Development of an Allogeneic, Anti-Inflammatory Bone Marrow Stromal Cell Therapy for Chronic Low Back Pain Patients with Modic Type 1 Changes

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Zusammenfassung

Chronische Rückenschmerzen sind nicht nur weit verbreitet, sondern auch extrem einschränkend. Modic Typ 1 Veränderungen (MC1) – schmerzhafte fibro-entzündliche Veränderungen im Wirbelknochenmark neben degenerierten Bandscheiben – werden häufig bei Patienten mit chronischen Rückenschmerzen (cLBP) beobachtet. Dennoch werden sie nicht als spezifische Schmerzquelle betrachtet. Trotz überzeugender Beweise für einen Zusammenhang zwischen MC1 und cLBP erhalten Patienten mit cLBP dieselbe Behandlung wie cLBP-Patienten ohne MC1. Die hohe Prävalenz von cLBP-Patienten mit MC1, kombiniert mit dem Fehlen einer anerkannten Behandlungsstrategie, verdeutlichen die Notwendigkeit einer gezielten Behandlung.

Diese Doktorarbeit legt die Grundzüge für die Entwicklung einer allogenen, entzündungshemmenden Therapie bestehend aus mesenchymalen Stromazellen (MSCs) für cLBP-Patienten mit MC1.

Wir haben festgestellt, dass die schmerzhafte Entzündung – ein zentrales pathologisches Merkmal von MC1 – durch die ausgeprägten entzündungshemmenden Eigenschaften von MSCs gezielt unterdrückt werden könnte. Wir haben die Vor- und Nachteile der Verwendung von stark entzündungshemmenden, allogenen MSCs zur Behandlung von MC1 diskutiert, unter Berücksichtigung früherer Untersuchungen zur Behandlung von cLBP-Patienten mit MSCs. Unsere Literaturrecherche verdeutlichte, dass eine hohe entzündungshemmende Potenz einer MSC-Therapie unerlässlich ist, um einen therapeutischen Nutzen zu erzielen. Pro-entzündliches und 3D-Kultur Priming zeigten eine erhebliche Wirksamkeit bei der Steigerung des entzündungshemmenden Potenzials von MSCs. Wir konnten zeigen, dass eine kontinuierliche 3D-Kultur in der Lage war, das gesteigerte entzündungshemmende Potential aufrechtzuerhalten, was die Verabreichung von MSC-Sphäroiden für eine nachhaltig verbesserte entzündungshemmende Potenz bekräftigt.

Die in dieser Doktorarbeit vorgestellten Ergebnisse legten den Grundstein für die Entwicklung einer allogenen, entzündungshemmenden MSC-Therapie für cLBP-Patienten mit MC1. Zukünftige Studien sollten sich auf die Reduzierung der inkonsistenten Potenzen von MSC-Kulturen konzentrieren.

Abstract

Chronic low back pain is not only a prevalent but also an extremely disabling condition. Modic type 1 changes (MC1) – painful fibro-inflammatory changes in the vertebral bone marrow adjacent to degenerated intervertebral discs – are frequently observed in chronic low back pain (cLBP) patients. Yet, they are not considered a specific source of pain. Despite solid evidence supporting a clear association of MC1 with cLBP, patients suffering from cLBP receive the same treatment as cLBP patients without MC1. The high prevalence of cLBP patients with MC1, combined with the absence of a recognized treatment strategy, underscores the need for a targeted treatment.

This doctoral dissertation, aimed to lay the foundation for the development of an allogeneic, anti-inflammatory bone marrow mesenchymal stromal cell (MSC) therapy for cLBP patients with MC1.

We identified that the painful inflammation – a pivotal pathologic feature of MC1 – could be targeted by the substantial anti-inflammatory properties of MSCs. We have discussed the advantages and disadvantages of using highly anti-inflammatory, allogeneic MSCs to treat MC1 in light of previous investigational MSC treatments for cLBP patients. Our literature review suggested that a high anti-inflammatory potency of an MSC therapy is essential to achieve a beneficial therapeutic effect. Pro-inflammatory and 3D culture priming demonstrated a considerable efficacy in enhancing the anti-inflammatory potential of MSCs. We demonstrated, that continuous 3D culture was able to maintain the enhanced anti-inflammatory potential, supporting the administration of MSC spheroids for a sustainably improved anti-inflammatory potency.

The achievements presented in this doctoral dissertation laid the foundation for the development of an allogeneic, anti-inflammatory bone marrow MSC therapy for cLBP patients with MC1. Future studies should focus on reducing the inconsistent potencies of MSC preparations.

Chapter 1

General introduction

1.1 Low back pain

1.1.1 Epidemiology

Approximately 60-80% of the general population experience non-specific LBP at some point in their lifetime, with 20% of individuals suffering from chronic LBP, and 10% being disabled by LBP. [1,2] With an estimated global incidence of 3.2%, prevalence of 7.6%, and 64.9 million disability-adjusted life years (DALYs) in 2020, LBP represents a major cause of disability worldwide. [3,4] In fact, LBP is the most common health condition that requires rehabilitation services in an alarmingly high number of 134 out of 204 countries studied. [5–8] The market size for chronic LBP treatments was valued at CHF 1.98 billion in 2022 and was projected to reach CHF 4.96 billion by 2030, corresponding to a compound annual growth rate (CAGR) of 9.5%. [9]

1.1.2 Guidelines for the management of chronic low back pain (LBP)

Most international clinical practice guidelines for the management of chronic LBP advise against the use of pharmacological treatments as a first-line therapy. [10] Before the administration of pharmaceuticals, most guidelines recommend exercise therapy, physiotherapy, and psychosocial interventions. [11,12] If medication is needed, non-steroidal anti-inflammatory drugs (NSAIDs) are recommended, while the use of opioid analgesics is discouraged. [12] Interestingly, a study on pain trajectories in LBP patients found that patients who took NSAIDs were at a higher risk of persistent pain. This finding raises concerns about the use of NSAIDs for managing acute LBP, as they might contribute to the development of chronic LBP. [13] Guidelines agree on a multimodal pain management approach combining non-pharmacological and pharmacological treatment strategies. [10,14]

1.1.3 Heterogeneity of chronic LBP patients

The multifactorial nature of chronic LBP can make it difficult to identify a single pain generator. Therefore, up to 90% of patients suffering from chronic LBP receive the unsatisfactory diagnosis “non-specific LBP”, meaning that the specific cause of pain remains unidentified. [15] Various spinal structures such as the intervertebral discs, vertebral bodies, facet joints, fasciae, ligaments, and muscles are plausible sources of pain but historically, standard diagnostic tests could not attribute the pain to those structures. [16,17] With the arise of advanced affordable diagnostic tools, the number of identifiable pain generators is expected to increase. [18] Common disorders present in specific LBP include spinal canal stenosis, vertebral compression fractures, spondyloarthritis, intervertebral disc degeneration, and cancer. This list is not exhaustive as clinical presentations such as myofascial pain and Modic changes are not yet considered a specific source of LBP and therefore categorized as non-specific. [17] If a specific pain generator can be identified, patients can benefit from non-pharmacological, pharmacological, or surgical interventions e.g. myorelaxants for LBP of myofascial origin or surgery for patients with a severe stenosis of the spinal canal. However, if the cause of LBP is unknown, patients disqualify from

targeted treatment approaches. Therefore, the treatment of patients with non-specific LBP focuses on pain and disability management. No treatment can cure persistent LBP, but interventions exist that can reduce pain and disability. [18,19] If exercise therapy, physiotherapy, and psychosocial interventions do not achieve the desired outcomes, pharmacological interventions will be used. However, the long-term use of analgesics for the management of chronic non-specific LBP can have serious side effects. [20]

The development of a universal disease-modifying treatment for chronic non-specific LBP is unlikely. The limited understanding of specific biological or mechanical pain generators largely contributes to the absence of disease-modifying treatments for patients with chronic non-specific LBP. More research should focus on the identification of pain generators that can be specifically treated by disease-modifying interventions. Stratification of non-specific LBP patients into subpopulations with targetable sources of pain might enable the development of disease-modifying treatments. Inflammatory Modic type 1 changes represent such a targetable source of pain, which might be treated with an immunomodulatory treatment approach. [21]

1.2 Modic changes

1.2.1 Definition

Modic changes (MC) are vertebral bone marrow lesions adjacent to degenerated intervertebral discs. [22,23] They are defined as signal intensity changes on T1w and T2w magnet resonance imaging (MRI) scans, and were named after Michael Modic in 1988. [23] MC can be stratified into three interconvertible types of MC based on their appearance on MRI (**Chapter 3 Figure 1**). Modic type 1 changes (MC1) – T1w: hypointense, T2w: hyperintense – are painful fibro-inflammatory changes with an abundance of granulation tissue, inflammatory infiltrates, and interstitial water. [22–25] Evidence strongly suggests that inflammation plays a pivotal role in MC1, as indicated by bone marrow edema-like changes in MRI, the presence of inflammatory dysmyelopoiesis, and elevated expression of pro-inflammatory cytokines in the adjacent intervertebral disc. [24,26–28] Additionally, T cell infiltration and dysregulated granulocyte maturation have been identified as potential contributors to MC1. [25,29] Our unpublished total RNA sequencing and single cell RNA sequencing data corroborate these findings and indicate that T cells are overrepresented and activated in MC1 lesions. It was previously shown that T cell infiltration was associated with MC1-like MRI changes in a rat-tail model of the vertebral bone marrow. [30] In Modic type 2 changes (MC2) – T1w: hyperintense, T2w: hyperintense – fatty bone marrow replaces the red hematopoietic bone marrow and the trabecular bone is thickened. [22,23,27,29] Modic type 3 changes (MC3) – T1w: hypointense, T2w: hypointense – represent subchondral bone sclerosis and possibly develop as a result of the abnormal mechanical loads on the vertebral endplates and vertebral bodies. [23] The vertebral bone will likely adapt to the altered mechanical stresses resulting in increased bone density or sclerosis. [27] Despite our growing understanding of the molecular and

cellular changes in MC bone marrow and discs, the underlying cause of MC remains elusive. Clinical and experimental studies support auto-inflammation against disc material and occult disc infection in the development of MC. [30–35] While infectious MC may be treated with antibiotics, there is no consensus on the treatment of auto-inflammatory MC.

1.2.2 Epidemiology

The reported prevalence of MC is inconsistent. A systemic literature review found a median prevalence of MSCs across 58 study samples to be 43% in non-specific LBP patients and 6% in the non-clinical population. [26] Among the three MC types, MC2 are the most common (≈72%), followed by MC1 (≈23%), with MC3 being the rarest type (≈5%). [26,36] The assignment of MC to one type is often ambiguous, resulting in a notable portion of MC with mixed types. [37] A major problem with MC diagnosis is the misclassification of MC subtypes. MRI field strength, acquisition matrix, and pulse sequence parameters influence image resolution, signal-to-noise, and contrast-to-noise ratios. [38] MRI precision is paramount to reliably distinguish MC subtypes. Therefore, the different prevalence of MC subtypes reported by various studies can partially be explained by the use of different MRI instruments and settings between the studies. [26,36,39]

The Swiss population has approximately 9 million people, 7.6% (684 thousand) suffer from LBP, and in 90% (616 thousand) of these people, no specific pain generator can be identified. [3,15] Among these 616 thousand Swiss people with LBP approximately 43% (265 thousand) suffer from any type of MC, with roughly 23% (61 thousand) being affected by MC1, the most painful type of MC. [26,36,40] Therefore, LBP patients with MC1 represent a considerable market. In the absence of a recognized treatment approach, an efficacious therapy could become the standard of care.

1.2.3 Association with LBP

The association of MC (any type) with LBP is controversial. Two recent systematic reviews found that the associations reported in previous literature between MC (any type) and LBP severity are inconsistent. [41,42] However, a significant association between MC and pain duration was observed across 17 studies. [42] When the study subjects were stratified for MC type, MC1 demonstrated a positive association with LBP, albeit not reaching statistical significance. [41] MC2 also positively associated with LBP but to a lesser extent, and MC3 showed no association with LBP. [41]

Importantly, a study investigated the impact of inter-rater agreement and the number of study participants on the relationship between MC and LBP. [38] They found that at least 200 participants must be tested in a study with an inter-rater agreement of Cohen's kappa coefficient = 0.79 (κ), to find a significant association between MC and LBP. In their simulation, they determined κ = 0.79 based on the mean inter-rater agreement of MC classification from six studies. In 2018, Herlin et al. summarized

the outcomes of 30 studies reporting on the association between MC (any type) and LBP. 15 studies found statistically significant positive associations, one found a statistically negative association, and the remaining 14 studies reported statistically non-significant findings. [41] The number of participants included in these studies varied from 36 to 2449 participants, with 15 of them including a meaningful >200 participants. Among these studies with a minimum of 200 participants, nine studies found a statistically positive association, and five studies reported statistically non-significant findings. Six studies included a minimum of 500 participants, with five of them reporting a statistically significant association between MC and LBP, whereas one study found no statistically significant association. Therefore, MC are likely associated with LBP, when only studies with a meaningful number of participants are considered. Another study with 50 chronic LBP patients investigated if vertebral endplate abnormalities, identified by MRI, associate with the outcomes of lumbar pain provocation tests (discography). [43] Discography was employed to assess if an IVD was painful. They assessed if IVDs adjacent to MC1 or MC2 are more likely to be painful than IVDs without adjacent MC. The authors found that MC1 and MC2 are useful in the prediction of painful IVDs in LBP patients. Patients with MC1 or MC2 adjacent to investigated IVDs demonstrated a positive predictive value of pain of 88% and even reached 100% when only moderate and severe cases were considered. This study showed that MC are local pain generators. Interestingly, MC1 patients have an inflammatory pain pattern, which is less responsive to standard treatments but more responsive to oral steroids. [26,44] This could be explained by the significantly increased number of immunoreactive nerve fibers and tumor necrosis factor-immunoreactive cells, in the vertebral endplates of patients with MC1 or MC2. [28,45]

In summary, the inconsistent associations of MC types with pain severity across all published studies might derive from substantial differences in study designs including different numbers of study participants, divergent inter-rater agreements, study population, imaging equipment, and scanning parameters. [38] There is solid evidence that MC are associated with LBP, when only studies with a meaningful number of participants are considered. The association of MC with LPB is further supported by a study showing that IVDs adjacent to moderate and severe cases of MC1 or MC2 were always painful, when assessed by discography. Detailed diagnostic guidelines defining painful MC types and standardized MC visualization protocols might be needed to identify a homogeneous population of clinically meaningful MC patients.

1.2.4 Treatment approaches

MC are not yet considered a specific source of LBP. Therefore, LBP patients with MC generally receive the same treatments as non-specific LBP patients without MC. There are few ongoing efforts towards developing targeted MC-specific treatments, with all of them focusing mainly on inflammatory MC1. TNF-α inhibitors and intradiscal steroids to suppress inflammation, antibiotics to target the potential

underlying bacterial infection, anti-nerve growth factor antibodies to terminate pathologic neo-innervation, and bisphosphonates to restore bone remodeling equilibrium, all represent valid treatment approaches. [21,46–54] Platelet-rich plasma (PRP) injection is a potential immunomodulatory and regenerative treatment approach with a limited duration of action, that has demonstrated promising outcomes in chronic LBP patients. [55,56] Hence, a randomized placebo-controlled clinical trial (NCT03712527) is currently investigating the efficacy of intradiscal PRP injections for chronic LBP patients with MC1. MSCs might represent an alternative treatment approach with an adaptive and multimodal mode of action. [57,58] Their favorable safety profile, potentially high efficacy, and their long duration of action, due to their ability to proliferate, make them interesting candidates for the treatment of MC1. MSCs have an exceptional potential to modulate a broad range of cells of the innate and the adaptive immune system, which might be needed to address inflammatory MC1 lesions.

1.3 Mesenchymal stromal cells

1.3.1 Definition

Mesenchymal stromal cells (MSCs) gained tremendous interest in translational medicine over the past decade, with an impressive number of over 450 completed interventional clinical trials using MSCs listed in the ClinicalTrials.gov database. Especially the field of regenerative medicine experienced a surge in MSCs, due to the translationally relevant properties of MSCs including a multi-lineage differentiation capacity, engraftment potential, self-renewal capacity, and a pronounced immunomodulatory potency. [21] Despite the wide use of MSCs in clinical settings, an accurate answer to the question: "What are MSCs?" proves to be difficult. The advent of MSCs dates back half a century when Friedenstein et al. discovered the presence of a cell population resident in the bone marrow, capable of connective tissue formation (e.g. bone, cartilage, adipose tissue, tendon, ligament). [59–61] As connective tissue originates from mesenchymal tissue, Caplan coined the term "mesenchymal stem cell" in 1991. [62] MSCs were thought to exclusively derive from the mesoderm, the germ layer that gives rise to the mesenchymal tissue. Interestingly, certain subtypes of MSCs derive from the ectoderm through a process called epithelial-mesenchymal transition (EMT). [63] These ectoderm-derived MSCs were found to be a non-proliferating subtype of MSCs involved in the formation of the hematopoietic niche. [64] In contrast, mesoderm-derived MSCs are associated with an intense proliferation capacity and seem to be involved in the embryo skeleton formation. [65] Interestingly, MSCs have been shown to be able to differentiate into cells originating from all three embryonic germ layers under specific cell culture conditions (**Figure 1**). [66]

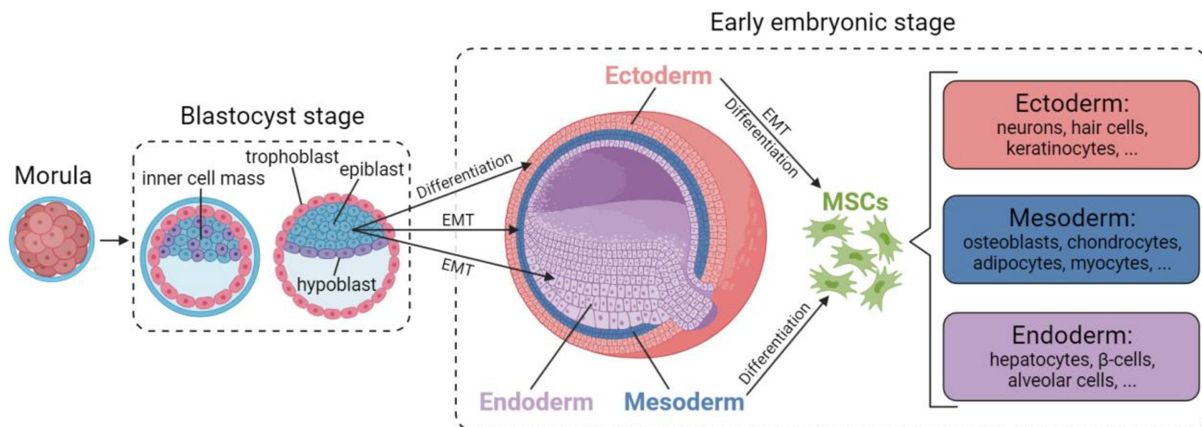

***Figure 1** Ontogenesis of MSCs. Adapted from: Li et al. (2021) [63]. Created with BioRender.com*

It is generally discouraged to use the term "stem cells" to describe multipotent stromal cells derived from mesenchymal tissue sources, unless there is proven evidence for self-renewal and multilineage differentiation capacity based on rigorous functional in vitro and in vivo tests. [67] As a consequence of conflicting data regarding the multi-lineage differentiation capacity and the self-renewal capacity of stromal cells isolated from mesenchymal tissues, the International Society for Cell and Gene Therapy (ISCT) published a position statement in 2006. [68] The ISCT recommended to use the term "mesenchymal stromal cells" to describe cells that fulfill three minimal criteria. First, MSCs must adhere to plastic in standard culture conditions. Second, MSC must express CD73, CD90, CD105, and lack the surface molecules CD14 or CD11b, CD19 or CD79α, CD34, CD45, and HLA-DR. Third, MSCs must be able to differentiate into adipocytes, chondroblasts, and osteoblasts in vitro.

1.3.2 Heterogeneity of MSC preparations

MSCs describe a bulk of non-hematopoietic and non-endothelial cells encompassing fibroblasts, myofibroblasts, and a small fraction of self-renewing stem cells. [69–71] The proportion of these cell types in isolated MSCs likely varies based on the tissue source from which they are harvested. [72,73] It is well established that MSCs have a cellular plasticity, and that their functions are tightly regulated by their surrounding microenvironment. [74] Therefore, MSCs isolated from different tissue sources may have distinct functions (**Figure 2**). Furthermore, MSC donor characteristics including age, sex, and health status have been shown to affect the proliferation rate, differentiation potential, and immunomodulatory properties of MSCs. [72,73] The inherent adaptability of MSCs, coupled with the absence of standardized in vitro culture protocols, contribute to the heterogeneity of MSC preparations. [75–77] This is particularly important regarding the development of MSC based therapeutics, where standardization of MSC preparations is crucial to ensure the quality of the product. Consequently, the marked heterogeneity of MSC preparations may result in inconsistent therapeutic potentials.

1.3.3 Translational landscape of MSC therapies

The multimodal properties of MSCs along their adaptability to pathologic microenvironments make them an attractive therapeutic option to treat a broad range of clinical conditions (**Figure 2**). Levy et al. gave in their detailed review an overview of ongoing and completed clinical trials using MSCs, and provided a comprehensive list of MSC products that have received regulatory approval. [57] Importantly, the majority of approved MSC products rely on the immunomodulatory properties of MSCs, not their regenerative capacities. This suggests that the translational potential of MSC therapies lies within the immunomodulatory capacity of MSCs. Most clinical trials on MSC therapies failed to meet their primary efficacy endpoints, likely due to the inconsistent efficacy of MSC preparations. [57] To address this challenge, bioengineering strategies could be employed to produce consistently effective MSC preparations.

As the immunomodulatory properties of MSCs appears to be the main driver of the translational potential of MSC therapies, the most commonly investigated indication of MSC-based therapies include osteoarthritis, ischemic hearth disease, and graft-versus-host disease. [78]

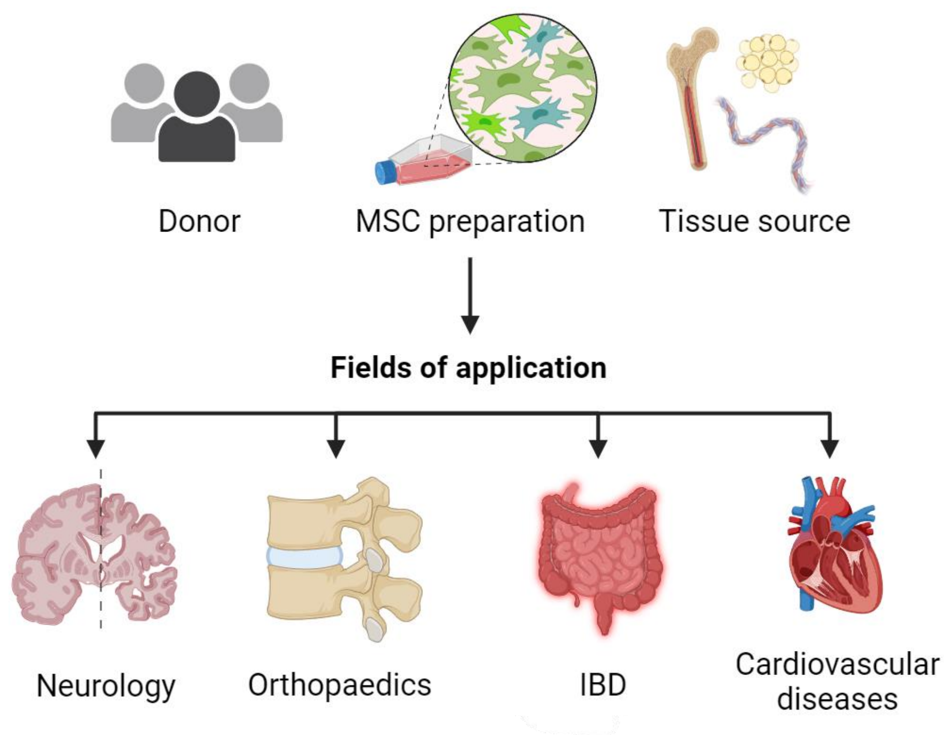

Figure 2 *Sources of heterogeneity in MSC preparations and their potential fields of application. IBD = inflammatory bowel disease. Created with BioRender.com*

Notably, inflammation plays a crucial role in the pathogenesis of all the aforementioned conditions. The potential advantages of MSC therapies over conventional drugs for immunomodulatory treatments are manyfold. While MSC therapies are associated with high costs and an invasive route of administration, their favorable safety profile, potentially high efficacy, and the long duration of action

might outweigh their disadvantages. Even though, tumorigenesis is a widely discussed safety concern of MSC therapies, no conclusive evidence could show an association between MSC administration and tumor formation in humans. [79] Allogeneic immunogenicity is a valid safety concern of allogeneic MSC therapies. Few pre-clinical studies observed an immune response following the infusion of allogeneic MSCs. [80–83] In contrast, more than 3000 human patients have received allogeneic MSCs for various indications, and no adverse event linked to the potential allogenicity of MSCs has been reported. [84–87]

The human leukocyte antigen (HLA) allows the immune system to distinguish between the body's own cells and foreign invaders. HLA class I molecules are found on most nucleated cells as well as platelets, whereas HLA class II molecules are exclusively expressed on antigen-presenting cells. [88] The marked HLA polymorphism enables the defense against invaders carrying distinct HLA molecules. A mismatch between the HLA molecules on grafted tissues and the recipients HLA molecules can result in alloreactions against the grafted tissues. [89,90] The matching of HLA molecules between organ donors and recipients plays a crucial role in organ transplantation to reduce the risk for transplant rejections. The same concerns might arise from the transplantation of allogeneic MSCs. Alloreactions against repeated intra-articular injections of HLA mismatched allogeneic MSCs, but not HLA matched allogeneic of autologous MSCs, were observed in an equine model. [91] The alloreactions resulted in local inflammation and reduced MSC therapeutic action, which were not observed in single HLA mismatched allogeneic MSC dose applications. On the other hand, the administration of HLA mismatched allogeneic MSCs for the treatment of osteoarthritis and degenerative disc disease in humans only mounted a weak and transient immune response against the MSCs, with no effect on therapy efficacy. [90] The limited immune reactions against HLA mismatched allogeneic MSCs might be explained by the immunosuppressive properties of MSCs and the low expression levels of HLA class I and II on MSCs, albeit HLA class II molecules are significantly upregulated following the exposure to inflammatory environments. [88,89] About 40% of all MSC-based clinical trials use allogeneic MSCs and most of them do not perform HLA matching. [88] Clinical trial results show that MSCs are generally well tolerated regardless of tissue source and whether or not HLA matching was performed. [84,86,88] Nevertheless, the potential risk of an immune response against HLA mismatched MSCs should be carefully considered and closely monitored in ongoing clinical trials using allogeneic MSCs.

