THERAPEUTIC POTENTIAL OF CYTOKINE-PRECONDITIONED HUMAN UMBILICAL CORD BLOOD DERIVED MESENCHYMAL STEM CELLS IN THE EXPERIMENTAL MODEL OF RHEUMATOID ARTHRITIS

by

MADINA A. SARSENOVA

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School of Medicine
Nazarbayev University

Supervisor: Arman Saparov, MD, PhD, DSc
Co-supervisor: Vyacheslav Ogay, PhD, Cand. Sc. (Biology)

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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the author's original work. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed 

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CONTENTS

ABSTRACT

Rheumatoid arthritis (RA) is a persistent autoimmune condition primarily marked by the generation of autoantibodies. RA causes the gradual deterioration of joints due to the breakdown of cartilage and bone tissues, resulting in to a debilitating illness for the patient. Approximately 30% of instances result in people becoming incapacitated within the initial 10 years if the condition is unmanageable. The pathophysiology of RA is linked to the imbalance of both innate and adaptive immune responses. Currently, therapeutic practice is using traditional therapies such as steroid medications, antirheumatic medicines, and biological agents. These treatments have shown to be effective and provide a longer time of remission. Nevertheless, prolonged use of these medications leads to adverse reactions, and some individuals with RA may develop resistance to these therapies.

Recent research has uncovered that mesenchymal stem cells (MSCs) have strong capabilities to regulate and suppress immune system activity. These cells possess significant potential for differentiation into several cell types, such as adipocytes, chondrocytes, and osteoblasts, among others. MSCs possess distinctive immunomodulatory features, in addition of their progenitor traits.

The MSCs can adopt either a pro-inflammatory or anti-inflammatory phenotype, depending on the surrounding environment. Given this fact, we propose that the cell preconditioning technique with pro-inflammatory cytokines can be utilized to enhance the therapeutic effectiveness of MSCs in treating RA.

This study aims to assess the immunomodulatory and immunosuppressive characteristics of MSCs in a laboratory setting. Furthermore, the impact of administering cytokine-preconditioned MSCs systemically will be evaluated in mice with collagen antibody induced arthritis. The study findings indicate that MSCs could be considered as a viable alternative for treating RA.

Keywords: mesenchymal stem cell, preconditioning, rheumatoid arthritis, cellular therapy.

1. INTRODUCTION

The Current Study: Hypothesis, Aims, Objects, Novelty and Significance

Hypothesis:

We hypothesize that cytokine-preconditioned (IL-1β, TNF-α, IL-17A) human umbilical cord blood-derived mesenchymal stem cells (hUCB MSCs) will exhibit enhanced therapeutic potential in alleviating the symptoms and progression of the disease in an experimental model of RA.

Aims:

The primary aim of this investigation is to identify the therapeutic effects of cytokine-preconditioned human UCB MSCs in mice with collagen antibody induced arthritis (CAIA).

Objectives:

1. To isolate and characterize hUCB MSCs primary cultures.
2. To determine the optimal preconditioning conditions for hUCB-MSCs with pro-inflammatory cytokines (IL-17A, IL-1β, TNF-α) to effectively increase their immunomodulatory properties *in vitro*.
3. To investigate the therapeutic effects of cytokine-preconditioned MSCs in the CAIA mouse model.

Novelty:

This study introduces the innovative approach of utilizing cytokine-preconditioned UCB-MSCs, aiming to implement their enhanced therapeutic potential for the management of RA. The emphasis on understanding the immunomodulatory mechanisms underlying the observed effects contributes novel insights into the cellular interactions mediating RA progression and resolution.

Significance:

Novel findings of the research may contribute to the scientific understanding of MSC-based immunomodulation and its application in RA. Additionally, the results may provide a foundation for further investigations into the therapeutic potential of cytokine-preconditioned MSCs in other autoimmune and inflammatory conditions.

Regarding potential clinical implication, preconditioned MSC-based therapy for RA could offer a safer and more effective alternative to current treatment modalities. Moreover, positive outcomes from this study could pave the way for the development of innovative and improved treatment strategies, ultimately enhancing the quality of life for individuals affected by RA.

Summary:

In this study, we will be testing the hypothesis that the strategy for enhancing the therapeutic potential of MSCs using preconditioning by pro-inflammatory cytokines will significantly increase their immunosuppressive and immunomodulatory capacities.

hUCB MSCs will be primed by IL-17A, IL-1β, and TNF-α proinflammatory cytokines. Cells will be primed both individually and in combination. In the next step of the research, we will study the therapeutic effects of systemic intravenous administration of selected cytokine-preconditioned MSC group, which showed the best results in the enhancement of immunomodulatory genes and proteins according to the in vitro study. Moreover, the in vivo assay will be performed in CAIA in mice, which provides the rapid arthritis induction and severe form of the disease development within a few days and allows quick screening and evaluation compared to collagen-induced arthritis. In this study for the first time the immunomodulatory effects of cytokine-preconditioned MSC group in suppressing inflammation and subsequent joint deterioration in CAIA model will be evaluated. The findings of this study may be used as a foundation for implementing an innovative cell-based therapy strategy for treating RA by using cytokine-preconditioned MSCs.

2. LITERATURE REVIEW

2.1 Rheumatoid Arthritis

2.1.1 Definition and Prevalence of RA

Rheumatoid arthritis (RA) is a persistent autoimmune disorder characterized by the immune system attack on the synovium, resulting in inflammation, as well as harm to cartilage, bone, and many internal organs such as the heart, kidney, lung, digestive system, eye, skin, and neurological system (Bottini & Firestein, 2013; Conforti et al., 2021; Radu & Bungau, 2021; Smolen et al., 2016).

Approximately 40% of individuals with RA experience consequences, with a prevalence of significant complications reaching 8.3%. Among them, cardiovascular disease, interstitial lung disease, osteoporosis, and metabolic syndrome are frequently observed RA (Nagy et al., 2022; Taylor et al., 2021). Complications significantly diminish the quality of life for those with RA and can potentially result in higher mortality rates among RA patients. The complications of RA are typically linked to the prognosis of the illness and necessitate prompt diagnosis and proactive action. The primary objectives of treatment are to decrease disease activity and manage the additional damage that occurs outside of the joints in RA (Wu et al., 2022). Two decades previously, more than fifty percent of patients with RA were disabled, incapable of working full-time, and experienced heightened mortality rates (Aletaha & Smolen, 2018).

The worldwide occurrence of RA is a half to one percent. Typically, the disease is more prevalent in women than men. The estimated lifetime risk of having adult-onset RA is around 3.6% for women and 1.7% for men (Crowson et al., 2011). Most of the studies consistently found that the prevalence of RA in females was three to five times higher than in males. The Argentinian study found the most notable disparity, with a prevalence of 3.2% among women and 0.6% among men. In contrast, Serbia had the closest numbers, with a prevalence of 0.29% among women and 0.09% among men. Moreover, small continuous variations and a visible increase from the southern to the northern regions, as well as from rural to urban areas, are observed (Radu & Bungau, 2021).

From an epidemiological point of view, the occurrence of RA fluctuates based on age and population demographics. Japan has recorded a lower incidence rate of 8 cases per 100,000 population, whereas France has reported a slightly higher rate of 8.8 cases per 100,000 inhabitants. The United States has the greatest incidence rate, with 44.6 cases per 100,000 inhabitants (Radu & Bungau, 2021). Various studies have documented variations in the occurrence rates at the regional level within countries. Potential causes that may contribute to these variations include exposure to

pollutants in the environment, changes in climate, infectious diseases, and dietary factors (Taylor-Gjevre et al., 2018). Moreover, there have been findings suggesting that persons hailing from a socioeconomically deprived background and being raised in rural locations have a higher susceptibility to developing RA later in life (Parks et al., 2013). Recent research indicate that the United Kingdom has the highest standardized incidence rate, with 27.5 cases per 100,000 population. Additionally, Canada has experienced the most significant increase in incidence rate over the past three decades (Almoallim et al., 2021; Safiri et al., 2019).

2.1.2 Clinical Signs of RA

The illness is distinguished by symmetrical inflammation that affects many joints on both sides of the body. People with RA commonly experience pain and swelling in the joints of their hands and feet. The clinical manifestation differs considerably between the first phase of RA and the later phases of the disease that have not been successfully managed. In the initial stages of rheumatoid arthritis (RA), individuals commonly experience symptoms such as fatigue, flu-like sensations, swollen and tender joints, and prolonged morning stiffness lasting over 30 minutes, often persisting for several hours. Furthermore, elevated levels of C-reactive protein (CRP) and an increased erythrocyte sedimentation rate (ESR) are associated with these symptoms (Brzustewicz et al., 2017). Both minor and major joints can be impacted, with small joints such as the metacarpophalangeal, metatarsophalangeal, proximal interphalangeal, and wrist joints being frequently afflicted. The ankle, knee, elbow, and shoulder, which are larger joints, are also prone to susceptibility (Aletaha & Smolen, 2018).

Conversely, inadequately managed RA manifests more intricate clinical presentation, characterised by notable systemic symptoms. The symptoms may include pleural effusions, lung nodules, interstitial lung disease, lymphomas, vasculitis in small or medium-sized arteries, keratoconjunctivitis, atherosclerosis, hematologic abnormalities (such as anaemia, leukopenia, neutropenia, eosinophilia, thrombocytopenia, or thrombocytosis), joint malalignment, loss of range of motion, bone erosion, cartilage destruction, and the development of rheumatic nodules (Aletaha & Smolen, 2018; Smolen et al., 2016). Collectively, these systemic consequences caused by the long-term inflammatory condition in individuals with RA contribute to a higher risk of death (Lin et al., 2020).

2.1.3 Pathophysiology of RA

The inflammation and swelling seen in RA are a result of the recruitment of immune system cells, such as T cells, B cells, and monocytes, into the synovium. Concurrently, endothelial cells experience stimulation, resulting in the creation of fresh blood vessels, a phenomenon referred to as neovascularization. This sequence of events adds to the rapid increase of synovial fibroblast-like and macrophage-like cells, resulting in the excessive expansion of the synovial lining layer. The occurrence is frequently known as the development of a "pannus," which essentially is an atypical proliferation of tissue. The pannus invades the periarticular bone where the cartilage and bone meet, causing bone erosion and cartilage degradation. Essentially, it involves an inflammatory force infiltrating the joints, stimulating the growth of fresh blood vessels, and ultimately leading to harm to both the bone and cartilage (Aletaha & Smolen, 2018).

The first stage of the illness, referred to as the pre-RA phase, spans from several months to several years prior to the manifestation of clinical symptoms. This phase is influenced by the existence of circulating autoantibodies, elevated levels of inflammatory cytokines and chemokines, and changes in cellular metabolism (Firestein & McInnes, 2017). The advanced form of the disease is characterized by severe and debilitating chronic pain that compromises patients' quality of life. Insufficient treatment also contributes to the advancement of diseases, finally resulting in the erosion, destruction, and deformities of joints.

Autoantibodies have the ability to create immune complexes, which can then trigger the activation of complement. This, in turn, leads to an escalation of inflammatory reactions. The combination of RF and ACPAs can induce a significant inflammatory reaction, but ACPAs alone elicit minimal inflammation. RFs increase the size of immune complexes that are produced by ACPAs and enhance the inflammatory reaction caused by immune complexes and complement activation. The mechanism leads to the alterations in the functions of pro-inflammatory T helper (Th) and anti-inflammatory T regulatory cells (Tregs) (Aletaha & Smolen, 2018; Alunno et al., 2015; Wang et al., 2012). The synovial fluid and the serum of the RA patients are usually characterized by the large quantity of Th1 and Th17 cells which mainly play the role in TNF-α, IL-6, IL-1 and IL-17 inflammatory cytokines overexpression (Gonzalez-Rey et al., 2007; Nakae et al., 2003). In contrast, the number and functional activity of immunosuppressive Tregs is significantly reduced in RA patients (Ehrenstein et al., 2004).

2.1.4 Genetic Factors

The precise etiology of RA remains unknown; nonetheless, it is well-established that a confluence of genetic, epigenetic and environmental variables contributes to its pathogenesis. The interaction between an individual's genetic factors and external environmental factors seems to contribute to the advancement of the disease. The hereditary factors that contribute to RA have a significant impact on the variety of disease progression, impacting a of cellular and complex set molecular pathways. Researchers have investigated the role of heritability in RA risk by conducting linkage and association studies. These studies have shown the molecular changes that are responsible for the diversity in RA risk. Crucial elements of this genetic impact involve: (i) class II HLA genes, specifically HLA-DRB1, carry polymorphisms that are linked to a 2–3-fold higher risk of developing RA due to aberrant presentation of antigens (Eyre et al., 2012); (ii) the 620W allele of the PTPN22 gene, which encodes for protein tyrosine phosphatase nonreceptor 22, has been found to have diverse effects on different types of immune cells and is correlated with a heightened susceptibility to autoimmune diseases (Okada et al., 2014); (iii) genes encoding chemokine receptors, such as C-C chemokine receptor 6 (CCR6) (K. Kim et al., 2015; Laufer et al., 2019); (iv) PADI4 is an enzyme which parcitipates in protein citrullination (Ikari et al., 2005; Suzuki et al., 2003); (v) the gene responsible for encoding the transcription factor protein known as Signal Transducer and Activator of Transcription 4 (STAT4) (Laufer et al., 2019), (vi) the gene CTLA4 encodes the protein cytotoxic T-lymphocyte antigen 4 (CTLA4), which is related with cytotoxic T-cells (Laufer et al., 2019; Márquez et al., 2018), and (vii) the gene responsible for the cell surface receptor on B-cells, known as CD40 (van der Linden et al., 2009). Although individuals are genetically predisposed to RA due to these variables, patients display a wide range of combinations of these genetic variations. The genetic diversity among individuals is thought to have a significant impact in the variability seen in the etiology, clinical manifestation, and treatment response of rheumatoid arthritis. The distinctive genetic composition of each individual contributes an additional level of intricacy to the comprehension of RA (Viatte et al., 2013).

The disease is linked to several gene loci (Viatte & Barton, 2017). However, specific HLA class II antigens, such as HLA-DRB1*01 and HLA-DRB1*04, possess a "shared" epitope. This epitope is a sequence of 5 amino acids located in the region that is responsible for presenting antigens to T cells.

The genetic variables that contribute to RA and affect molecular and cellular disturbances vary significantly among different ethnic origins. This attribute is not limited to RA alone, but is seen in

several autoimmune disorders, such as multiple sclerosis, systemic lupus erythematosus, and type 1 diabetes. Variations in the molecular pathways underpinning RA pathophysiology are found across distinct ancestral genetic backgrounds. For example, the frequency of the 620W allele of the PTPN22 gene differs significantly throughout Europe and is particularly rare outside of the continent (Coenen & Gregersen, 2009). Hence, the spatial arrangement of this gene variant affects its influence on vital immunological processes such as T-cell activation and B cell decrease. The effects of PADI4 on RA susceptibility, perhaps connected to citrullination of arginine residues and/or leukocyte formation, disproportionately impact individuals of East Asian heritage.

As medical treatments progress towards individualized methods, a thorough understanding of the molecular consequences becomes crucial for developing therapies and facilitating practical application by healthcare providers. Seropositive RA, which is characterized by the presence of anti-citrullinated protein antibodies (ACPA) and/or rheumatoid factor (RF), demonstrates a unique genetic susceptibility profile in contrast to seronegative RA. The primary reason for the variation in RA subtypes can be traced, at least partially, to genetic variations among the patients. HLA-DRB1 haplotypes and PTPN22 R620W are mostly linked to seropositive illness (Frisell et al., 2016), but other susceptibility variations are common to both seropositive and seronegative forms.

The collection of RA susceptibility variations is believed to contribute to the determination of the severity of the disease. A study conducted on the entire genome of 384 RA patients who tested positive for autoantibodies revealed that a specific variation in the genetic code, known as a single nucleotide polymorphism (SNP), located at location 2q34 of the sperm-associated antigen 16 (SPAG16) gene in monocytes, specifically identified as rs7607479, was found to have a beneficial impact on the health of joints. This single nucleotide polymorphism (SNP) largely controls the release of matrix metalloproteinases (MMPs), the production of SPAG16 protein, and the levels of mRNA in fibroblast-like synoviocytes (FLS) from the RA synovial membrane (Knevel et al., 2014). In addition, the existence of baseline ACPA-positive is correlated with heightened radiological harm, and the genetic variant rs2900180 in the TRAF1/C5 locus is connected to a more severe illness progression (Plant et al., 2011). A distinct genome-wide investigation was conducted on 262 RA patients who tested negative for ACPAs. This study revealed that 33 SNPs were linked to the occurrence of joint damage. The area including rs2833522 contains H3K4me3 histone marks, transcription factors, and long non-coding RNAs. These elements are associated with the extent of bone damage in ACPA-negative RA patients (de Rooy et al., 2015).

2.1.5 Environmental Factors

Environmental risk factors are important contributors to the management of RA. Like other diseases, smoking is linked to either the initiation or exacerbation of RA. The early evidence associating smokers with an increased incidence of RA was discovered by chance in a study that had a different objective (Radu & Bungau, 2021). Consequently, smoking has emerged as the risk factor for RA that has been subject to the most thorough research. Extensive research has been conducted on the toxic components found in tobacco products, indicating that smoking might be associated with a certain genetic background, perhaps initiating a distinct kind of RA (Liao et al., 2009). Research suggests that smoking mostly affects individuals with RF- or ACPA-positive RA, while having minimal to no impact on those with ACPA-negative RA (Ishikawa & Terao, 2020). Furthermore, smokers who possess HLA-DRβ1 shared epitope alleles have a significantly increased likelihood of developing ACPA-positive RA (Padyukov et al., 2004). There is no correlation between individuals who are exposed to secondhand smoke and the chance of developing RA (Mehri et al., 2020).

Occupational exposure to silica dust has been recognized as an additional environmental risk that affects RA, especially in cases of silicosis, which mostly affects RA patients who test positive for ACPAs. Long-term exposure to silica can result in rheumatoid pneumoconiosis, sometimes referred to as Caplan's syndrome, a rare disorder that affects individuals with silicosis who also have RA (Alaya et al., 2018).

Extensive research has shown that fasting periods and vegetarian diets may reduce the progression of RA. Furthermore, refraining from consuming red meat while simultaneously increasing the consumption of fruits and fatty fish has been linked to a decreased likelihood of developing RA (Jin et al., 2021). The drinking of coffee may present a possible risk for RA, possibly due to its involvement in the development of RF. On the other hand, it has been proposed that consuming alcohol may have a positive impact on RA by reducing the likelihood of developing ACPA-positive RA (Liao et al., 2009). However, this idea needs more research to be confirmed. Hence, it is imperative to take into account a customized dietary plan for every individual.

2.1.6 Bacterial and Infectious Agents

Infections, among other biological risk factors, can potentially initiate the onset of RA. A comparative cohort research observed an elevated risk of joint, skin, and bone infections in people

with RA in comparison to individuals with non-inflammatory rheumatic disorders (Mehta et al., 2019). Specific bacterial stimuli have also been identified which play a crucial role in the disease development and progression.

Multiple studies highlight the vital significance of the gut microbiota in the emergence and progression of RA, through mechanisms such as the creation of proinflammatory substances, damage to the protective lining of the intestines, and the resemblance of self-antigens at a molecular level. The gastrointestinal tract acts as the main site for immune cells in the body, and their function and characteristics are influenced by ongoing interactions with the gut microbiota. The connection between the host immune system and the microbiome is regulated to mitigate the risk of detrimental infections, while concurrently sustaining a symbiotic association inside the human organism (Shin & Kim, 2019). Within the gut-associated lymphoid tissue, innate immune cells serve as the initial barrier against foreign chemicals originating from the gastrointestinal system. Imbalances in the gut microbiota can cause abnormal activation of these natural immune cells. This results in an elevation of proinflammatory cytokines like as IL-12, IL-23, and type I interferons, while simultaneously reducing anti-inflammatory cytokines including TGF-β and IL-10 (Zhang et al., 2020). T and B cells, which are types of adaptive lymphocytes, have important functions in autoimmunity, and their abnormal activation is linked to RA. Proinflammatory gut pathogens have the ability to alter the immunological milieu by excessively activating the innate immune system, which is then followed by aberrant modulation of the adaptive immunity. Microbial antigens can stimulate CD4+ T cells, leading to the activation of inflammatory T cell subtypes such as Th17 cells, which are characterized by their production of IL-17 (Y. Wang et al., 2019). Tregs, which originate from CD4+ T cells, exert immunosuppressive effects and have the potential to hinder Th17 responses (Chen & Oppenheim, 2014). Research suggests that there is a considerable correlation between RA and an elevated Th17/Treg ratio. This equilibrium is significantly impacted by the gut microbiota and its metabolites (Cheng et al., 2019). The presence of microbial antigens can cause excessive activation of B lymphocytes, facilitated by T follicular helper (Tfh) cells. This leads to the transformation of B lymphocytes into plasma cells and the generation of harmful autoantibodies, which significantly impact the development of RA. The development of RA is influenced by the interaction between dysbiosis in gut microbiota, inflammatory factors, and immunological responses (Li et al., 2021).

Recent scientific investigations have shown a robust correlation between infections induced by the prevalent periodontal bacteria *Porphyromonas gingivalis* and the initiation of autoimmune

reactions via the mechanism of citrullination of host peptides (Lin et al., 2020). The enzymatic mechanism responsible for the conversion of positively charged arginine residues in "self" proteins into neutral citrulline residues is facilitated by the enzyme peptidyl arginine deiminase (PAD). This mechanism induces a reduction in surface charge, facilitating the degradation of citrullinated "self" proteins and leading to the formation of new antigenic sites (Littlejohn & Monrad, 2018).

The induction of local tolerance by the expression of PADi4 in *Porphyromonas gingivalis*, which facilitates the conversion of arginine to citrulline, not only elicits autoimmune responses but also triggers the subsequent formation of ACPAs (Wegner et al., 2010). Furthermore, the potential involvement of other bacterial species, including *Proteus mirabilis*, *Escherichia coli*, and *Prevotella copri*, has been proposed as potential causative agents in the onset of RA. This idea is derived on molecular mimicry processes, in which the presence of identical amino acid sequences between autoantigens and certain bacterial or viral proteins may initiate the development of RA (Aletaha & Smolen, 2018; Li et al., 2013).

Furthermore, viral infections operate as biological variables that could potentially trigger the development of RA. Specific human leukocyte antigen (HLA) types, namely HLA-DRB1*01 and HLA-DRB1*04, have been associated with particular bacteria such as *Prevotella* or the *Epstein-Barr virus* (Pianta et al., 2017; Tan & Smolen, 2016). A comparative cohort study has identified an increased susceptibility to joint, skin, and bone infections in Individuals diagnosed with RA compared to those with non-inflammatory rheumatic disorders. This highlights the significant influence of viral infections on the progression of RA, especially in individuals with specific genetic predispositions (Mehta et al., 2019).

Epithelial cells in the synovium and activated antigen-presenting cells (APCs) are responsible for initiating the process of inflammatory response in inflamed joints. In both lymph nodes and adjacent organs, these cells serve as the initiators of certain immunological responses. T cells, B cells, and monocytes are the main types of cells that infiltrate the inflamed joints (Brzustewicz et al., 2017).

2.1.7 Synovial Membrane in RA

Within the synovial membrane of individuals with RA, two significant pathological alterations take place. Initially, the intimal layer undergoes significant enlargement due to increased activity of two types of synoviocytes: FLSs and macrophage-like synoviocytes (MLSs). These cells are known to have a substantial impact on the cytokines and proteases synthesis, including integrins, selectins,

and the immunoglobulin superfamily. MLSs produce several pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α (Smolen et al., 2018). Apart from expressing IL-6, FLSs, also produce substantial amounts of matrix metalloproteinases (MMPs) and small-molecule signaling mediators like leukotrienes and prostaglandins (Bartok & Firestein, 2010). Additionally, FLSs augment the stimulation of the immune response activation by their interaction with cells of the immune system and promotion of the formation of ectopic lymphoid structures (ELS) inside the synovium (Yoshitomi, 2019). Another significant change occurs when adaptive immune cells infiltrate the synovial sublining. This infiltration leads to the characteristic formation of "pannus" at the interfaces between cartilage and bone (Ouboussad et al., 2019). Pannus is composed of many cellular components, including macrophages, FLSs, dendritic or plasma cells, and mast cells. During the advanced stages of the illness, it plays a role in the progression of tissue destruction and erosion. CD4+ memory T cells make up around 50% of the sublining cells. The aforementioned cells has the capacity to either disseminate over the various tissues of the body or generate anomalous germ centres. Within these germ centres, fully developed B cells undergo proliferation, undergo structural alterations, and produce antibodies. The sublinear layer comprises several cellular components, such as B cells, plasmablasts, and plasma cells. A considerable proportion of these cells generate RF or ACPAs (Kerkman et al., 2013).

2.1.8 T Cells Activation in RA

The observed association between the HLA-DRB1 in human leukocyte antigens and individuals with RA suggests that T cell selection and antigen presentation play a role in the activation of autoimmunity (Mellado et al., 2015). CD4+ T cells play a significant role in the development of RA, with IL-6 being a crucial element in causing bone destruction in this condition. The significance of IL-6 lies in its pivotal involvement in the regulation of T lymphocyte production and inflammation (Wong et al., 2006). While the amount of IFN-γ in the synovial membrane in the joints of people with RA may not be elevated, it is still regarded as essential in the progression of the condition. Th1 cells, responsible for IFN-γ production, and Th17 cells, responsible for interleukin-17 production, play crucial roles in the progression of rheumatoid arthritis (RA) (Komatsu & Takayanagi, 2012; Mellado et al., 2015).

Th17 cells may be classified into two primary categories: "pathogenic" Th17 cells and "non-pathogenic" Th17 cells. The classification of these kinds is dependent upon the particular cytokine

milieu encountered throughout the cellular differentiation process. The pathogenic Th17 cells have immune response boosting capabilities via the production of pro-inflammatory cytokines, including IL-17A, IL-17F, and IL-22. In contrast, Th17 cells classified as "non-pathogenic" have the potential to release immunosuppressive agents such as IL-10, which serve to modulate the immune system's reaction in a negative way (Wu et al., 2018). In rheumatoid arthritis (RA), inflammatory Th17 cells have a significant influence, producing two important cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-22. Numerous investigations have provided evidence suggesting that the IL-22 (GM-CSF) signalling pathway could possibly be involved in the pathogenesis of RA (Y. Li et al., 2017; Lotfi et al., 2019).

In a number of tissues a various cell types , including cartilage, synovium, macrophages, and osteocytes, IL-17 has a role in promoting the synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Onishi & Gaffen, 2010). Apart from that, it promotes the production of additional chemokines such as CXCL1, CXCL2, CXCL8, CCL2, CCL7, and CCL20. These chemokines contribute to inflammation by involving neutrophils, macrophages, and lymphocytes to the synovium (Shen & Gaffen, 2008). There exists a correlation between the absence of IL-17 and a reduction in the progression of RA (Nakae et al., 2003). Consequently, clinical trials have assessed other inhibitors of IL-17A, such as monoclonal antibodies targeting IL-17A and a monoclonal antibody targeting the IL-17 receptor component A. However, the aforementioned studies have not shown any further benefits for patients who were unresponsive to prior TNF-α inhibitors and are now receiving methotrexate (MTX) as a supportive therapy (Blanco et al., 2017).

IL-22, along with IL-17, contributes to the synovial inflammation through promoting the proliferation and release of chemokines by FLS (Ikeuchi et al., 2005). These inflammatory cytokines contribute to the T cell regulation, referred to as T cell plasticity, leading to alterations in the features and way they act. The increase in the number of Tregs is seen in the inflamed joints of individuals with RA, indicating the existence of an inflammatory environment (Wehrens et al., 2013). Nevertheless, in the context of autoimmune inflammatory condition, Tregs may exhibit diminished activity or even exhibit deleterious effects. For instance, the expression of Foxp3 is reduced by IL-1β and IL-6, resulting in a decline in the inhibitory function of Tregs (Mellado et al., 2015). Foxp3 plays a crucial role in controlling and inhibiting the immune response in Tregs, where it affects the expression of genes, their function, and their ability to survive. The gene in question is subject to

regulation via transcriptional, epigenetic, and post-translational pathways, and it serves a vital function in maintaining immunological self-tolerance (Jiang et al., 2021; Li & Zheng, 2015).

Overall, Th1, Th17, Tregs, and Th22 cells T cell populations, initiate a consecutive sequence of actions (rolling, arrest, spreading, crawling, and migration) and eventually leave the blood circulation and reach the inflamed joint (Plant et al., 2011). Studies have shown a notable degree of flexibility among different subsets of CD4+ T cells. Th1 and Th17 effector T cells in combination with FoxP3+ Tregs have notable flexibility, enabling them to effectively adjust to diverse physiological circumstances during the immune response. Cytokines including IL-1, IL-6, IL-12, IL-17, and IFN-γ stimulate this flexibility. Hence, the capacity of CD-4+ T cells to modify themselves has evolved to maintain both robustness and uniformity, so facilitating the immune system's optimal response to pathogens and environmental changes. On the other hand, this capacity to adapt makes the patient vulnerable to possible risks, since the disruption of this system increases the probability of autoimmunity activation. Therefore, regulating the adaptability of Treg and Th17 cells may be a promising strategy for immunotherapy in the management of autoimmune illnesses (Kleinewietfeld & Hafler, 2013).

