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CRISPR-Based Epigenome Editing of Cytokine Receptors for the Promotion of Cell Survival and Tissue Deposition in Inflammatory Environments

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Musculoskeletal diseases have been associated with inflammatory cytokine action, particularly action by TNF-α and IL-1β. These inflammatory cytokines promote apoptosis and senescence of cells in diseased tissue and extracellular matrix breakdown. Stem cell-based therapies are being considered for the treatment of musculoskeletal diseases, but the presence of these inflammatory cytokines will have similar deleterious action on therapeutic cells delivered to these environments. Methods that prevent inflammatory-induced apoptosis and proinflammatory signaling, in cell and pathway-specific manners are needed. In this study we demonstrate the use of clustered regularly interspaced short palindromic repeats (CRISPR)-based epigenome editing to alter cell response to inflammatory environments by repressing inflammatory cytokine cell receptors, specifically TNFR1 and IL1R1. We targeted CRISPR/Cas9-based repressors to TNFR1 and IL1R1 gene regulatory elements in human adipose-derived stem cells (hADSCs) and investigated the functional outcomes of repression of these genes. Efficient signaling regulation was demonstrated in engineered hADSCs, as activity of the downstream transcription factor NF-κB was significantly reduced or maintained at baseline levels in the presence of TNF-α or IL-1β. Pellet culture of undifferentiated hADSCs demonstrated improved survival in engineered hADSCs treated with TNF-α or IL-1β, while having little effect on their immunomodulatory properties. Furthermore, engineered hADSCs demonstrated improved chondrogenic differentiation capacity in the presence of TNF-α or IL-1β, as shown by superior production of glycosaminglycans in this inflammatory environment. Overall this work demonstrates a novel method for modulating cell response to inflammatory signaling that has applications in engineering cells delivered to inflammatory environments, and as a direct gene therapy to protect endogenous cells exposed to chronic inflammation, as observed in a broad spectrum of degenerative musculoskeletal pathology.

Keywords: CRISPR, epigenome editing, adipose derived stem cells, receptor, inflammation, intervertebral disc

Introduction

Musculoskeletal diseases are a major healthcare concern, ranking second in years lived with disability.[1] Disability due to musculoskeletal disease is increasing and is expected to continue increasing with the rise of a sedentary and aging population.[2] Current treatments for musculoskeletal diseases such as osteoarthritis and low back pain (LBP) are largely palliative, and fail to restore function or retard disease progression.[3–5] The progression of multiple musculoskeletal diseases has been associated with the action of inflammatory cytokines that signal the breakdown of the extracellular matrix (ECM) and promote apoptosis.[6–18] One approach for regulating this inflammation and regenerating ECM is cell delivery to the site of disease. For example, treatment by stem cell delivery to the intervertebral disc

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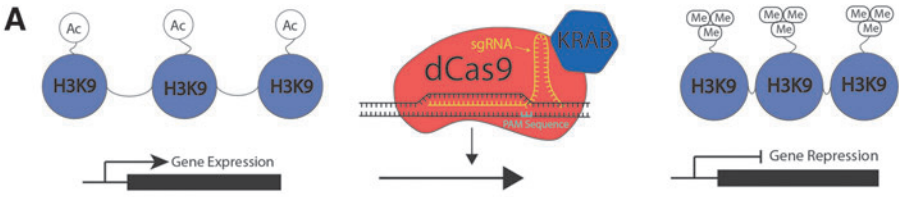
(IVD) has been investigated for degenerative disc disease (DDD) associated with LBP.[19–21] These delivered cells may function on a short-term basis but can succumb to the deleterious side effects of inflammatory signaling, which includes the induction of apoptotic pathways, protease expression, and inflammation maintenance.[6,8,14–16,22–24] Therefore, methods that protect therapeutically delivered cells from inflammatory environments are needed.

Here, we investigate the use of clustered regularly interspaced short palindromic repeats (CRISPR)-based epigenome editing to engineer cell response in inflammatory environments via modulation of inflammatory cytokine receptor expression. CRISPR-based epigenome editing provides highly targeted epigenome modifications, via methylation and acetylation, to regulate gene expression when targeted to regulatory elements and enhancers.[25–30] Briefly, this system requires the expression of a nuclease-deficient Cas9 protein (dCas9) fused to an effector domain (transcriptional activator or repressor) in combination with a single guide RNA (sgRNA).[31] The sgRNA targets a specific genomic site, via a 20 base pair sequence (gRNA) complementary to the genomic target, and is recognized and bound by dCas9. When dCas9 is fused to the transcription repression domain Krüppel Associated Box (KRAB) and coexpressed with a sgRNA, a complex forms that induces site-specific H3K9 methylation to repress gene expression (Fig. 1A).[25,29] Fusion of dCas9 to the acetyl transferase p300 core causes site-specific histone acetylation to upregulate genes.[27] Epigenome editing systems perform highly specific and effective gene modulation in mammalian cells,[25,32] can be multiplexed,[26,28] and perform more robust gene downregulation than RNAi.[29] In this study, we applied this technology for use in cell-based therapeutics and gene therapy to modulate cell response to inflammatory signaling, with broad applications for musculoskeletal pathology.

