THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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

by

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CONTENTS

1. ABBREVIATIONS

ADH - Alcohol DeHydrogenase
APC - Antigen-Presenting Cell
ATRA – All-Trans Retinoic Acid
BM-MSC – Bone-Marrow-derived Mesenchymal Stem/Stromal Cell
CCL - Chemokine (C-C motif) Ligand
CD - Cluster of Differentiation
CLP - Common Lymphoid Progenitor
CM - Conditioned Media
CMP – Common Myeloid Progenitor
CSF-1 - Colony-Stimulating Factor-1
CTLA-4 - Cytotoxic T-lymphocyte Antigen-4
CXCL - C-X-C Motif Chemokine Ligand
DC - Dendritic Cell
G-CSF - Granulocyte Colony-Stimulating Factor
GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor
GMP - Granulocyte and Macrophage Progenitor
HLA - Human Leukocyte Antigen
HSC - Hematopoietic Stem Cells
IFN - Interferon
IL - InterLeukin
IP-10 - Interferon gamma-induced Protein-10
LPS - LipoPolySaccharide
MDA-5 - Melanoma Differentiation-associated Antigen-5
MDC - Mature Dendritic Cells
MHC - Major Histocompatibility Complex
MMP - Matrix MetalloProteinase
moDC – monocyte-derived Dendritic Cell
MSC - Mesenchymal Stem/Stromal Cell
MSCl - Mesenchymal Stem/Stromal Cell-like cells
NCBI GEO - National Center for Biotechnology Information - Gene Expression Omnibus
PD-L1 - Programmed Death-Ligand-1
Poly(I:C) - Polyinosinic: polycytidylic acid
RA - Retinoic Acid
RALDH - Retinal DeHydrogenase
RAR - Retinoic-Acid Receptor
RIG-I - Retinoic acid-Inducible Gene-I
RXR - Retinoid X Receptor
SV-MSC - Saphenous vein-derived Mesenchymal Stem/Stromal Cell
TGF - Transforming Growth Factor
TIMP - Tissue Inhibitors of MetalloProteinases
TLR - Toll-Like Receptor
TNF - Tumor Necrosis Factor

2. 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[1]. Later, these cells were functionally characterized as essential cells to prime and polarize the specific immune responses[2]. 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[3]. 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.

3. THEORETICAL BACKGROUND

3.1. Mesenchymal Stem/Stromal Cells

Among various stem cells, i.e., embryonic, induced pluripotent, and hematopoietic ones, the MSCs show great interest as well. Until last year, the term 'mesenchymal stromal cell' was used parallel with 'mesenchymal stem cell' and 'multipotent mesenchymal stromal cell'. However, according to Viswanathan et al., the term 'mesenchymal stem cell' cannot be used unless the stemness of the cells is supported by both *in vitro* and *in vivo* data[4]. As essential cells in all tissues and organs, MSCs play a critical role during regeneration and in the regulation of tissue homeostasis. To confirm this statement, a body of evidence shows that depletion or dysfunction of MSCs may lead to the development of diverse diseases like lupus, diabetes, psoriasis, rheumatoid arthritis, and aging or frailty syndrome[5]. Based on these observations, the MSCs could provide promising alternatives to develop treatments for patients suffering from

chronic inflammatory or degenerative diseases. *In vivo* studies have already investigated the wide array of MSC-derived secreted mediators, expanding their potential clinical application in cell-free therapies[6].

The heterogeneous group of these multipotent cells form colonies and differentiate into mesenchymal lineages such as bone, fat, and cartilage cells, but they also have endodermic and neuroectodermic differentiation potential[7] ***(Figure 1)***.

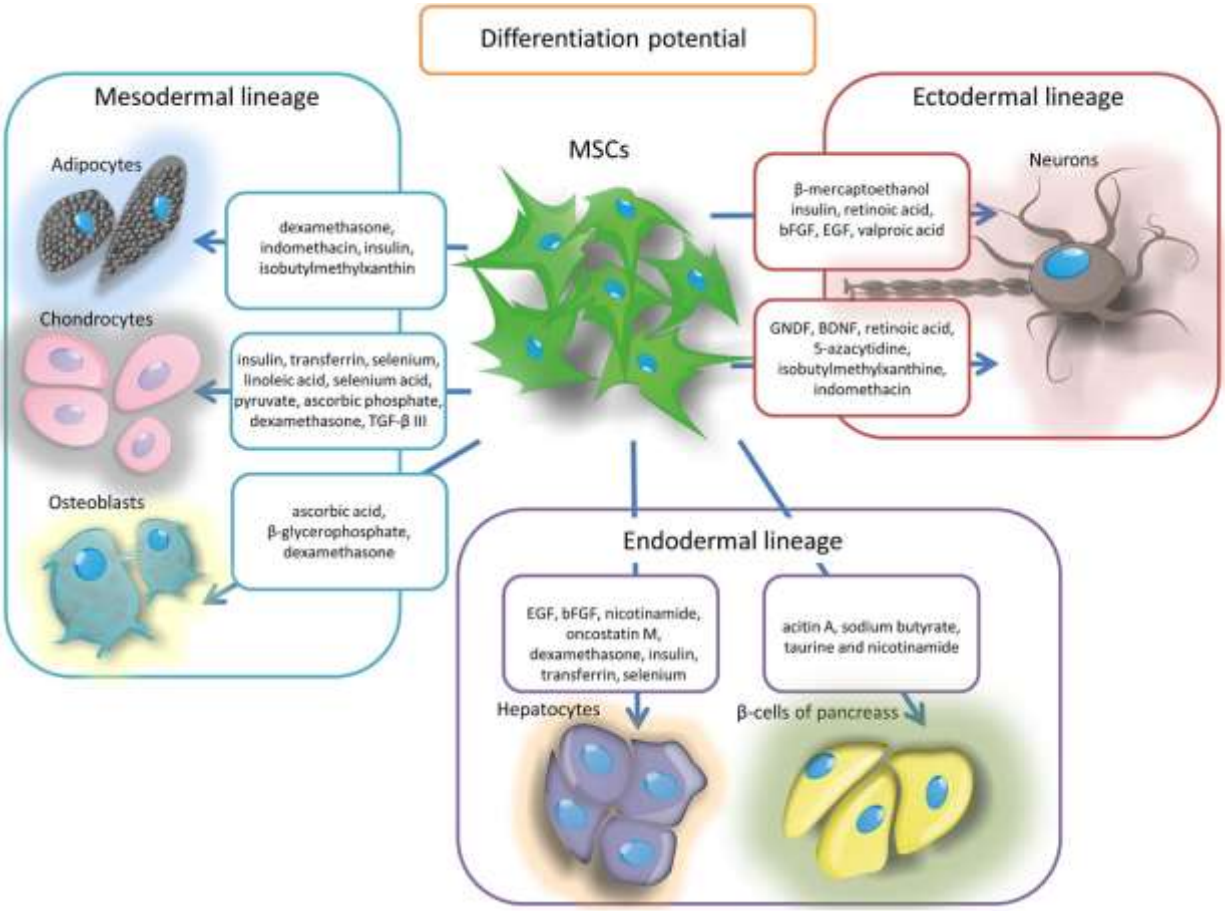


Figure 1. The differentiation potential of mesenchymal stem cells. *The capacity to differentiate into three types of cells, such as osteocytes, chondrocytes, and adipocytes, is one of the criteria for MSCs. This potential of MSCs can be traced in vitro by culturing the cells in a proper medium containing specific supplements detailed in this figure. Additionally, MSCs might also be a source of other cell types: hepatocytes and β-cells of pancreatic islets or neurons. MSC: mesenchymal stem/stromal cell; TGF: Transforming growth factor; EGF: epidermal growth factor; FGF: fibroblast growth factor; GDNF: glial cell line-derived neurotrophic factor; BDNF: brain-derived neurotrophic factor, doi:10.1002/stem.3016 (2019)*

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[8].

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[9,10].

3.2. MSCs in the vessel walls

MSCs with different origins and organ/tissue localization differ in their morphology and biological features[11]. Additionally, many experiments suggest variations in the differentiation capacity or immunomodulatory effect of MSCs from distinct tissue sources[12].

The establishment of an MSC niche in the vascular adventitia provides a basis for the rational design of additional *in vivo* therapeutic approaches. Although arteries and veins differ structurally and functionally, they share particular properties. For instance, they are composed of the same layers and populated by the same cell types ***(Figures 2 and 3)***. Additionally, they utilize similar mechanisms to maintain their stability under physiologic conditions or ensure the repair mechanisms[13].

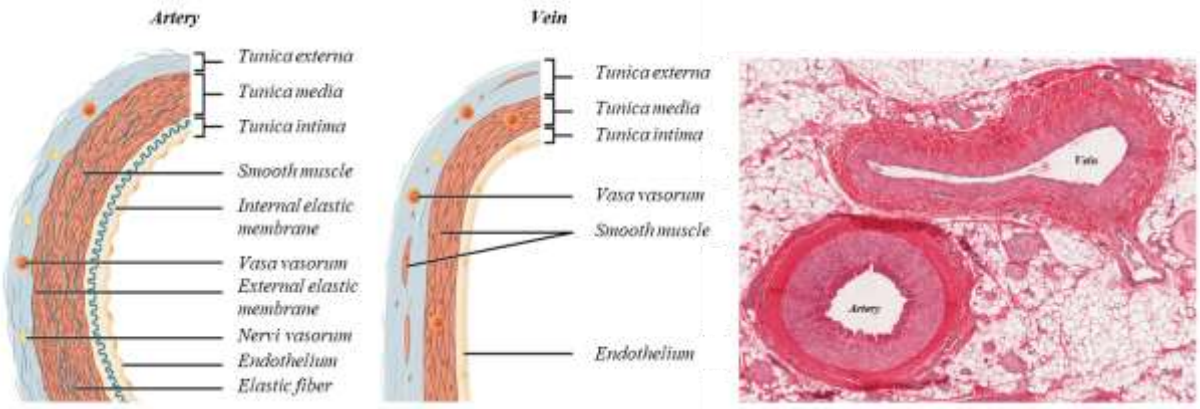


Figure 2. *The structures of the human blood vessels. Illustration from Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013*

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. Endothelial cells and pericytes are two essential components of vessel walls. Several studies have revealed new cell populations that can be a potential source of endothelial cells and pericytes supporting vascularisation and angiogenesis[14]. In 1973, Schwartz and colleagues detected proliferating endothelial cells after a vascular injury during rodent development[15]. In 1997 Asahara published circulating bone marrow-derived progenitor cells called putative endothelial progenitor cells (ECP) for the endothelial lineage[16]. Later, Minasi and colleagues have proposed that resident vascular progenitor cells can differentiate into endothelial cells[17].

In 2012, $CD31^{+}CD105^{+}SCA\text{-}1^{+}CD117^{+}$ stem cell population was isolated from lung vasculature, which could differentiate toward endothelial lineage. These cells are the vascular endothelial stem cells (VESCs) associated with self-renew capacity and could be sources of all cell populations within the tissue[18]. In 2016, in an *in vivo* study Qing proved the robust vessel formation capacity of Procr+ (Protein C Receptor also known as EPCR) VESCs[19].

The possible mesenchymal origin of pericytes and endothelial cells partially explains why the perivascular MSCs almost indistinguishable from the other vascular cell types ***(Figure 3)***.

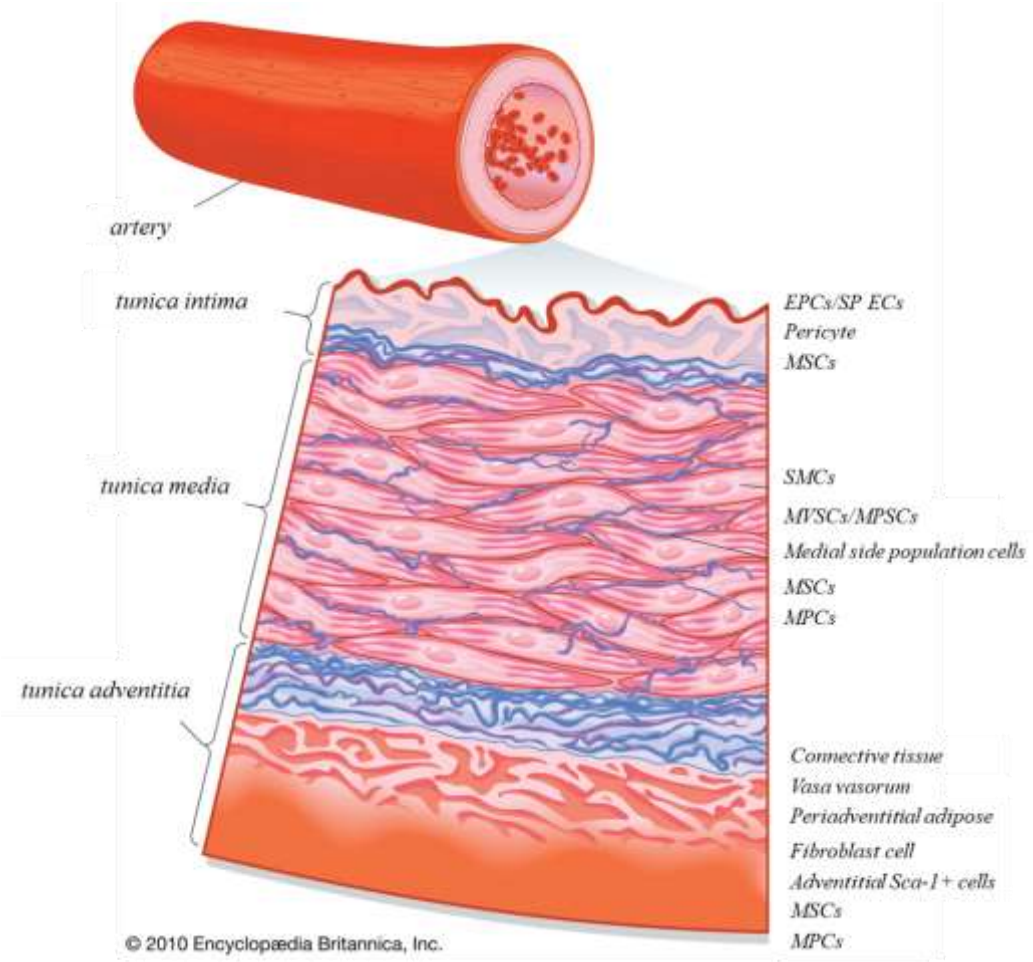


Figure 3. Diverse origin of adult resident vascular stem cells in different compartments of the blood vessel wall. *The presence of various vascular progenitor cells have been observed in the layers of the blood vessels. SMC indicates smooth muscle cell; MSC, mesenchymal stromal/stem cell; MVSC, multipotent vascular stem cell; MPSC, multipotent stem cells; SP/EC, side population endothelial cell; EPC, endothelial progenitor cell; MPC, macrophage precursor cell*[20].

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.

3.3. 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.

Expression of major histocompatibility complex II (MHCII) on their surface can be induced in the presence of interferon-γ (IFNγ) lending for the cells antigen-presenting property[21]. Additionally, MSCs express CD80, CD86 co-stimulatory, as well as low level of human leukocyte antigen (HLA) class I molecules. They seem to be weakly immunogenic, but MSCs could induce allogeneic MHCI mismatched memory $CD8^+$ T-cell-mediated lysis[22]. They are susceptible to lysis by autologous IL-2-activated natural killer (NK) cells due to the relatively low level of the NK cells inhibitory ligand (MHCI) on their surfaces[23]. Thus, it can be assumed that MSCs are not fully hypoimmunogenic, but they are less immunogenic than other allogeneic cell types[24]. Functionally, they should instead be seen as a controller of immune responses.

MSCs are not spontaneously suppressive cells; their immunomodulatory role depends on the actual environmental conditions. There are several strategies for immunomodulation ***(detailed in Table 1)*** and therapeutic use of MSCs.

	Priming reagent	Function
Cytokines	IFN-γ	upregulated secretion of PGE_2, HGF, TGF-β, and MCP-1
		upregulated secretion of PDL-1 & T cell suppression
		downregulated secretion of IFN-γ and TNF-α & T_H17 cells
		upregulated secretion of IL-6 and IL-10 & Tregs promotion
	TNF-α	upregulated survival, proliferation, migration, and immunosuppression
		upregulated secretion of PGE_2 & alternative macrophage
	IL-1β	upregulated expression of TGF-β1 and MMPs
	IL-17A	effector T cell suppression & Tregs promotion
		upregulated expression of MMP1, MMP13, and CXCL6
	IL-1α, β	upregulated secretion of G-CSF & microglial cell suppression
	IL-25	T_H17 cell suppression & Tregs promotion
	IL-2	upregulated expression of genes related growth, anti-inflammation, angiogenesis, and anti-apoptosis
Growth factors	TGF-β1	upregulated proliferation and *in vivo* survival
	HGF	upregulated secretion of albumin and α-fetoprotein
	FGF-2	upregulated chondrogenic differentiation
		upregulated secretion of VEGF and HGF & angiogenesis

Table 1. Priming effect of cytokines and growth factors on MSCs. *MSCs could perceive and subsequently respond to the microenvironment, known as "MSC licensing." IFN: Interferon; TNF: tumor necrosis factor; IL: Interleukin; TGF: Transforming growth factor; HGF: hepatocyte growth factor; FGF: fibroblast growth factor; PGE2: Prostaglandin E2; MCP-1: Monocyte chemotactic protein 1, PDL-1: Programmed death-ligand 1; MMP: matrix metalloproteinase, CXCL: C-X-C Motif Chemokine Ligand, G-CSF: Granulocyte colony-stimulating factor, VEGF: vascular endothelial growth factor. doi:10.1186/s13287-020-01920-3 (2020)*

Stimulation of MSCs by inflammatory cytokines[25] (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.

