Adapted from: Grabiec AM, Tak PP, Reedquist KA
Function of histone deacetylase inhibitors in inflammation.
INTRODUCTION

Histone deacetylases (HDACs) display multi-faceted roles in coordinating the interaction of intracellular signaling pathways with chromatin remodeling and transcription factor function to finely specify gene alterations and maintenance of gene expression during cellular activation, proliferation and differentiation. These processes, epigenetic and non-epigenetic, are critical to the development of both the adaptive and innate arms of the mammalian immune system, and the measured initiation and resolution of immune responses. Inflammatory gene responses are tightly regulated by the reversible acetylation and deacetylation of histones. In resting cells, transcription of specific genes is often negatively regulated by histone deacetylases (HDACs). HDACs are recruited to the vicinity of histones, often by promoter-specific and tissue-specific transcriptional repressor proteins. Here, HDACs suppress regional gene transcription by maintaining histones in a hypoacetylated state. Extracellular stimuli, for example lymphocyte antigen receptor ligation, TNFα and LPS, induce association of transcription factors with HATs. HAT activity is then targeted to gene promoter regions, and acetylates histones, a requisite initial step in a series of histone post-translational modifications needed to permit induction of gene transcription. HATs also acetylate their associated transcription factors, resulting in modification of transcription factor stability, nuclear retention, and transcriptional activity. HDACs are temporarily displaced from the promoter region, but are later recruited in complexes with transcription factors and co-repressors to terminate gene transcription.

Each of the above-mentioned epigenetic processes can regulate the stable, inheritable maintenance of cellular gene expression patterns, but there is a growing appreciation that modification of chromosomal DNA and histones is a reversible process, subject to extracellular stimuli and environmental cues. This aspect of gene expression regulation, while utilizing the same enzymatic machinery responsible for classical epigenetic regulation of gene expression, is an integrated and essential part of cellular signal transduction pathways. Exemplary of this is the case of HDACs, where contributions of these enzymes to cellular activation, proliferation, and survival can readily be distinguished from effects on chromatin structure. Inhibition of cellular HDAC function results in changes in the expression levels of only a relatively small subset of genes, yet, intriguingly, some changes in gene expression can be more sensitive to changes in HDAC activity than global or promoter-specific histone acetylation status. This latter observation can probably be ascribed to direct influences of HDACs on signaling proteins and transcription factors (Fig. 1). Indeed, in evolutionary terms, these non-histone targets of HDACs represent the primary substrates of HDACs, as HDACs evolved prior to histones. Recent proteomic analyses have indicated that more than 1700 mammalian proteins serve as HDAC substrates, and a substantial number of these proteins are already recognized as key signal transduction components, transcription factors, structural proteins as well as regulators of mRNA stability, protein degradation and secretion, many of which regulate immune responses and inflammation. Moreover, cellular HDAC expression and activity is acutely and directly regulated by inflammatory signals. This concept is important, as it suggests the possibility that pharmacological manipulation of HDACs can be utilized to correct or influence specific signaling pathways and their gene targets in inflammatory diseases.

Recent studies have identified key roles for specific HDACs in regulating immune function, as well as alterations in HDAC expression and function in a number of immune-
mediated inflammatory diseases (IMIDs), such as asthma, systemic lupus erythmatosus (SLE) and rheumatoid arthritis (RA), which may contribute to pathology in these diseases. On the other hand, pharmacological modulators of HDAC activity have demonstrated uniformly potent anti-inflammatory effects in experimental animal models of these diseases, in relevant immune and stromal cell populations from patients, as well as initial successes in the clinic. Studies presented in this thesis were undertaken to characterize the contribution of changes in protein acetylation and HDAC expression to RA pathobiology, to understand the effects of inhibiting HDAC activity on inflammatory responses of cells relevant to RA, and to identify molecular mechanisms responsible for HDACi regulation of cellular inflammatory activation.
PROTEIN ACETYLATION AND HDAC EXPRESSION IN RA SYNOVIAL TISSUE – BEYOND EPGENETICS

Epigenetic mechanisms regulate the alteration and stabilization of gene expression during the development of adaptive and innate immune cells from hematopoietic stem cells, as well as the temporal changes in gene expression needed for the initiation and resolution of productive immune responses. Our inability to completely ascribe the etiology of RA and other complex IMIDs to genetic inheritance, environmental influences and infectious agents has suggested the involvement of epigenetic processes in these diseases. One component of epigenetic enzymatic machinery receiving increasing attention for its role in modulating immune function is the HDAC superfamily.

Stimulated by studies suggesting that in addition to genetic predispositions and environmental factors, epigenetic mechanisms might contribute to disease onset and pathology, investigation has been initiated examining if similar alterations in HDAC biology might contribute to pathology in RA. A first study analyzing potential involvement of HDACs in RA indicated that HDAC activity in RA synovial tissue was depressed compared to patients with non-inflammatory osteoarthritis (OA) and healthy controls. This was associated with decreased synovial protein expression of HDAC1 and HDAC2 as monitored by immunoblotting and immunohistochemistry, respectively. In particular, HDAC2 was notably absent in RA synovial macrophages, suggesting this cell population might be subject to altered acetylation-dependent chromatin-remodeling or signaling. However, an independent study observed increased HDAC activity in RA synovial tissue compared to OA and healthy donor controls, which correlated with local production of TNFα production in synovial tissue. Here, HDAC1 mRNA and protein expression was selectively elevated in RA synovial tissue, while a significant decrease in HDAC4 mRNA expression was noted. Other studies observed elevated HDAC activity in RA patient peripheral blood mononuclear cells compared to healthy individuals, and increased HDAC1 expression in isolated, cultured RA FLS compared to OA FLS. However, due to the limited size of these studies, it is as yet difficult to reconcile the relationship between HDAC activity, global acetylation of histone and non-histone proteins, and inflammation in RA.

