

DNA Hypomethylation Ameliorates Erosive Inflammatory Arthritis by Modulating Interferon Regulatory Factor-8

Gaurav Swarnkar¹, Dorothy Mims¹, Syeda Kanwal Naqvi¹, Chia-Lung Wu², Yousef Abu-Amer¹

¹Washington University in Saint Louis, University of Rochester.

abuamery@wustl.edu; gauravswarnkar@wustl.edu

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INTRODUCTION: Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease considered as one of the leading causes of disability worldwide with a prevalence of approximately 1% of the world total population. The disease is characterized by synovitis and joint swelling affecting most of the small joints in the body. RA symptoms include variable degree of joints pain, impaired physical ability, fatigue, general sickness, and substantial mental, physical, psychological, social, and economic impact. RA is characterized by dysregulation of immune cells, hence, understanding the mechanisms underlying this pathology will advance therapeutic interventions. Recent work has affirmed that inflammation is governed by dysregulation of DNA methylation, an epigenetic mechanism that regulates gene expression which is presumed to play a crucial role in the pathogenesis of autoimmune diseases such as inflammatory arthritis. DNA hypomethylating agents, such as decitabine (DAC), have been shown to dampen inflammation and restore immune homeostasis. This study was designed to test if safe doses of DAC elicit effective anti-inflammatory effects and modify disease symptoms in several animal models of arthritis.

METHODS: (1) KBxN serum induced arthritis (STIA) and Collagen antibody induced arthritis (CAIA) murine model of RA were used in mice with CB57BL/6J background. (2) DBA/1 mice were used for collagen induced arthritis model. To investigate the role of Irf8 in mediating anti-osteolytic effect, STIA models were used in Irf8-KO and litter mate WT control. This study was approved by IACUC. More specifically, animals were divided into different groups (Control, DAC, RA and RA+DAC) and treated with low doses of DAC (0.125-0.25 mg/Kg) for different time points. To monitor RA progression in mice, we performed paw and ankle thickness measurement, behavioral (to correlate with RA pathology), micro-CT, histological, and FACS and sc-RNAseq analyses of synovial cells isolated from arthritic WT and *N/kb2*-null mice. (3) CD11b⁺ myeloid cells from different groups were used to perform methylseq analysis. (4) Other biochemical assays include, osteoclast differentiation assay, qPCR for osteoclast and inflammatory markers, and in depth-scRNAseq analysis were carried out.

RESULTS: Using different murine model of RA (STIA, CAIA and CIA) we showed that low dose administration of DAC inhibits RA. DAC treated groups, showed significant decrease in paw and ankle thickness ($p<0.0001$), decreased bone erosion and osteolysis using microCT analysis, decreased number of infiltrating cells in synovial joints ($p<0.0001$) and reduced number of OC. Further behavioral analysis, including grip strength test, thermal analgesia and open field movement analysis showed comparable measurement between Control and STIA+ DAC groups. Transcriptomic and epigenomic profiling showed that DAC-mediated hypomethylation regulates a wide range of cell types in arthritis, altering the differentiation trajectories of anti-inflammatory macrophage populations, regulatory T cells, and tissue-protective synovial fibroblasts. Mechanistically, we identified the transcription factor interferon regulatory factor-8 (Irf8) as a key component of this mechanism. DAC-mediated demethylation of intragenic CpG islands of *Irf8*, induced its re-expression and promoted its repressor activity. As a result, DAC restored joint homeostasis by resetting the transcriptomic signature of negative regulators of inflammation in synovial macrophages (MerTK, Trem2, Cx3cr1), TREGs (Foxp3), and synovial fibroblasts (Pdpn, Fapa). Finally, we demonstrated that delivery of viral Irf8 attenuated murine RA evident by inhibition of joint swelling, bone erosion and inflammation in mouse RA joints. In summary, we established that Irf8 is necessary for the inhibitory effect of DAC in murine arthritis and demonstrate that direct expression of Irf8 is sufficient to significantly mitigate arthritis.

DISCUSSION:

Under inflammatory conditions, the genomes of myeloid, lymphoid, and several other cell types undergo hypermethylation that hinders homeostatic functions by dampening cellular suppressors and exacerbating disease pathology. Therefore, we surmised that hypo-methylating agents hold promise to re-activate methylated suppressor genes and restore tissue homeostasis. Administration of low dose of the hypo-methylation drug DAC reversed these *in vitro* cellular changes and restored cellular homeostasis. Strikingly, administration of low doses of DAC *in vivo* ameliorated arthritis in STIA, CIA and CAIA mouse models and therapeutically restored tissue homeostasis. Mechanistically, scRNA-seq analysis revealed that DAC dramatically reduced myeloid cell populations in STIA joints. In total, twelve unique myeloid populations were detected in the synovium across all treatment group, of which four pro-inflammatory groups were present only in the arthritic joints, whereas DAC-treated STIA mice exhibited increased percentage of five anti-inflammatory macrophage populations compared to arthritic mice. Of interest, expression of members of the TAM family receptor tyrosine kinase MER (MerTK), essential to induce suppressors of cytokine signaling, is diminished in inflammatory arthritis, yet treatment with DAC restores its expression and its anti-inflammatory signature highlighted by elevated expression of IL-10, an anti-inflammatory cytokine. Collectively, these findings suggest that DAC acts systemically and at the local joint environment to rewire homeostatic immune and anti-inflammatory activities of tissue-resident and synovium-infiltrating cells. More importantly, DAC appears to restore tissue homeostasis by reversing the inflammatory phenotype of multiple cell types including myeloid, lymphoid, SFs, and potentially others cell types. Transcriptomic profiling further identified three novel inflammatory populations responsive to IFN γ and IL-1.

Notably, we identified hypermethylation of the suppressor gene *Irf8* as a key therapeutic target. Our transcriptomic studies unveiled several Irf8 expressing myeloid populations. Wdyf4/Trim35/Irf8⁺ Mo-Dc/Neu cells have the highest expression of Irf8. We also show that two anti-inflammatory macrophages (C1aq/Mcr1/Pf4 and Spp1/Cd3b/Arg1) have strong Irf8 expression. These two populations were largely increased in STIA+DAC group (55.3% and 74%, respectively).

Our findings indicate that DAC demethylates STIA-induced hypermethylation of the genome and *Irf8* promoter regions. More importantly, DAC restores Irf8 protein expression in myeloid cells from STIA mice, inhibits osteoclastogenesis, and most notably, Irf8 mediates DAC effect and directly inhibits inflammatory and erosive characteristics of RA. These findings are consistent with the established anti-osteoclast function of Irf8. Collectively, these observations suggest that the Irf8 regulatory network is far more elaborate than anticipated. Therefore, Irf8 appears as central intersection point for several regulatory elements and hence it is not surprising that re-expression of Irf8 directly or through DAC-mediated hypomethylation overrides proximal suppressions and inhibits inflammation and bone erosion in RA.

SIGNIFICANCE/CLINICAL RELEVANCE: In this study, we show that the clinically approved hypomethylation drug decitabine (DAC) inhibits murine RA. Furthermore, using transcriptomic screening, we identify the transcription factor interferon regulatory factor-8 (Irf8) as a key component of this mechanism. Specifically, DAC induces expression of Irf8, and direct transduction of Irf8 ameliorates murine RA. These findings position DAC and Irf8 as potential arthritis therapeutic targets.

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