MSC-derived extracellular vesicles (EVs) are an emerging cell-free alternative to MSC therapies with various advantages and disadvantages. [92] MSC-derived EVs have potent immunomodulatory properties and were shown to reduce pulmonary fibrosis and reduce the expression of inflammatory markers in the lungs of a systemic sclerosis mouse model by modulating anti-inflammatory and anti-fibrotic markers. [93,94] Furthermore, EVs have a favorable safety profile compared to MSCs as they are unable to differentiate into ectopic tissue. [95] However, allogeneic MSC-derived EVs were shown to carry

potentially immunogenic proteins, which suggests the potential of EVs to elicit an allogeneic immune response. [96] EVs carry a fixed and limited therapeutic cargo, which is incapable of adapting to pathologic microenvironments. In contrast, the cellular plasticity of MSCs make them highly adaptable to changing microenvironments, and their ability to proliferate potentially enable a long therapeutic action. MSCs could be regarded as "live drug factories" that can modulate a variety of immune cells and adapt to changing microenvironments. [57]

1.3.4 Immunomodulatory properties of MSCs

MSCs have an exceptional potential to modulate a broad range of immune cells of the innate and the adaptive immune system (**Figure 3**). Immune cells that can be modulated by MSCs comprise B-cells, dendritic cells, lymphocytes, monocytes/macrophages, mast cells, and natural killer cells. [97,98] Huang et al. have recently reviewed the immunomodulatory effects of MSCs on immune cells. [98] In summary, MSCs can inhibit the antibody production of B cells, suppress dendritic cell migration, suppress T lymphocyte proliferation, polarize macrophages towards the inflammation-resolving phenotype, deactivate mast cells, decrease the pro-inflammatory properties of neutrophils, and inhibit natural killer cell activation. [58] MSCs exert their immunomodulatory functions via various mechanisms including paracrine activity, cell-to-cell contact dependent interactions, mitochondrial transfer, and secretion of EVs. [99,100]

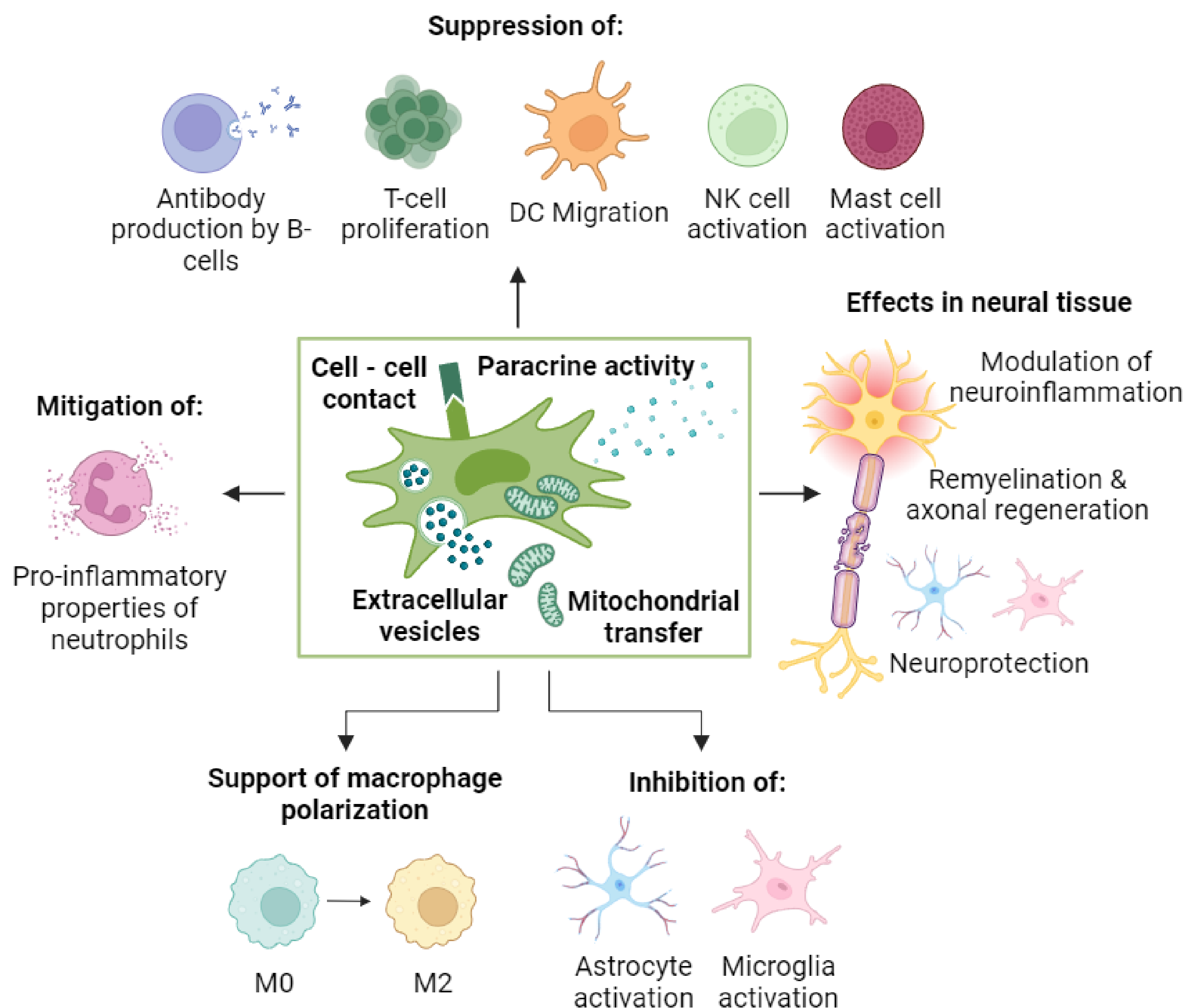

Figure 3 *Immunomodulatory and analgesic mechanisms of MSCs. DC = dendritic cells, NK cells = natural killer cells. Created with BioRender.com*

1.3.5 Analgesic effect of MSCs

In general, there are two types of pain – nociceptive pain (caused by tissue damage) and neuropathic pain (caused by nerve damage). [101,102] Nociceptive pain can either be acute or chronic and is frequently caused by musculoskeletal pain conditions including trauma, arthritis, or mechanical low back pain. [103,104] Neuropathic pain is usually chronic and can be caused by injury or diseases affecting the central nervous system (e.g. multiple sclerosis, diabetes, or neuropathic low back pain). [101] Inflammation is a key contributor to pain and can cause pain through various mechanisms. [105] Inflammation triggers an increased production of biochemical mediators, which are involved in direct activation of pain receptors, sensitization of nerve fibers, and the transmission of pain signals. [105] Many analgesics, like non-steroidal anti-inflammatory drugs (NSAIDs), primarily exert their effects by inhibiting enzymes involved in prostaglandin synthesis, thereby reducing inflammation and pain. However, some analgesics, including opioids, acetaminophen, and tricyclic antidepressants, operate differently. Opioids act on specific receptors in the central nervous system, acetaminophen likely

affects prostaglandin synthesis in the brain, and tricyclic antidepressants modulate neurotransmitter levels, all of which influence pain perception.

MSCs have mainly been investigated for the management of nociceptive pain and demonstrated promising results in patients and animal models of discogenic and osteoarthritis pain. [106] A phase I/II randomized controlled study investigated the analgesic effects of allogenic bone marrow-derived MSCs in patients with discogenic low back pain. [19,107] They observed a rapid and sustained reduction in pain, supporting the use of MSCs to treat chronic low back pain. The analgesic effects of MSCs can be attributed to multiple mechanisms. Especially, the modulation of neuroinflammatory components seems to be crucial for the analgesic effects of MSCs. [108] MSCs have also been investigated for the treatment of conditions leading to neuropathic pain, including spinal cord injury, diabetic neuropathy, and multiple sclerosis. [109–111] Additional analgesic mechanisms of MSCs include the inhibition of microglia and astrocyte activation, neuroprotection through the secretion of neurotrophic factors, and remyelination / axonal regeneration (**Figure 3**). [112–117] In summary, the encouraging outcomes from pre-clinical and clinical studies on the analgesic effects of MSCs may be attributed to their multimodal mode of action, primarily driven by their immunomodulatory properties.

1.3.6 Priming of MSCs

Priming is a widely used bioengineering strategy to enhance the therapeutic potential of MSCs. Various priming strategies exist aiming to improve specific properties of MSCs such as angiogenesis, engraftment, immunomodulation, migration, stemness, survival, and tissue regeneration. However, the main function of MSCs that priming aims to improve is their immunomodulatory capacity. Preconditioning MSCs with pro-inflammatory cytokines or exposing MSCs to hypoxia represent the most commonly used priming strategies. [118] Emerging MSC priming strategies comprise 3D culture conditions, exposure to growth factors and bioactive compounds, genetic modifications, and the co-culture with disease-associated effector cells. [119] The absence of standardized priming protocol is a serious hurdle to translate MSC priming into clinical practice. The countless available priming protocols make it difficult to compare the results of different studies. Unfortunately, many of the proposed priming protocols were insufficiently studied, resulting in a limited understanding of which priming-induced cellular adaptations of MSCs are translationally relevant. Understanding the immunomodulatory mechanisms underlying MSC priming is crucial as it can guide the development of standardized priming protocols to generate more effective and predictable immunomodulatory MSC therapies.

1.3.7 Standardization

Heterogeneity is a major translational hurdle for MSC therapies and can be introduced into an MSC product on multiple levels. As discussed in **Chapter 1.3.2**, MSCs describe a bulk of heterogeneous cells with a remarkable cellular plasticity. Matching of donor characteristics, tissue source, isolation method, and culture conditions does not guarantee functionally identical MSC preparations. [72] Inter-clonal variations combined with asymmetric cell division will likely yield functionally diverse MSC preparations. [73] Additionally, the myriad of priming strategies to improve the potency of MSCs introduce an additional level of heterogeneity into MSC preparations. Therefore, different MSC preparations are unlikely to have identical functional properties.

The definition of minimal criteria for defining MSCs by the ISCT in 2006 laid the foundation for standardized MSC products. [68] With the growing understanding of MSCs and the advent of omics technologies that enable analysis of MSC preparations on the single-cell level, the ISCT published an updated position paper to foster harmonization. [67] The ISCT amended to their original position paper to additionally i) disclose the tissue-source origin of MSCs, ii) refrain from using the term “stem cells” unless there is solid evidence for a self-renewal and multilineage differentiation potential, and iii) introduce a robust matrix of functional assays to demonstrate the intended functional properties of an MSC preparation. According to the Food and Drug Administration (FDA), there is likely no single biological/analytical assay that can adequately predict the clinical efficacy of MSC preparations. This is due to the complex mechanism of action (MOA) of MSCs, involving multiple bioactive factors. [120,121] The FDA also recommends to use an assay matrix (i.e. multiple complementary assays) to measure different MSC attributes associated with clinical efficacy. Unfortunately, neither the ISCT nor the FDA released protocols for relevant functional assays. Although, several functional in vitro assays measuring the potency of MSC preparations have been reported in literature, few publications provide detailed assay protocols. [122–125] Most in vitro functional potency assays do not mimic key features of a possible MSC recipient tissue such as hypoxia, 3D environment, or inflammation, which have a significant impact on MSC function. [126–128]

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Chapter 2

Aims and Motivation

Overall aim of the project

The overall aim of this project is the development of an allogeneic, anti-inflammatory bone marrow stromal cell therapy for chronic low back pain patients with Modic type 1 changes.

Within this larger project, the goal of this doctoral thesis was divided into two aims:

Aim 1

To discuss whether degenerated intervertebral discs of patients with Modic type 1 changes should be treated with MSCs.

– Chapter 3

Motivation

Over the past decade, intradiscal cell therapies, especially those involving MSCs, have made significant advancements. Yet, a persistent challenge is the inherent heterogeneity of the disc degeneration populations and the unclear mechanism of action of intradiscally injected MSCs. Evidence suggests that MSCs improve outcomes mainly by modulating discal inflammation. Vertebral bone marrow oedemas – known as "Modic type 1 changes" – are closely associated with disc degeneration. Patients with MC1 – a homogeneous subpopulation within the heterogeneous disc degeneration population – have a characteristic painful discal and vertebral inflammation. Harnessing the anti-inflammatory properties of MSCs to treat degenerated discs with MC1 is an intriguing concept.

In **Chapter 3**, we discussed the therapeutic potential of MSCs to treat patients with MC1. First, we summarized all pharmaceutical clinical trials on chronic low back pain patients with Modic changes. This enabled us to thoroughly understand the current therapeutic landscape, assess the efficacy and limitations of existing interventions, and to determine the most promising treatment approaches. Importantly, the anti-inflammatory effects of intradiscally administered MSCs have shown promising outcomes in completed clinical trials on degenerative disc disease. Hence, we explored the advantages and disadvantages of treating intervertebral discs of patients with MC1 with anti-inflammatory MSCs.

Chapter 3 underscores a critical clinical gap: the absence of a treatment consensus for patients with MC1, despite their high prevalence and association with pain. Various pharmaceutical interventions have been explored, but with largely inconclusive outcomes. Surprisingly, the potential of intradiscal MSC therapy to treat patients with MC1 remains unexplored.

In light of our findings, revealing a notable gap in leveraging intradiscal MSC therapy for MC1, combined with the compelling anti-inflammatory properties of MSCs, we will develop an anti-inflammatory MSC-based therapy for chronic low back pain patients with MC1.

Aim 2

To investigate the impacts of priming on distinct immunosuppressive mechanisms under translationally relevant conditions.

Aim 2 was divided into three sub-aims:

i) to study the effects of pro-inflammatory, hypoxic, and 3D culture priming on the in vitro immunosuppressive potential of MSCs

ii) to assess if immunosuppressive priming effects are temporally preserved under standard and translationally relevant culture conditions

iii) to investigate if the three priming strategies engage the same immunosuppressive mechanisms

– Chapter 4

Motivation

Priming is a promising strategy to improve the anti-inflammatory potency of MSCs. However, the lack of standardized priming protocols combined with a limited understanding of the effects of priming on the anti-inflammatory mechanisms of MSCs, hinder the translational potential of MSC priming. To consider a priming strategy for our therapy, MSCs must preserve the priming effects post-administration. Little information is available whether MSCs can preserve the priming effects over time in disease-specific microenvironments. To assess whether priming could be a suitable method for enhancing the anti-inflammatory potency of our MSC-based therapy, we investigated the translational value of different priming strategies.

We demonstrated that priming induced profound transcriptomic changes in MSCs resulting in a significantly enhanced immunosuppressive potential of pro-inflammatory and 3D culture primed MSCs. While priming effects rapidly faded under standard cell culture conditions, they were partially preserved under translationally relevant conditions. Continuous 3D culture completely rescued the enhanced immunosuppressive priming effects from fading, supporting the administration of MSC spheroids for a sustainably improved immunosuppressive potency. The cellular mechanisms leading to immunosuppression were priming strategy-specific, suggesting the engagement of distinct immunomodulatory mechanisms.

The insights we provide in **Chapter 4** could significantly influence the current understanding of MSC priming and will be applied to our anti-inflammatory MSC-based therapy for chronic LBP patients with MC1.

Chapter 3

Should Degenerated Intervertebral Discs of Patients with Modic Type 1 Changes Be Treated with Mesenchymal Stem Cells?

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3.1 Abstract

Low back pain (LBP) has been among the leading causes of disability for the past 30 years. This highlights the need for improvement in LBP management. Many clinical trials focus on developing treatments against degenerative disc disease (DDD). The multifactorial etiology of DDD and associated risk factors lead to a heterogeneous patient population. It comes as no surprise that the outcomes of clinical trials on intradiscal mesenchymal stem cell (MSC) injections for patients with DDD are inconsistent. Intradiscal MSC injections have demonstrated substantial pain relief and significant disability-related improvements, yet they have failed to regenerate the intervertebral disc (IVD). Increasing evidence suggests that the positive outcomes in clinical trials might be attributed to the immunomodulatory potential of MSCs rather than to their regenerative properties. Therefore, patient stratification for inflammatory DDD phenotypes may (i) better serve the mechanisms of action of MSCs and (ii) increase the treatment effect. Modic type 1 changes—pathologic inflammatory, fibrotic changes in the vertebral bone marrow—are frequently observed adjacent to degenerated IVDs in chronic LBP patients and represent a clinically distinct subpopulation of patients with DDD. This review discusses whether degenerated IVDs of patients with Modic type 1 changes should be treated with an intradiscal MSC injection.

Keywords: mesenchymal stem cell; stem cell therapy; Modic change; intervertebral disc; regeneration; immunomodulation

3.2 Introduction

A fundamental challenge for improving the lives of chronic low back pain (CLBP) patients is the lack of effective targeted treatments. The development of novel targeted therapies for CLBP patients is hampered in part by the heterogeneity of the CLBP population. Pain may arise from several anatomical structures, including the intervertebral disc (IVD), the endplate, the vertebral body, the facet joints, the spinal ligaments, and the muscles. Central pain sensitization and psychosocial factors can further complicate the diagnosis.

Degenerative disc disease (DDD) is one of the most common findings in CLBP patients. DDD is an inflammatory–catabolic process triggered by a long list of genetic, mechanical, and environmental factors that ultimately leads to the resorption of the IVD. Anti-inflammatory and regenerative approaches have been attempted to treat degenerated discs. In the past 15 years, many cell therapy approaches for DDD have been developed, several of which have reached phase I and II clinical trials, and a few phase III trials [1].

Patient stratification is critical for showing a clinically meaningful treatment effect. However, the high prevalence of disc degeneration (DD) in the heterogeneous CLBP population [2] and the high percentage

of asymptomatic individuals with DD (31.5–37.5%) [3] limit the sensitivity and specificity of DD for CLBP and hence impose a major challenge to stratify patients for a potential therapy.

Modic changes (MC) are vertebral bone marrow lesions that are almost exclusively present at levels with DD. MC are frequently observed in CLBP patients. A systematic review investigated the prevalence of MC and reported a median prevalence of 43% in CLBP patients and 6% in a non-clinical population [4]. Prevalence generally increases with age and peaks in the 60s [5]. Accumulating evidence shows that CLBP patients with DDD and MC are different from DDD patients without MC [6]. Patients with MC report a greater frequency and duration of low back pain (LBP) episodes, seek care more often, have a higher risk of a poor outcome, and have an 'inflammatory pain pattern' [4,7–11]. Larger lesions seem more painful and have a positive predictive value for pain of up to 100% [12,13]. Therefore, MC patients may in fact represent a clearly defined subpopulation of DDD patients. However, the effects of discal cell therapy at spinal levels of MC remain unknown. Thus, we discuss in this review whether patients with MC should be considered for a specific discal cell therapy.

Modic Changes

Vertebral bone marrow lesions adjacent to degenerated discs were first described by Assheuer et al. in 1987 [14] and later coined by Modic et al. in 1988 [15]. Three interconvertible types of MC have been defined based on their appearance in T1-weighted and T2-weighted magnetic resonance imaging (MRI) (**Figure 1**) [15,16]. Histological data of MC patient bone marrow are sparse [14,15,17,18]. In Modic type 1 changes (MC1), fibrosis, granulation tissue, lymphocytic and neutrophilic infiltrations, increased frequency of adipocytes, necrotic adipocytes, and interstitial water have been reported [14,15,19]. In Modic type 2 changes (MC2), the red hematopoietic bone marrow is replaced by fatty bone marrow and can contain displaced disc tissue along with fibrotic tissue [14,15,20]. Trabecular bone in MC1 is thinned, possibly due to osteoclastic activity, and thickened in MC2 [14,15,17]. Modic type 3 changes (MC3) represent extensive sclerotic changes [15,17]. Increased numbers of peptidergic nerve endings were found in MC1 and to a lesser extent in MC2 [18,19]. This may relate to the high specificity of MC for pain in discography [13,20].

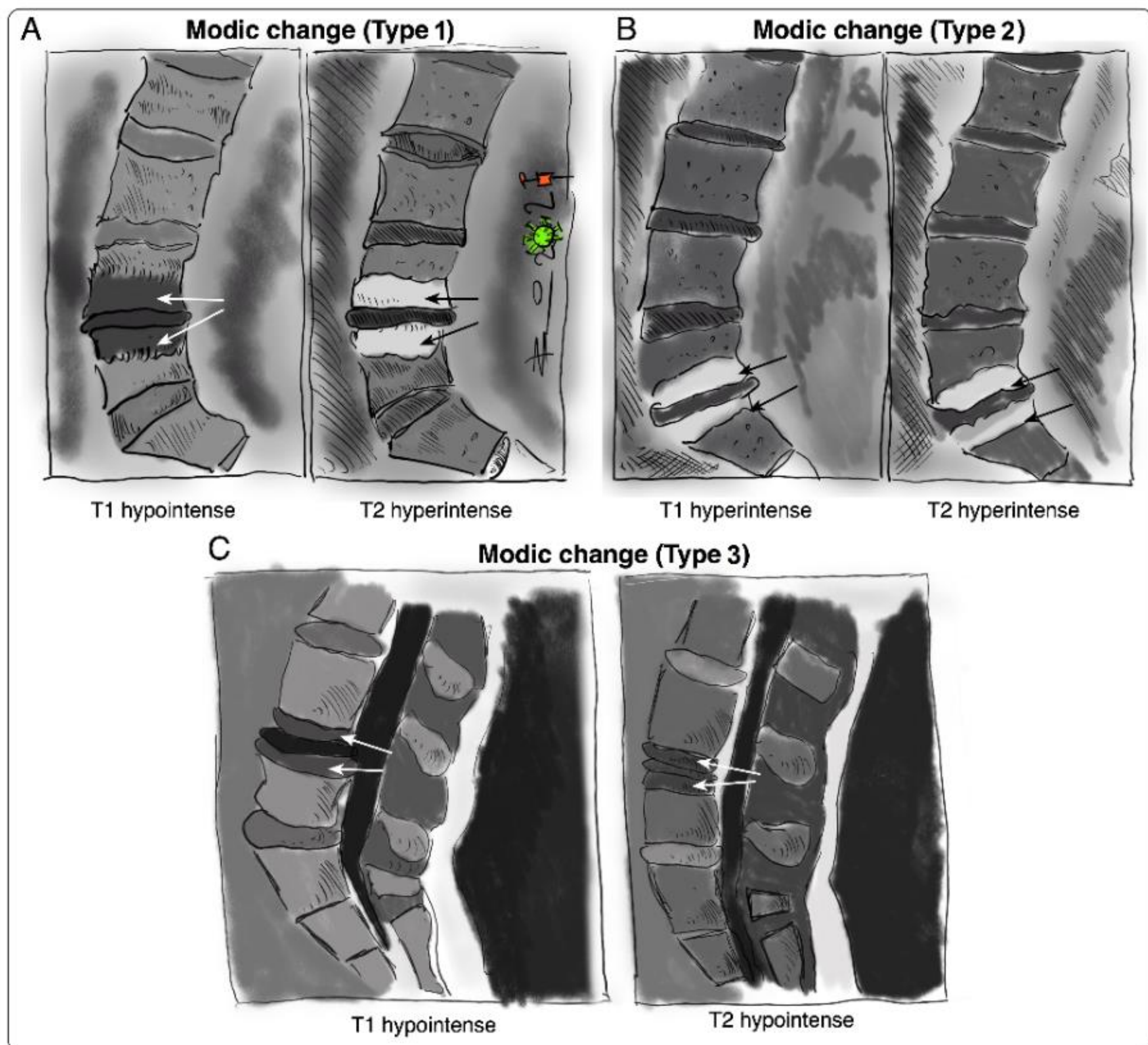

Figure 1 Sketches of intensity changes when scanning vertebral columns of human patients and classification of the three distinguishable MC according to T1- and T2-weighted sequences on MRI [15]. MC are classified into (A) MC type I, hypointense in T1 and hyperintense in T2, (B) MC type 2, hyperintense in T1 and T2, and (C) MC type 3, hypointense in T1 and T2.

The IVD and the vertebral endplate seem to play an important role in the pathomechanism of MC (**Figure 2**). MC only occur adjacent to degenerated discs and mostly develop simultaneously in the cranial and caudal vertebrae of the degenerated disc [16]. Progression of DD accompanies the progression or evolution of MC [21]. Vertebral endplate defects are strongly associated with MC and extensive endplate degeneration is a risk factor for the progression of DD and MC [21,22]. Endplate defects enhance the fluid flow between the disc and the bone marrow [23,24] and may provide a physical explanation for the inflammatory and pro-fibrotic cross talk between the disc and the bone marrow observed in MC [25]. This cross talk likely promotes MC development, thus representing an interesting treatment target. In vivo studies with mice, rats, and baboons confirm that disc injury can cause

changes in the adjacent vertebrae, with alterations in marrow composition and remodeling of trabecular bone [26–29]. Analysis of human disc samples revealed increased expression of pro-inflammatory, pro-osteoclastic, and neurotrophic cytokines (**Table 1**) [19,30,31]. Notably, many of them can affect hematopoiesis and contribute to the hematopoietic changes observed in MC bone marrow [25].

Table 1 List of pro-inflammatory, pro-osteoclastic, and neurotrophic cytokines with elevated expression levels in 'MC discs'.