We directly tested whether reported alterations in HDAC expression and activity are translated into changes in synovial protein acetylation status in RA, and analyzed potential correlations of protein acetylation and HDAC family member expression with local expression of inflammatory mediators and disease activity (chapter 4). We failed to find major differences in total protein and histone acetylation status in the synovial tissue of RA and OA patients, and cellular protein acetylation levels were not associated with RA patient clinical parameters of disease activity. Furthermore, we noted that inflammatory stimulation of RA FLS with cytokines or TLR ligands has no effect on global acetylation of histones and non-histone proteins (chapter 6). In light of studies demonstrating that cell exposure to HDACi affects expression levels of a relatively small proportion of expressed genes (2-5%), and the reported lack of correlation between histone acetylation and response to therapy in cancer patients treated with HDACi, our findings provide additional evidence suggesting that increased accessibility of gene promoter regions due to histone hyperacetylation is not sufficient to promote gene transcription. However, it remains an open question whether local alterations in histone acetylation (or other posttranslation modifications) at specific gene loci important to RA can be associated with disease pathology.
The data presented in chapter 4 indicate that protein acetylation status is associated with the composition of RA synovial tissue. We observed strong positive correlations between cellular protein and histone acetylation and the numbers of synovial macrophages, but not with FLS or T cell numbers. Consistent with this, we also demonstrated that acetylated proteins are most readily detected in macrophages infiltrating RA synovial tissue (chapter 3). While independent studies are needed to characterize molecular mechanisms underlying this phenomenon, these results indicate that among cell types detected in RA synovial tissue, macrophages express relatively highest levels of histone modifications characteristic for chromatin structure accessible to transcriptional machinery and, by extension, might represent the cell type most vulnerable to anti-inflammatory and/or pro-apoptotic activities of HDACi. Indeed, while 24 hour treatment with trichostatin A (TSA) failed to affect viability of RA FLS regardless of the

Figure 2. TNFα-induced expression of histone deacetylase 1 (HDAC1) might lead to perpetuation of inflammation in the rheumatoid joint via epigenetic and non-epigenetic mechanisms. Exposure of cells to TNFα causes activation of transcription factors (TF), their nuclear translocation, association with histone acetyl transferases (HATs), acetylation (Ac) of histones and binding of activated TF to DNA. These molecular events lead to transcriptional upregulation of HDAC1 expression. High levels of HDAC1 result in hypoacetylation and condensation of certain chromatin regions, which might in turn lead to epigenetic silencing of anti-inflammatory and pro-apoptotic genes. At the same time TFs which activity is modulated by reversible acetylation are in a non-acetylated state promoted by high HDAC1 activity which may induce expression of pro-inflammatory mediators as well as anti-apoptotic genes. Collectively, high expression of HDAC1 may promote survival and inflammatory activation of cells in the rheumatoid synovium. Treatment with TNF blockers might prevent upregulation of HDAC1, which could possibly explain reduced HDAC activity in patients treated with anti-TNF biologicals. Treatment with HDACi or silencing of HDAC1 causes hyperacetylation of chromatin and transcription factors, resulting in a shift in gene expression profile towards reduced production of inflammatory mediators and increased susceptibility to apoptosis.
dose, high concentrations of TSA induced apoptosis of macrophages and this effect was even more pronounced in the presence of inflammatory stimuli (Chapter 5). It remains to be verified experimentally whether these differences between FLS and macrophages in their susceptibility to pro-apoptotic properties of HDACi are functionally associated with HDAC activity or expression, and/or histone acetylation status.

We also noted complex relationships between HDAC family member expression in the synovial tissue and local expression of inflammatory mediators, as well as disease activity. While expression of class I HDACs positively correlated with TNFα and MMP-1 mRNA levels, we observed negative relationships between class II HDACs and disease parameters, as well as IL-6 mRNA expression. These observations suggested that class I HDAC activity might be involved in stimulating transcription of mediators of inflammation and tissue damage in RA. Interestingly, a recent report demonstrating that stimulation of RA FLS with TNFα leads to a transient increase in HDAC1 expression and activity raises the possibility that changes in the functional expression of HDACs in RA synovial tissue are secondary to local inflammatory input or patient treatment history (Fig. 2).20,27 However, RA FLS stimulation with IL-1β failed to significantly affect mRNA levels of any of the class I HDACs, indicating that regulation of HDAC expression might have a stimulus-specific character. It will therefore be important to conduct more extensive studies testing whether changes in synovial HDAC expression can be substantiated and, if so, whether this has functional consequences for synovial cell activations. On the other hand, IL-1β stimulation of FLS caused a rapid downregulation of HDAC5 and, to a lesser extent, other class II HDACs. This observation, together with negative correlations of synovial HDAC5 mRNA expression with RA patient disease activity, indicates that reduction of HDAC5 might serve as a potential biomarker of systemic inflammation. Indeed, reduced expression of HDAC5 has also been noted in peripheral lung tissue of COPD patients,28 raising the possibility that regulation of HDAC5 mRNA levels occurs in a myriad of inflammatory processes. Further studies are needed to characterize biological consequences of inflammation-mediated downregulation of HDAC5 in cell types relevant to RA pathology. Understanding the contribution of specific HDACs to regulating immune responses is essential to the design and therapeutic application of pharmacological inhibitors of HDAC (HDACi) activity, especially as compounds are just entering the clinic in the treatment of IMIDs.