MSCs are sensors and switchers of inflammation. They sense their surrounding microenvironment (inflammation vs. infection) and regulate the function of the host's immune cells[26]. MSCs display a set of functional pattern recognition receptors (PRR) in definite patterns depending on their localization[27], turning them selectively sensitive to dangerous compounds[28,29]. Upon activation, PRRs (TLR and Nod-like receptors /NLRs/) can control the proliferative and immunomodulatory potential of MSCs[30-32] ***(Table 2)***.

Target receptor	Priming reagent	Function
TLR3	Poly(I:C)	upregulated Notch signaling
		T_H17 cell suppression & Tregs promotion
		upregulated secretion of PGE_2
TLR4	LPS	upregulated secretion of IL-6 and IL-8
		upregulated expression of miR-146a and miR-155
		downregulated expression of miR-150
		upregulated expression of BAFF
		induction of alternative macrophage
TLR5	flagellin	upregulated expression of IL-10, CCL24, and TGF-β
		downregulated expression of expression of CCL5 and IP-10
		upregulated expression of antimicrobial protein & Treg promotion
NOD2	MDP	upregulated secretion of PGE_2

Table 2. Priming effect of PAMPs on MSCs. *In line with preconditioning with cytokines and growth factors, priming with other bioactive substances such as innate immune receptor agonists could boost the immunomodulatory effect of MSCs. TLR: Toll-like receptor; Poly(I:C): Polyinosinic: polycytidylic acid; LPS: lipopolysaccharide, MDP: muramyl dipeptide, PGE2: Prostaglandin E2; IL: Interleukin; miR: microRNA, BAFF: B-cell activating factor, CCL: Chemokine (C-C motif) ligand; TGF: Transforming growth factor; IP-10: Interferon gamma-induced protein 10. doi:10.1186/s13287-020-01920-3 (2020)*

After the ligation of TLRs, the expression of immunomodulatory genes encoding interleukin (IL)-6, IL/C-X-C motif chemokine ligand (CXCL)-8, and cyclooxygenase-2 (COX-2) is increased[33]. The more robust suppressive activity of MSCs after recognizing pathogen- or damage-associated molecular pattern (PAMP/DAMP) seems essential to generate a negative feedback loop for protecting tissues from unnecessary damage. However, there are cases when TLR activation may inhibit the typical immunosuppressive effect of MSCs, allowing T cell responses to build up[34].

Other agonists are boosting the immunomodulatory effect of MSCs as well; however, these are not used as therapeutically relevant "licensing" of MSCs. They express functional retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated antigen-5 (MDA-5) receptors. Their activation could induce the apoptosis of MSCs[35], or dependently on the concentration of stimuli or the origin of MSC, the soluble mediator expression could be triggered as well. Upon activation, they can produce leukemia inhibitory factor (LIF), indoleamine 2,3-dioxygenase (IDO), IL-6, IL-8, CCL5, TNF-α, IFN-β, and IFN-λ1[36]. Moreover, MSCs, depending on their tissue-origin, present several relevant receptors, including advanced glycation end-products (RAGE) receptor; C-type lectin receptors (CLRs, including

DECTIN-1, DECTIN-2, and MINCLE); leukotriene B4 (LTB4) receptors (BLT1 and BLT2); and cysteinyl leukotrienes (CysLTs) receptors (CYSLTR1 and CYSLTR2), which are involved in the modulation of immune responses[37].

Surprisingly, MSCs are phagocytes, which newly identified functional feature also could modify their immunomodulatory effects[32].

Molecules involved in extracellular matrix (ECM) turnover, such as matrix metalloproteinases (MMPs), and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), also alter the regulatory effects of MSCs. The proliferation (MMP1, MMP3, MMP2, MMP14, MMP16), migration (MMP1, MMP2, MMP14, TIMP1, TIMP2), angiogenesis (MMP2, MMP9, MMP14), and adipogenic (MMP2, MMP11, MMP13, TIMP1)/ chondrogenic (MMP2, MMP9, MMP13, MMP14)/ osteogenic (MMP3, 13, 14, TIMP1) differentiation of MSCs could be promoted by the fine-tuned expression of specific MMPs and/or TIMPs[38].

The background of the therapeutic effect as well as the MSC-driven immunomodulation is not clearly revealed yet. It is known that endogenous MSCs cannot move into the inflamed areas via the circulation throughout the body[39]. However, they migrate in the given tissue to support the processes in the damaged location. In contrast, the intravenously administrated MSCs with therapeutic purpose target the highly vascularized tissues throughout the body[40]. Dominantly because of their immunosuppressive activity MSCs have beneficial effects in multiple immunological diseases. Pre-clinical and clinical trials carried out so far have reported signs of immune modulation after MSC intravenous administration; like graft versus host disease (GVHD)[41], organ transplantation[42], inflammatory bowel disease (IBD)[43], systemic lupus erythematosus (SLE)[44], multiple sclerosis (MS)[31], rheumatoid arthritis (RA)[45] and chronic obstructive pulmonary disease (COPD)[34].

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.

3.4. 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[46], but they dominantly represent CD73 on their surfaces[47]. 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)[48,49], transforming growth factor β (TGFβ)[50], IL-6, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF)[51], vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1)[52] and the secreted form of PD-L1[53], cytotoxic T-lymphocyte antigen-4 (CTLA-4)[54], and HLA-G[55]. 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[56,57]. Production of IL-10 has been reported to be associated with induction of non-classic HLA class I molecule HLA-G[58]. 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[25].

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)[59]. 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. Effects of MSC-derived mediators on target cells are summarized in ***Table 3***.

Soluble mediators secreted by MSCs	Target cells
NO	Mo, DC, T
IDO	Mo, DCs, B, T, Tregs
TSG-6	Mo, Mph, DC
IL-10	Mph, Neu, DCs, Th1, Tregs, Tr1, tumor cells
TGFβ	Mph, NK, DCs, B, T, Tregs
IL-6	Neu, Mo, DCs, B, Th2, Tregs, Th17, CD8+FoxP3+
PGE2	Mph, Mo, NK, DCs, T, Tr1
HO-1	Tregs
CTLA-4, PD-L1	Mph, DC, T
HLA-G	T, NK
GALECTIN-1	Tc, Th
Growth factors	DCs, EC, Th1, Th17, Tregs, EC
ICAM-1, VCAM-1	T, MSCs, Mo
Chemokines	Neu, Mo, NK, Eo, Baso, DCs, Ly

Table 3. Effects of MSC-derived mediators on target cells showing the broad regulatory impact of these adult stem cells. *MSC: Mesenchymal stem cells; TGFβ: Transforming growth factor β; CCL: CC chemokine ligand; MCP-1: Monocyte chemotactic protein 1; RANTES: Regulated on activation, normal T cell expressed and secreted; IDO: Indoleamine-2,3-dioxygenase; VEGF: Vascular endothelial growth factor; ICAM: Intercellular adhesion molecule; VCAM: vascular cell adhesion molecule, PGE2: Prostaglandin E2; Mph: Macrophages; Neu: Neutrophils; DCs: Dendritic cells; Th: T helpers; Tregs: T regulatory cells; Tr1: T regulatory 1; Mo: Monocytes; B: B cells; NK: Natural killers; T: T cells; Eo: Eosinophils; Baso: Basophils; Ly: Lymphocytes; EC: Endothelial cells; PL: Plasma cells. Based on the references of Chapter 3.4.*

The therapeutic and regulatory effects of MSCs are also mediated by extracellular vesicles (EV)[60]. Exosomes produced by MSCs store cytokines, growth factors, signaling lipids, mRNAs, and regulatory miRNAs playing roles in physiological and pathological processes. MiRNAs involved in organism development, epigenetic regulation, immunoregulation (miR-155 and miR-146)[61], tumorigenesis, and tumor progression (miR-23b, miR-451, miR-223, miR-24, miR-125b, miR-31, miR-214, and miR-122)[62], DC maturation and functions (miR-21-5p, miR-142-3p, miR-223-3p, and miR-126-3p) were detected within the top 10 most enriched miRNAs in MSC-EVs[63].

The modulatory effect of MSCs on T cells is dependent on the ratio of the cells or the concentration of MSC-conditioned supernatant in the common T cell-MSC culture media. A high MSC : T-cell ratio commonly leads to T-cells inhibition, while a low MSC:T-cell ratio is associated with increased T-cell proliferation. Obermajer et al. revealed an important mechanism by which intravenously injected MSCs put their long-lasting modulatory effect on the T cell responses after organ transplantation; the Th17 into Treg conversion constituted an important immunological mechanism by which MSC-educated myeloid-derived immunosuppressive cells generate the transplant tolerance[64].

3.5. Immunomodulation by apoptotic and dead MSCs

Interestingly, administration of apoptotic adipose tissue-derived MSCs (A-ADMSC) seems to be more effective in the silencing of inflammation, oxidative stress, apoptosis, and sepsis-induced histopathological changes in the lung and kidney than the injection of living stromal cells[65]. Cells involved in the clearance of dying MSCs ensured the increased survival of hypoxic cardiomyocytes[66], in accordance with the "dying stem cell hypothesis" published by Thum in 2005. Based on that, the apoptosis of MSCs has a regulatory role in modulating the local immune responses via the alteration of both innate and adaptive immunity[67].

The viability of MSCs does not appear to be a prerequisite for some of their immunoregulatory capacity[68]. Within 24 hours after being trapped in the pulmonary system, a significant decrease in the number of viable MSCs can be observed[69]. Monocytes and neutrophils are the dominant cell types in the clearance of administered MSCs. While neutrophils appear to deposit in the lung after the engulfment of MSCs, monocytes migrate through the circulation to distant injury sites, where they exert their acquired immunoregulatory effect[70]. The partial immune regulatory activity can be explained by MSC-derived enzymatically active membrane particles, which could be engulfed by monocytes and became bound to their membranes inducing selective apoptosis of proinflammatory monocytes[71]. Furthermore, uptake of MSCs triggers expression of the regulatory markers CD163 and CD206 on monocytes and increases IL-10 and TGFβ expression and reduces TNFα, which suggests the adaptation of a regulatory function of monocytes upon engulfment of MSCs[70].

Heat-inactivation of MSCs (HI-MSC) (for 30min to 50°C) a possible method to examine the effects of dying MSCs. However, the heat inactivation evoked irreversible expiry of the metabolic activity and inhibited proliferation of MSCs[72], but they preserved their suppressive properties partially; they down-regulated the strength of immune response upon LPS stimuli through the increased IL-10 and decreased IFN production, but they could not inhibit the proliferation of T-cells anymore.

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.

3.6. 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 the '*classical*' $CD14^{(hi)}/CD16^{(neg)}$ / $CD14^{++}CD16^{-}$ phagocytes playing an essential role in the initiation and progression of the inflammatory responses. They have pro-inflammatory activity in multiple chronic diseases, including atherosclerosis[73], cancer[74], and rheumatoid arthritis[75]. 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. These cells seem to play a role in resolving inflammation and taking part in the clearance of dying cells[76], viruses[77], bacteria[78], and tumor cells[79] from the circulation. The '*intermediate*' $CD14^{(hi)}/CD16^{(med/high)}$ population is present in low numbers in the blood under physiological conditions. After stimulation, they are thought to represent more mature macrophage-like monocytes[80] showing both phagocytic, inflammatory[81-84], and anti-inflammatory[85] features as well. 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[86].

They observed that however the monocytes could be activated, monocytes respond differently upon TLR stimulation and appear to have a built-in capacity to produce a particular different secreted mediator. Variability among donors in the expression of multiple genes in monocytes has been previously described[87,88]. The mechanisms and stimuli that lead to the development of individual variations between monocyte phenotypes are mostly unknown. Pieces of evidence suggest that the alterations within the population of mononuclear cells are driven mainly by combinations of intrinsic and environmental factors such as epigenetic

modifications, age-related alterations, but also through viral infections, the composition of the microbiota, serum lipid levels, and lifestyle[89-95].

3.7. Origin and differentiation of human monocytes

Endo- and exogenous signaling leading to the generation of quantitative and qualitative changes in the gene expression profile determine alterations in the fate of myeloid-derived cells. During the differentiation processes, the well-tuned network of signals by direct and indirect regulators coordinates progenitor cell specifications.

Immune cells are differentiated from the hematopoietic stem cells (HSCs) in the bone marrow during the hemopoiesis. Transcription factors, cell surface receptors, and various environmental mediators, including colony-stimulating factor 1 (CSF-1), stress, cholesterol loading, hyperglycemia, and infections, play an essential role in determining the lineage commitment of HSCs[96]. These adult stem cells reside mainly in the bone marrow, but a low number of HSCs circulates in peripheral blood[97]. HSCs are a heterogeneous population that consists of three major subtypes: the self-renewing long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent HSCs (MPPs) associated with the ability of transient reconstitution[98]. The BM-resident and circulating HSCs could "collect" information from the periphery, thereby adapting to the given conditions by altered gene expression. The accumulation of information could be manifested in epigenetic modifications leading to the formation of a defined, dynamic chromatin structure and an "individual" gene expression pattern, which regulates the expression of genes and determines the self-renewal and differentiation potential of HSCs[96].

HSCs can produce either common lymphoid progenitors (CLPs) and myeloid progenitors (CMPs). CMPs differentiate into granulocyte and macrophage progenitors (GMPs ($CD123^+CD45RA^-CD135^+$ in the presence of Flt3L), critical for developing the myeloid-lineage such as granulocytes, monocytes, macrophages, and DCs[99]. Monocytes are produced under physiologic conditions during the steady-state monopoesis. However, pathological conditions (e.g., infections, inflammation, cancer, or stress) lead to emergency monopoesis and the generation of new monocyte subsets associated with altered functions. Monocyte precursors in humans are localized in the bone marrow. They traditionally are the monocyte-macrophage/dendritic cell precursor (MDP) and common monocyte progenitor (cMoP). Because the lifespans of monocyte subsets are short, their developmental programs and equilibrium must be tightly regulated. MDP was subsequently identified within, and probably derived from, the GMP population. This 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. Investigations into cMoP biology have uncovered that this population possesses a high proliferative capacity and is characterized by CD14 expression in humans[100]. Human cMoPs can be characterized by the expression of CD64 (FcγRI) and C-type lectin domain family 12 member A (CLEC12A). These markers are expressed by human monocytes and macrophages as well.Based on the expression of CD64 and CLEC12A , the human GMPs could be grouped into four populations. Only the $CD64^{high/int}CLEC12A^{high}$ cells are able to differentiate into monocytes, macrophages, or monocyte-derived DCs (moDC). Subsequently, cMoPs express CD64, CLEC12A, and in humans, these cells additionally positive for CD135 as well. Human cMoPs give rise to monocytes via monocyte precursors, i.e., pre-monocytes. The cell surface marker expression and differentiation potential of the pre-monocytes are almost the same as those of cMoPs. Still, they do not express CD34 stem cell marker have no colony-forming capacity[101].

In general, monocytes can migrate through the endothelium of the blood vessels. Before, during, and after the extravasation, they undergo several changes to become the diverse populations of macrophages or DCs. These cells represent subtype specification pathways resulting in the differentiation of classically and alternatively activated macrophages, or different tissue macrophages, or moDCs.

Monocyte-derived cells are exposed to a wide array of actual microenvironmental factors. Thus, their differentiation is significantly determined by nuclear receptor and TLR ligands, lipoproteins, lipids - derived from pathogens or dying cells -, and the panels of secreted mediators, including cytokines and chemokines. The circumstances primarily control the activation of the master transcription factors modifying the expression profile of a set of genes that have an essential role in the emergence of functional and phenotypical features of monocyte-derived immune cells ***(Figure 4)***.