MC Type	Pro-Inflammatory	Pro-Osteoclastic	Neurotrophic
MC1	CCL2, IL-6, IL-8, PGE2	OSCAR	NTRK1
MC2	CCL2, CXCL5, GM-CSF, IL-1β, M-CSF	RANKL, RUNX1, RUNX2	NTRK1

CCL2, C-C motif chemokine ligand 2; CXCL5, C-X-C motif chemokine ligand 5; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; M-CSF, macrophage colony-stimulating factor; NTRK1, neurotrophic receptor tyrosine kinase 1; OSCAR, osteoclast-associated Ig-like receptor; PGE2, prostaglandin E2; RANKL, tumor necrosis factor superfamily member 11; RUNX1, runt-related transcription factor 1; RUNX2, runt-related transcription factor 2.

Despite an increasing understanding of the molecular and cellular changes in MC bone marrow and discs, the etiology of MC remains largely unknown. Autoinflammation against disc material and occult disc infection are both supported by clinical and experimental studies [30–37]. While infectious MC may be treated with antibiotics[36,37], no approved treatment or treatment consensus exists for autoinflammatory MC. Standard treatments for CLBP are generally less effective in MC1 patients [8,38]. Treatment attempts with intradiscal steroids, bisphosphonates, and tumor necrosis factor alpha (TNF-α) inhibitors to control inflammation had limited short-term efficacy and lacked anatomical or biological specificity [39–44]. Spinal fusion surgery may relieve pain but can have serious risks besides those of surgery and anesthesia [45].

In summary, clinical and experimental data suggest that disc inflammation can affect the adjacent bone marrow via an enhanced cross talk through damaged endplates. Therefore, suppression of discal inflammation might represent a promising treatment strategy to protect the bone marrow from the vicious cross talk with the inflammatory disc.

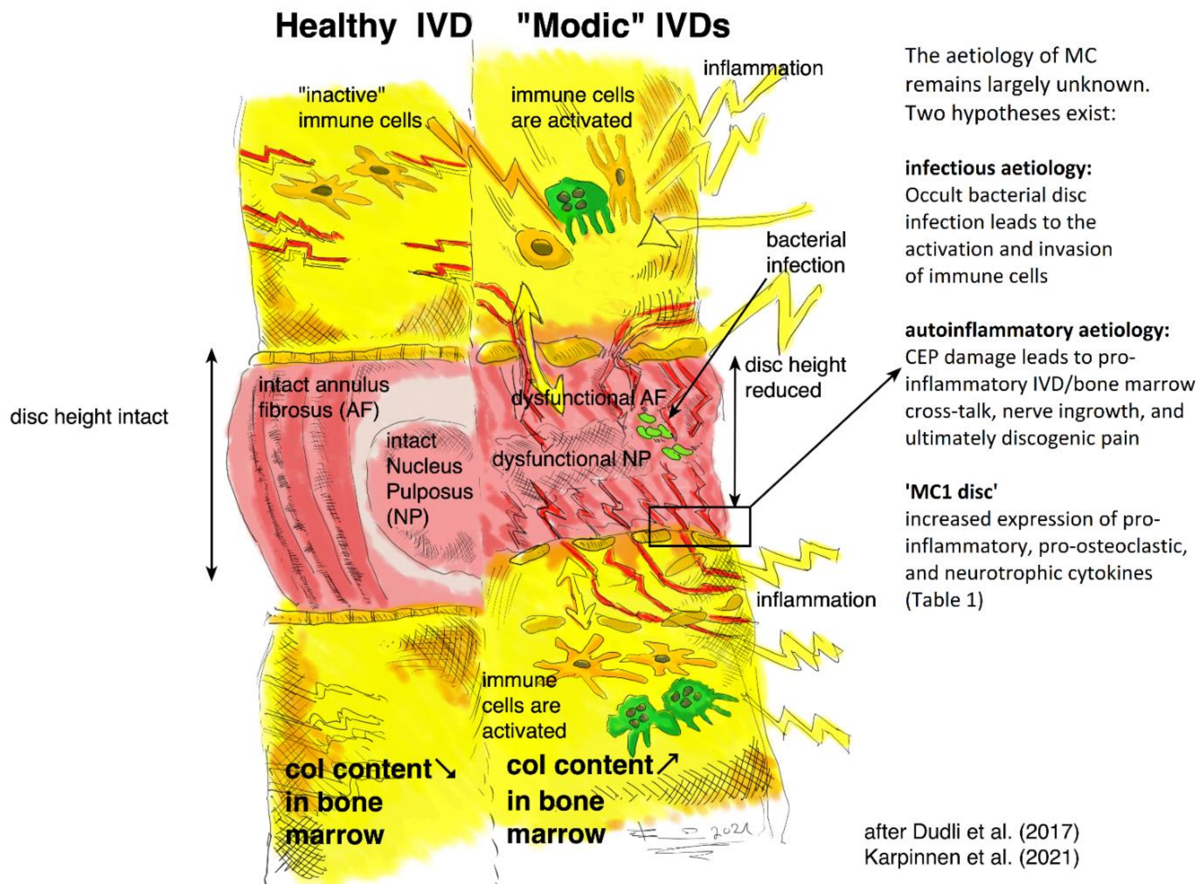

Figure 2 Schematic illustration of possible causes of pain and inflammation in 'MC discs', comparing a 'healthy disc' (on the left side) to a 'Modic disc' (on the right side). Note that the central role is given to the CEP: CEP damage possibly enables inflammation in the adjacent vertebrae, triggering a cross-talk to inflammatory cells. Ingrowth of nerve endings into the IVD might be responsible for pain development. Increased osteoclast activity might be responsible for the inflammatory trabecular bone resorption observed in MC1 [14,15,17]. MSCs in the bone marrow adjacent to 'MC1 discs' have a pro-fibrotic phenotype [46], possibly due to the pro-fibrotic and pro-inflammatory cross-talk with the 'MC1 disc'.

3.3 Clinical Trials for MC

A literature review was carried out in January 2022 across the ClinicalTrials.gov database. Keywords used in the selection of clinical trials were: "modic", "discopathy", and "endplate changes". All pharmaceutical interventional studies were considered and their relevance to the subject of this review was checked. Multiple clinical trials investigating the efficacy of antibiotics or intradiscal steroid injections for the treatment of CLBP patients with MC were not listed in the ClinicalTrials.gov database. Eligible studies were selected and are summarized in **Table 2**.

Various drugs have been tested in clinical trials to treat CLBP patients with and without MC. The AIM study investigated the use of amoxicillin to treat occult disc infections in a double-blind, randomized, phase III, placebo controlled, multicenter clinical trial (NCT02323412) [47,48]. The study showed that amoxicillin failed to provide a clinically meaningful benefit for CLBP patients with MC. Therefore, the study results did not support the use of amoxicillin for CLBP patients with MC. Interestingly, when the analysis was limited to the inflammatory MC1, they found significant improvements in pain and disability. However, the minor clinical benefit failed to reach a pre-defined threshold for clinically meaningful improvements. The treatment efficacy might have been limited to a group of responders, which would explain the weak overall efficacy. On the contrary, two randomized, placebo-controlled studies investigated the use of amoxicillin and clavulanate for the management of CLBP in patients with disc herniation and MC1 (NCT00302796) [49,50]. They found significant pain and disability related improvements in the cohort treated with antibiotics compared to the placebo cohort. A more recent prospective, open label study used the same treatment regimen yet failed to replicate the treatment outcomes [51]. In summary, studies using antibiotics to treat CLBP patients with MC are inconsistent. A potential explanation for these inconsistent study results might be the diverse percentages of patients with infectious MC between the different trials. While patients with infectious MC represent a valid target population, those with autoinflammatory MC might not benefit from antibiotics. This highlights the need for new diagnostics that enable the stratification of the MC study population into infectious and autoinflammatory subpopulations.

Bisphosphonates might consolidate inflamed vertebral bodies, thereby improving the tolerance for mechanical load. A phase II, randomized, placebo controlled, double blinded clinical trial investigated the efficacy of zoledronic acid (ZA) for CLBP patients with MC (NCT01330238) [43,52]. Besides a higher frequency of adverse events in the ZA group compared to the placebo group, this trial reported minimal pain and disability related improvements after one month in favor of the ZA group. No significant difference in pain and disability-related improvements was observed after one year between the ZA group and the placebo group. Interestingly, MC1 volumes tended to decrease in the ZA group, but increased in the placebo group. Furthermore, MC1 to MC2 conversion was more common in the ZA group, although statistical significance was not demonstrated. In summary, ZA tended to accelerate the conversion from MC1 to less painful MC2. Even though the study results are encouraging, larger studies are required to verify the efficacy of ZA in the treatment of CLBP patients with MC.

Glucocorticoids are potent anti-inflammatory and immunosuppressive drugs, frequently used to treat various inflammatory, allergic, and autoimmune disorders. Clinical trials investigated the efficacy of intradiscal steroid injection in a heterogeneous study population of patients suffering from discogenic

LBP. These trials reported no significant clinical benefit in the use of intradiscal steroids [53,54]. More recent clinical trials on intradiscal or epidural steroid injections recognized the importance of stratifying CLBP patients and focused specifically on CLBP patients with MC. The reported outcomes on intradiscal steroid injections across multiple clinical trials [39–41,55,56] and case reports [57,58] are consistent. Rapid pain- and disability-related improvements could be found but the long-term efficacy of intradiscal and epidural steroid injections could not be demonstrated. This reinforces the hypothesis that MC is a type of inflammation that can and should be treated. Glucocorticoids might not be the right treatment approach to target the discal inflammation because they failed to modify the disease, as indicated by the recurrence of pain after intradiscal and epidural steroid injection.

The BackToBasic study is currently recruiting CLBP patients with MC1 to compare the effect of Infliximab—an intravenously administered TNF-α inhibitor—to a placebo (NCT03704363) [59]. This pharmaceutical intervention aims to target the underlying discal inflammation in MC1.

Antibiotics to treat occult disc infections, anti-inflammatory drugs to suppress discal inflammation, and bisphosphonates to consolidate vertebral bodies all represent valid causal treatment approaches. Current clinical trials seem to have recognized the pivotal role of inflammation in MC1 and therefore are looking into anti-inflammatory treatment approaches.

A recent review highlighted the central role of inflammation in DDD independent of the presence of MC [60]. The review summarizes outcomes from anti-inflammatory clinical interventions in discogenic LBP patients [60]. TNF-α inhibitors were previously shown to be effective in the treatment of sciatica (NCT00364572) [61]. An open label study used Infliximab to treat disc herniation-induced severe sciatica and reported promising early results [62,63]. This study was followed up by a randomized, placebo-controlled trial (FIRST II) to validate these early results [44,64]. Unfortunately, the study results did not support the use of Infliximab for the treatment of patients with herniation induced sciatica. Interestingly, they showed that Infliximab treatment was beneficial if MC were present adjacent to the symptomatic level. This finding might have laid the foundation for the ongoing BackToBasic study that uses Infliximab for CLBP patients with MC1 [59]. Tanezumab—a potent anti-nerve growth factor (NGF) antibody—was used in several studies on CLBP (NCT00584870 [65], NCT00876187 [65,66], NCT00924664 [67,68]). Overall, tanezumab treatment led to a significant LBP reduction. However, adverse events including abnormal peripheral sensation, arthralgia, and accelerated osteoarthritis progression in affected patients were observed [65,68]. Platelet rich plasma (PRP) represents a multimodal treatment approach, as it contains growth factors, blood clotting factors, proteinase inhibitors, and immunomodulatory factors. Completed studies on PRP for CLBP patients consistently reported significant pain relief and disability-related improvements [69–71]. The promising study results might be

attributed to the multimodal effects of PRP, including the reduction of inflammation and promotion of tissue regeneration [60]. Since MC1 are highly associated with DD and inflammation, it might be insightful to consider these study outcomes for future clinical trials on anti-inflammatory interventions for DDD patients with MC1.

3.4 MSC Therapy for DDD

Mesenchymal stem cells (MSCs) have been used in numerous clinical trials for DDD independent of concomitant MC during the past decade. Clinical trials for DDD using MSCs have recently been reviewed [1]. MSCs are an attractive option for cell therapies because of their self renewal capacity, ease of isolation, multi-lineage differentiation potential, engraftment capacity, safety profile, and immunomodulatory properties. MSC treatments have been shown to be safe and well-tolerated, with no reported severe adverse events but with occasional mild pain related adverse events [1,72,73]. Previous studies observed no host immune rejection against allogeneic MSCs, indicating that allogeneic MSCs might avoid immunogenic reactions in humans [1,74,75]. Although concerns about MSC associated tumorigenesis, osteophyte formation, infection, and immune rejections are justified, none of these safety concerns have been confirmed in completed clinical trials on MSC injections in DDD patients.

The underlying concept of regenerative treatment approaches in DDD assumes that MSC injection reconstitutes the healthy disc anatomy and thereby restores the normal functioning of the motion segment [76]. The successful restoration of a functionally impaired motion segment could prevent the development of degenerative spinal pathologies [72,73,76]. The chance to disrupt the degenerative cascade led to the conduction of multiple clinical trials, investigating the use of intradiscal MSC injections to treat DDD [1]. Most completed clinical trials focused on IVD regeneration and the safety profile of MSC injections. Additionally, pain relief and disability related improvements have been frequently investigated yet controlling disc inflammation has not been a focus of these trials. Despite tremendous efforts, substantial IVD regeneration could not be shown in any of the completed clinical trials on intradiscal MSC injections in DDD patients, besides occasional IVD rehydration and deceleration of degenerative processes [1]. Interestingly, DDD patients treated with intradiscal MSC injections experienced substantial pain relief and showed significant disability related improvements in a group of responders [1,75,77]. These findings raised the question of whether IVD regeneration is essential for achieving favorable therapy outcomes and indicated that the analgesic effect of the MSC injections may be due to a regeneration independent mode of action.

Table 2 Summary of pharmaceutical interventional clinical trials on the treatment of CLBP patients with MC.

Clinical Trial/Study	Year	Aim	Treatment	Phase and Design	Inclusion Criteria	Number of Patients	Status and Outcome	Outcome Measures	References
Antibiotics in Modic changes (AIM) NCT02323412	2015 - 2018	Effects of amoxicillin in CLBP patients with MC at the disc herniation level	Amoxicillin 750 mg three times a day for three months	Phase III. Double-blind, multicenter, randomized, placebo-controlled	CLBP patients with disc herniation and MC1 and/or MC2 at the same level	180	Completed Without clinically important benefit	- LBP intensity scores - Oswestry Disability Index - Roland-Morris Disability Questionnaire	[47,48]
Antibiotic treatment of patients with low back pain NCT00302796	2006 - 2010	Effect of antibiotics in CLBP patients with MC1	Amoxicillin-clavulanate (500/125 mg) three times a day for 100 days	Phase IV. Double-blind, randomized, placebo-controlled	CLBP patients with disc herniation and MC1	162	Completed Clinically important benefit	- LBP intensity scores - Disease-specific disability - Global perceived health - MRI	[49]
Antibiotic treatment for the management of CLBP ACTRN12615000958583	2015 -	Efficacy of antibiotics in a broader subgroup of CLBP patients with disc herniation	Amoxicillin-clavulanate (500/125 mg) two times per day for 90 days	Double-blind, randomized, placebo-controlled	CLBP patients with disc herniation—with and without MC1 and MC2	170	Recruiting	- LBP intensity scores - Self-reported disability - Work absenteeism - Hindrance in work performance	[129]
Antibiotic treatment of CLBP patients with MC1	2016	Efficacy of antibiotic treatment of CLBP patients with MC1	Amoxicillin-clavulanate (500/125 mg) three times a day for 100 days	Prospective, open-label	CLBP patients with MC1	28	Completed No clinically important benefit	- LBP intensity scores - Self-reported improvement - Analgesics consumption - Spinal steroid injections	[51]
Antibiotics in CLBP patients with MC1	2014	Efficacy of antibiotics in CLBP patients with MC1	Amoxicillin-clavulanate (500/125 mg) two times per day for 100 days	Randomized, placebo-controlled	CLBP patients with disc herniation and MC1	71	Completed Clinically important benefit	- LBP intensity score - Roland-Morris Disability Questionnaire	[50]
PP353 for CLBP patients with MC1 NCT04238676	2020 -	Safety, tolerability, and efficacy of PP353	Intradiscal injection of the antibiotic PP353	Phase I/II. Randomized, placebo-controlled	CLBP patients with MC1	43	Recruiting	- Adverse events incidence - LBP intensity scores - Roland-Morris Disability Questionnaire	-
The efficacy of Zoledronic Acid in MC-related LBP NCT01330238	2008 - 2011	Efficacy of Zoledronic Acid in patients with CLBP and MC	Single infusion of 5 mg zoledronic acid	Phase II. Double-blind, randomized, placebo-controlled	CLBP patients with MC1 or MC2	40	Completed Reduced LBP and faster MC1 conversion to MC2	- LBP intensity scores - Oswestry Disability Index	[42,43]
Intradiscal steroid injection in CLBP with inflammatory MC	2007	Association between MC severity and response to intradiscal steroid injection	Intradiscal injection of 25 mg prednisolone acetate	Retrospective	CLBP patients with MC1, MC1/2, or MC2	74	Completed Significant short-term benefit	- LBP intensity score	[39]

Intradiscal steroid therapy for CLBP patients with MC	2011	Efficacy of various intradiscal steroid injection regimens for CLBP patients with MC	Intradiscal injection of normal saline, disprospan, or disprospan and songmeile	Double-blinded, randomized, placebo-controlled, prospective	CLBP patients with MC and positive discography	120	Completed Significant short-term benefit	- LBP intensity score - Oswestry Disability Index	[40]
Intradiscal steroid injection in CLBP patients with MC1	2012	Efficacy of intradiscal steroid injection on CLBP patients with MC1	Intradiscal injection of methylprednisolone	Retrospective	CLBP patients with and without MC1	97	Completed Significant short-term benefit	- Self-reported improvement	[41]
Intradiscal glucocorticoid injection for CLBP patients with active discopathy NCT00804531	2017	Efficacy of single intradiscal glucocorticoid injection in CLBP patients and active discopathy	Single intradiscal injection of 25 mg prednisolone acetate	Phase IV. Prospective, parallel-group, double-blind, randomized, placebo-controlled	CLBP patients with active discopathy	135	Completed Significant short-term benefit with no long-term benefit	- LBP intensity score - MRI - Disability - Quality of life - Use of analgesics	[55]
Epidural steroid injections in discogenic LBP NCT04930211	2020 -	Effectiveness of epidural steroid injections in DDD patients with/without MC1	Transforaminal epidural steroid injection of dexamethasone-lidocaine	Non-randomized without placebo	CLBP patients with/without MC1	40	Recruiting	- LBP intensity score - Oswestry Disability Index	-
BackToBasic: Infliximab in CLBP and MCs NCT03704363	2018 -	Efficacy of Infliximab in CLBP with MCs	Four intravenous Infliximab infusions (5 mg/kg)	Phase III. Double-blind, multicenter, randomized, placebo-controlled	CLBP patients with MC1	126	Recruiting	- LBP intensity score - Oswestry Disability Index - Incidence of adverse events - Roland-Morris Disability Questionnaire	[59]
Intradiscal injection of PRP for CLBP patients with MC1 NCT03712527	2018 -	Efficacy of intradiscal PRP injection at 3 months	Single intradiscal PRP injection versus normal saline	Randomized, placebo-controlled	Patients with at least 3 months LBP with MC1	126	Recruiting	- LBP intensity score - Roland-Morris Disability Questionnaire - Analgesics consumption	-

The multifactorial etiology of DDD could manifest in various sources of discogenic pain. The key processes of DDD that are related to discogenic pain have been reviewed elsewhere [74]. In summary, inflammation, an acidic IVD microenvironment, nerve ingrowth, and endplate damage were found to be closely linked to discogenic pain in DDD [60,74,75,77–80]. All these features are characteristics of MC, making MC one of the DDD-associated findings with the highest pain specificity [20,81]. These sources of discogenic LBP could potentially be targeted by the broad mode of action of MSCs, including the secretion of immunomodulatory factors, multi lineage differentiation potential, and the promotion of cell survival [82,83].

In order to assess whether MSCs should be considered for 'MC discs', we next review the regenerative and immunomodulatory mode of action of MSCs and their contribution to pain relief.

3.4.1 Regenerative Mode of Action

Structural damage of the IVD and cartilage endplate (CEP) might contribute to functional impairment of the vertebral motion segment, which in turn could result in painful discal inflammation [73,76]. Targeting the underlying biomechanical issue might alleviate or even prevent painful vertebral bone marrow inflammation and subsequent degenerative processes. MSCs can promote IVD regeneration by various mechanisms. Their ability to proliferate and differentiate into chondrocytes [84–88] could allow them to replace damaged IVD cells (IVDCs), thereby supporting chondrogenesis [89]. Additionally, MSCs can contribute to IVD regeneration by the de novo synthesis of the extracellular matrix (ECM) [90]. Paracrine secretion of anabolic growth factors, anti-catabolic factors, and immunomodulatory cytokines by MSCs influences the survival and function of resident IVDCs [90] and renders MSCs promising candidates for inducing IVD regeneration [85,90–94].

The cross talk between MSCs and IVDCs was shown to downregulate the gene expression of pro-inflammatory cytokines in IVDCs and to significantly increase their insoluble collagen synthesis and proliferation rate in vitro [92]. Furthermore, the co culture of MSCs with nucleus pulposus cells (NPCs) was shown to protect NPCs against compression induced apoptosis by reducing the concentration of reactive oxygen species and maintaining mitochondrial integrity [95]. On the contrary, the cross talk between MSCs and IVDCs did not lead to the significantly increased synthesis of insoluble collagen by MSCs, but clearly induced the gene expression of various growth factors [92]. The paracrine secretion of these growth factors might have led to the increased proliferation and collagen synthesis of IVDCs.

Unfortunately, in vitro studies are incapable of mimicking the complex IVD microenvironment. The hostile IVD microenvironment likely impairs the regenerative potential of MSCs [77]. To enable MSCs to contribute to substantial IVD regeneration, they must be adapted to or protected from the harsh IVD microenvironment, including nutrient deprivation, hypoxia, acidic pH, high osmolarity, and a

combination of inflammatory cytokines [61,86,96,97]. The use of MSC licensing strategies and biomaterial scaffolds may help to improve the survivability and the therapeutic potential of MSCs. Modulation of the inflammatory environment before attempting to regenerate the IVD and CEP using MSCs might be necessary. A non-inflammatory IVD microenvironment is more likely to support larger numbers of chondrogenic MSCs, which are needed to induce regeneration [77,95].

The following section summarizes the characteristics of the IVD microenvironment, which were discussed in a comprehensive review by Vadalà et al. [98]. We review the impact of the harsh IVD microenvironment on IVDCs and MSCs and discuss its relevance in MC.

3.4.1.1 Nutrient and Oxygen Deficiency

The avascular nature of IVDs creates a hypoxic microenvironment with limited nutrient availability. Hypoxia (2–5% O2) and low glucose (1 mg/mL) were found to have positive effects on MSC mediated IVD regeneration. MSCs cultured under hypoxia not only grew significantly faster than MSCs cultured under normoxia, but also had increased expression of genes associated with ECM assembly and improved differentiation potential [96,99–102]. The viability and proliferation of MSCs were maintained at IVD like low glucose levels, whilst ECM biosynthesis was significantly enhanced [97,103]. Similarly, hypoxia supports the survival of NPCs and significantly enhances ECM biosynthesis and NPC proliferation [104–107]. Nutrients and oxygen are transported in blood vessels to the CEP and small capillaries supply nutrients through the CEP to the CEP/annulus fibrosus (AF) interface [108]. The nutrient supply of IVDCs then depends on diffusion from the CEP/AF interface into the IVD [108]. Endplate calcification increases with ageing and progression of DD and likely limits the diffusion of nutrients as the capillaries can no longer penetrate the endplate or are damaged as a result of the calcification [25,97,109]. Endplate defects are frequently seen in MC and have been shown to be responsible for substantial changes in diffusion between the IVDs and adjacent vertebral bodies [24]. No data on nutrient and oxygen concentrations in 'MC discs' have been published, but it can be speculated that the increased diffusion through damaged endplates increases the nutrient concentration and oxygen tension in 'MC discs', with unknown consequences for IVDC behavior and intradiscally injected MSCs.

3.4.1.2 Acidity

Non degenerated IVDs have a pH between 7.1 and 7.4 but the pH can drop to 6.8 in mild DD and can reach values of 6.2 in severe DD [110,111]. The proliferation rate and viability of MSCs and NPCs decrease with increasing acidity [112]. Furthermore, an acidic pH stimulates NPCs to increase the secretion of pro-inflammatory cytokines, nerve growth factors, and catabolic enzymes [113–115]. The pH in 'MC1 discs' has not yet been investigated. Endplate leakage enhances the fluid flow between the IVD and the bone marrow, thus likely facilitating the efflux of acidic metabolites into the adjacent bone marrow. A low pH can lower the threshold for the activation of sensory nerve fibers through acid-sensing sodium

channels and is hence directly linked to nociceptive pain [116]. This might be relevant in MC, because more sensory nerve fibers were found in the bone marrow close to the endplates [18,19].

3.4.1.3 Hyperosmolarity

The IVD has a hyperosmolar environment. In non degenerated IVDs, osmolarity ranges between 430 and 500 mOsm/L [117] but steadily declines with the progression of DD due to loss of proteoglycans [117]. A hyperosmolar culture condition (485 mOsm/L) significantly decreases the gene expression of aggrecan and collagen 1 and decelerates MSC proliferation compared to standard cell culture conditions (280 mOsm/L) [103]. Therefore, reduced hyperosmolarity in degenerated IVDs might be beneficial for ECM deposition and the proliferation rate of intradiscally injected MSCs. Osmolarity in 'MC discs' has not been investigated; thus, the effect of osmolarity on intradiscally injected MSCs in 'MC discs' remains unknown.