**HDACi AS ANTI-INFLAMMATORY AGENTS IN RA AND OTHER IMIDs**

While details of the roles of specific HDACs in the differentiation and activation of immune cells are just emerging, considerable data is already available on the effects of HDACi on immune cell function, particularly in regard to myeloid-lineage cells and T lymphocytes. Several lines of experimental evidence suggest that multiple class I/II HDACi suppress production of inflammatory cytokines by immune cells: HDACi block LPS-induced production of TNFα, IL-1β, IL-6 and IFNγ by murine and human PBMC cultures, isolated monocytes and murine bone marrow-derived macrophages.29-32 HDACi also suppress production of chemokines regulating migration of myeloid cells and T cells by macrophages and dendritic cells,33 as well as dendritic cell differentiation and maturation,34-36 indicating that immunosuppressive activities of HDACi can be attributed to effects of these compounds on several components of the immune system.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Model</th>
<th>Species</th>
<th>Inhibitor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>Adjuvant-induced arthritis</td>
<td>rat</td>
<td>PheBut, TSA, FK228</td>
<td>Reduced paw swelling, inflammatory infiltration, synovial hyperplasia and bone damage, induction of cell cycle inhibitors, suppression of TNFα 90,91</td>
</tr>
<tr>
<td>Collagen antibody-induced arthritis</td>
<td>mouse</td>
<td>TSA, FK228</td>
<td></td>
<td>Ameliorated paw swelling, synovial inflammation and bone damage, induction of cell cycle inhibitors, reduced TNFα and IL-1β levels, VEGF expression and angiogenesis, suppressed MMP and elevated TIMP-1 production by chondrocytes 92-94</td>
</tr>
<tr>
<td>Collagen-induced arthritis</td>
<td>rat, mouse</td>
<td>MS-275, SAHA, VPA</td>
<td></td>
<td>Reduced disease incidence, protection from paw swelling and bone erosions, elevated numbers of splenic and synovial Tregs 65,66</td>
</tr>
<tr>
<td>SCW-induced arthritis</td>
<td>mouse</td>
<td>ITF2357</td>
<td></td>
<td>Reduced paw swelling and production of cytokines, restoration of chondrocyte proteoglycan synthesis 97</td>
</tr>
<tr>
<td>Asthma</td>
<td>Ovalbumin sensitization and challenge</td>
<td>mouse</td>
<td>TSA</td>
<td>Attenuated airway hyper-responsiveness, reduced numbers of inflammatory cells in lung tissue, suppression of IL-4, IL-5 and IgE production 98</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Experimental autoimmune encephalomyelitis</td>
<td>mouse</td>
<td>TSA, PheBut</td>
<td>Ameliorated disability, reduced spinal inflammation and demyelination, suppressed caspase activation 99,100</td>
</tr>
<tr>
<td>Lupus</td>
<td>NZB/W mice</td>
<td>mouse</td>
<td>TSA</td>
<td>Reduced kidney damage and autoantibody production, decreased IL-6 and increased TGF-β production by T cells, elevated numbers of Tregs 101</td>
</tr>
<tr>
<td>MRL/lpr mice</td>
<td>mouse</td>
<td>TSA, SAHA</td>
<td></td>
<td>Ameliorated proteinuria, pathogenic renal disease and reduced spleen weight, suppressed cytokine production by splenocytes and mesangial cells 102,103</td>
</tr>
<tr>
<td>Graft-versus-host disease</td>
<td>Allogenic bone marrow transplantation</td>
<td>mouse</td>
<td>SAHA, ITF2357</td>
<td>Ameliorated intestinal damage, clinical severity and mortality, reduced serum levels of TNFα, IL-1β and IFNγ 104,105</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>SAHA, ITF2357</td>
<td></td>
<td>Improved survival after injection of HDACi-treated DCs, associated with increased expression of IDO 106</td>
</tr>
<tr>
<td>Septic shock</td>
<td>LPS challenge</td>
<td>mouse</td>
<td>SAHA, ITF2357</td>
<td>Reduced serum levels of TNFα, IL-1β, IL-6 and IFNγ 30,31</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>ConA-induced liver injury</td>
<td>mouse</td>
<td>SAHA, ITF2357</td>
<td>Reduced release of alanine amino transaminase 30,31</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>dextran sulfate sodium-induced colitis</td>
<td>mouse</td>
<td>SAHA, VPA, TSA, ITF2357</td>
<td>Protection from weight loss and colon shortening, reduced inflammation, rectal bleeding and diarrhea, suppressed IFNγ and increased IL-10 production by colon cultures 107,108, reduced disease severity and elevated Treg numbers in lymphoid tissues 109</td>
</tr>
<tr>
<td></td>
<td>trinitrobenzene sulfonic acid-induced colitis</td>
<td>mouse</td>
<td>SAHA</td>
<td>Protection from weight loss and colon shortening, reduction in histological score, reduced IL-6 and IFNγ production by colon cultures 107</td>
</tr>
</tbody>
</table>