Despite their abundance, the precise purpose of T cells in the synovial environment remains incompletely understood. The efficacy of cyclosporine or T cell-depleting medications in directly targeting T cells has shown limitations or inefficiency (Panayi, 2006). In contrast, abatacept, which hinders the activation of T cells via CD28, has shown efficacy in controlling inflammation in RA. Abatacept's effectiveness in inducing remission in RA might be attributed, to some extent, to its capacity to enhance the presence of immunological regulatory cells, namely IL-35 + IL-10 + regulatory B (Breg) cells (Alenazy et al., 2021).

2.1.9 B Cells and Autoantibodies

The role of B lymphocytes, specifically in the production of autoantibodies, antigen presentation, and release of cytokines, is closely associated with the advancement of RA. The majority of autoantibodies are produced and released by autoreactive B cells, which are activated by Toll-like receptors (TLR) and have proceeded maturation into plasma cells (Hua & Hou, 2013). The increased number of autoreactive B cells in RA may be attributed to the capacity of some proteins to undergo post-translational modifications and interact with external antigens (Dekkers et al., 2017). In addition, the upregulation of an enzyme, known as cytidine

deaminase, in B cells is associated with increased concentrations of Th cell cytokines IFN-γ and IL-17. These cytokines are involved in the development of anti-CCP and RF antibodies (Xu et al., 2009).

The group of autoantibodies associated with RA includes RF, ACPA, anti-modified citrullinated vimentin antibody, anti-carbamylated protein antibody, anti-PAD-4 antibody, and anti-GPI antibody. Among the several autoantibodies associated with RA, ACPA has the most significant diagnostic potential in predicting the initiation of RA in symptomatic individuals who are at risk (Rönnelid et al., 2021).

RF and ACPA display clear differences. B cells expressing ACPA undergo many rounds of germinal centre responses, leading to a notable enhancement in somatic hypermutations and isotype shifting. One perspective is that B cells that are positive for RF undergo numerous cycles of germinal centre responses, characterised by a restricted set of genetic alterations. These alterations include two transcription factors, namely BACH2 and SOX11, and may be triggered by innate immune processes. Conversely, B cells that are positive for ACPA show a high number of genes that are produced differently and are associated with the development of B cells that depend on T cells. Therefore, the immune response that is unique to citrulline leads to the generation of plasma cells with an extended lifetime and the creation of ACPA autoantibodies. However, the reaction to RF is distinguished by the generation of plasma cells that exhibit a reduced lifetime and fluctuating levels of RF (Malmström & Grönwall, 2018).

The RF antibody selectively recognises the Fc region of the IgG antibody. The original type of autoantibody found in RA was RF, which was subsequently used in the 1987 ACR classification criteria for RA (van Delft & Huizinga, 2020). While the Waaler-Rose test for RF detection in human serum primarily relies on IgM antibodies, it is worth noting that RF activity may be seen in a wide range of immunoglobulins, including IgA, IgG, and IgM. A wide range of illnesses display different levels and types of RF, as shown by varied levels and types of RF (Ingegnoli et al., 2013). Specifically, there is a substantial correlation between increased levels of IgM and IgA and the existence of RA. The correlation between levels of RF and clinical disease activity is a subject that is now under debate and subject to disagreement. During the early phases of the illness, there may be fluctuations in RF levels, which may not always be associated with clinical outcomes (Barra et al., 2013). Nevertheless, RF remains a potent diagnostic marker for RA that is often used in routine clinical settings (Volkov et al., 2020).

Another prognostic marker, ACPA, plays a crucial role in aiding rheumatologists in promptly identifying RA and initiating first therapy (Pruijn et al., 2010). Due to the significant impact of inflammation on the advancement of RA, it has been proposed and shown that ACPAs have the ability to stimulate immune cells and boost the synthesis of pro-inflammatory cytokines (Volkov et al., 2020). Multiple studies have shown elevated levels of ACPA in persons with preclinical RA. The existence of ACPA serves as a robust predictor for the prospective advancement of RA, exhibiting a notable degree of precision. The specificity of the test ranges from 85% to 95%, while the sensitivity is 67% (Kwon & Ju, 2021). As a result, ACPAs have been incorporated into the widely accepted ACR/EULAR 2010 classification standards (Kay & Upchurch, 2012). ACPAs have a unique affinity for citrulline residues that are found on proteins or peptides. The process of citrullination involves the permanent modification of arginine via the enzymatic activity of PADs. Consequently, ACPA has been seen to identify many proteins that have undergone citrullination, including fibrinogen, β-enolase, vimentin, and collagen type II (Jang et al., 2022; Mahdi et al., 2009).

The correlation between the occurrence of murine and administered human ACPAs in mice models and the progression of arthritis is substantial. The biological impact of ACPAs has been shown in laboratory experiments, whereby they bind to Fc receptors present on immune cells of the myeloid lineage and elicit activation of the complement system via both traditional and alternative routes (Kurowska et al., 2017). The research findings indicate that the synovial membrane of persons with RA contains complexes consisting of citrullinated fibrinogen and ACPA. These complexes have the ability to stimulate macrophages by engaging in simultaneous interactions with TLR-4 and FcγR. The aforementioned relationship results in a synergistic impact that amplifies the synthesis of TNF-α. This suggests that the process of citrullination may play a significant role in augmenting the efficacy of endogenous immune ligands, hence providing insights into the potential contribution of autoimmune reactions against citrulline to the pathogenesis and advancement of inflammation in RA (Clavel et al., 2008; Sokolove et al., 2011).

It has been shown by FcR-mediated or complement-dependent pathways that immune complexes including ACPA and IgM or IgA RF, which are autoantibodies derived from individuals with RA, possess detrimental properties (Anquetil et al., 2015). Furthermore, an alternative pathway by which the generation of TNF-α takes place in RA via ACPA has been recorded. By adhering to the surface of peripheral blood mononuclear cells with an over-expression of citrullinated glucose-

regulated protein 78, ACPAs exhibit preferential activation of the ERK1/2 and JNK signalling pathways in RA. This process activates NF-κB and leads to the production of TNF-α (Lu et al., 2013).

The presence of ACPAs in RA is linked to the initiation of neutrophil extracellular traps, which include the release of intracellular substances such as DNA, histones, IL-17A, TNF-α, granular proteins, and cytoplasmic proteins by neutrophils. Numerous studies have provided evidence indicating that the presence of anti-citrullinated vimentin antibodies has a substantial influence on the facilitation of NET formation (Khandpur et al., 2013). The phenomenon of increased NET formation in RA serves as a provider of citrullinated autoantigens and PADs. Upon their release from intracellular compartments, these enzymes have the ability to catalyse the citrullation of extracellular proteins. This procedure contributes to the production of ACPAs. The stimulation of NET formation by ACPA may potentially sustain inflammatory and autoimmune pathways in RA (Kurowska et al., 2017).

ACPAs have the ability to bind to the surface of osteoclasts and osteoclast progenitor cells, hence facilitating the process of bone resorption, which further leads to progression of severe forms of RA (Harre et al., 2012).

2.1.10 Presentation of Antigens

There are three primary classifications of antigen-presenting cells found in the human body, namely dendritic cells (DC), macrophages, and B cells. The antigen receptor on B cells effectively delivers distinct antigens, leading to the activation of matched T lymphocytes (Chen & Jensen, 2008). During the disease, B cells function as APCs by presenting their own antigens to CD4+ T helper cells. There are two kinds of CD4+ helper T cells, Tfh and peripheral helper cells (Tph). Numerous studies have shown an increased prevalence of circulating Tph and Tfh cells in the bloodstream of RA patients (Lucas et al., 2020). The production of pro-inflammatory cytokines, such as IL-21, by pro-inflammatory Th17 cells plays a crucial role in the process of synovial inflammation. The activation of B cells and facilitation of several processes, including cell division, specialisation, enhancement of affinity, and antibody production, are mediated by these cytokines (van den Berg & McInnes, 2013). Additionally, the levels of IL-21 are strongly correlated with the ACPA, CRP, RF, and ESR levels in the serum of RA patients (Cao et al., 2019).

2.1.11 Cytokine Production in RA

The synovium of RA individuals exhibits a complex interplay of cytokines that have a role in the advancement of the illness. The serum of theses patients may contain different cytokines, such as, IFN-γ, IL-6, TNF-α, IL-1β, IL-17, IL-18, CCL3, and RANK-L (Yanaba et al., 2008).

The secretion of TNF-α, B cells, and NK cells plays a pivotal role in the initiation of joint inflammation in persons diagnosed with RA (Brennan & McInnes, 2008). Previous *in vitro* studies have shown that TNF-α had the capacity to induce both cartilage degradation and bone tissue atrophy (Bertolini et al., 1986). Recent studies have provided evidence suggesting that TNF-α is involved in the augmentation of RANK-L secretion by osteocytes, hence facilitating the process of bone resorption (Marahleh et al., 2019). Several studies have shown that TNF-α has the capacity to induce the conversion of monocyte/macrophage lineage cells into osteoclasts, independent of the RANK-L complex (Lotfi et al., 2019; Onishi & Gaffen, 2010; Shen & Gaffen, 2008). Moreover, the co-administration of TNF-α and IL-1β has been seen to enhance the synthesis of RANKL by B cells, hence promoting the differentiation of osteoclasts (Luo et al., 2018).

In addition, TNF-α plays a crucial role in the progression of RA by promoting the synthesis of other inflammatory cytokines, such as IL-1β and IL-6. The cytokines present in the synovium have the ability to recruit leukocytes and induce an inflammatory milieu (Brennan & McInnes, 2008).

Produced by Th17 cells IL-17A significantly contributes to the progression of RA. The process induces the production of pro-inflammatory cytokines, including IL-6, IL-8, and GM-CSF, from several cellular sources, and subsequently draws neutrophils, leading to the development of localised inflammation. IL-17A furthermore plays a role in the process of bone erosion, cartilage degradation, and the development of new blood vessels in individuals with RA. It elicits the differentiation of nascent osteoclast cells into mature osteoclasts, promotes the production of RANK-L by osteoblasts and synoviocytes, diminishes bone development, and exacerbates bone disintegration. Furthermore, IL-17A induces synoviocytes to generate MMP-1, hence facilitating the deterioration of cartilage. IL-17A enhances the movement of endothelial cells and promotes the production of vascular endothelial growth factor (VEGF) by synovial fibroblasts in the context of angiogenesis (Pickens et al., 2010; Robert & Miossec, 2018).

Elevated concentrations of IFN-γ have been also seen in the plasma, synovial tissue, and synovial fluid of RA patients (Kokkonen et al., 2010). IFN-γ is produced by different immune cells, such as T cells, B cells, NK cells, monocytes/macrophages, DCs, and neutrophils (Olalekan et al., 2015; Thanapati et al., 2017). The activation of IFN-stimulated genes occurs through various

pathways, including the JAK-STAT1 pathway, MAP kinase pathway, PI3K pathway, and NF-κB pathway (Tang et al., 2018).

IFN-γ acts by enhancing the process of antigen presentation and stimulating macrophages. Activated macrophages and monocytes secrete the CXCL10 chemokine. This chemokine facilitates the process of osteoclast formation via the induction of RANK-L and TNF-κ secretion by CD4+ T cells. Furthermore, previous studies have shown evidence that the production of IFN-γ by B cells impedes the maturation of Tregs in a murine model of proteoglycan-induced arthritis, hence augmenting autoimmune responses. In contrast, it has been suggested that IFN-γ may have a protective impact on the tissues throughout the advanced phases of the disease. The observed effects include the inhibition of osteoclastogenesis mediated by RANK-RANK-L, the suppression of synoviocyte proliferation based on TNF-α as well as the inhibition of the production of prostaglandin E2 and GM-CSF (Kim & Moudgil, 2017; Tang et al., 2018).

The active macrophages in the inflamed synovium produce additional pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These cytokines play a role in the inflammation process by recruiting and activating other immune cells to the site of inflammation. For instance, activated neutrophils release substantial quantities of cytokines and inflammatory agents in the joints impacted by RA. Furthermore, these components contribute to the deterioration of joints (Chen et al., 2019).

2.1.12 Osteoclast Activation

The maintenance of bone homeostasis is established by the balance between osteoblastic bone formation and osteoclastic bone degradation. The expression of RANKL, another important cytokine implicated in bone homeostasis, has been seen in memory B cells (Anquetil et al., 2015; Clavel et al., 2008; Sokolove et al., 2011).

Empirical investigations have shown that an increased abundance of plasma cells expressing RANKL facilitates the proliferation of osteoclasts, which play a crucial role in the process of bone resorption. These results suggest that plasma cells have a role in promoting the development of osteoclasts in RA (Komatsu et al., 2021).

2.1.13 Angiogenesis

Angiogenesis, the physiological process of generating new blood vessels from pre-existing ones, is also observed in RA. While angiogenesis is advantageous in certain physiological functions, it has

a crucial role in the development of RA. It promotes the movement of immune cells into the joints by enhancing the permeability of blood vessels and stimulating the production of adhesion molecules, such as vascular adhesion molecule 1 (Elshabrawy et al., 2015). Moreover, the presence of VEGF, a factor that promotes the creation of blood vessels, in the synovium of patients with RA, has a strong impact on the degradation of bones by stimulating the formation of osteoclasts (Radu & Bungau, 2021).

2.2 Current Approaches for Rheumatoid Arthritis Treatment and Their Limitations

2.2.1 DMARDs

Enhanced knowledge in the pathophysiological mechanisms and breakthrough in the RA cure resulted in the development of effective drugs that are able to control the disease activity and the level of joint damage and pain (Burmester & Pope, 2017). Currently, the most frequently used therapeutic approaches in RA include glucocorticoids (GCs) and synthetic and biologic disease-modifying anti-rheumatic drugs (DMARDs), and non-steroidal anti-inflammatory drugs (NSAIDs) (Guo et al., 2018).

DMARDs are the primary treatment for RA. They consist of a variety of treatments that hinder the course of the illness and manage symptoms (Bottini & Firestein, 2013). MTX, a common synthetic DMARD, has anti-proliferative effects similar to those of folic acid (van der Linden et al., 2009; Viatte et al., 2013). MTX produces the disruption of purine and pyrimidine metabolism, hinders the production of amino acids and polyamines, and triggers death in T cells and platelets (Viatte & Barton, 2017). Nevertheless, clinical practice has documented the presence of hazards associated with the development of skin cancer, impairments in bone marrow function, as well as gastrointestinal, infectious, pulmonary, and hematologic side effects (Coenen & Gregersen, 2009; Frisell et al., 2016).

The first treatment for patients with moderate or severe illness should include the administration of MTX as a monotherapy. If a condition does not react satisfactorily to MTX, it may be complemented with a complementing medicine or completely substituted with other DMARDs if any negative effects are noticed (Knevel et al., 2014). Nevertheless, the administration of MTX is often terminated in fewer than 5% of patients as a result of adverse effects, which may also be mitigated with the proactive incorporation of folates (Plant et al., 2011). Leflunomide, sulfasalazine, hydroxychloroquine, and chloroquine are among the synthetic DMARDs that are available as alternatives. Hydroxychloroquine may serve as an initial therapeutic option for individuals with a modest illness course (de Rooy et al., 2015). Leflunomide and sulfasalazine are often recommended

medications for the treatment of RA, particularly in situations when patients are unable to tolerate MTX (Liao et al., 2009). In some cases, a triple-drug treatment including the administration of MTX, sulfasalazine, and hydroxychloroquine may be used (Ishikawa & Terao, 2020). Significantly, MTX is favoured for utilisation in patients due to its cost-effectiveness and therapeutic effectiveness (Padyukov et al., 2004). Nevertheless, the use of MTX in conjunction with other medications has been shown as a superior therapeutic approach compared to MTX alone (Mehri et al., 2020; Nagy et al., 2022).

The American College of Rheumatology (ACR) and European League Against Rheumatism advocate for the use of MTX in conjunction with short-term GC application as a viable anti-inflammatory medication for the treatment of RA in individuals who have just been diagnosed with the condition (Alaya et al., 2018; Taylor-Gjevre et al., 2018). The immunomodulatory impact of GCs is achieved by the induction of death in immature CD4+CD8+ thymocytes, as well as the transformation of DCs into tDCs. tDCs stimulate the production of Tregs and enhance the phagocytosis of apoptotic cells by macrophages (Jin et al., 2021; Mehta et al., 2019; Shin & Kim, 2019). Nevertheless, the adverse effects after the use of GC are more pronounced when compared to other medications. Excessive administration of GCs results in the development of ecchymosis, cushingoid characteristics, parchmentlike skin, leg swelling, sleep disruption, and immunosuppression. Additional detrimental consequences include increasing body weight, epistaxis, glaucoma, depression, hypertension, and diabetes (Y. Wang et al., 2019; Zhang et al., 2020). Although GCs have negative effects, the concurrent use of MTX and GCs has been shown to decrease RA symptoms in around 25% of patients during a 6-month period of therapy. Furthermore, when combined with the systemic administration of gonadotropin-releasing agents, intra-articular (IA) injections have the potential to mitigate local joint inflammation (Chen & Oppenheim, 2014).

Approximately 30-50% of patients exhibit an overwhelming lack of response to standard DMARDs. In cases when a 2-6 month course of MTX monotherapy or combination therapy proves insufficient, the use of biologic DMARDs is recommended (Alaya et al., 2018; Cheng et al., 2019). The category of biologic disease-modifying antirheumatic medicines (DMARDs) includes TNF inhibitors, costimulation modifiers, IL-6 inhibitors, and B cell depleting medications. Biological drugs that are available for purchase, such as etanercept (Enbrel®), infliximab (Remicade®), adalimumab (Humira®), golimumab (Simponi®), and certolizumabpegol (Cimzia®), are all TNF inhibitors. These drugs work by blocking cytokine signalling, reducing cell recruitment, normalising IL-6 expression

in the blood and MMP expression in cartilage and bone. As a result, they slow down the process of bone destruction. In the context of biologic DMARDs, it is recommended that TNF inhibitors be considered as the primary pharmacological intervention for patients who have an insufficient response to traditional synthetic DMARDs. Additionally, TNF inhibitors are often used in conjunction with other DMARDs, particularly MTX (Li et al., 2021; Littlejohn & Monrad, 2018). However, these medications have significant adverse effects, including heightened susceptibility to infections and neurological disorders, as well as the potential for the development of multiple sclerosis and lymphoma (Li et al., 2013; Wegner et al., 2010). In addition, the suppression of TNF has previously led to the formation of Merkel cell carcinomas, which are skin tumours, in individuals with rheumatologic illnesses (Tan & Smolen, 2016). TNF inhibitors in clinical studies have shown a lack of therapy response in certain individuals (Pianta et al., 2017). Another DMARD, anakinra, which specifically binds to IL-1 receptors and inhibits inflammation, is being explored as a potential treatment option in this scenario. The use of Anakinra is often seen in conjunction with other DMARDs or as a standalone treatment. However, its utilisation is limited by the potential for opportunistic and latent infections (Alunno et al., 2015; Wang et al., 2012). Rituximab, an anti-CD20 monoclonal antibody, is often used to treat lymphomas, leukaemia, and autoimmune diseases. It may also be administered to individuals with RA when TNF medications fail to provide an adequate response (Gonzalez-Rey et al., 2007). Abatacept, a fusion protein encompassing the domain of cytotoxic T lymphocyte-associated antigen 4, has the ability to impede T cell activation. This is achieved through its binding to CD80 and CD86 receptors on APCs, as well as its ability to hinder the interaction between DCs and T cells. The clinical investigation of abatacept yielded noteworthy outcomes; yet, a subset of patients exhibited insensitivity to this therapeutic intervention, which was linked to the downregulation of CD28 expression on T cells (Ehrenstein et al., 2004; Nakae et al., 2003). Tocilizumab, a biologic DMARD, inhibits IL-6 receptors and substantially reduces the severity of the illness in RA patients who have not responded well to conventional DMARDs (Smolen et al., 2018). The clinical investigation of secukinumab, an anti-IL-17 antibody, and brodalumab, an anti-IL-17RA antibody, has shown a limited response in patients with RA in both instances (Bartok & Firestein, 2010; Yoshitomi, 2019). The effectiveness of biologic DMARDs as a standalone treatment is inferior to their combination with MTX (Ouboussad et al., 2019).

The induction of RA has been shown to include many cytokines, including TNF-α, IL-1, IL-6, IL-7, IL-15, IL-17, IL-18, IL-21, IL-23, IL-32, IL-33, and GM-CSF (Kerkman et al., 2013).

Nevertheless, clinical studies using therapeutic approaches that inhibit IL-1, IL-18, or IL-17 have shown limited advantages. In contrast, medication that targets TNF-α or IL-6 has shown efficacy in alleviating symptoms and instigating disease remission (Mellado et al., 2015). Targeting small molecules is an alternative strategy in RA treatment. Janus kinases (JAK) inhibitors are a specific class of synthetic DMARDs that specifically target and control the activity of the JAK family of non-receptor tyrosine kinases. These kinases are responsible for transmitting signals from various cytokine receptors via affecting the STAT family of transcription factors. Tofacitinib is a synthetic DMARD that specifically targets and suppresses the generation of IL-6 by inhibiting the JAK1 and JAK3 signalling pathways involved in the IL-6/gp130/STAT3 pathway (Wong et al., 2006). Consequently, it hinders the synthesis of IL-17 and IFN-γ, as well as the growth of CD4+ T cells in individuals with RA (Komatsu & Takayanagi, 2012; Wu et al., 2018). Baricitinib, a synthetic DMARD that targets JAK1/JAK2 inhibitors, shown higher efficacy compared to adalimumab, a TNF-α antagonist, in patients who did not respond well to the MTX (Y. Li et al., 2017). Upadacitinib, a JAK1 inhibitor, greatly enhances the effectiveness of rheumatoid arthritis therapy in individuals who do not respond to MTX or a TNF-α antagonist (Lotfi et al., 2019). Therefore, it is advisable to investigate targeted synthetic DMARDs as a standalone treatment or in conjunction with conventional synthetic DMARDs (Onishi & Gaffen, 2010).

2.2.2 NSAIDs and Glucocorticoids

NSAIDs and GCs are often used in combination with a basic treatment. NSAIDs are used to alleviate discomfort and inflammation in RA, however they do not possess the ability to mitigate bone and cartilage degradation (Shen & Gaffen, 2008). NSAIDs are often categorised into two distinct categories according to their chemical composition and selectivity. The first category comprises non-selective NSAIDs, which effectively block both cyclooxygenase-1 (COX-1) and COX-2. The second group consists of COX-2 specific inhibitors. COX-1 is involved in the maintenance of the lining of the gastrointestinal mucosa, the functioning of the kidneys, and the aggregation of platelets. On the other hand, COX-2 is produced specifically during an inflammatory reaction. Acetylsalicylate, naproxen, ibuprofen, and etodolac are the most frequently used NSAIDs for treating RA. In the past, NSAIDs were regarded as the primary treatment option. However, their poor efficacy in preventing the advancement of damage and the occurrence of side effects such as nausea, stomach discomfort,

ulcers, and gastrointestinal bleeding at high dosages hindered their widespread usage (Nakae et al., 2003).

Surgery serves as the ultimate therapeutic intervention for RA when the aforementioned nonsurgical modalities prove inadequate, a circumstance that is progressively diminishing in frequency. Currently, a range of surgical procedures are being used, including tenosynovectomy, radiosynovectomy, arthroscopy, osteotomy, and joint replacement. The primary objective of surgical intervention is to alleviate pain and restore optimal joint functionality (Blanco et al., 2017; Ikeuchi et al., 2005).

Among the aforementioned traditional therapies, DMARDs have shown a considerable capacity to mitigate disease symptoms and impede disease development in individuals diagnosed with RA. Nevertheless, these medicines are associated with substantial financial burdens and manifest grave adverse effects (Lin et al., 2020). Additional approaches strive to sustain illness remission or little advancement while also reducing the likelihood of therapy, hence exhibiting relative effectiveness. Approximately 20-30% of individuals with moderate-to-severe RA persist in experiencing clinically significant levels of residual pain while receiving therapy, hence demonstrating intolerant or resistant responses to various therapeutic interventions (Altawil et al., 2016; Singh et al., 2016). Stem cell-based therapy, with its ability to modulate and inhibit the immune system, has great potential in the treatment of RA.

2.3 Stem-Cell Based Therapy for the Treatment of RA

2.3.1 Mesenchymal Stem Cells in RA Treatment

Given the aforementioned evidence, it may be concluded that standard pharmacological interventions for the treatment of RA are insufficiently efficacious. This issue continues to be unresolved, necessitating the replacement of current therapeutic approaches with innovative ways aimed at restoring the functionality of immune cells. One of the innovative methodologies involves the use of mesenchymal stem cells (MSCs) in cellular treatment (Sarsenova et al., 2021; Shin et al., 2016). MSC-based therapy is rapidly recognising the potential of these entities because to their inherent capacity for self-renewal, migratory capabilities, potent immunosuppressive and immunomodulatory qualities, as well as their ability to facilitate tissue regeneration (Naji et al., 2019).

Adult MSCs have a morphology like that of fibroblasts. The potential therapeutic use of MSCs in the management of autoimmune disorders, such as RA, is being explored (Marinescu et al., 2021). Human MSCs are initially defined by their fibroblast-like appearance, which allows them to attach to

culture plastic and transform into mesodermal tissues including osteoblasts, chondrocytes, and adipocytes. Moreover, MSCs exhibit the expression of CD73, CD90, and CD105 cell surface markers, while lacking the presence of haematological and endothelial markers such as CD14, CD34, CD45, and HLA-DR (Dominici et al., 2006; Heo et al., 2016; Li et al., 2015; Mushahary et al., 2018). Different types of tissues, such as bone marrow (BM), gingiva, synovium, periosteum, adipose tissue (AT), dental pulp, umbilical cord (UC), and umbilical cord blood (UCB), serve as the origin of MSCs (K. Bieback & P. Netsch, 2016; De Bari et al., 2006; Gan et al., 2020; Gaur et al., 2017; Lanzillotti et al., 2021; Sun et al., 2019; Zupan, 2019). MSCs possess the capacity to undergo differentiation into various cell lines. Furthermore, MSCs exhibit the ability to regulate both innate and adaptive immune responses by mitigating the proinflammatory phenotype. This is achieved primarily through the reduction of populations of DCs, macrophages, natural killer (NK) cells, B and T cells, mast cells, and neutrophils. Additionally, MSCs promote an anti-inflammatory phenotype (Figure 1) (Fan et al., 2020; Luque-Campos et al., 2019; Luz-Crawford et al., 2016; Luz-Crawford et al., 2013). The immunomodulatory ability of MSCs is attributed to two main mechanisms: cell-cell contact and the production of soluble molecules (De Miguel et al., 2012).

Figure 1

Scheme of Immunomodulatory properties of MSCs (adapted from Sarsenova et al.)

Furthermore, Uccelli et al. (2008) demonstrated that MSCs have the capacity to migrate towards regions of inflammation. The cells have the ability to regulate the function of proinflammatory immune cells at the site of inflammation (Uccelli et al., 2008) of proinflammatory immune cells to the inflammation site.

Bernardo and Fibbe (2013) proposed that MSCs function as sensors in response to inflammation, depending on the inflammatory environment. Inhibition of pro-inflammatory cell activity and activation of anti-inflammatory cells of the immune system are facilitated by these features (Bernardo & Fibbe, 2013). When exposed to an inflammatory environment characterised by elevated levels of TNF-κ and IFN-γ, which are produced by immune cells, MSCs undergo activation and exhibit an anti-inflammatory phenotype. The activation of immunosuppressive mediators secreted by MSCs is influenced by pro-inflammatory cytokines generated by pro-inflammatory immune cells. Indolamine-2,3-dioxygenase (IDO), nitric oxide (NO), transforming growth factor β (TGF-β), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), hemeoxygenase (HO), COX-2, IL-6, and IL-10 are among the proteins that are included in this group. In response to the presence of a pro-inflammatory milieu, MSCs have the ability to inhibit the activation of DCs, M1-like proinflammatory macrophages, neutrophils, natural killer (NK) cells, T cells, and B cells. This activation leads to the creation of immune cells exhibiting anti-inflammatory characteristics (Fan et al., 2020; Luque-Campos et al., 2019; Luz-Crawford et al., 2016; Luz-Crawford et al., 2013).

