Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics
Grabiec, A.M.

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Rheumatoid arthritis (RA) is one of the most common autoimmune diseases worldwide affecting approximately 1% of the population, characterized by chronic inflammation of the joints that leads to progressive and irreversible destruction of bone and cartilage. In RA, large quantities of cytokines and chemokines, such as tumour necrosis factor-α (TNFα), interleukin (IL)-1β, IL-6 and IL-8, secreted by activated macrophages and other immune cells infiltrating the synovial tissue stimulate hyperplastic growth and activation of stromal fibroblast-like synoviocytes (FLS), which in turn produce matrix-degrading enzymes directly contributing to joint damage. Although recent increases in our understanding of how distinct components of this network of inflammatory mediators contribute to inflammation and joint destruction in RA have been translated into innovative and increasingly successful treatment of patients in the clinic, a substantial fraction of RA patients fails to respond to these novel therapeutic strategies. Therefore, there is a continuing need for characterizing mechanisms underlying pathological inflammatory gene regulation in RA, which might be translated into novel therapeutic strategies.

The production of inflammatory cytokines is tightly regulated by epigenetic mechanisms, among which acetylation and deacetylation of histones plays a prominent role. Inflammatory stimuli activate transcriptional coactivators possessing intrinsic histone acetyltrasferase (HAT) activity, leading to histone acetylation and increased accessibility of gene promoters for transcription. HAT activity is counteracted by histone deacetylases (HDACs) resulting in termination of transcription. HATs and HDACs also modulate gene expression via non-epigenetic mechanisms through reversible acetylation of the components of intracellular signalling pathways, transcription factors, and proteins regulating post-transcriptional mRNA processing and protein secretion. In this thesis we analyzed the contributions of HDAC activity to the regulation of inflammatory responses in RA synovial tissue, we investigated how inhibition of HDAC activity modulates inflammatory activation of RA synovial macrophages and FLS, and attempted to characterize molecular mechanisms underlying modulation of inflammatory mediator production by HDAC inhibitors (HDACi).

Aberrant activity and expression of HDACs contributes to pathology in inflammatory lung diseases, and initial reports have suggested alterations of HDAC activity in RA. In chapter 4 we analyzed potential associations of synovial protein acetylation and HDAC expression, with RA patient disease activity and synovial expression of inflammatory mediators. We failed to detect major differences in the levels of acetylated histone and total protein in RA and osteoarthritis (OA) synovial tissue. We found no correlation of protein acetylation levels with patient clinical parameters and synovial T cell or FLS numbers, while we observed a strong positive association of acetylated lysine, acetylated histone 3 (H3), acetylated H4 and dimethylated H3 levels with synovial macrophage numbers. Expression of TNFα and matrix metalloproteinase-1 (MMP-1) in RA synovial tissue positively correlated with mRNA levels of HDAC1 and other class I HDACs. On the other hand, a negative correlation was observed between HDAC5 expression and patient disease activity, and mRNA expression of HDAC5 in RA FLS was rapidly suppressed following IL-1β stimulation. These findings provide novel insights into the relationship between HDAC-dependent epigenetic regulatory mechanisms and inflammation in RA. Our data indicate that differences in HDAC expression or activity that might be present in RA synovial tissue are not sufficient to cause global changes in total protein or histone acetylation. Furthermore, we demonstrate that high levels of cellular protein acetylation are not directly related to diagnosis and disease activity in RA, but represent a cell type-specific phenomenon characteristic for
synovial macrophages. Finally, we confirm previous studies suggesting an association between class I HDAC expression in the synovial tissue and local expression of inflammatory cytokines, but identify cytokine-mediated reduction of HDACs as a potential marker of systemic inflammatory processes.

Cytokines, chemokines and other inflammatory mediators secreted by synovial macrophages contribute significantly to the pathology of RA. While HDACi demonstrate therapeutic effects in animal models of several chronic and acute inflammatory diseases, contributions of depressed macrophage HDAC activity to inflammation and glucocorticoid resistance have been suggested in asthma and COPD, potentially contraindicating therapeutic application of HDACi in inflammatory disorders. In chapter 5 we directly examined HDACi effects on activation and survival of macrophages derived from the inflamed joints of RA patients. Inhibition of either class I/II HDACs or class III sirtuin HDACs suppressed production of IL-6 and TNFα, but not IL-8, by both healthy donor and RA patient synovial macrophages at concentrations insufficient to induce cell death. High concentrations of the class I/II HDACi trichostatin A and the sirtuin inhibitor nicotinamide selectively induced macrophage apoptosis associated with specific down-regulation of the anti-apoptotic protein Bfl-1/A1, and inflammatory stimuli sensitized macrophages to HDACi-mediated apoptosis. HDACi also blocked the production of a broad range of inflammatory and angiogenic cytokines by intact RA synovial biopsy explants. This study provides novel findings regarding essential roles for class I/II and class III HDACs in regulating inflammation and cell survival in macrophages, and suggest that therapies targeting HDAC activity may be useful in suppressing macrophage inflammatory activation in RA.