A number of inflammatory cytokines have been implicated in musculoskeletal pathology including TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IL-17, and interferon-γ.[17,18,33–39] Two primary cytokines associated with musculoskeletal disease, TNF- α and IL-1β, signal ECM degradation, propagation of proinflammatory signaling, apoptosis, and senescence.[10,15,17,18,40,41] This signaling is mediated by NF-κB, a transcription factor that induces expression of many proinflammatory and catabolic target genes involved with ECM breakdown and cell survival.[42–44] It is hypothesized that TNF-α and IL-1β play important roles in NF-κB-mediated tissue breakdown, cell apoptosis, and senescence, as NF-κB inhibition demonstrated reduced degradation in musculoskeletal disease models.[44–48] The known presence of TNF-α and IL-1β within diseased musculoskeletal tissue could threaten survival and anabolic activities for any stem cell therapy for musculoskeletal pathologies.[19,21,49,50]

TNF-α and IL-1β exert their action on cells through their receptors and exhibit varied responses through different receptors. TNF-α can exhibit proinflammatory, catabolic, and apoptotic signaling through TNFR1, but confers anti-apoptotic effects through TNFR2.[40] Furthermore, TNFR2 promotes an immunomodulatory phenotype in stem cells within inflammatory environments.[51,52] IL-1β induces pro-inflammatory signaling via IL1R1, while IL1R2 acts as a decoy receptor.[53] Methods antagonizing inflammatory signaling by direct inhibition of TNF-α and IL-1β via monoclonal antibodies or receptor antagonists broadly block cytokine action, but also eliminate antiapoptotic and immunomodulatory signaling. In this regard, methods capable of targeted inhibition of inflammatory cytokine/receptor



B

Target Gene	Name	Sequence
TNFR1	gRNA 1	5′ – CAGTGTTGCAACAGCGGGAC – 3′
TNFR1	gRNA 2	5′ – AGACTCGGGCATAGAGATCA – 3′
TNFR1	gRNA 3	5′ – GAATGGCAGGCACCCAGTCA – 3′
TNFR1	gRNA 4	5′ – ATAAGCGTCCGACACATGAT – 3′
TNFR1	gRNA 5	5′ – GCAAGGGGCTTATTGCCCCT – 3′
IL1R1	gRNA 1	5′ – GGAGTCGCCAACTCAATTCG – 3′
IL1R1	gRNA 2	5′ – TGGGGTCCTTGGGCGACTGC – 3′
IL1R1	gRNA 3	5′ – AGAGGCATTTCCCGGACTCG – 3′
IL1R1	gRNA 4	5′ – AGCACAAAGTTGGCTGCGCC – 3′
IL1R1	gRNA 5	5′ – GGGAGGTGACACCCAGTTTA – 3′
IL1R1	gRNA 6	5′ – AAAGTAGCCTGACGTATCCG – 3′
N/A	Nontarget gRNA	5′ – TTTTTAATACAAGGTAATCT – 3′

FIG. 1. **(A)** General mechanism of gene repression by CRISPR-based epigenome editing. Gene expression occurs when chromatin is maintained in the euchromatin (open) configuration by acetylated H3K9 histones. Binding of dCas9-KRAB fusion protein via the single guide RNA (sgRNA) and recognition of the protospacer adjacent (PAM) sequence by dCas9 recruits endogenous factors that replace acetylation of H3K9 with trimethylation, shifting chromatin to the heterochromatic state, therefore silencing gene expression. **(B)** Guide RNA sequences for each gene and the control nontarget gRNA sequence with no complementary target. **(C)** Lentiviral cassette demonstrating components of the epigenome editing system and how their expression is driven. CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, nuclease-deficient Cas9 protein.

(e.g., TNF-α/TNFR1) interactions, which maintain and promote the cell-protective and immunomodulatory interactions (e.g., TNF-α/TNFR2), are beneficial. CRISPR epigenome editing can be used for highly specific modulation of receptor expression, resulting in abrogated proinflammatory and apoptotic cytokine interactions (e.g., TNFR1 and IL1R1), while protecting anti-inflammatory and antiapoptotic interactions (e.g., TNFR2 and IL1R2). These systems can be used to protect endogenous cells from apoptosis and senescence and antagonize propagation of inflammatory signaling and/or engineer therapeutic cells delivered to inflammatory environments.

The objective of this study was to explore the novel application of CRISPR-based epigenome editing to modulate inflammatory signaling via targeted KRAB-mediated repression of gene regulatory elements for TNFR1 and IL1R1. We describe the design, construction, and screening of a library of TNFR1 and IL1R1 epigenome targeting vectors. Furthermore, we demonstrate how successful epigenome editing of human adipose-derived stem cells (hADSCs), a cell type currently used in clinical trials for musculoskeletal disease treatment,[54–56] protects them from inflammatory signaling during culture under inflammatory challenge. This work demonstrates that the epigenome edits promote cell survival, ECM deposition, stem cell differentiation, and immunomodulation under inflammatory conditions and provides a platform for broad scale cell protection in inflammatory environments.

Materials and Methods

Experimental overview

Experiments were conducted to build and verify lentiviral vectors encoding TNFR1 and IL1R1 targeting CRISPR-based transcriptional repressors, and test their ability to engineer human cells capable of thriving in inflammatory environments. The verified lentiviral vectors were used to produce hADSCs with targeted repression of TNFR1 or IL1R1 promoters, which was verified via quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR). Effects of this targeted repression on inflammatory signaling, cell proliferation, and ECM deposition in inflammatory conditions were tested in undifferentiated and chondrogenically differentiated hADSCs. Additionally, we tested the effects of the targeted repression on the immunomodulatory properties of hADSCs.

TNFR1 and IL1R1 epigenome editing construct design and cloning

For each gene, five to six gRNAs along with a nontargeting gRNA (Fig. 1B) were designed for screening in HEK293T cells (described in Supplementary HEK293T Screen: Materials and Methods and Results section; Supplementary Data are available online at www.liebertpub.com/tea). Based on screening results, four total lentiviral CRISPR epigenome editing vectors were built, two each for TNFR1 and IL1R1 (Supplementary Data HEK293T Screen: Materials and Methods and Results section). Final lentiviral constructs simultaneously expressed a single gRNA under the control of the hU6 promoter and dCas9-KRAB-T2A-GFP under the control of the hUbC promoter (Fig. 1C).