The release of IDO is believed to be triggered by the inflammatory cytokine IFN-γ. The factor exerts its activity by the conversion of the crucial amino acid tryptophan to kynurenine, resulting in the disruption of cellular protein synthesis and subsequent inhibition of T cell proliferation. IDO is implicated in the production of Tregs and tDCs triggered by MSCs (Hong et al., 2016). In addition, MSCs generate many factors, one of which is iNOS. This enzyme stimulates the synthesis of NO from macrophages, hence impeding the proliferative, secretory, and cytolytic activities of T cells. In the process of immunosuppression, both soluble components have a role. Nevertheless, previous studies have shown evidence that iNOS is responsible for immunosuppression in mouse MSCs, but IDO has a comparable function in human MSCs (Ling et al., 2014). In conjunction with the continuous release of TGF-β by MSCs, the environment promotes the production of Tregs (Kalinski, 2012; Maffioli et al., 2017; Shin et al., 2017; Spaggiari et al., 2008; Vizoso et al., 2017; Wang et al., 2018).

Without an inflammatory environment characterised by low levels of TNF-α and IFN-γ, MSCs have the potential to assume a pro-inflammatory phenotype and augment T cell responses through the

release of chemokines such as MIP-1a and MIP-1b, RANTES, CXCL9, and CXCL10. These chemokines have the ability to attract lymphocytes to the specific locations of inflammation. Furthermore, these chemokines have the ability to bind to CCR5 and CXCR3 receptors that are expressed on T cells. When the pro-inflammatory phenotype is adopted, the levels of immune suppressive mediators, such as IDO and NO, are reduced (S. Liu et al., 2020).

The regulatory effects of MSCs on the functioning of both the innate and adaptive immune systems have been shown by several in vitro, preclinical, and clinical studies.

2.3.2 In Vitro Studies

B cells in RA undergo differentiation to generate RF and ACPAs, and then function as APCs to activate T cells (Volkov et al., 2020). Nevertheless, when AT MSCs were co-cultured with T cells, B cells, and Tregs, there was a significant doubling in the quantity of CD4+CD25+FoxP3 Tregs. In addition, MSCs decreased the release of TNF-κ by CD3+T cells, increased the synthesis of IL-10, and blocked the creation of ACPAs by B cells (Usha Shalini et al., 2017). In a separate investigation concerning the impact of MSCs on B cells, it was shown that MSCs had a substantial role in impeding the production of plasmablasts and exerting an influence on the differentiation of B cells. This effect was facilitated by the secretion of an IL-1 receptor antagonist (IL1RA) by MSCs (Luz-Crawford et al., 2016). According to existing literature, the impact of MSCs on B cells is facilitated by both direct intercellular contacts and the secretion of soluble substances by MSCs.

The immunosuppressive capacity of secretory factors produced by AT MSCs, which disrupt the Th17/Treg balance and promote Treg accumulation, as well as downregulate the production of major effector cytokines implicated in disease progression, has been extensively elucidated in an in vitro study conducted by Vasilev et al. (Vasilev et al., 2019). The production of TNF-κ, IL-1β, and IL-6 in mice macrophages primed with lipopolysaccharide (LPS) was downregulated by human AT MSCs via cell-cell contacts. Additionally, the proliferation of human primary T cells in response to mitogens was suppressed (L. Zhang et al., 2017).

In contrast, MSCs possess the capacity to impede the growth of effector memory T cells, which were seen in significant quantities within the peripheral blood and synovial fluid of individuals with RA. The effector memory T cells have the capability to release proinflammatory cytokines, including IFN-γ, IL-4, and IL-17. On the other hand, MSCs have the ability to regulate the immune response in RA by suppressing the growth of γε effector T cells and their production of inflammatory cytokines

(X. Liu et al., 2015). The modulation mechanism is regulated by PGE2, which is influenced by the presence of COX-2, which is secreted by MSCs (Martinet et al., 2009). The immunosuppressive action shown by MSCs may be attributed to the creation of a suitable inflammatory milieu by immune system cells that release pro-inflammatory cytokines.

Recent studies have extensively examined the therapeutic potential of MSCs in the treatment of RA using experimental animal models, with a particular focus on a collagen-induced arthritis (CIA) model in mice.

2.3.3 Preclinical Studies

Preclinical investigations have shown that the administration of allogeneic MSCs is more advantageous compared to the administration of autologous MSCs. For example, the injection of MSCs via intravenous (IV) routes has shown distinct advantages in facilitating a systemic distribution. This approach enables the cells to specifically target damaged tissue by leveraging the gradient of inflammatory cytokines (Cohn Yakubovich et al., 2017). Nevertheless, the administration of autologous MSCs systemically had a detrimental effect on the illness outcome in a well-defined CIA model in mice. Conversely, the administration of allogenic MSCs had more favourable outcomes, indicating that allogenic MSCs have a qualitative influence on the therapy of RA (Hwang et al., 2021).

Regarding the impact of MSCs on adaptive immune cells, the therapy yielded favourable outcomes. The presence of phagocytic, proinflammatory HLA-DR+ macrophages in the synovium of individuals with RA affects the activation of T cells and the subsequent migration and activation of B cells, leading to the development of an inflammatory response (Mulherin et al., 1996; Shapouri-Moghaddam et al., 2018; Udalova et al., 2016). In this context, MSCs exert influence on the polarisation of macrophages, hence regulating the equilibrium between pro-inflammatory and anti-inflammatory phenotypes. MSCs consistently generate IL-6, which, either alone or in conjunction with LPS and/or pro-inflammatory cytokines like IFN-γ, induces a shift in pro-inflammatory M1 macrophages towards anti-inflammatory M2 macrophages that produce IL-10. The secretion of elevated quantities of IL-10 and TGF-β1 by M2 macrophages serves to inhibit inflammation and facilitate the process of tissue regeneration (Bernardo & Fibbe, 2013). Polarisation is believed to be triggered by a confluence of intercellular contact mechanisms and the synthesis of soluble molecules, including IDO, PGE2, IL-10, and COX-2. UCB MSCs inhibited the growth of M1 macrophages and encouraged the generation of M2 macrophages via the activation of COX-2 and TSG-6 by TNF-α. In

addition, UCB MSCs suppressed the release of IL-1β and caspase-1 in macrophages via the IL-1β feedback loop in CIA mice, which is mediated by the nucleotide-binding domain and leucine-rich repeat pyrin 3 (NLRP3) inflammasome (Shin et al., 2016). Moreover, the activity of osteoclasts is increased in RA, leading to heightened bone degradation. Conversely, in individuals with good health, the activity of osteoblasts and osteoclasts is in equilibrium, allowing a typical equilibrium between bone creation and resorption (Steffen et al., 2019). In addition, pro-inflammatory macrophages emit TNF-κ and IL-1β, which stimulate synovial fibroblasts to release receptor activator of RANKL and M-CSF. These elements play a crucial role in the process of osteoclast development (Bozec et al., 2018). In relation to this issue, AT MSCs demonstrated inhibitory effects on both RANKL-induced osteoclastogenesis and the reduction of osteoclast precursors in the bone marrow, so effectively mitigating systemic bone loss in mice with CIA (Garimella et al., 2015).

DCs are a kind of cells found in the innate immune system that function as a proficient APC. These entities have a stellate form and demonstrate a notable expression of major histocompatibility complex (MHC) class II. Additionally, they possess the ability to internalise antigens and move towards lymph nodes for the purpose of priming naïve T lymphocytes (Worbs et al., 2017). Nevertheless, in RA, DCs also play a role in triggering inflammation by presenting antigens to autoreactive T cells, leading to the generation of cytokines that promote the development of Th cells. The formation of the pannus in RA is likely facilitated by the participation of circulating dendritic cells via chemokine signalling. Despite the absence of conclusive evidence, the pathophysiology of CIA has been linked to the involvement of many chemokine receptors that are expressed on DCs. The aforementioned receptors include CX3CR1, CCR9, CXCR4, and CCR2 (Worbs et al., 2017). Furthermore, another research shown that the treatment of FTY720, an immune-modulator derived from myriocin, effectively inhibited the migration of CCR7-mediated DCs towards draining lymph nodes. This intervention was found to improve CIA (Han et al., 2015). The method by which MSCs exert their effects on DCs is mostly understood via studies conducted on various autoimmune disorders. This understanding is based on the capacity of MSCs to impede the maturation process of DCs and promote the development of tDCs. This is achieved by lowering the activation of TLRs and regulating the synthesis of IL-12 by DCs (Shi et al., 2018). Furthermore, the combination of MSCs and tDCs has shown a synergistic immunosuppressive impact in a model of CIA via the polarisation of T cells, suppression of proinflammatory cytokine synthesis, and mitigation of cartilage degradation (R. Li et al., 2017).

T cells have a significant influence and play a pivotal part in the development of RA. MSCs possess the capacity to regulate the growth, specialisation, and functionality of T cells while diminishing the synthesis of pro-inflammatory cytokines. The regulating powers of MSCs have been verified by recent investigations. For example, several studies have shown that the use of MSCs effectively reduced levels of pro-inflammatory cytokines (IL-1β, IL-6), while increasing the production of anti-inflammatory cytokines (IL-10). In addition, there was a notable rise in the number of Tregs after the treatment with human UCB MSCs (L. Liu, H. P. Farhoodi, et al., 2020; Yu, Yoon, et al., 2019). The examination of CD4 T cell populations from mice with CIA after treatment with human embryonic stem cell-derived (hESC) MSCs improved CIA by stimulating IFN-γ+ Th1 cells and IDO1, and demonstrated an augmentation in the quantity of FoxP3+ Tregs (Gonzalo-Gil et al., 2016). In addition, the administration of hAT MSCs resulted in the proliferation of Tregs in both the peripheral blood and the spleen (L. Zhang et al., 2017). Furthermore, the introduction of hUC MSCs in a mouse CIA model effectively hindered the progression of the illness by diminishing the occurrence and capabilities of Tfh cells via IDO activity (R. Liu et al., 2015). Therefore, MSCs have the ability to inhibit the development of Tfh cells into effector subsets, including Tfh1, Tfh2, and Tfh17. Consequently, this suppression leads to a decrease in the generation of autoreactive antibodies.

The investigation of the pathogenic involvement of cytotoxic CD8+ T lymphocytes (CTLs) in RA has not been extensively explored. Nevertheless, empirical data suggests a clear association between the severity of RA and the quantity of cytotoxic CTLs present in the joint. Notably, a notable prevalence of CD8+ T cells has been documented at the site of inflammation (Petrelli & van Wijk, 2016). In addition, a recent research conducted by Vohra and colleagues has verified that UC MSCs have the ability to decrease the activities of activated CD4+ and CD8+ T cells seen in both the peripheral blood and synovial fluid of patients with RA. Furthermore, they have the capacity to inhibit the release of pro-inflammatory cytokines and promote the growth of Tregs. The administration of UC MSCs by intraperitoneal (IP) injection in CIA rats shown a consistent effect in terms of decelerating the advancement of the illness (Vohra et al., 2020). Research shown that providing hAT MSCs intravenously to mice with CIA resulted in a reduction in CD4+ T cells expressing GM-CSF in both the blood and spleen. These CD4+ T cells are recognised as crucial effector cells in the pathogenesis of RA (Lopez-Santalla et al., 2015). Furthermore, the aforementioned information supports the inference that RA is a disease generated by Th1/Th17 cells, and Tregs possess the ability to regulate inflammatory processes. MSCs play a crucial role in maintaining a balance between T cells

and Tregs, which is regulated by many soluble factors including IDO, PGE2, IL-10, NO, and HGF (Chen et al., 2020; Liu et al., 2010).

Therefore, it can be inferred that a substantial amount of data about the use and potential immunomodulatory impacts of MSCs in RA is now being studied. The extant study data exhibit variations among themselves as a result of diverse defining factors. Several factors play a crucial role in determining the therapeutic success of MSCs, including their origin (human or mouse), tissue source, method of administration, timing of therapy, number of repeats, dose, and the specific strains used (human or mouse). These factors have distinct impacts on the therapeutic outcome. Table 1 adapted from Sarsenova et al. provides a summary of the *in vivo* research conducted on the impact of MSCs on innate and adaptive immune cells, as detailed in this section (Sarsenova et al., 2021).

Table 1

In Vivo Studies on the MSCs Utilization and Their Effects on the Cells of Innate and Adaptive Immunity

RA Model	**Source and Tissue Origin of MSCs**	**Route of Administration /Number of Repetitions**	**Dosage**	**Mechanism of Action**	**Therapeutic Outcome**	**Reference**
CIA in DBA1 /J mice	hUCB MSCs	IP injections for 5 days after the RA score reached 3 or more	1×10^6 cells	hUCB MSCs polarized M1 macrophages toward M2 phenotype through TNF-α-mediated activation of COX-2 and TSG-6	Amelioration of the severity of CIA	(Shin et al., 2016)

RA Model	Source and Tissue Origin of MSCs	Route of Administration /Number of Repetitions	Dosage	Mechanism of Action	Therapeutic Outcome	Reference
CIA in DBA/ 1J mice	hBM MSCs	IP injection on day 22 after primary immunization	2×10^6 cells	hBM MSCs inhibited RANKL induced osteoclastogenesis	Amelioration of inflammation induced systemic bone loss in CIA	(Garimella et al., 2015)
CIA in DBA1 /J mice	hUC MSCs	IV injection on day 28 after RA score reached 1 or more	1×10^6 cells	hUC MSCs reduced number and downregulated function of Tfh cells in the spleen accompanied with decreased Th1 and Th17 cells	Prevention of CIA progression	(R. Liu et al., 2015)
CIA in DBA/ 1OlaHsd mice	hESC MSCs	Single-dose IP injection on the day of immunization (prophylaxis) or	1×10^6 cells	hESC MSCs increased the number of FoxP3(+) Tregs and IFN-γ+ Th1 cells but not Th17,	Reduction of disease progression and severity of CIA	(Gonzalo-Gil et al., 2016)

RA Model	Source and Tissue Origin of MSCs	Route of Administration /Number of Repetitions	Dosage	Mechanism of Action	Therapeutic Outcome	Reference
		with three doses of hESC MSCs every other day starting on the day of arthritis onset (therapy)		additionally induced the expression of IDO1 in inguinal lymph nodes		
CIA in DBA1 /J mice	hUCB MSCs	IV injection of three different doses every 2 weeks, overall, three times	1×10^6 cells, 3×10^6 cells, 5×10^6 cells	hUCB MSCs decreased IL-1β and IL-6 levels; concentration of 5_106 hUCB MSCs increased the level of IL-10 production and the expansion of Tregs	Alleviation of RA symptoms in a CIA model	(Yu, Yoon, et al., 2019)
CIA in DBA1 /J mice	hUC MSCs	IV injection after 24 days after RA induction	2×10^6 cells	hUC MSCs reduced the level of IL-6 by 80% 2 days after treatment and by 93.4% at the endpoint	Relief of RA Disease symptoms in a CIA model	(L. Liu, H. P. Farhoodi, et al., 2020)

RA Model	Source and Tissue Origin of MSCs	Route of Administration /Number of Repetitions	Dosage	Mechanism of Action	Therapeutic Outcome	Reference
CIA in DBA/ 1 mice	hAT MSCs	IV injection on day 28 after arthritis induction for the next five days	2×10^6 cells	hAT MSCs induced the expansion of Tregs both in the peripheral blood and spleen (in vivo); and downregulated the level of TNF-α, IL-1β and IL-6 in mouse macrophages and inhibited the proliferation of human primary T cells (*in vitro*)	Attenuation of Systemic inflammation in mice with CIA	(L. Zhang et al., 2017)
CIA in Balb/c mice	Murine BM MSCs	IV injection of MSCs and IP injection of IL-4 at day 21	5×10^6 cells	BM MSCs in combination with IL-4 treatment decreased the levels of RF, CRP and anti-nuclear antibodies; TNF-α and	Reduction of joint inflammation , synovial cellularity, vascularizatio n	(Haikal et al., 2019)

RA Model	Source and Tissue Origin of MSCs	Route of Administration /Number of Repetitions	Dosage	Mechanism of Action	Therapeutic Outcome	Reference
				monocyte chemoattractant protein-1 (MCP-1) levels. Additionally, BM MSCs decreased the levels of cartilage oligomeric matrix protein (Comp), tissue inhibitor metalloproteinase -1 (Timp1), MMP-1 and IL-1 receptor	and bone destruction in a CIA model	
CIA in female Wistar rats	hUC MSCs	IP injection on days 16 and 18	2×10^6 cells	hUC MSCs downregulated the functions of activated CD4+ and CD8+ T cells, suppressed the secretion of proinflammatory	Slowing down the progression of disease activity	(Vohra et al., 2020)

RA Model	Source and Tissue Origin of MSCs	Route of Administration /Number of Repetitions	Dosage	Mechanism of Action	Therapeutic Outcome	Reference
				cytokines and induced the expansion of Tregs		

Furthermore, there has been new research conducted on the potential therapeutic uses of apoptotic MSCs. In a research conducted by Galleu et al., it was shown that infused MSCs experience significant caspase activation and death when exposed to cytotoxic cells. This phenomenon is crucial for the immunosuppressive role of MSCs, as evidenced by both preclinical and clinical investigations (Galleu et al., 2017). The process is elucidated by the phagocytosis of apoptotic MSCs and the subsequent synthesis of IDO, which is essential for facilitating immunosuppression. Another group also observed similar findings. The action of MCSs is predicated upon the activation of caspase 3-mediated apoptosis generated by hypoxia, the recruitment of immune cells at the site of transplantation, and their subsequent engulfment by macrophages present in the local circulation (Preda et al., 2021).

Therefore, it can be inferred that a substantial amount of data about the use and potential immunomodulatory impacts of MSCs in RA is now being studied. The extant study data exhibit variations among themselves as a result of diverse defining factors. Several factors play a crucial role in determining the therapeutic success of MSCs, including their origin (human or mouse), tissue source, method of administration, timing of therapy, number of repeats, dose, and the specific strains used. These factors have distinct impacts on the therapeutic outcome.

2.3.4 Clinical Studies

In contrast to the favourable outcomes shown in preclinical investigations, the number of clinical trials investigating the use of MSC treatment in patients with RA is rather few. Nevertheless,

the safety and effectiveness of cell-based therapy have been substantiated via the completion of nine research. Furthermore, there are now thirteen ongoing clinical studies that are being investigated. The primary objective of clinical studies examining the therapeutic capabilities of MSCs derived from different tissue sources was to assess the safety and effectiveness of MSC transplantation in the context of RA. The first clinical trial was initiated in 2010 by Ra et al. (Ra et al., 2011). The researchers examined the safety and effectiveness of intravenous and intraarterial infusion of autologous AT MSCs in patients with RA. The participants were divided into three groups, each receiving varying doses of AT MSCs. The first cohort was given two distinct intravenous dosages of 3×10^8 AT MSCs. The second group had two injections of AT MSCs: (1) IV injection of 2×10^8 and IA injection of 1×10^8 AT MSCs into finger, wrist, elbow and knee joints; (2) IV injection of 3.5×10^8 and IA injection of 1.5×10^8 AT MSCs. The third group was given four intravenous injections of 2×10^8 AT MSCs at one-month intervals. The research findings indicate that autologous AT MSCs are both safe and effective in enhancing the clinical outcomes of individuals with RA.

For a comprehensive randomised multicenter clinical study, a total of 53 patients with refractory RA were enlisted to assess the effectiveness of various doses of allogeneic AT MSCs (Álvaro-Gracia et al., 2017). RA patients were categorised into three groups and administered IV injections of allogeneic AT MSCs at dosages of 1, 2, or 4×10^6 cells per kilogramme of body weight. These injections were given three times, with a one-week gap between each injection. The research findings revealed that intravenous administration of allogeneic AT MSCs led to a 2% clinical enhancement in 20-45% of patients with RA, as per the criteria set by the ACR, after one month, irrespective of the dosage of MSCs delivered. In 15-25% of RA patients who received MSCs, the therapeutic benefit remained after 3 months, but it did not continue in the placebo control group. The usage of MSCs was shown to be well tolerated, with no evidence of dose-dependent harm.

A randomised, triple-blind placebo-controlled phase 1/2 clinical trial was conducted from 2011 to 2013 to investigate the safety and tolerability of IA injection of autologous BM MSCs in patients with RA (Shadmanfar et al., 2018). The findings reported in 2018 indicate that the treatment of MSCs did not elicit any detrimental consequences in individuals with RA. Furthermore, it was found that patients who received intraarterial injection of BM MSCs exhibited better clinical outcomes compared to those in the placebo group. These outcomes were assessed using the Western Ontario and McMaster Universities Arthritis Index (WOMAC), visual analogue scale (VAS), time to jelling, and pain-free

walking distance for a duration of up to 12 months. According to the results, the authors propose that injecting BM MSCs into the knee is typically safe and well tolerated in patients with RA.

Ghoryani and colleagues recently conducted a successful clinical experiment to investigate the impact of intravenous infusion of autologous BM MSCs on the immunological, clinical, and paraclinical markers linked to the development of RA in patients with refractory RA (Ghoryani et al., 2019). They demonstrated that administering a solitary intravenous injection of 1×10^6 BM MSCs per kilogramme led to a significant reduction in the quantity of Th17 cells, disease activity score 28-erythrocyte sedimentation rate (DAS28-ESR), and VAS after 12 months of MSC treatment. Nevertheless, the administration of autologous BM MSCs had no notable impact on the levels of serum CRP and anti-CCP in patients with refractory RA. Collectively, the clinical findings indicate that the use of autologous BM MSCs has the potential to effectively improve the severity and activity of refractory RA.

A phase 1/2 clinical study was conducted in 2013 by a team of researchers to assess the safety and effectiveness of intravenous injection of allogeneic hUC MSCs in patients with active RA (Wang et al., 2013). The research included 172 individuals with RA who did not show improvement with traditional therapy. The patients in the control group were given culture media devoid of UC MSCs. The group of patients in the trial was given a solitary dosage of 4×10^7 UC MSCs. DMARD medication was administered to all cohorts of patients. The clinical study findings demonstrated that the treatment with UC MSCs did not cause any negative effects and led to the following clinical enhancements: a moderate decrease in inflammatory cytokines and chemokines, an elevation in the proportion of Tregs in the bloodstream, and an increase in the production of IL-4-producing Th2 cells. Furthermore, the ACR improvement criteria, the DAS28 score, and the Health Assessment Questionnaire (HAQ) demonstrated a notable remission of the illness. This remission was sustained for a duration of 3-6 months without the need for recurring intravenous injection of UC MSCs. Furthermore, an additional clinical investigation has provided evidence that the therapy of UC MSCs may provide enduring advantageous outcomes in patients with RA for a duration of up to three years (L. Wang et al., 2019). Therefore, the findings of this clinical study demonstrate that the intravenous injection of allogeneic UC MSCs in conjunction with DMARDs was both safe and efficacious in improving disease activity in patients with refractory RA, as compared to the control group that received culture media without UC MSCs.

Unlike the clinical trials stated above, researchers from KangStem Biotech administered a single intravenous infusion of 2.5×10^7, 5×10^7, or 1×10^8 allogeneic UCB MSCs to RA patients who had not previously been treated with any biologic medicines (Park et al., 2018). The results of a phase Ia clinical study indicated that there were no instances of significant adverse events or abnormalities in hematologic profiles seen in any of the patients during and after the therapy. The study found that administering UCB MSCs intravenously (1×10^8 cells per patient) effectively decreased the levels of inflammatory cytokines in the peripheral blood of patients with RA within 24 hours. Additionally, the average DAS28-ESR, HAQ, and VAS scores showed a decrease by week 4. Although there are certain limitations, such as a limited sample size and a brief follow-up period following the administration of UCB MSCs, the clinical evidence obtained indicates that a solitary high dosage of allogeneic UCB MSCs is both safe and efficacious in treating patients with refractory RA.

The aforementioned preliminary clinical investigations suggest that both autologous and allogeneic MSC transplantation are deemed safe and efficacious in the management of recalcitrant RA patients. There have been no significant negative effects seen in any of the individuals with RA throughout these clinical studies. The individuals who had MSC therapy exhibited a modest decrease in serum inflammatory markers, clinical amelioration, and notable disease remission. Furthermore, there have been reports demonstrating that the therapeutic benefits of MSC treatment for patients with RA may be sustained for a duration of up to 3 years, resulting in a consistent clinical outcome. This suggests that MSC-based therapy is both safe and effective in the long run. The latest concluded clinical trials have shown very encouraging outcomes; yet, it is crucial to take into account some significant factors (Lopez-Santalla et al., 2020). For example, a clinical trial conducted over a period of 24 months, involving the administration of BM or UC MSC infusion at a dosage of 1×10^6/1 kg, demonstrated a transient effect in three patients. However, one patient out of four did not exhibit any response to the EULAR response. This finding suggests that relying solely on single and low-dose administration may not be adequate for the treatment of rheumatoid arthritis.

Ultimately, there exist some constraints in the use of MSCs for the therapy of RA. Primarily, the majority of studies have focused on RA patients who were recruited from a single medical facility, sometimes without including a placebo control. Furthermore, there was a low rate of patient participation in several clinical studies aimed at assessing the safety and effectiveness. MSC-treated groups consisted of one or three patients in some instances. Hence, in order to validate the existing clinical evidence about the effectiveness of MSC treatment, it is essential to carry out a multi-center,

randomised study including a substantial number of patients with RA. Furthermore, at now, there is no established procedure for the best treatment of RA with MSCs. This is attributed not only to the disparity in MSC origins, but also to the varying methods of delivery, treatment protocols, and dosage used in the clinical trials. The presence of these variations in research designs has posed challenges in the comparison of therapy effects. However, the majority of clinical investigations have shown that the therapeutic effectiveness of MSCs is attained when administered at a dose of at least 1×10^6 cells per kilogramme of body weight, whether by single or repeated injections. While there is evidence of a dose-dependent association between MSC therapy and response, there is a lack of a well defined and efficacious therapeutic range for RA when using MSCs. Therefore, it is essential to conduct comprehensive investigations on treatment regimens and dose changes in forthcoming clinical trials. Furthermore, it is well acknowledged that MSC therapy incurs higher costs in comparison to DMARDs or biologics. Nevertheless, during an extended period of pharmacological treatment, a significant proportion of RA patients, ranging from 15% to 40%, acquire resistance to these medications and encounter a rise in the occurrence of side effects that have a negative impact on their well-being. Concluded clinical trials have shown that the administration of MSCs is a secure and efficient method for managing patients with RA and does not result in significant negative consequences. MSCs have the potential to serve as a therapeutic option for RA patients who do not respond to DMARDs. Furthermore, the use of cell preconditioning techniques to boost the immunomodulatory and anti-inflammatory characteristics of MSCs may not necessarily enhance therapeutic effectiveness. However, it might potentially decrease the manufacturing expenses associated with producing MSCs, which could be a valuable therapy option for patients with RA. Therefore, conducting future research using cutting-edge cell technology may play a crucial role in enhancing scientific knowledge about the effectiveness of MSC-based treatment.

The data presented suggest that MSCs have the potential to be effectively used in the management of autoimmune illnesses such as RA, owing to their immunomodulatory and immunosuppressive properties. Nevertheless, the inquiry about the extended therapeutic efficacy and the enduring functionality of these cells remains unresolved. In this context, researchers and medical professionals have focused on the matter of seeking a strategy to enhance the capabilities of MSCs and their subsequent utilisation in clinical settings (Hu & Li, 2018). Despite of the available of therapeutic effects of MSCs (Liu et al., 2019; Nam et al., 2018; Yu, Yoon, et al., 2019; Zhou et al.,

2011), the issue regarding the enhancement of therapeutic effectiveness of MSCs in the context of RA remains unresolved.

2.4 Strategies for Preconditioning of MSCs

2.4.1 Culture Condition Modification

There are several strategies to strengthen and restrain the immunomodulatory and anti-inflammatory effects of MSCs in RA. The well-described methods include (i) cytokines and factors, (ii) immune receptor agonists, (iii) culture condition modification, (iv) hypoxia, (v) autophagy, (vi) genetic modifications, and (vii) other agents for the improvement of the therapeutic potential of MSCs (Lee & Kang, 2020; Saparov et al., 2016; Sarsenova et al., 2022).

Miranda et al. (2019) suggested a strategy called 3D culture-based priming, which demonstrated the effectiveness of using conditioned medium/secretome from a 3D spheroid culture of hUCB-MSCs. The researchers observed that administering conditioned media obtained from 3D spheroid MSC culture inside the joint was more successful in suppressing joint inflammation and reducing the production of pannus, as compared to conditioned media produced from 2D MSC culture (Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019)(Miranda et al., 2019). A comparative analysis between the utilisation of spheroids and 2D conditions for cell culture revealed that the 3D conditions align with physiological conditions. Spheroids replicate the *in vivo* milieu for cells by creating favourable circumstances for both cell-to-cell and cell-to-matrix interactions, enabling proliferation in both directions. Multiple research have provided evidence indicating that the use of 3D models significantly enhances the immunomodulatory and anti-inflammatory capabilities of MSCs by promoting the production of TSG-6 and COX-2 factors (Bartosh et al., 2013; Yuriy Petrenko et al., 2017). Furthermore, it has been shown that 3D culturing yields a significantly elevated secretion rate of IL-6, IDO, TGF-β, and PGE-2 in comparison to 2D culturing. This finding serves as further support for the notion that the 3D culture environment has the ability to stimulate the immunomodulatory capabilities of MSCs (Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014)(Zimmermann & Mcdevitt, 2014). Although 3D spheroid culture offers many benefits for the treatment of RA, there exist some

technical constraints related to the integration, optimisation, and cost reduction of 3D MSC culture production and growth.