ConA: concanavalin A; DC: dendritic cell; IDO: indoleamine 2,3-dioxygenase; IFNγ: interferon gamma; IgE: immunoglobulin E; IL-1β: interleukin-1beta; MMP: matrix metalloproteinase; PheBut: phenylbutyrate; SAHA: suberoylanilide hydroxamic acid; SCW: Streptococcus pyogenes cell wall TGFβ; transforming growth factor beta; TIMP-1: tissue inhibitor of metalloproteinases-1; TNFα: tumor necrosis factor-alpha; TSA: trichostatin A; VEGF: vascular endothelial growth factor; VPA: valproic acid; Treg: regulatory T cell.
system. In all cases, HDACi blocked inflammatory mediator production at concentrations insufficient to cause apoptosis, providing evidence that anti-inflammatory effects of HDACi can be achieved in the absence of general cellular toxicity. Consistently, many HDACi have demonstrated therapeutic potential in experimental models of human acute and chronic IMIDs, including colitis, multiple sclerosis, graft-versus-host disease, endotoxic shock and arthritis. A concise overview of their reported efficacy can be found in Table 1.

Initial surprising observations that HDAC2 expression was decreased in RA synovial macrophages raised the possibility that these cells might be refractory to anti-inflammatory effects of HDACi. This would be important in considering therapeutic application of HDACi in RA, as changes in synovial macrophage activation and numbers are tightly associated with RA patient response to therapy. We directly compared the influence of class I/II HDACi, as well as the inhibitor of class III HDACs nicotinamide (NIC), on inflammatory activation and survival of macrophages derived from healthy donor peripheral blood (PB) and RA patient synovial fluid (chapter 5). Treatment of macrophages derived from the synovial fluid of RA patients with HDACi suppresses TNFα- and LPS-induced IL-6 production, and LPS-induced TNFα production to a similar extent as that observed in healthy donor PB-derived macrophages. In line with previous reports, HDACi suppressed cytokine production at concentrations insufficient to induce apoptosis. Additionally, RA synovial fluid and healthy donor PB-derived macrophages demonstrate equivalent apoptosis responses to higher concentrations of TSA, which are further enhanced in the presence of TNFα or LPS.

Surprisingly, treatment with NIC also blocked cytokine production by macrophages, and the anti-inflammatory effect of NIC was even more pronounced than that of class I/II HDACi. It is currently difficult to reconcile this finding with the number of in vivo studies indicating an immunoregulatory role for Sirt1, and may suggest that NAD+-dependent enzymes other than sirtuins mediate the NIC effect. However, treatment of macrophages with NIC partly phenocopies TSA treatment in regard to changes in profiles of gene expression regulating cell survival. Furthermore, a recent report indicates that sirtuin expression is elevated in RA synovial tissue and stimulation with TNFα upregulates Sirt1 expression in RA FLS and monocytes in vitro. Notably, overexpression of Sirt1 not only protects FLS and monocytes from apoptosis, consistent with involvement of sirtuin activity in cell survival and longevity, but also potentiates IL-6 and TNFα production by FLS and monocytes in response to LPS. Pro-inflammatory effects of sirtuins are not restricted to myeloid and stromal cells as sirtinol, a specific inhibitor of Sirt1, also downregulates expression of adhesion molecules and prevents cytokine-induced production of chemokines CCL2 and CXCL10 by human dermal microvascular endothelial cells. Collectively, these observations indicate that regulation of inflammatory responses by Sirt1 strongly depends on cellular and environmental contexts. Results presented in chapter 5 support the idea that sirtuin activity might have a proinflammatory role in RA, and that specific targeting of sirtuin activity might be therapeutically beneficial. It remains unknown however how Sirt1 inhibitors affect inflammation and disease severity in animal models of arthritis, and such experiments are eagerly awaited.

We next extended our analyses of HDACi effects on cellular inflammatory responses to RA FLS, which are potent effector cells in RA synovial tissue and, next to macrophages, serve as a primary source of cytokines and mediators of bone and cartilage destruction. We demonstrated that HDACi reduce IL-6 production by FLS induced by cytokines and TLR ligands (chapter 6). We next performed broader analyses of HDACi modulation of genes...
involved in immunity, cell survival, angiogenesis, regulation of extracellular matrix and adhesion \((\text{chapter 7})\). FLS exposure to ITF2357 reduced the mRNA levels of approximately 85% of genes induced by IL-1\(\beta\), including cytokines (TNF\(\alpha\), IL-1\(\alpha\), IL-1\(\beta\)), chemokines (CCL2, IL-8, CXCL6, CXCL9-11) and matrix-degrading enzymes (MMP-1, MMP-3, MMP-12), without affecting expression of genes non-responsive to IL-1\(\beta\). At the same time, although a small number of studies reported the ability of HDACi to enhance certain cellular proinflammatory functions under restricted experimental conditions,\(^43-45\) we failed to identify any IL-1\(\beta\)-responsive genes super-induced in the presence of ITF2357. Curiously, while HDACi did not affect LPS-induced IL-8 expression in macrophages, an effect independently noted in other studies,\(^30,31,33\) they prevented IL-8 mRNA induction in FLS in response to IL-1\(\beta\). This observation indicates that HDACi effects on certain genes are cell type- and stimulus-specific, and indirectly suggests that distinct mechanisms unrelated to changes in chromatin architecture may be responsible for HDACi suppression of cytokines and chemokines. Collectively, these findings clearly demonstrate that HDACi block the production of a broad range of inflammatory mediators not only by macrophages, but also other cell types present in the synovial tissue and contributing to RA pathology.