Epigenome editing of inflammatory cytokine receptors in human ADSCs

Generation of engineered human ADSCs. To generate hADSCs expressing epigenome editing tools and appropriate controls, immortalized hADSCs (SCRC-4000, ATCC) were separately transduced with each of the four lentiviral vectors encoding repressors targeted to the TNFR1 and IL1R1 promoters and the nontarget control vector using lentiviral transduction methods (Supplementary Materials and Methods), and cultured in manufacturer recommended expansion media (PCS-500-030, PCS-500-040, ATCC), until analysis. Transduced hADSCs were analyzed for TNFR1/IL1R1 expression via qRT-PCR (Supplementary Materials and Methods; $n=3$), and the vectors showing greatest TNFR1/IL1R1 downregulation were used in remaining experiments.

Inflammatory challenge: undifferentiated engineered human ADSCs. As undifferentiated stem cell delivery is of clinical interest for musculoskeletal disease,[49,50,57,58] we investigated the effects of repressing the target genes in undifferentiated hADSCs in an inflammatory environment.

NF-κB activity

To allow measurement of NF-κB activity, engineered and control hADSCs were transduced with an NF-κB reporter (Addgene, 49343, Supplementary Materials and Methods). Before dosing, NF-κB reporter expressing hADSCs were plated (5000 cells/well) in white 96-well plates in 100 μL of expansion media and allowed to attach overnight. The following day, engineered hADSCs were treated with a range (0, 0.15, and 10 ng/mL) of TNF-α or IL-1β, respectively ($n=4$). Non-transduced cells and cells transduced with the control nontarget vector were used as controls. After 24 h of cytokine treatment, luminescence (Bright Glo, Promega) and cell number (Real-Time Glo, Promega) were measured. NF-κB activity was quantified as a fold-change in luminescence relative to the untreated (0 ng/mL) cells, with NF-κB activity normalized to cell number.

Inflammatory pellet culture challenge

Engineered and control hADSCs were maintained in pellet culture in the presence of TNF-α or IL-1β to observe cell proliferation and survival under inflammatory conditions ($n=7$–8/group). 250,000 cells/pellet were pelleted in 200 μL of media at 142 G for 5 min. Pellets formed after 24 h and media was replaced with media treated with 1 ng/mL of TNF-α (nontarget and TNFR1-engineered cells) or IL-1β (nontarget and IL1R1-engineered cells). Serum-free pellet culture media consisted of DMEM-HG with pyruvate (Thermofisher), insulin (5 μg/mL), transferrin (5 μg/mL), selenous acid (5 ng/mL), bovine serum albumin (1.25 mg/mL), 0.17 mM ascorbic acid 2-phosphate, 0.35 mM proline, 0.1 μm dexamethasone, and 1% antibiotic/antimycotic (unless specified reagents were purchased from Sigma). Pellets were cultured for 28 days with media changed every 3 days. After 28 days, pellets were either fixed for histology in 10% neutral buffered formalin (NBF) ($n=2$–3) or frozen at −80°C for quantitative biochemistry ($n=5$).

Pellet size analysis

After 28 days of culture, pellets were uniformly imaged (Pentax K5) while still in wells. Cross-sectional area of pellets ($n=6$) were calculated from images using ImageJ.[59]

DNA quantification

Pellet cultures ($n=5$/group) were analyzed for DNA content using Hoechst dye assay.[60]

Histology

Pellet culture samples ($n=3$/group) were fixed in 10% NBF for 24 h, embedded in paraffin, and 5 μm sections were cut and stained with Haematoxylin (Fisher) and Eosin (Thermoscientific) (hematoxylin and eosin [H&E]).

Immunomodulation

To measure the effect of repression of inflammatory cytokine receptors on the immunomodulatory properties of hADSCs, we performed a peripheral blood mononuclear cell (PBMC) proliferation assay ($n=4$/group), which analyzes immunomodulation by looking at the ability of ADSCs to inhibit PBMC proliferation in coculture.[61–63] Mitomycin C-treated hADSCs were plated (12,800 cells/well) in a 96-well plate in RPMI1640 (Thermofisher) with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma). The following day PBMCs (PCS-800-011, ATCC) were added (102,400 cells/well), to wells with or without hADSCs and activated with 5 μg/mL phytohemagglutinin (Sigma). After two days, EdU (5-ethynyl-2-deoxyuridine) was added to 10 μM at 18 h before PBMC harvest. EdU incorporation was quantified (Click-iT EdU flow cytometry kit, Thermofisher) in CD45 stained (560178, BD Biosciences) PBMCs by flow cytometry. Decreases in proliferation were calculated as the fold change in EdU and CD45-positive PBMCs in cocultures relative to PBMCs cultured alone.

Inflammatory challenge: chondrogenically differentiated human ADSCs.

Due to interest in delivering differentiated stem cells or to differentiate stem cells in inflammatory environments,[64–67] we investigated the effects of targeted gene repression on chondrogenic differentiation of hADSCs in inflammatory conditions.

Pellet culture

Engineered and control hADSCs were chondrogenically differentiated in pellet cultures treated with TNF-α or IL-1β to investigate effects of TNFR1/IL1R1 repression on chondrogenesis, proliferation, and ECM deposition under inflammatory conditions. Chondrogenic media consisted of DMEM-HG with pyruvate (Thermofisher), 1X ITS+ premix (Corning), 0.1 μM dexamethasone, 0.35 mM proline, 0.17 mM ascorbic acid 2-phosphate, 1% antibiotic/antimycotic solution, and 10 ng/mL of TGFβ-3 and BMP-6 (Peprotech) (unless specified all reagents were purchased from Sigma).[68–70] 250,000 cells/pellet were pelleted in 200 μL of media at 142 G for 5 min. After 24 h, treatment with 1 ng/mL of TNF-α (controls and TNFR1-engineered cells) or IL-1β (controls and IL1R1-engineered cells) began. Pellets were cultured for 21 days with media replaced every 3 days after which pellets were either fixed in 10% NBF for histology ($n=2$–3/group) or frozen at −80°C for biochemical quantification ($n=5$/group). Additionally, supernatant was collected at each media change for biochemical quantification ($n=7$–8/group).