Lim et al. offered an additional approach that demonstrates how the effectiveness of cell treatment in RA may be enhanced by combining co-cultured MSCs and Tregs that generate IL-10. The implementation of this particular technique successfully mitigated the inflammatory response in the joints of mice, while also exhibiting a significant reduction in the likelihood of detrimental arthritis development when compared to the therapy using non-combined cells (Lim et al., 2016). Nevertheless, one may argue that while Tregs possess the ability to augment the immunosuppressive properties of MSCs, this approach is subject to certain constraints pertaining to the separation, propagation, induction, and secure utilisation of these regulatory cells.

2.4.2 Immune Receptor Agonists

A different method to enhance the therapeutic application of MSCs is to impact on the specific receptor proteins by targeting them with chemicals (agonists). Toll-like receptors (TLRs), especially TLR3 and TLR4, localized on the surface of MSCs can detect structurally conserved molecules of foreign agents, thus representing a potential target in the approach for increasing MSCs properties (Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016)(Contreras et al., 2016). Targeting these receptors can be performed by agonists ligation using polyinosinic:polycytidylic acid and lipopolysaccharides. TLR3 ligation activates the Notch signaling, stimulates Tregs production and Th1/Th17 cells dysfunction. Moreover, TLR3 stimulation participates in PGE2 secretion, which is one of the well-described immunoregulatory factor in MSCs (Kim, Lee, et al., 2018; Rashedi et al., 2017).

2.4.3 Other Agents

Moreover, recent noteworthy study has shown that the administration of caffeine to MSCs led to a reduction in the release of pro-inflammatory cytokines *in vitro*. In the rat arthritic model, the use of caffeine preconditioned MSCs resulted in a notable decrease in the production of CRP, NO, myeloperoxidase, and TNF-α, while significantly increasing the expression of IL-10 (Ghaffary & Froushani, 2020).

2.4.4 Autophagy and Hypoxia

Other promising approaches to improve the the rapeutic capacities of MSCs are autophagy and hypoxia. Autophagy, which is known as a mechanism that controls the elimination of dysfunctional or unnecessary cell components through lysosomes, preserves the cells from the products of metabolism as reactive oxygen species after irradiation or oxidative stress (Chen et al., 2016). As an option, the starvation or mTOR protein kinase inhibition can be used for autophagy stimulation. Priming by promoting hypoxia condition stimulates the secretion of IDO and PGE-2 immunomodulatory factors (Gorgun et al., 2021; Ogay et al., 2021; Rhijn et al., 2013; Xu et al., 2016; Z. Zhang et al., 2017). Additionally, the recent study evaluated the preconditioning by the combined application of hypoxia and IFN-γ pro-inflammatory cytokine (Wobma et al., 2018). MSCs preconditioning with hypoxia alone resulted in CD4+ and CD8+ T cell expansion. However, dual IFN-γ and hypoxia application decreased T cell activation and significantly upregulated HLA-G and IDO expression. Preconditioning with one of the mentioned options demonstrated a weak effect on MSCs, assuming that the dual application of hypoxia and pro-inflammatory cytokine has a synergistic effect.

2.4.5 Pro-Inflammatory Cytokines

An optional strategy to improve the therapeutic impact of MSCs is preconditioning by pro-inflammatory cytokines. The strategies of preconditioning MSCs with proinflammatory cytokines have proven to improve the therapeutic potential of MSCs by affecting their immunosuppressive properties including the production of anti-inflammatory molecules and increased homing to the site of injury (Baldari et al., 2017; Najar et al., 2018; Petrenko et al., 2017; Saparov et al., 2016; Zhou et al., 2017). The method relies on the phenomena that MSCs play the role of sensors to inflammation (Bernardo & Fibbe, 2013). In the inflammatory environment with a high rate of soluble pro-inflammatory cytokines, MSCs become activated and switch on anti-inflammatory mode by expressing a high amount of immunomodulatory regulators such as TGF-β, IDO, NO, PGE-2 and HO.

IFN-γ is an immunomodulatory cytokine that exerts its effects on both innate and adaptive immune responses via the induction of inflammatory processes, therefore safeguarding organisms against a range of diseases (Alspach et al., 2019). The synthesis of this substance is often limited to activated lymphocytes, such as CD4+ Th1 cells, CD8+ T cells, γι T cells, and NK cells (Burke & Young, 2019). Nevertheless, B cells, DCs, macrophages, and monocytes are also capable of secreting IFN- γ (Castro et al., 2018). The first research investigations have recognised the pivotal function of

this cytokine in the activation and alteration of the phenotype of MSCs, rendering it one of the extensively studied cytokines used for MSC preconditioning.

The exposure to IFN-γ at the molecular level results in the phosphorylation of STAT1/3 and the suppression of the ERK1/2-dependent mTOR signalling cascade in MSCs. Additionally, it triggers the nuclear translocation of pSTAT1, which subsequently enhances the expression of immunoregulatory genes in MSCs (Vigo et al., 2017). The research conducted by Kim et al. provided evidence that IFN-γ triggers the activation of the JAK signal transducer and STAT1 signalling cascade, resulting in the generation of IDO by MSCs (Kim, Jang, et al., 2018). Nevertheless, it is important to note that prolonged administration of IFN-γ might hinder the activity of STAT3, hence compromising the growth and specialisation of MSCs. This highlights the need for more investigation and modifications in its use.

Furthermore, recent research has shown that IFN-γ has the ability to enhance the production of HLAI/II and their associated proteins, while also promoting the release of immunosuppressive molecules such as IDO, TGF-β, PGE-2, CCL2, and HGF (de Witte et al., 2015). These modifications decrease the vulnerability of MSCs to NK cells. Preconditioned MSCs inhibit the generation of IFN-γ by NK cells via IDO and PGE2. Additionally, they safeguard themselves from NK cell cytotoxicity by enhancing HLA-I and decreasing NKG2D, the ligand necessary for NK cell activation (Noronha et al., 2019). Furthermore, it has been shown that secreted IDO has inhibitory effects on T cell proliferation and CD8+ T cell degranulation, hence indicating an increased level of protection against T cell cytotoxicity (Chinnadurai et al., 2016).

Wang et al. showed that IFN-γ priming increases the expression of HLA-G5 and IDO genes, as well as immunosuppressive factors, in MSCs derived from Wharton's jelly. This finding aligns with earlier research conducted on MSCs from other tissue sources (Wang et al., 2016). The findings of this study demonstrate that preconditioning with IFN-γ effectively inhibits the release of IFN-γ and TNF-α, while concurrently augmenting the synthesis of IL-6 and IL-10 in MSCs. Preconditioning also facilitates homing via the upregulation of chemokine ligands CXCL9, CXCL10, and CXCL11, as well as adhesion proteins, including vascular cell adhesion protein-1 (VCAM-1) and intercellular adhesion molecules-1 (ICAM-1). Furthermore, when IFN-γ primed MSCs are co-cultured with lymphocytes, it results in the suppression of Th1 and Th17 cell growth, while having no impact on Th2 and Treg populations in a laboratory setting. Furthermore, the administration of IFN-γ alone led to the stimulation of Tregs proliferation and functionality via the upregulation of immunomodulatory

cytokines (Chinnadurai et al., 2014). The observed alterations in the phenotypic of MSCs together demonstrate an enhanced immunosuppressive capability of these cells (Noronha et al., 2019; Park et al., 2021; Wang et al., 2016).

Therefore, our data unequivocally validate the significance of using IFN-γ for the activation of MSCs in order to enhance their therapeutic efficacy. It is crucial to acknowledge that the overexpression of HLAI/II by MSCs in response to exposure to IFN-γ may enhance the immunogenicity of MSCs, rendering them vulnerable to death by host immune cells subsequent to in vivo delivery (Burand et al., 2017).

TNF-α is a prominent pro-inflammatory cytokine that is predominantly present at sites of injury. It is recognised for its wide range of pleiotropic effects. Additionally, it plays a crucial role in regulating the expression of genes associated with cell survival, proliferation, differentiation, and migration through the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signalling pathway (Jang et al., 2021; Liu et al., 2017; You et al., 2021).

The expression of TNF-receptor (TNFR) 1 and 2 by MSCs leads to the activation of downstream IκB kinase proteins via the phosphorylation of IκBa, resulting in the release of NF-κB (Kaltschmidt et al., 2021). The upregulation of NF-κB target genes, such as VCAM1, CD44, and (MMP9, is facilitated by this mechanism. Additionally, it significantly enhances the migration capacity of MSCs and promotes MSC proliferation by upregulating cyclin D1, a protein gene associated with the G1-phase of the cell cycle (Kaltschmidt et al., 2021; Shioda et al., 2017; You et al., 2021). Furthermore, the process of TNF-α priming leads to the upregulation of immunosuppressive components IDO and PGE2, as well as an increase in the production of cytokines, including CXCL5, CXCL6, HGF, IL-8, insulin-like growth factor-1, and VEGF (Jauković et al., 2020). Putra et al. reported that the activation of MSCs by priming with TNF-α leads to the secretion of substantial quantities of TGF-b and IL-10, hence augmenting their immunosuppressive character (Putra et al., 2018). Animal tests have also detected and validated the anti-inflammatory priming effect of TNF-α. The clinical outcome was significantly improved by the topical administration of TNF-α-activated MSCs to mice with experimental allergic conjunctivitis. This was achieved through a reduction in IL-4 and TNF-α levels, as well as a decrease in the number of inflammatory immune cells and NF-kB p65 expression. These effects were observed through COX-2-dependent mechanisms (Su et al., 2015). These findings provide convincing evidence of the possible use of TNF-α in MSC treatment. Nevertheless, its impact

is much diminished when compared to IFN-γ priming (Noronha et al., 2019). Consequently, TNF-α is mostly used in conjunction with other cytokines at present.

IL-1β is a pivotal pro-inflammatory cytokine that is synthesised by several cell types, including innate immune cells like macrophages and monocytes. It plays a crucial role in the host's immunological response against a range of infections (Dinarello, 2018). Consequently, it underwent thorough investigation for MSC priming. Recent studies have shown that the pretreatment of IL-1β has a substantial impact on the immunomodulatory ability of MSCs and promotes their migration towards the site of inflammation. Liu et al. have shown that the activation of the NF-κB, TSG-6, and COX-2 pathways in MSCs by IL-1β is responsible for their heightened immunomodulatory capacity (Liu et al., 2021). Another research corroborated these results by demonstrating that IL-1β stimulates the COX-2-PGE2 signalling pathway in MSCs, leading to an upregulation in the production of PGE-2, SDF-1, and VEGF (L. Liu, Y. R. He, et al., 2020). A separate investigation revealed that the application of IL-1β as a priming agent led to an increase in the secretion of trophic factors and extracellular matrix adhesion factors. Additionally, it resulted in an upregulation of the secretion of angiogenic factors. Consequently, this process facilitated the migration ability of MSCs towards sites of inflammation by means of CXCR4 expression (Alvites et al., 2022).

Yao et al. revealed a distinct method wherein pretreatment with IL-1β leads to an upregulation of miR-21 expression in MSCs. then, these microRNAs are enclosed inside exosomes, which are then released to convert pro-inflammatory macrophages into M2 anti-inflammatory macrophages. These results are confirmed by both in vitro and *in vivo* tests (Yao et al., 2021). This demonstrates that MSCs preconditioned with IL-1β have the ability to have diverse effects on the immune system and effectively modulate inflammation via different pathways. In general, the findings presented in this study indicate that the use of IL-1β as a priming agent might serve as a viable strategy for the activation of MSCs, hence enhancing their anti-inflammatory and migratory properties.

IL-17 is a cytokine that promotes inflammation and is produced by different types of lymphocytes, such as CD4+ Th17, CD8+ Tc17, γδ T, NK cells, and ILC3s cells. It is released in response to external pathogens or injury and plays a role in tissue inflammation by stimulating the release of other cytokines and chemokines that promote inflammation (McGeachy et al., 2019). Upon interaction with its receptors, IL-17 initiates a cascade of signalling events, leading to the activation of many pathways including MAPK/AP-1 and NF-κB. This activation then affects the expression of

target genes, therefore elucidating the involvement of IL-17 in the processes of immune cell proliferation, differentiation, and migration (McGeachy et al., 2019).

Huang et al. conducted a molecular-level investigation to examine the involvement of IL-17 in the microenvironment of the bone marrow, which serves as the primary site of interaction with MSCs. Research indicates that macrophages in the bone marrow spontaneously generate GM-CSF upon direct interaction with Th17 cells, leading to the release of IL-17. The cytokine subsequently triggers the activation of TRAF6/Act and Rac-dependent Nox1 protein, leading to an augmentation in the generation of reactive oxygen species inside MSCs located in the bone marrow. This particular procedure is accountable for the hyperphosphorylation of MEK/ERK, which subsequently results in the proliferation, migration, and differentiation of MSCs. Hence, the use of IL-17 for priming MSCs may be regarded as an artificial method to replicate the natural process (Huang et al., 2009). As anticipated, the use of IL-17 for MSC preconditioning has shown to efficiently stimulate the growth of MSCs in a way that is dependent on the dosage (Noronha et al., 2019).

In contrast to IFN-γ pre-activated MSCs, the utilisation of IL-17 does not have an influence on the expression levels of HLA I/II and associated costimulatory components. This observation implies that the hypoimmunogenicity is preserved (Sivanathan et al., 2017). It is beneficial since MSCs are often administered either injection or transplantation. IL-17 treatment does not impact the morphology of MSCs or hinder the growth of MSC-co-cultured lymphocytes. However, it does stimulate the production of significant quantities of IL-8 and IL-6, and increases the expression of IDO, TLR3/4, and PD-L1 by MSCs. These factors play a role in enhancing the anti-inflammatory capabilities of MSCs (Du-Rocher et al., 2020). Significantly, IDO is a prominent immunomodulatory cytokine that protects MSCs from the harmful effects of NK and T cells, as previously stated (Chinnadurai et al., 2016; Noronha et al., 2019) and TLR3/4 and TLR3/4 are receptors that detect danger and trigger the release of cytokines by MSCs. Notwithstanding the aforementioned advantageous impacts of IL-17 on the immunomodulatory capabilities of MSCs, more investigation is necessary to address unforeseen safety apprehensions associated with its use. Recent research has shown that the administration of IL-17 induces the buildup of NO inside MSCs, hence increasing the expression of PD-L1 on their cellular membrane. This upregulation has the potential to transform MSCs into protumorigenic MSCs (Wang et al., 2020) and promote the process of osteogenic development (Liao et al., 2020), promoting some limitations for their use.

Du-Rocher et al. shown that the immunosuppressive effects of MSCs are enhanced by IL-17, not via direct mechanisms, but rather by facilitating greater proximity between MSCs and lymphocytes. In particular, IL-17 facilitates the migration of MSCs towards the site of inflammation via the upregulation of crucial migratory chemokines, including CXCL8 and CCL8, as well as metalloproteases such as MMP2 and MMP9 (Du-Rocher et al., 2020). In addition, the preconditioned MSCs enhance the proliferation of Tregs compared to Th1 cells by inhibiting the synthesis of Th1-associated cytokines such as IFN-γ, IL-2, and TNF-α. Remarkably, the immunosuppressive impact was strongly associated with an elevated level of IL-6 in the supernatant of primed MSCs with T cells, rather than with IDO, TGF-β, or COX-2 (Sivanathan et al., 2017). Therefore, our findings indicate that the process of priming MSCs with IL-17 is a very effective approach to enhance the effectiveness of MSC treatment while minimising the risk of immune response.

The investigation of pro-inflammatory cytokine mixtures as a supplementary approach to prime MSCs for improved therapeutic results is a subject of extensive research. For example, whereas the suppressive function and secretion of immunomodulatory factors (PGE2, ICAM-1, CXCL16) of MSC T cells may vary across different cell lines and passages, the co-administration of TNF-α and IFN-γ demonstrated the ability to mitigate this heterogeneity (Andrews et al., 2022). Barrachina et al. reported that the expression of IDO, iNOS, IL-6, COX-2, and VCAM-1 is upregulated by MSCs after combination treatment with TNF-α and IFN-γ (Barrachina et al., 2017). Additionally, it facilitated the preservation of the differentiating capacity and viability of MSCs (Huang et al., 2009). The synovial inflammation in the horse model with osteoarthritis was seen to diminish over a period of two months after the infusion of TNF-α/IFN-γ-primed murine MSCs. The treatment resulted in a rise in COL2A1 (collagen type II), cartilage oligomeric protein, aggrecan, MMP2, and TGF-b1, while causing a reduction in COX-2 and IL-1b, indicating an enhanced therapeutic outcome (Barrachina et al., 2018).

In addition, the concurrent administration of TNF-α and IFN-γ enhances the synthesis of IDO, which promotes the transformation of monocytes into immunosuppressive macrophages. This, in turn, leads to an augmented production of IL-10, ultimately suppressing the proliferation of T cells stimulated by CD3/CD28 (Domenis et al., 2018; Hagmann et al., 2016; Jauković et al., 2020). It is noteworthy that the administration of TNF-α and IFN-γ results in an augmentation of extracellular vesicle production by MSCs, hence exerting a dose-dependent inhibitory effect on T cell proliferation (Cheng et al., 2020).

The study conducted by Hackel et al. examined the preconditioning of MSCs using a combination of TNF-α/IFN-γ and IL-1β. The findings of the study indicate that the therapy effectively increases the expression of IFN-γR in MSCs, a process that is facilitated by the NF-κB signalling pathway. This enhances the sensitivity of MSCs to IFN-γ, therefore promoting the activation of the STAT5 and p38-MAPK signalling cascade, leading to heightened production of IL8 and recruitment of PMNs (Hackel et al., 2021). The results indicate that priming MSCs enhanced their capacity to react to lymphocytes that are summoned to the injury site during advanced stages of inflammation, perhaps leading to an immunosuppressive impact (Hackel et al., 2021; Petri et al., 2017; Vigo et al., 2017).

The effective preconditioning of MSCs with IL-1β and IFN-γ was observed. The result was an increase in the amounts of NO, IL-6, and PGE-2 release, which controlled the polarisation of macrophages and had an immunosuppressive impact (Philipp et al., 2018). The concurrent administration of IL-1β and IFN-γ also suppresses the growth of peripheral blood mononuclear cells. The MSCs that were primed exhibited an increase in the expression of COX-2 and IDO mRNA in comparison to MSCs that were preconditioned by just one of the cytokines (Yu, Yoo, et al., 2019). In the mouse model of colitis, the secretion of PGE-2/IDO resulted in the inhibition of Th1 differentiation and the promotion of Tregs development. These findings demonstrate the therapeutic potential of using IL-1β/IFN-γ for priming MSCs, as shown by the beneficial clinical results seen. Another research found that MSCs treated with IFN-γ and/or IL-1β reduced the growth of T cells, restricted the activation of macrophages and NK cells, and blocked the production of pro-inflammatory cytokines such IL-2, IFN-γ, and TNF-α. The preconditioning of MSCs with IFN-γ and TNF-α in various studies demonstrated an upregulation of immunomodulatory factors, including IDO and PD-L1. This upregulation resulted in the inhibition of T cell activity, an increased secretion of IL-10, and a shift in macrophage polarisation towards the M2 anti-inflammatory phenotype (Alvites et al., 2022).

In light of the limited available data, it is essential to conduct more research on the utilisation of pro-inflammatory cytokines and their combinations for preconditioning MSCs in order to enhance MSC therapy for the treatment of RA.

CHAPTER 1
METHODS

Isolation and Expansion of hUCB MSCs

The umbilical cord blood sampling was performed by an obstetrician at the National Scientific Center for Mothers and Children of Astana city after the informed consent of mother. Immediately after the birth of the child and cutting off the umbilical cord, the umbilical cord vein was punctured by additional puncture with a sterile syringe. Then blood in a volume of 40-50 ml was collected into a sterile disposable vacutainer containing an anticoagulant solution. The material was delivered at room temperature in a special shipping box.

At the next stage, under sterile conditions, hUCB MSCs were isolated by centrifugation in a density gradient using Ficoll-Paque® PLUS (Cytiva) according to the protocol (Karen Bieback & Philipp Netsch, 2016).

At the next stage, the cells were seeded and cell cultivation was carried out expanded in MSC NutriStem® XF culture medium (Sartorius) containing 2.5% FBS and 1% antibiotic-antimycotic solution (Gibco) according to standard methods in 25 cm^2 culture flasks in a CO_2 incubator (37ºC, 5% CO_2). After 72 hours, unattached cells were removed by thorough washing with PBS three times. When the monolayer reached 80% confluence, the cells were suspended using a mixture of 0.25% trypsin solution (Gibco) and dispersed into 75 cm^2 flasks. The nutrient medium was changed every 3 days.

UCB MSCs Characterization

MSCs showed an ability to form colonies from single cell by colony forming unit (CFU) assay, and to differentiate into adipocytes, osteoblasts, and chondrocytes. The analyses were performed according to the protocols (Ciuffreda et al., 2016; Pochampally, 2008). Immunophenotyping for CD105, CD73 and CD90 surface markers expression was performed by flow cytometry using BD StemFlow® Human MSC Analysis kit (BD Biosciences) following the instructions of the manufacturer. The data was analyzed by Attune NxT Flow Cytometer (ThermoFisher, USA), and processed using Attune™ NxT software. MSCs were used between passages three and six in all the experiments.

Cytokine Preconditioning of hUCB MSCs

To increase the immunosuppressive properties of MSCs, cells were seeded at 5000 cells/cm^2 in 100 mm Petri dishes. After 24 hours, the medium was changed with either no cytokines or supplemented with IL-17A (50 ng/ml) (Abcam), IL-1β (10 ng/ml) (Cusabio), and TNF-α (20 ng/ml) (Abcam) within 48 hours both individually and in various combination. Table 2 represents different concentrations of cytokines applied alone or in combination for MSC preconditioning.

Table 2

Pro-Inflammatory Cytokine Concentrations for hUCB MSCs Preconditioning

#	Group (cytokine alone or in combination)	Concentration (ng/ml)
1	Untreated control (UT CT)	-
2	IL-17A	50 ng/ml
3	IL-1β	10 ng/ml
4	TNF-α	10 ng/ml
5	IL-17A + IL-1β + TNF-α	50 ng/ml of IL-17A, 10 ng/ml of others
6	IL-17A + IL-1β	50 ng/ml of IL-17A, 10 ng/ml of IL-1β
7	IL-17A + TNF-α	50 ng/ml of IL-17A, 10 ng/ml of TNF-α
8	IL-1β + TNF-α	10 ng/ml of each cytokine

***In Vitro* Evaluation of the Immunosuppressive Properties of Cytokine-Preconditioned MSCs**

Enzyme-linked Immunosorbent Assay

For IL-6, TGF-β1, and IDO immunosuppressive mediators' identification, MSCs were primed with pro-inflammatory cytokines for 48 hours. Next, cells were cultured in the complete culture medium without cytokines for 24 hours. After 24 hours, we evaluated the secretion level of IL-6 and TGF-β1 proteins in cell culture supernatants and IDO level from lysed cells. Analyses were performed with commercial ELISA kits (BD OptEIA, Thermo Fisher, Abcam) according to the manufacturer's instructions.

Colorimetric Assay

For the determination of NO expression level, MSCs were cultured in the complete culture medium without cytokines for 24 hours after 48 hours of preconditioning. After 24 hours, we lysed

cells for the evaluation of NO, which is rapidly oxidized to nitrites and nitrates. These two molecules, in turn, are used to quantitate NO production. All the procedures were performed according to the manufacturer's instructions (Abcam).

RNA Isolation and qRT-PCR Analysis

For the quantification of immunomodulatory gene expression level, we isolated total RNA from MSCs after 48 hours of preconditioning and purified it using TRIZOL method. Next, we synthesized complementary DNA using High-Capacity RNA-to-cDNA Kit (Applied Biosystems) following the instructions of the manufacturer. In the last stage, we performed qRT-PCR using FAM fluorophore (Applied Biosystems) and primers for HGF, COX2, iNOS, IDO, IL-10, TGF- β, TSG-6, galectin-1, and IL-6. The Laboratory of Chemical Synthesis of National Center for Biotechnology kindly produced the primers (Astana, Kazakhstan). Table 3 indicates the primer sequences of the selected genes.

Table 3

Primer Sequences for Immunomodulatory and Immunosuppressive Genes of MSCs

Gene	Primer sequence (forward)	Primer sequence (reverse)
TGF-β	ACACCAACTATTGCTTCA	CTTGCGGAAGTCAATGTA
IDO	CTTGCCAAGAAATATTGC	CGTCCATGTTCTCATAAG
IL-6	CAACCTGAACCTTCCAAA	ACCTCAAACTCCAAAAGAC
TSG-6	CCATCTCGCAACTTACAA	GCTTCACAATGGGGTATC
Gal-1	CCTGAATCTCAAACCTGGA	GGTTGTTGCTGTCTTTGC
HGF	CATAATATGCTACTCGGACA	TTACGAGTGGCACATCTC

Stimulation of Macrophages and Co-Culture with Cytokine-Preconditioned MSCs

To evaluate the effects of cytokine-preconditioned MSCs on the secretory activity of innate immune system cells such as macrophages, the transwell assay was performed. For that, human monocytes of the THP-1 line (ATCC) were first differentiated into macrophages with the M0 phenotype by treatment with 100 ng/ml PMA (Sigma) on day 0. The next day, to activate the pro-

inflammatory M1 phenotype, M0 macrophages were treated with 100 ng/ml LPS (Sigma) and 40 ng/ml IFN-γ (Abcam). Next, 8×10^5 M1 macrophages were dispersed into the upper chamber of a 12-well plate with insert chambers, and MSCs at a concentration of 1×10^5 were dispersed into the lower well of the plate. After 25 hours of incubation, supernatants were collected and analyzed by ELISA to determine the level of human TNF-α secretion from macrophages.

Mixed Lymphocyte Reactions (MLRs)

To determine the effects of cytokine-preconditioned MSCs on T lymphocytes, MLRs was performed. For analyzing T cell proliferation, paired (from two different donors) peripheral blood mononuclear cells (MNCs) were isolated from human peripheral blood by density gradient centrifugation using Ficoll-Paque Plus (Cytiva). Next, MNCs from one donor were stimulatory and treated with Mitomycin C at a concentration of 10 mg/ml, and MNCs from another donor were responder and stained with Violet Proliferation Dye (BD) to further determine the number of proliferating T cells. At the next stage of the experiment, MSCs in an amount of 1×10^6, as well as stimulating and responder MNCs in an amount of 2.5×10^6 were scattered into the wells of a round-bottomed 96-well plate in AIM-V:M199 (1:1) complete nutrient medium (Gibco). On the third day, the nutrient medium was replenished with fresh one. On the fifth day of the experiment, supernatants were collected for human IL-10 and IFN-γ evaluation using ELISA (Abcam). Additionally, to determine the number of proliferating CD4+CD197+ T cells the flow cytometer analysis was performed using an Attune NxT. Experiments were performed in three independent replicates.

Collagen Antibody Induced Arthritis (CAIA) Model in Mice

In this study male Balb/c mice 6-8 weeks old were used. The animals were kept in vivarium conditions, with a 12-hour day/night cycle, at a temperature of 22-23ºC. Access to food and water was unlimited.

CAIA was induced in male Balb/c mice using a combination of monoclonal antibodies to type II collagen (Chondrex) according to the manufacturer's protocol (Maleitzke et al., 2022). On day 0 of the experiment, mice were given an intravenous injection of a cocktail of monoclonal antibodies in an amount of 1.5 mg. To achieve a severe course of the disease, on day 3, mice were additionally injected with lipopolysaccharide (LPS) from Escherichia coli (E. coli): O111:B4 (Chondrex, Inc.) at a concentration of 50 μg. In order to evaluate therapeutic efficacy, treatment was started only after

determining the clinical severity of CAIA on a 4-point scale (0-4) (Delgado et al., 2001). Shortly, the severity of arthritis was scored as follows: 0 - no detectable symptoms of arthritis (normal joint appearance); 1 - mild swelling and/or erythema confined to the toes or fingers, with slight joint stiffness; 2 - moderate swelling and erythema extending to the ankle or wrist, with noticeable joint stiffness and movement difficulty; 3 - severe swelling and erythema affecting the entire limb, with pronounced joint stiffness, significant movement impairment, and potential deformation; 4 - very severe swelling and erythema affecting the entire limb and beyond, loss of joint function, and severe pain. The observations were monitored by three independent examiners. Intact mice and mice with grades 3 and 4 RA were selected for preclinical research.

In Vivo Studies

Next, CAIA mice on day received two doses of systemic injections of unpreconditioned and preconditioned human umbilical cord blood MSCs within 3 days interval (Figure 2).