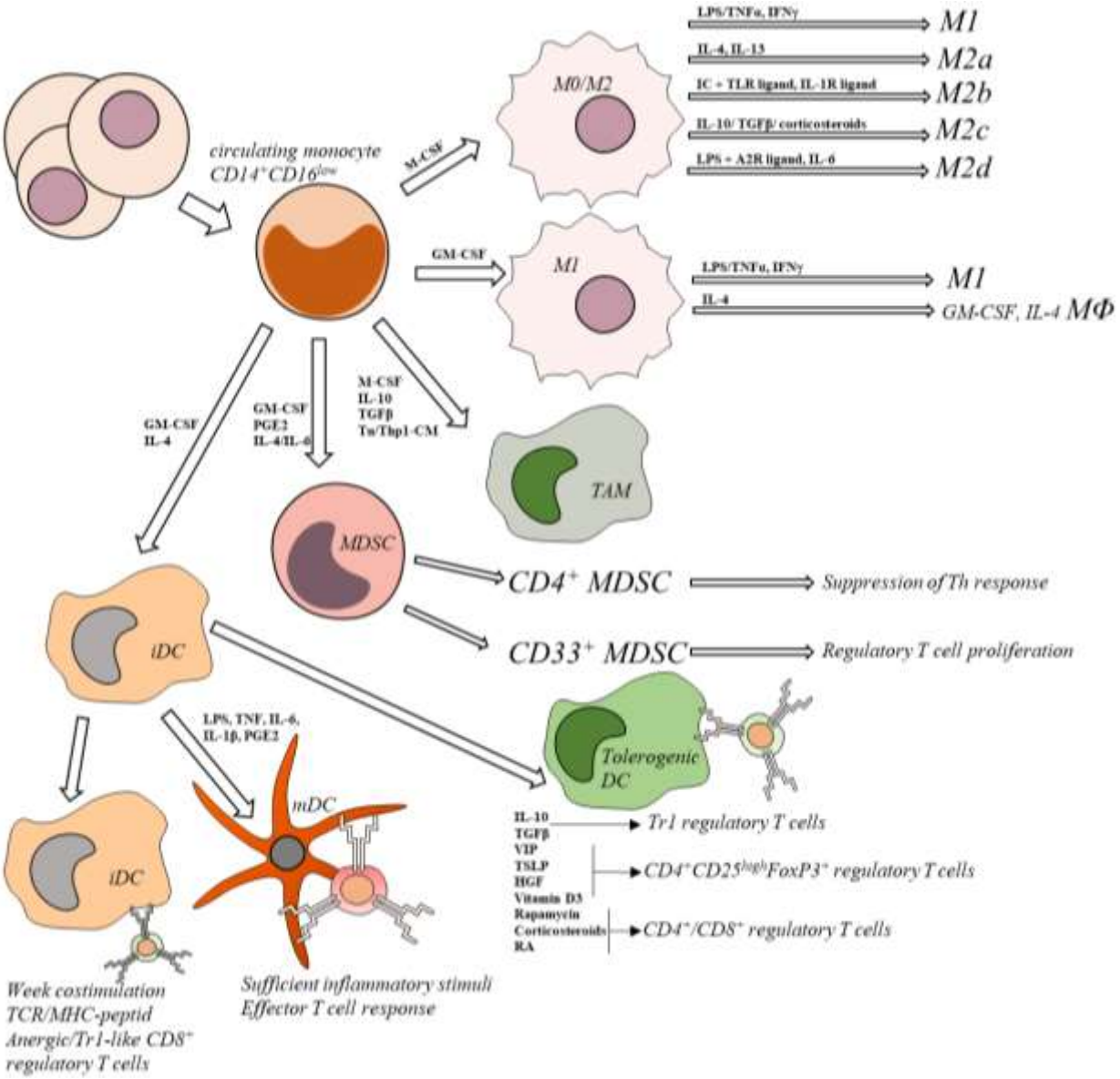


Figure 4. The landscape of human monocyte differentiation and polarization. *$CD14^+$ monocytes could differentiate into cell types depending on the presence of a wide array of soluble mediators. The presence of IL-4 and GM-CSF from the beginning of the differentiation process is essential for the differentiation of monocytes toward dendritic cells. GM-CSF or M-CSF mediates the differentiation of monocytes into M1 or M2 macrophages, which cells could be polarized into subpopulations associated with various functions. These cytokines support the generation of tumor-promoting myeloid-derived cells such as tumor-associated macrophages (TAM) or myeloid-derived suppressor cells (MDSC). As antigen-presenting cells (APC), these cell populations are regulating T cell responses. Lipopolysaccharides (LPS), Tumor Necrosis Factor-alpha (TNFα), Interferon-gamma (IFNγ), Interleukin- (IL-), Immune complex (IC), Toll-like Receptor (TLR), Transforming Growth Factor beta (TGFβ), Macrophage Colony Stimulating Factor (M-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Tumor (Tu), Conditioned Media (CM), Tumor-associated macrophage (TAM), Myeloid-derived suppressor cells (MDSC), Dendritic cell (DC), Prostaglandin E2 (PGE2), T Cell Receptor (TCR), Major Histocompatibility Complex (MHC), Regulatory T cell (Tr), vasoactive intestinal peptide (VIP), thymic stromal lymphopoietin (TSLP), hepatocyte growth factor (HGF), Retinoic acid (RA)*

As ***Figure 4*** shows, 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.

3.8. 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 APCs, they constitutively express MHCII molecules on their surface; however, its expression level could be altered by environmental factors[102]. Under physiological conditions, DCs are considered "immature" and shift the T cell response toward peripheral tolerance by initiating T-cell anergy, deletion, or generation of regulatory T-lymphocytes[103].

As phagocytes, DCs gather pathogenic and “danger signals” from microbes and tissues, leading to their increased migratory capacity toward T-cell zones. At the onset of an inflammatory response, DCs differentiate into mature DCs (MDC). The maturation process includes cytoskeletal rearrangement, up-regulation of MHC and co-stimulatory molecules, and secretion of a wide array of cytokines to trigger the activation and polarization of naïve T cells[104].

Activation of T cells occurs by recognizing peptides presented on MHC I or II molecules of APCs to the T cell receptor (TCR) expressing $CD4^+$ or $CD8^+$ T lymphocytes. Another possible antigen presentation mode is when the conserved/nonclassical MHC I-like CD1 molecules display lipid antigens to Natural Killer T cells (NKT) with a limited diversity of TCR repertoire. Some species have limited CD1 repertoires, but on human immune cells, these molecules play a role in the fine-tuning of anti-microbial and tumor-specific immunity *via* acting on CD1-restricted T cell subsets. $CD1a^+$ and $CD1a^-$ moDCs differ in their antiviral capacity, level of cytokine and chemokine production, phagocytic potential, and T cell polarizing capacity but possess similar T cell activating abilities[105]. The $CD1a^{high}$ moDCs are considered inflammatory cells *in vitro* and *in vivo*[106], while the $CD1a^{low}$ moDCs are rather tolerogenic.

Antigen presentation, antigen recognition, and costimulation are the three essential phases of naïve T cell activation. TCR engagement provides the first signal for the activation

of naïve T cells, but it is not enough to trigger an efficient response. Full activation of naïve T lymphocytes requires additional co-stimulatory signals from APCs ***(Figure 5)***. TCR engagement in the absence of costimulation leads to a hyporesponsive state, called anergy.

APC	T cell
B7-1 (CD80)	CD28
B7-2 (CD86)	CTLA-4 (CD152)
CTLA-4 (CD152)	
4-1BBL	4-1BB (CD137)
OX-40L	OX-40 (CD134)
LIGHT (TNFSF14)	HVEM
ICOSL (B7h/LICOS)	ICOS
GITRL	GITR
CD27L (CD70)	CD27
CD40	CD40L (CD154)
PD-L1	PD-1
PD-1	
B7.x	B7-H4
B7-H4	
MHCII	LAG-3 (CD223)

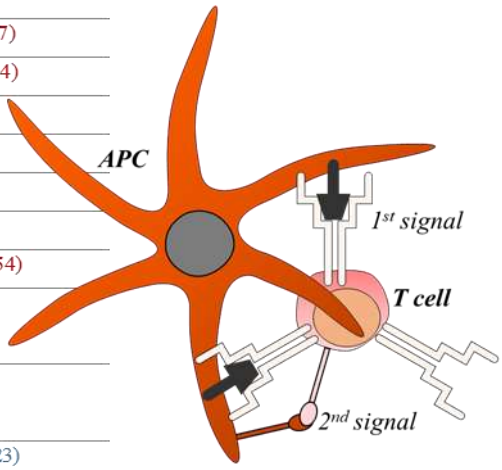


Figure 5. T cell activation by professional APCs. *Naïve T cell activation by DCs requires two signals, termed signal-1 and signal-2. Signal-1 is equivalent to the binding of TCR to the peptide-MHC complex. Signal-2 requires the interaction of co-stimulatory molecules at the interface between DCs and T cells. The onset of strong adaptive immunity crucially depends on the activation of DC. Positive (red) and negative (blue) signals have been identified*[107]*. Antigen-Presenting Cell (APC), Cytotoxic T-lymphocyte antigen 4 (CTLA-4), herpesvirus entry mediator (HVEM), Inducible T-cell costimulator (ICOS), Programmed cell death protein 1 (PD-1), Programmed cell death protein ligand 1 (PD-L1)*

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[108]. 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[109]. CTLA-4 is expressed by lymphoid cells to limit the co-stimulatory potential of myeloid APCs[110]. 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[111,112]. 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*[113]. The regulatory role of CTLA-4 in autoimmune diseases and antitumor therapies is still controversial in both mice and humans.

3.9. Subsets of human DCs

Dendritic cells can be divided into resident lymphoid tissue DC and migratory non-lymphoid tissue DC, while in blood, they represent a rare population. In lymphohematopoietic tissues, DCs commitment to a subpopulation is mainly defined by ontogeny. In contrast, in non-lymphohematopoietic tissues, DCs can shape their phenotype under microenvironmental pressure. Both subpopulations are heterogeneous, including subsets with well-distinct but sometimes overlapping phenotypic markers and genetic profiles.

MDPs could differentiate into common-DC-progenitor (CDP). DCs could be classified into different categories: plasmacytoid DC (pDC/ $CD123^{+}CD11c^{-}$) or the classical/myeloid DC (cDC/$CD123^{-}CD11c^{+}$ cells). Pre-cDCs are the progenitors of the two major cDC subgroups named cDC1 (BDCA-3/$CD141^{+}$) and cDC2 (BDCA-1/$CD1c^{+}$). The development and expansion of DC subpopulations are determined by transcription factors and the combination of cytokines. Stromal-, endothelial- and activated T-cells produce Fms-related tyrosine kinase 3 ligand (Flt3L), functioning as an essential cytokine for the differentiation of myeloid DCs. The main phenotypical and functional characteristics of DC subsets are summarized in ***Table 4***.

DC subset	‚master' transcription factors	Selected surface markers	Main secreted cytokines	Main functional specialization	Localization
cDC1 *Flt3L-dependent*	STAT5 C/EBP2 IP2 BATF3 IRF8 ID2 NFIL3	CD11c/CR4 (iC3bR) BDCA-3/CD141 Clec9A/DNGR1 XCR1 CADM1 HLA-DR DEC205 CD103* CD1c$^-$	TNFα CXCL10 IL-12p70 Type III IFN-λ IL-1β IL-6 IL-8 IL-15 IL-23	Cross presentation Priming of virus or tumor derived antigen-specific CD8$^+$ and *Th*1 response Type I IFNs increase their cross-presenting capacity *Activation of CD4$^+$ immunogenic (*Th*17) and induce switching of specific B cells to IgA in the MLN	Lymphoid-resident (tonsils, lymph nodes,dermis upper layer) *dominant population in the PPs and colon LP peripheral tissues (lung, liver, skin) Blood
cDC2 *Flt3L-dependent*	STAT5 C/EBP2 ID2 ZEB2 Notch/KLF4 IRF4 RelB	CD11c/CR4 (iC3bR) BDCA-1 (CD1c) CD172/SIRP1α HLA-DR CD11b/CR3 CD103* BDCA3/CD141$^-$ Inducible CD14	TNFα IL-10 IL-23 IL-1β IL-6 CXCL8/IL-8 IL-10 IL-21	Direct exogenous-derived antigen presentation through MHCII *Production of TGF-β and ATRA *Activation of CD4$^+$ immunogenic (*Th*17) and *regulatory* T cells and induce switching of specific B cells to IgA in the MLN	Lymphoid-resident (skin: dermis lower layer, MALT; *lamina propria*) *In SI-lamina propria (LP) as major migratory DC subset peripheral tissues Blood
double negative DC (DN-DCs) *Flt3L-dependent*		CD11c/CR4 (iC3bR) HLA-DR CD206 CD40 Sometimes CD1c BDCA-3/CD141$^-$	IL-12 p70	Not well defined They trigger CD4$^+$ and CD8$^+$ T cell response	Lymphoid-resident (Peyer's patches: subepithelial dome and interfollicular regions within the follicle-associated epithelium) peripheral tissues Blood
pDC **pDC-like DC*** *Flt3L-dependent*	E2.2 STAT3 Bcl11a Runx2 IRF8	BDCA-2/CD303 BDCA−4/CD304 CD123/IL-3R HLA-DR^{low} CD11c$^-$ CD56*	Type I IFN(α,β) production IL-6 TNF Less type I IFN* IL-1β* IL-6*	Anti-viral response (TLR7/9) Increase the cross presenting activity of cDC1 T*reg* induction NK cell activation CD56+ γδ T-cell and NK cell activation*	Lymphoid-resident (tonsil) Blood
Langerhans cell (LC)	PU.1 ID2 Runx3	CD11c$^{+/low}$ HLA-DR Langerin CD1a E-cadherin EpCAM	TNFα IL-1β IL-6 IL-8 IL-12 IL-15 IL-23	Clearance of apoptotic cells Antigen presentation to CD8+, *Th*1, *Th*17, regulatory and follicular T cells	Epidermis, mucosa
moDC *(from CD14+ monocytes in the presence of Flt3L, IL-4/13 and GM-CSF/M-CSF)*	STAT5 C/EBP2 IP2 PU.1 MAFB KLF4 IRF4 IRF8 Runx3 PPARγ RXR RAR	CD11c HLA-DR Often: DC-SIGN/CD209 CD16 CD1a, b, c SIRP1α CD11b S100A8/A9 CD206 CD14 CX3CR1 *(Flt3L+M-CSF)* CD141$^-$	TNFα IFNγ IL-12 IL-1β IL-6 IL-15 IL-23 IL-27 IL-10 TGFβ	Context dependent	Mainly induced upon inflammatory signals in peripheral tissues

Table 4. Classification of human dendritic cell subsets[114-122].

Generally, human DCs are differentiated from CDPs or from monocytes. The two most abundant DC subtypes are the cDC1 and cDC2 cells; CDP-derived $CD141^+$ cDCs are mostly localized in the circulation, tonsils, lymph nodes, lung, skin, and liver. They are efficient $CD8^+$ T cell activators due to their cross-presenting activity. Additionally, cDC1 cells produce a relatively large amount of tumor necrosis factor (TNF)-α, IL-12, and interferon (IFN)-γ cytokines. CD1c-expressing cDC2 cells are positive for CD172, CD11b, and CD11c markers. They are dominantly present in the blood, the skin, and the mucosal-associated lymphoid tissues (MALT). Upon activation, they produce TNF-α, IL-10, and IL-23, which cytokine-secreting profile could support the activation and polarization of Th17 cells. As was described before, CD14+ monocytes derived from cMoPs could differentiate into moDCs as well. The phenotype and function of these cells are very diverse depending on the actual environmental cues. However, they dominantly could enroll a tolerogenic or inflammatory phenotype. In the presence of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), IL-4, VitaminD3, rosiglitazone (RSG), Transforming Growth Factor (TGF)-β and IL-10 monocytes differentiate into anti-inflammatory cells. In contrast, in the presence of GM-CSF, IL-4, and TNFα they give rise to inflammatory cells. These cells express up-regulated level of HLA-DR, activator markers (CD83), and costimulatory molecules, like B7 family members. The development of these cells is modulated by the circumstances which determine the activation of different master transcription factors and nuclear receptors *(based on the references of Table 4)*.

3.10. 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 like Interferon regulatory factors 4 (IRF4), IRF8, and Runx3. Additionally, ligand-dependent nuclear receptors have a major role in the regulation of myeloid cell differentiation as well and could be classified into four major groups:

1. homodimer steroid hormone receptors *(estrogen, testosterone receptors)*
2. RXR heterodimers *(PPARs, retinoid X receptors or RXRs, retinoic-acid receptors or RARs, liver X receptors or LXRs, vitamin-D receptors or VDRs)*
3. RXR/RXR and orphan receptor homodimers
4. monomeric orphan receptors

Inactive forms of nuclear receptors are localized in the nucleus repressed by co-receptor complexes or found in the cytoplasm where heat-shock proteins bind them. In the presence of their ligands, the nuclear receptors are released from the inhibition, and the binding to their specific response elements in the genome enables the increased transcription of target genes.

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. Vitamin A or retinol has a structure depicted in ***Figure 6.*** Retinol is the immediate precursor to two critical active metabolites: retinal and retinoic acid, regulating the transcription of a number of genes. Vitamin A does not occur in plants, but many plants contain carotenoids such as beta-carotene that can be converted to vitamin A within the intestine ***(Figure 6)***.

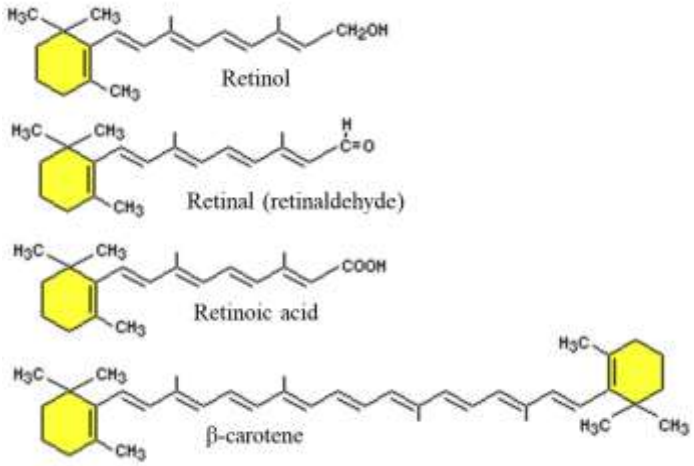


Figure 6. *Retinol is the precursor to two active metabolites: retinal and retinoic acid. Retinol can be converted from beta-carotene to produce ligand for nuclear receptors.*

Showing the role of these factors in the development of myeloid-derived cells, receptors of Vitamin A derivates drive the myeloid cell differentiation into $CD103^{+}CD11b^{+}$ DC population[123]. *In vivo* vitamin A deficiency (VAD) is linked with the diminished size of intestinal CD103 and CD11b expressing DC subset but expands $langerin^{+}$ DCs in mucosal tissues.

3.11. Role of retinoic acid in the differentiation process of DCs

Retinyl esters stored in chylomicrons and are transported into the liver, where the hepatocytes capture and store them as retinol. The molecule forms complexes with the retinol-binding protein (RBP) and is carried through the circulation. The RBP-retinol complexes are

recognized by the retinoic acid 6 (STRA6) receptor, which initiates the absorption of extracellular vitamin A derivate into the cytosol, where retinoic acid (RA)/all-*trans* retinoic acid (ATRA) is generated. Retinol could be oxidized into retinal/retinaldehyde by the enzyme alcohol dehydrogenase (ADH) ***(Figure 7)***. Further, the retinal is oxidized by the enzyme retinal dehydrogenase[124] (RALDH) with three possible isoforms: RALDH1, RALDH2, and RALDH3[125] encoded by the ALDH1A1, ALDH1A2, and ALDH1A3 genes to produce ATRA.