DNA and GAG quantification

Chondrogenic hADSC pellets ($n=5$/group) and supernatant ($n=7$–8) were analyzed for DNA content using the Hoechst dye assay[60] and/or GAG content using the modified DMMB assay.[71]

Histology

Pellet culture samples ($n=3$/group) were fixed in 10% NBF for 24 h, embedded in paraffin, and 5 μm sections were cut and stained with alcian blue and nuclear fast red (Newcomer Supply).

Statistics

All statistical analyses were performed using JMP pro software (SAS). Quantitative RT-PCR data were analyzed by one-way analysis of variance (ANOVA), using Dunnet's *post hoc* test (nontarget cells treated as control), using engineered cell groups as the factor. PBMC proliferation assay was analyzed by one-way ANOVA with Tukey's *post hoc* test, treating cell groups as the factor. NF-κB activity was analyzed by two-way ANOVA with Tukey's *post hoc* test, treating cell groups and cytokine dose as factors. Pellet size, DNA content, and GAG content data were non-normal and analyzed by a two-way ANOVA on ranks, with Tukey's *post hoc* test, using cell groups and cytokine dose as factors. Alpha level was set at 0.05 for all tests.

Results

Repression of inflammatory receptors in human ADSCs

Human ADSC transduction and qRT-PCR.

Lentiviral TNFR1 and IL1R1 targeting vectors were built based on HEK293T screen (Supplementary Data HEK293T Screen: Materials and Methods and Results section). Both TNFR1 and IL1R1 gene expression were downregulated in hADSCs by CRISPR-based repression, with TNFR1 repressed 90% ($p=0.0005$) by gRNA 1 and IL1R1 repressed 88% ($p<0.0001$) by gRNA 2 relative to nontarget controls (Fig. 2A). Nontarget controls showed no differences in receptor gene expression from nontransduced cells ($p=0.65$ [TNFR1], $p=1.00$ [IL1R1]).

Inflammatory challenge: undifferentiated human ADSCs

NF-κB activity. Repression of TNFR1 expression resulted in significant decreases in TNF-α-induced NF-κB activity at all TNF-α doses compared to nontransduced cells ($p=0.017$ at 150 pg/mL, $p<0.0001$ at 10 ng/mL, Fig. 2B). NF-κB activity remained at untreated (0 ng/mL) cell levels at both 150 pg/mL ($p=1.00$) and 10 ng/mL ($p=0.99$) doses. Repression of IL1R1 showed significantly less IL-1β-induced NF-κB activity at 150 pg/mL of IL-1β ($p<0.0001$, Fig. 2B) compared to nontransduced cells. NF-κB activity was equivalent to controls at 10 ng/ml IL-1β. The nontarget control showed no significant differences in NF-κB activity

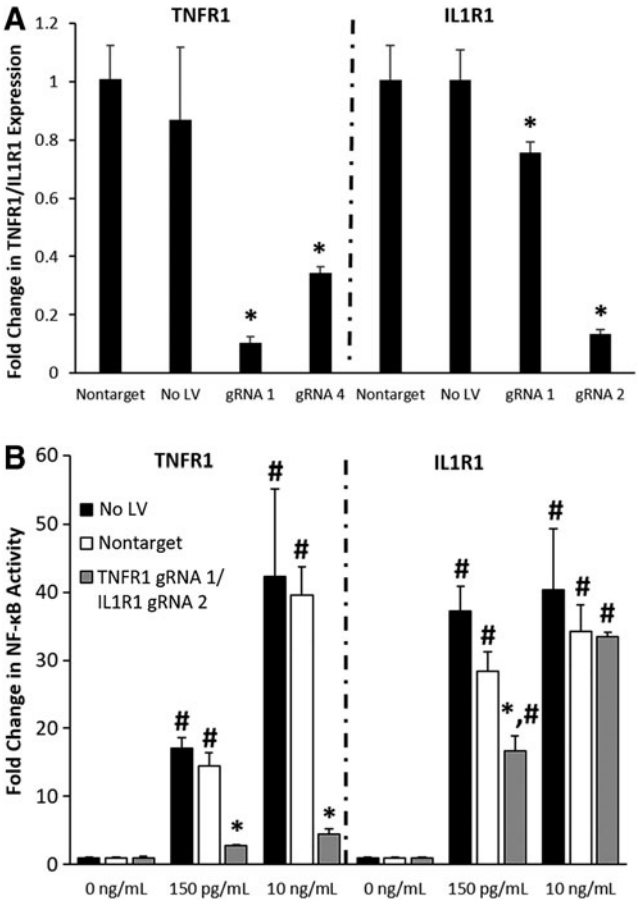


FIG. 2. Verification of lentiviral mediated gene and receptor signaling downregulation in hADSCs. Nontransduced groups abbreviated as ''No LV.'' **(A)** TNFR1 and IL1R1 expression in hADSCs post-transduction of epigenome editing system under the control of selected gRNAs ($n=3$, $*=p<0.05$ [TNFR1/IL1R1-engineered vs. Nontarget control cells]). **(B)** Fold changes in NF-kB activity post TNF-α/IL-1β dosing in engineered hADSCs that express the most efficient gRNAs (TNFR1: gRNA 1, IL1R1: gRNA 2) and in control cells ($n=4$, $*=p<0.05$ (cytokine-treated TNFR1/IL1R1-engineered cells vs. cytokine-treated No LV cells), $^{\#}=p<0.05$ (TNF-α/IL-1β treated cells vs. untreated controls). hADSC, human adipose-derived stem cells.

compared to nontransduced cells at any cytokine concentration ($p=1.00$ for TNF-α dosing, $p=0.13$ at 150 pg/mL IL-1β, $p=0.55$ at 10 ng/mL IL-1β).