The mice were divided into the following groups:

1) negative control group (intact mice) (n = 3);

2) positive control group (mice with CAIA) (n = 4);

3) positive control group (mice with CAIA, which were intravenously injected with PBS) (n = 4);

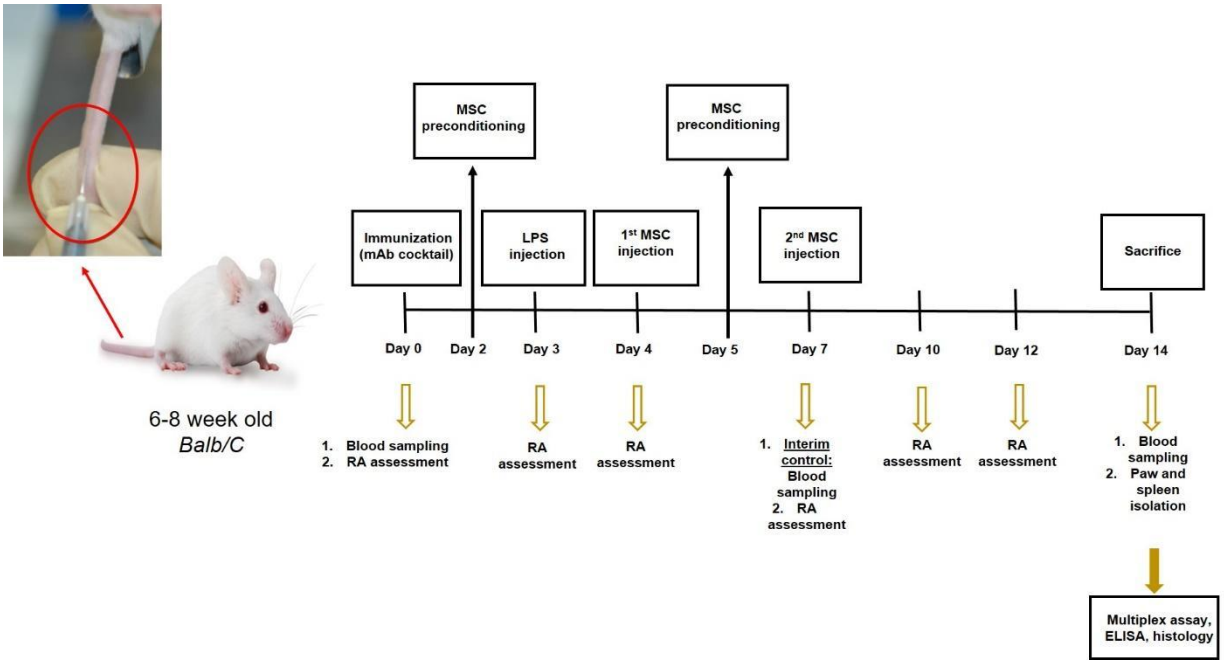
4) MSC group (mice with CAIA, which received an intravenous injection of 1.5×10^6 cells) (n = 7).

5) a group of cytokine-preconditioned MSCs (mice with CAIA that received an intravenous injection of 1.5×10^6 cells) (n = 7).

On days 0, 7 and 14, blood was taken from experimental animals for ELISA. All mice were then euthanized. Formalin-fixed limb specimens were prepared for histology according to standard procedures. Next, standard hematoxylin and eosin (H&E) and Safranin O staining was performed to assess cartilage destruction. Spleens were also removed from mice for lymphocyte isolation and subsequent analysis by flow cytometry (Figure 2).

Figure 2

Scheme of the Protocol for CAIA Induction in Mice and Systemic Administration of hUCB MSCs



The therapeutic efficacy of MSCs was assessed using ELISA, flow cytometry, histological and clinical analysis methods.

Flow Cytometry

Murine lymphocytes were isolated from homogenized mouse spleens. RBC contaminants were removed using hemolysis buffer (BD Biosciences). Next, 1×10^6 cells were stained with the corresponding antibodies to CD4 and CD25. Cytofluorimetic analysis was performed using AttuneNxT (ThermoFisher). Experiments were performed in three independent replicates.

Histological Analysis

The limbs, fixed with 10% formaldehyde, were decalcified within 10 days. The decalcified limbs were then embedded in paraffin blocks and cut using a microtome (SLEE) into thin sections 5 µm thick. Histological sections were further stained with H&E for general morphology and Safranin O for cartilage destruction . Inflammation assessment was based on inflammation (immune cell infiltration), joint destruction, and bone erosion, each on a scale of 0-4: 0 – no changes, 1 – minimal damage, 2 – mild changes, 3 – moderate damage, 4 – severe changes. Each section was scored independently by three blinded observers. The mean score for each histopathological feature was calculated for each animal. The overall mean histological score was determined by averaging the mean

scores of all animals within each experimental group. The images were taken using an AxioScopeA1 microscope (CarlZeiss) and processed using the ImageJ program.

Multiplex Assay

Multiplex assay was performed using Milliplex MAP Mouse High Sensitivity T Cell Magnetic Bead Panel (Millipore) according to the instructions of manufacturer. The principle of the method is based on flow cytometry instruments that integrate detection components. The test is based on the immunoassay technique, which involves using fluorescent-coded magnetic and non-magnetic bead microspheres coated with a specific capture antibody.

Briefly, after sacrificing mice, the blood was collected. The mouse serum samples were stained by multiplex immunofluorescence for 11 pro-inflammatory and anti-inflammatory markers, including GM-CSF, IF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12 (p70), MCP-1, TNF-α, IL-10, to evaluate the cytokine response in CAIA mice. For that, after an analyte from a test sample was captured by the bead, a biotinylated detection antibody was introduced. The reaction mixture was then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. Luminex 200 instrument was used to acquire and analyze data using flow cytometry-based detection method.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software. All experiments was replicated independently three times. Any statistically significant data was identified through two-tailed Student's t-test for the comparison of the data from two groups, or one-way and two-way ANOVA tests followed by Bonferroni's post hoc test for the comparison of the data from multiple groups.

Ethics Concerns

All procedures in the studies with human sampling were performed according to the ethical approval obtained from the IRB of National Scientific Center of Motherhood and Childhood (1/14112022). Informed consent was obtained from all participants involved in the study. Participants were provided with detailed information about the study objectives, procedures, potential risks, and benefits. Written consent was obtained, and participants were given a copy of the consent form for

their records. All data collected was kept confidential. Participation was voluntary, and participants were informed that they could withdraw from the study at any time without any consequences.

All experiments with animals were carried out only after the approval by Nazarbayev University IACUC (1/29092023).

CHAPTER 2

RESULTS

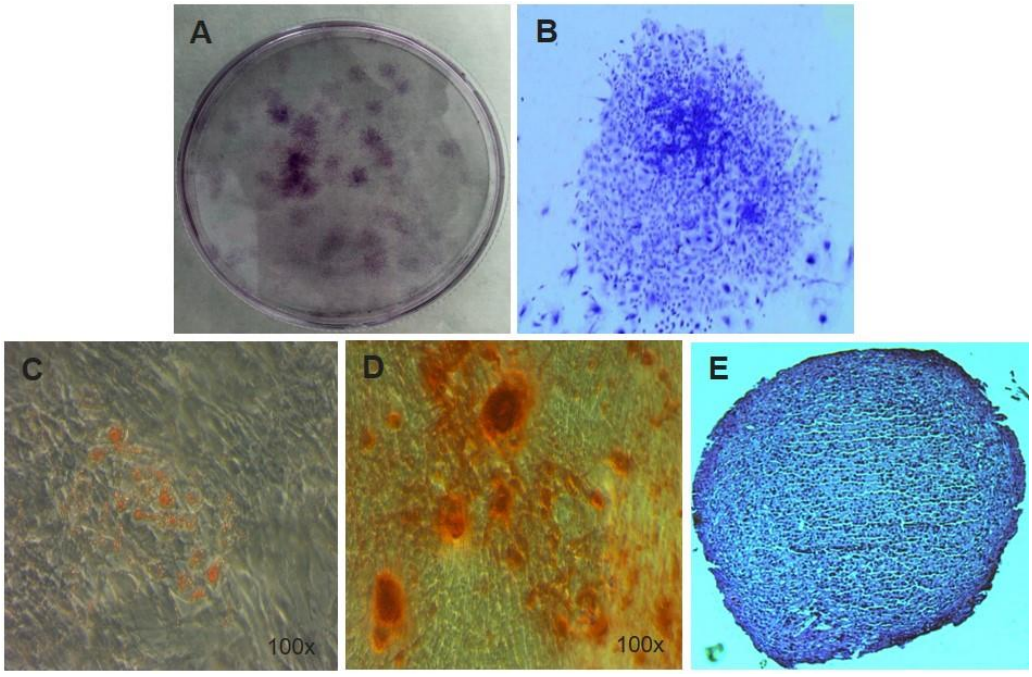
UCB MSCs Characterization

Multilineage Differentiation and the Formation of Colonies

Once we obtained a primary cell culture, we performed the analyses for characterization of hUCB MSCs. After isolation UCB MSCs, we confirmed the characteristic features of MSCs by the following tests: CFU-assay and multilineage differentiation assay. On day 14, crystal violet staining showed that cells were adherent to culture plastic and could generate colonies when plated at low densities. Figure 3A shows that 100 cells seeded in 100 mm Petri dish formed a number of colonies. Additionally, on day 21, multilineage differentiation test after functional staining demonstrated that UCB MSCs at passage 4 differentiated into adipocytes and osteocytes. Figure 3C represents adipocytes staining with Oil Red O, which determined the formation of lipid droplets like vacuoles. Alizarin Red staining, in turn, confirmed calcium deposition, and Toluidine Blue staining visualizes the formation of proteoglycans in the chondrogenic micropellets (Figure 3D-E).

Figure 3

UCB MSCs Proliferation and Differentiation Potential



Note. (A) The formation of MSC colonies stained with crystal violet; (B) enlarged representation of the colony; (C) adipocytes containing lipid vacuoles, stained with Oil Red O; (D) osteoblasts

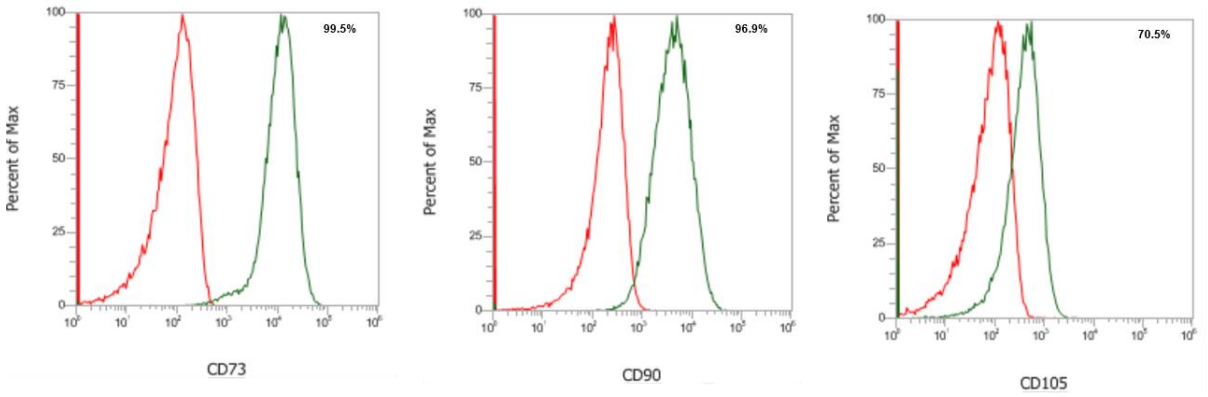
encapsulated with orange-red stained calcium deposits after Alizarin Red staining; (E) chondrogen micropellet, stained with Toluidine Blue.

Expression of MSCs Surface Markers

In the next stage of the MSC characterization, we evaluated the expression of characteristic surface markers such as CD105, CD73 and CD90 by flow cytometry. Figure 4 shows the expression of CD105, CD73 and CD90 markers at the level of 99,5%, 96,9% and 70,5%, respectively. Negative control included the markers for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR, which are expressed on the surface of hematopoietic stem cells, endothelial cells, monocytes, T and B cells, DCs, and NK cells.

Figure 4

UCB MSCs Surface Markers Expression



Note. UCB MSCs express CD73, CD90 and CD 105 (indicated by green peak). K-S D-value = 0.995, 0.969, and 0.705, respectively. Green peaks represent positive markers and red peaks represent isotype controls.

Cytokine-Preconditioned MSCs Express Immunomodulatory Genes

After isolation and characterization of hUCB MSC primary culture, we performed cell preconditioning with 50 ng/ml IL-17A, 10 ng/ml IL-1β and 10 ng/ml TNF-α pro-inflammatory cytokines for 48 hours. The factors were used separately and in various combinations (Table 1), and the untreated cells were used as a control group. After RNA isolation followed by cDNA synthesis, we performed RT-PCR for relative mRNA expression level.

TGF-β1, IDO, TSG-6, galectin-1, HGF and IL-6 are one of the well-described immunomodulatory factors produced by MSCs. Figure 5A shows that TGF-β expression in TNF-α,

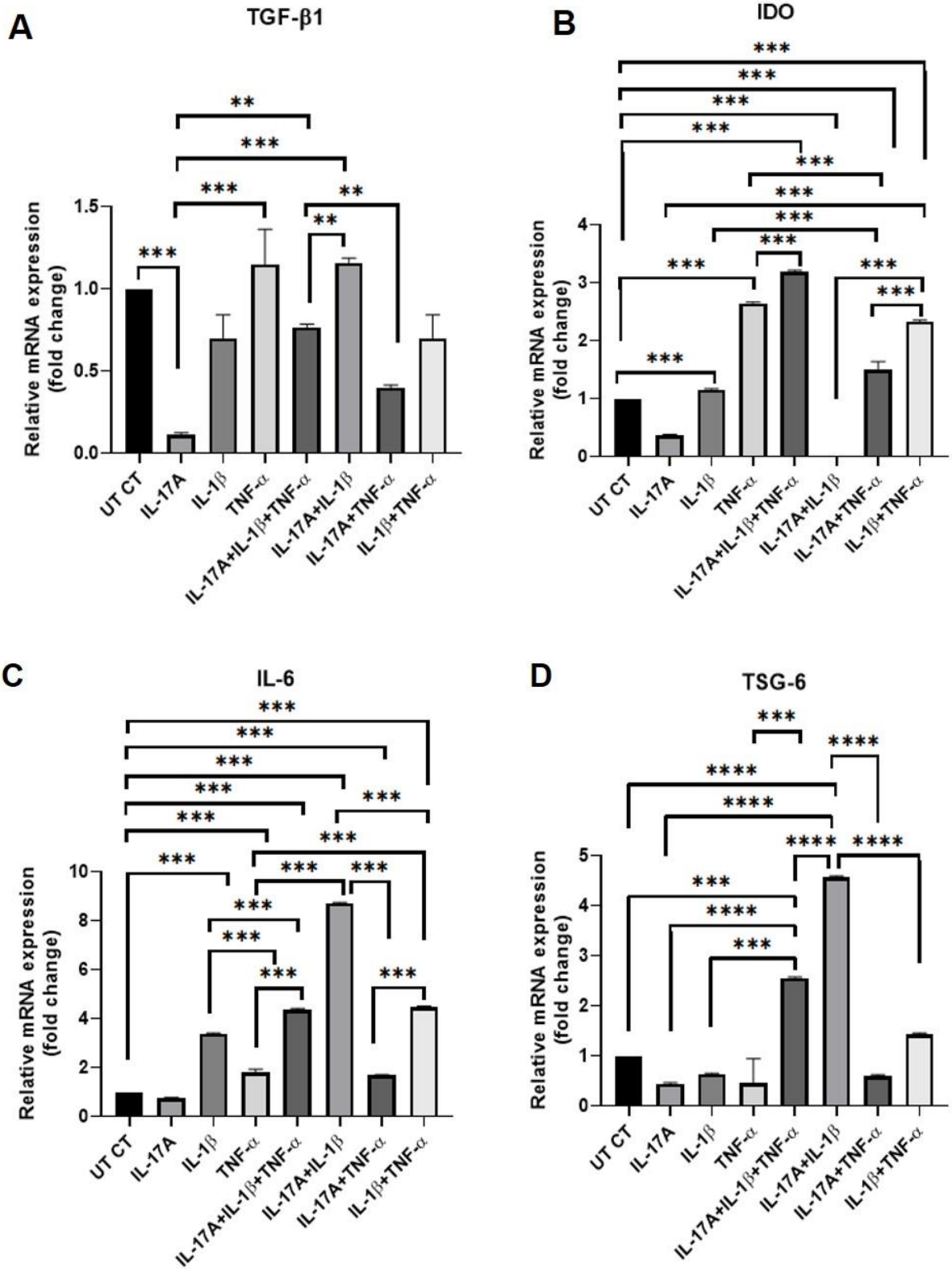
IL-17A+IL-1β and untreated control group did not have any significant difference confirmed by one-way ANOVA followed by Bonferroni's test with p = 0.4505. However, slightly higher difference in TGF-β expression was observed in the IL-17A+IL-1β preconditioning group. This varies from the protein expression evaluated by ELISA where the TNF-α priming showed significantly higher expression of TGF-β.

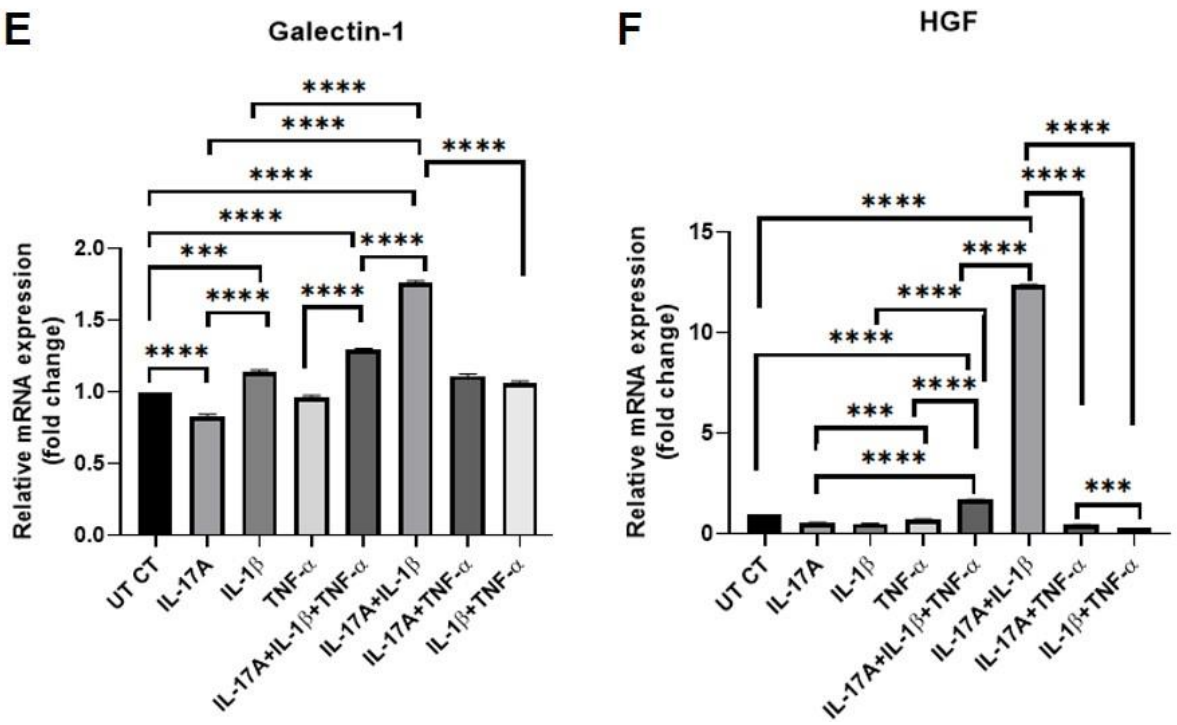
Next, Figure 5B represents that IDO mRNA expression was three fold higher in combination group compared to untreated MSCs with a significant difference at p < 0,0001, 95% CI [2,130 to 2,260]. The expression correlates with the IDO protein secretion. Additionally, there was significant difference in combination group compared to TNF-α treated group at p = 0.0015, 95% CI [0,4587 to 0,6413]. Interestingly, the cells primed with IL17A+IL-1β did not show any gene expression level.

Logarithm change was observed in IL-6 relative mRNA expression level. IL-6 was increased in the MSCs primed with IL-17A+IL-1β cytokines compared to untreated control group with a significance confirmed by two-tailed Student's t-test at p < 0.0001, 95% CI [8,644 to 8,816] (Figure 5C). We identified similar trend in TSG-6 gene expression. Figure 5D demonstrates that the preconditioning with IL-17A+IL-1β increased the relative mRNA synthesis almost five times (p < 0.0001, 95% CI [3,510 to 3,640]).

Figure 5

Immunomodulatory Gene Expression by Cytokine-Preconditioned MSC


A
TGF-β1
Relative mRNA expression (fold change)
UT CT
IL-17A
IL-1β
TNF-α
IL-17A+IL-1β+TNF-α
IL-17A+IL-1β
IL-17A+TNF-α
IL-1β+TNF-α
B
IDO
C
IL-6
D
TSG-6



Note. qRT-PCR for the relative mRNA expression level of (A) TGF-β, (B) IDO, (C) IL-6, (D) TSG-6, (E) Galectin-1, and (F) HGF in hUCB MSCs after 48h preconditioning with pro-inflammatory cytokines. Error bars represent the means ±S.D. from three separate experiments (n = 3). One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; 95% CI of difference.

Galectin-1 and HGF mRNA expression was also increased in the MSCs preconditioned with IL-17A+IL-1β treatment group (Figure 5E-F). One-way ANOWA followed by Bonferroni's test demonstrated significant difference in 17A+IL-1β group compared to untreated cells for both genes with p = 0.0002, 95% of CI [0,7170 to 0,8030] and p < 0.0001, 95% of CI [11,34 to 11,42], respectively. Additionally, galectin-1 and HGF expression was significantly higher in IL-17A+IL-1β group compared with combination group with p = 0.0009, 95% of CI [0,4092 to 0,5308] and p < 0,0001, 95% of CI [10,62 to 10,77], respectively.

Thus, the amount of relative mRNA was at a higher level in five out of six genes. These results suggest that IL-17A+IL-1β group can be selected as an optimal treatment for MSC preconditioning.

Cytokine-Preconditioned MSCs Secrete Immunomodulatory Molecules *In Vitro*

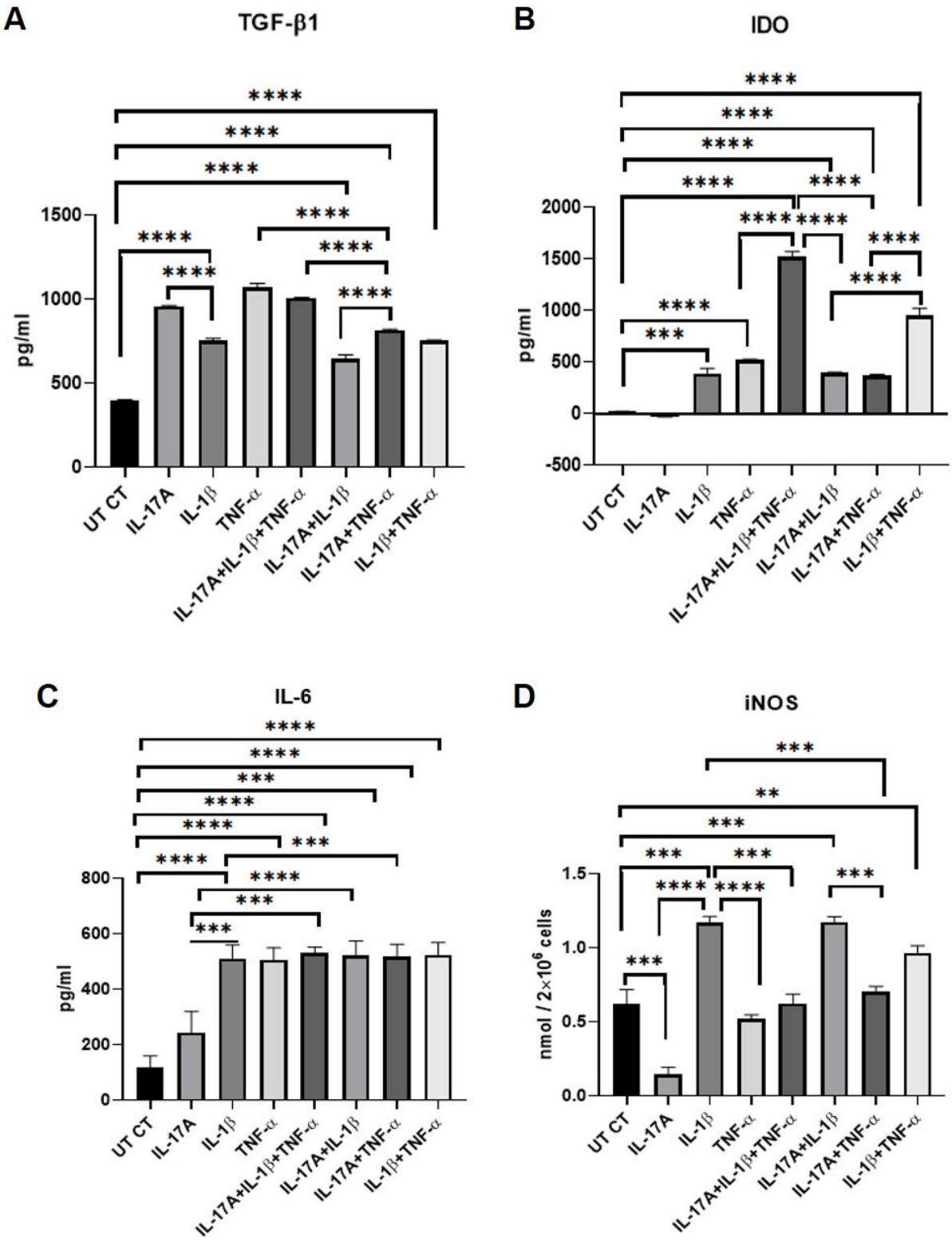
To evaluate the impact of cytokine preconditioning on the secretion of immunomodulatory factors, we collected the cell culture supernatants for the assessment of TGF-β1 and IL-6 expression level. Cells were detached from the culture plastic and further lysed for the evaluation of NO and IDO levels. Figure 6A shows the expression level of TGF-β1 after 48-hours preconditioning of hUCB MSCs. The treatment with TNF-α alone resulted in the highest expression of TGF-β1 with the concentration over 1000 pg/ml. These findings produced a statistically significant interaction between the TNF-α and the untreated control group, which were confirmed by one-way ANOVA test, $p = 0.0006$, 95% CI [605,4 to 748,4]. Interestingly, the impact of all three cytokines lead to the similar expression of the immunomodulatory factor and was not significant compared to TNF-α treatment at $p = 0.0563$, 95% CI [-129,9 to 4,193]. Additionally, in both of the preconditioning groups the expression of TGF-β1 was twice as high compared to the untreated control group. The lowest level of immunomodulatory factor expression showed the IL-17A+IL-1β group of preconditioning.

Next, we performed the ELISA for the IDO immunoregulatory factor. Figure 6B demonstrates that the highest concentration of IDO was after the combination of three pro-inflammatory cytokines together, IL-17A+IL-1 β+TNF-α, for hUCB MSCs preconditioning. The expression level was about 1500 pg/ml and was significantly higher compared to untreated MSCs. The statistically significant result was revealed with the $p = 0.0006$, 95% CI [1345 to 1653]. Additionally, IL-1β+TNF-α preconditioning also was also statistically reliable with the value of $p = 0.0024$, 95% CI [740,0 to 1131]. In contrast, the IL-17A treatment group did not show any expression of IDO.

In the next stage of optimizing the preconditioning with cytokines, we analyzed the level of IL-6 expression (Figure 6C). Cytokine priming lead to high levels of IL-6 secretion in the presence of almost all the pro-inflammatory cytokines, except of IL-17A group. The one-way ANOVA followed by Bonferroni's test showed statistically significant difference between all the treated groups compared to untreated control with $p < 0.0001$. Additionally, the statistical tests did not showed any significant result between the treated groups with $p = 0{,}9844$. However, slightly higher IL-6 concentration was in the combination group. Finally, priming separately with IL-17A showed the least level of IL-6 expression at the concentration of 300 pg/ml. However, the expression was not significant with $p = 0.0641$, 95% CI [-11,89 to 264,6]. In the final stage of immunomodulatory factors expression by preconditioned MSCs, we evaluated the NO production (Figure 6D).

Figure 6

Immunomodulatory and Immunosuppressive Molecules Secretion by Cytokine-Preconditioned MSCs



Note. ELISA for the secretion level of (A) TGF-β1, (B) IDO, (C) IL-6, and (D) iNOS in hUCB MSCs after 48h preconditioning with pro-inflammatory cytokines. Error bars represent the means ±S.D. from

three separate experiments (n = 3). One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*p < 0.05, **p <0.01, ***p < 0.001, and ****p < 0.0001; 95% CI of difference.