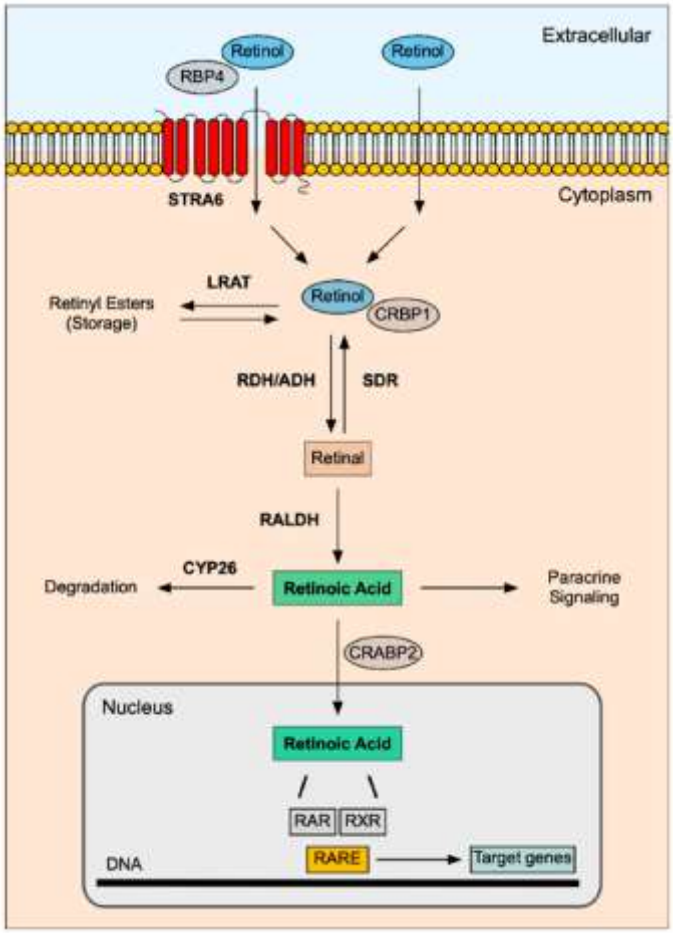


Figure 7. Retinoic acid, as nuclear receptor ligand. *Retinol binds to retinol-binding protein (RBP). This complex is recognized by the retinoic acid 6 (STRA6) receptor, which initiates its absorption. Retinol oxidized into retinal/retinaldehyde by alcohol dehydrogenase (ADH). The retinal is oxidized by the enzyme retinal dehydrogenase (RALDH), producing retinoic acid (RA). RA binds to nuclear receptors RAR and RXR, regulating the transcription of the target genes' set by binding the retinoic acid-responsive elements (RAREs) in DNA[124].*

ATRA can be produced in $CD103^+$ DCs, macrophages, or mucosal epithelium and stromal cells. RALDH isoforms are expressed differently in different cell types ***(Table 5)***. The primary source of RAR ligands is the intestine, the blood, but it could be produced endogenously as well. Interestingly, in the gut *lamina propria*, DC subpopulations and MSCs

constitutively express enzymatic machinery for ATRA production even in the absence of dietary vitamin A[126].

	CD103+ DC	CD103-CD11b+ DC	Stromal cell	Macrophage	moDC
localization	GALT	lymph nodes in skin and lungs	Througout the body dominantly in the gut	colon	pheriphery
enzyme	RALDH2 RDH10	RALDH2	RALDH1 RALDH2 RALDH3	RALDH1	RALDH2 RDH10
activating factors	• retinol • RA • GM-CSF • gut microbiom	?	• retinol • RA	• microbiota	• exrtacellular lipids • GM-CSF • gut microbiom

Table 5. Known cell types expressing retinal dehydrogenase isoforms. *DC: dendritic cell; GALT: gut-associated lymphoid tissue; RALDH: retinal dehydrogenase; RA: retinoic acid; ADH/RDH: alcohol dehydrogenase; GM-CSF: granulocyte-macrophage colony-stimulating factor*

Released ATRA interacts with nuclear receptors, including RAR and RXR. ATRA and ATRA receptors link environmental sensing with immune cell fate. The RAR family includes three members: RARα (isoforms α1–2), RARβ (isoforms β1–4), and RARγ (isoforms γ1–2). Ligands of the RAR family can be either ATRA or its isomer 9-cis RA. The RXR family also includes three members (RXRα, -β, and -γ) and interacts at the physiological level only with 9-cis RA[127]. RARs and RXRs could form heterodimers and function as ligand-dependent transcription factors that bind to specific RAREs in the gene promoter and enhancer region[128]. Most cells express more types of RAR and RXR receptors in various combinations. Thanks to their overlapping ligation (e.g., ATRA as a ligand of RAR and RXR), retinoic signaling is very complex. ATRA responsible for regulating a wide array of genes in moDCs, which was analyzed in a study published by Lajos Széles et al. They identified genes regulated by agonists for RXR and RAR but were not affected by LXR and PPAR agonists[129]. The gene set is available on NCBI GEO under serial number GSE23073, including genes involved in regulating T cell responses by moDCs.

ATRA, as the physiologically active form of vitamin A, plays a crucial role in embryonic development and the determination of cell fate[130]. The molecule shapes early intestinal immune responses by promoting interleukin (IL)-22 synthesis by γδ T cells and innate lymphoid cells[131] and regulates DC and macrophage differentiation from circulating peripheral blood monocytes[132-134]. 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[135,136]. Importantly, in case of moDCs, the expression of CD1a was down-modulated by RARα, in contrast to CD1d which molecule' expression was elevated according to the findings of Szatmari et. al. Based on these observations, RARα nuclear receptor did not confer up-regulated antigen presentation by moDCs, but the stimulated cells acquired a selectively enhanced NKT cell activating capacity due to elevated levels of CD1d accompanied by diminished expression of CD1a[137].

3.12. 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 ***(Figure 8)***, such as granulocytes[138], natural killer (NK) cells[139], monocytes[140], DCs[141], and macrophages[142].

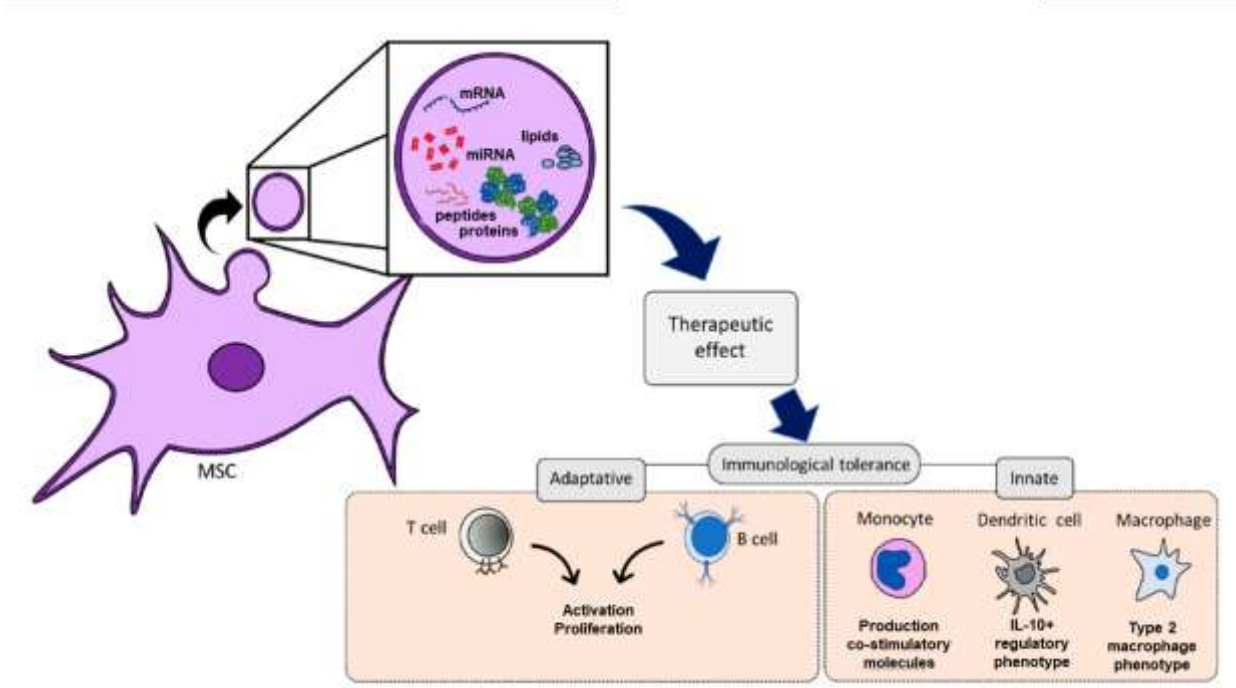


Figure 8. MSCs have an impact on the differentiation and functions of adaptive and innate immune cells. *For years was thought that MSC-derived secreted factors do not affect immune cells. Today we get to know their immunomodulatory activities via released mediators. The increasing number of studies reveal mechanisms by which the MSCs may alter the differentiation and functional activity of T-, B-lymphocytes and innate immune cells, such as monocytes, dendritic cells, and macrophages. (https://www.mdpi.com/2077-0383/9/2/445/htm)*

During homeostasis and inflammation, monocytes leave the blood circulation and move into tissues to differentiate toward DCs[143]. 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. There is a study where bone marrow-derived MSCs were co-cultured with monocytes in the presence of GM-CSF and IL-4. Here, MSCs significantly inhibited monocyte differentiation into immature DCs[144]. Bigger ratio of the cultured cells in the presence of MSCs did not express CD1a, indicating that the cells had not differentiated into immature DCs; however, MSCs did not alter the expression of CD14.

There is another project where coculturing monocytes with MSCs, the stromal cells substantially prevented the enhancement of the co-stimulatory molecules CD40, CD86, and CD80 and diminished HLA-DR expression on moDCs. On the contrary, MSC-derived supernatants had no effects on DC differentiation[144]. However, in another study, where the transwell chamber system was used to separate monocytes from bone marrow-derived MSCs during differentiation[141], MSC completely prevented monocyte differentiation into moDCs. In the presence of MSC-produced factors, monocytes retained as $CD14^+$ cells without acquisition of CD1a and displayed no up-regulated expression of CD80 and CD83[141]. In a latter study, in the presence of GM-CSF, IL-4, and BM-MSCs, monocytes also did not acquire the typical phenotype of immature ($CD14^-$, $CD1a^+$) DCs[145]. Later, it has been investigated that after coculture with MSCs (differentiated from human induced pluripotent stem cells), monocytes displayed a low expression of CD1a, but an increased expression of CD14, while exhibiting down-regulated expression of CD40, CD80, CD83, and HLA-DR[146].

4. AIMS OF STUDY

4.1. 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.

4.2. 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.

5. MATERIALS AND METHODS

5.1. 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.

For this study the monocyte separation was performed and the 90% of the samples were processed by the author of present thesis.

5.2. 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.

For this study 70% of the isolation techniques and the maintaining of the cells was performed by the author of present thesis.

5.3. 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.

For this study the cells were cultured, maintained and treated by the author of present thesis.

5.4. 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.

For this study the trilineage differentiation potential of MSCs was tested by the author of present thesis and Zoltán Veréb (50-50%).

5.5. 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.

For this study the *in vitro* activation of MSCs was performed by the author of present thesis and Zoltán Veréb (50-50%).

5.6. 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).

For this study the co-cultures were prepared by the author of present thesis.

5.7. 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).

FITC	PE	APC
CD34	CD14	CD45
CD44	CD49a	CD36
CD49f	CD31	CD69
CD90	CD73	CD56
CD105	CD117	CD146
HLA-DR	CD104	CXCR4
CD106	PDGFRb	CD47
CD147	CD144	CD133
CD54	CD29	CD49b
VEGFR2/KDR	CD166	

Labeling panel of multiparameter analysis of BM-and SV-MSC-associated cell surface antigens.

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.

For this study the labeling of cell surface-associated antigens and the flow cytometry measurements were performed and evaluated by the author of present thesis.

5.8. 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).

For this study the effect of MSCs on the proliferation of PBMC was tested by the author of present thesis and Zoltán Veréb (50-50%).

5.9. 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.

For this study the measurement of the cytokine concentrations and the protein array were performed and the results were evaluated by the author of present thesis.

5.10. 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).

For this study the T cell polarizing activity of moDCs by flow cytometry was tested by the author of present thesis.

5.11. 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.

The isolation of naïve T cells from human blood was performed by the author of present study.

5.12. 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).

The effect of MSCs on the T cells polarizing effect on moDCs by ELISPOT assays was performed and evaluated by the author of present thesis.

5.13. 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).

TaqMan Assay	ID
CTLA-4	Hs00175480_m1
RDH10	Hs00416907_m1
RALDH1A1	Hs00946916_m1
RALDH1A2	Hs00180254_m1
RALDH1A3	Hs00167476_m1
RIG-I	Hs01058986_m1
MDA-5	Hs01070332_m1
IL-6	Hs99999032_m1
IFNβ	Hs02621180_s1
CXCL-10	Hs00171042_m1

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.

The collection and preparation of the samples as well as the RNA isolation and RT-PCR were performed by the author of present thesis and Zoltán Veréb (50-50%). Affymetrix Gene Chip Human Gene 1.0 ST Array was performed by Zoltán Veréb and Szilárd Póliska. The gene expression profile of MSCs was compared and analyzed by Szilárd Póliska.

5.14. 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 *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$P < 0.0001$.

The statistical analysis was performed by Zoltán Veréb, Szilárd Póliska, Attila Bácsi and the author of present thesis.

6. RESULTS

6.1. Part I.

6.1.1. 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) ***(Figure 9)***. 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.

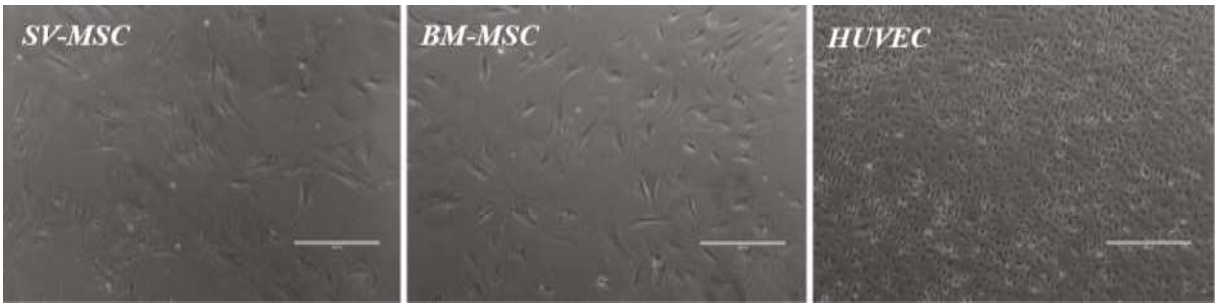


Figure 9. Morphology of SV-, BM-MSCs, and HUVEC cells. *Comparison of the morphology of vessel wall- and bone marrow-derived MSCs. HUVEC cells were used as control vein endothelial cells. All three populations exhibited spindle-shaped morphology. The morphology of bone marrow, saphenous vein, and umbilical cord samples were investigated using an OLYMPUS IX-81 microscope. Images were taken at 200x magnification.*

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 ***(Figure 10)***. Based on these observations, SV-MSCs are fit to the criteria of ISCT regarding the differentiation potential.

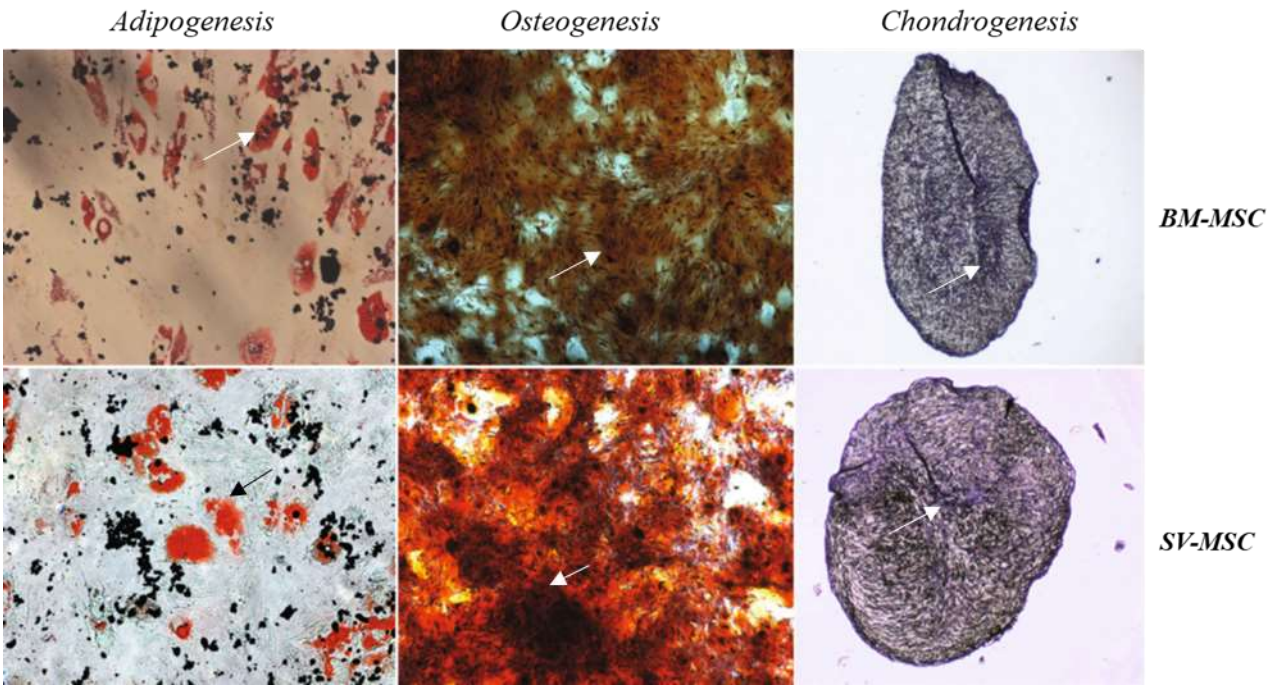


Figure 10. Comparison of multilineage differentiation potential of BM- and SV-MSCs. *BM- and SV-derived MSCs are able to differentiate toward fat, bone, and cartilage directions. Adipogenic, osteogenic, and chondrogenic differentiation were performed using StemPro® Adipogenesis, Osteogenesis, and Chondrogenesis Differentiation Kits. The oil red positivity for adipogenic, the presence of calcium deposits for osteogenic and toluidine-blue staining were used to certificate the multilineage differentiation potential of MSCs. The images about bone marrow or saphenous vein samples were investigated using an OLYMPUS IX-81 microscope.*

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.