Inflammatory cell pellet culture. Repression of TNFR1 and IL1R1 expression demonstrated protection of undifferentiated hADSCs from deleterious effects of TNF-α or IL-1β treatment. Both TNF-α ($p=0.024$) or IL-1β ($p=0.016$) treatment resulted in decreased cross-sectional area in nontarget control cells, while cytokine-treated engineered cells demonstrated no significant differences in cross-sectional area compared to untreated (0 ng/mL) groups (TNF-α: $p=0.94$, IL-1β: $p=0.90$) (Fig. 3A, B). Changes in DNA content due to TNF-α treatment demonstrated similar effects, with significant increases in cytokine-treated TNFR1-engineered cells compared to the nontarget control ($p=0.0021$) (Fig. 3C). IL-1β treatment showed similar trends for DNA content between groups, but differences were not statistically significant ($p=0.57$) (Fig. 3D). H&E staining of cell pellets demonstrated a decreased presence of cell nuclei (hematoxylin) and eosin staining in nontarget control cytokine-treated hADSC pellets (Fig. 3G, H) compared to all other pellet types and conditions.

Immunomodulation. Coculture of engineered hADSCs with PBMCs demonstrated maintenance of immunomodulatory properties after epigenome editing (Fig. 4). Relative to PBMCs cultured with nontransduced hADSCs, PBMCs cultured with TNFR1-engineered hADSCs showed no significant increase in proliferation ($p=1.00$). PBMCs cultured with IL1R1-engineered hADSCs showed a small (11%) but significant ($p=0.0059$) increase in PBMC proliferation relative to nontransduced hADSCs. However, there was a significant 43% decrease in PBMC proliferation in coculture with IL1R1-engineered hADSCs relative to PBMCs alone ($p<0.0001$) indicating maintenance of immunomodulatory properties after IL1R1 repression.

Inflammatory challenge: chondrogenically differentiated human ADSCs

GAG and DNA. Chondrogenic differentiation by TGFβ3 and BMP-6 was observed in both engineered and control hADSC cells with no significant differences in GAG/pellet observed between the untreated non-transduced control and nontarget control ($p=0.98$), TNFR1-engineered ($p=1.00$), or IL1R1-engineered cells ($p=1.00$) (Fig. 5). When treated with TNF-α or IL-1β in chondrogenic media, chondrogenesis was inhibited in nontransduced and nontarget control hADSCs, as noted by significant decreases in GAG content relative to respective untreated groups (Fig. 5). These decreases were observed for GAG/pellet (TNF-α treated: $p<0.0001$ (nontransduced), $p=0.0002$ (nontarget), IL-1β treated: $p<0.0001$ (non-transduced), $p=0.0043$ (nontarget)), and GAG released (TNF-α treated: $p<0.0001$ (nontransduced), $p=0.0012$ (nontarget), IL-1β treated: $p<0.0001$). Repression of TNFR1 resulted in the protection of chondrogenesis, by returning GAG released ($p=1.00$) to untreated cell levels. GAG/pellet measures were not fully returned to untreated levels in TNFR1-engineered cells, but were significantly elevated above the TNF-α treated nontransduced cell group ($p=0.0075$). IL1R1 repression resulted in elevated levels of GAG released compared to both treated controls ($p=0.0001$ [nontransduced], $p=0.045$ [nontarget]). No differences were observed between the IL1R1-engineered group for the GAG/pellet measure compared to nontransduced cells treated with IL-1β ($p=0.76$). The transduction of epigenome editing systems had no consistent effect on cell proliferation and no significant changes in DNA content were observed between TNF-α or IL-1β treated and untreated cells (TNF-α treated: $p=0.51$ [nontransduced], $p=1.00$ [TNFR1-engineered and nontarget], IL-1β treated: $p=0.82$ [nontransduced], $p=1.00$ [IL1R1-engineered and nontarget]) (Data not shown).

Histology. Histology was consistent with GAG/pellet quantitative measures indicating improved pellet GAG deposition in TNFR1-engineered cells, but not in IL1R1-engineered cells (Fig. 6). As expected from quantitative data, TNFR1 repression resulted in similar alcian blue staining between treated and untreated pellets indicating their improved ability to differentiate and deposit ECM in the presence of

