# IL1-β + TGF-β2 dual licensed mesenchymal stem cells have reduced MHC class I expression, enhance tenocyte migration and metabolism, and blunt IL-1β-induced tenocyte expression of *MMP13*

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**INTRODUCTION**: Intralesional mesenchymal stem cell (MSC) therapy to treat superficial digital flexor tendon (SDFT) injuries in equine veterinary patients has shown clinical and experimental benefit.[1,2] Unfortunately, MSC therapeutic efficacy has not translated to human tendon injury and is likely due to variability in MSC quality due to donor age and health status. Recent evidence indicates that cytokine stimulation (deemed licensing) of unstimulated, naïve MSCs may provide an avenue to enhance MSC function and reduce immune recognition, which may yield a source of allogenic cells capable of more consistent and repeatable treatment of tendon injuries in human and veterinary patients. The study objectives were to 1) determine if MSCs dual licensed with both IL-1β and TGF-β2 had reduced MHC class I expression and superior tendon-relevant transcriptional changes and 2) determine if dual licensed MSCs had greater ability to positively modulate tenocyte function over naïve MSCs.

**METHODS**: Equine bone marrow derived MSCs (n=6) and SDFT tenocytes (n=3) were isolated from donors, expanded, and frozen similar to previous protocols.[3,4] MSCs were grown in standard MSC growth media as naïve MSCs or standard MSC media containing IL-1β (2 ng/ml) and TGF-β2 (1 ng/ml) for 72 hours as dual licensed MSCs. Following 72 hours of growth in their respective medias, tenocytes, naïve MSCs, or dual licensed MSCs were switched to a media formulation containing low glucose DMEM, 5% fetal bovine serum (FBS), 1% l-glutamine, and 1% penicillin-streptomycin for 24 hours to generate conditioned media (CM) and used immediately for experiments. Cell surface expression of MHC class I was examined with flow cytometry and reported as geometric mean fluorescence intensity (GMFI).[5] Total RNA was extracted from paired MSCs and outsourced to Azenta for bulk RNA sequencing. Tenocyte migration (scratch wound) assays underwent 12 hours of serum deprivation on confluent tenocyte wells, a vertical scratch was created with a 200 ul pipette tip, wells washed twice with PBS, and appropriate CM added to each well. Each well was imaged every 12 hours for 36 hours and percent wound closure calculated as previously reported.[3] Tenocyte metabolism was determined by the reduction of a yellow tetrazolium salt (MTT) within each well and absorbance measured at 450 nm. Tenocytes were grown for 24 hours in CM, MTT added to each well, and absorbance measured every 24 hours for 72 hours. Finally, tenocytes were stimulated for 24 hours with IL-1β (10 ng/ml) and 12 hours into stimulation, appropriate CM added to each well until RNA was extracted. Gene expression was determined through a custom multiplex gene expression assay (Nanostring nCounter MAX) where extracted RNA was hybridized with target-specific reporter and capture probes and a digital detection assay used to determine mRNA counts within each sample. Differentially expressed genes (DEGs) were identified from RNAseq data using DESeq2 and the Wald test to generate log2 fold changes and p values. mRNA counts from the nCounter MAX system were normalized to three housekeeping genes. Each horse's expression levels were normalized to their own baseline expression. A one-way ANOVA with Tukey's test for multiple comparisons was utilized for MSC MHC expression and tenocyte migration, metabolism, and gene expression with $p<0.05$ deemed significant.

**RESULTS SECTION**: IL-1β + TGF-β2 dual licensed MSCs had significantly reduced surface expression of MHC class I compared to their naïve counterparts (p=0.0034). Additionally, transcriptome-wide analysis indicated that dual licensing significantly upregulated multiple genes specific for immunomodulation and tissue repair (*NOS2, HIF1A, PTGS2, TNFAIP6, IL11*), extracellular matrix production and remodeling (*ADAMTS4, COL3A1 MMP3 TIMP1, TNC*) and vascular development (*VEGFA, ANGPTL4*) that could provide positive reparative tendon-relevant effects (data not shown). When conditioned media from naïve or dual licensed MSCs was applied to tenocytes, a significant improvement in tenocyte migration was observed for dual licensed CM groups at 12 and 24 hours and for both naïve and dual licensed MSC CM at 36 hours (Figure 1). Similarly, tenocyte metabolism was significantly improved with the addition of dual licensed MSC CM over both naïve MSC CM and control tenocyte CM (Figure 2). Finally, IL-1β induced tenocyte gene expression of matrix metalloproteinase 13 (*MMP13*). Both naïve and dual licensed MSC CM significantly blunted IL-1β-induced MMP13 expression, and while dual licensed did to a greater degree, this was not to a significantly greater degree than naïve MSC CM (Figure 3).