As summarized in ***Table 6 and Figure 11***, expression 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 ***(Table 6 and Figure 11)***.

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 ***(Table 6 and Figure 11)***. 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 ***(Table 6 and Figure 11)***.

		BM-MSC	SV-MSC	HUVEC
		Percentage of positive cells (%)		
Hematopoietic markers	CD14	0.22±0.11	1.37±1.15	0±0
	CD34	0±0	0±0	4.62±2.05
	CD36	32.51±8.18	18.12±5.28	36.6±17.60
	CD45	0±0	0±0	0±0
	CD47	97.00±0.86	96.65±1.55	85.06±12.49
	CD69	0±0	0±0	27.24±10.93
	CD133	0±0	0±0	0±012.03
	CD117	0±0	0.02±0.02	81.57±11.26 ***
	CXCR4	0±0	0±0	37.37±8.18 **
	HLA-DR	0±0	0±0	0.19±0.12
Endothelial markers	CD31	0±0	0±0	96.78±0.82 ***
	CD144	45.33±12.61	61.55±18.18	93.91±2.45
	VEGFR2/KDR	0±0	0±0	0.75±0.41
	CD104/Integrin β4	28.25±12.20	34.42±17.82	76.42±11.50
MSC/Fibroblast markers	CD73	91.99±1.92	97.90±0.80	97.85±0.94
	CD90/Thy-1	89.05±1.49	89.68±3.63	2.86±1.55 ***
	CD105/Endoglin	82.64±2.56	89.62±2.54	97.94±0.52 ***
	CD147/Neurothelin	77.33±8.87	81.11±13.59	98.31±0.91
	PDGF Rβ	78.01±8.28	90.77±3.74	54.67±11.90
Cell adhesion molecules	CD29/Integrin β1	92.96±1.71	97.02±1.87	98.77±0.64
	CD44/H-CAM	87.28±2.87	88.66±2.38	79.28±5.06
	CD49a	79.60±7.77	94.25±1.55	89.44±1.64
	CD49b	68.52±7.95	48.44±12.25	85.32±5.42 *
	CD49f	0±0	0±0	2.21±1.27
	CD54/ICAM	14.95+8.36	19.89+8.59	34.29±7.24
	CD56/NCAM	20.53±8.41	19.18±9.10	50.33±7.94
	CD146/MCAM	77.54±5.14 ***	7.09±6.56	96.68±1.02 ***
	CD166/ALCAM	89.57±6.27	96.22±2.13	98.54±0.45

Table 6. Summary about the phenotypic analysis of BM-MSCs, SV-MSCs, and HUVECs. *The expression of surface antigens associated with different cell types was measured by flow cytometry. The percentage of positive cells in SV-MSC culture was compared to that of BM-MSCs and HUVECs, as vein endothelial control. (Data are presented as means ± SEM; N = 5 for SV-MSC, N = 12 for BM-MSC, N = 7 for HUVEC. $p < 0.05*$, $p < 0.01**$, $p < 0.001***$ vs. SV-MSCs determined by Student t-test).*

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 ***(Figure 11)***. These observations indicate that our isolation technique with the applied phenotype analysis is suitable to identify MSCs isolated from the vessel wall.

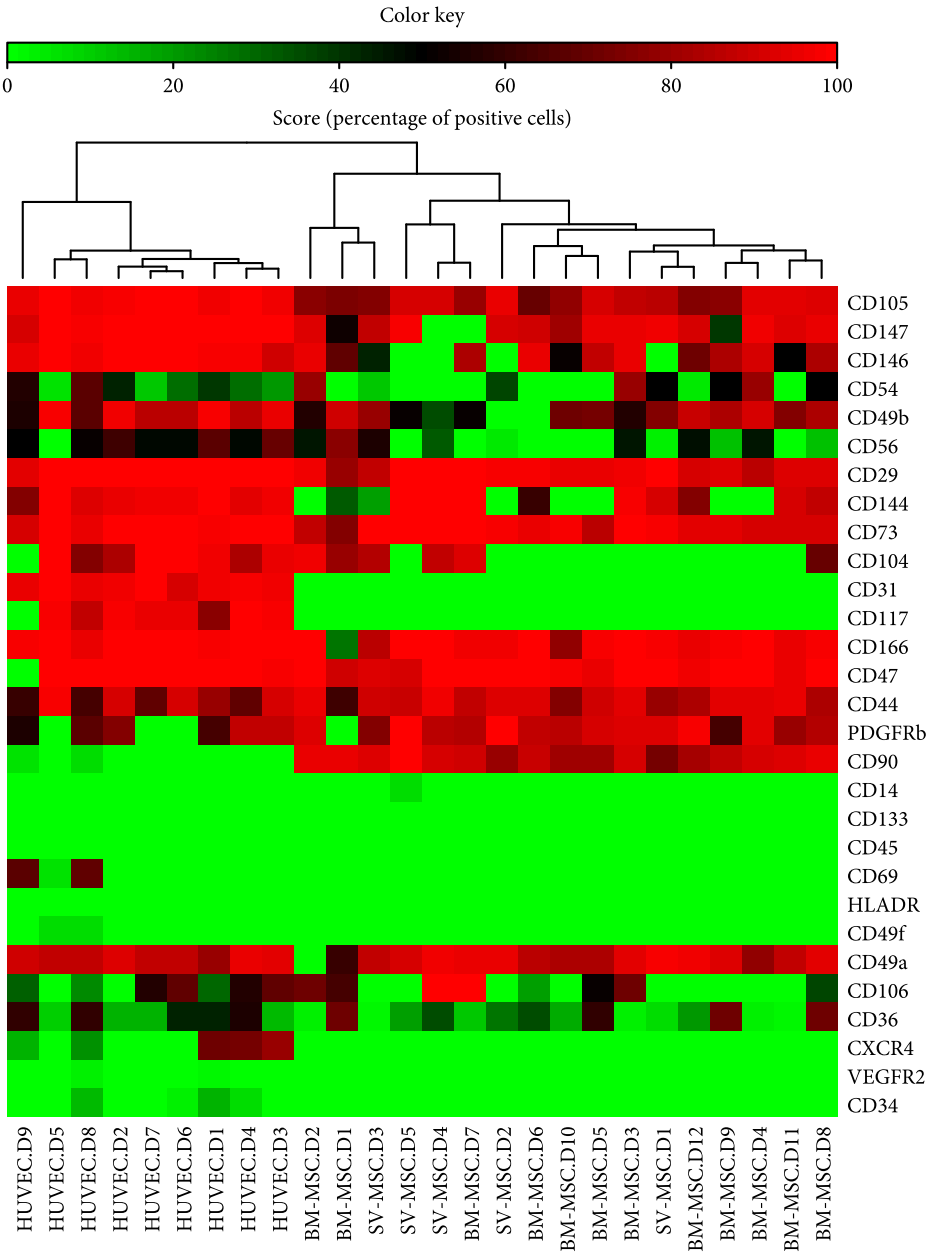


Figure 11. Hierarchical clustering of surface markers expressed by BM-MSCs, SV-MSCs, and HUVECs. *Hierarchical clustering of cell surface molecules' expression divided the stem cells of different tissue origin from the endothelial cells. SV-MSCs were more similar to BM-MSC than endothelial cells (color key represents the percentage of positive cells).*

6.1.2. 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 ***(Figure 12, Table 7-8)***.

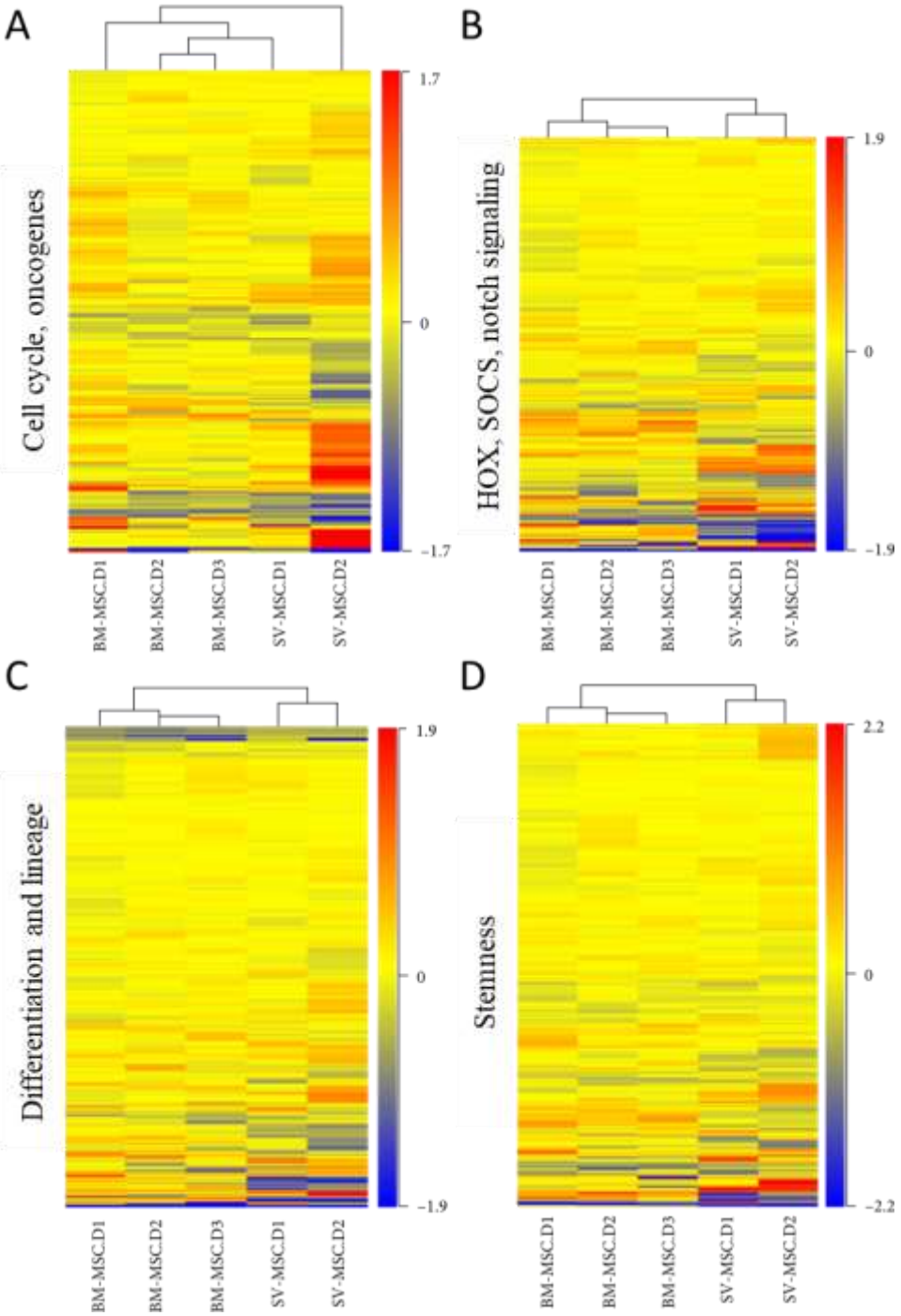


Figure 12. Heat maps about genes associated with particular biological function in BM-MSCs and SV-MSCs. *Genes linked to* ***(A) cell cycle***, ***oncogenes****;* ***(B) HOX*** *(homeobox),* ***SOCS*** *(suppressor of cytokine signaling), and* ***Notch signaling; (C) differentiation and lineage****; and* ***(D) stemness*** *were collected into functional groups and analyzed. The functional cluster analysis of the different expressions of selected genes shows the difference between the cell types suggesting different tissue origins. (Color key represents relative gene expression levels.) To identify the relationships between the selected genes, the Ingenuity Pathway Analysis (IPA) was used. For the representation of the relationships between the genes, the "Pathway Designer" tool of the IPA software was applied.*

The gene expressions in SV-MSCs in the custom group ***cell cycle and oncogenes*** was not differed significantly from their BM-MSC counterpart ***(Figure 12 panel A, Table 7-8)***; 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 ***(Figure 12 panel A, Table 7-8)***.

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 ***(Figure 12 panel B, Table 7-8)***.

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) ***(Figure 12 panel C, Table 7-8)***.

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 ***(Figure 12 panel D, Table 7-8)***. These results highlight the importance of the MSC origin, which seems to be determining in the pattern of observed genes.

Symbol	Entrez gene name	Fold change	p-value	Molecule type	Group
Fold Change up-regulated					
S100A4	S100 calcium binding protein A4	2.8	0.0426795	calcium binding protein	Cell cycle and oncogenes
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	1.6	0.0221517	cell adhesion molecule	
BRCA2	breast cancer 2, early onset	1.4	0.0158221	DNA repair	
SMG6	Smg-6 homolog, nonsense mediated mRNA decay factor (C. elegans)	1.2	0.0169296	enzyme	
FGF9	fibroblast growth factor 9 (glia-activating factor)	6.6	0.0116976	growth and differentiation factor	HOX, SOCS, Notch signaling
IL33	interleukin 33	2.9	0.0380778	cytokine	
HOXA11	homeobox A11	2.0	0.0164048	transcription factor	
BMP4	bone morphogenetic protein 4	1.8	0.0429056	growth and differentiation factor	
PODXL	podocalyxin-like	4.6	0.0230397	cell differentiation	Differentiation and lineage
CTSK	cathepsin K	2.6	0.0201834	lysosomal cysteine protease	
CSF1	colony stimulating factor 1 (macrophage)	2.2	0.0155533	cytokine	
TGFB3	transforming growth factor, beta 3	1.9	0.0230397	growth and differentiation factor	
NRP1	neuropilin 1	1.8	0.0058021	membrane-bound coreceptor	
GDF10	growth differentiation factor 10	1.7	0.0155533	growth and differentiation factor	
FGF9	fibroblast growth factor 9 (glia-activating factor)	6.6	0.0143408	growth and differentiation factor	Stemness
ZFPM2	zinc finger protein, multitype 2	5.2	0.0195738	transcription factor	
MME	membrane metallo-endopeptidase	3.2	0.0245681	enzyme	
FZD4	frizzled homolog 4 (Drosophila)	2.5	0.0143408	receptor	
ACVRL1	activin A receptor type II-like 1	1.9	0.0143408	enzyme	

Table 7. Upregulated genes in SV-MSCs compare to BM-MSCs. *Genes linked to* ***cell cycle***, ***oncogenes***, ***HOX*** *(homeobox),* ***SOCS*** *(suppressor of cytokine signaling), and* ***Notch signaling, differentiation and lineage***, *and* ***stemness*** *were grouped based on functions, and expression levels were analyzed. Genes were selected by significance. Robust microarray analysis (RMA) was applied for normalization. Stem/stromal cell-related genes were selected, and statistical analysis was performed (Oneway ANOVA with Tukey post hoc test and Benjamini-Hochberg FDR) to calculate p-value and fold change.*