The highest amount of nitrates and nitrites was after IL-17A+IL-1β priming. Single IL-1β preconditioning also resulted in a higher NO production, which is equal to IL-17A+IL-1β level, with the concentration of 1.1 nmol compared to the untreated cells. Bonferroni's test indicated that the difference between two groups was not significant with the value of p = 0.9098, 95% CI [-0,1630 to 0,1730]. Additionally, statistical test for the comparison of IL-17A+IL-1β and IL-1β priming with untreated control group showed that the data was significant with the specified p = 0.0069, 95% CI [-0,8283 to -0,2817] and p = 0.0071, 95% CI [-0,8233 to -0,2767], respectively.

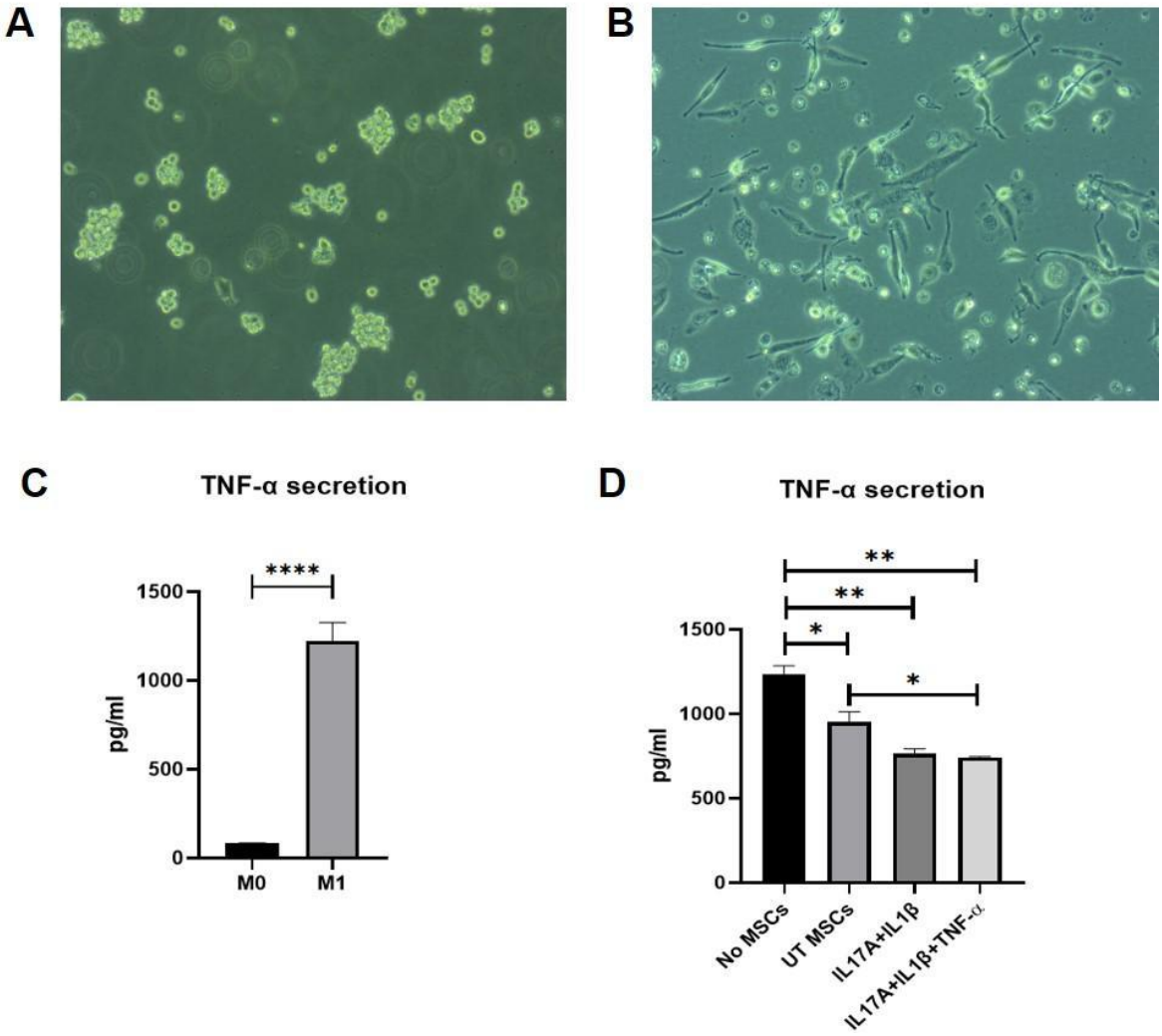
Preconditioned MSCs Co-Cultured with Macrophages Suppress their Secretory Activity

After 24 hours of PMA treatment, THP-1 derived M0 macrophages were observed (Figure 7A-B). Following that, as shown in Figure 7B, in order to activate pro-inflammatory phenotype of macrophages, on day 3, IFN-γ and LPS exposure activated M1 phenotype. The secretion of TNF-α in M1 macrophages was significantly higher at the level of 1300 pg/ml compared to M0 macrophages (p < 0.0001) (Figure 7C).

At the next stage, to evaluate the secretory activity of M1 macrophages co-cultured with preconditioned MSCs, the transwell assay was performed. Subsequent ELISA after 24-hour co-cultivation of macrophages with preconditioned MSCs in two different combinations (IL-17A+IL-1β and IL-17A+IL-1β+TNF-α) showed that the treatment of MSCs with a combination of three factors reduced the secretion of human TNF-α by almost three times compared to untreated MSCs (p = 0.0406) (Figure 7D).

Figure 7

Activation of Macrophages M1 Phenotype and the Secretion of TNF-α



Note. (A-B) Differentiation of THP-1 monocytes toward M0 macrophages; (C) activation of M1 phenotype; (D) the secretion level of human TNF-α. Error bars represent the means ±S.D. from three separate experiments (n = 3). Unpaired t test was used to used for the comparison of the data between two groups. One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*p < 0.05, **p <0.01, ***p < 0.001, and ****p < 0.0001; 95% CI of difference.

Preconditioned MSCs Co-Cultured with T-lymphocytes Suppress their Proliferative Activity

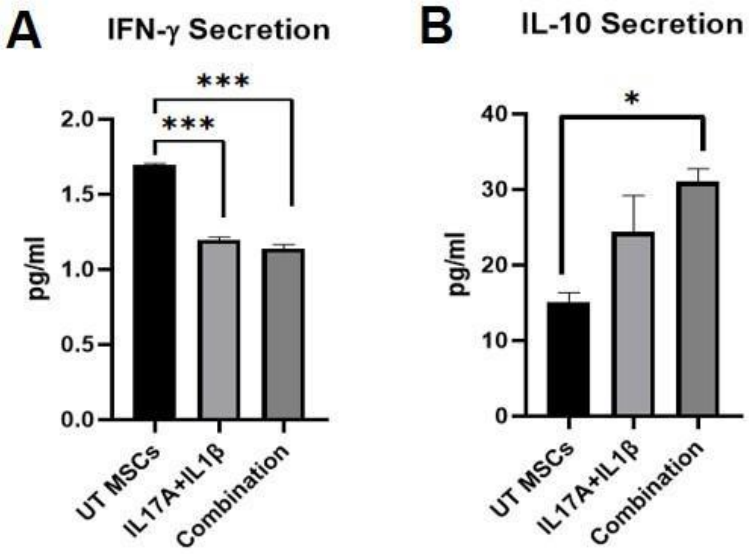
Subsequently, in order to examine the activation patterns of T cells in the MLR experiment, we analysed the T cell activation indicator, the levels of pro-inflammatory and anti-inflammatory cytokines in the supernatants on the fifth day of the experiment.

Differences in the levels of pro-inflammatory and anti-inflammatory cytokines are obvious and indicate that both groups of preconditioned MSCs suppress IFN-γ secretion in T lymphocytes to an almost equal extent (p = 0.101) compared to untreated MSCs (p = 0.001) (Figure 8). Likewise, IL-10 production was significantly increased in the IL-17A+IL-1β+TNF-α-treated MSC group (p=0.009).

In addition, to evaluate the differentiation of T cells, we analyzed the expression of CCR7 and CD45RA markers using flow cytometry. On day 5 of the experiment, in the group with non-preconditioned MSCs (negative control), the level of expression of CD45RA-CCR7+ naive T cells was 37.1% of the entire population of CD4+ T cells, while in the group of preconditioned MSCs with three cytokines (IL-17A +IL-1β+TNF-α), CD45RA-CCR7+ was only 10.2%. It is interesting that in the group of MSCs treated with IL-17A+IL-1β, the number of CD45RA-CCR7+ was higher and amounted to 49.5% (Supplementary Figure 4).

Figure 8

IFN-γ and IL-10 Secretion Level in Co-Cultures of PBMC with MSCs



Note. ELISA results from supernatants of co-cultured PBMC with MSCs. Error bars represent the means ±S.D. from three separate experiments (n = 3). One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*p < 0.05, **p <0.01, and ***p < 0.001; 95% CI of difference.

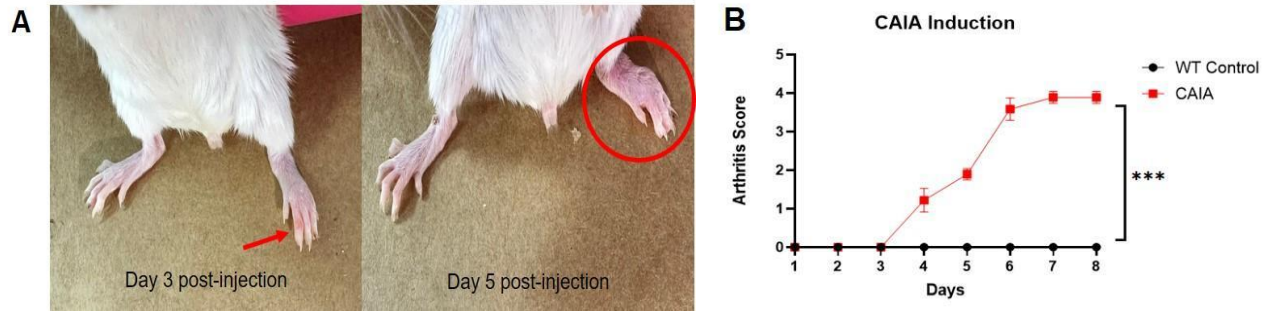
Thus, the results of MLR analysis demonstrated that the combination of IL-17A+IL-1β+TNF-α for MSC preconditioning has led to the optimal enhancement of the immunomodulatory and immunosuppressive properties of the cells.

Systemic Delivery of Preconditioned hUCB MSCs Exerts Therapeutic Effect Against Mouse CAIA Model

Based on the outcomes of in vitro studies, we chose a combination of three cytokines for subsequent animal studies. At the first stage of the study, experiments on the induction of CAIA in Balb/c mice were performed. Groups of mice injected with a cocktail of anti-collagen monoclonal antibodies developed inflammatory arthritis after LPS administration with a three days interval (Figure 9). RA developed in 90% of mice within 24-48 hours after LPS injection and reached its maximum within 6-7 day.

Figure 9

Establishment and Assessment of Clinical Severity of RA Model in Balb/c Mice



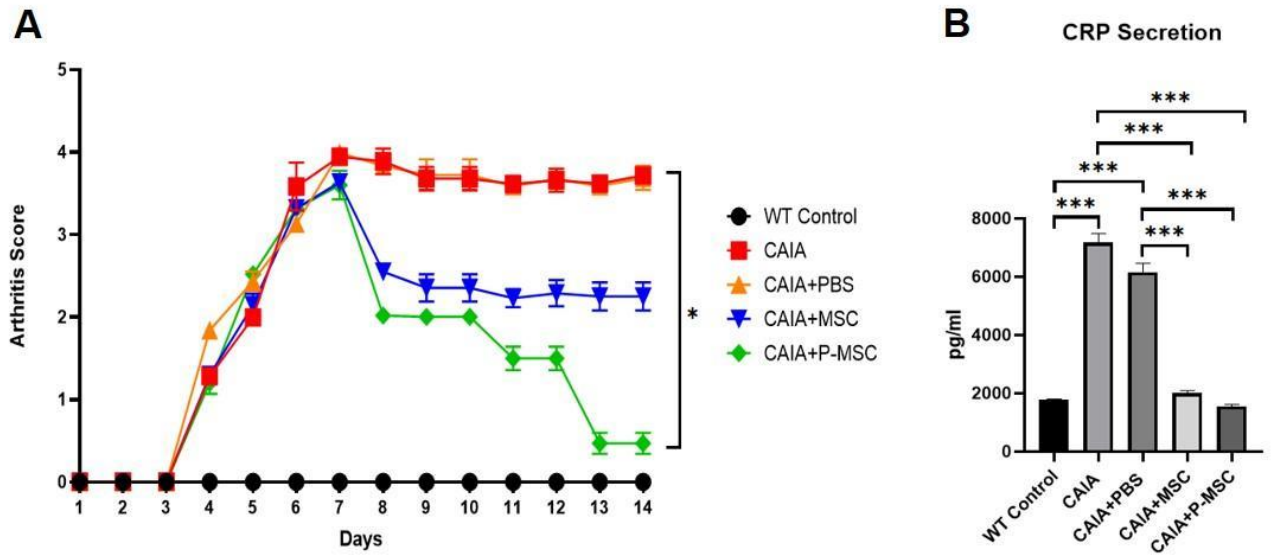
Note. (A) Joint inflammation developed on day 3 following antibodies injection and on day 5 after LPS administration; (B) arthritis score reflecting joint swelling in CAIA mice. Clinical severity was graded from 0 (no swelling) to 4 (erythema and severe swelling of the entire ankle joint). Error bars represent the means ±S.D. from three separate experiments (n = 3). Two-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple mice groups.*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; 95% CI of difference.

MSCs significantly reduced the degree of inflammation in mice with CAIA model (Figure 10A-B) compared to mice that did not receive any treatment. Figure 10A demonstrates that the arthritis score of CAIA mice treated with non-preconditioned MSCs underwent significant changes starting from day 5, remained stable until day 10, and subsequently decreased. On the other hand, the arthritis score of CAIA mice injected with preconditioned MSCs experienced a sharp decrease starting from day 4 and continued to decrease until reaching a stable level from day 10. The RA scores of untreated CAIA mice and mice treated with PBS were relatively constant at a level of 4 throughout the duration of the experiment.

These findings also align with the release of CRP. Following the introduction of MSCs into the tail vein, the mice who were given preconditioned MSCs had the lowest protein level (1800 pg/ml) compared to the untreated group and the group who received PBS (Figure 10B) ($p < 0.0001$). Following the intravenous delivery of non-preconditioned MSCs in a group of mice with CAIA, a comparable therapeutic outcome was seen, demonstrating minimal distinction from the group treated with MSCs.

Figure 10

Evaluation of Clinical Severity of CAIA in Mice Treated with hUCB MSCs and Untreated Animals



Note. (A) Mean arthritis score in mice for the control and experimental groups ; (B) secretion of CRP from mouse blood serum with CAIA model. Error bars represent the means ±S.D. from three separate experiments (n = 3). Two-way (for panel A) and one-way ANOVA (for panel B) tests followed by Bonferroni's post hoc test were used for the comparison of the data from multiple mice groups.*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; 95% CI of difference.

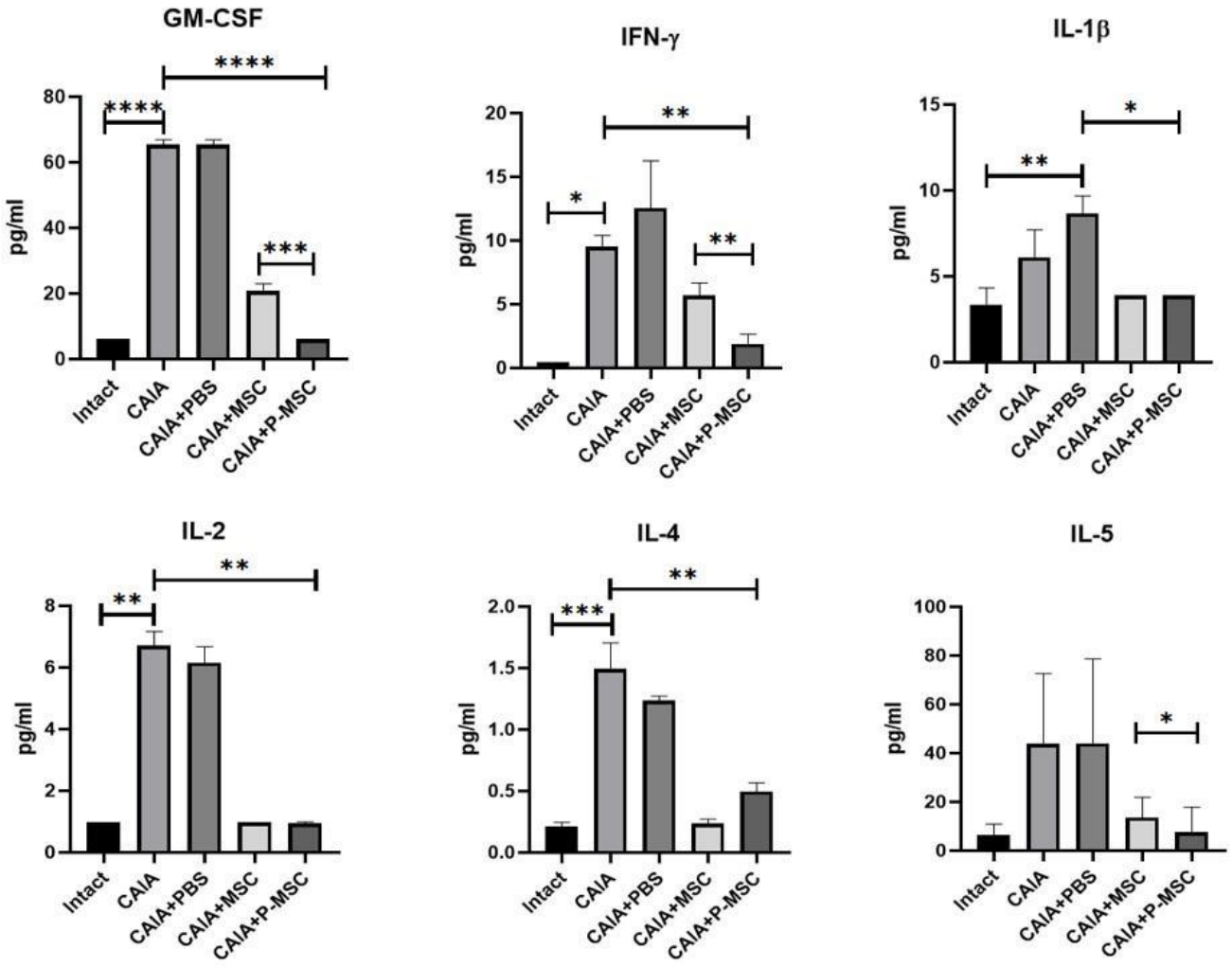
Multiplex Assay

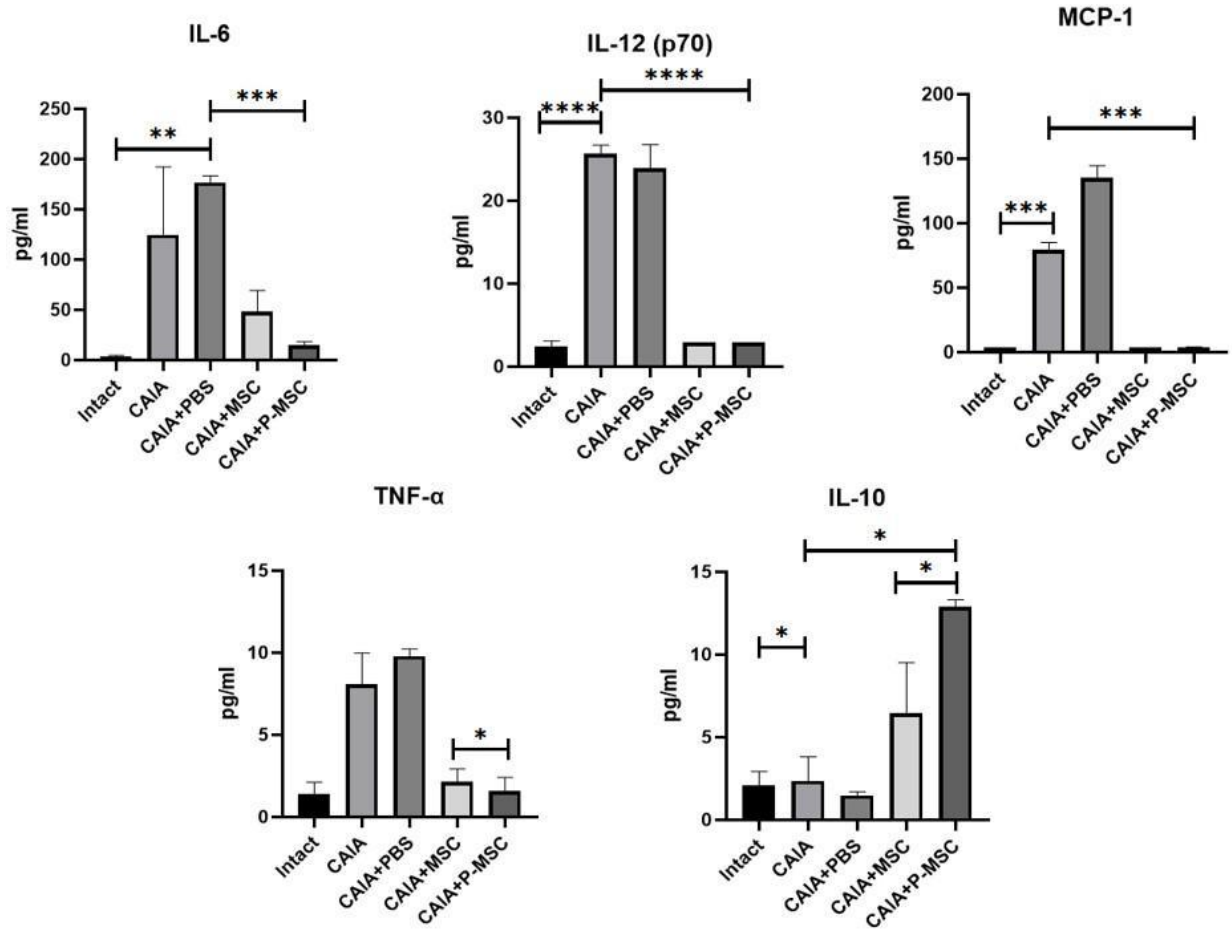
Following the assessment of clinical severity in CAIA mice, the serum samples were subjected to analysis using a 16-plex assay, and the resulting data were subjected to statistical analysis. The performance characteristics of the multiplex test for each pro-inflammatory cytokine and one anti-inflammatory cytokine were determined based on the results. Figure 11 demonstrates notable differences in the levels of eight inflammatory cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6,

IL-12 (p70), and MCP-1 and one anti-inflammatory cytokine (IL-10) produced by T cells across the test groups ($p < 0.05$).

Figure 11

Results of the Inflammatory and Anti-Inflammatory Cytokine Multiplex Assay





Note. There were significant differences among the five groups with respect to cytokine production (all $p < 0.05$). Error bars represent the means ±S.D. from three separate experiments (n = 3). One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, , and ****$p < 0.0001$; 95% CI of difference.

Histological Analysis

Moreover, joint structures in mice with an CAIA model that received systemic administration of MSCs were preserved better compared to control mice (Figure 12). Histological studies (Figure 13A-B) on sections from control CAIA mice revealed significant accumulation of inflammatory synovial cells and proinflammatory innate and adaptive immune cells, as well as pannus formation. Also, histological sections showed a higher level of cartilage destruction in control mice with the CAIA model compared to mice that received injections of MSCs, and the use of preconditioned MSCs had a more noticeable therapeutic effect and reduced the degree of pannus formation to the level of intact mice.

The Safranin-O staining revealed the presence of glycosaminoglycans in the MSC and preconditioned MSC injected mice, as shown by the red color (Figure 13A). This staining also showed the presence of exudate in the joint cavity, synovial hyperplasia of the synovial lining layer, joint destruction, erosion of cartilage and bone, and the creation of pannus as signs of RA in untreated mice CAIA. After being administered by systemical intravenous injection with preconditioned MSCs, the CAIA mice with preconditioned MSCs demonstrated a reduction in pannus development, hence preventing invasion and destruction of the underlying joints. Additionally, the CAIA mice injected with untreated hUCB MSCs showed similar but less significant results. The outcome is also confirmed by the histologic score (Figure 13B).

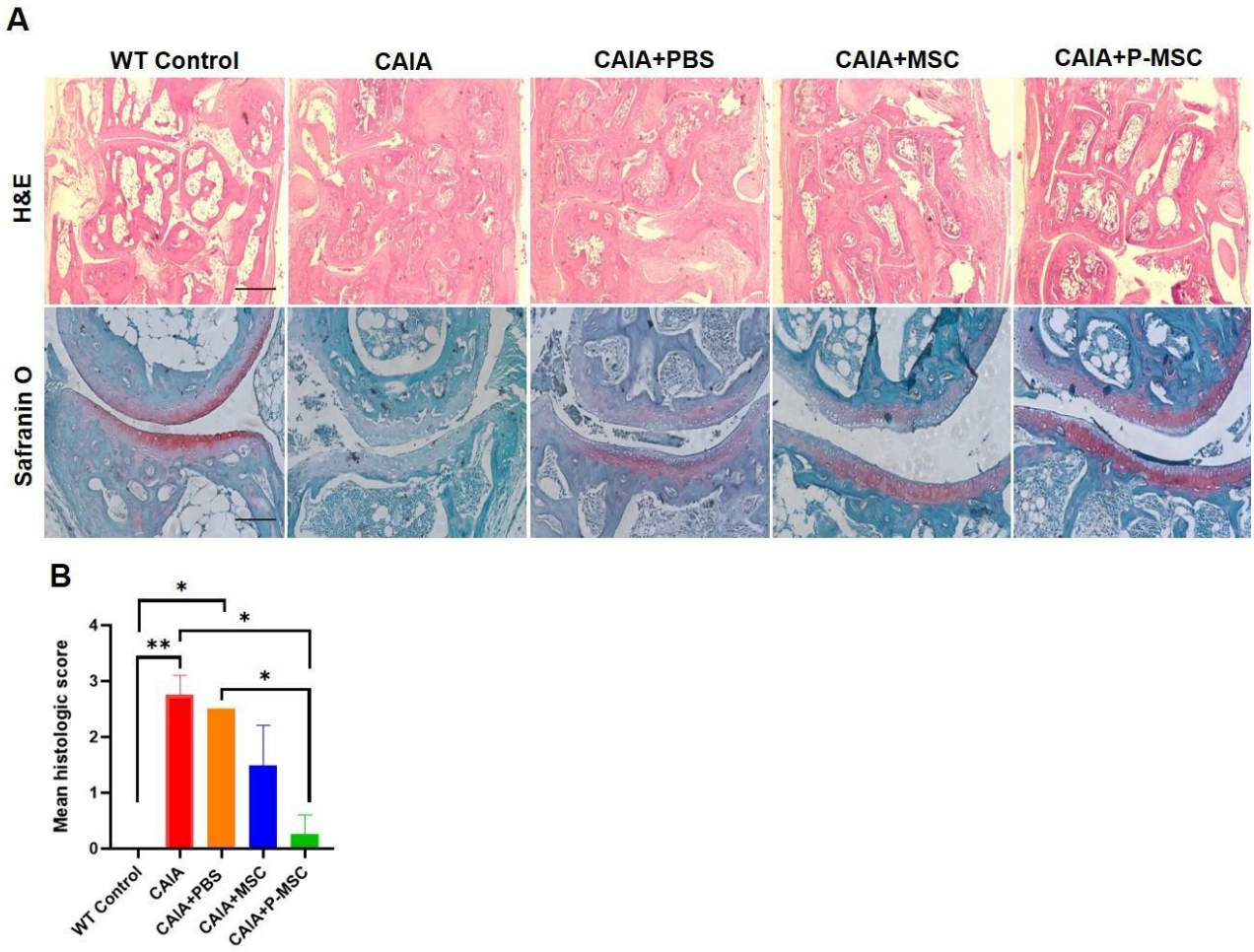
These results indicate a beneficial effect of hUCB MSCs in reducing the severity of inflammatory arthritis in this experimental model.

Figure 12

Macroscopic Images Hind Limbs of Mice with the CAIA Model

Figure 13

Histological Sections of the Hind Limbs of Mice with the CAIA Model



Note. The hind paws from each group were subjected to histologic examination on day 14. (A) H&E and safranin O staining for the transverse sections of tarsal joints. (B) Histopathological evaluation using a numerical score ranging from 0 to 3. The histology score was calculated by averaging the ratings given by three independent examiners. One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*$p < 0.05$, and **$p < 0.01$; 95% CI of difference.