**DISCUSSION**: These results indicate that dual licensing of MSCs with both IL-1β and TGF-β2 result in MSCs with a reduced MHC class I expression profile and secretion of paracrine factors that significantly enhance tenocyte parameters—such as migration, growth, and secretion of remodeling enzymes—that may be important for tendon healing following injury. With reduced MHC class I expression, this may also allow for MSCs to be used in an allogenic fashion. Limitations include lack of immune cell assays to determine immune response, utilization of CM instead of co-culture, and short duration of both IL-1β stimulation and treatment of tenocytes with CM which could prevent identifying more robust changes in tenocyte gene expression. However, these data provide strong support for further research examining whether IL-1β + TGF-β2 dual licensing is a relevant technique to improve MSC therapy of tendon injuries.

**SIGNIFICANCE/CLINICAL RELEVANCE**: Dual cytokine licensing of MSCs with IL-1β and TGF-β2 may provide an avenue to enhance MSC therapy for tendon injuries through improved secretion of paracrine factors and through reduced immune recognition allowing for a source of allogenic cells.

**REFERENCES**: [1] Godwin et al. 2012, *Equine Vet J*. doi: 10.1111/j.2042-3306.2011.00363.x [2] Schnabel et al. 2009, *J Orthop Res*. doi: 10.1002/jor.20887 [3] Koch et al. 2022, *Stem Cell Res Ther*. doi: 10.1186/s13287-022-03172-9. [4] Koch et al. 2022, *Front Vet Sci*. doi: 10.3389/fvets.2022.963759 [5] Berglund et al. 2017, *Front Vet Sci. d*oi: 10.3389/fvets.2017.00084

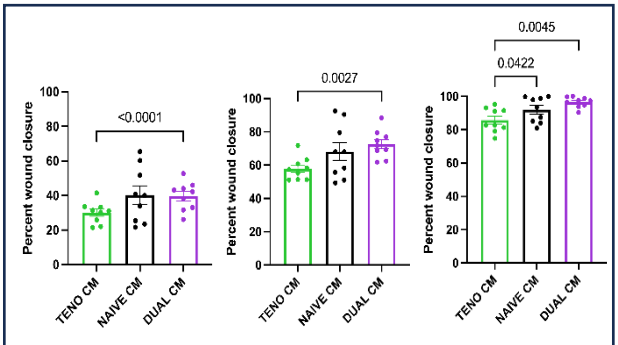


**Figure 1.** Tenocyte migration at 12, 24, and 36 hours (left to right). Dual licensed CM significantly enhanced migration over control tenocyte CM at all time points while naïve MSC CM finally provided a significant improvement at 36 hours. Data analyzed by one-way ANOVA with Tukey's multiple comparisons, n=9.

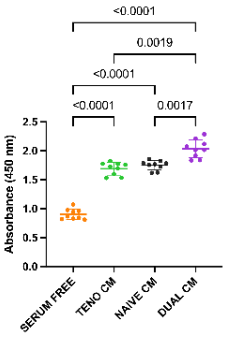


**Figure 2**. Tenocyte metabolism as determined by MTT reduction was significantly enhanced with dual MSC CM over naïve CM, tenocyte CM, and serum free media (negative control). Data analyzed by one-way ANOVA with Tukey's multiple comparisons, n=9.

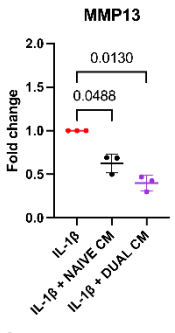


**Figure 3**. Tenocyte expression of MMP13 was significantly reduced with MSC CM, but not to a significantly greater degree in dual CM over naïve CM. Data analyzed by one-way ANOVA with Tukey's multiple comparisons, n=3.