Symbol	Entrez gene name	Fold change	p-value	Molecule type	Group
Fold Change down-regulated					
SMAD3	SMAD family member 3	-2.6	0.0108465	transcriptional modulator	Cell cycle and oncogenes
CDK6	cyclin-dependent kinase 6	-2.2	0.0237225	enzyme	
KRAS|LYRM5	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog | LYR motif containing 5	-1.9	0.0461384	proto-oncogene	
TGFB1	transforming growth factor, beta 1	-1.7	0.0158221	growth and differentiation factor	
RARA	retinoic acid receptor, alpha	-1.6	0.0337291	nuclear receptor	
HUS1	Checkpoint protein HUS1	-1.3	0.0108465	genotoxin-activated checkpoint complex	
SUN1|C7orf20	Sad1 and UNC84 domain containing 1 | chromosome 7 open reading frame 20	-1.3	0.0108465	nuclear envelope protein	
PURA	purine-rich element binding protein A	-1.3	0.0108465	multi-functional DNA- and RNA-binding protein	
CDON	Cdon homolog (mouse)	-1,6	0,0691849	cell surface receptor	HOX ,SOCS, Notch signaling
HOXA2	homeobox A2	-1,6	0,0280393	transcription factor	
SNAI1	snail homolog 1 (Drosophila)	-1,6	0,0442530	transcription factor	
PYGO1	pygopus homolog 1 (Drosophila)	-1,7	0,0924799		
NOTCH2	Notch homolog 2 (Drosophila)	-1,7	0,0177410	transmembrane protein	
MAML2	mastermind-like 2 (Drosophila)	-1,7	0,0481715	transcriptional co-activator	
VCAM1	vascular cell adhesion molecule 1	-17,4	0.0058021		Differentiation and lineage
ACAN	aggrecan	-5,6	0.0192411		
EGR2	early growth response 2	-4,4	0.0191151		
TGFB2	transforming growth factor, beta 2	-3,9	0.0058021		
IGF2|INS-IGF2	insulin-like growth factor 2 (somatomedin A) | INS-IGF2 readthrough transcript	-3,5	0.0192411		
BMP2	bone morphogenetic protein 2	-3,3	0.0230696		
BDNF	brain-derived neurotrophic factor	-3,3	0.0155533		
JAG1	jagged 1 (Alagille syndrome)	-3,0	0.0058021		
INHBA	inhibin, beta A	-2,9	0.0422998		
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	-2,8	0.0155533		
SMAD3	SMAD family member 3	-2,6	0.0078861		
HES1	hairy and enhancer of split 1, (Drosophila)	-2,2	0.0192411		
EFNB2	ephrin-B2	-2,1	0.0358564		
PTN	pleiotrophin	-2,1	0.0155533		
PDGFA LOC100132080	platelet-derived growth factor alpha polypeptide | hypothetical LOC100132080	-2,0	0.0155533		
LIF|MGC20647	leukemia inhibitory factor (cholinergic differentiation factor) | hypothetical protein MGC20647	-9.5	0.0154874		Stemness
CXCL12	chemokine (C-X-C motif) ligand 12 | chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-8.5	0.0414186		
MCAM	melanoma cell adhesion molecule	-5.9	0.0080718		
ACAN	aggrecan	-5.6	0.0193145		
LTBP1	latent transforming growth factor beta binding protein 1	-4.4	0.0398936		
BMP2	bone morphogenetic protein 2	-3.3	0.0236414		
SMAD3	SMAD family member 3	-2.6	0.0087946		
ALCAM	activated leukocyte cell adhesion molecule	-2.1	0.0080718		
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	-2.1	0.0143408		
GDF6	growth differentiation factor 6	-2.1	0.0427049		
FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	-2.0	0.049803		
FGFR2	fibroblast growth factor receptor 2	-1.9	0.0324610		

Table 8. Downregulated genes in SV-MSCs compare to BM-MSCs. *Genes related to* ***cell cycle****,* ***oncogenes****,* ***HOX*** *(homeobox),* ***SOCS*** *(suppressor of cytokine signaling), and* ***Notch signaling, differentiation and lineage****, and* ***stemness*** *were collected into functional groups and analyzed. Genes were selected by significance. Robust microarray analysis (RMA) was applied for normalization. Stem/stromal cell-related genes were selected, and statistical analysis was performed (Oneway ANOVA with Tukey post hoc test and Benjamini-Hochberg FDR) to calculate p-value and fold change.*

6.1.3. 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 ***(Figure 13)***.

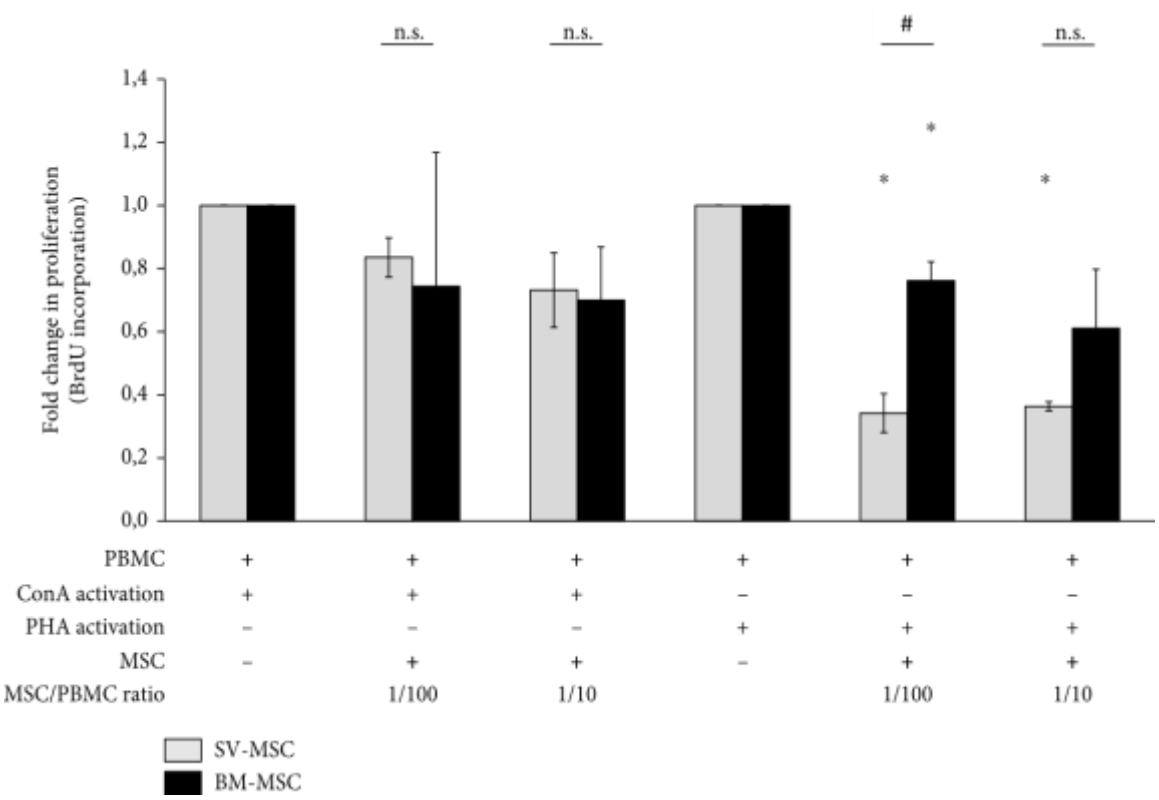


Figure 13. Immunosuppressive activity of SV- and BM-MSCs in vitro. *Mitogen-induced T cell proliferation was triggered by the treatment of the cells with concanavalin A (ConA) or phytohemagglutinin (PHA) at a final concentration of 10 µg/ml and 1 µg/ml. The extent of the proliferation was monitored by a BrDU colorimetric assay. To compare the immunosuppressive effect of SVMSCs and BM-MSCs, the proliferation of mitogen-stimulated PBMCs (OD values, BrdU incorporation) was taken as value 1, and changes in BrdU incorporation caused by MSCs were compared. (Data shown are mean ± SEM, N = 3; p <0.05∗ (compare to the control ConA or PHA activated PBMC) or p <0.05 # (between BM- and SV-MSCs)). Statistically significant differences were determined with two-way ANOVA analysis.*

6.1.4. 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 ***(Figure 14)***.

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 ***(Figure 14)***. Based on our results, MSCs originated from bone marrow or saphenous vein respond to priming inducers in slightly different ways.

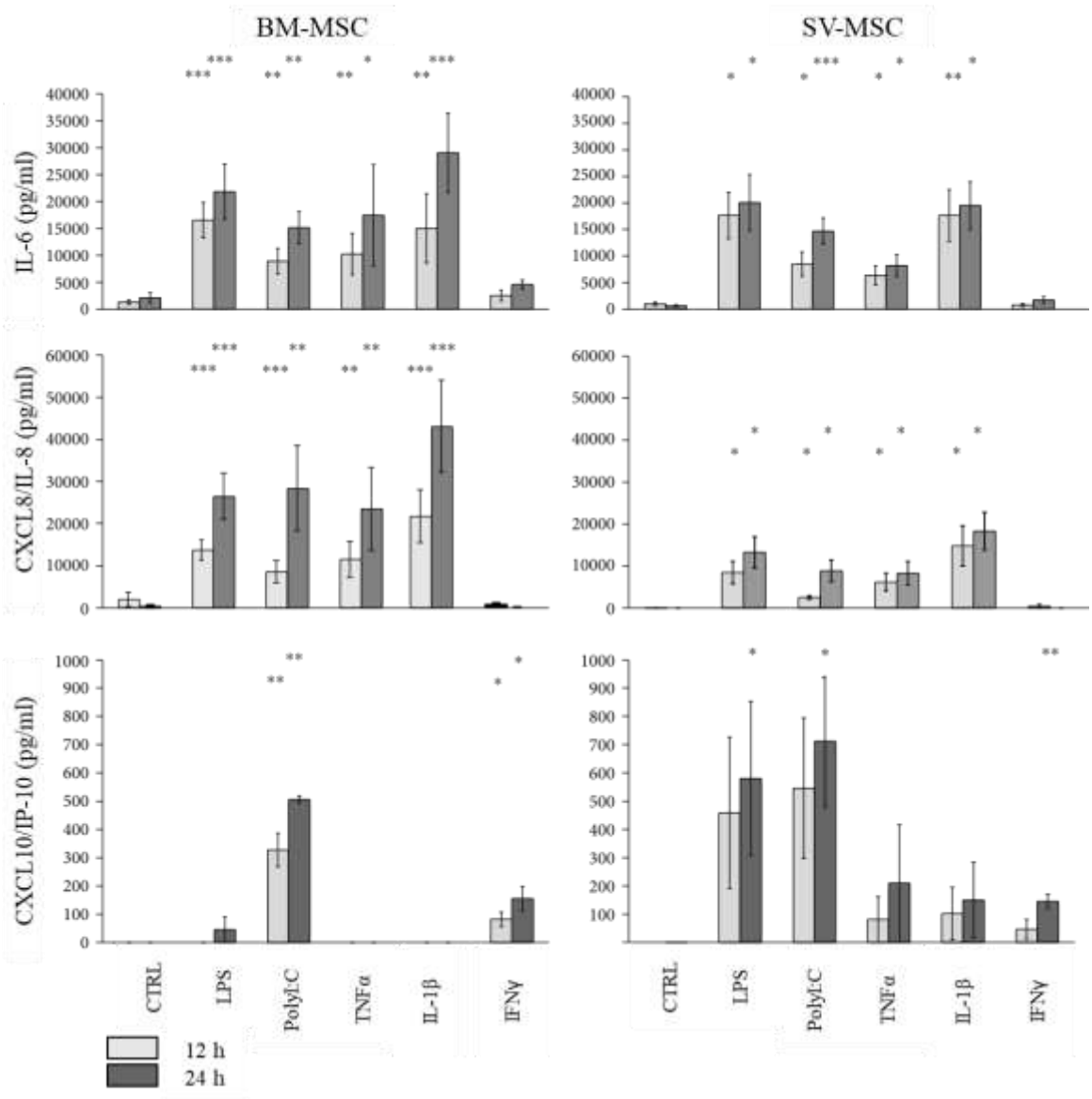
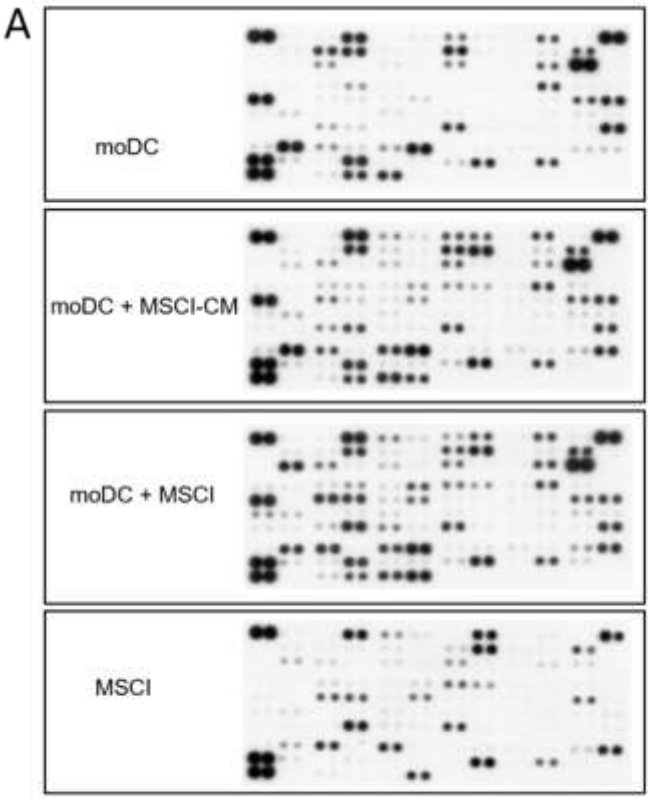


Figure 14. Secretion of soluble mediators by activated MSCs derived from bone marrow or saphenous vein. *IL-6, CXCL8/IL-8, and CXCL10/IP-10 production of stromal cells were determined upon their stimulation with TRL ligands (LPS (100 ng/ml), Poly I:C (25 μg/ml)), or pro-inflammatory cytokines (TNFα (100 ng/ml), IL-1β (10 ng/ml), IFNγ (10 ng/ml)). In vitro, cultured cells were treated in 12 h and 24 h intervals. (Data shown are mean±SD; p<0.05 *, p<0.01 **, p<0.001 *** compare to the ctrl (non-treated) BM- or SV-MSCs; N=6 for the BM-MSCs and N=3 for the SV-MSCs, respectively).*

6.2. Part II.

6.2.1. 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 ***(Figure 15)***.



A1	A2	Reference Spots	C15	C16	Flt-3 Ligand	E23	E24	IL-18 Bpa	H5	H6	PDGF-AA
A3	A4	Adiponectin	C17	C18	G-CSF	F1	F2	IL-19	H7	H8	PDGF-AB/BB
A5	A6	Apolipoprotein A-1	C19	C20	GDF-15	F3	F4	IL-22	H9	H10	Pentraxin-3
A7	A8	Angiogenin	C21	C22	GM-CSF	F5	F6	IL-23	H11	H12	PF4
A9	A10	Angiopoietin-1	D1	D2	GRO-α	F7	F8	IL-24	H13	H14	RAGE
A11	A12	Angiopoietin-2	D3	D4	Growth Hormone	F9	F10	IL-27	H15	H16	RANTES
A13	A14	BAFF	D5	D6	HGF	F11	F12	IL-31	H17	H18	RBP4
A15	A16	BDNF	D7	D8	ICAM-1	F13	F14	IL-32α/β/γ	H19	H20	Relaxin-2
A17	A18	Complement Component C5/C5a	D9	D10	IFN-γ	F15	F16	IL-33	H21	H22	Resistin
A19	A20	CD14	D11	D12	IGFBP-2	F17	F18	IL-34	H23	H24	SDF-1α
A21	A22	CD30	D13	D14	IGFBP-3	F19	F20	IP-10	I1	I2	Serpin E1
A23	A24	Reference Spots	D15	D16	IL-1α	F21	F22	I-TAC	I3	I4	SHBG
B3	B4	CD40 ligand	D17	D18	IL-1β	F23	F24	Kallikrein 3	I5	I6	ST2
B5	B6	Chitinase 3-like 1	D19	D20	IL-1ra	G1	G2	Leptin	I7	I8	TARC
B7	B8	Complement Factor D	D21	D22	IL-2	G3	G4	UF	I9	I10	TFF3
B9	B10	C-Reactive Protein	D23	D24	IL-3	G5	G6	Lipocalin-2	I11	I12	TfR
B11	B12	Cripto-1	E1	E2	IL-4	G7	G8	MCP-1	I13	I14	TGF-α
B13	B14	Cystatin C	E3	E4	IL-5	G9	G10	MCP-3	I15	I16	Thrombospondin-1
B15	B16	Dkk-1	E5	E6	IL-6	G11	G12	M-CSF	I17	I18	TNF-alpha
B17	B18	DPPIV	E7	E8	IL-8	G13	G14	MIF	I19	I20	uPAR
B19	B20	EGF	E9	E10	IL-10	G15	G16	MIG	I21	I22	VEGF
B21	B22	EMMPRIN	E11	E12	IL-11	G17	G18	MIP-1α/MIP-1β	J1	J2	Reference Spots
C3	C4	ENA-78	E13	E14	IL-12 p70	G19	G20	MIP-3α	J5	J6	Vitamin D BP
C5	C6	Endoglin	E15	E16	IL-13	G21	G22	MIP-3β	J7	J8	CD31
C7	C8	Fas Ligand	E17	E18	IL-15	G23	G24	MMP-9	J9	J10	TIM-3
C9	C10	FGF basic	E19	E20	IL-16	H1	H2	Myeloperoxidase	J11	J12	VCAM-1
C11	C12	FGF-7	E21	E22	IL-17A	H3	H4	Osteopontin	J23	J24	Negative Controls
C13	C14	FGF-19									

Figure 15. The soluble mediator secretion of moDCs is modulated by MSCl cells and MSCI-CM. *The modulatory role of MSCI cells or the MSCI-CM in the regulation of protein production of moDC, were examined on day 4 of moDC differentiation; the integrated density of produced mediators was measured by Human XL Cytokine Array Kit.* ***(A)*** *The kit contained four nitrocellulose membranes, each containing 105 different capture antibodies printed in duplicate. Panel* ***B*** *shows a transparency overlay template for coordinate reference. Panel* ***C*** *shows a table for the Human XL Cytokine Array coordinates.*

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 ***(Figure 15, Figure 16 panel A)***. 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 ***(Figure 15, Figure 16 panel A)***. 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 ***(Figure 15, Figure 16 panel A)***. Next, concentrations of some inflammatory (IL-6) and anti-inflammatory (IL-10 and TGFβ) mediators were measured by ELISA ***(Figure 16 panel B)***. 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 ***(Figure 16 panel B)***. 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.