We also provided evidence indicating that HDACi can reduce inflammatory parameters in intact RA synovial tissue \((\text{chapter 5})\). Treatment of RA synovial explants with HDACi reduces production of many of the cytokines, chemokines, and growth factors thought to contribute to the pathology of RA, including IL-6, IL-8, CCL2, CCL5 and VEGF. However, some selectivity is observed, as production of IL-12, IL-15, IL-17, and IL-23 is unaffected. Thus, despite the ability of HDACi to elicit both pro-inflammatory and immunosuppressive effects of isolated cell populations \(\text{in vitro}\), these compounds have a globally therapeutic effect within the complex tissue of the inflamed RA synovium. Translation of these findings to clinical application has proceeded with caution however, in part due to uncertainties about the potential role of altered HDAC activity in the pathogenesis of RA, safety concerns regarding extended patient treatment with HDACi, and the limited information available regarding whether HDACi might influence inflammation via epigenetic or non-epigenetic mechanisms in RA patient-derived synovial cells.\(^9,27\) Recently, 17 patients with systemic onset juvenile idiopathic arthritis (SOJIA) were enrolled in an open-label Phase II study in which they were treated twice per day for 12 weeks with orally administered ITF2357 (givinostat). The compound was well-tolerated and no patients were withdrawn from the study due to drug-related adverse effects. Patients treated with ITF2357 demonstrated a significant improvement in disease activity, as measured by the number of clinically involved joints, and patient mobility and well-being.\(^46\) In this study, improvement of patient clinical parameters was associated with reduced levels of IL-1\(\alpha\), IFN\(\gamma\) and CD40L in whole blood lysates of treated patients.\(^47\) The data presented in \text{chapter 6} and \text{chapter 7} clearly demonstrate that ITF2357 prevents inflammatory activation of RA FLS and macrophages \(\text{in vitro}\) at concentrations that are achievable in humans after oral administration.\(^48\) Likewise, in a phase I clinical trial of ITF2357 in healthy subjects, reduced production of TNF\(\alpha\), IL-1\(\beta\), IL-6 and IFN\(\gamma\) was observed in LPS-stimulated whole blood cultures,\(^49\) confirming that immunosuppressive effects of HDACi observed \(\text{in vitro}\) are reproduced at the systemic level in humans, and indicating that the efficacy of ITF2357 in patients with SOJIA at least partly results from its anti-inflammatory activity.\(^46\) This initial study is the first to specifically attempt to inhibit HDAC activity in a human IMID, has shown encouraging results, and larger, independent, placebo-controlled studies are eagerly awaited.
Several lines of experimental evidence suggest that HDACs regulate gene expression not only by modulation of histone acetylation and resulting changes in chromatin structure, but also through deacetylation of non-histone proteins, including components of intracellular signaling pathways and transcriptional regulators. Notably, many of the signaling proteins and transcription factors regulated by reversible acetylation are also involved in modulation of expression of genes identified in chapter 5 and chapter 6 as suppressed by HDACi. Therefore, we directly evaluated the effects of HDACi on key signaling cascades activated in RA FLS after inflammatory stimulation (chapter 6).

Mitogen-activated protein kinases (MAPKs) are critical intermediaries in coupling diverse inflammatory stimuli, such as antigen receptor ligation, cytokine exposure, TLR signaling and environmental stress, to gene transcription. In general, receptor ligation leads to activation of Ras superfamily GTPases, initiating a serine-threonine kinase cascade that climaxes in phosphorylation and activation of the MAPKs p38, extracellular signal regulated kinase (ERK) and c-jun N-terminal kinase (JNK). Activated MAPKs in turn phosphorylate transcription factors, inducing their nuclear translocation and transcriptional activity. ERK and p38 are negatively regulated by MAPK phosphatase-1 (MKP-1). A recent report has suggested that in murine macrophages and macrophage cell lines broad pharmacological inhibition of cellular HDACs induces acetylation of MKP-1, which in turn enhances MKP-1 association with p38 and blocks p38 activation by LPS. Other investigators however have been unable to reproduce this observation. Reversible acetylation can also regulate JNK signaling, doing so at multiple levels. First, decreases in cellular HDAC activity can suppress induction of c-fos and c-jun, two important components of the heterodimeric AP-1 transcription factor. On the other hand, acetylation of c-jun is also required for AP-1 transcriptional activity. This requirement has been demonstrated in both T cells and macrophages, where HDAC3 and Sirt1 suppress AP-1 transcriptional activity by deacetylating c-jun. However, we find no evidence for HDACi regulation of IL-1β-induced p38, ERK and JNK phosphorylation in RA FLS. FLS treatment with TSA also modulated neither the induction of c-Jun mRNA expression, nor DNA binding of c-jun and other Jun family members after cell exposure to IL-1β. These negative findings demonstrate that HDACi effects on early signaling events after inflammatory stimulation have a cell type-specific character, and indicate that different mechanisms underlay anti-inflammatory effects of HDACi in stromal cells from RA synovial tissue.

