SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

CHARACTERIZATION OF VESSEL WALL-DERIVED MESENCHYMAL STROMAL CELLS AND INVESTIGATION OF THE EFFECTS OF MESENCHYMAL STROMAL CELL-LIKE CELLS ON THE DIFFERENTIATION OF MONOCYTES

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Characterization of vessel wall-derived mesenchymal stromal cells and investigation of the effects of mesenchymal stromal cell-like cells on the differentiation of monocytes

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The Examination takes place at the Department of Immunology, Faculty of Medicine, University of Debrecen, at 10.00 a.m. on 5th of October, 2021

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1.1.Introduction

Homeostasis is the phenomenon by which the organisms maintain the self's internal environment, not as a closed system but through tight interaction with the exterior world. The dynamic communication between the cells is the basis of adaptation to the continuously changing environment. The cells involved in the efficient protection of our integrity and maintaining homeostasis could be immune and non immune cells as well. The two main arms of defense are innate and adaptive immune systems, including cells responsible for the recognition, effector mechanisms, and regeneration. Dendritic cells (DC) are innate immune cells identified in 1973 by Steinman and Cohn as a small population in secondary lymphoid tissues. Later, these cells were functionally characterized as essential cells to prime and polarize the specific immune responses. DCs are critical regulatory elements at the crossroads between immunity and tolerance determined by their dynamic interaction with other tissue-resident cells. A great target to examine the fine-tuned synergy of DCs with other immunomodulatory cells is the population of mesenchymal stem/stromal cells (MSC). Revealing the complex network of the DCs and MSCs communication is essential for understanding the molecular mechanisms in the pathogeneses of immune-related diseases. Despite many studies about the MSC-driven modifications, the results are often inconsistent. The possible explanation for differences can be found in the general health status of the individual, the source, or the culture condition of MSCs. These circumstances determine their diverse phenotype, epigenetic background, viability, as well as the quantity and quality of their secreted mediators.

1.2.Mesenchymal Stem/Stromal Cells

Among various stem cells, i.e., embryonic, induced pluripotent, and hematopoietic ones, the MSCs show great interest as well.

Despite being a heterogeneous population, MSCs share a common characteristic: they express CD73, CD90, CD105, and lack the hematopoietic lineage markers, like CD45, CD34, CD11c, CD14, CD19, CD79A. MSCs could be isolated from numerous organs and tissues, such as bone marrow, adipose tissue, dermis, synovial membrane, umbilical cord, uterus, peripheral blood, periodontal ligament, dental pulp, muscle, tonsils, and vessel wall. MSCs with different origins and organ/tissue localization differ in their morphology and biological features. Additionally, many experiments suggest variations in the differentiation capacity or immunomodulatory effect of MSCs from distinct tissue sources.

The establishment of an MSC niche in the vascular adventitia provides a basis for the rational design of additional *in vivo* therapeutic approaches. The cellular components of vessel walls are well characterized. However, many questions have arisen about the progenitor and stem/stromal cells responsible for replacing the cells of vessel walls. The possible mesenchymal origin of pericytes and endothelial cells partially explains why the perivascular MSCs almost indistinguishable from the other vascular cell types. Independently from the origin of MSCs, they generally have immunomodulatory activity. Their effect on the immune responses is extremely complex and diversified. MSCs could modulate the differentiation and functional properties of immune cells through direct cell-cell interaction or via the production of variable soluble mediators. Additionally, their immunoregulatory activity could be altered and determined by environmental factors as well.

1.3.The immunologically relevant activity of MSCs

MSCs play a central role in immune responses. This raises the academic question of whether MSCs are immune cells or are tissue precursor cells with immunoregulatory capacity. MSCs do not fit the traditional definition of an immune cell; however, they display a set of mechanisms that endow them with immune-like properties. MSCs are not spontaneously suppressive cells; their immunomodulatory role depends on the actual environmental conditions. There are several strategies for immunomodulation and therapeutic use of MSCs. Stimulation of MSCs by inflammatory cytokines (cytokines, growth factors, or Toll-like Receptors/TLRs) is an inflammatory cytokine-licensing process for MSCs. During an immune response, T cells and antigen-presenting cells produce inflammatory cytokines leading to (i) release of immunosuppressive factors, (ii) altered expression of surface molecules, and (iii) production of growth factors by MSCs.

Because of the relatively short half-life of MSCs after their intravenous injection, and because they cannot pass through the lung capillaries, the question is raised: how could they have long-term immunomodulatory effects.

1.4.Immunomodulation by MSCs

MSCs play an essential role in maintaining immune homeostasis by interacting with immune cells via a plethora of mechanisms, via cell surface-associated molecules or soluble mediators.

MSCs are proficient at converting adenosine-triphosphate (ATP) into adenosine by expressing the ectonucleotidases CD39 and CD73, thereby taking away the inflammation-promoting effect of ATP. MSCs could co-express these two molecules, but they dominantly

represent CD73 on their surfaces. In MSC-mediated regulation, the release of immunoregulatory paracrine factors play a crucial role, including nitric oxide (NO), IDO, tumor necrosis factor-stimulated gene 6 (TSG-6), interleukin-10 (IL-10), transforming growth factor β (TGFβ), IL-6, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1) and the secreted form of PD-L1, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and HLA-G. Although IL-10 has been implicated in MSC-mediated immunosuppression, it has been found that MSCs induce IL-10 production of antigen-presenting dendritic cells or monocytes. Production of IL-10 has been reported to be associated with induction of non-classic HLA class I molecule HLA-G. Additionally, MSCs highly express galectin-1, which could be detected as intracellular, cell surface-associated, and also as a secreted molecule. Furthermore, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), angiopoietin-1 (Ang-1), stromal-derived factor-1 (SDF-1) growth factors are also important molecules involved in the MSC-driven immunoregulation.

In vitro cultured MSCs constitutively secrete a set of chemokines, such as CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL20 (MIP-3α), CCL26 (eotaxin-3), CXCL1 (GROα), CXCL2 (GROβ), CXCL5 (ENA-78), CXCL8 (IL-8), CXCL10 (IP-10), CXCL11 (i-TAC), CXCL12 (SDF-1) and CX3CL1 (fractalkine). Through the synergistic action of these chemokines and adhesion molecules (ICAM- and VCAM-1), immune cells accumulate close to the MSCs, where the high concentration of secreted factors can suppress the immune cells. The therapeutic and regulatory effects of MSCs are also mediated by extracellular vesicles (EV).

Based on the above-detailed mechanisms, the immunoregulatory role of MSCs is unarguable. One arm of their activity is the ability to modulate the differentiation and functional activity of myeloid-derived cells, such as monocytes, dendritic cells (DC), or macrophages.

1.5.Monocytes

The myeloid progenitor cell-derived monocytes contribute to immune responses as sources of subsets of DCs and macrophages. Considering the importance of monocytes in the pathogenesis of inflammatory disorders, the phenotypic and functional characterization of monocyte populations has emerged as a critical requirement.

Human circulating monocytes could be categorized into three different subsets based on the expression of cell surface markers, corresponding to three main functional subpopulations.

90–95% of the circulating monocytes are '*classical*' $CD14^{(hi)}/CD16^{(neg)}$ / $CD14^{++}CD16^{-}$ cells. The remaining *'nonclassical'* $CX3CR1^{+}$ monocytes are $CD16^{+}$ and have been further divided into two subsets based on the level of CD14 expression: $CD14^{(dim)}/CD16^{(hi)}$ subset exhibits pro- or anti-inflammatory properties and capable of presenting antigens. The '*intermediate*' $CD14^{(hi)}/CD16^{(med/high)}$ population is present in low numbers in the blood under physiological conditions. However, the particular functional properties of these heterogeneous subtypes are hard to define due to conflicting results and significant overlaps.

Hamers et al. utilized the high dimensionality of mass cytometry together with the FlowSOM clustering algorithm to accurately identify and define monocyte subsets regarding 34 phenotypic markers in healthy individuals. They identified seven distinct human monocyte subpopulations based on their phenotypic characteristics. They found three subsets can be grouped into the $CD16^{+}$ nonclassical monocyte population, and four subsets belong to the $CD14^{+}$ classical monocytes, illustrating significant monocyte heterogeneity in humans.

1.6. Origin and differentiation of human monocytes

HSCs can produce either common lymphoid progenitors (CLPs) and myeloid progenitors (CMPs). CMPs differentiate into granulocyte and macrophage progenitors (GMPs), which cells traditionally are the monocyte-macrophage/dendritic cell precursor (MDP) and common monocyte progenitor (cMoP). MDP population was proposed to give rise to monocytes and classical DC but not neutrophils in mice and humans. cMoP have also been identified in human BM, where they were found amongst the GMP population. These cells together critical for developing the myeloid-lineage such as granulocytes, monocytes, macrophages, and DCs.

In the presence of IL-4 and GM-CSF cytokines, CD14-expressing monocytes could differentiate into moDCs. DCs continuously monitor their microenvironment; they collect antigens, process and transport them into the secondary lymphoid tissues, and present the peptides to the specific receptor-bearing T lymphocytes.