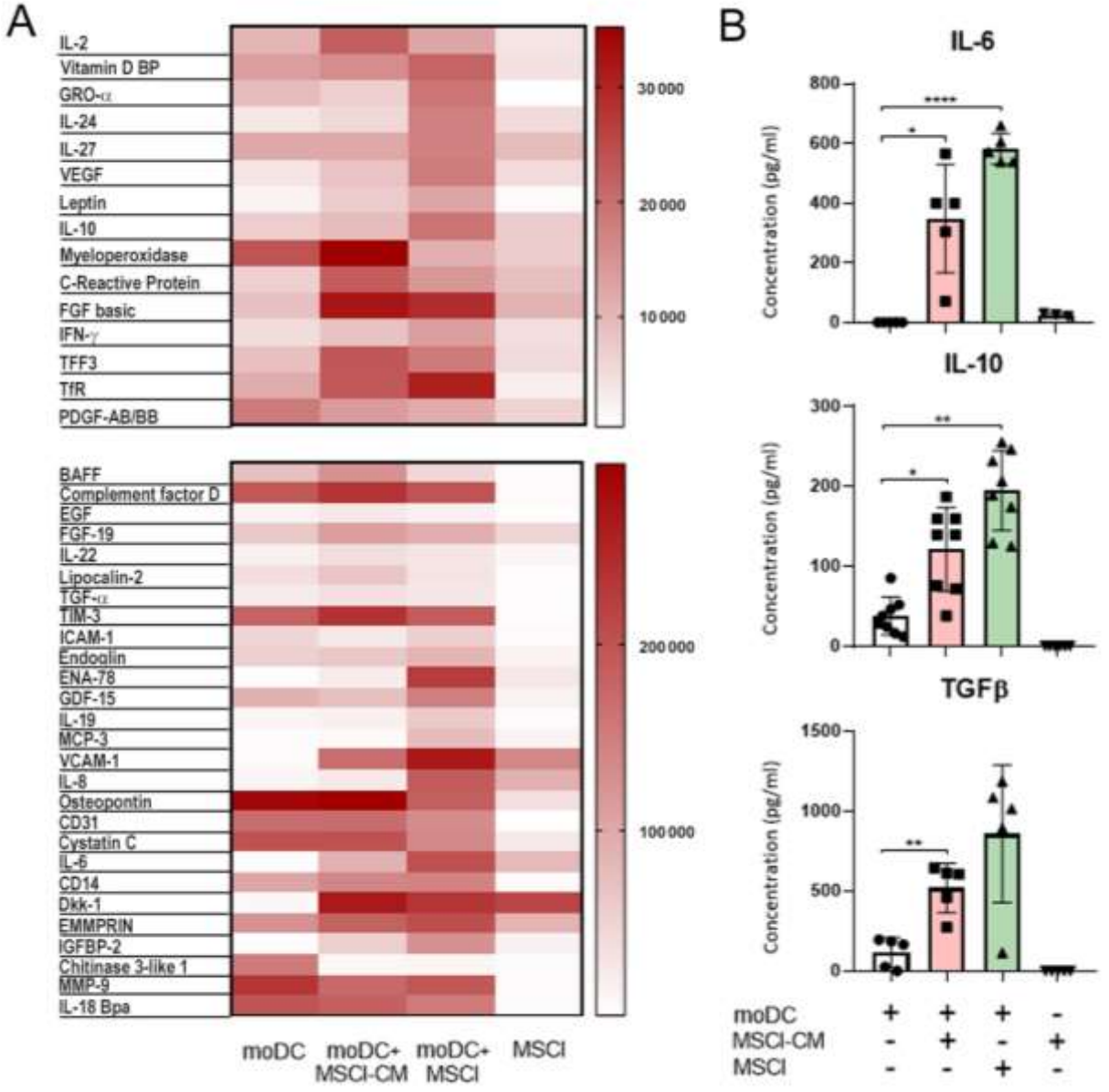


Figure 16. The soluble molecule production of moDCs is modified by MSCl cells and MSCI-CM. ***(A)****To investigate the modulatory role of MSCI cells or the MSCI-CM on the cytokine and chemokine production of moDCs, on the fourth day of moDC differentiation, the integrated density of soluble mediators was measured from the supernatants of cells by Human XL Cytokine Array Kit****, and* ***(B)*** *the concentration of secreted cytokine IL-6, IL-10, TGFß was detected by ELISA to validate and to complete the array-measurements. In the case of co-culture samples, the diagrams represent the secretion of cytokines released by cocultured moDCs and MSCI cells. Mean values of relative cytokine levels and concentrations were calculated from 4 or more independent experiments +SD. In the statistical analysis, ANOVA followed by Bonferroni's multiple comparison tests was used with significance defined as *P < 0.05, **P < 0.01 and ****P < 0.0001.*

6.2.2. 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 ***(Figure 17 panel A)***. 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 ***(Figure 17 panel A)***.

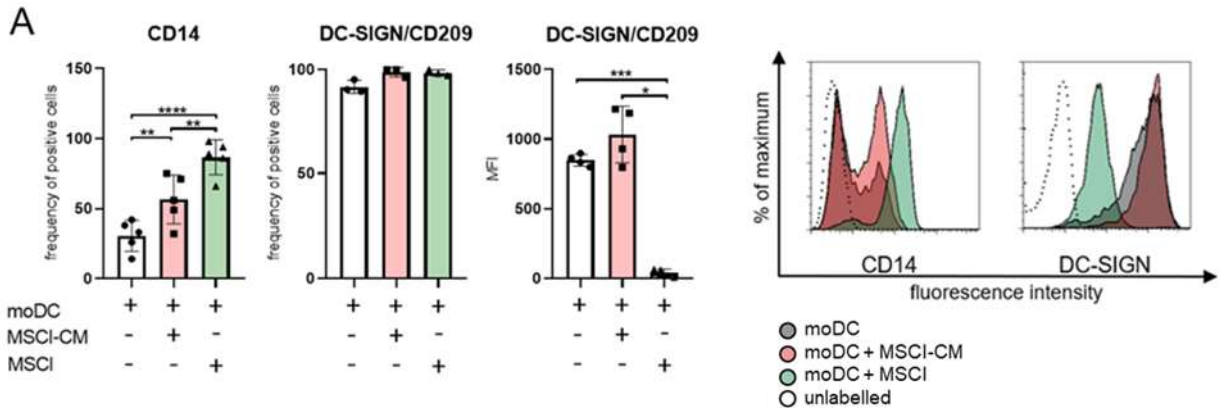


Figure 17. MSCl cells and MSCl-CM alter the surface expression of antigens CD14, DC-SIGN/CD209 and group 1 CD1 molecules (CD1a, b, c). *Freshly isolated CD14$^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF ± MSCl cells or MSCl-CM for 4 days.* ***(A)****On the fourth day of the differentiation process, the surface expression of CD14 and DC-SIGN was measured by flow cytometry on moDCs. Continued on next page.*

Under our experimental conditions, approx. 40% of untreated cells expressed CD1a, b, and c molecules ***(Figure 17 panel B (next page))***. 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 ***(Figure 17 panel C)***.

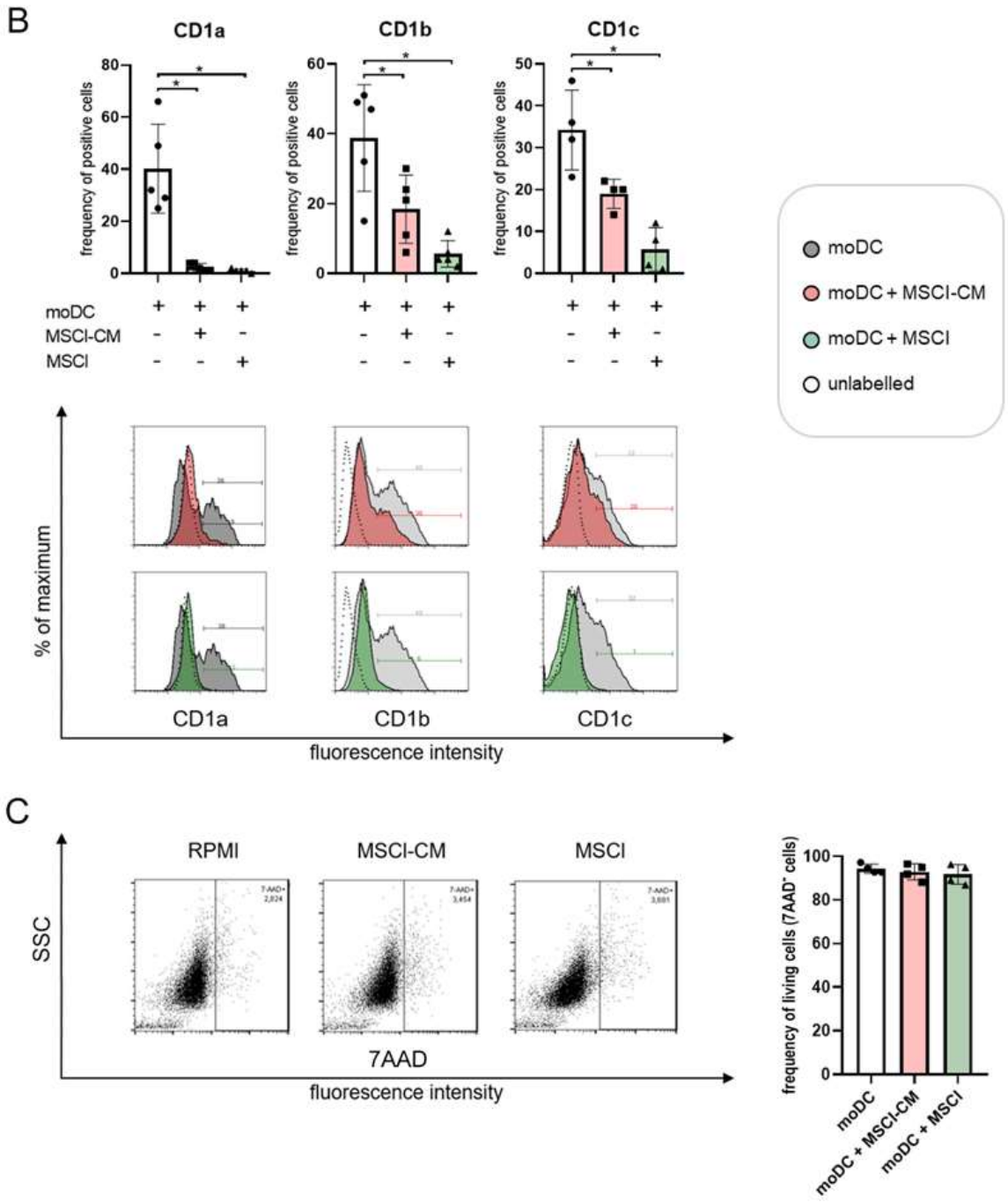


Figure 17. ***(B)*** *On the fourth day of the differentiation process, the surface expression of CD1a, CD1b and CD1c molecules were measured by flow cytometry on monocyte-derived cells.* ***(C)*** *To exclude the unspecific staining because of the presence of dead cells in the culture, the viability of the cells was observed by flow cytometry after 7-aminoactinomycin D (7-AAD) staining. The MFI (median fluorescence intensity) and the mean values of the ratio of cells positive for the examined surface markers were calculated from at least three independent experiments. Data are represented as individual data points with the mean ± standard deviation. Histograms show one of at least four independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.*

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 ***(Figure 18)***. 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 ***(Figure 18 panel A, B)***. 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 ***(Figure 18 panel C)***.

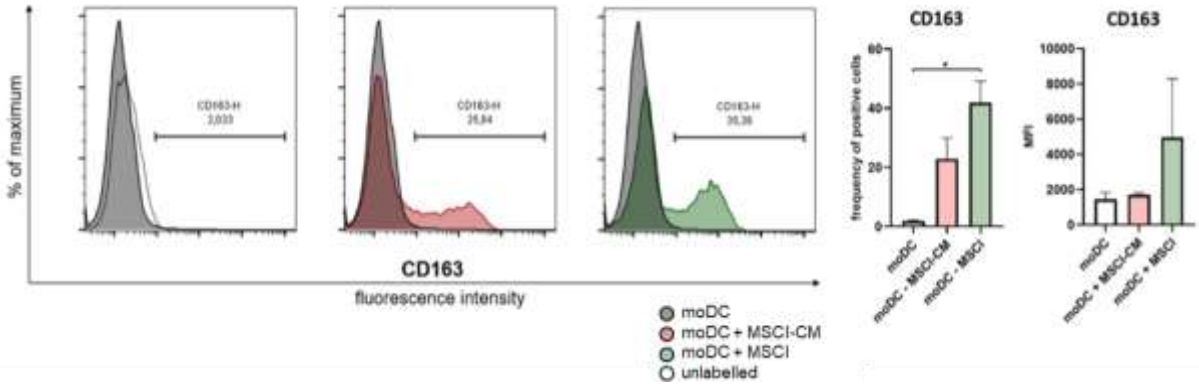


Figure 18. Expression of CD163 marker on the cell surface of monocyte-derived cells differentiated under the effect of MSCl cells or MSCl-CM. *Freshly isolated $CD14^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF ± MSCl cells or MSCl-CM for 4 days. On the fourth day of the differentiation process, the expression of CD163 macrophage marker was measured by flow cytometry on the surface of monocyte-derived cells.* ***(B)*** *The mean values of the ratio of cells positive for the measured cell surface molecules and* ***(C)*** *the median values were calculated from three independent experiments +SD. Histograms show one of three independent experiments* ***(A)****. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *P < 0.05.*

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 ***(Figure 17 panel A, B and Figure 18)***.

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 ***(Figure 19 panel A)***. In the presence of MSCl cells, increased expression of co-inhibitory molecule PD-L1 could also be found on monocyte-derived cells ***(Figure 19 panel B)***. Notably, an upregulated expression of another co-inhibitory molecule CTLA-4 on monocyte-derived cells was triggered exclusively by MSCl-CM ***(Figure 19 panel B)***. 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 ***(Figure 19 panel C)***. 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 *(**Figure 19 panel C**)*.

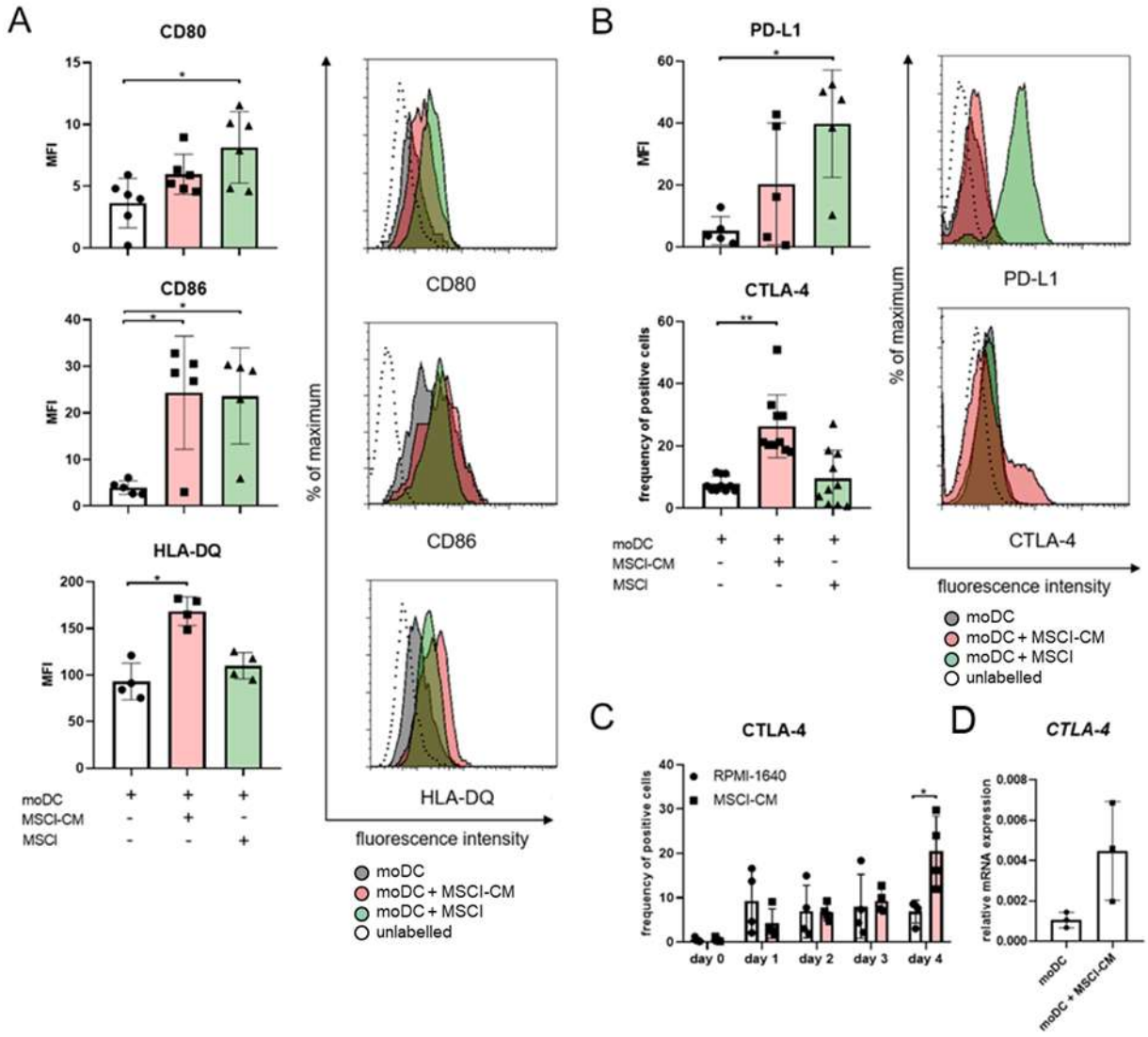


Figure 19. MSCl cells and MSCl-CM change the cell surface expression of costimulatory and negative costimulator molecules involved in T cell priming. *Freshly isolated CD14$^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF ± MSCl cells or MSCl-CM for 4 days. On the fourth day of the differentiation process, monocyte-derived cells were analyzed (**A**) for the cell expression profile of the T-cell stimulatory CD80, CD86, HLA-DQ, and (**B**) the regulatory CTLA-4 and PD-L1 molecules by flow cytometry. (**C**) The ratio of CTLA-4$^+$ cells was measured each day during the moDC differentiation. **(D)** The mRNA expression level of CTLA-4 was measured by qPCR on the fourth day of the moDC-differentiation process. Amplification of h36B4 housekeeping gene was used as normalizing controls. The MFI and the mean values of the ratio of cells positive for the measured cell surface molecules were calculated from at least four independent experiments. Mean values of relative mRNA levels were calculated from three independent experiments. Data are represented as individual data points with the mean ± standard deviation. Histograms show one of at least four independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test (**A** and **B**) as well as Student's t-test and Mann–Whitney rank sum test (**C**) were used with significance defined as *P < 0.05, **P < 0.01.*

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 ***(Figure 20)***. 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 ***(Figure 20)***.