In summary, it is challenging to restore a healthy IVD microenvironment by addressing single components of the complex network of cytokines, growth factors, catabolic enzymes, and neurotrophic factors found in degenerated IVDs [118]. An adaptable multimodal therapeutic approach might be needed to suppress the discal inflammation and to restore a healthy IVD microenvironment.

3.4.2 Immunomodulatory Mode of Action

The exceptional potential of MSCs to modulate a broad range of immune cells makes them an interesting candidate for the treatment of inflammatory disorders. The impact of MSCs on the functional properties of various cells from the innate and adaptive immune system has been thoroughly reviewed elsewhere [109,119]. In summary, MSCs can modulate immune cells through a paracrine mode of action. The secretion of immunomodulatory factors, including indoleamine-2,3-dioxygenase (IDO), TNF-α inducible protein 6 (TSG-6), PGE2, IL-10, and transforming growth factor-beta (TGF-β), has distinct effects on various cell types. MSCs can regulate the antibody secretion of B cells, suppress T-cell activation and proliferation, and prevent the activation of neutrophils [119]. Furthermore, MSCs can inhibit the maturation of dendritic cells and polarize macrophages towards immunomodulatory M2 macrophages. It has been shown that macrophages and other leukocytes [118] can infiltrate contained IVDs during degeneration and the number of infiltrated macrophages positively correlated with the progression of DD [113,120]. This might indicate the importance of MSCs in modulating a broad range of immune cells to resolve discal inflammation. Besides the secretion of immunomodulatory factors, MSCs interact with immune cells via cell–cell contact, mitochondrial transfer, and extracellular vesicles [1,109,114,115,121]. Regarding intradiscal MSC therapy, it is important that the immunomodulatory action of MSCs is not limited to leukocytes but also affects disc cells.

The secretion of inflammatory factors by IVD cells and infiltrating immune cells not only shifts the balance between anabolic and catabolic processes towards ECM degradation but also promotes the secretion of neurotrophic factors by IVD cells, ultimately leading to nerve ingrowth and discogenic pain [60,118,120,122–125]. Interestingly, co culture of MSCs with degenerative IVDCs significantly downregulated the gene expression of pro-inflammatory cytokines (interleukin 1α (IL-1α), IL-1β, IL-6, TNF-α) [92]. Moreover, the co culture of MSCs with degenerative NPCs significantly upregulated the gene expression of various growth factors (epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1), osteogenic protein-1 (OP-1), growth differentiation factor-7 (GDF-7), and TGF-β in MSCs. This study demonstrated the immunomodulatory potential of MSCs to modulate the degenerative IVDCs. To consider MSCs for the treatment of MC, MSCs must suppress the inflammation in the 'MC disc'. Elevated levels of inflammatory molecules including TNF-α, IL-1β, IL-6, interferon-gamma (IFN-γ), and interleukin-17 (IL-17) are common findings in degenerating IVDs [60,113,126–129]. The effect of these elevated inflammatory molecules on IVDCs was investigated by Gabr et al. [129]. IVDCs from patients undergoing surgery for DD or scoliosis were stimulated with IL-17 in combination with IFN-γ or TNF-α. The stimulation of IVDCs significantly increased the secretion of inflammatory molecules (nitric oxide (NO), PGE2, IL-6, and intercellular adhesion molecule-1 (ICAM-1)), thereby indicating the potential of stimulated IVDCs to recruit immune cells to the IVD tissue [129].

3.4.3 Immunomodulatory vs. Regenerative Mode of Action in DDD

An ex vivo experimental study—using a bovine model of IVD degeneration—investigated the effect of a degenerative IVD microenvironment on the regenerative and immunomodulatory potential of human MSCs in co culture with the bovine IVD [130]. No notable effect on ECM remodeling by MSCs was found, but evidence was presented for an immunomodulatory paracrine effect of MSCs, suggesting a predominant cytokine feedback loop between MSCs and disc cells [130]. In summary, the co culture of human MSCs with bovine IVDs in an inflammatory environment led to the significant downregulation of bovine pro-inflammatory cytokines, including IL-6, IL-8, and TNF-α [130]. This study indicated that the immunomodulatory potential of MSCs might be more relevant than the regenerative capacity in an inflammatory IVD microenvironment. Furthermore, it raised the question as to what extent the regenerative capacity of MSCs contributed to the favorable outcomes of previous clinical trials on intradiscal MSC injections in DDD patients. In conclusion, the immunomodulatory potential of MSCs is an important factor in discal MSC therapy that deserves more attention.

3.5 Patient Stratification: MSC Therapy for DDD Patients with MC1

The clinical benefit of intradiscal MSC injections in clinical trials was inconsistent and had occasionally been reported to be restricted to a group of responders [1,131]. These inconsistent study outcomes can partially be explained by inconsistent study designs. Most of the clinical trials on MSC injections in DDD

patients had low patient numbers and lacked a placebo control group and a standardized cell preparation [1,86,132]. Another important reason for the inconsistent clinical trial results might be the highly heterogeneous study populations. The multifactorial etiology of DDD makes it difficult to select a homogeneous and representative study population. Inclusion and exclusion criteria differed from trial to trial, thus leading to heterogeneous study populations and large inter study variations. Stratification of patients into clinically meaningful subpopulations is essential for demonstrating the efficacy of MSC treatments in the highly heterogeneous DDD population. Selecting a cohort of potential responders based on clinical presentation could help to achieve consistently high therapeutic efficacy. DDD patients with MC are clinically different from DDD patients without MC in terms of pain perception and severity, standard treatment efficacy, and duration of LBP episodes [6,8,9,11,40,133]. Therefore, MC patients may represent a more homogeneous subpopulation of DDD patients and should be specifically investigated. Unfortunately, DDD patients with MC were not differentiated from DDD patients without MC in clinical trials on intradiscal MSC injections in DDD patients. In fact, patients with MC were occasionally excluded from the clinical trials, thereby possibly omitting a group of potential responders to MSC injections [73,75,134]. Therefore, the efficacy of discal MSC treatments against MC1 has yet to be investigated in future clinical trials.

The three different types of MC may represent different stages of the same pathology [25]. However, a stronger stratification might be beneficial. Assuming that the anti-inflammatory action of MSCs is a main contributor to the beneficial effects seen in the trials, it might be reasonable to focus on MC1. MC1 are the MC type with the strongest inflammation and with the highest pain association [13]. MC1 account for around 20% of all MC cases and appear as bone marrow edema like changes in MRI, indicating areas of inflammation [15,135]. Investigating MC1 patients, as a clearly defined subpopulation of DDD patients, could significantly reduce study population heterogeneity. MRI could serve as a reliable technique to specifically include MC1 patients and to monitor treatment efficacy as a companion diagnostic. While infectious MC1 may be treated with antibiotics [36,37] and should be excluded from clinical trials on MSC injections, the main focus should be on the autoinflammatory MC1. Therefore, it is important to develop diagnostic tools to distinguish infectious from autoinflammatory MC1, enabling a causal treatment. The shape of the MC lesion seen on MRI has been shown to correlate with the infectious etiology of MC1. The absence of a 'claw-sign' of the MC1 lesion suggests an infectious etiology [136–138]. Magnetic resonance spectroscopy of the disc is another promising tool that may be able to identify MC1 related to infection of the disc with Cutibacterium acnes [139,140]. No study has reported a serum biomarker for MC1 that stratifies for infectious and autoinflammatory MC1, yet recent studies indicate different pathomechanisms in the bone marrow [141].

3.6 Possible Mode of Action of MSCs in MC1

To address the complex pathogenesis of MC1, a broad treatment approach targeting multiple aspects of the disease might be favorable. Multimodal MSCs seem ideal to address the vicious IVD/bone marrow cross talk through immunomodulation of the inflammatory DD environment, regeneration of the degenerated IVD, and repair of the CEP through chondrogenic differentiation or stimulation of host repair responses [1,109]. However, MSCs have not been used to specifically treat CLBP patients with MC1. A major advantage of MSC based treatment approaches over steroids, bisphosphonates, and TNF-α inhibitors is the long-acting multimodal action of MSCs. As MC1 is a multifactorial disease with characteristic CEP defects, DD, nerve ingrowth, thinned trabecular bone, and active vertebral inflammation, short-term immunosuppression, inhibition of TNF-α, or suppression of osteoclast activity alone might not be sufficient to alleviate CLBP.

Discal inflammation seems to play an important role in MC1 pathology, as the increased diffusion of inflammatory molecules from 'MC1 discs' [25,142] through the damaged endplates into the bone marrow might promote intensified inflammation in adjacent nutrient rich vertebral bodies. Therefore, suppressing the inflammation in 'MC1 discs' could represent a valid treatment strategy to disrupt the inflammatory and pro-fibrotic feedback loop between the 'MC1 disc' and the bone marrow. The immunomodulatory cytokines TSG-6, IL-10, and TGF-β secreted by MSCs counteract the pro-inflammatory effects of TNF-α and IL-1β in IVDs. TNF-α and IL-1β are key inflammatory mediators found in DD [60] and are frequently used in in vitro experiments to investigate the effect of discal inflammation on IVDCs [132–134]. IL-1β stimulation of IVDCs induces the downregulation of stemness associated genes and upregulates pro-inflammatory, pro-angiogenic, and catabolic genes [140,142,143]. Treating IL-1β stimulated NPCs with TSG-6 reduced IL-6 and TNF-α secretion, increased the proliferation rate of NPCs, and promoted ECM synthesis [132]. IL-10 was found to increase the expression of ECM associated genes and decrease the expression of inflammatory genes in IL-1β-stimulated NPCs [134]. TGF-β stimulation of degenerative AF cells grown in micromass culture increased the ECM production [144]. Moreover, TGF-β1 stimulation was shown to significantly increase the ECM production in NP cells isolated from degenerated human IVDs [145]. TGF-β3 treatment of degenerative human NP cells stimulated NP cell proliferation and induced an anti-catabolic gene expression profile, highlighting the regenerative potential of TGF-β [146]. In addition, co culture experiments of degenerative IVDCs with MSC showed downregulation of IL-6 and IL-8 gene expression levels—pro-inflammatory cytokines that were found to be elevated in 'MC1 discs' [25,142]. Altogether, these studies provide compelling evidence that MSC can suppress discal inflammation. Moreover, suppression of discal inflammation might terminate nerve ingrowth into the IVD, as IL-1β was shown to stimulate the expression of vascular endothelial growth factor (VEGF), NGF, and brain derived neurotrophic factor (BDNF) in degenerative

IVDCs, resulting in angiogenesis and innervation [147]. Microvascular blood vessels expressing NGF were shown to enter painful IVDs from the adjacent bone marrow through the CEP [148]. These microvascular blood vessels were accompanied by neurotrophic receptor tyrosine kinase 1 (TrkA)-expressing nerve fibers. Thus, the suppression of discal inflammation might ultimately help to relieve discogenic pain.

The CEP is damaged in MC1, allowing a pro-inflammatory 'MC1 disc'/bone marrow cross-talk. Repair of the leaky CEP might restore the IVD/bone marrow barrier, thereby disrupting the pro-inflammatory 'MC1 disc'/bone marrow cross talk. MSCs can promote cartilage regeneration by differentiating into chondrocytes to replace damaged cells or by promoting the proliferation and ECM deposition of resident chondrocytes through the secretion of cytokines, growth factors, and extracellular vesicles [89]. There are no studies on vertebral CEP regeneration. However, clinical trials on MSCs for cartilage repair in patients with cartilage degeneration—including osteoarthritis—have demonstrated encouraging results [149]. Single intra-articular administration of MSCs into the knee of patients suffering from degenerative joint disease or osteoarthritis showed cartilage maintenance or growth. Functionality of the joint was improved and pain relief was achieved in most patients [150–152]. CAR-TISTEM®—a composite of allogenic MSCs and hyaluronic acid—has already been approved by the Korean Ministry of Food and Drug Safety in 2012 for the treatment of cartilage degeneration including degenerative OA. A 7-year follow-up study demonstrated the persistence of the regenerated cartilage [143].

The trabecular bone is thinned in MC1, possibly due to osteoclast activity. Suppression of osteoclast activity might consolidate inflamed vertebral bodies, thereby improving the tolerance for mechanical load. MSCs support osteoclastogenesis under physiological conditions through the secretion of RANKL and M CSF [153]. On the contrary, MSCs suppress osteoclast formation and activation under inflammatory conditions, through the secretion of osteoprotegerin (OPG) and IL-10 [154–156]. Therefore, MSCs might suppress the inflammatory trabecular bone resorption observed in MC1.

In summary, the regenerative and immunomodulatory properties of MSCs might counteract the painful molecular changes observed in DDD patients with MC1 (**Figure 3**). Firstly, regeneration of degenerated IVD and suppression of inflammatory bone resorption might restore normal functioning of the motion segment, thereby disrupting the degenerative cascade [78,80,157]. Secondly, CEP regeneration might repair the leaky IVD/bone marrow barrier, thereby disrupting the pro-inflammatory cross talk observed in MC1. Thirdly, suppressing disc inflammation and the cross talk with the adjacent bone marrow might suppress vertebral inflammation, nerve ingrowth, and ultimately discogenic pain [19,61,86].

We next discuss whether patients with MC1 should be treated with MSCs.

3.7 PRO—MSCs for Patients with MC1

MSCs represent a valid treatment option for patients with MC1 for several reasons. Firstly, patients with MC1 represent a homogeneous subpopulation of DDD patients with a clear inflammatory phenotype. Treatment approaches in the homogeneous patient populations can result in larger and more consistent treatment effects, as seen in clinical trials on intradiscal and epidural steroid injections in CLBP patients with MC [39–41,55,56]. Secondly, MSCs were shown to have a favorable safety profile, with no reported serious adverse events in completed clinical trials on intradiscal MSC injections in DDD patients [1,72,73]. Besides the absence of serious adverse events, DDD patients treated with MSCs experienced substantial pain relief and disability-related improvements [1]. Thirdly, MSCs can break the inflammatory 'MC1 disc'/marrow cross-talk and heal MC. The multimodal mode of action of MSCs might play a crucial role in the treatment of MC1. MSCs are potent immunomodulators [124,125,140] with the potential to suppress the inflammation in the 'MC1 disc' and the cross-talk with the adjacent bone marrow. Suppression of the inflammatory and pro-fibrotic cross-talk could also suppress nerve ingrowth into the IVD, thereby suppressing discogenic pain [19,61,86]. The structural damage of the IVD and the CEP in MC1 could lead to altered biomechanics of the vertebral motion segment, which in turn could result in discal inflammation [76]. MSCs could target the underlying biomechanical issue by regenerating the degenerated IVD and the disrupted CEP, thereby alleviating or even preventing vertebral inflammation and subsequent degenerative processes. CEP regeneration might repair the leakage in the IVD/bone marrow barrier, thereby disrupting the inflammatory cross-talk between the 'MC1 disc' and the adjacent bone marrow. Furthermore, MSCs might suppress inflammatory trabecular bone resorption [154–156], thereby consolidating the inflamed vertebral bodies and improving the tolerance for mechanical load.

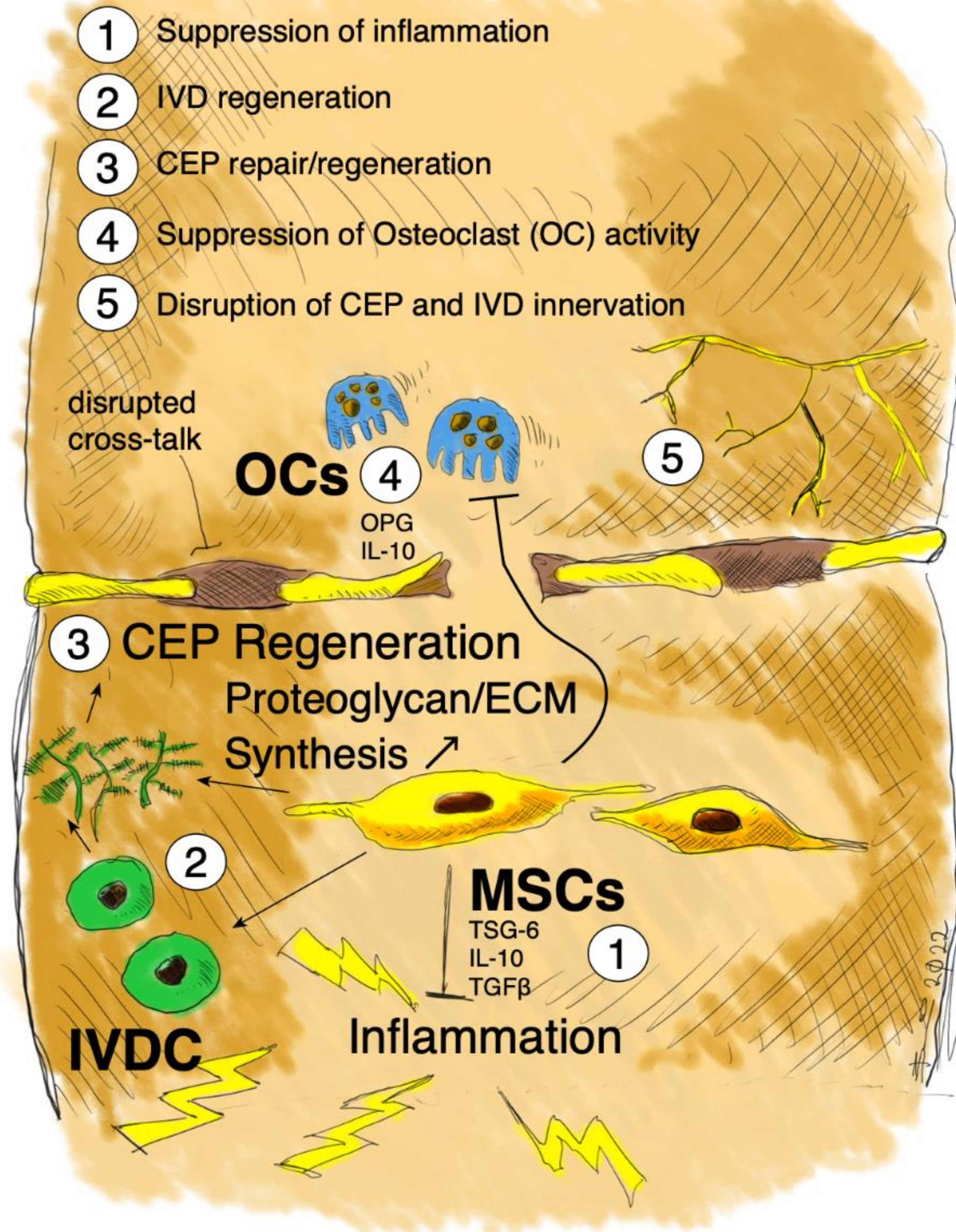

Figure 3 Schematic illustration of the possible multimodal mode of action of intradiscally injected MSCs in MC1. MSCs might regenerate the 'MC1 disc', repair the CEP leakage, and suppress osteoclast activity, thereby improving the tolerance for mechanical load. Suppression of discal inflammation and sealing of the CEP leakage might disrupt the inflammatory 'MC1 disc'/bone marrow cross-talk, thereby suppressing nerve ingrowth and discogenic pain. OCs = osteoclasts.

3.8 CONTRA—MSCs for Patients with MC1

A major concern regarding the use of MSCs for IVD repair is the harsh IVD microenvironment [77]. Although MSCs might counteract DD by chondrogenic differentiation, the differentiation of MSCs into chondrocytes is inhibited by IL-1β and TNF-α [158]—key inflammatory mediators found in DD [60]. This implies that IVD regeneration might be inhibited in the inflammatory 'MC1 disc'. The number of viable MSCs needed to induce significant regeneration of the IVD is high and unlikely to be achieved in the hostile 'MC1 disc' [77]. Therefore, suppression of discal inflammation prior to the regeneration of the IVD and CEP might be a more promising treatment approach [158].

The positive outcomes in clinical trials on intradiscal MSC injection in DDD patients pointed to a regeneration independent mode of action. Patients experienced substantial pain- and disability related improvement despite the absence of discal regeneration [1,76]. However, if MSCs do not regenerate damaged IVD structures, the structural damages might worsen functional impairment and potentially lead to secondary pathologies of the motion segment.

As infectious MC1 may be treated with antibiotics [36,37] and should be excluded from clinical trials on MSC injections, the main focus should be on the autoinflammatory MC1. Unfortunately, no imaging or serum biomarker for MC1 exists that reliably differentiates between infectious and autoinflammatory MC1. Therefore, it is important to develop diagnostic tools to distinguish infectious from autoinflammatory MC1 that enable a causal treatment.

3.9 Conclusions

The efficacy of intradiscal MSC injections against MC1 has yet to be investigated in future clinical trials. The main goal of future trials on MC1 patients—as a homogeneous population with a clear inflammatory phenotype—should be to alleviate pain and slow down disease progression. These studies should focus on immunomodulation of the 'MC1 discs' and the repair of CEP damage to disrupt the inflammatory IVD/bone marrow cross talk. MSCs might represent a safe and multimodal treatment approach with promising immunomodulatory and regenerative properties. Regeneration of structural damage to the IVD and CEP could prevent discal inflammation and possibly cure MC1 by disrupting the inflammatory IVD/bone marrow cross talk. Advanced stages of DD might be an exclusion criterion for MSC injections, as the loss of structural integrity of the IVD and poor nutrient supply could impair the therapeutic efficacy of MSCs in this hostile IVD environment. No data on pH, osmolarity, and oxygen concentration in 'MC1 discs' are published, which might hamper the development of discal treatments. Assessing the condition of the CEP could be particularly important and should be considered when selecting patients for an MSC based therapy. Therefore, novel tools to identify non-infectious MC1 and to assess CEP defects are required. Patient stratification, standardization of MSC

preparation techniques, and selection of immunomodulation related endpoints might pave the way for efficacious MC1 treatments.

3.10 Personal contribution

This work would not have been possible without the collaborative efforts of my esteemed co-authors. Their insights and perspectives not only enriched the content and quality of this work but also expedited its journey from conception to publication. I extend my sincere gratitude to each contributor.

In leading the writing process for this chapter, my role encompassed guiding the conceptualization, preparing the initial draft, and overseeing the alignment of our thoughts and ideas. Responsibilities extended to writing and editing the final version, as well as summarizing clinical trial outcomes. I am grateful to Prof. Benjamin Gantenbein for generously providing the visualizations that enhance the visual appeal and clarity of this chapter.

The seamless collaboration among our team members has made this publication possible, and I am privileged to have worked alongside such dedicated and talented individuals.

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Chapter 4

Impacts of Priming on Distinct Immunosuppressive Mechanisms of Mesenchymal Stromal Cells under Translationally Relevant Conditions

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4.1 Abstract

Background: The multimodal properties of mesenchymal stromal cells (MSCs), particularly their ability to modulate immune responses is of high interest in translational research. Pro-inflammatory, hypoxic, and 3D culture priming are promising and often used strategies to improve the immunosuppressive potency of MSCs, but the underlying mechanisms are not well understood. Therefore, the aims of this study were i) to compare the effects of pro-inflammatory, hypoxic, and 3D culture priming on the in vitro immunosuppressive potential of MSCs, ii) to assess if immunosuppressive priming effects are temporally preserved under standard and translationally relevant culture conditions, and iii) to investigate if the three priming strategies engage the same immunosuppressive mechanisms.

Methods: Functional in vitro T cell suppressive potency measurements were conducted to assess the impact of pro-inflammatory, hypoxic, and 3D culture priming on the immunosuppressive potential of human bone marrow-derived MSCs. Primed MSCs were either cultured under standard cell culture conditions or translationally relevant culture conditions, and their transcriptomic adaptations were monitored over time. Next-generation sequencing was performed to assess if different priming strategies activate distinct immunosuppressive mechanisms.

Results: i) Pro-inflammatory, hypoxic, and 3D culture priming induced profound transcriptomic changes in MSCs resulting in a significantly enhanced T cell suppressive potential of pro-inflammatory and 3D culture primed MSCs. ii) Priming effects rapidly faded under standard cell culture conditions but were partially preserved under translationally relevant conditions. Interestingly, continuous 3D culture priming of MSCs maintained the immunosuppressive potency of MSCs. iii) Next-generation sequencing revealed that priming strategy-specific differentially expressed genes are involved in the T cell suppressive capacity of MSCs, indicating that different priming strategies engage distinct immunosuppressive mechanisms.

Conclusion: Priming can be a useful approach to improve the immunosuppressive potency of MSCs. However, future studies involving primed MSCs should carefully consider the significant impact of translationally relevant conditions on the preservation of priming effects. Continuous 3D culture could act as a functionalized formulation, supporting the administration of MSC spheroids for a sustainably improved immunosuppressive potency.