The capacity and complexity of reversible acetylation processes in the regulation of signal transduction pathways is perhaps best exemplified by the role of HDACs in NF-κB signaling. Nuclear accumulation and transcriptional activity of the NF-κB p65 subunit is required in coupling antigen, inflammatory cytokine and TLR stimulation to transcription of many genes regulating inflammatory activation, proliferation, and survival. Upstream IκBα kinases IKKα and IKKβ are activated by receptor ligation and phosphorylate IκBα. This leads to dissociation of IκBα from p65/RelA, and the ubiquitination and degradation of IκBα, allowing p65 to translocate to the nucleus. Five lysine residues on p65 have been identified, acetylation of which by the HAT p300/CPB differentially regulates p65 subcellular localization and activity. Dissociation of p65 from IκBα is promoted by p65 acetylation on lysine 221. Acetylation at this residue allows nuclear import of p65 and enhances p65 DNA binding affinity without...
affecting p65 transcriptional activity. Instead, acetylation of p65 at lysine 310 enhances its transcriptional activity, at least in part by promoting p65 association with the bromodomain-containing transcriptional co-activator Brd4. Acetylation events can also negatively regulate p65, as acetylation at lysines 122 and 123 decreases p65 DNA binding affinity, promotes its nuclear export, and stabilizes p65 interactions with IKBa. HDAC1, HDAC2, and HDAC3 have been identified as candidates which mediate p65 deacetylation at lysine 221. Additionally, SIRT1 can mediate deacetylation of p65 at lysine 310. Evidence has also emerged that HDACs can modulate NF-kB signaling upstream of p65. In osteoclasts, broad inhibition of Class I and Class II HDACs prevents TNFα-induced activation of IKKa and IKKβ, although the mechanism by which this occurs, and the HDACs responsible for maintaining IKK signaling capacity, remain to be identified. We observed complex regulation of NF-kB activation in FLS by HDACi: while TSA slightly increased DNA binding and transcriptional activity of NF-kB subunits at early time points following IL-1β stimulation, it reduced nuclear retention of p65 and p50 24 h post treatment (chapter 6). These changes might be consistent with time-dependent changes in NF-kB acetylation at distinct residues, but more detailed analyses are needed to verify this interpretation. However, HDACi effects on the induction of IL-6 and vast majority of other IL-1β-responsive genes are detectable within the first 1-4 h after stimulation (chapter 7), arguing against involvement of acetylation-mediated changes in NF-kB signaling in this process in FLS. Although alterations in NF-kB activation cannot explain acute suppression of cytokine expression by HDACi in FLS, additional experiments are needed to analyze consequences of prolonged HDACi treatment on NF-kB-mediated inflammatory responses. This is especially important in the context of potential application of HDACi in humans, and may provide relevant information about long-term effects of HDACi treatment on the immune system. Of note, amelioration of mucosal inflammation in patients with distal ulcerative colitis has been observed following local administration of butyrate, a short-chain fatty acid displaying HDACi activity, and this effect is associated with suppressed NF-kB nuclear translocation in lamina propria macrophages.

Receptor tyrosine kinases, receptor-associated tyrosine kinase activity and G-coupled protein receptors can stimulate the lipid kinase activity of phosphoinositide (PI) 3-kinase (PI3-K) family members. The resulting accumulation of PI(3,4,5) phosphate (PIP3) on the inner leaflet of the cell membrane attracts and contributes to the activation of pleckstrin-homology (PH) domain-containing proteins, including protein kinase B (PKB). Key substrate targets of PKB are the forkhead box O (FoxO) transcription factors FoxO1, FoxO3a and FoxO4. In the absence of PI3-K/PKB signaling, FoxO proteins induce expression of gene products which regulate cell cycling, survival responses to stress, or promote apoptosis. PKB-dependent phosphorylation of FoxO proteins disrupts their interaction with DNA, and promotes nuclear export via association with 14-3-3 proteins. Activation of JNK by extracellular stimuli or stress promotes JNK-dependent phosphorylation of FoxO proteins. This induces nuclear import of FoxO proteins, where subsequent serial acetylation and deacetylation steps by Class I/II HDACs and Sirt1 not only regulate transcriptional activity and nuclear retention of FoxO proteins, akin to p65/RelA, but also influence target gene specificity of FoxO proteins. Initial studies suggest that acetylated FoxO proteins can induce transcription of pro-apoptotic gene products but are susceptible to PKB-dependent nuclear export, while deacetylated FoxO proteins promote transcription of gene products inducing cell cycle arrest and survival. This ability of FoxO proteins to integrate multiple extracellular signaling pathways confers a central role for them in regulating immune cell function.
fate decisions under homeostatic immune conditions, as well as in chronic inflammatory diseases.\textsuperscript{65,66} Furthermore, several cytokines and other inflammatory mediators have recently been identified as FoxO transcriptional targets,\textsuperscript{69} and substantial experimental evidence for important contributions of aberrant FoxO signaling to pathology of IMIDs has accumulated.\textsuperscript{70} Initial studies reported reduced activity and expression of FoxO family members in RA compared to healthy individuals and/or disease controls,\textsuperscript{71,72} indicating that FoxO transcriptional activity might be required for prevention of autoimmune processes.