1.7. Dendritic cells – costimulation – T cell activation

Dendritic cells are the most potent professional antigen-presenting cells, capable of initiating adaptive responses and tolerance induction, maintaining and restoring the sensitive balance between tolerance and immunity. An increasing number of DC subpopulations are described in association with unique functional attributes. Ontogenically distinct DC subtypes represent different functional specializations to orchestrate T cell responses.

As the most important costimulatory pathway, B7-1/B7-2/CD28 ensures the generation of the primary co-stimulatory signal provided by APC to enhance naive T cell activation. Carensa et al. developed a flow cytometry panel that directly compares the stimulatory/regulatory phenotype of DC-lineage and inflammatory DC subsets. Based on their results, the phenotype of DC subsets, especially the expression of co-stimulatory and inhibitory molecules, could be extremely diverse. Human monocyte-derived Dendritic Cells (moDCs) can take up and present antigens to T cells and can either activate or inhibit them through the regulated expression of co-stimulatory and co-inhibitory molecules such as CTLA-4. CTLA-4 is expressed by lymphoid cells to limit the co-stimulatory potential of myeloid APCs. Interestingly, an increasing number of studies demonstrate the expression and production of CTLA-4 protein in myeloid lineage cells, indicating that it could be a possible target of the moDC mediated immune regulation. It has also been demonstrated that myeloid cell-derived CTLA-4 can prevent the binding of CD28 receptors to CD80 and CD86 ligands, resulting in negative regulation of effector T cell responses *in vitro* and antitumor immunity *in vivo*. The regulatory role of CTLA-4 in autoimmune diseases and antitumor therapies is still controversial in both mice and humans.

1.8.Role of nuclear receptor RARα in the differentiation of DCs from monocytes

MoDCs could differentiate toward regulatory, tolerogenic, or inflammatory cells with unique characteristics to orchestrate immune responses. The well-tuning regulation of DC differentiation genes is determinative in the appropriate modulation of lineage determining master transcription factors. Additionally, ligand-dependent nuclear receptors have a major role in the regulation of myeloid cell differentiation.

The development of monocytes into moDCs remains plastic until their terminal differentiation ensues to acquire a mature phenotype, which is determined by a set of multiple extracellular signals like growth factors, cytokines, microbes, and metabolites or the components of the diet. Vitamin A could be taken up by consuming foods containing its precursors, like β-carotene and in the form of retinyl esters**.** Retinol is the immediate precursor to two critical active metabolites: retinal and retinoic acid, regulating the transcription of a number of genes. Retinol binds to retinol-binding protein (RBP). This complex is recognized by the retinoic acid 6 (STRA6) receptor, which initiates its absorption. Retinol oxidized into retinal/retinaldehyde by alcohol dehydrogenase (ADH). The retinal is oxidized by the enzyme retinal dehydrogenase (RALDH), producing retinoic acid (ATRA). Released ATRA interacts

with nuclear receptors, including RAR and RXR. ATRA and ATRA receptors link environmental sensing with immune cell fate.

ATRA shapes early intestinal immune responses by promoting interleukin (IL)-22 synthesis by γδ T cells and innate lymphoid cells and regulates DC and macrophage differentiation from circulating peripheral blood monocytes. Together with GM-CSF, ATRA induces the differentiation of monocytes into dendritic-like (ATRA-DC) cells exhibiting DC morphology. The molecule, completed with inflammatory cytokines, increasing the expression of MHC-II and CD86 on moDCs. In parallel, retinoids cooperated with inflammatory signals (cytokines and CD40 signaling) to improve the ability of moDCs to present antigens. These changes may explain the enhanced allogeneic T cell proliferation seen when retinoid-treated moDCs were used in the cocultures. Additionally, they can trigger a proliferative and cytokine-producing response in naive $CD4^+$ T cells. Importantly, in case of moDCs, the expression of CD1a is down-modulated by RARα, in contrast to CD1d which molecule' expression is elevated according to the findings of Szatmari et. al. Based on these observations, RARα nuclear receptor does not confer up-regulated antigen presentation by moDCs, but the stimulated cells acquire a selectively enhanced NKT cell activating capacity due to elevated levels of CD1d accompanied by diminished expression of CD1a.

1.9.Effect of MSCs on the differentiation of moDCs

MSCs have the potential to modulate the differentiation and the functions of T and B lymphocytes as well as innate immune cells, such as granulocytes, natural killer (NK) cells, monocytes, DCs, and macrophages.

During homeostasis and inflammation, monocytes leave the blood circulation and move into tissues to differentiate toward DCs. Several studies have investigated the effects of MSCs on the differentiation of moDCs. However, the results about the impact of MSCs on monocyte differentiation are inconsistent. The regulation of CD1a, CD14, CD40, CD80, CD86, CD83 and MHCII is modulated by MSCs, but the indirect and indirect effect of stromal cells on these molecules among others is not clear yet.

2. AIMS OF STUDY

2.1.Part I

The greatest known reservoir of MSCs is the bone marrow, but MSCs can be isolated from all vascularized organs. Because of the presence of a wide variety of cellular components in the vessel wall, there is not yet an accepted protocol for MSC isolation/differentiation from readily-available vessels. Our study attempted to elaborate an isolation technique and a morphology/phenotype analysis that is suitable to detect MSCs from vessel walls (saphenous vein / SV-MSC). We wanted to attempt comparing:

- the multilineage differentiation potential of SV- and BM-MSCs,
- SV- and BM-MSC-surface antigen expression profiles in a frame of a multiparameter analysis,
- the gene expression profile of SV- and BM-MSCs to identify differences or similarities in the expression of genes groups linked with particular biological function by microarray analysis,
- the effect of SV- and BM-MSC's on the proliferation of PBMC,
- the secretion of soluble mediators by activated/"primed/licensed" SV- and BM-MSCs.

2.2.Part II

To overcome the MSC isolation-related difficulties, we plan to characterize the use of MSC-like (MSCl) cell lines. This cell type seemed to be an appropriate model to examine the functional properties of MSCs *in vitro*[147]. We planned to examine the effect of MSCl cells on the DC functions, notedly on the monocyte differentiation process and its consequences on their functional properties;

- we wanted to examine the protein secretion and phenotypic profile of MSCl-modulated moDCs,
- we attempted to identify mediators, which could be involved in the modulation of DC differentiation.
- to dissect the functional activity of MSCl-CM-exposed monocyte-derived cells, we examined the moDC-mediated allogeneic naive T-lymphocyte polarization.

To further examine the mechanisms guiding the modification in the phenotype of moDCs in the presence of MSCl-conditioned media (MSCl-CM), we wanted to analyze the role of a nuclear receptor RARα in the MSCl cell-mediated moDC manipulation.

3. MATERIALS AND METHODS

3.1.Human moDC cultures

Heparinized buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Research Ethical Committee of the University of Debrecen, Faculty of Medicine (Debrecen, Hungary). Written, informed consent was obtained from the blood donors before the blood donation; their data were processed and stored following the directives of the European Union.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation. Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation and anti-CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), based on the manufacturer's instruction. After separation on a VarioMACS magnet, 96–99% of the cells were shown to be $CD14^+$ monocytes, as measured by flow cytometry. Isolated monocytes were plated at $1.5x10^6$ cell/ml concentration in RPMI (Sigma-Aldrich, Schnelldorf, Germany) supplemented by 10% FCS (Gibco, Paisley, Scotland) and 1% anti-mycotic/antibiotic solution (Hyclone, South Logan, Utah) in the presence of 100 ng/ml IL-4 (PeproTech EC, London, UK) and 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) added on day 0. Monocytes were cultured for four days in 12-well tissue culture plates at a density of 1.5×10^6 cells/ml in conditioned MSCI medium or in RPMI-1640 medium or were cultured for 4 days in T75 culture flasks at a density of 1.5×10^6 cells/ml in RPMI-1640 medium (Sigma-Aldrich).

To determine the role of nuclear receptors, freshly isolated monocytes were treated with or without 1 μM BMS-195614 specific RARα-antagonist (Sigma-Aldrich). After 75 min incubation the supernatant was changed to RPMI-1640 or MSCl-CM and the cells were differentiated in the presence or absence of natural RARα- agonist, ATRA (Sigma-Aldrich) and IL-4 and GM-CSF at 37°C atmospheres containing 5% CO_2.

3.2.Bone marrow, saphenous vein, and umbilical cord samples

Collection of bone marrow, umbilical cord, and saphenous vein samples complied with the directive of the Helsinki Declaration were approved by the institutional ethical review board (Medical Research Council) of the Medical and Health Science Center of the University of Debrecen (Ethical protocol numbers: UD MHSC REC/IEC No. 2754-2008, OSTRAT/1210-

1/2008/OSTR). Tissue samples were obtained corresponding to the EU Member States' Directive 2004/23/EC on tissue isolation[148].