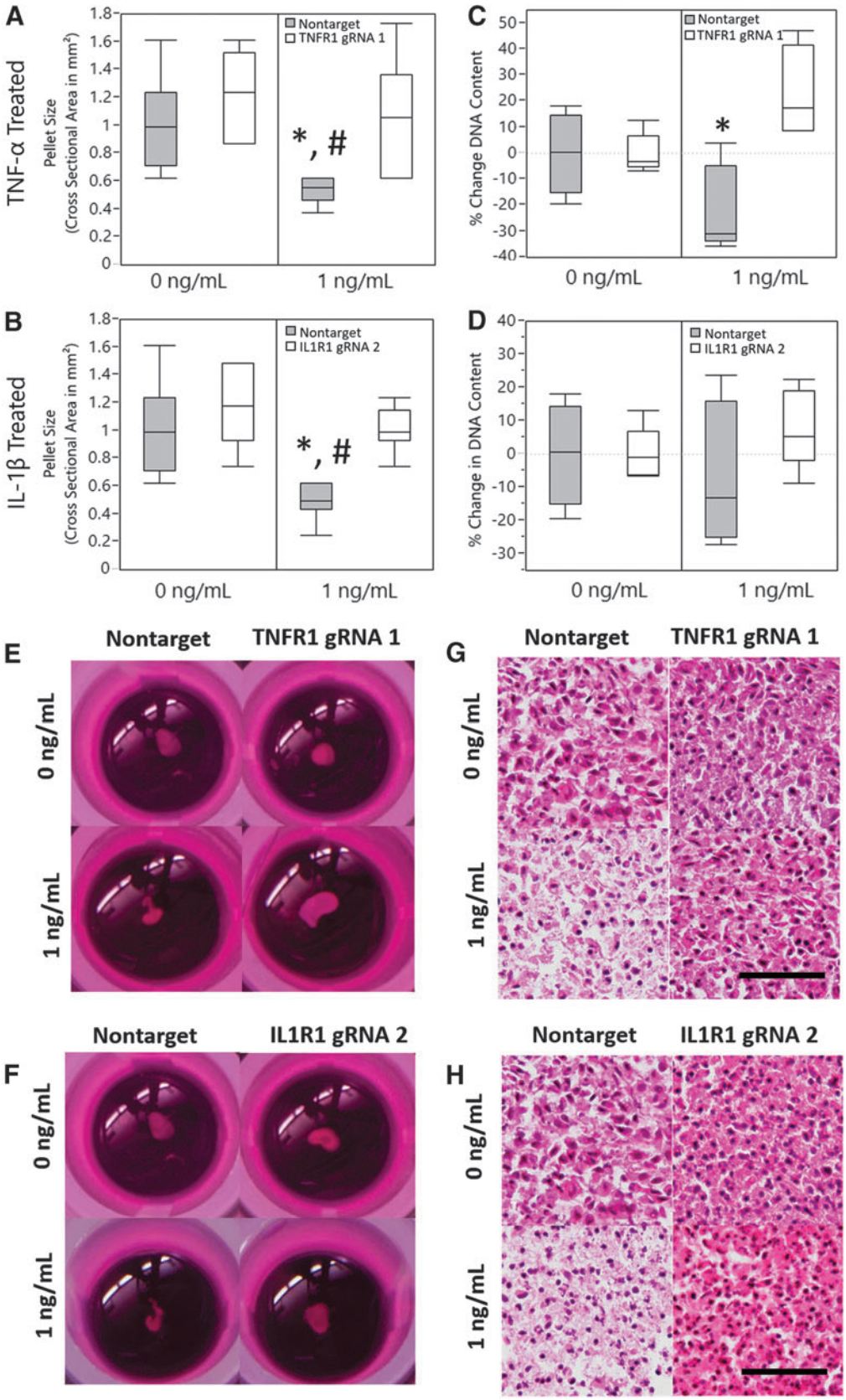


FIG. 3. Investigation of cell survival and proliferation and ECM deposition in 3D culture by hADSCs in the presence of cytokines. **(A, B)** Cross-sectional area of cell pellets in mm^2, cultured with or without TNF-α/IL-1β ($n=6$). **(C, D)** Percent changes in DNA content relative to untreated controls ($n=4$–5). **(E)** Representative images of untreated and TNF-α treated pellet cultures. **(F)** Representative images of untreated and IL-1β treated pellet cultures. **(G)** H&E staining of untreated and TNF-α treated cell pellets. **(H)** H&E staining of untreated and IL-1β treated cell pellets. ($^*=p<0.05$ compared to cytokine-treated TNFR1/IL1R1-engineered cells, $^{\#}=p<0.05$ compared to untreated control, scale bars are 50 μm). ECM, extracellular matrix; H&E, hematoxylin and eosin.

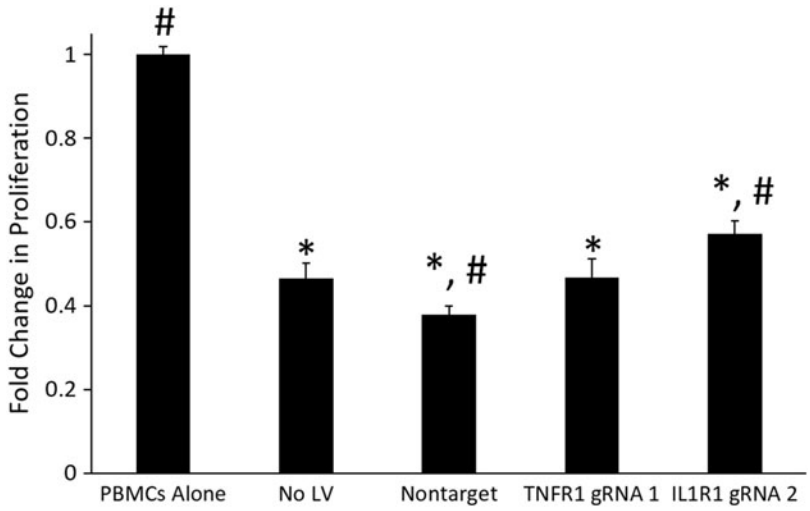


FIG. 4. Ability of engineered hADSCS to suppress PBMC proliferation in coculture. Graph shows fold change in proliferation of each coculture relative to PBMCs cultured alone ($n=4$), ($*=p<0.05$ relative to PBMCs alone, $^{\#}=p<0.05$ relative to coculture with nontransduced (No LV) hADSCs). PBMC, peripheral blood mononuclear cell.

TNF-α. IL1R1 repression results also qualitatively matched quantitative results, with visible decreases in GAG content in IL1R1-engineered cells after IL-1β treatment.

Discussion

CRISPR-based epigenome editing is a highly versatile tool with the ability to robustly upregulate and downregulate endogeneous gene expression with minimal offtarget effects.[30] With these features and ability to be multiplexed with orthogonal systems[72] it provides a powerful tool for regulating cell function. In this study we developed CRISPR-based epigenome editing systems that modulate inflammatory signaling by repressing TNFR1 and IL1R1 expression. These editing systems have the potential to protect both endogenous and exogenous cells within inflammatory environments by regulating receptor expression.