Since FoxO proteins are regulated by reversible acetylation, and FoxO1 is the most abundant FoxO family member in stromal cells in RA synovial tissue,\textsuperscript{72} we tested whether HDACi can modulate the activity of FoxO1 (chapter 8). In line with previous reports, we found reduced levels of FoxO1 mRNA in RA FLS compared to FLS isolated from OA patients, and FoxO1 expression as well as DNA binding activity was further reduced after inflammatory stimulation. Curiously, FoxO3a and FoxO4 transcript levels were largely non-responsive to IL-1\textbeta\textsuperscript{β} stimulation, suggesting that among FoxO family members, FoxO1 is most susceptible to dynamic regulation by inflammatory triggers in FLS. HDACi treatment not only prevented IL-1\textbeta\textsuperscript{β}-mediated inactivation and transcriptional downregulation of FoxO1, but even increased FoxO1 DNA binding and mRNA accumulation above the levels detected in resting FLS. More detailed analyses are needed to verify whether these effects of HDACi can be attributed to maintaining FoxO1 in the acetylated state, or result solely from its transcriptional induction.

Restoration of FoxO1 activity in RA FLS by HDACi might overcome their relative resistance to apoptosis through modulation of pro- and anti-apoptotic genes.\textsuperscript{73} Indeed, constitutive activation of FoxO1 reduces FLS viability, and this effect is associated with changes in expression of Bcl-2 family members Bcl-XL and Bim. It is however unclear how maintenance of FoxO1 activity affects production of inflammatory cytokines and chemokines. Results of our preliminary experiments suggest that overexpression of a constitutively active FoxO1 mutant in RA FLS might suppress expression of some inflammatory mediators (CCL2, CXCL6, PDGF and ICAM1), while promoting others (IL-8, CXCL3 and BMP-2) in IL-1\textbeta\textsuperscript{β}-stimulated cells, emphasizing the complex roles of FoxO in the regulation of cellular inflammatory responses. These observations indicate that FoxO1 re-activation can explain only a proportion of suppressive HDACi effects on IL-1\textbeta\textsuperscript{β} inducible genes, and additional studies using systematic complementary overexpression and gene silencing experiments will be necessary to identify subsets of genes expression of which is critically dependent on HDACi prevention of FoxO1 inactivation.

Collectively, the data presented in chapter 6 and chapter 8 clearly show that inhibitors of class I/II HDAC activity can modulate intracellular signal transduction in FLS at multiple levels, including transcriptional activity, nuclear retention and expression of transcription factors. However, temporal changes in the activation status of these molecules generally do not coincide with suppression of IL-6 and other mediators of inflammation and tissue damage by HDACi, suggesting that alternative mechanisms are responsible for immediate effects of HDACi on expression of genes induced by inflammatory stimulation in stromal synovial cells.

### HDACs AS REGULATORS OF mRNA STABILITY

While in the majority of previous reports HDACi regulation of cellular inflammatory responses has been explained either by influences on epigenetic events or by effects on intracellular signaling pathways, initial studies of cancer cells identified regulation of mRNA
stability as an additional mechanism by which HDACi can modulate gene expression. In these studies, HDACi uniformly accelerated degradation of target mRNAs through effects on their 3' untranslated regions (UTRs), resulting in reduced protein expression. Here we reported for the first time that IL-6 mRNA undergoes rapid decay in the presence of HDACi. Accelerated degradation of the IL-6 transcript after TSA treatment was observed both in RA FLS and macrophages, suggesting that regulation of mRNA stability might represent a shared molecular mechanism underlying suppression of IL-6 production by HDACi (chapter 6). We next extended our analyses of HDACi effects on mRNA stability of cytokines, chemokines and other inflammatory mediators, and identified a subset of genes, including IL-8, CXCL2, cyclooxygenase-2 (PTGS2) and BCL2L1 (Bcl-XL), which are regulated by HDACi in a similar fashion in RA FLS (chapter 7). While this observation indicates acceleration of mRNA degradation as an important general mechanism by which HDACi mediate their anti-inflammatory effects, expression profiles of other HDACi-sensitive genes suggest that distinct mechanisms by which HDACi can regulate gene expression in RA FLS are also present, awaiting further molecular characterization. Future experiments will also be needed to test whether similar sets of genes are regulated by HDACi through effects on mRNA degradation in other types of immune cells.

