytokine suppression. Butyrate-similar synthetic compounds, on the contrary, have exhibited potent anti-inflammatory effects at nanomolar concentrations.

From class to isoform selectivity of HDAC inhibitors

Compounds with higher selectivity, targeting selective HDAC family members, rather than HDAC classes, are currently in development. The HDAC2-3 inhibitor MI-192 was reported to suppress IL-6 production in RA but not healthy donor PBMCs, and we have

<table>
<thead>
<tr>
<th>Table 2. IC50 values (nM) of different HDACi for HDAC1-11 isoforms</th>
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<tbody>
<tr>
<td><strong>Compound name</strong></td>
</tr>
<tr>
<td>TSA</td>
</tr>
<tr>
<td>SAHA (Vorinostat)</td>
</tr>
<tr>
<td>FK228 (Romintens)</td>
</tr>
<tr>
<td>Butyrate</td>
</tr>
<tr>
<td>Phenylbutyrate</td>
</tr>
<tr>
<td>LBH589 (Panobinostat)</td>
</tr>
<tr>
<td>ITF2377 (Givinostat)</td>
</tr>
<tr>
<td>Valproic acid</td>
</tr>
<tr>
<td>MS-275 (Orentinostat)</td>
</tr>
<tr>
<td>MPT0G009</td>
</tr>
<tr>
<td>IT-8</td>
</tr>
<tr>
<td>NW-21</td>
</tr>
<tr>
<td>HDAC12/16 inhibitor</td>
</tr>
<tr>
<td>MI-192</td>
</tr>
<tr>
<td>HDAC3/11 inhibitor</td>
</tr>
<tr>
<td>HDAC6 inhibitor</td>
</tr>
<tr>
<td>Tubostatin</td>
</tr>
<tr>
<td>ACY-738</td>
</tr>
<tr>
<td>HDAC8 inhibitor</td>
</tr>
<tr>
<td>PCI30451</td>
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</tbody>
</table>
recently demonstrated that selective HDAC3/6i treatment potently impairs pro-inflammatory gene expression in RA FLS. While we have not observed effects of HDAC1 or HDAC2 inhibition on RA FLS cytokine production, siRNA-targeting techniques, or use of the NW-21 inhibitor preferentially targeting HDAC1 and HDAC2 have shown reduction of RA FLS proliferation and osteoclast formation, respectively. These results suggest that inhibition of these HDACs could be beneficial in RA treatment, potentially limiting synovial hyperplasia and bone erosion.

We have found that an HDACi targeting HDAC8 could not suppress cytokine expression in RA synovial fibroblasts. In PBMCs, inhibition of HDAC8 was shown to limit LPS-induced IL-1β production at high concentrations, compared to the anti-inflammatory nanomolar concentrations used for other class I HDACi. Similar results were reported elsewhere, showing that impairment of HDAC8 limits IL-1β synthesis and the accumulation of its intracellular precursor, rather than total IL-1β. Recently, the HDAC8 inhibitor PCI34051 was found to confer a limited acetylation signature in human cells, providing evidence that this HDAC family member functions as a restricted, target-specific deacetylase.

Tubastatin A, a class IIb HDACi targeting HDAC6, was used in different murine arthritis models, in all cases reducing typical disease manifestations. However, the most prominent clinical results were obtained only at high concentrations of the compound, which were considerably higher compared to the therapeutic doses used for other pan-HDACi. More recently, low doses of ACY-738, a compound with preferential inhibitory activity for HDAC6, were shown to decrease autoantibody production and boost Tregs induction in a SLE murine model. These results support the critical role of HDAC6 in the regulation of T cell-dependent immune responses, and suggest the possibility that compounds targeting HDAC6 have therapeutic potential in chronic inflammatory diseases in which improved Treg function is desirable. With regard to class Ila HDACs, it is frequently assumed that class Ila HDACs display minimal enzymatic activity and function as scaffolding proteins for class I HDACs. However, recent reports have demonstrated that the lack of reported class Ila HDAC activity may be due to inappropriate probes, and that an improved chemical structure for class II inhibitors could make them better pharmacological drugs.