For the separation of BM-MSCs, 10 ml of bone marrow aspirate was collected from the donors, which were diluted with saline in the ratio of 1:3. The cells were separated by Ficoll Histopaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. The viability was measured by Trypan blue exclusion assay. Bone marrow-derived nucleated cells (BMNC) were maintained in 25 cm^2 flasks at a density of $2x10^5$ cells/cm^2 and cultured in DMEM-LG medium (DMEM with 1 g/L glucose, Gibco/Invitrogen, London, UK), supplemented with 10% FSC and 1% anti-mycotic/antibiotic solution (PAA Laboratories GmbH, Pasching, Austria). 3 – 4 days later, the nonadherent cells were washed away, and a fresh medium was added to the adherent cells. The cultures' media was refreshed every 3 – 4 days. According to the confluency, the cells were passaged after 0.025% trypsin-EDTA (both Sigma-Aldrich, Budapest, Hungary) application and replated into new 25 cm^2 flasks. Bone marrow-derived MSCs were purchased from PromoCell, and they were maintained under the same conditions. At passage five, the cells were tested for cell surface antigen expression by flow cytometry, their differentiation potential was examined by using *in vitro* differentiation assays, and mycoplasma-test (Lonza, Basel, Switzerland) was used to exclude the infection of the cells.

Saphenous vein samples were isolated from saphenectomies. Saphenous veins were transported in ice-cold PBS and processed within 4 hours after the collection. The sample was cleaned from adipose or connective tissues, then it was cut into small pieces. These segments were washed in PBS, then enzymatically digested by 0.2 mg/mL collagenase type XI (Sigma-Aldrich, Budapest, Hungary) dissolved in DMEM-LG medium for 60 minutes at 37ºC. Cells were centrifuged at 1000 rpm for 20 minutes and washed by DMEM-LG medium after two washing steps; cells were plated and cultured, and maintained as described for BM-MSC.

The separation and *in vitro* culture of HUVEC cells was described elsewhere[149]. Briefly, HUVEC was collected from the umbilical cord with 1% collagenase type XI. (Sigma-Aldrich, Budapest, Hungary) digestion, and maintained in M199 medium (Sigma-Aldrich, Budapest, Hungary) supplemented with 20% FCS (Gibco, London, UK), 1% anti-mycotic/antibiotic solution (PAA), and 1% L-glutamine (Gibco, London, UK), in a CO_2 incubator at 37°C. According to the confluence, the cells were trypsinized and transferred into new culture flasks.

3.3.Generation of MSCl cells

Mesenchymal stromal cell-like cells (MSCl) originated from the human embryonic stem cell lines HUES9 and HUES1 were provided by Douglas Melton, HHMI (Howard Hughes Medical Institute). MSCl cells were used based on the ethical permission 6681/2012/EHR. The cells were maintained on mitotically inactivated mouse embryonic fibroblast (MEF) until they formed embryoid bodies (EB). To obtain single-cell cultures, the cells were trypsinized and further cultured on gelatin-covered 10 cm plates in DMEM (Gibco, Waltham, Massachusetts, U.S.) containing 10% FBS. MSCl cells associated fibroblast-like morphology were further characterized in a collaborative study with the laboratory of Balázs Sarkadi, Membrane Research Group of the Hungarian Academy of Sciences, Semmelweis University and National Blood Service, Budapest, Hungary[147]. MSCl cell passages in optomechanical-treated polystyrene flasks (TPP, Trasadingen, Switzerland) provided coherent cell layers in the presence of L-glutamine, 10% FCS (Gibco), and 1% anti-mycotic/antibiotic solution (Hyclone) in low glucose DMEM (Gibco). The confluent cell layer was cultured in 12 ml RPMI (Sigma-Aldrich) completed with 10% FCS (Gibco) and 1% anti-mycotic/antibiotic solution (Hyclone) for 48 hours to get the MSCl cell-derived conditioned media (MSCI-CM). Synthesis of ATRA by MSCl cells was inhibited specifically by 1 μM N, N-diethylaminobenzaldehyde (DEAB) (Sigma-Aldrich) in the MSCI cultures for 24 hours. After this incubation time, MSCl cells were washed, and fresh RPMI media was added to the cells for 48 hours.

3.4.Multilineage differentiation

To undertake trilineage differentiation, the isolated cells were applied to commercially available Gibco's StemPro® Adipogenesis, Osteogenesis and Chondrogenesis Differentiation Kits (Gibco). All differentiation patterns were evaluated according to the manufacturer's guidelines. Oil red O staining was used to detect the lipid-laden particles in the differentiated adipocytes. The mineral deposits during osteogenesis could be demonstrated by Alisarin red staining, while toluidine blue staining was used to label the chondrogenic mass formed by CSMSCs.

3.5.In vitro activation of MSC

To examine the role of TLR ligands and pro-inflammatory cytokines on the functional properties of MSCs, cells were plated to 24 well plates at $5x10^4$ cell density, and then the cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich, Budapest, Hungary), 25 μg/ml PolyI:C

(InvivoGen, San Diego, CA), 100 ng/ml TNFα, 10 ng/ml IL-1β or 10 ng/ml IFNγ (all from Preprotech, Rocky Hill, NJ, USA). After 12 or 24 hours, the supernatant was collected for ELISA.

3.6. Co-cultures of moDC and MSCl cells

Adherent MSCl cells were collected by using 0.05 –0.02% Trypsin/EDTA solution in Dulbecco's PBS (DPBS), washed, and cultured at a cell density of $3.6x10^6$ cells in T75 flask (TPP). MSCl cells were cultured in 12 ml RPMI (Sigma-Aldrich) supplemented with 10% FCS (Gibco) and 1% anti-mycotic/antibiotic solution (Hyclone). $1.8x10^7$ freshly isolated monocytes were placed directly on the top of the MSCl cells and differentiated for four days in the presence of 80 ng/ml GM-CSF (Gentaur Molecular Products) and 100 ng/ml IL-4 (PeproTech) into moDCs. After the differentiation process moDCs were separated from MSCI cells by positive selection using magnetic cell separation and anti-CD209/DC-SIGN-conjugated microbeads, based on manufacturer's instruction (Miltenyi Biotec).

3.7. Flow cytometry

Phenotyping of resting, conditioned moDCs in the presence of MSCI-CM or with MSCI cells was performed by flow cytometry using anti-human CD14-fluorescein isothiocyanate *(FITC*), CD209/DC-SIGN-phycoerythrin (PE), CD1a-*FITC*, CD80-FITC, CD86-PE, PD-L1-PE (BioLegend, San Diego, CA, USA), CTLA-4-PE, CD1b-FITC, CD1c-allophycocyanin (APC) (Sony Biotechnology Inc., San Jose, USA), HLA-DR-FITC (BD Biosciences, Franklin Lakes, NJ, USA).

Multiparameter analysis the expression of surface antigens on BM- or SV-MSCs and HUVEC cells was performed by flow cytometry: CD34 - FITC, CD44 - FITC, CD45 - APC, CD49f - FITC , CD73 - PE, CD106 - FITC, CD144, CD147 - FITC (All from BD Biosciences, San Jose, CA, USA), CD49a - PE (Biolegend, San Diego, CA, USA), CD14 - PE, CD29 - PE, CD31 - PE, CD36 - APC, CD47 – APC, CD49b - APC, CD54 - FITC, CD56 - APC, CD69 - APC, CD90 - FITC, CD104 -PE, CD105 - FITC, CD117 - PE, CD146 - APC, CD166 - PE, CXCR4 - APC, HLA-DR - FITC, PDGFRb - PE, VEGFR2 - FITC (All from R&D Systems, Minneapolis, MN, USA) and CD133 - APC (Miltenyi Biotech, Gladbach, Germany).

After trypsinization, the cells were washed with FACS buffer. Cells were labeled with antibodies on ice for 30 minutes according to the manufacturers' instructions. After the incubation, the cells were washed again with FACS buffer and fixed in 1% PFA/PBS. Samples were measured by FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ), and data were analyzed by FlowJo software. Results were expressed as medians of positive cells (%) ±SEM/SD.

3.8.Mixed lymphocyte reaction (MLR) and mitogen-induced cell proliferation

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll gradient centrifugation (Amersham Biosciences). Mitogen-induced T cell proliferation was triggered by concanavalin A (ConA), or phytohemagglutinin (PHA, all from Sigma-Aldrich) used at a final concentration of 10 μg/mL and 1 μg/mL, respectively. SV-MSCs were added to $1x10^6$ PBMCs at $10^{4,}$ and 10^5 cell numbers, and the cells were co-cultured for 3 days. Next, the proliferation was observed by a Bromodeoxyuridine (BrdU) colorimetric assay in the culture plate according to the manufacturer's instructions (Roche, Budapest, Hungary).

3.9.Measurement of the cytokine concentrations

The concentration of secreted IL-1β, IL-6, IL-8, IL-10, IL-12, IFNγ, TNFα, and IP10/CXCL10 cytokines were measured using OptEIA kits (BD Biosciences San Jose, CA) according to the manufacturer's protocol.