To gain more insight into potential mechanisms responsible for HDACi-mediated mRNA destabilization, we analyzed 3' UTRs of the monitored genes for the presence of AU-rich element (ARE) motifs. Our analysis revealed that of most of the ARE sequences are enriched in transcripts regulated by HDACi through reduction of mRNA stability compared to transcripts non-responsive to HDACi or regulated through different mechanisms (chapter 7). Since these sequences are typically recognized by ARE-binding proteins (ARE-BPs), such as AU-rich binding factor-1 (AUF1), tristetraprolin (TTP), KH-type splicing regulatory protein (KSRP) and HuR, this observation suggests that HDACi might mediate their effects on mRNA stability through regulation of subcellular localization, expression, or activity of ARE-BPs. In line with this possibility, butyrate, a short-chain fatty acid with HDACi activity, promotes the expression of the TTP family member BRF-1 which results in reduced TNFα production by macrophage-like synovial cells derived from RA patient synovial tissue. However, since most ARE-BPs bind several classes of ARE motifs, it is not yet possible to determine which of these proteins might be responsible for HDACi-induced acceleration of mRNA decay using bioinformatic tools. Finally, mRNA stability is regulated not only by ARE-BPs, but also by miRNAs. Studies in tumour cells have provided initial evidence that HDACi can differentially modulate miRNA expression, raising the possibility that inhibition of HDAC activity might promote target mRNA degradation through upregulation of specific miRNAs. Furthermore, miRNAs can indirectly affect mRNA stability through regulation of ARE-BP expression. Further experiments are therefore necessary to characterize molecular events underlying HDACi-mediated mRNA degradation of IL-6 and other inflammatory mediators, and to verify potential involvement of ARE-BPs and miRNAs in this process.

**CONCLUDING REMARKS**

In this thesis we have provided the most comprehensive insight to date into the involvement of HDAC activity in inflammatory responses of synovial cells relevant to RA pathology. We demonstrate that HDACi suppress production of a broad range of cytokines, chemokines,
matrix-degrading enzymes and other inflammatory mediators by RA patient synovial macrophages, FLS, and intact synovial tissue explants. We also identify acceleration of mRNA degradation and modulation of FoxO1 signaling as two novel mechanisms contributing to anti-inflammatory effects of HDACi. The data presented in this thesis, in combination with the weight of evidence from experimental models of inflammation suggests a broad potential for HDACi in the treatment of human IMIDs. Moreover, the first clinical trial modulating HDAC activity in SOJIA has provided initial indications of both safety and efficacy, although more extensive validation is required. Several challenges need to be addressed as work presses forward to apply HDACi toward the treatment of inflammatory diseases. First, it is apparent that pharmacodynamic aspects of specific compounds will need to be monitored closely in each disease setting, as at least in vitro, pro- and anti-inflammatory effects of HDACi can be segregated over compound concentration ranges.

Second, it is unclear whether, and for which diseases, HDACi which more selectively target specific HDAC family members need to be developed. Most studies examining the effects of HDACi in animal models of human IMIDs have utilized HDACi which, at least in vitro, display pan-HDAC or class I-selective HDAC inhibitory activity. If HDACs demonstrate redundancies in promoting inflammation, more specific HDACi may actually demonstrate lower efficacy in the clinic. Such a possibility may be observed in recent, and disappointing, attempts to treat RA with isoform-specific p38 MAPK inhibitors. However, increasingly elegant techniques for assessing the specificity of HDACi indicate that the selectivity of “pan-HDACi” may be greater than previously appreciated. A recently reported chemoproteomic approach, involving, in its simplest terms, precipitation of HDACi-binding targets and their associated proteins from cellular lysates with immobilized HDACi, reveals that pan-HDACi have surprisingly relatively few off-target binding partners, and provides indications of HDAC selectivity, even within classes. Such an approach, combined with our structural knowledge of HDACi-HDAC interactions and the use of chemical library screens, should rapidly accelerate the development of compounds targeting specific HDACs. Additional validation, as well as fundamental knowledge of HDAC function in the immune system in vivo, will be provided by the continued development of tissue-specific HDAC-deficient mice and, ultimately, the generation of knock-in mice expressing catalytically inactive HDACs. This latter approach has proven extremely useful in understanding the biology of specific PI3-K catalytic subunits in vivo, as well as examining the specificity of inhibitory compounds targeting specific PI3-K isoforms. HDAC knock-in mice would be extremely useful not only in assessing the specificity of HDACi in vivo, but also provide an invaluable tool for dissecting contributions of HDACs to inflammatory signaling complexes that operate independently of HDAC catalytic activity. These protein-protein interactions would in turn represent a novel class of therapeutic targets. In this regard, an important set of proof-of-principal experiments have already been performed, in which small molecular weight compounds designed to interfere with the association of bromodomain-containing proteins with acetylated histones have been shown to prevent the inflammatory activation of innate immune cells in vitro and in vivo. The encouraging introduction of pharmacological HDAC modulators into the clinic for the treatment of inflammatory lung diseases and SOJIA represent an important milestone in the field, and this, combined with increasingly sophisticated technological approaches, is paving the way for more intense development of HDACi with increasing specificity and clinical application.
REFERENCES

25. Klimek VM, Fircanis S, Maslak P, Guernah J, Baum M, Wu N et al. Tolerability, pharmacodynamics, and pharmacokinetics studies of depsipeptide (romidepsin) in


50. Cao W, Bao C, Padalko E, Lowenstein CJ. Acetylation of mitogen-activated protein

GENERAL DISCUSSION


77. Zhou Q, Shaw PG, Davidson NE. Inhibition of histone deacetylase suppresses EGF signaling


84. Gebeshuber CA, Zatloukal K, Martinez J. miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. *EMBO Rep* 2009; 10(4):400-405.


102. Mishra N, Reilly CM, Brown DR, Ruiz P, Gilkeson GS. Histone deacetylase inhibitors modulate...