Keywords: Mesenchymal stromal cells, MSC, priming, 3D culture, hypoxia, immunomodulation

4.2 Introduction

Mesenchymal stromal cells (MSCs) have an exceptional clinical potential due to their multi-lineage differentiation capacity, ease of isolation, engraftment potential, self-renewal capacity, favorable safety profile, and immunomodulatory properties. [1] Over 450 completed interventional clinical trials using MSCs have been listed in the ClinicalTrials.gov database (accessed on 10 August 2023). The fields of application of MSCs encompass a wide range of diseases, including degenerative disorders and chronic inflammatory conditions, cancer, COVID-19, graft-versus-host disease, and the repair of fractures and articular cartilage defects. Despite the large number of completed clinical trials on MSCs, only a handful of MSC-based products made it on the market. The reasons are manifold. Briefly, the potency of MSCs and, consequently, the outcomes of clinical trials is influenced by donor selection, tissue origin, lack of standardized isolation methods and culture protocols, insufficient MSC characterization, the absence of meaningful critical quality attributes, and the administration route. [2,3] In 2019, the International Society of Cell and Gene Therapy (ISCT) refined their existing definition of MSCs with the goal to address the emerging clinical challenges and to standardize the use of MSCs. [4] In addition to fulfilling the three minimal criteria for MSC definition (plastic adherence, surface marker expression, trilineage differentiation), the ISCT recommended to additionally demonstrate the functional properties of MSCs based on functional assays. [5,6]

Priming has been suggested as a promising bioengineering strategy to overcome challenges encountered in MSC therapies. It may enhance several therapeutically relevant properties of MSCs such as immunomodulation, migration, tissue regeneration, survival and engraftment, angiogenesis, and stemness. [7] Pre-conditioning MSCs with pro-inflammatory cytokines or exposing MSCs to hypoxia represent the most commonly used priming strategies to improve the therapeutic potential of MSCs. [7–9] 3D culture priming represents a non-genetic emerging MSC priming strategy that was shown to enhance the immunomodulatory, pro-angiogenic, and regenerative properties of MSCs. [10–14]

The ability of MSCs to modulate a wide range of immune cells is not only pivotal for their therapeutic promise in treating immune disorders, but it also holds great potential for tissue regeneration. Besides the high cellular plasticity and self-renewal capacity of MSCs, the immunomodulatory capacity is likely a crucial contributor to their regenerative potential. [15,16] The immunomodulatory potential of MSCs is typically assessed by in vitro T cell suppression assays. [17] Interferon gamma (IFN-γ) is the most commonly used pro-inflammatory agent for MSC priming to increase their immunomodulatory potency. [7,11,15] The combination of IFN-γ with tumor necrosis factor alpha (TNF-α) was shown to synergistically improve the therapeutic potential of MSCs. [18–21] The exposure of MSCs to oxygen concentrations as low as 1% O_2 is a commonly applied priming strategy to activate anti-apoptotic and angiogenic pathways. [22,23] Recent data demonstrated that hypoxia also enhances the

immunomodulatory capacity of MSCs. [9,24] The aggregation of MSCs into 3D spheroids is an emerging priming strategy, which was shown to enhance the immunomodulatory capacity of MSCs. [12] The immunomodulatory mechanism of 3D culture priming is likely distinct from those of pro-inflammatory and hypoxic priming. [25]

The main goal of priming is to solve the clinical challenges faced by MSC products to ultimately administer them to patients in need. However, the lack of standardized priming strategies combined with a limited understanding of the effects of priming on MSC function, hinder the translational potential of MSC priming. To consider a priming strategy for clinical application, MSCs must preserve the priming effects post-administration. Little information is available whether MSCs can preserve the priming effects over time. If primed MSCs are administered to patients, they will be exposed to disease-specific microenvironments. The influence of local microenvironments on the temporal preservation of priming effects is not well studied. Therefore, the aims of this study were i) to investigate the effects of pro-inflammatory, hypoxic, and 3D culture priming on the in vitro immunosuppressive potential of MSCs, ii) to assess if immunosuppressive priming effects are temporally preserved under standard cell culture conditions, or if translationally relevant conditions are necessary to maintain the priming effects over time, and iii) to clarify if pro-inflammatory, hypoxic, and 3D culture priming engage the same immunosuppressive mechanisms.

4.3 Materials and methods

Experimental design

Commercial human iliac crest-derived bone marrow MSCs from three donors were used for this study. The MSCs were either primed by pro-inflammatory, hypoxic, or 3D culture priming for 48 h (**Fig. 1**). Unprimed MSCs served as comparator. Following priming, RNA was isolated from a fraction of the MSCs for RNA sequencing and quantitative real-time polymerase chain reaction (qPCR). The remaining MSCs were either used for the in vitro functional immunosuppressive potency assay or cultured under two separate culture conditions (standard conditions or translationally relevant conditions). The translationally relevant conditions were priming approach-specific, i.e. following hypoxic priming, MSCs were cultured in oxygen concentration levels of a possible MSC recipient tissue in vivo (5% O_2); following pro-inflammatory priming, MSC were cultured in recipient tissue typical inflammation level (50 pg/mL TNF-α); and following 3D culture priming, MSC were kept in spheroids, because this

represents the translational scenario of spheroid injection. On days 4 and 8, the primed MSCs were harvested and analyzed by qPCR and with the in vitro immunosuppressive potency assay.

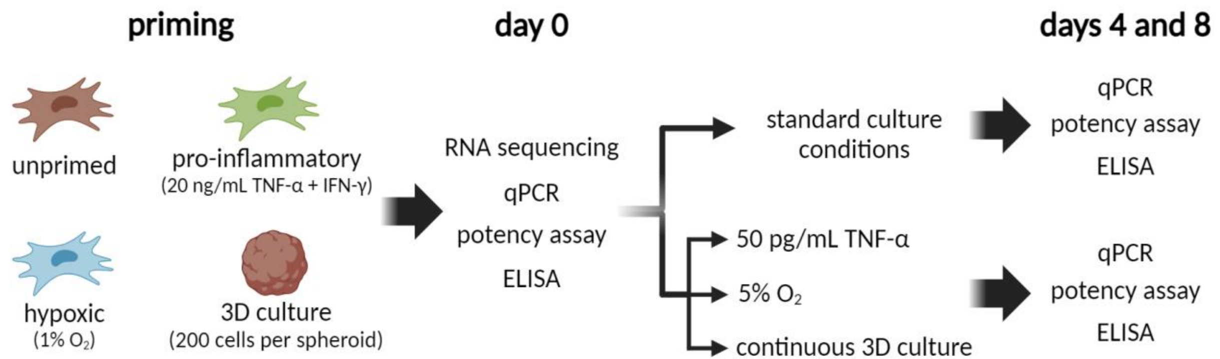


Fig. 1_Illustration of the experimental setup. Created with BioRender.com_

Preparation of peripheral blood mononuclear cells and expansion of MSCs

The study was conducted in accordance with the declaration of Helsinki and approved by the local ethics commission (ethical approval: BASEC-Nr. 2018-01873). Commercial human iliac crest-derived bone marrow MSCs from three donors were used (RoosterBio, Maryland, USA). MSC donor information is detailed in the supplementary Table 1. The MSCs conform with the ISCT minimal criteria for human MSC identity and were between population doubling levels 12 and 14 for all conducted experiments. MSCs were expanded and primed in minimum essential medium alpha (Biowest, Nuaillé, France) supplemented with 5% human platelet lysate (STEMCELL Technologies, Vancouver, Canada) and 50 U/mL penicillin/streptomycin (Life Technologies, California, USA) in a humidified incubator at 37 °C, 21% O_2, and 5% CO_2. Media change was performed twice a week. Whole blood was obtained from a single healthy donor and peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (STEMCELL Technologies, Vancouver, Canada) density gradient medium. The PBMCs were subsequently aliquoted and frozen in 10% dimethyl sulfoxide (Carl Roth, Karlsruhe, Germany) in heat-inactivated fetal calf serum (Life Technologies, California, USA) for 24 h at -80 °C, in a CoolCell LX cell freezing container (Corning, New York, USA), before being transferred to a liquid nitrogen tank. For the co-culture of MSCs with PBMCs, RPMI 1640 (Biowest, Nuaillé, France) supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL penicillin/streptomycin (Life Technologies, California, USA), 200 nM L-glutamine (Life Technologies, California, USA), and 2.5 ng/mL recombinant human basic fibroblast growth factor (Peprotech, New Jersey, USA) was used. No media change was performed throughout the co-culture experiment.

MSC priming and culture

Pro-inflammatory priming – MSCs were treated with a combination of 20 ng/mL TNF-α (Peprotech, New Jersey, USA) and 20 ng/mL IFN-γ (Peprotech, New Jersey, USA) in the priming medium for 48 h according to a previously published priming protocol. [26] Following pro-inflammatory priming, MSCs were either cultured in standard cell culture medium without any supplemented inflammatory cytokines, or supplemented with 50 pg/mL TNF-α to mimic the inflammation level of a possible MSC recipient tissue in vivo. [27,28]

Hypoxic priming – MSCs were exposed to 1% O_2 in a humidified incubator at 37 °C and 5% CO_2 for 48 h. Following hypoxic priming, MSCs were either cultured in standard cell culture medium under normoxia (21% O_2) or under 5% O_2 to simulate the oxygen concentration of possible recipient organs, e.g. bone, brain, heart, and liver. [29,30]

3D culture priming – SP5D plates (Kugelmeiers, Zurich, Switzerland) were used for the 3D culture priming of MSCs (**supplementary Fig. 1**). 1.5 x 10^5 MSCs were seeded per SP5D well and incubated for 48 h to generate 750 spheroids each containing approximately 200 MSCs. MSC spheroids were dissociated by digestion in 500 µg collagenase P (Hoffmann LA Roche, Basel, Switzerland) dissolved in 1 mL Hank's balanced salt solution (Sigma-Aldrich, Missouri, USA) for 1 h at 37 °C, resulting in a single cell suspension. MSCs cultured as spheroids were viable and metabolically active for the duration of culture (**supplementary Fig. 2**). Following the 48 h 3D culture priming, MSC spheroids were dissociated and cultured in monolayers under standard cell culture conditions (37 °C, 21% O_2, and 5% CO_2). To simulate the translational scenario of spheroid injection, the 3D culture primed MSCs were continuously cultured as spheroids.

In vitro functional immunosuppressive potency assay

Pro-inflammatory and hypoxic primed MSCs were harvested using Accutase (Innovative Cell Technologies, California, USA) and 3D culture primed MSCs were dissociated as described above. 3 x 10^4 primed MSCs were seeded in F-bottomed 96-well plates and cultured for 24 h in co-culture medium to allow MSCs to adhere to the plate. PBMCs were thawed, resuspended in co-culture medium, and incubated overnight under standard cell culture conditions. The PBMCs were labeled with 2.3 µM CellTrace CFSE Cell Proliferation Kit (Life Technologies, California, USA) in Dulbecco's phosphate-buffered saline (PBS) (PAN-Biotech, Bayern, Germany) for 5 min at room temperature, washed, and 10^5 CFSE-PBMCs were directly added to the adherent MSCs in the 96-well plate. The MSC:PBMC ratio of 30:100 was determined based on a preliminary titration experiment (supplementary Fig. 3). To activate T cells and induce T cell proliferation 1 µL/100 µL TransAct (Miltenyi Biotec, Nordrhein Westfalen, Germany) – a nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists – was directly added to the co-culture. The co-culture of MSCs with PBMCs was

preferred over the co-culture with isolated T cells to more closely mimic physiological conditions. Additionally, the co-culture of MSCs with PBMCs preserves the natural interactions between MSCs and cell types other than T cells, which allows for the detection of indirect T cell suppressive effects, for example via the modulation of monocytes. MSCs were co-cultured with CFSE-PBMCs for 3 days in a humidified incubator at 37 °C, 21% O_2, and 5% CO_2. CFSE-PBMCs were subsequently harvested and processed for flow cytometry analysis. CFSE-PBMCs were stained with anti-CD3 PE-Cy5, anti-CD4 BV711, and anti-CD8 PE (all from BioLegend, California, USA) and a LIVE/DEAD fixable viability dye (Life Technologies, California, USA) was used to exclude dead cells from the analysis. CFSE-PBMCs were stained for 1 hour at room temperature. Stained CFSE-PBMCs were washed with PBS and analyzed using a BD LSRFortessa cell analyser (BD Biosciences, New Jersey, USA). Data was analyzed with FlowJo v10.8 software. Cell doublets and dead cells were excluded from the analysis. The proliferation of viable T cells ($CD3^+$), helper T cells ($CD4^+$), and cytotoxic T cells ($CD8^+$) was calculated based on the CFSE fluorescence intensity. T cells cultured in the absence of co-cultured MSCs served as proliferation control. FlowJo's proliferation modeling tool was utilized for calculating the proliferation index (total number of cell divisions divided by the number of cells that went into cell division). Proliferation index is presented on a scale from 0% (complete suppression of CFSE-PBMC proliferation) to 100% (CFSE-PBMCs proliferation in co-culture with unprimed MSCs). It is important to highlight that a low proliferation index indicates strong immunosuppression.

RNA sequencing and data analysis

RNA was isolated from unprimed, pro-inflammatory, hypoxic, and 3D culture primed MSCs directly following 48 h priming, using the RNA isolation protocol described below. Library preparation was performed with 500 ng total RNA using the poly(A)-based TruSeq Stranded mRNA kit (Illumina, California, USA). Libraries were sequenced using the NovaSeq 6000 (Illumina, California, USA) sequencing system (16 million reads per sample). The quality of the data was assessed using FastQC and the readings were mapped to the reference genome hg38 using STAR v.2.7.10b. Genes of primed MSCs with FDR ≤ 0.1 and $|\log_2$ fold change$| \geq 1.5$ ($\log_2$FC) relative to the gene expression of unprimed MSCs were considered differentially expressed. Principle component analysis (PCA) was calculated based on the top 2000 genes ranked by standard deviation. A Venn diagram of significantly differentially expressed genes (DEGs) of primed versus unprimed MSCs was generated. For the volcano plots, DEGs of primed MSCs versus unprimed MSCs were ranked from highest $|\log_2 FC|$ values to lowest. Afterwards, the list was filtered by all genes that were part of GO:0006955 (immune response) or GO:0006954 (inflammatory response). The top 10 DEGs from the resulting list were identified and highlighted in the volcano plots and listed in the supplementary Table 2. Hypergeometric over-representation analyses (ORA) were performed separately for upregulated and downregulated DEGs

using the R package clusterProfiler v.3.17. Top 5 over-represented biological processes were selected based on adjusted p-values.

Quantitative real-time polymerase chain reaction

A fraction of primed MSCs was resuspended in buffer RLT (Qiagen, Venlo, Netherlands) supplemented with 10 µL/mL 2-mercaptoethanol (Merck, Darmstadt, Germany) for RNA isolation. RNA was isolated from MSCs using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and cDNA was synthesized using SensiFAST cDNA Synthesis Kit (Solis BioDyne, Tartu, Estonia) according to the manufacturer's instructions. qPCR was performed in 384 white well qPCR plates on a CFX Opus Real-Time qPCR System (Bio-Rad, California, USA) using the HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia) using primers listed in supplementary Table 3. We used a qPCR reaction volume of 10 µL and the following cycling protocol: 95 °C for 12 min followed by 45 cycles performed at 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 30 s. Relative fold gene expressions were calculated using the $2^{-\Delta\Delta Cq}$ method and TATA-box binding protein (TBP) served as housekeeping gene.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of MSC cultures were collected at days 0, 4, and 8. Media changes were performed at days 2 and 6 to ensure that each collected supernatant was conditioned for 48 h. The concentration of PGE_2, CXCL9, and kynurenine were quantified in undiluted supernatants and their concentration per 10^5 MSCs was calculated. For each priming strategy, one representative protein was selected, which demonstrated strong upregulation immediately following priming on the transcriptomic level. PGE_2 and CXCL9 ELISA kits were acquired from Abcam (Cambridge, United Kingdom), the kynurenine EILSA kit was acquired from AssayGenie (Dublin, Ireland).

Selection of signature genes

Three gene sets were assembled to reflect the immunomodulatory mechanisms of pro-inflammatory, hypoxic, and 3D culture primed MSCs. Each gene set contained five immunomodulation-associated signature genes (DEGs of GO:0006955 or GO:0006954) that were significantly upregulated after respective priming. The selection of the signature genes was based on our RNA sequencing data of primed versus unprimed MSCs and has been cross-referenced with immunomodulatory potency markers published in literature. [31–39] Only DEGs and immunomodulation-associated genes were considered. A fourth gene set was created containing five immunomodulatory potency markers, which are frequently used in literature. [40–44] This gene set was investigated in all primed MSCs, regardless of which priming strategy was used. We quantified the signature genes using qPCR to evaluate the temporal stability of different priming approaches and to study how translationally relevant microenvironmental factors influence the priming effects over time. To test if the immunomodulation-

associated genes correlate with T cell suppression, we quantified all twenty signature genes (5 pro-inflammatory, 5 hypoxic, 5 3D culture, and 5 frequently used) for pro-inflammatory, hypoxic, and 3D culture primed MSCs. We hypothesized that different priming mechanisms engage different immunomodulatory mechanisms and hence correlate to varying degrees with T cell suppression.

Statistical analysis

Statistical analysis was performed with GraphPad PRISM v.10.0.1. Significance level was $\alpha = 0.05$, if not stated otherwise. To compare the effects of priming, time, and culture condition on T-cell suppression, a three-way ANOVA was calculated followed by Tukey post-hoc test corrected for multiple testing using Bonferroni p-value adjustment. To investigate which priming strategy (represented by the signature genes) shows best correlation with T cell suppression, Pearson correlations between the expression levels of signature genes and T cell suppression were computed. Priming strategies with strongest correlations are expected to have the highest T cell suppressive effects.

4.4 Results

First, we conducted an immune functional potency assay on primed and unprimed MSCs. Our main goal was to evaluate if pro-inflammatory, hypoxic, and 3D culture priming can enhance the in vitro functional immunosuppressive potency of MSCs. Second, we assessed if the in vitro functional immunosuppressive potencies of differently primed MSCs are stable over time. We hypothesized that priming effects fade over time but can be partially preserved by translationally relevant conditions i.e. pro-inflammatory environment, hypoxic environment, and continuous 3D culture. Third, we tested if the three priming strategies engage the same immunosuppressive mechanisms.

Pro-inflammatory and 3D culture priming enhance the T cell suppressive capacity of MSCs

Differently primed MSCs have different in vitro T cell suppressive potencies ($p < 0.001$, **Fig. 2 a**). The T cell suppressive capacities of differently primed MSCs were compared to unprimed MSCs. Pro-inflammatory priming significantly enhanced the immunosuppressive capacity of MSCs compared to unprimed, hypoxic primed, and 3D culture primed MSCs, directly following 48 h of priming. Pro-inflammatory primed MSCs achieved a complete suppression of T cell proliferation (mean difference ± standard error = -99% ± 11%, $p < 0.001$). Hypoxic priming had no beneficial effect on the T cell suppressive potential of MSCs (mean difference ± standard error = +21% ± 11%, $p > 0.48$). 3D culture priming significantly increased the immunosuppressive capacity of MSCs (mean difference ± standard error = -35% ± 11%, $p = 0.03$), but to a lesser extent than pro-inflammatory priming ($p < 0.001$).

Priming effects fade over time but are partially preserved by translationally relevant conditions

Priming effects were not stable over time under standard cell culture condition ($p < 0.001$, **Fig. 2 b**). The strongly enhanced immunosuppressive capacity of pro-inflammatory primed MSCs relative to

unprimed MSCs entirely disappeared already four days after priming (mean difference ± standard error for days 4 and 8: +15% ± 11% ($p = 0.84$), +2% ± 11% ($p > 0.99$)). 3D culture primed MSC lost their significantly enhanced immunosuppressive capacity as well at later time points (mean difference ± standard error for days 4 and 8: -9% ± 11% ($p > 0.99$), -5% ± 11% ($p > 0.99$)). Hypoxic primed MSCs even favored T cell proliferation at day 4 (mean difference ± standard error: +42% ± 11% $p = 0.005$).

Translationally relevant culture conditions had a significant impact on the temporal preservation of priming effects ($p < 0.001$, **Fig. 2 b**). The T cell suppressive capacities of MSCs cultured under translationally relevant conditions were compared to the T cell suppressive conditions of MSC cultured under standard cell culture conditions. A continuous stimulation of pro-inflammatory primed MSCs with as little as 50 pg/mL TNF-α was enough to partially preserve immunosuppressive potency up to four days (mean difference ± standard error for days 4 and 8: -35% ± 11% ($p = 0.04$), -22% ± 11% ($p = 0.46$)). No notable effect of translationally relevant 5% O_2 was found (mean difference ± standard error for days 4 and 8: -13% ± 11% ($p = 0.92$), -11% ± 11% ($p = 0.96$)). Continuous spheroid culture completely rescued the enhanced immunosuppressive priming effects from fading (mean difference ± standard error for days 4 and 8: -27% ± 11% ($p = 0.21$), -41% ± 11% ($p = 0.007$)). We observed no noticeable differences regarding the potency of MSCs to suppress total T cells, T-helper cells, and cytotoxic T cells (**supplementary Fig. 4 and 5**).

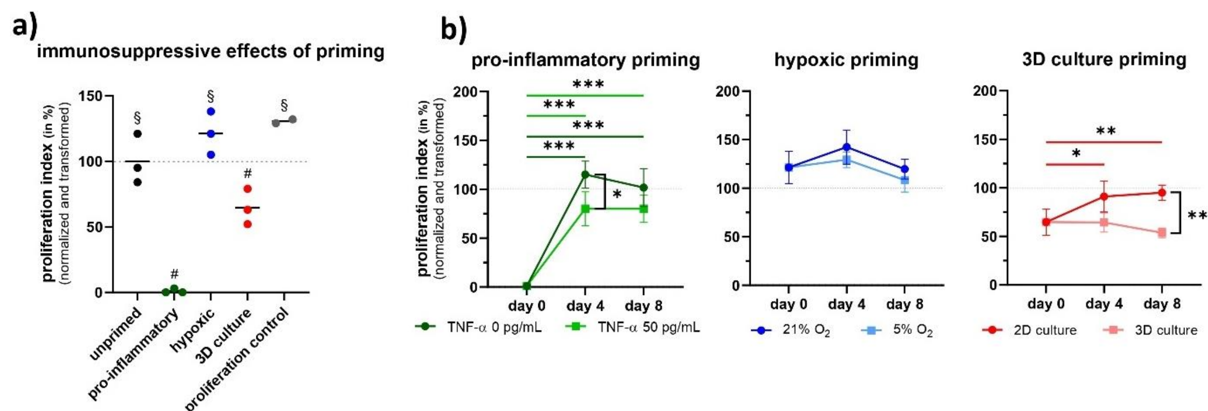


Fig. 2 The effects of each priming strategy on the immunosuppressive potential of MSCs were measured by an in vitro functional potency assay following 48 h of priming. A normalized transformed proliferation index of 0% corresponds to the complete suppression of $CD3^+$ T cell proliferation. A value of 100% indicates that the T cells proliferated equivalent to T cells co-cultured with unprimed MSCs (grey dashed horizontal lines). ***a)*** *Different priming strategies enhance the T cell suppressive capacity of MSCs.* ***b)*** *Priming effects fade over time but are partially preserved by translationally relevant conditions. The proliferation control represents the proliferation of T cells in the absence of co-cultured MSCs. Data points represent averages ± standard deviations of the MSCs from three healthy donors. # indicate significant differences ($p < 0.001$, except unprimed vs. 3D culture primed $p = 0.03$) against all other conditions. § indicate significant differences ($p < 0.001$) against pro-inflammatory and 3D culture priming. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$*

Different priming strategies induce distinct cellular mechanisms

In order to evaluate if pro-inflammatory, hypoxic, and 3D culture priming engage the same immunosuppressive mechanisms, we performed RNA sequencing after priming. We observed largely different effects of the three priming strategies on the transcriptome of MSCs as evidenced by the

distinct clustering of the priming strategies in the PCA plot (**Fig. 3**). Hypoxic priming induced the largest transcriptomic changes, whereas pro-inflammatory primed MSCs clustered closest to unprimed MSCs. The distinct clustering of the differently primed MSCs in the PCA plot is also reflected by the individual numbers of DEGs compared to unprimed MSCs, as shown in the Venn diagram. Several genes were differentially expressed in more than one priming strategy, suggesting possible similarities between the priming strategies (supplementary Tables 4, 5, 6, 7).

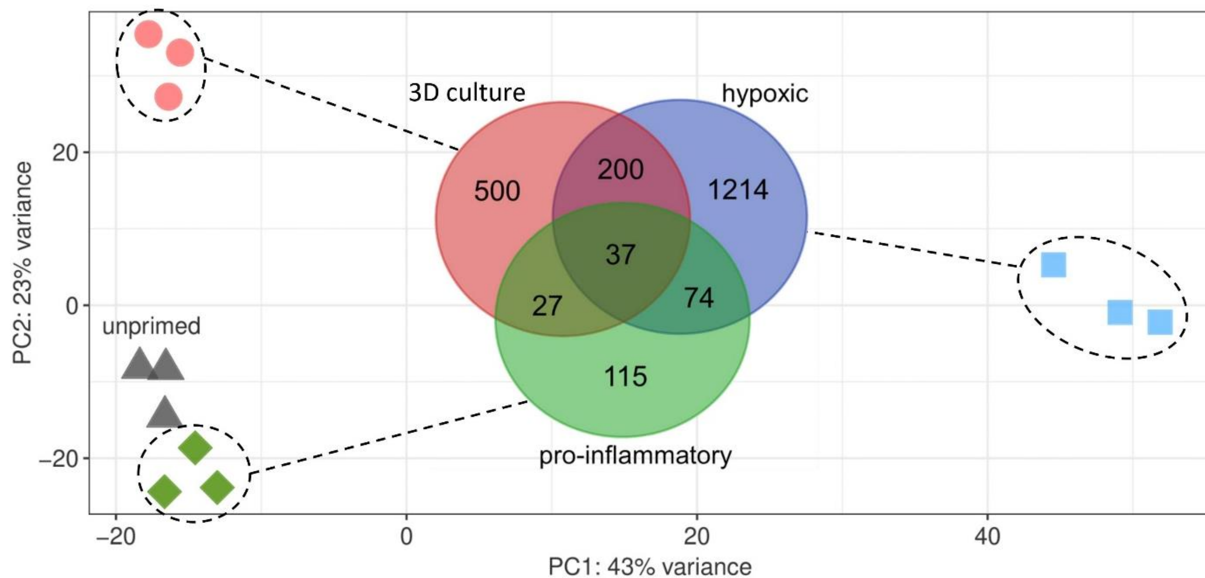


***Fig. 4** The PCA plot revealed a distinct clustering of pro-inflammatory, hypoxic, 3D culture primed, as well as unprimed MSCs from three donors. Notably, the MSCs clearly cluster by priming strategy and not by donors. The top 2000 genes ranked by standard deviation were considered for this PCA plot. The Venn diagram shows for each priming strategy, the numbers of significant DEGs (FDR ≤ 0.1, |log_2 ratio| ≥ 1.5) compared to unprimed MSCs. Additionally, the Venn diagram highlights the numbers of overlapping DEGs between priming strategies to indicate similarities or dissimilarities between different priming strategies.*

To better understand the transcriptomic changes induced by priming, we performed an ORA with the DEGs to identify the underlying biological processes (**Fig. 4**).