Culture supernatants from moDCs, CM-moDCs, or co-cultures containing moDCs and MSCI cells were harvested 4 days after monocyte separation, and the relative levels of selected human cytokines chemokines were determined by Human XL Cytokine Array Kit following the manufacturer's instructions (R&D Systems). Culture supernatants from moDCs, CM-moDCs, or co-cultures containing moDCs and MSCI cells were harvested 4 days after monocyte separation. The concentration of IL-6, IL-10, and TGFβ cytokines and chemokine IL-8 was measured and validated using OptEIA kits (BD Biosciences) following the manufacturer's instructions.

3.10. Treatment of moDCs to measure T-lymphocyte polarization

Control, CM-moDCs were counted, washed, and co-cultured with allogeneic naïve T cells for three, five, or nine days in RPMI-1640 medium (Sigma-Aldrich) at a moDC : T-cell ratio

of 1 : 10 at 37°C. Control and CM-moDCs were incubated with 2.5 μg/ml anti-CTLA-4 neutralizing antibodies (LifeSpan BioSciences, San Jose, CA, U.S.) on ice for 1.5 hours, then the cells were washed and co-cultured with PBL for three, five, or nine days in RPMI-1640 medium (Sigma) at a moDC : T-cell ratio of 1 : 10 at 37°C. The T cells were analyzed for IL-17 and IL-10 secretion by the avidin-horseradish peroxidase-based enzyme-linked ImmunoSpot (ELISPOT) system (NatuTec GmbH, Frankfurt am Main. Germany). Cultures including PBL or moDCs alone served as negative controls. To detect the secretion of IFNγ, IL-4, IL-17, or IL-10, the plates were coated with 0.5 μg/ml mouse anti-hCD3 antibody (BD Biosciences). The plates were analyzed using the ImmunoScan plate reader (Cell Technology Limited, Shaker Heights, OH, USA).

To determine which T-lymphocyte populations are responsible for the cytokine production, after three, five or nine days, the T cells were stimulated with 1μg/ml ionomycin and 20 ng/ml phorbol myristate acetate (PMA) for 4 hours, and the vesicular transport was inhibited by BD GolgiStop™ protein transport inhibitor (BD Biosciences) four hours before the cell staining. The cells were labeled with anti-human CD4-Peridinin Chlorophyll Protein Complex (PerCP) conjugated antibodies (BioLegend). Following this, they were fixed and permeabilized by using BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences) and labeled with anti-human IFNγ-APC (BD Biosciences), anti-human IL-4-PE (R&D Systems), anti-human IL-10-Alexa Fluor 488, and anti-human IL-17-PE antibodies (BioLegend). Fluorescence intensities were measured by FACS Calibur (BD Biosciences), and data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA).

3.11. Naïve $CD4^+$ T-cell isolation

Naive T-cells were separated from human blood mononuclear cells using the naive CD4+ T cell isolation kit based on negative selection according to the manufacturer's instruction (Miltenyi Biotec). Using the $CD4^+$ T Cell Isolation Kit, human $CD4^+$ T helper cells are isolated by negative selection. Non-target cells are labeled with a cocktail of biotin-conjugated monoclonal antibodies and the $CD4^+$ T Cell MicroBead Cocktail. The magnetically labeled non target T cells are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled T helper cells pass through the column.

3.12. ELISPOT assays

Cells were collected, counted, and subjected to IFNγ, IL-4, IL-17A or IL-10 Ready Set Go ELISPOT assays according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Briefly, 200 000 cells/well (IL-17A) or 400 000 cells/well (IL-10, IFNγ, IL-4) were

incubated in CTL medium for 48 hours at 37°C in MultiScreen-HTS PVDF plates (Millipore S.A., Molsheim, France) pre-coated with capture antibodies specific for IL-17A, IFNγ, IL-4 or IL-10. Together with the cytokine-specific capture antibody, 0.5 µg/ml purified anti-human CD3 antibody (BD Biosciences) was added to the coating buffer for the mitogenic stimulation of $CD3^+$ T cells. The detection of the cytokine release was performed by biotinylated IL-17A, IFNγ, IL-4 or IL-10-specific antibodies in the presence of horse-radish peroxidase enzyme (HRP) conjugated to avidin. Soon after the addition of hydrogen peroxide and the colorigenic substrate, the color development was stopped by tap water, and air-dried plates were analyzed by a computer-assisted ELISPOT image analyzer (Series 1 ImmunoSpot Analyzer, ImmunoSpot Version 4.0 Software Academic, Cellular Technology).

3.13. RNA isolation, cDNA synthesis, real-time quantitative PCR (RT-qPCR) and microarray analysis

Briefly, mRNA was isolated by TriReagent (Molecular Research Centr, Inc., Cincinnati, OH, USA) and reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene-specific TaqMan assays (Applied Biosystems) were used to perform qPCR in a final volume of 12.5 µl in duplicates using DreamTaq DNA polymerase and ABI StepOnePlus real-time PCR instrument. Amplification of h36B4 housekeeping gene was used as normalizing controls using specific primers and probes (Integrated DNA Technologies, Coralville, IA, USA). Cycle threshold values were determined using the StepOne Software, version 2.1 (Applied Biosystems).

To compare the gene expression profiles of the different cells isolated, an Affymetrix Gene Chip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) was used. 150 ng of total ribonucleic acid (RNA) was subjected to an Ambion WT Expression Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a GeneChip WT Terminal Labeling Kit (Affymetrix) according to the manufacturers' protocol, then washed and stained on FS-450 fluidics station (Affymetrix). The signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7 G (Hewlett Packard, Palo Alto, CA, USA). The scanned images were processed using GeneChip Command Console Software (AGCC) (Affymetrix) and the CEL files were imported into GeneSpring GX 12.6 software (Agilent Technologies Inc, Santa Clara, CA, USA). Robust microarray analysis (RMA) was applied for normalization. Gene transcripts with a maximal signal values less than 32 across all arrays were removed to filter for low and non-expressed genes, reducing the number of gene transcripts to 23,190. Based on the literature, stem cells-related genes were selected and statistical analysis was performed (One-way ANOVA with

Tukey post hoc test and Benjamini-Hochberg FDR; fold change cut off being set at 2) to calculate p values and fold change. To identify the relationships between the selected genes, the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) was used. Excel datasheets containing gene IDs with the assigned gene expression values were uploaded into the program. The Ingenuity Pathways Knowledge Base (IPKB) provided all known functions and interactions which were published in the literature. For the representation of the relationships between the genes, the "Pathway Designer" tool of the IPA software was used. Clustering analysis was made using the same name module in a Partek Genomics Suite Software.

3.14. Statistical analysis

Comparisons between two groups were performed using unpaired two-tailed Student's t-test for normally distributed variables with equal variance and Mann–Whitney rank-sum test for data that did not pass normality or equal variance test. Normality was assessed both visually (Q-Q plots) and through the Shapiro-Wilk test. One-way ANOVA followed by Bonferroni's post hoc test was used for comparisons. The results were expressed as mean + standard deviation (SD). Analyses were performed by using Excel (Microsoft Corporation) and GraphPad Prism Version 6.0 (GraphPad Software Inc.) software. Statistica 7.0 software (StatSoft Inc., USA) was used as well for the statistical analyses. The normality of the distribution of data was tested by Kolmogorov-Smirnov and Lilliefors test. Non-normally distributed parameters were transformed logarithmically to correct their skewed distributions. R software was used for hierarchical clustering. Each experiment was performed at least three times, and each sample was tested in triplicate. Data are expressed as mean$\pm$SD or SEM. Statistically, a significant difference was determined with two-way ANOVA analysis when there were more than two groups; for analysis between two groups paired student-test was used. Differences were considered to be statistically significant at $p < 0.05$. Significance was indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$P < 0.0001$.

4. RESULTS

4.1. Part I.

4.1.1. Morphology, Differentiation Potential, and Phenotype of Saphenous vein-derived MSCs

Saphenous vein-derived MSCs (SV-MSC) showed similar morphology to MSCs isolated from bone marrow (BM-MSCs). The cells did not form a cobblestone pattern, and they were

much larger than endothelial cells (HUVECs). We used HUVEC cells as vein vessel wall-derived endothelial cell controls.

MSCs have to fit the criteria defined by the ISCT regarding plastic adherence and differentiation potential[8]. We investigated whether the SV-MSC cultures could be differentiated toward canonical mesodermal directions in the proper adipogenic, osteogenic or chondrogenic induction media. Comparing the differentiation potential of SV-MSCs to BM-MSCs, it was revealed that both cell types showed oil red positive staining as a characteristic for the adipocytes in the presence of adipogenic induction media. Additionally, the presence of dense calcium deposits was detected after osteogenic differentiation, and in sections made from chondrogenic mass culture, metachromasy was detected upon toluidine-blue staining. Based on these observations, SV-MSCs are fit to the criteria of ISCT regarding the differentiation potential.

SV-MSCs have to fit the criteria of ISCT regarding the phenotype as well. We characterized and compared the cell surface molecule expression of the cells by flow cytometry.

The xpression of **hematopoietic markers** CD34, CD45, CD69, CD133, and the chemokine receptor CXCR4 could not be detected on the surface of MSCs. A low proportion of SV-MSCs was positive for CD117/c-kit (0.02 ± 0.02%), while BM-MSCs did not express that. Neither BM-MSCs nor SV-MSCs expressed HLA-DR involved in exogenous antigen presentation.