Repression of inflammatory cytokine receptors TNFR1 and IL1R1 was successfully performed in multiple cell types with repression reaching 90% in hADSCs (Fig. 2A), and 99% in HEK293T cells (Supplementary Fig. S1). The degree of repression corresponded to DNase I hypersensitivity

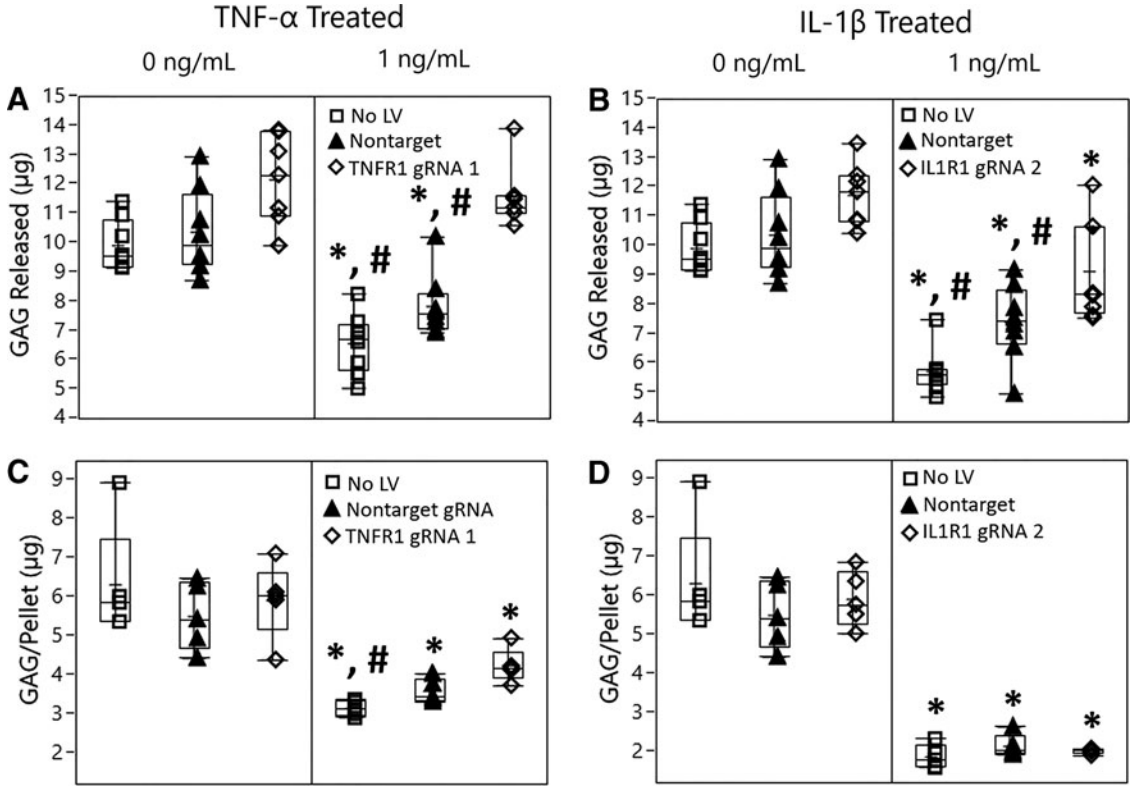


FIG. 5. Quantification of GAG content in pellet cultures that have undergone chondrogenic differentiation for 3 weeks with or without the presence of TNF-α/IL-1β. Nontransduced groups abbreviated as ''No LV.'' **(A, B)** Amount of GAG released into media during culture ($n=7$–8). **(C, D)** GAG content per pellet ($n=5$). ($*=p<0.05$ relative to 0 ng/mL control, $^{\#}=p<0.05$ relative to cytokine-treated TNFR1/IL1R1-engineered cells).

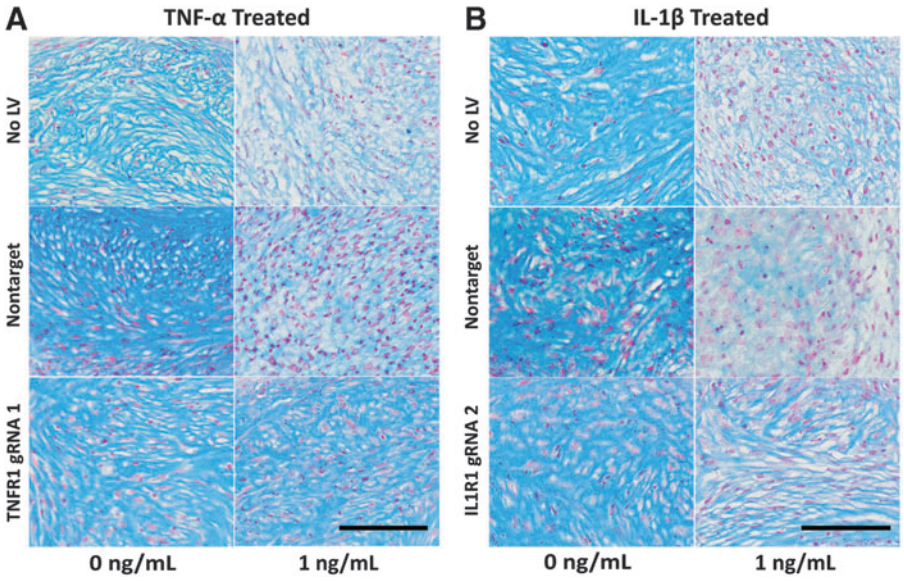


FIG. 6. Alcian blue and nuclear fast red staining of hADSC pellet cultures that have undergone chondrogenic differentiation for 3 weeks with or without treatment of **(A)** TNF-α or **(B)** IL-1β (Non-transduced groups abbreviated as ''No LV,'' scale bars are 100 μm).

levels at the gRNA target site (Supplementary Fig. S1), demonstrating the importance of targeting active promoter regions to achieve efficient gene repression.[32] It is important to note, while trends for TNFR1 regulation were similar between HEK293T and hADSCs, IL1R1 showed opposite trends between gRNA 1 and gRNA 2. This indicates cell type dependence in gene repression, and it may represent varying epigenomic regulation of IL1R1 between cell types.