Spleen Isolation and Flow Cytometry

Furthermore, during this phase of in vivo research on the utilization of MSCs derived from umbilical cord blood, T cells were extracted from the spleens of mice and the expression of CD4+CD25+ Tregs was examined using flow cytometry. The analysis of spleen morphometrics demonstrated that the spleens of mice exhibited progressive structural alterations as a result of CAIA induction, indicating an increase in the formation of clusters of mononuclear cells. The spleen sizes of the mice group that received preconditioned MSCs were identical to those of the control intact

group (Figure 13A). The mean weight of the spleens in mice that were administered preconditioned MSCs via injections was 0.15 grams, representing a reduction of 2.5-fold compared to the untreated groups of mice with the CAIA model and PBS injected mice (Figure 13B). In addition, the spleens of mice injected with untreated MSCs weighed 0.8 gram.

Figure 13

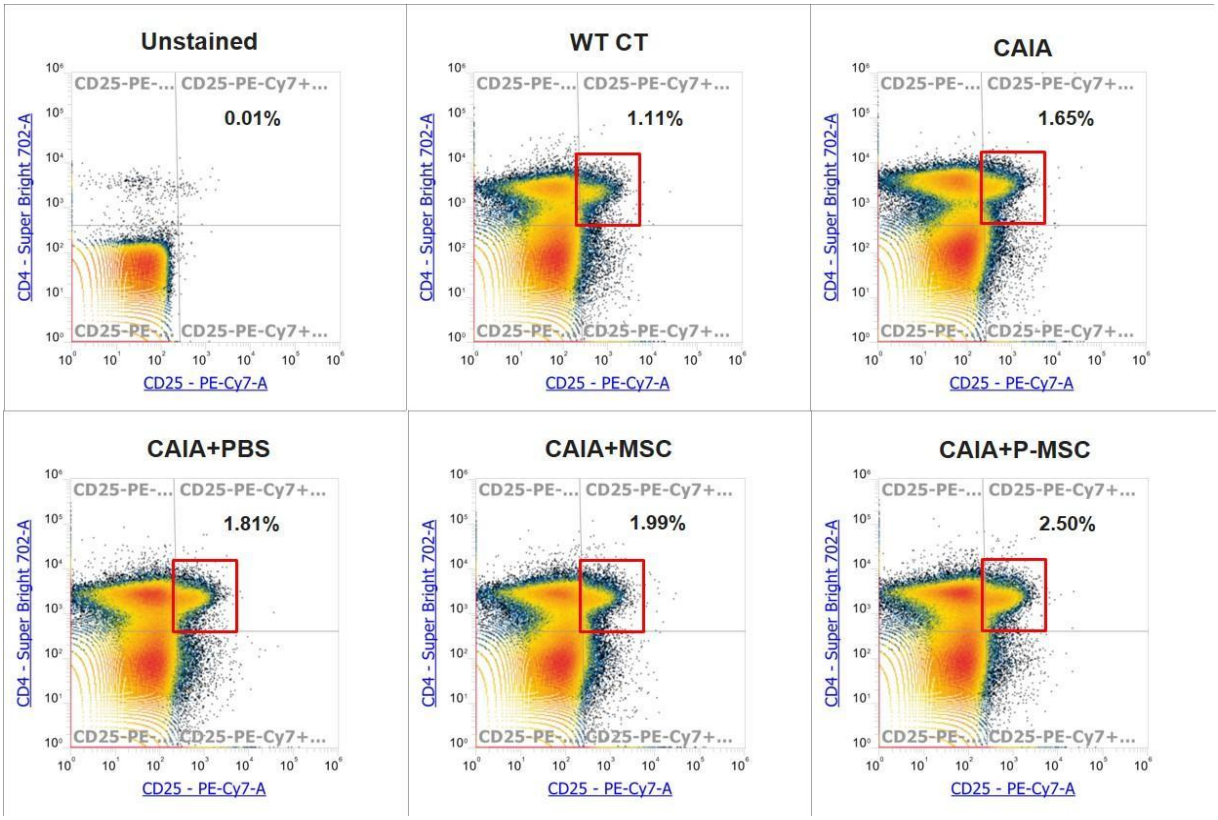
Morphometric Data of the Spleens of Mice from the Control and Experimental Groups

Note. (A) Spleen sizes of untreated animals and treated with MSCs; (B) average spleen weight. One-way ANOVA followed by Bonferroni's post hoc test were used for the comparison of the data from multiple groups.*p < 0.05 and **p <0.01; 95% CI of difference.

During the last phase of the experiment, the measurement of T-regulatory lymphocyte expression in the spleens of mice was conducted. The flow cytometry results revealed that the group of mice injected with non-preconditioned MSCs exhibited a CD4+CD25+ cell expression level of 1.99%, while the animals injected with preconditioned MSCs showed a level of immunoregulatory cells at 2.5% (Figure 14). The intact mice exhibited an expression level of 1.1%, while CAIA group and CAIA+PBS group had a level of 1.65% and 1.81%, respectively. Flow cytometry studies validate that the utilization of cytokine-preconditioned MSCs yields a comparable therapeutic outcome to that of untreated MSCs.

Figure 14

The Results of Flow Cytometry of Splenocytes on the Expression of CD4+CD25+ T-Lymphocytes


Unstained
0.01%
WT CT
1.11%
CAIA
1.65%
CAIA+PBS
1.81%
CAIA+MSC
1.99%
CAIA+P-MSC
2.50%
CD25-PE-...
CD25-PE-Cy7+...
CD4 - Super Bright 702-A
CD25 - PE-Cy7-A

DISCUSSION

Currently drugs that provide symptomatic treatment and slow the progression of the disease, which are quite effective and provide long-term remission, are used to treat RA. Used treatment approaches in clinical practice include non-steroidal anti-inflammatory drugs and glucocorticoids, disease-modifying drugs such as methotrexate and leflunomide, biological agents such as TNF-alpha and other protein inhibitors, and single-piece interleukin-6 (IL-6) antagonists such as like toquilizumab. However, prolonged use of the abovementioned drugs leads to the development of treatment resistance. Moreover, these drugs cause serious adverse effects, including metabolic changes, allergic reactions, increased risk of infections due to suppression of the immune system, and, in some cases, may promote the development of certain types of tumors. Hence, in recent times, there has been a growing optimism over the potential of cell therapy, namely mesenchymal stem cells (MSCs), in the management of rheumatoid arthritis (RA). MSCs have been recognised for their significant contribution in restoring immunological homeostasis by modulating T-cell populations.

Numerous research have been undertaken so far to investigate the therapeutic efficacy of MSCs in the context of rheumatic illnesses, including RA. However, results report mixed findings, ranging from no visible effect to significant improvement. Systemic administration of human adipose tissue MSCs has been shown to have significant therapeutic effects in the classical model of collagen-induced arthritis (Lopez-Santalla et al., 2015). Data from other studies using allogeneic bone marrow MSCs indicate opposite results, where no effect was observed from the use of MSCs (Chen et al., 2010; Djouad et al., 2005). These conflicting results may be due to several factors, including the origin of the MSCs, the number of cells administered, and the route of administration. In this study, we used umbilical cord blood MSCs, since previously published studies have reported the anti-inflammatory and immunomodulatory abilities of these cells in an experimental model of colitis and atopic dermatitis (Kim et al., 2013; H. S. Kim et al., 2015). Also, for the first time in Kazakhstan, we induced a modern model of RA, which takes only two weeks from the moment of administration of a cocktail of antibodies against collagen to a decrease in the severity of the disease after systemic administration of cytokine-preconditioned MSCs and their exosomes, probably due to the restoration of the homeostatic inflammatory environment.

Over the past 10 years, it has become known that MSCs in the initial state have virtually no immunosuppressive properties and must be “primed” in advance, i.e. preconditioned, in order to accept environmental conditions under the influence of certain environmental signals, for example,

inflammation (Krampera et al., 2013; Zimmermann et al., 2017; Zimmermann & Mcdevitt, 2014). However, to date, clinical trials still use unpreconditioned MSCs cultured in standard media, which do not secrete immunosuppressive and immunoregulatory proteins when administered to patients. This means that the cells rely only on signals received from the patient itself, which, given the individual characteristics of the body, makes therapy less optimal and less predictable, since the introduction of MSCs may not bring the same therapeutic benefit from patient to patient. The main goal of our work was to develop a strategy for preconditioning hUCB MSCs with proinflammatory cytokines, which could subsequently be used for testing in clinical studies. To do this, we first studied from the literature which factors and cytokines play an important role in inflammation during RA, when immune evasion and immune tolerance are present. These pro-inflammatory cytokines turned out to be IL-17A, IL-1β and TNF-α (Mateen et al., 2016), which were used both individually and in various combinations.

During the first phase of this study, we isolated and expanded the primary culture of hUCB MSCs. From our experience, the success rate of MSC isolation from human umbilical cord blood was approximately 30%, which coincides with some data in the literature (Bader et al., 2015). The choice of the source was UCB. It might be explained that this source is a neonatal tissue with an oxygen level of about 5%. Firstly, according to the data, it is considered that hUCB MSCs are highly promising due to their superior plasticity and shorter doubling time. Secondly, cells in this hypoxic tissue are originally physiologically adapted to these conditions – roughly "initially preconditioned" in physiological environment (Noronha et al., 2019), which means that these cells deliberately have enhanced immunomodulatory capacities.

Following the isolation and growth of the cells, we proceeded to characterise them. In accordance with the International Society for Cellular Therapy, MSCs should meet required "minimal criteria" which are known as important features for the identification and characterization of these cells (Dominici et al., 2006). Except for the capacity of the cells to be adhesive to culture plastic and the potential to differentiate to osteoblasts, adipocytes, and chondrocytes, MSCs must express characteristic surface markers as CD105, CD73 and CD90. In this study, the criteria were confirmed during the characterization of primary culture isolated from UCB.

In the present study, we found that *in vitro* preconditioning with pro-inflammatory cytokines significantly increased the immunomodulatory and immunosuppressive capacities of hUCB MSCs. Although MSCs cultured under normal condition without any pretreatment have therapeutic effect

and secrete immunomodulatory factors, we hypothesized that the preconditioning with IL-17A, IL-1β and TNF-α separately or in different combinations can stimulate immunoregulatory properties of hUCB MSCs. The choice of the cytokines is justified by the fact that these are key pro-inflammatory cytokines involved in the pathogenesis of RA. The application of different concentrations of factors alone or in combinations did not change the cell morphology and phenotype.

Under the inflammatory environment, MSCs have the ability to become polarized and develop either pro-inflammatory (MSC1) or anti-inflammatory (MSC2) characteristics (Bernardo & Fibbe, 2013). The MSC phenotype is determined by the circulating soluble substances in the milieu. The cells of the adaptive immune system release inflammatory molecules, such as TNF-α and IFN-γ, which activate MSCs and promote an anti-inflammatory phenotype. The immune suppression method facilitated by MSCs involves the release of many soluble substances, including enzymes, cytokines, and growth factors. These factors include TGF-β1, IL-6, IDO, COX-2, PGE2, NO, TSG-6, HGF, and IL-10 (English, 2013; Ren et al., 2008). Cord blood MSCs have been shown in recent research to possess the ability to create an anti-inflammatory phenotype. This phenotype is regulated by COX-2 and TSG-6, and is further intensified by TNF-α (Shin et al., 2016).

TNF-α, originally produced by macrophages during an acute inflammation, significantly contributes to TGF-β expression (Jiang & Xu, 2020). This was confirmed in the present study by evaluating the secretion level of TGF-β after the TNF-α treatment. The similar effect was obtained when the MSCs were preconditioned with the combination of all three cytokines together. Moreover, recent research has shown that the presence of pro-inflammatory cytokines leads to an increase in the expression of 11β-hydroxysteroid dehydrogenase type 1 in MSCs, which in turn enhances the production of TSG-6 via the classical NF-κB pathway (Huang et al., 2020). In our study the mRNA level of TSG-6 was enhanced in the presence of IL-17A in combination with IL-1β.

IDO and iNOS produced by MSCs also play a role in the immune regulation (McInnes & Schett, 2007). iNOS can be induced by inflammatory cytokines and is a dominant enzyme mediating the immunoregulatory effects of MSCs from rodents, while MSCs from other mammalian species preferentially use IDO (Su et al., 2014). The IL-17 pretreated MSCs acquire more potent immunosuppressive capacity, an effect likely attributed to IL-17 modulated mRNA stability through degrading ARE/poly(U)-binding/degradation factor 1 (AUF1) (Han et al., 2014). In our study, iNOS showed enhanced level of protein production in the presence of IL-17A and IL-1 β cytokines.

The IDO enzyme, which is responsible for the degradation of tryptophan into N-formylkynurenine, has been seen to cause apoptosis and cell cycle arrest in activated conventional T-cells. Additionally, it has been found to stimulate the development of Tregs. MSCs also need IDO for the facilitation of monocyte differentiation into immunosuppressive macrophages, hence mitigating inflammatory reactions. Human MSCs mostly produce IDO when exposed to IFN-γ in conjunction with TNF-α or IL-1, resulting in the manifestation of immunosuppressive effects (Han et al., 2022). According to our observations, stimulation of MSCs by IL-17A, IL-1β, and TNF-α, elevated IDO levels, which was confirmed both by gene expression and protein secretion.

The role of IL-6 in the regulation of the immune system has been described both as pro-inflammatory and anti-inflammatory. As an anti-inflammatory factor, it is also involved in the secretion of well-known anti-inflammatory molecules, such as the IL-1 receptor antagonist or IL-10, and reduces the abundance of TNF-α. IL-6 silencing reduces MSC proliferation by blocking the progression of the cell cycle through an intracellular signaling pathway (Dorronsoro et al., 2020). The stimulation of MSC by IL-17A and IL-1β and almost all the single or different combinations of pro-inflammatory factors revealed high IL-6 expression at the gene expression and protein secretion, respectively. Single application of IL-17 did not show a significant difference in IL-6 expression. This might be explained by the synergistic effect of IL-17A in combination with other cytokines compared with the application as a separate agent.

HGF serves as a crucial effector factor that not only facilitates tissue healing but also exerts regulatory control on the development and functionality of MSCs. The upregulation of HGF expression in MSCs was seen via the JNK signaling pathway, hence playing a role in tissue healing and the inhibition of fibrogenesis (Han et al., 2022). According to the gene expression analysis, the preconditioning with IL-17A, IL-1β and TNF-α in combination elevated the relative mRNA expression of HGF and galectin-1, which mediates wound healing, motility, and adhesion.

Our main results from gene expression and protein secretion analyses were that preconditioning of MSCs with combinations of cytokines, namely IL-17A+IL-1β and IL-17A+IL-1β+TNF-α, resulted in the cells being almost equally more effective in increasing the expression of immunosuppressive and immunomodulatory genes and proteins.

Some evidence indicates that macrophages play a significant role in the development of chronic inflammatory responses with direct tissue damage. Hence, in the subsequent stage of the research, we examined the impact of MSCs on macrophages, since the control of macrophages might potentially

serve as a therapeutic approach for RA. Previous studies have demonstrated that MSCs reduce the inflammatory cytokine TNF-α produced by activated macrophages (Choi et al., 2011). Moreover, there is evidence that MSCs stimulate the polarization of macrophages into the M2 phenotype through soluble mediators such as IDO, PGE2, IL-6, granulocyte-macrophage colony-stimulating factor and IL-1RA (Han et al., 2022; Luz-Crawford et al., 2016; Maggini et al., 2010; Zhang et al., 2010). Therefore, to study the effect of preconditioned MSCs on proinflammatory macrophages, combinations of cytokines were used, which, based on the results of the expression of immunosuppressive and immunomodulatory genes and proteins, had the best effect. Assessing the expression of TNF-α secreted by inflammatory macrophages suppressed the activation of pro-inflammatory M1 macrophages.

It is also known from the literature that MSCs have therapeutic potential in experimental arthritis by suppressing Th1 and Th17 responses and stimulating regulatory T cell responses (Chen et al., 2013). Therefore, in this work, we further studied the effect of preconditioned MSCs on T lymphocytes from human peripheral blood. The two above-mentioned combinations of cytokines were also used in the experiment. It turned out that the use of all three cytokines together suppressed the differentiation of central memory T cells, which play a key role in the progression of RA. In addition, preconditioning of MSCs with this combination of proinflammatory cytokines suppressed the secretion of IFN-γ and IL-10 in T cells after co-culture. Thus, the combination of IL-17A+IL-1β+TNF-α had an additional enhancing effect on innate and adaptive immune cells, both through cellular interactions and through a paracrine effect, compared to untreated naïve MSCs. These results also confirm that MSCs preconditioned with inflammatory cytokines can effectively respond to the specific immune microenvironment associated with RA, with a subsequent reduction in the inflammatory response and clinical manifestations of the disease.

For *in vivo* studies, we used a model of CAIA in Balb/c mice. Induction of CAIA bypasses all upstream primary immune responses, activating the activity of antibodies against type II collagen, causing complement activation. The pathogenic characteristics of the CAIA model exhibit notable resemblances to those seen in human rheumatoid arthritis (RA). These parallels include synovitis characterized by the invasion of polymorphonuclear and mononuclear cells, the creation of pannus, the deterioration of cartilage, and the erosion of bone. CAIA is a modified version of the traditional CIA model, which has been widely used in rats, mice, and primates. It emphasizes the use of type II collagen as an adjuvant for immunization. The presence of a commercially available combination of

four collagen antibodies offers a direct approach that eliminates the need for the host to produce autoantibodies against type II collagen (epitopes F10, A2, D8, and D1). Out of the four monoclonal antibodies, three specifically target conserved auto-antigenic epitopes situated within an 83-amino-acid fragment (LysC2) of the CB11 area, which is the CNBR-digested segment of type II collagen. The epitope located inside the LysC1 region in CB11 is recognized by the fourth monoclonal antibody (Khachigian, 2006). The main advantage of using the CAIA model in these experiments is that there is a short time interval between the administration of monoclonal antibodies and the development of arthritis.

Despite the short experimental period, we confirmed that MSC injection can induce changes in mice spleen size and cause alterations in the T cell populations. The results showed that preconditioned MSCs were more beneficial in suppressing systemic inflammation, tissue destruction and pannus formation compared to untreated MSCs. Synovitis associated with RA characterized by hyperplasia of the lining layer and infiltration of immune cells in the sublining were decreased after the preconditioned MSC administration.

Currently a great amount of evidence indicates that a specific group of newly developed CD4 T cells, which consistently express CD25, plays a crucial role in regulating various immune responses. These responses include preventing organ-specific autoimmunity and rejection of transplanted organs, inhibiting the immune response against tumors, and limiting the host's defense against antimicrobial agents. The CD4+CD25+Treg cell was first recognised as a "professional suppressor" by Sakaguchi (Sakaguchi et al., 1995). The study provided evidence that the introduction of BALB/c splenic cell suspensions, which were lacking in CD4+CD25+ cells, into an immunocompromised individual resulted in the development of autoimmune disorders. However, the cotransfer of CD4+CD25+ T cells effectively suppressed these autoimmune illnesses (Chen et al., 2005). In our study, the number of CD4+CD25+ cells from intact mice was significantly lower compared to CAIA mice injected with preconditioned MSCs. Systemic intravenous administration of MSCs to mice with CAIA stimulated the activation and Tregs. The expression of regulatory lymphocytes from mice administered with unstimulated MSCs was lower, indicating that preconditioning of MSCs with inflammatory cytokines induce the activation of immune regulatory cells. Thus, preconditioning of MSCs results in a higher anti-inflammatory effect by inducing CD4+CD25+ T-regulatory cells.

Furthermore, T lymphocytes are thought to secrete different pro-inflammatory and anti-inflammatory cytokines in a polarized manner towards the responding cells. Due to the fact that

cytokines level was measured from the blood serum, the data of Multiplex assay did not show TNF-α expression at a high level. Based on these results and on the observation that TNF-α upregulates the production of other proinflammatory cytokines, including IL-1β, IL-6 and GM-CSF. IL-1 has several proinflammatory effects that may be important in the pathogenesis of RA. IL-1β induces PGE-2 and collagenase production by synovial cells (Isomäki, 2012). In addition, the ability of IL-1 to induce fever and to promote the production of acute-phase proteins by liver cells may play a role in the systemic manifestations of RA. Similar to IL-1β, IL-6 enhances the secretion of acute-phase proteins by liver cells. IL-6 also induces osteoclast differentiation from haematopoietic precursors (Dorronsoro et al., 2020), and may therefore play a role in bone metabolism. GM-CSF activates macrophages, for example to produce cytokines, and by inducing differentiation of myeloid cells they may increase the number of mature macrophages in the inflamed synovium (Hamilton, 1993). IFN-γ is also T-lymphocyte-derived cytokine which directs the development of naive T cells into pro- inflammatory Thl phenotype. IFN-γ is present in rheumatoid joints at mRNA level, but is detected at relatively low quantities at protein level (Isomäki, 2012), which is confirmed by Multiplex assay. IL-12, observed in a serum from Balb/c mice promotes IFN-γ production and the generation of Thl cells. The proportion of aforementioned cytokines was significantly reduced after the preconditioned MSC administration into CAIA mice.

Thus, MSCs offer therapeutic effects in RA through several mechanisms. They have immunomodulatory properties, suppressing T cells activation and inducing Tregs. Additionally, the cells suppress B cells differentiation and modulate dendritic cells (Sarsenova et al., 2022). MSCs produce anti-inflammatory cytokines like IL-10, while inhibiting pro-inflammatory cytokines such as GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12 (p70), MCP-1, and TNF-α. MSCs also promote tissue repair and regeneration by secreting angiogenic factors like VEGF and remodeling the extracellular matrix through matrix metalloproteinases MMPs. They can home to injury sites via chemokine receptor-ligand interactions and modulate the synovial environment by interacting with synovial fibroblasts and macrophages, thereby reducing inflammation and joint damage (Sarsenova et al., 2021).

Therefore, the use of pro-inflammatory cytokines for hUCB MSCs preconditioning is a method to improve their therapeutic characteristics, particularly their immunomodulatory capabilities. Preconditioning may cause physiological alterations, including early senescence and apoptosis, which might affect the survival and functionality of the cells. Oxidative stress is a possible cause of

premature senescence. Pro-inflammatory cytokines, including TNF-α, may enhance the generation of reactive oxygen species (ROS) in MSCs, resulting in oxidative stress (Yang et al., 2015). In addition, the process of aging causes a reduction in the length of telomeres and impairs epigenetic modifications of DNA, such as methylation or histone acetylation (Y. Li et al., 2017). Moreover, senescent MSCs that have reached the end of their lifespan might contribute to a process known as inflammaging. This term describes a chronic, mild inflammation that occurs as part of the natural aging process (Lee & Yu, 2020). Senescent MSCs can develop a senescence-associated secretory phenotype, characterized by the secretion of pro-inflammatory cytokines, chemokines, and proteases. The cells emit an excessive amount of secretome, which includes IL-6, IL-8, IFN-γ, MCP-1, and MMPs. The secretion of these molecules leads to a systemic inflammatory response, which hinders the immunomodulatory activity of MSCs and facilitates the growth of cancer, exacerbates local inflammation and contributes to tissue damage (Mattiucci et al., 2018). The optimized cytokine dosing and duration of cytokine exposure can help balance the enhancement of therapeutic properties with the prevention of senescence in future experiments.

The study limitations include a limited sample size, selected animal model and duration of the study. Larger sample sizes in future experiments could provide more robust and statistically significant data. Furthermore, while the CAIA model in mice is a well-established model for studying RA, it may not fully replicate the complexity of human disease. Translation of results must take into account variations between mice and human immune systems and disease pathophysiology. Additionally, the study length may not have been sufficient to track long-term therapy effects. Longer follow-up times should be included in future research to evaluate the treatment impact longevity. It is important to mention that this research has possible bias, namely observer bias. Three independent observers conducted histology and clinical grading, for example. Nevertheless, the outcomes may still be influenced by inherent biases, and using automatic or semi-automated scoring methods in future research might provide more objective assessments.

Additional constraints of the research include the absence of the experiments aimed on elucidation of the specific mechanisms of preconditioned hUCB MSCs on the behavior of immune cells, particularly macrophage differentiation. MSCs may significantly enhance their ability to modulate macrophage characteristics by preconditioning with IL-1β and IFN-γ. The inhibition of M1 polarization during inflammation and the development of M2b markers are highly reliant on the effective signaling of IL-6 in macrophages (Philipp et al., 2018). Additionally, TNF-α primed MSCs

secrete PGE_2, which exert anti-inflammatory effects on macrophages via the cyclic AMP-responsive element (CRE) binding proteins. These proteins regulate the transcription rates of several immune-related genes, including TNF-α and IL-10, upon binding to CRE present in their promoter regions (Saldaña et al., 2019). Addressing this gap through future studies is crucial for gaining a comprehensive understanding of the therapeutic mechanisms of preconditioned MSCs and optimizing their use in RA treatment. Future in vitro co-culture studies, in vivo tracking, and molecular pathway analyses such as NF-κB, STAT3, and TGF-β signaling will provide valuable insights into the complex interactions between preconditioned MSCs and immune cells, particulary macrophages.

Moreover, to support the translational potential of this research it is crucial adding evidence on persistence and homing of preconditioned hUCB MSCs. Due to the limited effectiveness of MSCs natural homing ability, the cells were modified to improve their capability to home in on certain targets. Cells are able to target certain organs by expressing various combinations of chemokine receptors (CCR2, CCR4, CCR7, CCR10, CXCR4, CXCR5, CXCR6, CXCR7, CD29, CD44, and ULBP1), as well as via the use of MMP-1/2/9 enzymes, which are produced by the entering cells (Guan et al., 2018; Nitzsche et al., 2017). Compared to conventionally grown cells, hypoxia-derived or spheroid-derived AT MSCs exhibit increased expression of CXCR4, MMP-2, and MMP-9. In addition, when MSCs under low oxygen conditions were injected into mice with diabetes, they migrated to areas of injury, actively regulate the nearby inflammatory milieu, and aid in the healing of tissues (Yin et al., 2019). Thus, in future experiments the processes of hUCB MSCs migration towards inflammatory joints have to be examined for the expression of chemokine receptors on MSCs and their corresponding ligands in the tissues they are meant to interact with. Additionally, the evaluation of the duration of preconditioned hUCB MSCs presence in the in vivo system and their ability to successfully attach to the desired tissues have to be performed.

In this thesis, we investigated the therapeutic potential of preconditioned hUCB MSCs in a RA model. Our findings demonstrated significant improvements in clinical and histological outcomes, proposing that systemic administration of preconditioned hUCB MSCs may represent a potential therapeutic alternative for RA treatment. The strategy for preconditioning with pro-inflammatory cytokines can serve as an alternative way to enhance and modulate the immunomodulatory effects of MSCs.

Future research should focus on elucidating the molecular and cellular mechanisms underlying the interactions between preconditioned hUCB MSCs and immune cells. This will provide a deeper

understanding of their therapeutic potential and pave the way for optimizing MSC-based therapies for RA and other inflammatory diseases.

CONCLUSION

In conclusion, we demonstrated the advantages of the application of all three pro-inflammatory cytokines, IL-17A, IL-1β and TNF-α that could effectively enhance the immunomodulatory and immunosuppressive capacities of hUCB MSCs. Additionally, the macrophage co-culture and MLR analyses were performed for *in vitro* evaluation of MSCs preconditioning. In the final stage, we performed an *in vivo* study to assess preconditioned MSCs effect in the CAIA model in mice. The proposed strategy can serve as a tool for strengthening the MSCs effect in cellular therapy of various autoimmune disorders treatment including RA.

According to the objectives of the study, the following results were obtained:

1. Cultures of human umbilical cord blood MSCs were isolated and characterized. According to the results of the colony formation test, as well as the three-lineage differentiation test, it was revealed that MSCs had the ability to form colonies and differentiate into adipocytes, osteoblasts and chondrocytes. Flow cytometry analysis showed that MSCs expressed characteristic cellular markers such as CD73, CD90 CD105. Next, primary cultures were propagated for further in vitro and in vivo studies.

2. The optimal conditions for cytokine preconditioning of human umbilical cord blood MSCs were determined to effectively increase their immunomodulatory properties in vitro. The optimal concentrations and combinations of pro-inflammatory cytokines, such as IL-17A, IL-1β, and TNF-α, were determined to increase the immunosuppressive properties of MSCs. Preconditioning with cytokines was carried out both individually and in combination. In addition, the optimal time for preconditioning MSCs to effectively increase immunomodulatory properties has been identified. Based on the results of ELISA, PCR, and analysis of co-culture of MSCs with macrophages and T-lymphocytes, it was found that the optimal combination for preconditioning MSCs was the use of all three cytokines (IL-17A+IL-1β+TNF-α) at concentrations of 50 ng/ ml, 10 ng/ml and 10 ng/ml, respectively, for 48 hours.

3. The therapeutic effects of systemic intravenous administration of cytokine-preconditioned MSCs in mice with CAIA were studied. Flow cytometry, as well as clinical and histological evaluation methods showed that cytokine-preconditioned MSCs are more effective in activating and differentiating T-regulatory cells, as well as suppressing inflammation and joint destruction in CAIA compared with untreated MSCs.