To exclude the possible endothelial cell contamination in SV-SMC cultures, we also monitored **endothelial-specific markers'** expression. PEKAM (CD31) expression was detectable on HUVEC cells' surface, while the molecule was absent both in the BM- and SV-derived MSC cultures. The level of VEGFR2/KDR was very low in HUVEC cultures, and it lacked in MSC cultures. Integrin β4 (CD104) expression was high in HUVEC culture; however, it was also present on MSCs. The percentage of VE-Cadherin (CD144) positive cells in SV-MSC cultures was between HUVEC and BM-MSCs.

All the expected **MSC markers**' expression, such as ecto-5′-nucleotidase (CD73), Thy-1 (CD90), and endoglin (CD105), could be observed both on BM-MSCs and SV-MSCs. Although HUVECs also expressed CD73 and CD105, the ratio of CD90 expressing cells was low in the HUVEC cultures. We did not identify statistically significant differences in the expression of neurothelin (CD147) and PDGFRβ among the three cell types. None of the ISCT defined markers are exclusively expressed by MSCs; therefore, we further observed the expression pattern of other molecules as well.

Next, the expression of **adhesion molecules** was investigated. Only the percentage of the melanoma cell adhesion molecule (CD146/MCAM) positive cells were found to be significantly different in BM- (77.54 ± 5.14%) and SV-MSCs (7.09 ± 6.56%) cultures. Besides CD146, the expression of intercellular adhesion molecule 1 (ICAM-1/CD54), activated leukocyte cell adhesion molecule (ALCAM/CD166), neural cell adhesion molecule (NCAM/CD56), and homing-associated cell adhesion molecule (H-CAM/CD44) could be observed on the surface of all three cell types; however, no significant differences were detected in the expression of these markers. The expression of integrin (Itg β1/CD29) and (Itg α1/CD49a) was similar in BM-MSC, SV-MSC, and HUVEC cultures. In the case of Itg α2 /CD49b we measured significantly lower expression on SV-MSCs than HUVEC cells ($p = 0.0186$). The Itg α6 /CD49f is mostly expressed by smooth muscle stromal cells, fibroblasts, and epithelial cells. MSCs did not positive for CD49f.

Using cluster analysis, we found a clear division difference between the MSCs. Results on SV-MSCs from different donors integrated well into the BM-MSC cluster despite inter-donor variability. These observations indicate that our isolation technique with the applied phenotype analysis is suitable to identify MSCs isolated from the vessel wall.

4.1.2. Gene expression analysis of SV-MSCs and BM-MSCs

Next, the gene expression patterns of BM-, and SV-MSCs were compared by microarray analyses. Genes related to cell cycle, oncogenes (242 genes), HOX (homeobox), SOCS (suppressor of cytokine signaling), and Notch signaling (380 genes), differentiation and lineage (489 genes), and stemness (422 genes) were collected into functional groups and analyzed.

The gene expressions in SV-MSCs in the custom group ***cell cycle and oncogenes*** was not differed significantly from their BM-MSC counterpart; however, several genes related to this biological function group were differentially expressed in the two MSC groups. In SV-MSCs, the expression level of S100 calcium-binding protein A4 (S100A4) was significantly higher (2.8-fold change), whereas that of SMAD family member 3 (SMAD3) and cyclin-dependent kinase 6 (CDK6) was considerably lower (-2.6- and -2.2-fold change, respectively) than in BM-MSCs.

In the ***HOX, SOCS,*** and ***Notch signaling***, superfamily FGF9, IL-33, and homeobox A11 (HOXA11) genes were determined as significantly upregulated (≥2-fold) ones in SV-MSCs.

Significantly upregulated (≥2-fold) genes related to ***differentiation and lineage*** in SV-MSCs were found to be podocalyxin-like (PODXL), cathepsin K (CTSK), and colony-stimulating factor 1/macrophage (CSF1), while vascular cell adhesion molecule 1 (VCAM1),

aggrecan (ACAN), early growth response 2 (EGR2), transforming growth factor-beta 2 (TGFB2), insulin-like growth factor 2 (IGF2), bone morphogenetic protein 2 (BMP2), brain-derived neurotrophic factor (BDNF), jagged 1 (JAG1), inhibin-beta A (INHBA), integrin-alpha 3 (ITGA3), SMAD3, hairy and enhancer of split 1 (HES1), ephrin-B2 (EFNB2), pleiotrophin (PTN), and platelet-derived growth factor-alpha (PDGFA) genes were significantly downregulated (≤ -2-fold).

An SV-MSC-specific pattern of ***stemness*** could be characterized with high expression of fibroblast growth factor 9 or glia-activating factor (FGF9), zinc finger protein, multitype 2 (ZFPM2), membrane metalloendopeptidase (MME), and frizzled homolog 4 (FZD4) genes, together with low expression of leukemia inhibitory factor or cholinergic differentiation factor (LIF), hypothetical protein MGC20647 (MGC20647), chemokine C-X-C motif ligand 12 or stromal cell-derived factor 1 (CXCL12), melanoma cell adhesion molecule (MCAM), ACAN, latent transforming growth factor-beta binding protein 1 (LTBP1), BMP2, SMAD3, ALCAM, integrin, alpha V, or vitronectin receptor (ITGAV), growth differentiation factor 6 (GDF6), and fibroblast growth factor 7 (FGF7) genes. These results highlight the importance of the MSC origin, which seems to be determining in the pattern of observed genes.

4.1.3. Immunomodulatory activity of BM-MSCs and SV-MSCs

The immunosuppressive features of MSCs have been widely studied due to their promising therapeutic potential. In this study, mitogen-activated mixed lymphocyte reaction (MLR) was used to compare the anti-proliferative activity of SV- and BM-MSCs. As expected, human PBMCs from healthy donors showed increased proliferation in response to polyclonal T cell activators Concanavalin A (ConA) or phytohemagglutinin (PHA) (data not shown). Co-culturing MSCs with PBMC resulted in a moderated reduction in T cell proliferation upon ConA stimulation. In contrast, both BM-MSCs and SV-MSCs significantly inhibited the proliferation of PHA-activated T lymphocytes. At both cell ratios (MSC/PBMC 1 : 100 and 1 : 10), the silencing of PBMC proliferation by SV-MSCs was more intense; however, a significant difference between SV-MSCs and BM-MSCs in their anti-proliferative activity was detected only at a ratio of 1 : 100.

4.1.4. Stimulation of BM-MSCs and SV-MSCs

Although the immunosuppressive activity of MSCs is well described, much less data are available about their response to proinflammatory cytokine or TLR ligand exposure, especially in the case of SV-MSCs. In their unlicensed state, MSC exhibits immune homeostatic activity. This could be enhanced by exposure of the cells to pro-inflammatory cytokines such as IFNγ

and TNFα and/or TLR ligands such as LPS and dsRNA as evidenced by increased production of cytokines, chemokines, and lipid mediators[150,151]. Therefore, in the next series of our experiments, BM-MSCs and SV-MSCs were stimulated with LPS, PolyI:C, TNFα, IL-1β, or IFNγ for 12 and 24 hours and secreted cytokine (IL-6) and chemokine (IL-8/CXCL8 and CXCL10/IP-10) concentrations were determined from supernatants of MSCs.

Under normal conditions BM-MSCs and SV-MSCs constantly secreted proinflammatory cytokine IL-6. The secretion profile of IL-6 cytokine and IL-8 chemokine were similar in both MSC cultures. Activation of cells with LPS, PolyI:C, TNFα, or IL-1β for 12 and 24 hours resulted in a significant increase in the concentrations of both IL-6 and IL-8, whereas activation with IFNγ did not alter their secretion by BM-MSCs or SV-MSCs. More intense IL-6 and IL-8 production were observed in BM-MSC than SV-MSC culture. Both types of MSCs secreted CXCL-10/IP-10 chemokine upon TLR- and cytokine receptor ligation. In contrast to IL-6 and IL-8 levels, SV-MSCs secreted more CXCL-10/IP-10 in response to activation than BM-MSCs. According to our findings, in BM-MSCs cultures, PolyI:C and IFNγ were potent inducers of CXCL-10/IP-10 release, while SV-MSCs produced this chemokine in higher concentrations as a consequence of any applied stimuli; however, the changes were statistically significant only when the SV-MSCs were exposed to the TLR ligands, LPS, or PolyI:C and cytokine IFNγ. Based on our results, MSCs originated from bone marrow or saphenous vein respond to priming inducers in slightly different ways.

4.2. Part II.

4.2.1. Production of soluble mediators by moDCs is modulated by MSCl cells

To analyze the immunomodulatory effects of MSCl cells, moDCs were differentiated in the presence of MSCl cell-derived conditioned media (MSCl-CM) or MSCl cells. The presence and levels of soluble mediators in the cell culture supernatants were monitored by a protein array.