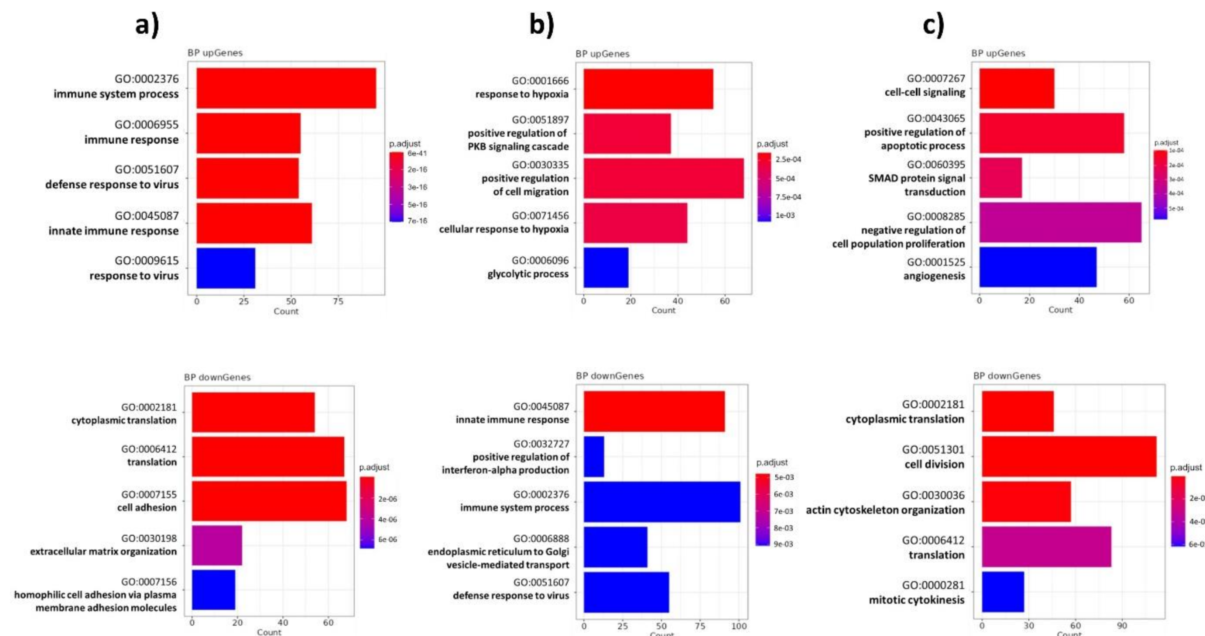


***Fig. 4** The bar plots show the top five upregulated and top five downregulated biological processes of **a)** pro-inflammatory, **b)** hypoxic, or **c)** 3D culture primed MSCs compared to unprimed MSCs. The upper bar plots reflect the upregulated biological processes, and the lower bar plot represents the downregulated biological processes. Only enriched biological processes based on DEGs with p-values ≤ 0.01 are shown.*

Pro-inflammatory priming led to the upregulation of genes involved in immune system responses and defense response to virus. In contrast, genes associated with protein synthesis, cell adhesion, and extracellular matrix organization were significantly downregulated. Hypoxic priming mainly induced adaptations to an anaerobic metabolism as well as an increased cellular migration potential. In contrast to pro-inflammatory priming the transcriptome of hypoxic primed MSCs revealed a downregulation of genes involved in immune system responses, corroborating findings from T cell suppression. MSCs cultured as spheroids exhibited substantial transcriptomic changes involving the cytoskeleton and cellular signaling. Furthermore, cell division was downregulated likely due to contact-inhibition, while apoptotic and angiogenic processes were upregulated. It is well established that MSCs cultured in self-aggregated spheroids form necrotic cores, due to limited nutrient availability at the core of spheroids. [45] Our small MSC spheroids (200 MSCs per spheroid, approximately 120 µm diameter) had few apoptotic cells and almost no necrotic cells (**supplementary Fig. 2**). Yet, upregulation of angiogenic processes could be a possible reaction to the oxygen and nutrient deprived spheroid core.

To investigate if differently primed MSCs engage distinct immunomodulatory mechanisms, we first identified DEGs involved in immunomodulation. DEGs that were either part of GO:0006955 (immune response) or GO:0006954 (inflammatory response) were considered immunomodulation-associated. The two selected gene ontologies represent high-level gene ontologies encompassing the majority of sub-ontologies involved in immunomodulation. The top 10 immunomodulation-associated DEGs were identified for each priming strategy separately (supplementary Table 2) and highlighted in volcano

plots (**Fig. 5**). 19% (49/253) of DEGs in pro-inflammatory primed MSCs were immunomodulation-associated. This fraction was lower in hypoxic (5%, 69/1525) and 3D culture (6%, 45/764) primed MSCs. In pro-inflammatory primed MSCs, all 10 immunomodulation-associated DEGs were upregulated. In hypoxic and 3D culture primed MSC, 7 were up- and 3 were down-regulated. None of the top 10 immunomodulation-related DEGs were shared between all three priming strategies and only two were shared between two priming strategies, suggesting that differently primed MSCs engage distinct immunomodulatory mechanisms. Prostaglandin-endoperoxide synthase 2 (*PTGS2)* was strongly upregulated in hypoxic primed and 3D culture primed MSCs. Major histocompatibility complex class II DR alpha *(HLA-DRA)* was upregulated in pro-inflammatory primed MSCs but downregulated in hypoxic primed MSCs. This aligns with the observations from the ORA where pro-inflammatory priming and hypoxic priming showed opposing immune system response-related transcriptional changes. The top 10 immunomodulation-associated DEG of pro-inflammatory primed MSC were mainly involved in immune cell trafficking, antigen presentation, and immune response activation. The immunomodulatory DEGs of hypoxic primed MSCs have diverse roles in inflammatory responses ranging from pathogen recognition and presentation to immune cell attraction and migration. Interestingly, several of the top 10 immunomodulation-associated DEGs of 3D culture primed MSCs are not only involved in the modulation of an immune response but also in cell differentiation.

In summary, PCA revealed distinct clustering of the different priming strategies. This indicates specific effects of the priming strategies on the transcriptome of MSCs. Different priming strategies activated separate biological processes as evidenced by the ORA. Most of the top 10 immunomodulation-associated DEGs were unique to the priming strategies. These findings suggest that differentially primed MSCs engage distinct immunomodulatory mechanisms.

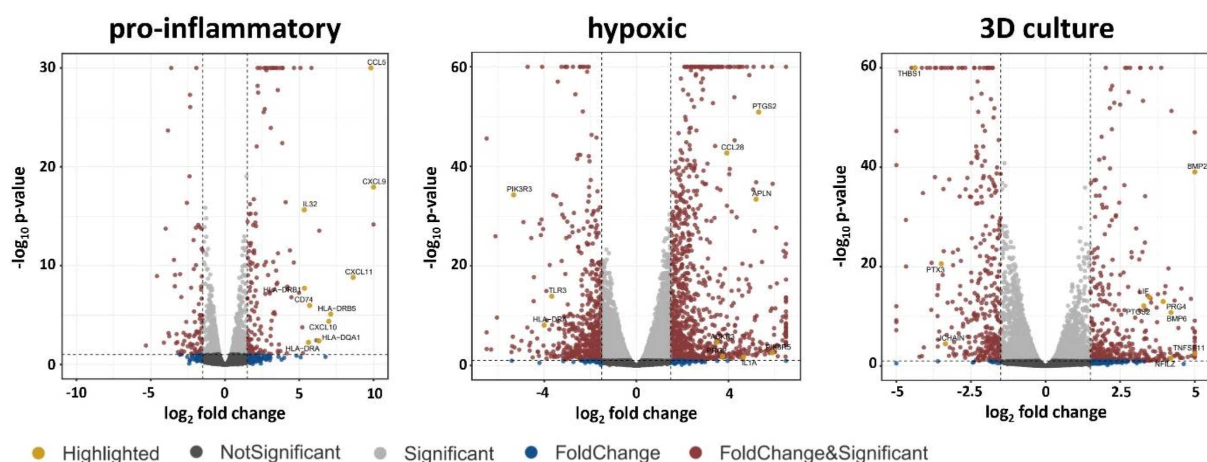


Fig. 5 *The volcano plots show the p-values and fold changes of the DEG analysis of primed versus unprimed MSCs. The horizontal dotted line represents the p-value threshold of 0.05 and the two vertical dotted lines represent the log_2FC thresholds of ± 1.5. The DEGs were ranked from highest $|log_2FC|$ values to lowest and the top 10 immune-response related DEGs were identified for each priming strategy separately. DEGs that were part of either GO:0006955 (immune response) or GO:0006954*

(inflammatory response) were considered immune-response related and the top 10 immune-response related DEGs were highlighted in the volcano plots.

The transcriptomic changes of primed MSCs fade rapidly but can be partially preserved by translationally relevant conditions

The expression levels of the immunomodulatory signature genes of pro-inflammatory, hypoxic, and 3D culture primed MSCs were analyzed at day 0, 4, and 8 to evaluate the temporal stability of different priming approaches and to study how translationally relevant microenvironmental factors influence the priming effects over time (**Fig. 6**). The pro-inflammatory priming effect was consistently strongest directly after priming at timepoint day 0. However, the expression levels of all signature genes rapidly faded, and reached expression levels comparable to those of unprimed MSCs. Fading of the pro-inflammatory priming effect was not significantly rescued by the continuous inflammatory stimulation but showed a trend towards improved temporal preservation. The expression levels of the immunomodulatory signature of hypoxic primed MSCs demonstrated moderate fold changes compared to unprimed MSCs. *PTGS2* and leptin (*LEP*) were notably upregulated directly following priming but rapidly faded under normoxic conditions. Physiological oxygen concentrations partially preserve the expression levels of multiple signature genes. Surprisingly, despite the significant differential expression of the 3D culture priming-specific signature genes in RNA sequencing, qPCR revealed weak differential expressions of these genes in 3D culture primed MSCs. Interestingly, when MSCs were continuously cultured in spheroids the gene expression levels of chemotactic factors and immunoregulatory secretory proteins further increased over time. This was not observed for MSCs that were dissociated after 3D culture priming and subsequently cultured as monolayers. We observed comparable outcomes for the frequently used priming strategy-independent immunomodulatory potency markers as shown in **supplementary Fig. 6**. In general, the priming effects rapidly faded over time, but they were partially preserved under translationally relevant conditions, in particular under continuous 3D culture.

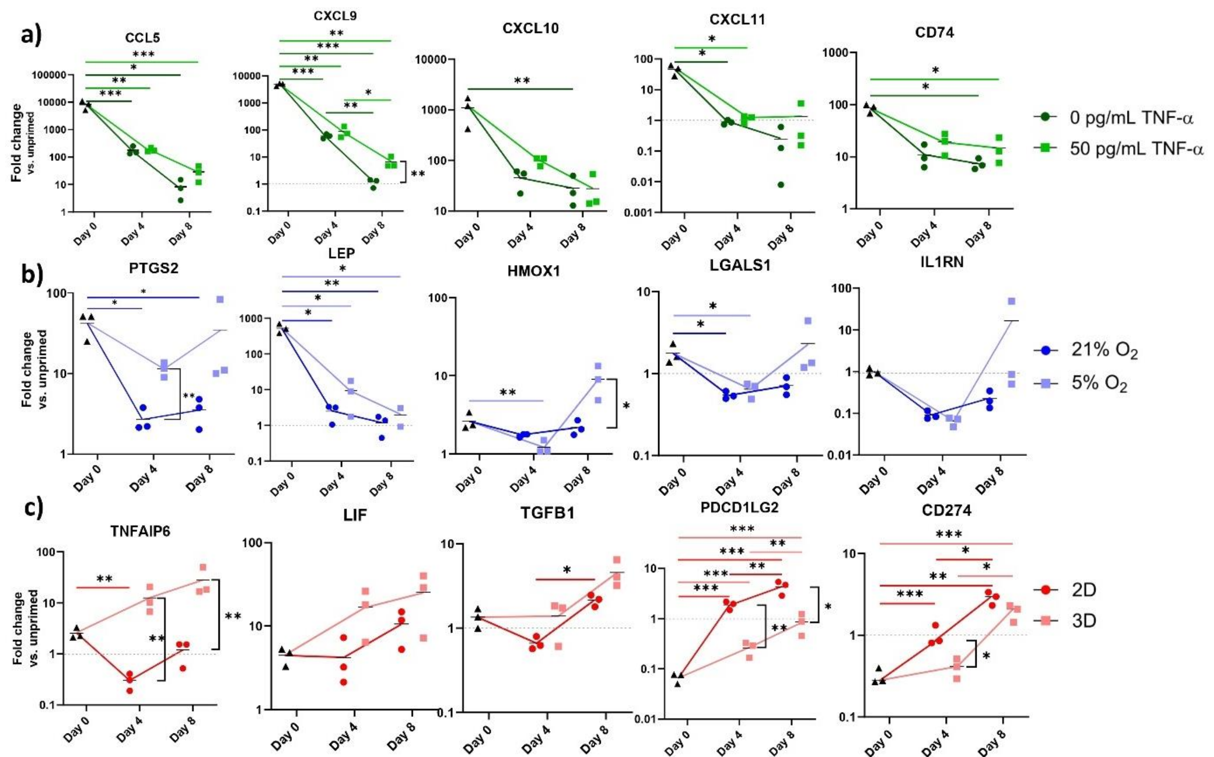


*Fig. 6 The effects of pro-inflammatory **a)**, hypoxic **b)**, and 3D culture priming **c)** on the transcriptome of MSCs were semi-quantified by qPCR at three time points: day 0 (directly following 48 h of priming), day 4, and day 8. Fold changes are shown and represent the gene expression levels of primed MSCs compared to the gene expression levels of unprimed MSCs at time point day 0. The significance levels for the gene expression differences between each time point and condition, compared to the gene expression directly after priming at day 0 are shown. Additionally, for each time point separately, significant gene expression differences between primed MSCs cultured under standard conditions and primed MSCs cultured under translationally relevant conditions are highlighted by significance indicators (not significant: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).*

The rapid fading of priming effects extended beyond the transcriptomic level, as evidenced by declining CXCL9 concentrations in the supernatants of pro-inflammatory primed MSC cultures (Fig. 7). Furthermore, the concentrations of PGE_2 and kynurenine – indicative of IDO1 activity – mirrored the gene expression patterns.

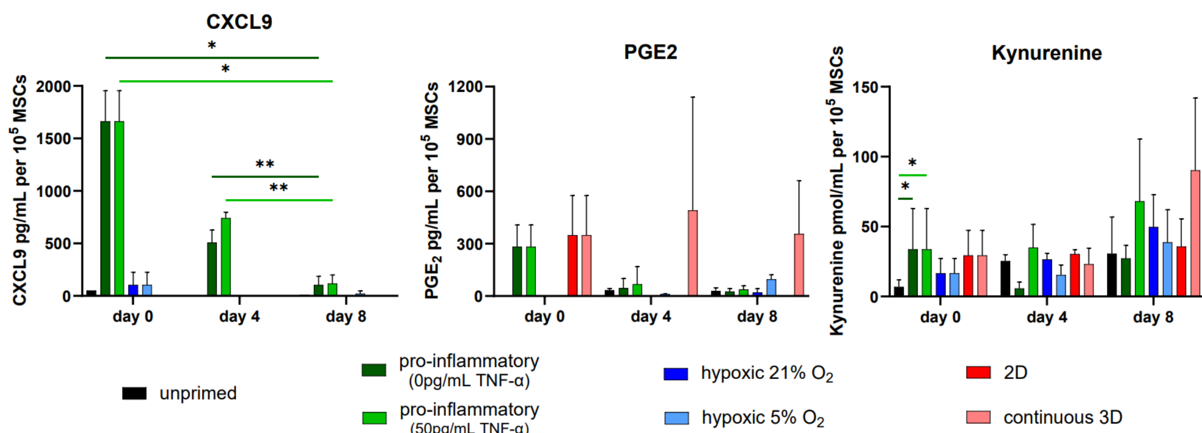


*Fig. 7 The effects of different priming strategies and culture conditions on the temporal stability of selected proteins in the supernatants of MSC cultures. Proteins were quantified in the supernatants of MSC cultures using ELISA. Supernatants were collected 48 h of culture with MSCs at day 0, day 4, and day 8. Media changes were performed at days 2 and 6. Not significant: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$*

The T cell suppressive mechanism of 3D culture primed MSCs is not reflected by the immunomodulatory potency markers

To identify potentially different T cell suppressive mechanisms, the expression levels of all signature genes (5 pro-inflammatory, 5 hypoxic, 5 3D culture, and 5 literature-based genes) were correlated with the corresponding T cell suppressive potentials. Fourteen genes had a correlation coefficient $|r| \geq 0.5$ (Table 1). 11 of them correlated with the pro-inflammatory priming effect, suggesting that the T cell suppression of pro-inflammatory primed MSCs is mediated by the signature genes. Only one gene correlated with T cell suppression of 3D culture primed MSC. This indicates that the T cell suppressive mechanism of 3D culture primed MSCs involves genes and mechanisms different from the gene ontologies GO:0006955 (immune response) and GO:0006954 (inflammatory response).

Table 1 Correlation for each priming strategy separately

gene	r	p-value	p/h/3D
CD74	-0.93	<0.001	p
CXCL9	-0.90	<0.001	p
IDO1	-0.88	<0.001	p
ICAM1	-0.87	<0.001	p
CXCL11	-0.86	<0.001	p
CCL5	-0.86	<0.001	p
TNFAIP6	-0.80	<0.001	p
IL1RN	-0.80	<0.001	p
CXCL10	-0.80	<0.001	p
CXCL8	-0.77	<0.001	p
PDCD1LG2	+0.72	0.002	3D
LGALS1	-0.59	0.02	p
CCL2	-0.57	0.03	h
PDCD1LG2	-0.51	0.05	h

Sorted by descending |r|. CCL5 (C-C motif chemokine ligand 5), CXCL9 (C-X-C motif chemokine ligand 9), CXCL10 (C-X-C motif chemokine ligand 10), CXCL11 (C-X-C motif chemokine ligand 11), CXCL8 (C-X-C motif chemokine ligand 8), CD74 (major histocompatibility complex class II invariant chain), IL1RN (interleukin 1 receptor antagonist), PDCD1LG2 (programmed cell death 1 ligand 2), IDO1 (indoleamine 2,3-diogygenase 1), CCL2 (C-C motif chemokine ligand 2), TNFAIP6 (TNF alpha induced protein 6), LGALS1 (galectin 1), ICAM1 (intercellular adhesion molecule 1). p: pro-inflammatory priming, h: hypoxic priming, 3D: 3D culture priming

4.5 Discussion

Priming holds great potential in overcoming clinical challenges faced by MSC therapies. Nevertheless, the absence of standardized priming protocols along with a limited understanding of the cellular adaptations to priming represent serious translational hurdles. A clear understanding of the effects of different priming strategies on MSC function is key for the selection of an appropriate priming approach. Importantly, the influence of translationally relevant in vivo environments on the preservation of priming effects likely impacts treatment efficacy and hence, should be carefully considered. Therefore, the aims of our study were i) to evaluate if pro-inflammatory, hypoxic, and 3D culture priming can enhance the in vitro functional immunosuppressive potency of MSCs, ii) to assess

if the in vitro functional immunosuppressive potencies of differently primed MSCs are temporally preserved under standard and translationally relevant culture conditions, and iii) to clarify if the three priming strategies engage the same immunosuppressive mechanisms.

First, pro-inflammatory and 3D culture priming significantly enhanced the in vitro T cell suppressive properties of MSCs. Conversely, hypoxic priming led to a notable loss of the inherent T cell suppressive capacity of unprimed MSCs. Therefore, our data showed that different priming strategies have distinct effects on the immunosuppressive potentials of MSCs. Interestingly, a previous study found that hypoxic priming improves the in vitro immunosuppressive capacity of MSCs. [9] A possible explanation for these contradictory results could be the differences in MSC tissue source and passage number as well as differences in the in vitro immunosuppressive potency assay setup. This highlights the need for a standardized approach to assess MSC potency. Efforts towards standardized in vitro potency assays are ongoing but final assessments of the correlation between the in vitro measured immunosuppressive capacity and in vivo clinical efficacy are pending. [17] We measured the functional immunosuppressive potency of MSCs based on their ability to suppress the proliferation of activated T cells. This approach is widely used but only captures a fraction of the immunomodulatory capacity of MSCs, as it was shown that MSCs have the ability to modulate a wide range of immune cells including macrophages, B cells, T cells, natural killer cells, mast cells, neutrophils, dendritic cells, and likely additional cell types. [15,16] While, assays to measure the immunomodulatory potential of MSCs on cell types other than T cells have been reported in literature, few publications provide detailed assay protocols. [42,46] Additionally, the pivotal role of T cells in various chronic inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis is undisputed. [47–49] Therefore, we focused on the frequently assessed ability of MSCs to suppress the proliferation of activated T cells.

Second, priming effects fade over time but are partially rescued from fading by translationally relevant culture conditions. To consider a priming strategy for clinical applications, MSCs must preserve the priming effect after administration to exert their enhanced therapeutic functions. Only few studies investigated the temporal stability of priming effects and discussed the relevance of temporal stability for clinical translation. [2,50] Pro-inflammatory, hypoxic, and 3D culture priming induced transient cellular adaptations under standard cell culture conditions, as shown by monitoring priming signature genes over time. All priming approaches induced substantial transcriptomic changes in MSCs directly following priming, but these changes rapidly faded only a few days after the priming. The rapid fading of the priming effects extended beyond the transcriptomic level, as evidenced by declining CXCL9 concentrations in the supernatants of pro-inflammatory primed MSC cultures. The fading of the priming effects was accompanied by the loss of enhanced in vitro functional immunosuppressive

potentials of both pro-inflammatory and 3D culture primed MSCs. This signifies that MSC therapies utilizing pro-inflammatory or 3D culture priming to enhance the immunosuppressive potency of MSCs should minimize the time between priming and injection to maximize their therapeutic effects.

It is well established that MSCs have a cellular plasticity, and that their functions are tightly regulated by their surrounding microenvironment. [18] However, the influence of translationally relevant factors such as inflammation, hypoxia, and 3D tissue environments on the preservation of priming effects is notably understudied. As microenvironmental factors might alter priming effects and thereby affect MSC potency, we investigated the effects of translationally relevant in vivo conditions on the temporal preservation of priming effects. To maintain the changes introduced by priming and to avoid superimposing other immunosuppressive mechanisms, the translationally relevant culture conditions were aligned with the individual priming strategies. Hence, pro-inflammatory primed MSCs were exposed to pathologic inflammatory TNF-α levels, hypoxic primed MSCs were cultured under physiological O_2 concentrations, and 3D culture primed MSCs were continuously cultured as spheroids to simulate the translational scenario of spheroid injection. Our data show that a weak continuous inflammatory stimulation of 50 pg/mL TNF-α was sufficient to demonstrate a trend towards decelerating the fading of the pro-inflammatory priming effect, both on the transcriptomic and functional levels. We selected 50 pg/mL TNF-α for the continuous inflammatory stimulation based on the lower limit of reported TNF-α concentrations in the synovial fluid of patients with rheumatoid arthritis. [27,28] Higher concentrations of TNF-α or the combination with IFN-γ may have preserved the pro-inflammatory priming effect even better. Nevertheless, TNF-α concentrations as low as 50 pg/mL demonstrated a promising trend towards partially preserving pro-inflammatory priming effects. This suggests that the administration of pro-inflammatory primed MSCs into an inflamed target tissue could preserve the priming effect and enhanced therapeutic potency. Priming MSCs for 48 h at 1% O_2 induced substantial transcriptomic adaptations to the low O_2 levels, including a reduced responsiveness to oxidative stress. Therefore, hypoxic priming may prepare MSCs for O_2 partial pressures present in frequently targeted tissues, consequently improving cell survival post-administration. [29,30] Our study shows that the exposure of hypoxic primed MSCs to a translationally relevant O_2 concentration better preserves the expression of signature genes compared to hypoxic primed MSCs exposed to an atmospheric O_2 concentration. It could be hypothesized that hypoxic priming pre-administration might be irrelevant, as unprimed MSCs are likely to undergo hypoxic priming following administration into the hypoxic tissue. Further research is needed to conclusively determine whether the in vitro adaptation to hypoxic conditions is necessary to improve cell survival post-administration. Few days following 3D culture priming, MSCs cultured in monolayers lost their significantly enhanced immunosuppressive capacity. In contrast, when MSCs were continuously

cultured in spheroids the gene expression levels of chemotactic factors and immunoregulatory secretory proteins further increased over time and fully preserved the enhanced T cell suppressive potential. The signature genes of pro-inflammatory and hypoxic primed MSCs experienced temporal fading, even under translationally relevant conditions. The continuous exposure of MSCs to high pro-inflammatory cytokine concentrations or low oxygen concentrations, as used during priming, would not have reflected translationally relevant conditions, and would potentially have affected cell viability. Complete preservation of the priming effect was a unique property of 3D culture priming. Maintaining MSCs in a continuous 3D culture could act as a functionalized formulation, supporting the administration of MSC spheroids for a sustainably improved immunosuppressive potency, independent of microenvironmental factors necessary to preserve the priming effect.

Third, different priming strategies engage distinct immunosuppressive mechanisms. We observed significant differences in the T cell suppressive potential of differently primed MSCs. While pro-inflammatory and 3D culture priming significantly enhanced the T cell suppressive potential of MSCs, hypoxic primed MSCs had no beneficial effect. These findings can be partially explained by the distinct transcriptomes of the differently primed MSCs. Pro-inflammatory priming clearly led to an upregulation of genes involved in immune system responses.

Hypoxic priming mainly led to adaptations to an anaerobic metabolism and increased the cellular migration potential. In contrast to pro-inflammatory priming, DEGs involved in immune system responses were clearly downregulated in hypoxic primed MSCs and showed an opposing immunomodulation-associated DEGs expression pattern. This aligned with the outcomes of the in vitro immunosuppressive potency measurements. According to literature, cell survival after in vivo administration might benefit from hypoxic priming. [7] Indeed, the transcriptome of hypoxic primed MSCs revealed a reduced response to oxidative stress, which might improve cell survival post-administration.