**SIRT1 activators rather than inhibitors?**

Available results regarding the therapeutic potential of targeting sirtuins in RA remain questionable. On one hand, SIRT1 was found to be overexpressed in cells derived from RA patients, and genetic silencing of SIRT1 is protective in the CIA model; on the other hand, different studies support that “activators” of SIRT1, such as resveratrol (RSV), could be beneficial in rheumatic diseases. One important question relies on the specificity of resveratrol for SIRT1, as this might act on other sirtuins, or be implicated in different processes. While an effort is being made to identify selective class II HDACi, and differentiate between the protective and pro-inflammatory roles of sirtuins, evidence from single knockdown and/or knockout experiments prevalently support a role of class I HDACs in rheumatic pathologies.
CONCLUSION AND FUTURE PERSPECTIVES

The introduction of biologic therapies in the treatment of RA, such as those targeting TNF or IL-6, have led to a better control of inflammation and disease progression in a subset of patient. As an unsatisfactory proportion of patients fails to respond to treatment, or encounters disease relapse soon after tapering of conventional therapies, the development of novel small molecule inhibitors has increased in the last years. Drugs modulating the epigenetic machinery have become of interest, following the growing recognition that the epigenome in RA patients is altered at multiple levels. Rapid progression in technological developments and high-throughput screening studies have supported systematic approaches and facilitated the identification of epigenetic marks in multiple rheumatic diseases. Specifically, studies documenting changes in DNA methylation in RA FLS have highlighted some potential biomarkers that could help to identify disease progression and response to treatment, and reversible acetylation of histone proteins has emerged as a potentially targetable epiphenomenon.

Here we describe that global or loci-specific alterations in histone acetylation are found in the myeloid, lymphocytic and stromal compartments derived from patients suffering from rheumatic diseases, including RA, OA, SLE and SSc. While outcomes of total cellular changes in histone acetylation results are difficult to interpret, altered acetylation levels at specific promoter regions allows a better understanding of the pathological processes that could be triggering disease outcome or progression. Detection of histone modifications at specific sites is still technically challenging, and interpretation is limited by the multiple post-translational modifications often occurring at concomitant loci and influencing chromatin regulatory activities in a combinatorial way. However, progress in the development of synthetic histones is providing a better understanding of sophisticated histone modifications. In addition, as acetylation marks are reversible, pharmaceutical targeting of HDAC activity, or stabilization of HAT activity, could provide new therapeutic opportunities.

Interest in histone acetylation has now led to the appreciation that the enzymatically-mediated acetylation of non-histone proteins regulates a wide variety of cellular and inflammatory processes. Protein acetylation has emerged as a critical post-translational modification that can affect protein function and activity, and subsequently modulate cell signal transduction and gene expression, in a fashion similar to protein phosphorylation. Here we describe that aberrant protein acetylation was found in samples derived from RA patients and could critically influence pro-inflammatory and anti-apoptotic responses in RA FLS.

As the net 'global' acetylation is orchestrated by the delicate balance of HAT and HDAC activities, aberrancies in the editing, or reading of these acetylation marks may represent a critical determinant for pathological cell behavior. While the activity and the expression of specific class I HDACs was found increased in RA synovial cells, suggesting a principal role of these enzymes in the pathological manifestations associated with this disease, HAT activity has been reported to be altered in other rheumatic disorder, suggesting that distinct HAT/HDAC unbalances could help distinguishing between different diseases. Techniques to determine global HDAC activity are already available,
and have been useful in discerning the HAT/HDAC profiles in healthy individuals and in patients with RA. However, given the identification of the involvement of specific HDACs in different inflammatory diseases, it seems necessary that highly selective compounds should be further developed, together with new accurate means to discern the activity of individual HDACs. In the past years, advances in the development of isoform-specific HDACi have helped to understand the specific contribution of HDACs family classes to inflammatory activation, providing an opportunity to target individual HDACs implicated in pathological processes.

In conclusion, while the “acetyl code” is not yet fully deciphered, its association with pathogenesis in RA and other chronic inflammatory diseases merits further investigation as to how pharmacological modulation of acetylation marks by the use of selective HDACi provide an opportunity to study the distinct role of HDACs in disease conditions, reverse inflammation, and limit potential side-effects caused by off-target specificity.

EXECUTIVE SUMMARY

• Histone and non-histone protein acetylation is tightly integrated in the control of cellular events critical to inflammatory processes.
• The acetylation code is the result of the dynamic regulation by writing, erasing and reading enzymes, namely histone acetyltransferases (HATs), histone deacetylases (HDACs) and bromodomain proteins (BRDs).
• HDAC expression and activity are deregulated in RA and in other rheumatologic diseases, and first and second generation HDACi have demonstrated promising results in in vitro and in vivo models of arthritis.
• Broad-specificity and possible off-targets effects of early generation pan-HDACi might be overcome by newly synthetized, class- and isoform- specific HDACi.
• Preliminary evidence in RA disease models indicate that specific epigenetic intervention of class I HDACs could result in the amelioration of disease activity, and suggest a wider applicability of these compounds to other forms of chronic inflammation.

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The acetyl code in rheumatoid arthritis

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

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