Soluble factors produced by MSCl cells remarkably changed the protein secretion profile of moDCs. Upregulated levels of BAFF, Complement factor D, EGF, IL-2, IL-22, Lipocalin-2, TGFα, TIM3, Myeloperoxidase, C-Reactive Protein, and Dkk-1, while the diminished release of ICAM-1 and EMMPRIN was measured in the supernatant of moDCs differentiated in the presence of MSCl-CM. In the supernatant of moDC-MSCl cell co-cultures, increased levels of Vitamin D BP, Endoglin, ENA78, GDF-15, GRO-α, IL-24, MCP-3, VEGF, IL-8, IL-10, and IFNγ, but decreased amounts of FGF-19, Osteopontin, CD31, and IL-18 Bpa were observed. Both exposures to MSCl-CM and direct moDC-MSCl cell-cell contact elevated the production

of IL-19, VCAM-1, Leptin, IL-6, CD14, FGF basic, IGFBP2, TFF3, and TfR, while the secretion of IL-27, Cystatin C, Chitinase 3-like 1, MMP-9, and PDGF-AB/BB was downregulated in the presence of either MSCl-CM or MSCl cells. Next, concentrations of some inflammatory (IL-6) and anti-inflammatory (IL-10 and TGFβ) mediators were measured by ELISA. MoDCs secreted significantly more IL-6, IL-10, and TGFβ in the presence of MSCl-CM than control cells, while significantly more IL-6 and IL-10 productions were detected in the supernatant of moDC-MSCl cell co-cultures. These results demonstrate that the baseline levels of both inflammatory and anti-inflammatory mediators in moDCs cultures can be altered by MSCl cells in direct and indirect ways as well.

4.2.2. MSCl cells change the phenotype of monocyte-derived cells

To get insight into how the presence of MSCl-CM or MSCl cells affects the phenotype of moDCs, we observed the expression of cell surface molecules by flow cytometry. During the process of moDCs differentiation in the presence of GM-CSF and IL-4, the expression of CD14 is down-regulated[152], while that of DC-SIGN/CD209[153] and CD1 family members (CD1a, b, c)[152] is increased. In our experimental setup, the percentage of the CD14-expressing population was higher in the presence of MSCl-CM or MSCl cells than in the case of control cells. While the ratio of DC-SIGN$^+$ cells *(%)* was similar in MSCl-exposed and control cell cultures, the cell surface expression of CD209 was significantly reduced on MSCl-treated cells.

Under our experimental conditions, approx. 40% of untreated cells expressed CD1a, b, and c molecules. Exposure to either MSCl-CM or MSCl cells significantly downregulated these glycolipid receptors' cell surface expression on monocyte-derived cells. At the same time, these treatments do not affect their viability.

For further investigation of phenotypic characteristics of MSCl-CM- and MSCl cell-treated monocyte-derived cells, expression of CD163, a macrophage scavenger receptor[154], was observed. During the monocyte differentiation, the presence of MSCl-CM triggered only a slight increase in the frequency of CD163$^+$ cells. In contrast, exposure to MSCl cells induced a significant rise in the ratio of CD163-positive cells. However, the level of the cell surface expression of CD163 was upregulated on MSCl-treated cells in oppose to the MSCl-CM-conditioned monocyte-derived cells.

As a summary, MSCl-CM can induce the differentiation of monocytes toward CD1a$^-$ DC-SIGN$^+$CD163^{low} semi-matured monocyte-derived cells, whereas MSCl cells support the differentiation of M2 macrophage-like cells associated with CD1a$^-$DC-SIGN^{low}CD163^{high} phenotype.

We also found that MSCl-CM significantly enhanced the expression of HLA-DQ and the co-stimulatory molecule CD86, whereas direct contact with MSCl cells significantly upregulated the expression of both CD80 and CD86 on the surface of moDCs. In the presence of MSCl cells, increased expression of co-inhibitory molecule PD-L1 could also be found on monocyte-derived cells. Notably, an upregulated expression of another co-inhibitory molecule CTLA-4 on monocyte-derived cells was triggered exclusively by MSCl-CM. MSCl-CM induced elevated expression of CTLA-4 in a time-dependent manner, and a significant upregulation in CTLA-4 levels on treated and control cells was found on day 4 of treatment. CTLA-4 mRNA expression showed a four-fold enhancement in four-day-old monocyte-derived cells differentiated in the presence of MSCl-CM compared to control moDCs.

When four-day-old, differentiated immature moDCs were co-cultured with MSCl cells for 48 h, and an enhancement in the expression of CD80, CD86, and PD-L1 on moDCs was detectable. However, when immature, differentiated moDCs were exposed to MSCl-CM for 48 hours, no or minor alterations in the levels of co-stimulatory and co-inhibitory molecules on moDCs could be observed. These results suggest that MSCl-derived secreted mediators affect the generation of immune regulatory moDC phenotype only at the initial stage of their development.

4.2.3. MSCl cells alter the phenotypic features of moDCs at least partially via the production of ATRA

RARα nuclear receptor plays an important role in the regulation of specialized DC differentiation from human blood monocytes[123]. To examine the mechanisms guiding the modification in the phenotype of moDCs in the presence of MSCl-CM, we investigated the role of RARα using ATRA as an agonist and BMS614 as a selective antagonist of this nuclear receptor.

The expression of CD1a on moDCs was significantly silenced by MSCl-CM and when ATRA was added to the monocytes. As expected, BMS614 prevented the effect of ATRA on the expression of CD1a. BMS614 slightly upregulated the expression of CD1a on monocyte-derived cells differentiated in the presence of MSCl-CM, but this was statistically insignificant. Blocking of RARα prevented the effect of ATRA or MSCl-CM on HLA-DQ expression; however, the observed differences were statistically non-significant. The enhancement in T-cell co-stimulatory or -inhibitory molecule expression on moDCs by ATRA or by MSCl-CM was dependent on functional RARα receptor. Indeed, the increased expression of CD86 on moDCs triggered by ATRA or MSCl-CM was significantly downregulated when

the function of RARα was blocked. Furthermore, the elevated ratio of CTLA-4-expressing monocyte-derived cells induced by ATRA or MSCl-CM was also significantly downregulated by the selective blockade of RARα.

Next, we analyzed whether MSCl cells alter the immune regulatory potential of monocyte-derived cells via ATRA production. Thus, the expression of genes known to play a role in ATRA synthesis was examined in MSCl cells compare to monocytes and differentiated moDCs. We found that MSCls express retinol dehydrogenase 10 (*RDH10*) and aldehyde dehydrogenase 1 family members *ALDH1A1* and *ALDH1A3,* but not *ALDH1A2*.

In the next series of experiments, we inhibited the aldehyde dehydrogenase isoenzymes using a highly selective inhibitor DEAB[155] in MSCl cells. In the following setup, the differentiation of monocytes was observed in the presence of ATRA deficient MSCl-CM, IL-4, and GM-CSF. MSCl-CM collected from cultures of MSCl cells with blocked ATRA synthesis was still able to significantly increase HLA-DQ expression on monocyte-derived cells. In contrast to HLA-DQ, modifications in the expression of CD1a, CD86, and CTLA-4 on myeloid-derived cells exposed to MSCl-CM were dependent on ATRA production by MSCl cell.

These results suggest that MSCl cells bring about monocytes' phenotypic changes at least partially through ATRA production.

4.2.4. CTLA-4 expression on monocyte-derived cells differentiated in the presence of MSCl-CM is essential to initiate the development of IL-17- and IL-10- producing T cells

As APCs, the primary function of DCs to prime, activate, and polarize the T cells. To dissect the functional activity of MSCl-CM-exposed monocyte-derived cells, enzyme-linked immune absorbent spot (ELISPOT) assays were used to examine the moDC-mediated allogeneic T-lymphocyte activation. We found that MSCl-CM-treated moDCs can induce the polarization of T cells secreting IL-17 and IL-10 at a significant level. To reveal a possible role of CTLA-4 in moDC-mediated T cell polarization, the coinhibitory molecule was blocked by specific neutralizing anti-CTLA-4. Unexpectedly, the inhibition of CTLA-4 signaling in MSCl-CM-treated moDCs robustly diminished their ability to induce the development of IL-17- and IL-10-secreting T cells.

Moreover, flow cytometric analysis revealed that MSCl-CM-exposed monocyte-derived cells induced the polarization of IL-10^{+}IL-17^{+} double-positive $CD4^{+}$ T cells. Additionally, the generation of IL-10^{+}IL-17^{+} double-positive $CD4^{+}$ T cells was dependent on CTLA-4 expression

by moDCs, because when the molecule was neutralized with anti-CTLA-4 the T cell polarizing activity of MSCl-CM-conditioned moDCs was significantly reduced.

Our results demonstrate that MSCl cell-released soluble mediators can regulate the T cell-polarizing capacity of monocyte-derived cells. Furthermore, the ability of MSCl-CM-exposed moDCs to drive the polarization of IL-17^+ and IL-10^+ helper T cells is dependent on the level of CTLA-4 molecules on their surface.