While pro-inflammatory and 3D culture primed MSCs significantly enhanced in vitro T cell suppressive capacity, albeit not to the same extent, they likely operate through separate immunosuppressive mechanisms. The transcriptomic changes induced by 3D culture priming were substantially different from those caused by pro-inflammatory priming. 3D culture primed MSCs mainly experienced changes to the cytoskeleton, cellular signaling, and the cell cycle. Our RNA sequencing data revealed that none of the top 10 immunomodulation-associated DEGs was shared between 3D culture primed and pro-inflammatory primed MSCs, indicating the activation of separate immunomodulatory mechanisms. Importantly, while the expression levels of multiple signature genes showed strong correlations with the T cell suppressive capacity of pro-inflammatory primed MSCs, the expression levels of the same signature genes demonstrated no significant correlation with the T cell suppressive potential of 3D

culture primed MSCs. Surprisingly, 3D culture primed MSCs demonstrated a strong positive correlation between the gene expression levels of *PDCD1LG2* and the proliferation index, suggesting that 3D culture primed MSCs with lower *PDCD1LG2* expression levels are likely to have a higher immunosuppressive capacity. This finding was unexpected as the binding of PD-L2 (encoded by the *PDCD1LG2* gene) to programmed cell death protein 1 (PD-1) expressed on T cells usually leads to a negative regulation of T cell proliferation. [51] A possible explanation for this unexpected finding could be that the gene expression level of *PDCD1LG2* is downregulated by PD-L2-independent immunosuppressive mechanisms or that binding of PD-L2 to PD-1 induces a negative feedback loop in MSCs i.e. downregulation of *PDCD1LG2*.

ORA showed an upregulation of the SMAD protein signal transduction in 3D culture primed MSCs, which is typically initiated by the binding of transforming growth factor-beta (TGF-β) ligands to their respective receptors. Bone morphogenic proteins (BMPs) – members of the TGF-β superfamily – were shown to inhibit T cell activation and promote macrophage polarization towards an inflammation-resolving phenotype. [52–55] *BMP2* and *BMP6* were highly upregulated in 3D culture primed MSCs and among the top 10 immunomodulation-associated DEGs (supplementary Table 2). This suggests a potential role of the TGF-β/SMAD signaling pathway in the T cell suppressive mechanism of 3D culture primed MSCs. A previous study suggested the involvement of a specific anti-inflammatory mechanism in 3D culture primed MSCs. The proposed mechanism operates at the post-transcriptional level by destabilizing the mRNAs encoding pro-inflammatory cytokines. [25] However, further studies are needed to unravel the immunomodulatory mechanisms of 3D culture primed MSCs.

Moreover, we found an enhanced differentiation potential of 3D culture primed MSCs on transcriptomic level. Angiogenesis (GO:0001525, GO:0045766), cartilage development (GO:0051216), and osteoblast differentiation (GO:0001649) were significantly enriched biological processes in 3D culture primed MSCs. This is in agreement with previous studies[11,41,56–58]. Therefore, 3D culture priming might be an interesting tool to simultaneously enhance the immunomodulatory and regenerative potential of MSCs.

In summary, we showed that three MSC priming strategies to increase the immunomodulatory potential result in different immunosuppressive potencies: pro-inflammatory priming induced an almost complete T cell suppression in vitro, 3D culture priming around 40%, and hypoxic priming had no effect. However, priming effects were transient and rapidly lost, yet continuous 3D culture was able to maintain the immunosuppressive potential. The cellular mechanisms leading to T cell suppression were different in pro-inflammatory and 3D culture priming. Pro-inflammatory priming activated inflammatory and immune mechanisms, 3D culture priming induced mechanisms involving the cytoskeleton, cellular signaling, and the cell cycle.

This study had several limitations. The experimental setup of our in vitro functional immunosuppressive potency assay had two major limitations. First, MSCs were co-cultured with PBMCs isolated from a single donor. The activation of T cells via a mixed lymphocyte reaction using PBMCs from multiple donors could have ensured that the assay results reflected more accurately the effects on a representative population. Second, MSC spheroids had to be dissociated prior to the immunosuppressive potency measurements. No suitable in vitro functional assay to measure the immunosuppressive potency of MSC spheroids exists. Therefore, we had to enzymatically digest the spheroids prior to the potency measurements. We decided to strictly adhere to a single in vitro potency assay to ensure comparability of the immunosuppressive potential measurements between the different priming strategies.
The selection of signature genes followed strict criteria including a comparison of potential signature genes with published immunomodulatory potency markers of MSCs. The majority of published immunomodulatory potency markers were identified in studies using pro-inflammatory priming. [8,34,42] This possibly introduced a bias into the selection of our signature genes, as we favored the selection of immunomodulatory signature genes that are mainly relevant for pro-inflammatory primed MSCs. This would explain the large number of signature genes that demonstrated a very strong correlation with the T cell suppressive capacity of pro-inflammatory primed MSCs and might explain the absence of signature genes that demonstrated relevant correlations with the T cell suppressive capacity of 3D culture primed MSCs. Nevertheless, it shows that 3D culture priming engages a different mechanism. The assessed signature genes might not reflect the immunosuppressive mechanism of 3D culture primed MSCs, as 3D culture is an emerging priming strategy, and only few potential immunomodulatory potency markers have been identified. [12,42,59]. The top 10 immunomodulation-associated DEGs (supplementary Table 2), could serve as unbiased potential immunomodulatory potency markers to bypass the selection bias of immunomodulatory signature genes.

In conclusion, we demonstrated that priming can be used to improve the immunosuppressive potency of MSCs and that translationally relevant in vivo conditions impact the preservation of priming effects. Continuous 3D culture could act as a functionalized formulation, supporting the administration of MSC spheroids for a sustainably improved immunosuppressive potency. Further studies are needed to evaluate the efficacy of 3D culture primed MSCs in pre-clinical and clinical settings.

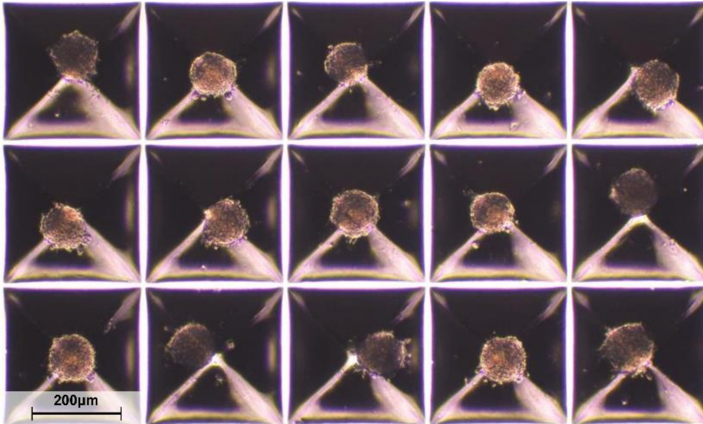
4.6 Abbreviations

3D	three dimensional
BMP	bone morphogenic protein
BP	biological processes
CCL2	C-C motif chemokine ligand 2
CCL5	C-C motif chemokine ligand 5
CD274	programmed cell death 1 ligand 1
CD74	major histocompatibility complex class II invariant chain
cDNA	complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CO_2	carbon dioxide
CXCL10	C-X-C motif chemokine ligand 10
CXCL11	C-X-C motif chemokine ligand 11
CXCL8	C-X-C motif chemokine ligand 8
CXCL9	C-X-C motif chemokine ligand 9
DEGs	differentially expressed genes
ELISA	Enzyme-linked immunosorbent assay
FDR	false discovery rate
GO	gene ontology
HLA-DRA	major histocompatibility complex class II DR alpha
HMOX1	heme oxygenase 1
ICAM1	intercellular adhesion molecule 1
IDO1	indoleamine 2,3-diogygenase 1
IFN-γ	interferon gamma
IL1RN	interleukin 1 receptor antagonist
IL6	interleukin 6
ISCT	International Society of Cell and Gene Therapy
LEP	leptin
LGALS1	galectin 1
LIF	leukemia inhibitory factor
mRNA	messenger ribonucleic acid
MSCs	mesenchymal stromal cells
O_2	oxygen
ORA	over-representation analysis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCA	principle component analysis
PD-1	programmed cell death protein 1
PDCD1LG2	programmed cell death 1 ligand 2 (gene)
PD-L2	programmed cell death 1 ligand 2 (protein)
PTGS2	prostaglandin-endoperoxide synthase 2
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
TBP	TATA-box binding protein
TGFB1	transforming growth factor beta 1
TNFAIP6	TNF alpha induced protein 6
TNF-α	tumor necrosis factor alpha

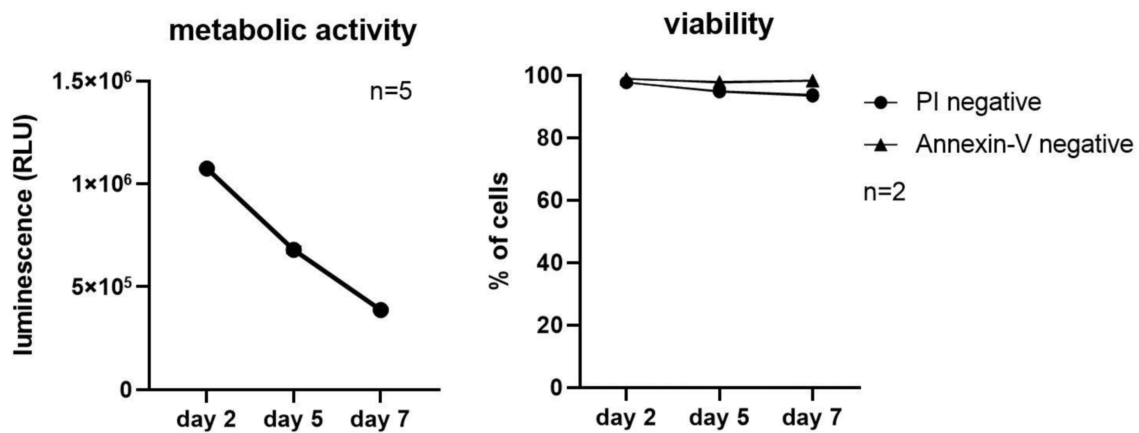
4.7 Supplementary Information

Supplementary Table 1 MSC donor information

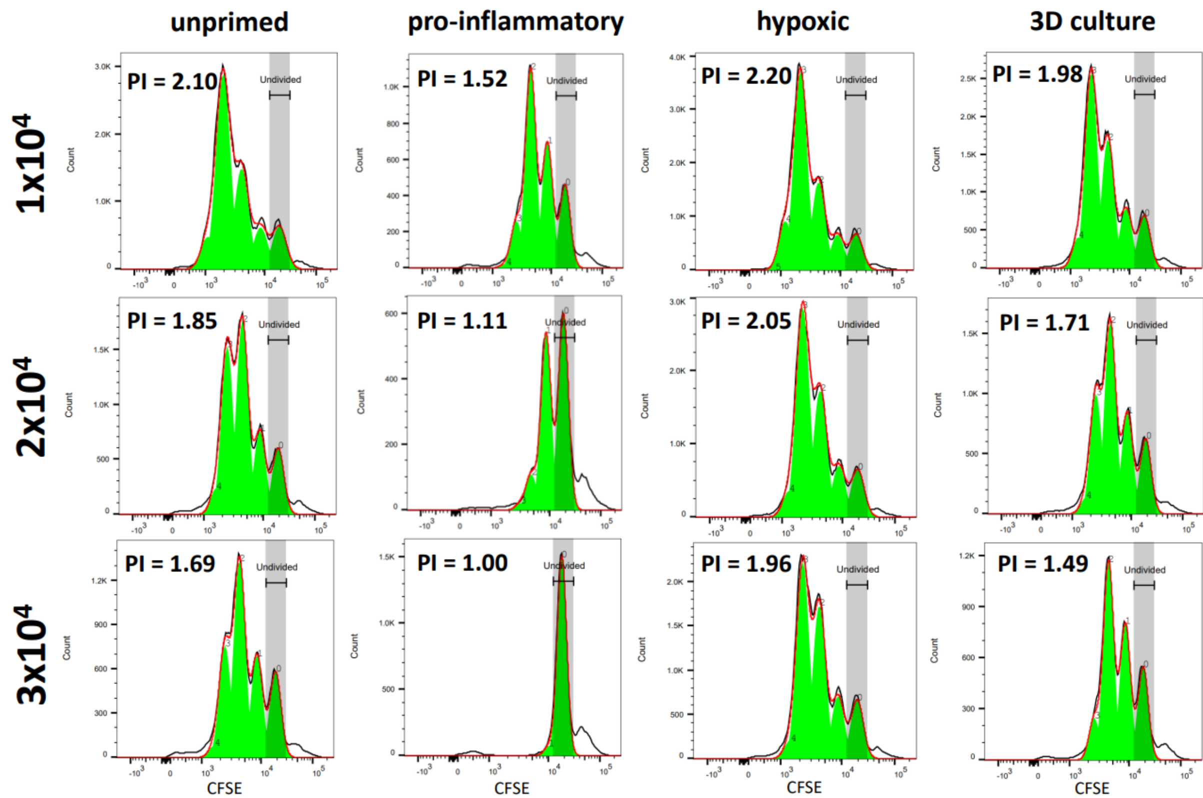
	Age	Sex
Donor 1	20	f
Donor 2	19	m
Donor 3	25	m



***Supplementary Fig. 1** Representative image of 3D culture primed MSCs in Kugelmeiers SP5D at 48 h post-seeding.*



***Supplementary Fig. 2** Metabolic activity and viability of MSCs cultured as spheroids over one week.*



Supplementary Fig. 3 *Identification of an optimal MSC:PBMC ratio for the in vitro functional potency assay. 1 x 10^4, 2 x 10^4, or 3 x 10^4 primed or unprimed MSCs were co-cultured with 10^5 CFSE-PBMCs. Their T cell suppressive potencies were assessed directly following priming. MSCs from donor 1 (supplementary Table 1) were used for this titration experiment, and co-cultured with CFSE-PBMCs isolated from an additional healthy donor. PI = proliferation index*

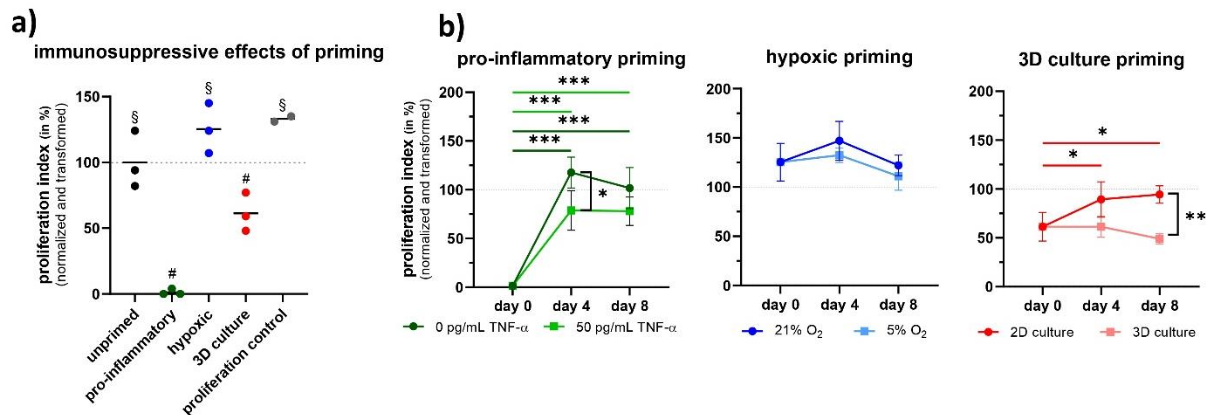
Supplementary Table 2 Top 10 immunomodulation-associated DEGs

pro-inflammatory			hypoxic			3D culture		
gene	**log_2FC**	**FDR**	**gene**	**log_2FC**	**FDR**	**gene**	**log_2FC**	**FDR**
CXCL9	13.92	1.13E-18	*PIK3R5*	5.91	2.76E-3	*TNFSF11*	6.37	3.32E-3
CCL5	9.82	3.49E-60	*PTGS2*	5.31	1.23E-51	*BMP2*	5.56	9.41E-40
CXCL11	8.63	1.51E-9	*APLN*	5.20	4.03E-34	*BMP6*	4.20	1.75E-11
HLA-DRB5	7.12	8.08E-6	*IL1A*	4.65	1.86E-2	*NFILZ*	4.20	2.64E-2
CXCL10	6.99	4.34E-5	*CCL28*	3.93	2.01E43	*PRG4*	3.94	1.09E-13
HLA-DQA1	6.32	4.07E-3	*PRG2*	3.74	1.41E-2	*LIF*	3.48	1.46E-14
CD74	5.70	1.06E-6	*ACKR3*	3.50	2.22E-5	*PTGS2*	3.29	8.12E-13
HLA-DRA	5.62	5.79E-3	*PIK3R3*	-5.33	5.41E-35	*THBS1*	-4.37	<1E-99
HLA-DRB1	5.34	1.93E-8	*HLA-DRA*	-4.00	7.99E-9	*PTX3*	-3.49	2.72E-21
IL-32	5.34	2.24E-16	*TLR3*	-3.67	1.22E-14	*JCHAIN*	-3.36	3.49E-5

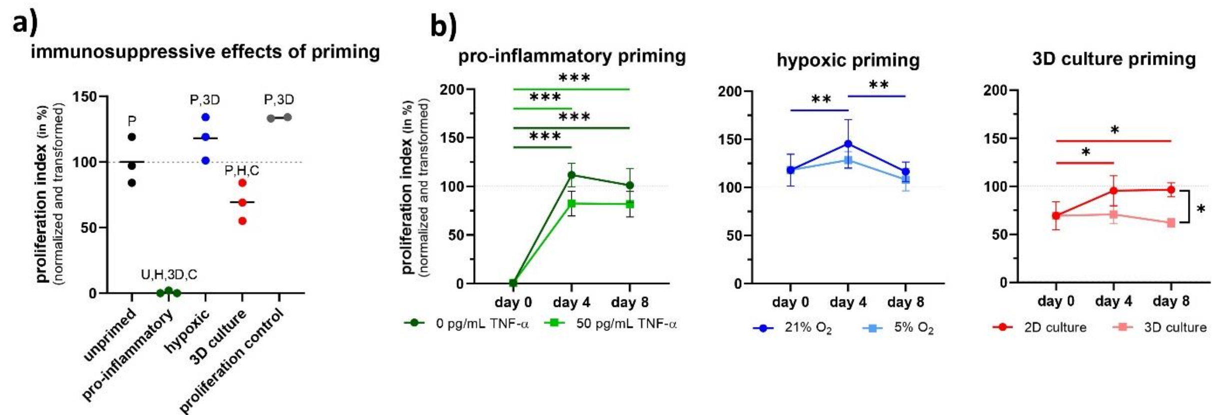
Supplementary Table 3 Primers used for qPCR amplification

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
CCL5 (RANTES)	CCTGCTGCTTTGCCTACATTGC	ACACACTTGGCGGTTCTTTCGG
CXCL9 (MIG)	CCAGTAGTGAGAAAGGGTCGC	AGGGCTTGGGGCAAATTGTT
CXCL10 (IP10)	GGTGAGAAGAGATGTCTGAATCC	GTCCATCCTTGGAAGCACTGCA
CXCL11 (I-TAC)	AAGGACAACGATGCCTAAATCCC	CAGATGCCCTTTTCCAGGACTTC
CXCL8 (IL8)	GAGAGTGATTGAGAGTGGACCAC	CACAACCCTCTGCACCCAGTTT
IL6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
CD74 (HLA-DR)	GATGACCAGCGCGACCTTATC	GTGACTGTCAGTTTGTCCAGC
PTGS2 (COX-2)	CGGTGAAACTCTGGCTAGACAG	GCAAACCGTAGATGCTCAGGGA
LIF	CCAACGTGACGGACTTCCC	TACACGACTATGCGGTACAGC
IL1RN	CATTGAGCCTCATGCTCTGTT	CGCTGTCTGAGCGGATGAA
CD274 (PD-L1)	TGCCGACTACAAGCGAATTACTG	CTGCTTGTCCAGATGACTTCGG
PDCD1LG2 (PD-L2)	ATTGCAGCTTCACCAGATAGC	AAAGTTGCATTCCAGGGTCAC
IDO1	TCATCTCACAGACCACAAGTCA	GCAAGACCTTACGGACATCTCC
CCL2 (MCP1)	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
TNFAIP6 (TSG6)	TCACCTACGCAGAAGCTAAGGC	TCCAACTCTGCCCTTAGCCATC
LEP	GCTGTGCCCATCCAAAAAGTCC	CCCAGGAATGAAGTCCAAACCG
HMOX1	CCAGGCAGAGAATGCTGAGTTC	AAGACTGGGCTCTCCTTGTTGC
LGALS1	TCGCCAGCAACCTGAATCTC	GCACGAAGCTCTTAGCGTCA
TGFB1	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA
ICAM1	AGCGGCTGACGTGTGCAGTAAT	TCTGAGACCTCTGGCTTCGTCA
TBP	TGTATCCACAGTGAATCTTGGTTG	GGTTCGTGGCTCTCTTATCCTC

CCL5 (C-C motif chemokine ligand 5), CXCL9 (C-X-C motif chemokine ligand 9), CXCL10 (C-X-C motif chemokine ligand 10), CXCL11 (C-X-C motif chemokine ligand 11), CXCL8 (C-X-C motif chemokine ligand 8), IL6 (interleukin 6), CD74 (major histocompatibility complex class II invariant chain), PTGS2 (prostaglandin-endoperoxide synthase 2), LIF (leukemia inhibitory factor), IL1RN (interleukin 1 receptor antagonist), CD274 (programmed cell death 1 ligand 1), PDCD1LG2 (programmed cell death 1 ligand 2), IDO1 (indoleamine 2,3-diogygenase 1), CCL2 (C-C motif chemokine ligand 2), TNFAIP6 (TNF alpha induced protein 6), LEP (leptin), HMOX1 (heme oxygenase 1), LGALS1 (galectin 1), TGFB1 (transforming growth factor beta 1), ICAM1 (intercellular adhesion molecule 1), TBP (TATA-box binding protein)



***Supplementary Fig. 4** CD4⁺ T-helper cells: The effects of each priming strategy on the immunosuppressive potential of MSCs were measured by an in vitro functional potency assay following 48 h of priming. A normalized transformed proliferation index of 0% corresponds to the complete suppression of T cell proliferation. A value of 100% indicates that the T cells proliferated equivalent to T cells co-cultured with unprimed MSCs (grey dashed horizontal lines). **a)** Different priming strategies enhance the T cell suppressive capacity of MSCs. **b)** priming effects fade over time but are partially preserved by translationally relevant conditions. The proliferation control represents the proliferation of T cells in the absence of co-cultured MSCs. # indicate significant differences ($p < 0.001$, except unprimed vs. 3D culture primed $p = 0.03$) against all other conditions. § indicate significant differences ($p < 0.001$) against pro-inflammatory and 3D culture priming. not significant: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$*



Supplementary Fig. 5 *CD8⁺ cytotoxic T cells: The effects of each priming strategy on the immunosuppressive potential of MSCs were measured by an in vitro functional potency assay following 48 h of priming. A normalized transformed proliferation index of 0% corresponds to the complete suppression of T cell proliferation. A value of 100% indicates that the T cells proliferated equivalent to T cells co-cultured with unprimed MSCs (grey dashed horizontal lines).* ***a)*** *Different priming strategies enhance the T cell suppressive capacity of MSCs.* ***b)*** *priming effects fade over time but are partially preserved by translationally relevant conditions. The proliferation control represents the proliferation of T cells in the absence of co-cultured MSCs. Letters indicate significant differences (p < 0.001, except unprimed vs. 3D culture primed p = 0.03) between conditions. U = unprimed, P = pro-inflammatory, H = hypoxic, 3D = 3D culture, C = proliferation control. not significant: p > 0.05; *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001*