We demonstrated that TNFR1 and IL1R1 regulation via epigenome editing downregulated proinflammatory NF-κB activity in inflammatory environments. Repression of TNFR1 signaling demonstrated a return of NF-κB activity to baseline levels (Fig. 2B). Repression of IL1R1 led to decreased NF-κB induction at 150 pg/mL, but not at 10 ng/mL IL-1β (Fig. 2B). Given that TNF-α and IL-1β levels are reported in the range of 0.1–400 pg/mL (TNF-α) and 1–800 pg/mL (IL-1β) in musculoskeletal pathology,[34,35,73–76] both observed responses suggest that the therapeutic range demonstrated in this study is consistent with musculoskeletal inflammation. Overall, this decrease in NF-κB signaling demonstrates the ability of CRISPR-based gene repression to downregulate this key proinflammatory signaling mediator directly linked to musculoskeletal diseases, through the propagation of inflammatory signaling, apoptosis, and protease production.[43–46,77]

To verify that TNFR1/IL1R1 repression is protective in inflammatory environments, we simulated hADSC delivery to such environments by TNF-α/IL-1β treatment of engineered cells in pellet culture. Cells were undifferentiated in this experiment, as current practice delivers progenitor cells to pathological musculoskeletal environments.[50,54–56,58] Our results demonstrated that TNFR1 and IL1R1 repression has a protective effect in these cultures (Fig. 3). Significantly larger cross-sectional areas in cytokine-treated TNFR1 and IL1R1 engineered pellets, indicated protection from the degenerative pellet phenotype. DNA content and histology demonstrated that this was an effect of cell numbers being maintained at untreated levels and increased ECM deposition in engineered cell pellets. Changes in DNA content between cytokine-treated and untreated cells demonstrated significantly enhanced survival of TNFR1-engineered hADSCs, when treated with TNF-α. This effect was not significant in IL1R1-engineered hADSCs, which is consistent with the less robust regulation of NF-κB activity observed in IL1R1-engineered cells compared to TNFR1-engineered cells. However, the overall increased pellet size and ECM deposition was observed in response to IL1R1 repression, indicating protection from the degenerative phenotype.

A major concern in altering the inflammatory receptor expression of ADSCs is the effect it may have on their inherent immunomodulatory properties. Stem cells from multiple sources have been shown to regulate immune cell activity and downregulate immune cell-mediated inflammation.[62,78–80] The ability for these cells to immunomodulate has been ascribed as a possible mechanism for stem cell action when delivered to musculoskeletal inflammatory environments.[81–84] Our data demonstrate that the immunomodulatory capacity of hADSCs is maintained after TNFR1 or IL1R1 repression (Fig. 4). However, it has been shown that immunomodulation is induced in stem cells via IFN-γ stimulation in combination with TNF-α/IL-1β.[62,85–88] Therefore, future work considering multiplex repression of TNFR1 and IL1R1 will need to consider its effect on stem cell immunomodulation.

In addition to the immunomodulatory properties of ADSCs, the ability to differentiate and promote ECM deposition is useful for ADSC delivery to inflammatory environments. However, inflammatory conditions associated with musculoskeletal disease inhibit chondrogenesis.[89–92] Our studies investigating hADSC chondrogenesis under inflammatory conditions, demonstrate improved chondrogenesis of hADSCs with TNFR1 or IL1R1 repression in the presence of TNF-α/IL-1β (Fig. 5). While TNFR1 repression showed more robust protection of chondrogenesis, both gene modifications showed improvements over nontransduced and nontarget controls. Overall, we demonstrated beneficial outcomes in the ability of CRISPR-based TNFR1/IL1R1 repression to allow chondrogenesis in inflammatory environments, which has applications for stem cells delivered to musculoskeletal inflammatory environments where differentiation is desired.

It is recognized that IL1R1 regulation was less protective than TNFR1 regulation. One possible explanation is that gene expression only explains 30–40% of the variance in protein abundance,[93] which could indicate greater IL1R1 presence than expected and reduced protection. Another potential mechanism is endogenous epigenome upregulation of IL1R1 by IL-1β stimulation (as demonstrated in other cell types[94–96]) overcoming our targeted epigenome modifications downregulating IL1R1. Finally, decreased levels of IL-1β stimulation may be needed to drive inflammatory signaling, compared to TNF-α. Future work will further investigate these mechanisms to better understand the therapeutic potential of these systems and improve our targeting of IL-1β signaling. One potential method to obtain more robust IL1R1 signaling inhibition is gene editing by CRISPR/Cas9 systems as their use in knocking out IL1R1 has recently shown success in potently inhibiting IL-1α signaling.[97]

While hADSCs were used in this study, appropriately designed CRISPR epigenome editing systems may be applied to other stem cells of interest, as these systems have shown efficacy in multiple cell types including embryonic and induced pluripotent stem cells.[98,99] Regarding clinical application of this technology, these lentiviral systems would be injected into a diseased joint or used to engineer therapeutic cells *ex vivo* before injection into the site of interest. The vectors in their current lentiviral form have the potential for clinical application, as several clinical trials with lentivirus have demonstrated promising results.[100–102] If alternatives are needed to improve the safety delivery profile, incorporation into safe harbor sites (i.e., AAVS1, ROSA26)[103] via CRISPR/Cas9 gene editing, nonintegrating adeno-associated virus,[104] or integrase deficient lentivirus[105] can be considered.

Conclusion

In this study we developed tools that utilize epigenome editing to downregulate TNFR1 and IL1R1 signaling, pathways associated with musculoskeletal diseases. We tested this regulation in hADSCs and demonstrated that repression of TNFR1 and IL1R1 inhibits NF-κB activation, promotes cell survival, protects ECM deposition, protects differentiation, and allows maintenance of immunomodulatory properties in inflammatory conditions. Overall it was demonstrated that TNFR1 and IL1R1 repression by CRISPR-based epigenome editing provides broad application to cell survival and function in inflammatory conditions, with potential utility for treating multiple musculoskeletal conditions.

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Disclosure Statement

No competing financial interests exist.

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