5. DISCUSSION

The mesenchymal stem/stromal cells (MSCs) are awakening extraordinary interest among various stem cell types. They express a wide array of receptors involved in recognizing cytokines, chemokines, or invaders, endowing them to sense and react to environmental changes. By active communication, MSCs strongly interact with other cell types, including immune cells. The immunomodulatory capacity of MSCs resulted in the inhibited proliferation of lymphocytes and suppressed inflammatory cells' function after the activation. Furthermore, they can drive the differentiation of monocytes or the polarization of the T cell response. Understanding the sensitivity of the diverse and complex networks that maintain homeostasis is essential to discover the mechanisms mediated by MSCs and characterize these stromal cells throughout the body.

The greater reservoir of MSCs is the bone marrow. The vast number of studies focused on investigating bone-marrow-derived MSCs (BM-MSCs)-biology. To broaden MSC biology knowledge, we elaborated an isolation technique and a morphology/phenotype analysis that is suitable to detect MSCs from saphenous vessel walls (SV-MSC). We managed to separate MSCs from the saphenous vein vessel wall by enzymatic digestion to reveal the background behind these differences. To ensure the efficiency of the isolation method, we compared the morphology, differentiation potential, phenotype, and functions of SV-MSCs to the well-characterized bone-marrow-derived MSCs counterparts.

There are reports published about perivascular cells that appear indistinguishable from MSCs. Because the pericytes and the perivascular mesenchymal stem or progenitor cells share a common phenotype, the MSCs can be characterized by a combination of MSC (CD29, CD44, CD73, CD90, CD105) and perivascular markers (PDGFRβ). Additionally, they do not express hemato-endothelial cell markers (CD31, CD34, CD45, CD144). As BM-MSC, the SV-MSC are also fit the criteria of MSC defined by ISCT, which means that the expression of all expected markers was detectable on their surfaces. SV-MSCs showed similar morphology to BM-MSC.

In the proper induction media, SV-MSCs could be differentiated into adipocytes, osteoblasts, and chondrocytes. Both MSC types are well distinguishable from the control HUVEC cells. However, we detected a higher expression of MCAM (CD146) on BM-MSCs and up-regulated level of CD49b/Itgα2 on SV-MSCs. *In vitro* and *in vivo* experiments suggest that increased expression of CD146 on the surface of MSCs makes the cells more plastic and enhances their ability to migrate through endothelial cells, although they almost lose their regenerative potential.

Our results from Affymetrix GeneChip Human Gene 1.0 ST Arrays give evidence that the isolation technique used by our group is suitable to collect a pure vessel wall-derived mesenchymal stem cell population. To compare the gene expression profile of BM-MSCs and SV-MSCs we investigated the genes related to cell cycle and oncogenes, HOX, SOCS, Notch signaling, differentiation and lineage, and stemness. These data were collected into functional groups to reveal the functional properties of the MSCs with a different origin. Based on the hierarchical clustering data, the genes associated with the cell cycle and oncogenes custom group regulated genes did not observe any significant difference between the BM- and SV-MSCs. On the contrary, the HOX, SOCS, and Notch signaling differentiation and lineage, and stemness, groups were divided into the two MSCs groups with different origins. These divergences could be explained by the variant origin and localization of MSCs where they have different functions. BM-MSCs in the bone marrow contribute to the differentiation of HSCs, while the SV-MSCs are responsible for angiogenesis and neovascularization.

The immunomodulatory activity of BM-MSCs is well known. In the present study, we attempted to observe the potential of SV-MSCs to suppress the mitogenic activation of PBMCs. Surprisingly, SV-MSCs had a stronger inhibitory effect suppressing PHA-induced T cell proliferation. Based on these observations, the SV-MSCs, as the BM-MSCs could function as a potent immunomodulatory cell type.

MSCs could efficiently perceive and subsequently respond to the microenvironment. In this study, we tested the consequences of MSC priming on cytokine and chemokine production. We used well-known and widely used priming stimuli, such as LPS, Poly(I:C), TNFα, IL-1β, and IFNγ to trigger the activation of BM- and SV-MSCs. In addition to the similarities, we observed differences as well.

One of the strategies to overcome the limitations of MSCs obtained from a diverse source, culture conditions, phenotype, epigenetic background, proliferating capacity, and secreted factors is the use of MSC-like (MSCI) cell lines, generating unlimited numbers of early passage MSCs with consistent quality and immunomodulatory properties. In our previous studies, we

described the characterization of a human MSCI cell line generated from pluripotent HUES9 embryonic stem cellsand their capacity to modulate the responses moDCs to RIG-I receptor-mediated stimulation. Based on these results and their phenotypical and functional features, such as differentiation potential and immunomodulatory effect, MSCI cells are considered an appropriate cell line to model mesenchymal stem cells' behavior *in vitro*[147]. However, the ability of MSCl cells and MSCl cell-derived conditioned media (MSCl-CM) to influence the initial differentiation of monocytes has not been explored yet.

Our results showed that MSCI cells could guide monocytes' differentiation into a semi-mature CD14 and DC-SIGN expressing moDC subtype associated with unique phenotypical and functional properties. In our experimental setup MSCl cell-derived conditioned media had only a minor effect on the expression level of CD209 (DC-SIGN), while upon the exposure of monocytes to MSCl cells, the expression of the marker was significantly reduced. Based on the literature and our findings, MSCl-derived secreted mediators drive monocytes' differentiation toward a DC-like cell type. In contrast, the direct interaction with MSCl cells regulates the differentiation of monocytes into M2-like cells. In line with our data, it was discovered that BM-MSCs induce the differentiation of monocytes into macrophages. An interesting question that why the effect of MSCl and MSCl-CM on monocyte fate could be different. Regulatory cytokines such as IL-6 and IL-10 could evoke the upregulated expression of CD163[176]; therefore, these cytokines may have a role in the differentiation of M2 macrophage-like cells from monocytes in our study. On the other hand, cytokine-independent pathways may also be involved in the MSC-mediated monocyte or macrophage polarization. In an asthma model, the engulfment of MSCs triggered lung-resident macrophages to turn into an immunosuppressive phenotype. It has been found that the engulfment of MSCs induced $CD14^{++}CD16^{-}$ classical monocytes to differentiate into a $CD14^{++}CD16^{+}CD206^{+}$ intermediate subtype associated with immune regulatory and anti-inflammatory properties. Since Notch-mediated signals play a role in the protection against inflammation by MSCs, it is possible that the Notch pathway becomes stimulated during the direct cell-cell contact between MSCl cells and monocytes.

Additionally to the modified cytokine and chemokine producing profile of moDCs, the up-regulated expression of the MHC class II-associated molecule HLA-DQ and the co-stimulatory molecules CD80 and CD86 enabled CM-moDCs to preserve their T-lymphocyte activating capacity. In line with the literature, the expression of CD1 molecules CD1a, b, and c was also modulated by MSC cells. One possible regulatory mechanism of MSCl cells contributing to the down-modulation of autoreactive lymphocytes' activity is the shift of T cell

responses via modulation of moDC differentiation to a less inflammatory but adaptive response stimulatory phenotype.

Monocyte-derived cells differentiated in the presence of MSCI cells or MSCI-derived CM have shown altered secretion of certain inflammatory and anti-inflammatory factors, proving the complexity of mechanisms by which moDCs can determine the polarization of T cell responses. The protein-producing profile of moDCs showed the promoted secretion of IL-6, IL-23, IL-27, and TGF-β in the presence of stromal-derived factors, which may explain the enhanced number of IL-10 and IL-17 producing Th cells induced by CM-moDCs. Traditionally, MSCs inhibit the proliferation and polarization of T-cells. However, Gao et al. have described that when MSCs do not create direct contact with the target cells (e.g., in the transwell system), they partially lose their T cell-proliferation inhibiting activity. According to our results, the number of IL-17 secreting T cells could be elevated by moDCs in the presence of MSCl-derived soluble factors. Besides, we found that MSCI-CM-moDCs could increase the number of IL-10 producing T cells as well.

Based on our results, moDCs differentiated in MSCI-CM could trigger the IL-10 and IL-17 production simultaneously by $CD4^+$ T cells in a CTLA-4 dependent manner. The co-inhibitory molecule CTLA-4 can be expressed or produced by cells from both myeloid and lymphoid origins and competes with CD28 expressed by T lymphocytes to inhibit co-stimulatory interaction B7 family members and CD28. CTLA-4 expressing DCs was detected in patients suffering from certain carcinomas, highlighting this molecule's importance during tumor progression. Furthermore, genetically modified DCs expressing CTLA-4-Ig fusion protein prevented alloimmune activity in inflammatory conditions. They ensured the survival of allografts by introducing IL-10 production by Th17 cells, proving the tolerogenic role of CTLA-4 expressing APCs in chronic inflammation. Besides DCs, monocytes can also express CTLA-4, which can be down-modulated during the differentiation process into moDCs[112]. However, our results showed that the cell surface expression of CTLA-4 was enhanced from the first day of the moDC differentiation period and was further increased in the presence of MSCI cell-derived factors on day 4. Additionally, we proved that MSCI-CM has the potential to generate semi-mature moDCs/monocyte-derived APCs associated with enhanced expression of co-inhibitory molecules CTLA-4 and PD-L1 as well as essential proteins in T cell activation such as co-stimulatory B7 family members and class II MHC molecules. These changes were induced only in the presence of stem cell-derived factors from the beginning of the moDC differentiation process. When the MSCl conditioned media were added to the differentiated moDCs, we could not generate $CTLA4^+$ cells. To further confirm the observed distinct effect

of MSCl cells and MSCl-CM on the *in vitro* monocyte-derived cells generation, there are no available results in the literature about the CTLA-4 expressing macrophages.

