3D Culture Priming Sustainably Improves the Immunosuppressive Potential of Mesenchymal Stromal Cells

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INTRODUCTION: The multimodal properties of mesenchymal stromal cells (MSCs), particularly their ability to modulate immune responses gained notable interest in translational research. Preconditioning of MSCs also referred to as MSC priming is widely used to improve the function, survival, and immunomodulatory potency of MSCs. Numerous promising priming strategies exist but no standardized priming protocols are available, which leads to heterogeneous MSC products and ultimately to inconsistent outcomes of clinical trials. The functional effects of pro-inflammatory and hypoxic priming – the most common priming strategies – as well as the emerging 3D culture priming on the immunosuppressive properties of MSCs are not well understood. Especially, the translationally relevant influence of physiological environments on the preservation of priming effects are unknown. Therefore, the aim of this study was to investigate: i) the effect of different priming strategies on the transcriptome and *in vitro* T cell suppressive capacity of MSCs, ii) the temporal stability of priming effects, and iii) the effect of physiologically relevant conditions on the temporal stability.

METHODS: This study was approved by the local ethics commission. Culture conditions and experimental analyses performed in this study are visualized in **Figure 1**. *Cell culture* – Commercial human iliac crest-derived bone marrow MSCs from three donors were used. MSCs were expanded and primed in minimum essential medium alpha supplemented with 5% human platelet lysate and 50 U/mL penicillin/streptomycin in a humidified incubator at 37 °C, 21% O_2, and 5% CO_2. MSCs were primed for 48 h with either pro-inflammatory (20 ng/mL TNF-α and 20 ng/mL IFN-γ), hypoxic (1% O_2), or 3D culture priming (200 MSCs per spheroid in Kugelmeiers SP5D®). Following priming, MSCs were cultured either under standard conditions (0 pg/mL TNF-α, 21% O_2, and monolayer culture) or translationally relevant conditions (50 pg/mL TNF-α, 5% O_2, or 3D culture). *RNA sequencing* – Transcriptomic differences of the priming strategies were analyzed with RNA sequencing using NovaSeq 6000 sequencing system (16 million reads per sample) and visualized by a principal component analysis (PCA). Genes of primed MSCs with FDR ≤ 0.1 and $|\log_2 \text{fold change}| \geq 1.5$ compared to the gene expression of unprimed MSCs were considered as differentially expressed genes (DEGs) and analyzed with an over-representation analysis (ORA). *Quantitative real-time polymerase chain reaction (qPCR)* – To investigate the persistency of priming, immunomodulation-associated DEGs were selected from the gene ontology terms *immune response* and *inflammatory response* as priming signature genes and their expression was semi-quantified with qPCR at days 0, 4, and 8. Three gene sets, each containing five signature genes were assigned to a separate priming strategy. *In vitro functional immunosuppressive potency assay* – To assess the immunomodulatory effect of priming, T cell suppression of MSC was quantified using an *in vitro* functional potency assay at days 0, 4, and 8. Briefly, PBMCs were labeled with a cell proliferation tracer (CellTrace®), activated with a T cell activator (TransAct®), and co-cultured with primed MSCs for three days before quantifying the proliferation state of the T cells using flow cytometry. *Statistical analysis* – Two-way ANOVA followed by Tukey post-hoc test and adjusted for multiple comparisons were calculated in GraphPad PRISM v.10.0.1.

RESULTS: i) Pro-inflammatory, hypoxic, and 3D culture priming induced profound transcriptomic changes in MSCs, with hypoxic and 3D culture priming inducing more extensive changes compared to pro-inflammatory priming (**Figure 2a)**. Directly following priming, the *in vitro* T cell suppressive effect was significantly enhanced in pro-inflammatory primed MSCs (-99.00% ± 11.02%, p = 0.02), trended towards enhancement in 3D culture primed MSCs (-35.33% ± 13.48%, p = 0.13), and was not different in hypoxic primed MSCs (+21.33% ± 14.53%, p = 0.40) (**Figure 2b**). ii) The differential expression of the signature genes for each priming strategy faded rapidly over time (**Figure 3a**). Additionally, pro-inflammatory and 3D culture priming lost their *in vitro* T cell suppressive effects few days following priming (**Figure 3b**). iii) When primed MSCs were cultured under physiological conditions, they demonstrated a decelerated fading of the priming effects (**Figures 3a,b**). Physiological culture after priming (50 pg/mL TNF-α) partially preserved the T cell suppression capacity (day 4: -35.00% ± 12.78%, p = 0.11, day 8: -19.33% ± 12.52%, p = 0.37). Only 3D culture primed MSCs demonstrated a preserved potency to suppress T cell proliferation (physiological conditions versus standard conditions, day 4: -26.67% ± 10.85%, p = 0.16, day 8: -34.33% ± 4.88%, p = 0.009).

DISCUSSION: In this study, we demonstrated that i) pro-inflammatory and 3D culture priming but not hypoxic priming of MSC enhances T cell suppressive potency although hypoxic priming caused greatest transcriptomic changes. ii) The pro-inflammatory and 3D priming effect is lost within few days, and iii) translationally relevant tissue inflammation level (50 pg/ml TNF-α) cannot preserve the pro-inflammatory priming effect, yet 3D culture can preserve the effect of 3D priming over time. This signifies that MSC therapies utilizing pro-inflammatory or 3D culture priming to enhance their immunosuppressive potencies should minimize the time between priming and administration to maximize their therapeutic potential. Our data support the administration of MSC spheroids for a sustainably improved immunosuppressive potency. Guidelines on MSC priming, highlighting such clinically relevant implications, could contribute to more standardized and potent MSC products.

SIGNIFICANCE/CLINICAL RELEVANCE: Priming holds great potential in overcoming clinical challenges faced by MSC therapies, but the absence of standardized priming protocols along with a limited understanding of the cellular adaptations to priming represent serious translational hurdles. This work highlights the transient nature of priming strategies and the significant influence of physiological environments on the immunosuppressive potency of MSCs, that must be considered in future clinical trials using primed MSCs.

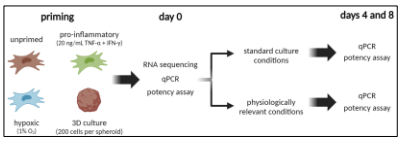

Figure 1: *Illustration of the experimental setup*

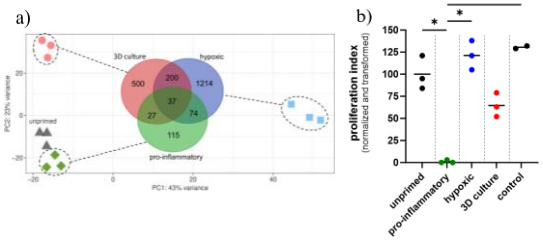

Figure 2: ***a)*** *Priming strategies induce distinct transcriptional changes in MSCs.* ***b)*** *in vitro functional immunosuppressive potency of differently primed MSCs*

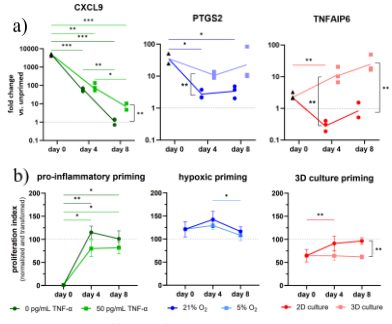

Figure 3: *Effect of culture conditions on the temporal preservation of* ***a)*** *transcriptional changes and* ***b)*** *in vitro immunosuppressive potentials of differently primed MSCs.*