The accumulation and persistence of activated FLS makes pivotal contributions to the pathology of RA. While HDACi effects on FLS proliferation and survival are well-characterized, little is known about regulation of FLS inflammatory activation by HDACi. In chapter 6 we examined the effects of HDACi on IL-6 production by RA FLS, and analyzed the molecular mechanism(s) underlying regulation of IL-6 expression by HDACi. HDACi TSA and ITF2357 dose-dependently suppressed RA FLS IL-6 production induced by IL-1β, TNFα and TLR ligands LPS and poly(I-C) without affecting cell viability. Treatment with HDACi did not modulate early signalling events occurring after IL-1β receptor triggering: phosphorylation of IκBα, as well as p38, ERK and JNK MAP kinases remained unaffected by TSA. Furthermore, changes in activity of AP-1 components c-Jun, JunB and JunD in response to IL-1β were not modulated by TSA. While TSA also failed to affect NF-κB activity at early time points following stimulation, it induced a significant reduction in nuclear retention of NF-κB subunits p65 and p50 in FLS stimulated with IL-1β for 24 hours. However, TSA-mediated inactivation of NF-κB did not coincide with reduction of IL-6 mRNA accumulation, detectable 4 hours following stimulation with IL-1β. Analysis of mRNA stability revealed accelerated degradation of IL-6 mRNA in the presence TSA. Similar effects of TSA were observed in macrophages, indicating regulation of IL-6 mRNA stability as a common mechanism responsible for suppression of IL-6 production by HDACi. These results demonstrated that inhibition of HDAC activity in RA FLS efficiently blocks the production of IL-6, and identified modulation of IL-6 mRNA stability as a potential mechanism underlying this regulation both in RA FLS and macrophages. Furthermore, the delayed reduction of NF-κB nuclear accumulation mediated by HDACi might lead to suppression of other inflammatory mediators regulated by NF-κB.

In chapter 7 we used low density quantitative PCR arrays to extend our analyses of HDACi effects on mRNA expression and stability of inflammatory mediators in RA FLS. FLS exposure to ITF2357 reduced the mRNA levels of approximately 85% of IL-1β-inducible genes, including
cytokines (IL-6, TNFα, IL-1α, IL-1β), chemokines (CCL2, IL-8, CXCL2, CXCL3, CXCL6, CXCL9, CXCL10), matrix-degrading enzymes (MMP1, MMP3, MMP13), and intracellular molecules regulating cellular inflammatory responses (COX-2, IRAK2, NFKB1, IRF1), while leaving the expression of genes non-responsive to IL-1β largely unaffected. Analyses of mRNA stability identified a number of transcripts, including IL-8, COX-2, CXCL2, Bcl-XL and ADAMTS1, which were, next to IL-6, regulated by HDACi through acceleration of mRNA decay. Importantly, significantly higher frequencies of AU-rich elements (AREs) were found in 3' untranslated regions of these transcripts. These results demonstrate that transcriptional induction of the majority of genes induced by inflammatory stimulation of RA FLS is potently blocked by HDACi. Our data also show that HDACi suppress a subset of these genes via the acceleration of mRNA degradation, suggesting that modulation of mRNA stability, possibly through regulation of ARE binding proteins, is an important general mechanism by which HDACi mediate their anti-inflammatory effects.

Forkhead box O (FoxO) transcription factors, regulated by phosphatidylinositol 3-kinase and reversible acetylation, integrate environmental signals to orchestrate inflammatory responses, cell cycle and apoptosis. In chapter 8 we examined the relationship between inflammation and FoxO expression and activity in RA, determined the effects of HDACi on FoxO expression and activity in RA FLS, and identified gene products relevant to RA regulated by FoxO proteins in RA FLS. Negative correlations were observed between RA synovial tissue expression of FoxO1 and clinical parameters of disease activity. A strong negative correlation was also observed between synovial FoxO1 and IL-6 mRNA levels, but not TNFα or MMP-1. FoxO1, FoxO3a and FoxO4 mRNA were each detected in RA and OA FLS, and FoxO1 mRNA levels were significantly reduced in RA FLS compared to OA FLS. IL-1β and TNFα suppressed FoxO1 DNA binding activity in RA FLS, and selectively downregulated FoxO1 mRNA expression in a time-dependent manner. HDACi reversed IL-1β-mediated reduction of FoxO1 DNA binding and mRNA expression. Overexpression of the constitutively active FoxO1 mutant (FoxO1ADA) in RA FLS suppressed expression of antiapoptotic Bcl-XL, and enhanced expression of proapoptotic Bim and the cell cycle inhibitor p27Kip1. FoxO1ADA also suppressed IL-1β-mediated induction of inflammatory mediators, including CCL2, CXCL6, PDGF and ICAM1. These findings indicate that inflammatory stimuli decrease FoxO1 expression and DNA binding activity, effects reversed by exposure of RA FLS to HDACi. Restoration of FoxO1 activity suppresses a subset of genes regulated by HDACi during FLS activation, raising the possibility that enhancing FoxO1 function might be one of the mechanisms by which HDACi mediate their anti-inflammatory effects in RA synovial cells.

In summary, the studies presented in this thesis demonstrate essential roles for HDAC activity in regulating inflammatory responses of myeloid and stromal cells in RA, and suggest that therapeutic targeting of HDACs with small molecule inhibitors might be useful in suppressing inflammation and joint destruction in RA patients. Our findings also identify two novel non-epigenetic mechanisms, namely reduction of target mRNA stability and modulation of FoxO1 expression and activity, which are at least partly responsible for anti-inflammatory effects of HDACi. In light of a recent clinical trial demonstrating initial clinical efficacy of ITF2357 in patients with systemic onset juvenile idiopathic arthritis, future studies should be aimed at characterizing the specific roles of HDAC family members in cellular inflammatory activation, and verifying the suitability of either inhibiting total HDAC activity or targeting specific HDACs in therapeutic strategies in RA and other inflammatory disorders.