According to our observations, the modifications induced by MSCI cell-derived mediators can be limited by the selective inhibition of the nuclear hormone receptor RARα, which plays an essential role in moDC differentiation initiated by IL-4 and GM-CSF. In the very early phase of moDC differentiation, the monocytes remain programmable at a physiologically relevant dose of environmental cues, such as in the presence of ATRA[195]. The molecule derived from retinol acts as an interaction partner of RARα to drive the differentiation program of moDCs[105,196]. Interestingly, in the gut *lamina propria*, the presence of DC subpopulations and MSCs could be detected, which cells could constantly express the enzymes for ATRA production even in the absence of dietary vitamin A[126]. Therefore, we examined the mRNA levels of *RDH10*, *ALDH1A1, ALDH1A2,* and *ALDH1A3* genes encoding RDH10, RALDH1, RALHD2, and RALDH3, respectively. It is known that stroma-cell-derived ATRA has a pivotal role in the differentiation of peritoneal macrophages toward a non-strongly inflammatory phenotype. Thus, licensed stromal cells after an inflammatory period could prevent the development of inflammatory monocyte-derived cells via ATRA production. We observed that in the absence of ATRA, the expression of CD86 and CTLA-4 was lower on monocyte-derived cells. The ability of MSCl-CM to induce the modification in the phenotype of moDCs was significantly diminished by either selective inhibition of RARα in moDCs or by that of ATRA synthesis in MSCl cells. Thus, we proved the MSCI cell-derived RARα ligands'/ATRA's regulatory role in moDC differentiation.

Collectively, our observations give novel evidence for the molecular mechanisms regulating the effects of MSCI cells on the differentiation and T-lymphocyte polarizing capacity of moDCs. Based on these results, monocytes differentiated in the presence of MSCI cells or MSCI-CM into monocyte-derived cells characterized by a unique phenotype and functional properties, opening new perspectives in treating patients who have cancer, autoimmune or inflammatory diseases. Our results suggest a new level of MSC and consequently moDC control over T cell responses and may have a remarkable implication for novel uses of CTLA-4 agonist and antagonist drugs. Mapping the molecular mechanisms of indirect regulation by MSCs on monocyte differentiation could expand the clinical application of stromal cells in cell-free therapies.

6. SUMMARY

Based on the ongoing clinical trials, mesenchymal stem/stromal cells (MSCs), due to their multipotent differentiation and efficient immunomodulatory potential, are promising candidates for cell-based therapies of immune-mediated diseases. MSCs are crucial players in regulating immune responses, namely if the communication between the stromal and other tissue-resident cells is impaired, resulting in functional failures causing diseases.

The greatest known reservoir of MSCs is the bone marrow, but MSCs reside in more organs and tissues like in the wall of blood vessels. The sensitivity of the diverse and complex networks that maintain homeostasis is essential to discover the mechanisms mediated by MSCs and characterize these stromal cells throughout the body. Despite many studies about the modifications in cells and tissues after MSC administration, the results are often inconsistent. The possible reason is the differences in the individual general health, source, or culture condition of MSCs, leading to their diverse phenotype, epigenetic background, viability, and secreted mediators. During our investigations, we attempted to elaborate an isolation technique and a morphology/phenotype analysis which is suitable to characterize vessel wall-derived MSCs (SV-MSCs) and discriminate them from the other vessel-wall or endothelium-related cells in the subendothelial area. In a comparative analysis, our project revealed the multilineage differentiation potential, antigen and gene expression profile, the immunomodulatory effect of SV-MSCs as well as their response to priming with different stimuli.

One of the strategies to overcome the limitation of MSCs with different origins is the use of MSC-like (MSCl) cell lines generating unlimited numbers of early passage MSCs with consistent quality and immune-modulatory features. *In vitro* generated MSCl cells seemed to be an appropriate model to examine the functional properties of MSCs. We investigated the effect of MSCI cells on the DC functions, notedly on the monocyte differentiation process. Dendritic cells (DCs) are critical players in the communication between innate and adaptive immune cells. Due to their plasticity, their differentiation processes from monocytes can be strongly modulated, resulting in cells with the most appropriate functions adapting to the given conditions. The immune-modulating MSCl cells indirectly promote the differentiation of CTLA-4 expressing DCs by producing the RARα ligand, all-trans retinoic acid (ATRA). These monocyte-derived cells are potent activators of allogeneic, IL-10, and IL-17 producing helper T lymphocytes in a CTLA-4-dependent manner.

Expanding the knowledge of the molecular background of MSC-driven immune modulation may help make safer the clinical application of MSCs.

7. PUBLICATIONS

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List of publications related to the dissertation

1. **Türk-Mázló, A.**, Kovács, R., Miltner, N., Tóth, M., Veréb, Z., Szabó, K., Bacskai, I., Pázmándi, K. L., Apáti, Á., Bíró, T., Bene, K., Rajnavölgyi, É., Bácsi, A.: MSC-like cells increase ability of monocyte-derived dendritic cells to polarize IL-17-/IL-10-producing T cells via CTLA-4. *iScience. 24* (4), 1-25, 2021.
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8. KEYWORDS

Bone marrow-derived Mesenchymal Stem/Stromal Cell, Saphenous vein-derived Mesenchymal Stem/Stromal Cell, Monocyte, differentiation, Dendritic cell, Mesenchymal Stem/Stromal Cell-like cell, stem cell line, CTLA-4, all-trans retinoic acid/ATRA, RAR, T cell, IL-10, IL-17

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Oral presentations:

Anett Mázló, *Novel mechanisms of mesenchymal stem cell-mediated immune suppression*, 8th Winter Symposium of Molecular Cell- and Immunobiology Doctoral School, Debrecen, Magyarország, 2015

Anett Mázló, *New means how mesenchymal stromal cell like cells can modulate dendritic cell functions,* 9th Molecular Cell and Immune Biology Winter Symposium, Debrecen, Magyarország, 2016

Anett Türk-Mázló, *Novel mechanisms of mesenchymal stem cell-mediated immune suppression,* 3rd Meeting of Middle-European Societies for Immunology and Allergology, Budapest, Hungary, 1-3. december, 2016

Anett Türk-Mázló, *Novel mechanisms of mesenchymal stem cell-mediated immune suppression*, 10th Molecular Cell and Immune Biology Winter Symposium, Debrecen, Magyarország, 2017

Anett Türk-Mázló, *Role of CTLA-4 in mesenchymal stem cell-mediated immune modulation,* 11th Molecular Cell and Immune Biology Winter Symposium, Debrecen, Magyarország, 2018

Türk-Mázló Anett, *Felnőtt szöveti őssejtek „checkpoint" mechanizmusok szabályozása révén megvalósuló immunválaszokat meghatározó működésének molekuláris biológiai vizsgálata*, ÚNKP Konferencia, Magyarország, Debrecen, 2018. június 18.

Türk-Mázló Anett, *Mezenhimális őssejtek dendritikus sejtek keresztprezentáló képességére gyakorolt hatásának vizsgálata*, ÚNKP Konferencia, Magyarország, Debrecen, 2019. június 17.

Anett Türk-Mázló *Differences in the sensitivity of classically and alternatively activated macrophages to TAK1 inhibitor-induced necroptosis,* International Cancer Research Conference, Dec 15-16, 2020

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Anett Mázló, Ildikó Bacskai, Veronika Makó, Ágota Apáti, Balázs Sarkadi, Éva Rajnavölgyi: *Inhibitory role of NLRX1 in human dendritic cells immunosupressed by MSCI,* 9th Winter Symposium of Molecular Cell- and Immunobiology Doctoral School, Galyatető, Magyarország, 2014

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Anett Türk-Mázló, Szilárd Poliska, Ildikó Bacskai, Ágota Apáti, Éva Rajnavölgyi: *Mesenchymal stromal cell like cells could regulate the immune response,* 44th Symposium of Hungarian Society for Immunology, Velence, Magyarország, 14-16. October, 2015

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Anett-Türk Mázló, Márta Tóth, Ramóna Kovács, Krisztina Szabó, Ágota Apáti, Tamás Bíró, Krisztián Bene, Attila Bácsi, Éva Rajnavölgyi: *MSCs have the potential to induce the generation of CTLA-4+ human moDCs*, 47th Annual Meeting of the Hungarian Society for Immunology, 17-19th October 2018, Bükfürdő

Ramóna Bíró-Kovács, Anett Türk-Mázló, Yair Tzikinovsky, Attila Bácsi, Éva Rajnavölgyi: *The effect of soluble factors produced by tumor cells on the differentiation of dendritic cells,* 47th Annual Meeting of the Hungarian Society for Immunology, 17-19th October 2018, Bükfürdő

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