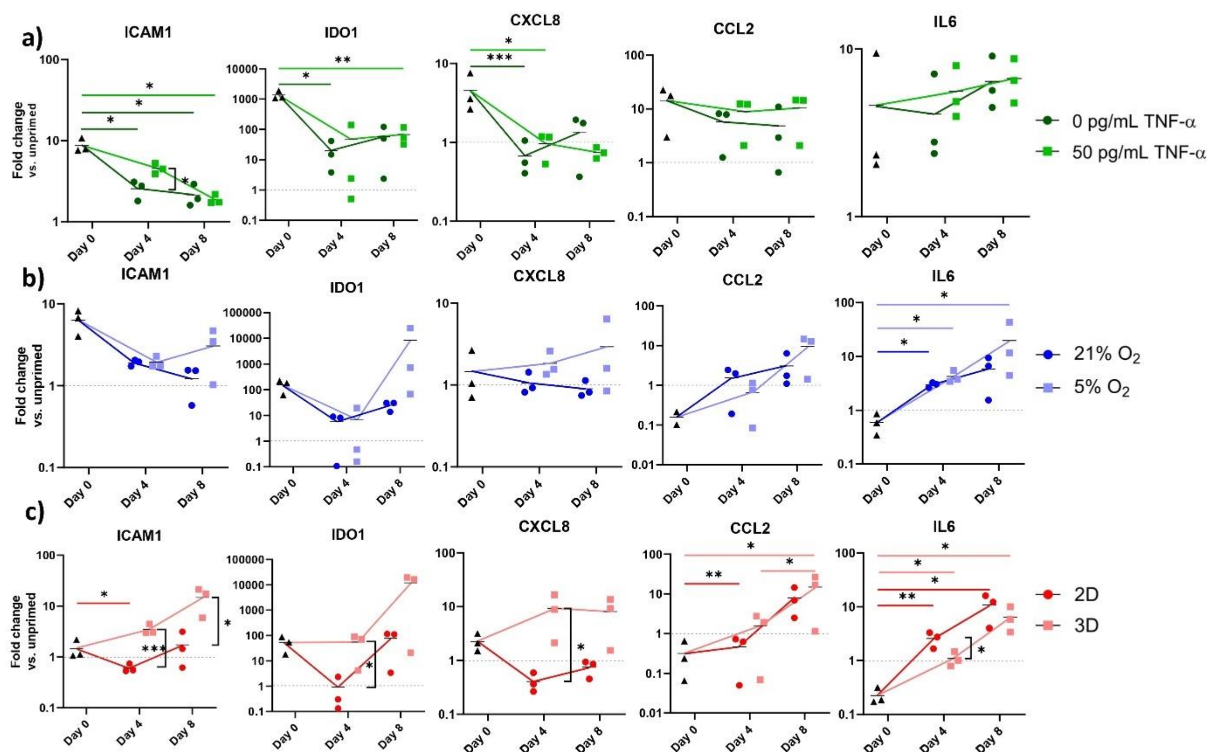
Supplementary Table 4 shared DEGs: pro-inflammatory and hypoxic primed MSCs				
ACSL5	CXCL2	IFIH1	PLCL1	SULF2
ADH1B	CXCL6	IFIT2	PLD6	SYTL3
ADRA2A	DDIT4	IL11	PPL	TENM2
ANGPT2	DDIT4L	IL15RA	PRSS35	TMEM150C
APOB	EFHC2	JCHAIN	PSMB9	TNFSF13B
APOE	EGR3	KCND2	PWWP2B	TP63
APOL1	EPSTI1	KCNK15	QPCT	TRIM14
APOL3	ESM1	LRP4	RCOR2	TSPAN11
ARMCX5-GPRASP2	ETV7	MAF	REPS2	WARS1
ASPN	FGF7	MGLL	RIPOR3	XAF1
BDKRB1	FMO2	MGP	RPP25	ZCCHC2
BST2	FMO3	MX2	RTN4RL1	
C15orf39	GJD3	NCKAP5	SELENOP	
C15orf48	GPR88	NPIPB13	SEMA6B	
CD74	H1-0	NTN1	SEMA6D	
CFB	H4C14	OAS2	SERPINB2	
CFI	HDAC9	OASL	SFRP4	
CHI3L1	HERC6	OGN	SLC15A3	
CIITA	HHIP	OMD	SLC40A1	
CLCA2	HLA-DRA	OSCAR	SLC44A3	
CLDN1	IBSP	PDE1A	SMAD9	
COL14A1	ICAM1	PDE2A	SNED1	
COL21A1	ICAM5	PDE5A	ST8SIA2	
CXCL1	IFI35	PDGFD	STAT1	
CXCL12	IFI44L	PDGFRL	STC2	

Supplementary Table 5 shared DEGs: pro-inflammatory and 3D culture primed MSCs				
ADH1B	CMPK2	IL11	NTN1	SELENOP
ADRA2A	CTAGE4	IL1B	OAS1	SEMA6B
AMIGO2	CXCL6	IL4I1	ODF3B	SERPINB2
ANKRD1	DCLK1	IL6	OGN	SFRP4
APCDD1	DDIT4L	JCHAIN	OMD	SLC40A1
APOB	DLX2	KCND2	PDCD1LG2	SYTL2
APOE	FGF7	KCNK15	PDE2A	SYTL3
APOL1	FMO2	LEPR	PDE5A	TENM2
ASPN	FMO3	LIF	PLCL1	TFPI2
BDKRB1	GDF15	MGP	QRICH2	TMEM132A
C15orf48	GPC4	NDNF	RGS4	TSPAN11
CFI	HLA-DRB1	NPIPB13	RPP25	TYMP
CHI3L1	ICAM5	NPR3	RSAD2	

Supplementary Table 6 shared DEGs: hypoxic and 3D culture primed MSCs				
ABAT	CPE	GPR68	NEDD9	SERPINE1
ADCY8	CRLF1	GRAMD2B	NEXN	SFRP4
ADGRG1	CTXN1	GREB1	NGFR	SGMS2
ADH1B	CXCL6	GRIA3	NKAIN1	SHC3
ADM	CYGB	GYG2	NLGN1	SLC16A6
ADRA2A	DAAM2	H2AC18	NLRP10	SLC17A7
AKAP5	DAPK1	H2AC19	NMB	SLC22A23
ALPK2	DCHS1	H2BC4	NOTCH3	SLC22A3
ANGPT1	DDIT3	HAPLN1	NPAS1	SLC23A3
ANGPTL4	DDIT4L	HIC2	NPIPB13	SLC37A2
APOB	DMD	HMOX1	NPTX1	SLC40A1
APOE	DSG2	HS3ST2	NR4A2	SLC46A3
APOL1	E2F7	HSF4	NR4A3	SLC5A3
ARNT2	ECM2	ICAM5	NTN1	SLC6A15
ARRDC2	EFEMP1	IER3	NUAK2	SLC7A14
ARRDC4	ENPP5	IGFBP5	OGN	SMCO4
ASGR1	ENSG00000279117	IL11	OMD	SMIM43
ASPN	ENSG00000279118	INSYN2A	OSGIN1	STXBP6
ATP6V0D2	ENSG00000279773	ITGA2	P2RY11	SYNPO2
B3GALT2	ENSG00000279881	JAM2	PCSK1	SYT7
B4GALNT2	ENSG00000280138	JCHAIN	PDE2A	SYTL3
BAIAP2L1	ENSG00000280351	KCNC3	PDE4C	TC2N
BCL11B	ENSG00000284540	KCND2	PDE5A	TENM2
BCL2L11	ERMN	KCNK15	PGF	TENT5B
BDKRB1	ESCO2	KCNK2	PHLDA1	TGFB3
BMP2	ETV4	KCNS1	PLAAT4	THBD
C11orf87	FCGBP	KCTD4	PLCL1	THBS1
C12orf56	FER1L6	KDM7A	PLEKHG4B	TICRR
C15orf48	FGD4	KIRREL3	PLIN2	TLL1

C1orf115	FGF7	KLRD1	PLPP2	TLR3
C1orf198	FHIP1A	KRT19	PODN	TM4SF19
C4orf47	FILIP1L	KRTAP1-1	PPFIA3	TMEM240
CA9	FKBP5	KSR1	PSAT1	TMEM26
CAMK2N1	FMO2	KY	PTGES	TNFAIP6
CAPS	FMO3	LACC1	PTGS2	TNFRSF11B
CASP1	FMO4	LEF1	PTPRN	TPBGL
CCN2	FOXQ1	LMOD1	PTPRQ	TPD52L1
CD200	FRY	LOX	RANBP3L	TRIM9
CDKN2B	FZD5	LRRC66	RASEF	TSPAN11
CEMIP	FZD8	LSAMP	RCAN2	UAP1L1
CFI	GALNT18	MALL	RIMS1	VLDLR
CH25H	GBP1	MARCHF4	RNF144A	VSTM4
CHI3L1	GDF5	MCHR1	ROS1	WNK4
CHL1	GDF6	MCM10	RPP25	WNT7B
CLK1	GEM	MEGF6	SCRG1	ZNF423
CMKLR1	GFRA1	MEOX2	SELENOP	
COL24A1	GOLGA8T	MGP	SEMA6B	
CPA4	GPR35	MYBL1	SERPINB2	

Supplementary Table 7 shared DEGs: pro-inflammatory and hypoxic and 3D culture primed MSCs				
ADH1B	CFI	IL11	OMD	SFRP4
ADRA2A	CHI3L1	JCHAIN	PDE2A	SLC40A1
APOB	CXCL6	KCND2	PDE5A	SYTL3
APOE	DDIT4L	KCNK15	PLCL1	TENM2
APOL1	FGF7	MGP	RPP25	TSPAN11
ASPN	FMO2	NPIPB13	SELENOP	
BDKRB1	FMO3	NTN1	SEMA6B	
C15orf48	ICAM5	OGN	SERPINB2	



Supplementary Fig. 6 *Fading of frequently used immunomodulatory potency markers. The effects of each priming approach on the transcriptome of MSCs were semi-quantified by qPCR at three time points: day 0 (directly following 48 h of priming), day 4, and day 8. Fold changes are shown and represent the gene expression levels of primed MSCs compared to the gene expression levels of unprimed MSCs at time point day 0. The significance levels for the gene expression differences between each time point and condition, compared to the gene expression directly after priming at day 0 are shown. Additionally, for each time point separately, significant gene expression differences between primed MSCs cultured under standard conditions and primed MSCs cultured under translationally relevant conditions are highlighted by significance indicators (not significant: p > 0.05; *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001).*

4.8 Personal contribution

This chapter owes its existence to the collaborative efforts of my co-authors, and I extend my sincere gratitude for their significant contributions. Their insights not only enriched the content but also enabled a swift submission of this work to the Stem Cell Research and Therapy journal. The collaborative efforts of my co-authors including their suggestions on data visualization and analysis were crucial to effectively conveying our findings.

My responsibilities included guiding conceptualization, preparing the initial draft, and ensuring alignment of thoughts and ideas. I took the lead in writing the original manuscript and conducting the manuscript revision. Furthermore, I actively contributed to various aspects, including cell culture, qPCR, ELISA, in vitro functional potency measurements, and performing statistical analyses.

I want to acknowledge the pivotal role of the Functional Genomics Center Zurich in this project. Their contribution, spanning RNA sequencing and foundational statistical analysis, was essential for this chapter.

Working alongside such committed and skilled individuals has been a privilege, with each person making a meaningful mark on this chapter.

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Chapter 5
General Discussion

5.1 General discussion

This chapter will evaluate the relevance of our findings from **Chapters 3 and 4** in relation to our overarching aim to develop an allogeneic, anti-inflammatory bone marrow stromal cell therapy for chronic low back pain patients with Modic type 1 changes. Essential next steps to advance this project will be discussed in a subsequent outlook section (**Chapter 5.1.1**).

To progress toward our overall aim, it is fundamental to ascertain whether patients with MC1 represents a valid target population. The identification of targetable features of the MC1 pathobiology is essential for the development of a disease-modifying treatment. Furthermore, the appropriateness of utilizing MSCs in this context must be critically evaluated. Identifying an overlap between the therapeutic potential of MSCs, and targetable pathologic features of MC1, could provide a roadmap to progress our project.

Chronic LBP patients with MC1 represent a valid target population. The high prevalence of chronic LBP patients with MC1, coupled with the lack of a recognized treatment strategy, underscores the need for a targeted treatment. MC1 patients represents a homogeneous subpopulation within the heterogeneous disc degeneration population, with a characteristic painful discal and vertebral inflammation. [1] Inflammatory MC1 may represent a targetable source of pain, which might be treated with an anti-inflammatory treatment approach. Our thorough literature review gave us valuable insights into the current clinical trial landscape of interventional MC1 therapeutics. This allowed us to assess the efficacy and limitations of investigational interventions and to pinpoint the most promising treatment approaches. Various pharmaceutical interventions have been explored to treat MC1, but with largely inconclusive outcomes. We observed a similar trend in the efficacy of investigational therapeutics targeting the heterogeneous degenerative disc disease population. However, in certain degenerative disc disease patients, MSC therapies seemed to improve the outcomes. The positive therapeutic results were mainly attributed to the substantial anti-inflammatory properties of MSCs. [2,3] Surprisingly, the potential of intradiscal MSC therapy to treat patients with MC1 remained largely unexplored.

The therapeutic potential of MSCs in addressing MC1-related chronic LBP is deeply rooted in their substantial immunomodulatory capacity. Modulation of the underlying inflammation in MC1 lesions is pivotal for the effective treatment of MC1. Inflammation is involved in multiple pathological features of MC1 including “MC1 disc”/marrow cross-talk, discal innervation, the degeneration of the IVD, and the inflammatory bone resorption. While the pathobiology of MC1 is multifactorial and might suggest a multimodal therapeutic approach, targeting inflammation through the substantial immunomodulatory actions of MSCs represents a valid treatment approach. Therefore, we will

leverage the anti-inflammatory potential of MSCs to develop a therapy for chronic LBP patients with inflammatory MC1.

Investigational MSC therapies consistently failed to reach predefined efficacy endpoints in chronic LBP patients. [4] The multifactorial etiology of chronic LBP – which results in heterogeneous study populations and large inter-study variability – may be one reason why clinical trials failed to demonstrate consistent therapeutic efficacy. Another reason might be attributed to the lacking or inconsistent anti-inflammatory potencies of MSC preparations used in the clinical trials. While patient stratification into chronic LBP subpopulations with targetable pathologic features could improve treatment consistency, enhancing the potency of MSC preparations would require a separate approach. Priming is a widely used strategy to enhance the anti-inflammatory potency of MSCs. [5,6] Yet, the absence of standardized priming protocols, combined with a limited understanding of the cellular mechanisms underlying priming, limits its translational value. To assess whether priming is a valid approach to enhance the anti-inflammatory potential of our MSC therapy, we thoroughly investigated its translational potential.

We showed that different priming strategies induce distinct anti-inflammatory effects in MSC preparations: pro-inflammatory priming induced an almost complete T cell suppression in vitro, 3D culture priming around 40%, and hypoxic priming had no effect. An enhanced T cell suppressive potential of our MSC therapy is desirable, as the involvement of T cells in the pathobiology of MC1 is supported by solid evidence. [7–9] However, the degree to which T cells contribute to the pathogenesis of MC1 remains to be elucidated. The enhanced T cell suppressive potentials of primed MSCs were transient and rapidly lost, even under in vitro conditions mimicking specific aspects of a possible MSC recipient tissue. Yet continuous 3D culture was able to maintain the enhanced immunosuppressive potential. The cellular mechanisms leading to T cell suppression were different in pro-inflammatory and 3D culture priming, suggesting that differently primed MSCs engage distinct immunosuppressive mechanisms. Maintaining MSCs in a continuous 3D culture could act as a functionalized formulation, supporting the administration of MSC spheroids for a sustainably improved immunosuppressive potency, independent of microenvironmental factors necessary to preserve the priming effect. This study helped us to pinpoint translationally relevant priming strategies among the myriad of available strategies. We identified pro-inflammatory and 3D culture priming as promising strategies to improve the efficacy of our anti-inflammatory MSC therapy.

In summary, several insights from **Chapters 3 and 4** have played pivotal roles in advancing our MSC therapy. First, we identified that MC1 patients constitute a prevalent, homogeneous, and targetable subpopulation of chronic LBP patients. Their inflammatory bone marrow lesions present a potential

target for anti-inflammatory interventions. Second, MSCs are well-suited to suppress the painful inflammation seen in MC1 lesions, due to their favorable safety profile and exceptional anti-inflammatory properties. Finally, enhancing the anti-inflammatory potency of our MSC therapy may be crucial to achieve a beneficial therapeutic outcome. Pro-inflammatory and 3D culture priming can significantly improve the anti-inflammatory properties of MSCs in vitro, supporting the development of a highly anti-inflammatory therapy – using pro-inflammatory primed MSC spheroids – to treat chronic LBP patients with inflammatory MC1.

Consequently, our therapy will target MC1 patients, a homogeneous subpopulation of chronic LBP patients characterized by a painful inflammatory bone marrow lesion. Anti-inflammatory MSCs will be administered to suppress the vertebral inflammation. A combination of pro-inflammatory and 3D culture priming will ensure a high anti-inflammatory potency of our therapy. Furthermore, we will use allogeneic over autologous MSCs because i) allogeneic MSCs can be isolated, in vitro expanded, and biobanked, making them readily available off-the-shelf. This can substantially reduce treatment times. ii) Allogeneic MSCs can be expanded to produce a large quantity of MSCs, which can be used to treat multiple patients. Hence, patients do not require an invasive MSC harvesting procedure to be eligible for the therapy. iii) The quality of a dose of allogeneic MSCs from a single donor is equal across all biobanked doses. In contrast, the efficacy of an autologous MSC therapy partly depends on the quality of the patients own MSCs. Therefore, patient-specific MSC preparations may have inconsistent potencies and could contribute to inconsistent treatment outcomes. Importantly, elderly patients may disqualify from an autologous MSC therapy, as MSC potency was shown to be reduced in aged MSCs. [10] On the other hand, an allogeneic MSC therapy not only enables to treat patients who disqualify as MSC donors, but also allows for the selection of an optimal MSC donor. iv) Autologous MSC therapies are likely more expensive than allogeneic MSC therapies. This is mainly because allogeneic MSCs therapies do not require a laborious custom preparation for each patient. The main argument supporting the use of autologous over allogeneic MSCs is safety-related, but as discussed in **Chapter 1**, no allogenicity-related adverse events have been reported. [11–14]

A high anti-inflammatory potency of our MSC therapy is essential because i) highly potent MSCs are more likely to achieve the desired therapeutic outcome, by effectively exerting their intended function. ii) The administration of only a few highly potent MSCs might achieve the same therapeutic outcome as administering large quantities of MSCs. Reducing the number of cells needed to be administered may reduce the cost of the therapy, as less MSCs are needed per dose, more doses can be produced from a single MSC preparation. iii) A low number of cells is more likely to survive post-administration. This is because intradiscal injection is the most commonly used administration route in clinical trials on MSC-based treatments for LBP. [2] The microenvironment of degenerated IVDs is harsh with a limited

nutrient and oxygen availability. Therefore, degenerated IVDs can only support a limited number of MSCs. [15,16] This makes the administration of few potent MSCs more favorable than the administration of a large number of regular MSCs.

The achievements presented in this doctoral dissertation laid the foundation for the development of an allogeneic, anti-inflammatory bone marrow MSC therapy for chronic LBP patients with MC1.

5.2 Outlook

Priming, to enhance the anti-inflammatory potency of MSC preparations, might not be sufficient for our therapy. The enhancement of MSCs' anti-inflammatory potency upon priming depends on how well they can adapt to a priming strategy. If an MSC preparation does not adapt well to a priming strategy, the benefit of priming might be small. MSCs are a highly heterogeneous population of cells, composed of a mix of cells with high and low anti-inflammatory potentials. [17–19] Therefore, it is likely that both responder and non-responder MSCs to priming co-exist within a single MSC preparation. Importantly, this heterogeneity of MSC preparations could be leveraged to identify homogeneous populations of MSCs with a high baseline anti-inflammatory potential, which are also likely to show enhanced anti-inflammatory potency upon priming. Therefore, the identification and isolation of MSCs with a high anti-inflammatory potential within heterogeneous MSC preparations, could enable the production of homogeneous MSC preparations exclusively composed of highly potent MSCs. The characterization of highly anti-inflammatory MSC subtypes – responsible for the anti-inflammatory potency of MSC preparations – could advance the development of standardized anti-inflammatory MSC therapeutics. Utilizing anti-inflammatory MSC subtype preparations may ensure that priming consistently enhances their efficacy, yielding highly potent MSC products.

To advance the development of our MSC therapy, future studies should prioritize identifying specific markers of highly anti-inflammatory MSC subtypes to enable their targeted isolation. The identification of specific markers of highly anti-inflammatory MSC subtypes would allow us to harness their therapeutic potential. This could enhance the efficacy and improve the quality of our therapy. For instance, batch-to-batch consistency could be improved by adhering to specific identity attributes, thereby ensuring functionally equivalent batches. [20,21] Furthermore, identity markers would allow for stringent quality control. MSC preparations that do not meet specific identity attribute requirements could be excluded from therapeutic use, thereby improving quality and overall efficacy of our therapy.

The subsequent section will outline a potential approach to identify the identity attributes of highly anti-inflammatory MSC subtypes. First, a methodology must be developed to identify the highly anti-inflammatory MSC subtypes relevant to the inflammatory features in MC1 lesions. Ideally, MSCs which strongly suppress the inflammatory MC1 lesions in vivo should be considered for this project.

Unfortunately, MSC preparations with an in vivo anti-inflammatory efficacy readout against MC1 lesions are unlikely to be available for this project. Instead, the in vitro anti-inflammatory potentials of MSC preparations should be utilized to define relevant MSC subtypes. This approach sacrifices translational relevance, as in vitro anti-inflammatory readouts are only approximations of the in vivo anti-inflammatory potentials. In vitro assays often focus on specific anti-inflammatory mechanisms of MSCs, neglecting their multimodal anti-inflammatory properties. [20,22–24] To counteract this limitation, an assay matrix should be utilized, combining multiple complementary potency assays, to provide a more comprehensive picture of the anti-inflammatory capacity of MSCs. [20,25] The in vitro assays should aim to measure the potency of MSC preparations to modulate cell types involved in the pathobiology of MC1. A more detailed characterization of cell types involved in the pathomechanism of MC1 would be desirable, as it would enable the identification of MSC subtypes tailored to modulate these specific cells.

The in vivo anti-inflammatory potentials of MSC preparations are likely affected by the formulation of the MSC preparation (pro-inflammatory primed MSC spheroids) and the target tissue environment. To enhance the likelihood of in vitro assay results to align with the anticipated effects post in vivo administration, it will be crucial that the in vitro potency assays closely mimic the conditions of in vivo administration. This includes i) evaluating anti-inflammatory mechanisms of MSCs relevant to the pathobiology of MC1, ii) utilizing the correct MSC formulation, and iii) replicating the tissue environment of the intended administration site. Therefore, to assess the anti-inflammatory potentials of pro-inflammatory primed MSC spheroid preparations, a "near-physiological" in vitro model of the target administration site should be utilized.

Available in vitro potency assays for measuring the anti-inflammatory potencies of MSC preparations do not replicate the MC1 microenvironment, and a suitable assay to assess the anti-inflammatory potency of MSC spheroids does not exist. A "near-physiological" in vitro functional potency assay would represent a promising approach to measure relevant anti-inflammatory potencies of pro-inflammatory primed MSC spheroids, with the goal of identifying anti-inflammatory MSC subtypes. Therefore, the immediate next steps of this project should focus on the development of a "near-physiological" in vitro model for co-culturing MSC spheroids with cell types involved in the pathobiology of MC1. Given that various cell types are likely involved in the pathobiology of MC1 – including T cells, neutrophils, and macrophages – MC1-relevant MSC subtypes should be identified based on their ability do modulate several cell types. This will require the development of multiple "near-physiological" in vitro model-based functional potency assays to assess the potency of MSC preparations to modulate several relevant cell types.

As most substantial evidence suggests the involvement of activated and highly proliferative T cells in inflammatory MC1 lesions, it would be best to begin with the identification of MSC subtypes, which strongly suppress T cell activation and proliferation. [8,9] Different MSC preparations could be tested, and their T cell suppressive potentials quantified. This would allow to distinguish MSC preparations with high T cell suppressive potentials – MSC preparations with a high concentration of potent MSC subtypes – from MSC preparations with low T cell suppressive potentials – MSC preparations with a low concentration of potent MSC subtypes.

In a subsequent step, the functionally tested MSC preparations should be thoroughly characterized to identify MSC subtype-specific identity attributes. Targeted isolation of potent MSC subtypes from heterogeneous MSC preparations, requires the identification of potent MSC subtype-specific surface markers. Only cell surface markers enable the targeted isolation of living cells using gentle cell isolation methods such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). Therefore, the characterization of potent MSC subtypes should focus on the identification of a surface marker expression pattern that is exclusive to the potent MSC subtypes.

In summary, the immediate next steps of this project should focus on the development of a "near-physiological" in vitro model. This in vitro model would enable to design functional assays to measure the potential of MSC preparations to modulate MC1 pathobiology-specific inflammatory cell types. MSC preparations with a high anti-inflammatory potential – containing high concentrations of potent MSC subtypes – could subsequently be characterized. The characterization should focus on the identification of potent MSC subtype-specific surface marker expression patterns, to enable their targeted isolation. The isolation of highly potent MSC subtypes is crucial for the successful development of an effective MC1 therapy. Achieving this could ensure the production of consistently efficacious doses, thereby addressing the inconsistencies observed in investigational MSC treatments for chronic LBP patients.

Future directions may comprise proof-of-concept studies, followed by a safety and feasibility study, before transitioning into first-in-human trials. Once potent MSC subtypes are isolated, a study should be conducted to confirm their enhanced in vitro anti-inflammatory potency. The logical progression from there would be to demonstrate efficacy in modulating MC1 lesions in a pre-clinical animal model. No in vivo model exists that accurately reproduces the complex MC1 pathobiology. [26,27] However, efficacy may be demonstrated in vitro by modulating MC1 lesion-derived cells, further emphasizing the need to develop a "near-physiological" in vitro model. In preparation for human trials, the safety and feasibility of implanting MSC spheroids into the target tissue should be validated in animal models, a step which is required by regulatory authorities.

5.3 Conclusion

The overarching goal of this project is the development of an allogeneic anti-inflammatory MSC-based therapy for chronic LBP patients with MC1. The high prevalence of chronic LBP patients with MC1, coupled with the absence of a recognized treatment strategy, underscores the need for a targeted treatment. We have thoroughly discussed the reasons why chronic LBP patients with MC1 are a valid target population and why MSCs are the favored therapeutic approach. Briefly, MC1 patients represent a homogeneous subpopulation of chronic LBP patients with a clear inflammatory phenotype. Inflammation is a pivotal pathologic feature in painful MC1 lesions. Targeting this inflammation, by harnessing the substantial anti-inflammatory properties of MSCs, could potentially lead to a sustained suppression of the painful inflammatory MC1 lesions. We have discussed the merits of using highly anti-inflammatory, allogeneic MSCs in light of our translational project. We identified pro-inflammatory and 3D culture priming as promising strategies to improve the efficacy of our anti-inflammatory MSC therapy. However, priming alone may not be sufficient to guarantee consistent potency of our therapy, due to the probable co-existence MSC subtypes that respond or do not respond to priming within a single MSC preparation. Consequently, future studies should focus on developing a method to identify, characterize, and isolate highly anti-inflammatory MSC subtypes. This would pave the way for developing an efficacious MSC therapy for chronic LBP patients with MC1.

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Appendix

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Sep 2017 – Feb 2019 **ETH Zurich, M.Sc. in Pharmaceutical Sciences**

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