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APPENDIX A

List of Reagents

Purpose	Name of Reagent	Supplier	Additional Information
MSC isolation	Ficoll-Paque Premium	Cytiva	10315590
	MSC NutriStem XF Medium	Sartorius	05-200-1A
	MSC NutriStem® XF Supplement Mix	Sartorius	05-201-1U
	Fetal Bovine Serum, qualified, one shot	ThermoFisher	A3160801
	Antibiotic-Antimycotic (100X)	ThermoFisher	15240096
	TrypLE Express	Gibco	12604-021
	Dimethyl Sulfoxide	Sigma-Aldrich	67-68-5
MSC characteriza-tion	Human MSC Analysis Kit	BD Stemflow	562245
	Oil Red O	Sigma-Aldrich	O0625-25G
	Alizarin Red	Sigma-Aldrich	A5533-25G
	Toluidine Blue	Fluka Analytical	89640-25G
Cytokine-precondition-ing of MSCs	Recombinant human IL-17A protein (Active)	Abcam	ab282392
	Recombinant Human Interleukin-1 beta protein (Active)	Cusabio	CSB-AP001671HU
	Recombinant human TNF alpha protein (Active)	Abcam	ab259410
ELISA, colorimetric assay for precondition-ing evaluation	Human IL-6 ELISA Kit	BD OptEIA	555220
	Human TGF-β1 ELISA Kit	ThermoFisher	BMS249-4
	Human IDO ELISA Kit	Abcam	ab245710
	Nitric Oxide Assay Kit (Colorimetric)	Abcam	ab65328
PCR, qRT-PCR	PureZOL RNA Isolation Reagent	Bio-Rad	732-6890
	High-Capacity RNA-to-cDNA Kit	Applied Biosystems	4387406

	6-FAM Dye Phosphoramidite	Applied Biosystems	403169
	TaqMan Fast Advanced Master Mix	ThermoFisher	4444557
	Taq 2X Master Mix	New England BioLabs	M0270L
	Ethidium Bromide Solution	Bio-Rad	1610433
	Agarose for DNA SDS	Sigma-Aldrich	K50246836827
	DNA Gel Loading Dye (6X)	ThermoFisher	R0611
Transwell assay	THP-1 cell line	ATCC	ATCC-TIB-202
	RPMI Medium 1640	Gibco	21875-034
	β-Mercaptoethanol	Gibco	21985-023
	Phorbol 12-myristate 13-acetate	Sigma-Aldrich	16561-29-8
	Recombinant Human Interferon gamma protein (Active)	Abcam	ab259377
	Lipopolysaccharides from Escherichia coli O111:B4	Sigma-Aldrich	MFCD00164401
	Human TNF-α ELISA Kit	Abcam	ab181421
MLR analysis	Ficoll-Paque Plus	Cytiva	10303396
	AIM V Medium	Gibco	12055-091
	Medium 199	Gibco	12340-030
	Mitomycin C	Sigma-Aldrich	M5353
	Lysing Buffer	BD Pharmingen	555899
	Stain Buffer (FBS)	BD Pharmingen	554656
	V500 Mouse Anti-Human CD45RA	BD Horizon	561640
	FITC Mouse anti-Human CD197 (CCR7)	BD Pharmingen	561271
	Human IFN-γ ELISA Kit	Abcam	ab174443
	Human IL-10 ELISA Kit	Abcam	ab185986

***In vivo* study**	Arthrogen-CIA 5-Clone Cocktail Kit	Chondrex	53040
	Anti-Mo CD4	ThermoFisher	67-0042-80
	Anti-Mo CD25	ThermoFisher	25-0251-81
	Mouse C Reactive Protein ELISA Kit	Abcam	ab222511
	Milliplex MAP Mouse High Sensitivity T Cell Magnetic Bead Panel	Millipore	3914751
	Xylene	Sigma-Aldrich	1.08298.4000
	Weigert's iron hematoxylin kit	Sigma-Aldrich	1.15973.0002
	Fast Green	Sigma-Aldrich	2353-45-9
	Safranin O	Sigma-Aldrich	477-73-6

APPENDIX B

List of primers for qRT-PCR

TGF-β

Forward - CTTTCCTGCTTCTCATG

Reverse - GCAGAAGTTGGCATGGT

Fam probe – CGCACGCAGCAGTTCTTCTC

IDO

Forward - CAAGGTCATGGAGATG

Reverse - CCTTGAATACAGTAGGAATTAC

Fam probe - TTCCTTACTGCCAACTCTCCAAG

IL-6

Forward - CAACCTGAACCTTCCAAA

Reverse - ACCTCAAACTCCAAAAGAC

Fam probe - CCAGGCAAGTCTCCTCATTGAATCC

TSG-6

Forward - TGTACCACAGAGAAGCAC

Reverse - CATCTTTCACTCCTATTGAGA

Fam probe - TCCATCCAGCAGCACAGACA

Gal-1

Forward - CCTGAATCTCAAACCTGGA

Reverse - TTGCACACGATGGTGTT

Fam probe - TCTTAGCGTCAGGAGCCACC

HGF

Forward - CATAATATGCTACTCGGACA

Reverse - TTACGAGTGGCACATCTC

Fam probe - TCACGAGCATGACATGACTC

β-actin

Forward - TCACCATTGGCAATGAG

Reverse - CCACGTCACACTTCATG

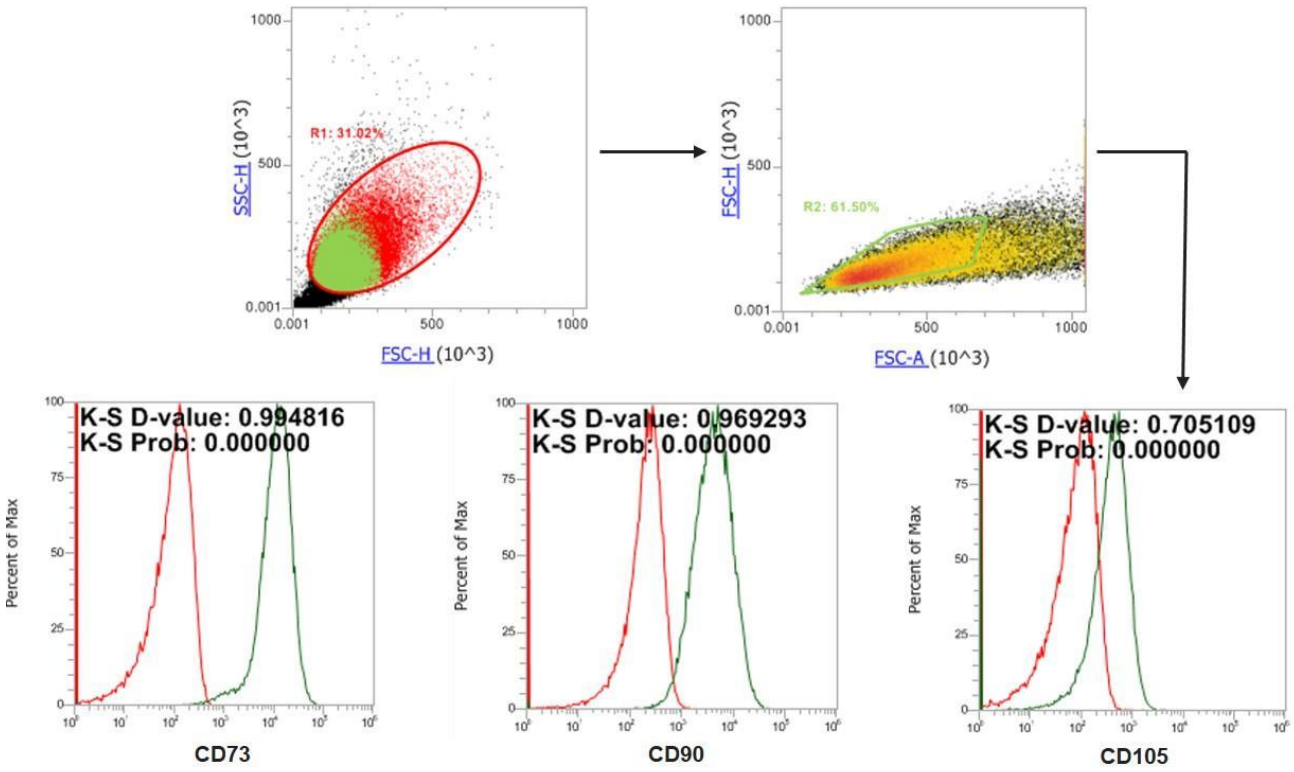
Cy5 probe - ACTCTTCCAGCCTTCCTTCC

APPENDIX C

Supplementary Figures and Tables

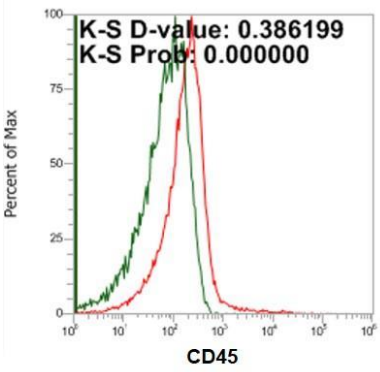
Supplementary Figure 1

Gating Strategy and K-S D-values for MSC Cell Surface Markers Expression



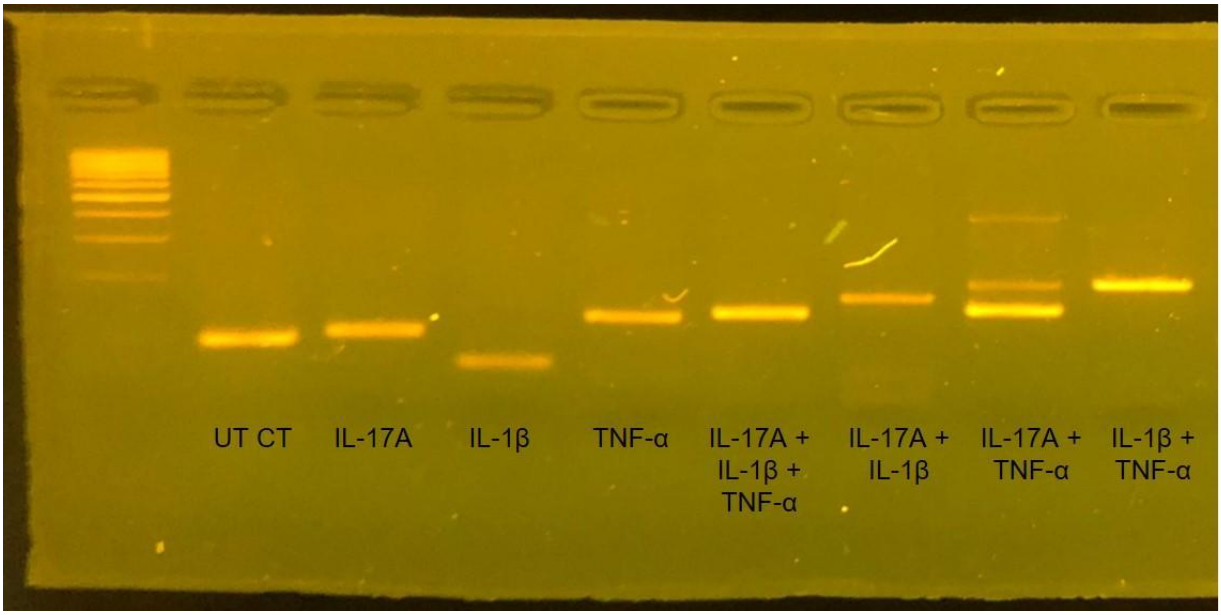
Supplementary Figure 2

MSC Negative Marker and K-S D-value



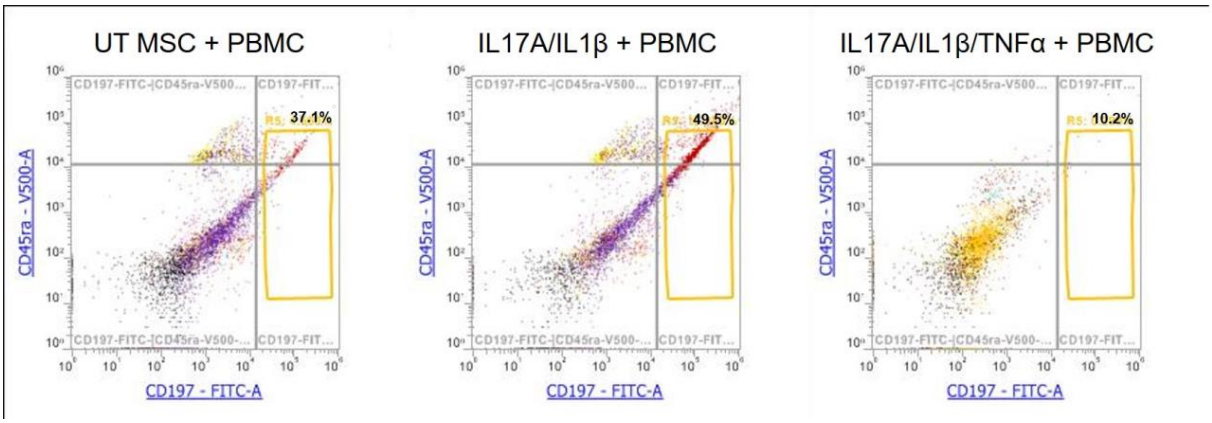
Supplementary Figure 3

Agarose Gel-Electrophoresis Results for Isolated RNA from MSCs by TRIZOL Method



Supplementary Figure 4

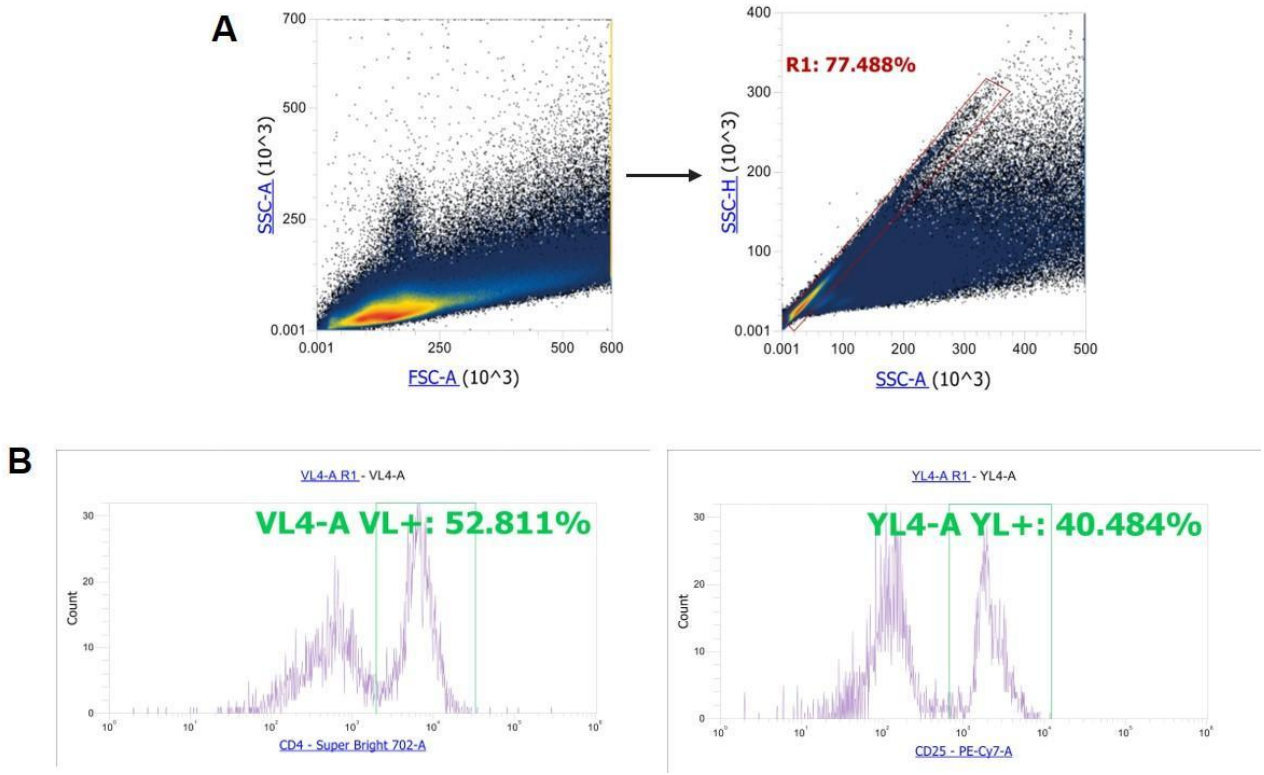
CD197 Expression in PBMC Co-Cultured with MSCs



Note. Flow cytometry results after co-cultivation of preconditioned MSCs with T-lymphocytes.

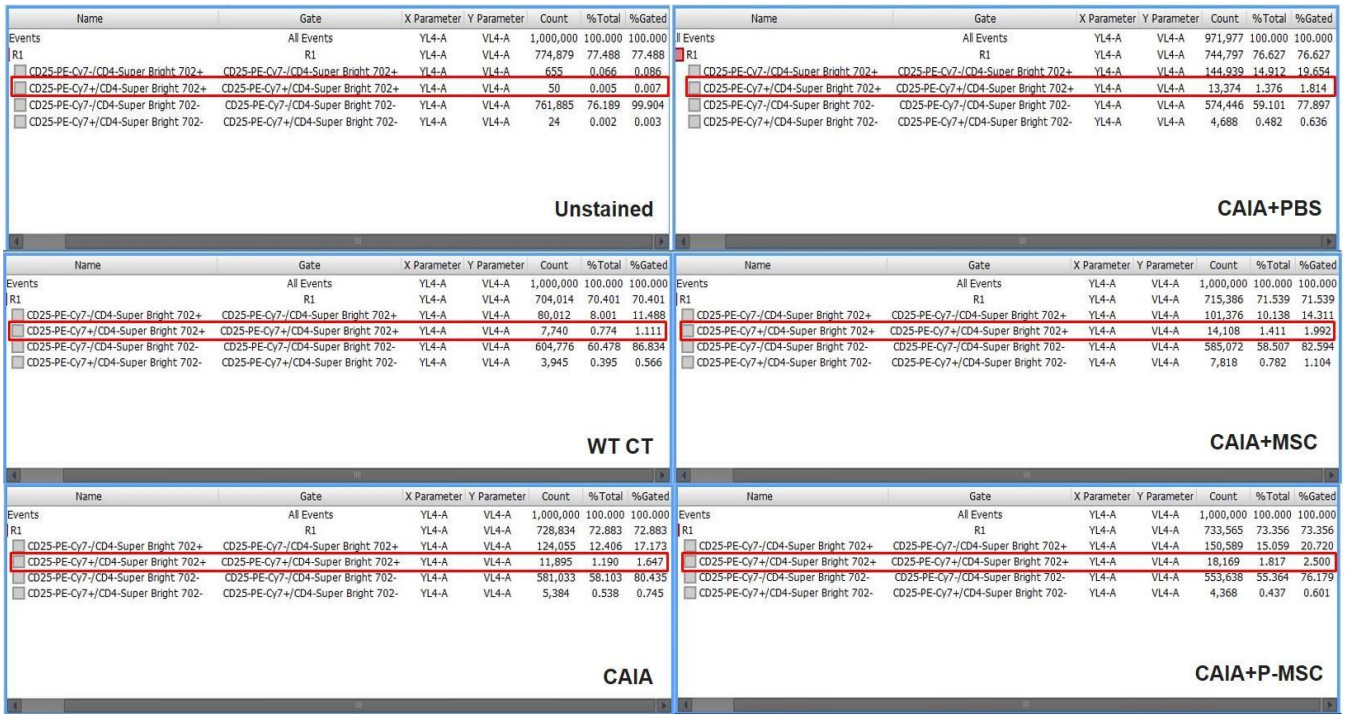
Supplementary Figure 5

(A) Gating Strategy for Mice Splenocytes for CD4+CD25+ Cells Expression and (B) Compensational Histograms



Supplementary Figure 6

Tables for Percentage of CD4+CD25+ cells



Supplementary Table 1

RNA Concentrations from Unstimulated MSCs and Preconditioned with Cytokines in Different Combinations

#	Group (cytokine alone or in combination)	Concentration (ng/ul)
1	UT CT	724 ng/ul
2	IL-17A	2248 ng/ul
3	IL-1β	2259 ng/ul
4	TNF-α	1720 ng/ul
5	IL-17A + IL-1β + TNF-α	546 ng/ul
6	IL-17A + IL-1β	1307 ng/ul
7	IL-17A + TNF-α	1411 ng/ul
8	IL-1β + TNF-α	1668 ng/ul

APPENDIX D

Statistical Data for Immunomodulatory Gene Expression and Molecules Secretion

Statistical Analysis of MSCs' Immunomodulatory Gene Expression by One-way Anova followed by Bonferroni's Multiple Comparisons Test (only statistically significant data included)

Gene	Comparison	Significant (p<0.05)	Adjusted P-value	Summary
TGF-β1	UT CT vs. IL-17A	Yes	0,0008	***
	UT CT vs. IL-17A+TNF-α	Yes	0,0118	*
	IL-17A vs. IL-1β	Yes	0,0132	*
	IL-17A vs. TNF-α	Yes	0,0002	***
	IL-17A vs. IL-17A+IL-1β+ TNF-α	Yes	0,0066	**
	IL-17A vs. IL-17A+IL-1β	Yes	0,0002	***
	IL-17A vs. IL-1β+TNF-α	Yes	0,0132	*
	TNF-α vs. IL-17A+TNF-α	Yes	0,0026	**
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	0,0023	**
IDO	UT CT vs. IL-17A	Yes	<0,0001	****
	UT CT vs. TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	0,0003	***
	UT CT vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β	Yes	0,0033	**
	IL-17A vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****

	IL-1β vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-1β vs. IL-17A+TNF-α	Yes	0,0054	**
	IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	0,0002	***
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	0,0100	**
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
IL-6	UT CT vs. IL-1β	Yes	<0,0001	****
	UT CT vs. TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β	Yes	<0,0001	****

	IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
TSG-6	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	0,0006	***
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-1β+TNF-α	Yes	0,0144	*
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	0,0001	***
	IL-1β vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	0,0149	*
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	0,0001	***
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	0,0059	**
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+TNF-α vs. IL-1β+TNF-α	Yes	0,0440	*
	UT CT vs. IL-17A	Yes	<0,0001	****
	UT CT vs. IL-1β	Yes	0,0002	***

Galectin-1	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	0,0009	***
	IL-17A vs. IL-1β	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	0,0003	***
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-1β vs. IL-1β+TNF-α	Yes	0,0086	**
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	0,0018	**
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A	Yes	<0,0001	****
	UT CT vs. IL-1β	Yes	<0,0001	****
	UT CT vs. TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	0,0004	***

HGF	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****

Statistical Analysis of MSCs' Immunomodulatory and Immunosuppressive Molecules Secretion by One-way Anova followed by Bonferroni's Multiple Comparisons Test (only statistically significant data included)

Protein	**Comparison**	**Significant (p<0.05)**	**Adjusted P-value**	**Summary**
	UT CT vs. IL-17A	Yes	<0,0001	****
	UT CT vs. IL-1β	Yes	<0,0001	****
	UT CT vs. TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-1β+TNF-α	Yes	<0,0001	****

TGF-β1	IL-17A vs. IL-1β	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	0,0009	***
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+TNF-α	Yes	0,0002	***
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β	Yes	0,0011	**
	IL-1β vs. IL-17A+TNF-α	Yes	0,0404	*
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	0,0463	*
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	0,0012	**
	IL-17A+TNF-α vs. IL-1β+TNF-α	Yes	0,0376	*
IDO	UT CT vs. IL-1β	Yes	0,0001	***
	UT CT vs. TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	0,0002	***
	UT CT vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****

	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-17A+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+IL-1β vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A+TNF-α vs. IL-1β+TNF-α	Yes	<0,0001	****
IL-6	UT CT vs. IL-1β	Yes	<0,0001	****
	UT CT vs. TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A+IL-1β	Yes	<0,0001	****
	UT CT vs. IL-17A+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-1β	Yes	0,0001	***
	IL-17A vs. TNF-α	Yes	0,0002	***
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	<0,0001	****
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+TNF-α	Yes	0,0001	***
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	UT CT vs. IL-17A	Yes	0,0007	***
	UT CT vs. IL-1β	Yes	0,0002	***
	UT CT vs. IL-17A+IL-1β	Yes	0,0002	***
	UT CT vs. IL-1β+TNF-α	Yes	0,0064	**

iNOS	IL-17A vs. IL-1β	Yes	<0,0001	****
	IL-17A vs. TNF-α	Yes	0,0036	**
	IL-17A vs. IL-17A+IL-1β+TNF-α	Yes	0,0006	***
	IL-17A vs. IL-17A+IL-1β	Yes	<0,0001	****
	IL-17A vs. IL-17A+TNF-α	Yes	0,0002	***
	IL-17A vs. IL-1β+TNF-α	Yes	<0,0001	****
	IL-1β vs. TNF-α	Yes	<0,0001	****
	IL-1β vs. IL-17A+IL-1β+TNF-α	Yes	0,0002	***
	IL-1β vs. IL-17A+TNF-α	Yes	0,0008	****
	TNF-α vs. IL-17A+IL-1β	Yes	<0,0001	****
	TNF-α vs. IL-1β+TNF-α	Yes	0,0011	***
	IL-17A+IL-1β+TNF-α vs. IL-17A+IL-1β	Yes	0,0002	***
	IL-17A+IL-1β+TNF-α vs. IL-1β+TNF-α	Yes	0,0071	**
	IL-17A+IL-1β vs. IL-17A+TNF-α	Yes	0,0007	***

APPENDIX E

Statistical Data for CAIA Induction and Treatment in Balb/c Mice

Statistical Analysis of CAIA Induction by Two-way Anova followed by Bonferroni's Multiple Comparisons Test

Parameter	Significant (p<0.05)	P-value	Summary
Arthritis Score	Yes	<0,001	***

Statistical Analysis of CAIA Treatment by Two-way Anova followed by Bonferroni's Multiple Comparisons Test

Comparison	Significant (p<0.05)	Adjusted P-value	Summary

WT Control vs. CAIA+PBS	Yes	<0,0001	***
WT Control vs. CAIA+MSC	Yes	<0,0001	***
WT Control vs. CAIA+P-MSC	Yes	<0,0001	***
CAIA vs. CAIA+PBS	No	>0,9999	ns
CAIA vs. CAIA+MSC	No	0,5893	ns
CAIA vs. CAIA+P-MSC	Yes	0,0332	*
CAIA+PBS vs. CAIA+MSC	No	0,4210	ns
CAIA+PBS vs. CAIA+P-MSC	Yes	0,0199	*
CAIA+MSC vs. CAIA+P-MSC	No	>0,9999	ns

Statistical Analysis of Mice Average Spleen Weights by Two-Way Anova followed by Bonferroni's Multiple Comparisons Test

Comparison	**Significant (p<0.05)**	**Adjusted P-value**	**Summary**
WT Control vs. CAIA	Yes	0,0019	**
WT Control vs. CAIA+PBS	Yes	0,0035	**
WT Control vs. CAIA+MSC	No	0,2171	ns
WT Control vs. CAIA+P-MSC	No	0,5736	ns
CAIA vs. CAIA+PBS	No	>0,9999	ns
CAIA vs. CAIA+MSC	Yes	0,0132	*
CAIA vs. CAIA+P-MSC	Yes	0,0076	**
CAIA+PBS vs. CAIA+MSC	Yes	0,0311	*
CAIA+PBS vs. CAIA+P-MSC	Yes	0,0164	*
CAIA+MSC vs. CAIA+P-MSC	No	>0,9999	ns

Statistical Analysis of Average Histologic Score by One-Way Anova followed by Bonferroni's Multiple Comparisons Test

Comparison	**Significant (p<0.05)**	**Adjusted P-value**	**Summary**
WT Control vs. CAIA	Yes	0,0086	**
WT Control vs. CAIA+PBS	Yes	0,0133	*
WT Control vs. CAIA+MSC	No	0,1172	ns
WT Control vs. CAIA+P-MSC	No	>0,9999	ns
CAIA vs. CAIA+PBS	No	>0,9999	ns
CAIA vs. CAIA+MSC	No	0,2327	ns
CAIA vs. CAIA+P-MSC	Yes	0,0133	*
CAIA+PBS vs. CAIA+MSC	No	0,4931	ns
CAIA+PBS vs. CAIA+P-MSC	Yes	0,0213	*
CAIA+MSC vs. CAIA+P-MSC	No	0,2327	ns

Sarsenova, M., Issabekova, A., Abisheva, S., Rutskaya-Moroshan, K., Ogay, V., & Saparov, A. (2021). Mesenchymal Stem Cell-Based Therapy for Rheumatoid Arthritis. *Int J Mol Sci*, *22*(21). https://doi.org/10.3390/ijms222111592

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