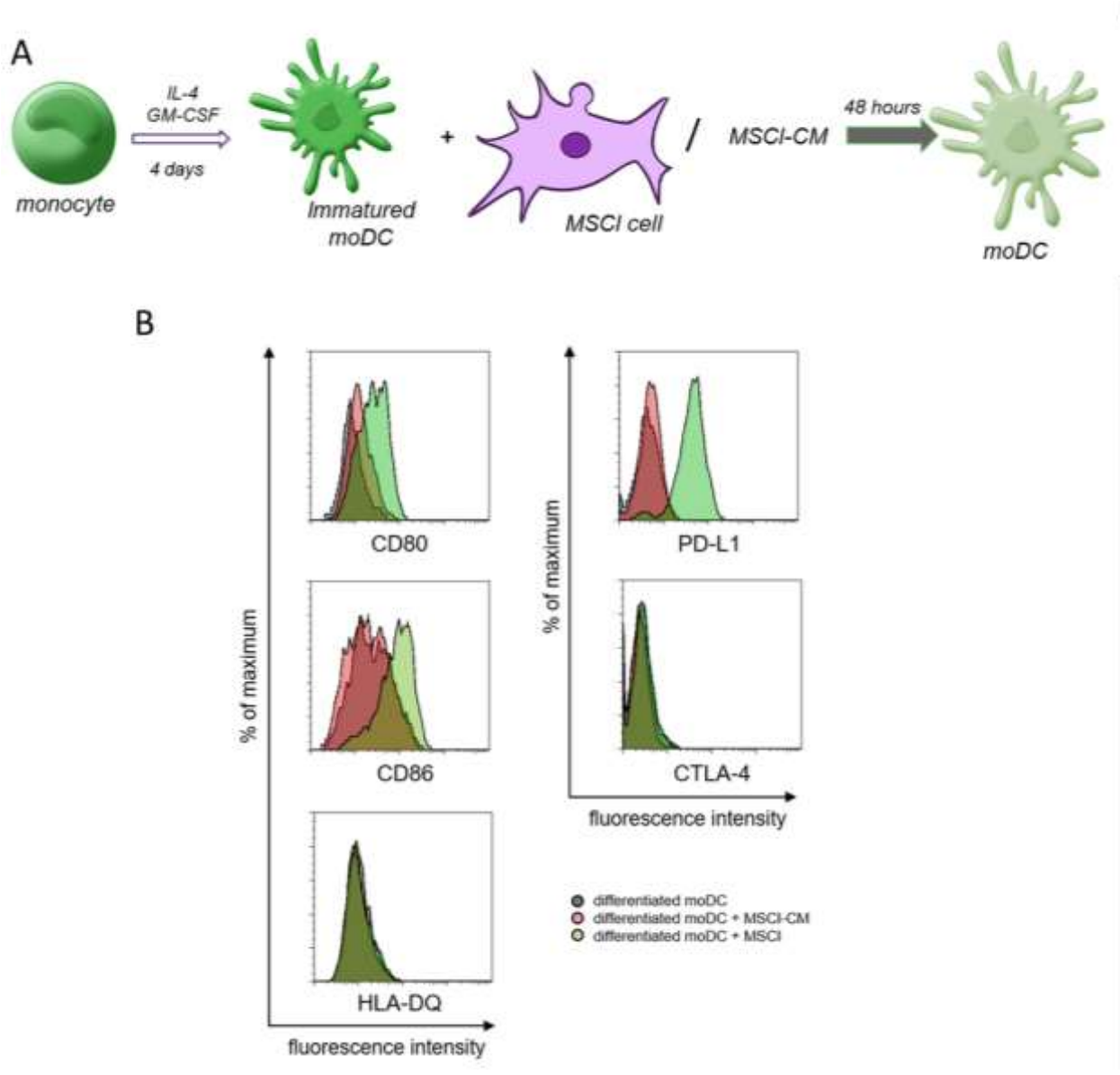


Figure 20. The effects of MSCl-CM on the phenotype of immature, differentiated moDCs. (A) *Freshly isolated CD14^{+} monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF for 4 days. On day 4, differentiated moDCs were were co-cultured with MSCl cells or treated with MSCl-CM.* ***(B)*** *After 48 hours, moDCs were washed from MSCl-CM or separated from MSCl cells by using DC-SIGN-specific antibodies conjugated with paramagnetic beads. The cell surface expression of CD80, CD86, HLA-DQ, PD-L1, and CTLA-4 was measured by flow cytometry.*

6.2.3. 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 ***(Figure 21)***.

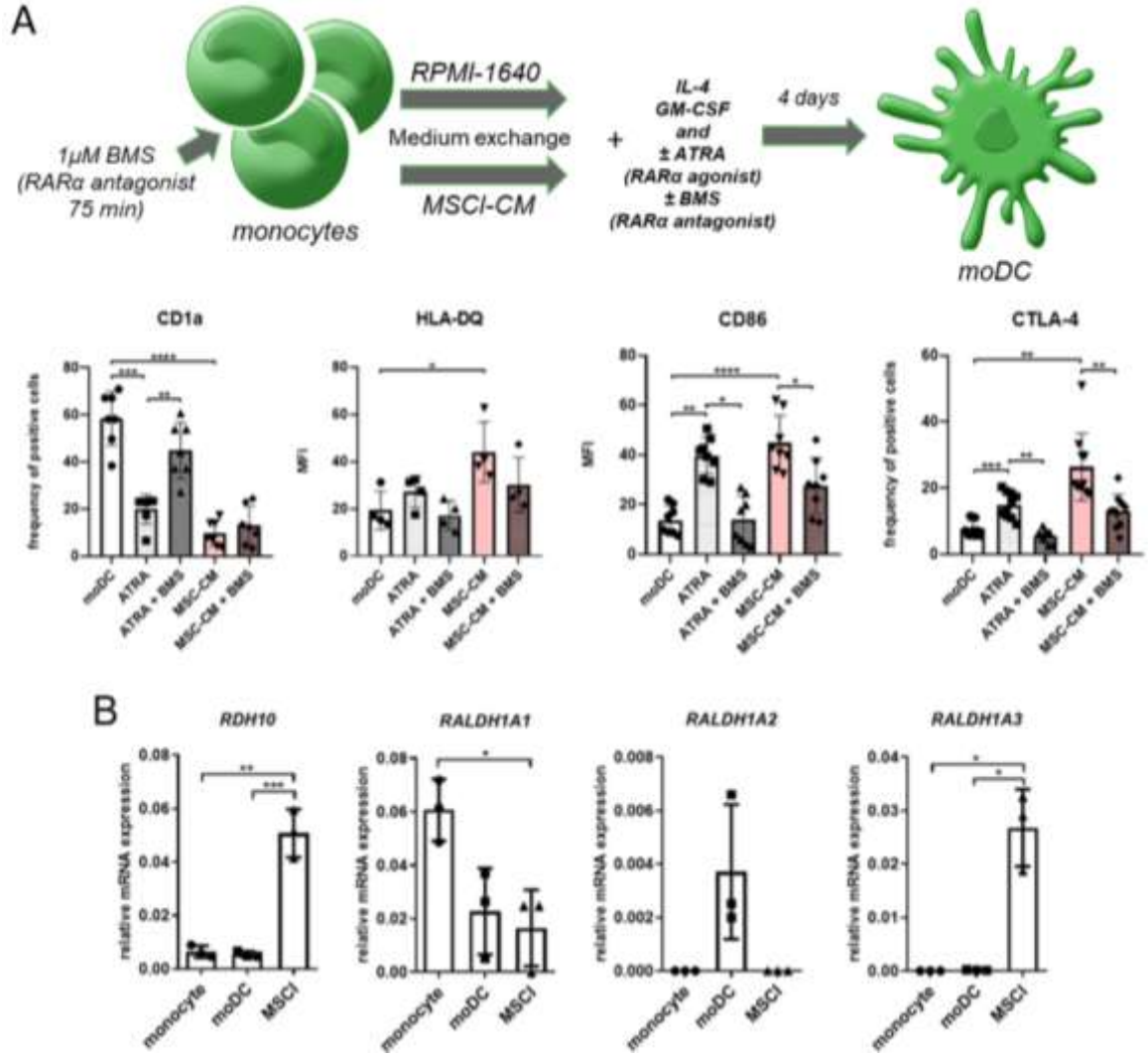


Figure 21. MSCl cells alter the immune modulatory activity of moDCs at least partially via nuclear receptor RARα and ATRA. (A) *To examine how MSCl-CM modulate the differentiation process of moDC, freshly isolated monocytes were treated with or without 1 μM BMS614 (BMS) specific RARα-antagonist. After 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 and IL-4 and GM-CSF. On the fourth day of the differentiation process, the expression level of CD1a, the T-cell stimulatory HLA-DQ, CD86, and CTLA-4 were monitored by flow cytometry on the surface of monocyte-derived cells.* ***(B)*** *To investigate and compare the ability of freshly isolated monocytes, differentiated moDCs and MSCl to generate ATRA, mRNA was isolated from the cells. The relative mRNA expression level of target genes RDH10, ALDH1A1, ALDH1A2 and ALDH1A3 was measured by RT-PCR.* *Continued on next page.*

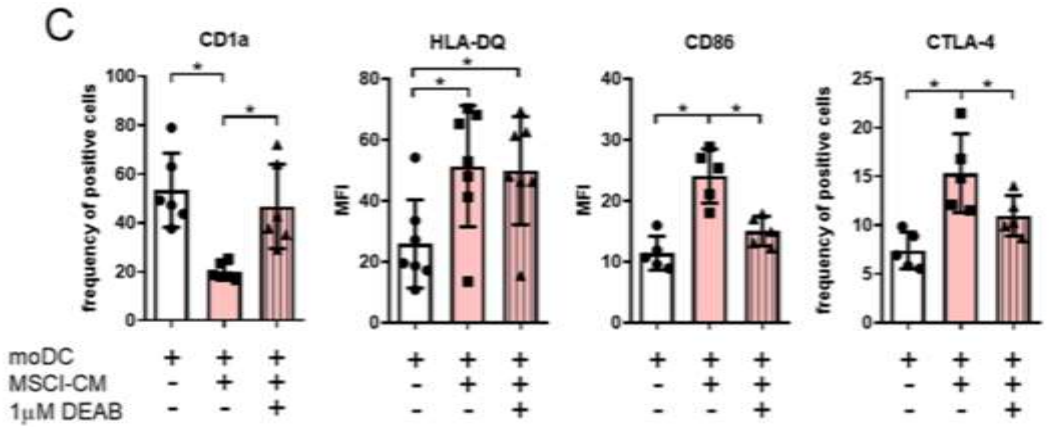


Figure 21. (C) *To test the effect of MSCl cell-derived ATRA on the differentiation of monocytes, the RALDH enzymes involved in ATRA synthesis were blocked by 1μM DEAB in the MSCl cultures for 24 h. After this incubation time, the supernatant was changed to fresh RPMI. After 48 hours the MSCl-CM was collected. Freshly isolated CD14$^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF ± MSCl-CM with or without ATRA for 4 days. On the fourth day of the differentiation process, expression of CD1a, HLA-DQ, CD86, and CTLA-4 was analyzed by flow cytometry on the surface of moDCs. Mean values of MFI and moDCs positive for the measured cell surface antigen were calculated from at least four independent experiments. Mean values of relative mRNA levels were calculated from three independent experiments. Data are represented as individual data points with the mean ± standard deviation. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *P < 0.05, **P < 0.01 and ***P < 0.001, ****P < 0.0001.*

The expression of CD1a on moDCs was significantly silenced by MSCl-CM ***(Figure 17 panel B and Figure 21 panel A)*** and when ATRA was added to the monocytes (***Figure 21 panel A)***. 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 (***Figure 21 panel A)***. Blocking of RARα prevented the effect of ATRA or MSCl-CM on HLA-DQ expression; however, the observed differences were statistically non-significant (***Figure 21 panel A)***. 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 ***(Figure 21 panel A)***. 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 ***(Figure 21 panel A)***.

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* ***(Figure 21 panel B)***.

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 ***(Figure 21 panel C)***. 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 ***(Figure 21 panel C)***.

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

6.2.4. 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. The presence of MSC-CM during the differentiation process of moDCs did not trigger the polarization of IFNγ (T*h*1) or IL-4 (T*h*2) production by helper T cells ***(Figure 22 panel A)***. We found that MSCl-CM-treated moDCs can induce the polarization of T cells secreting IL-17 ***(Figure 22 panel B)*** and IL-10 ***(Figure 22 panel C)*** 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 ***(Figure 22 panel B, C)***. Isotype control antibodies do not affect the IL-10 and IL-17 secretion by T lymphocytes triggered by MSCl-CM-educated moDCs ***(Figure 22 panel D)***.

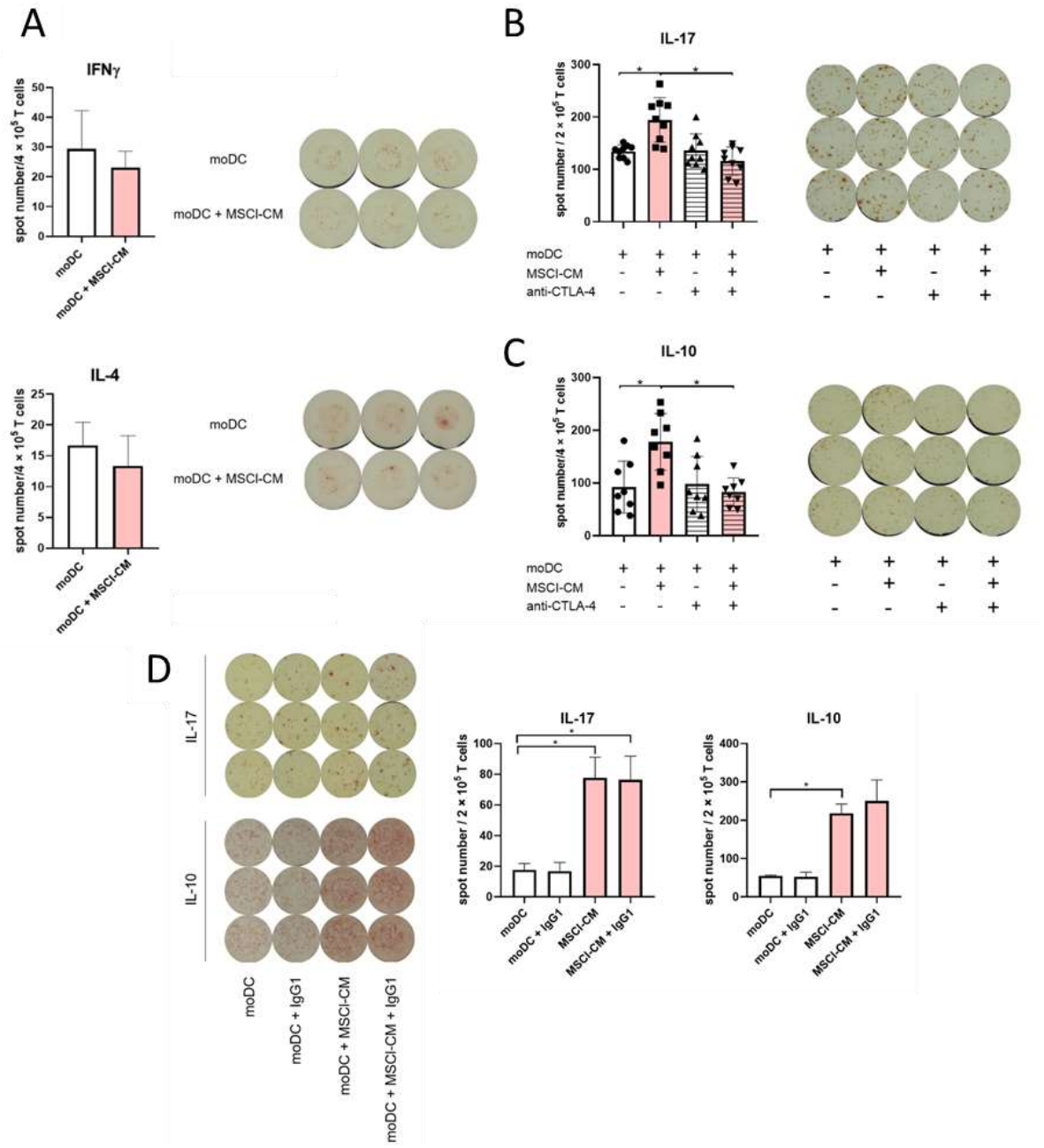


Figure 22. Expression of CTLA-4 on moDCs educated by MSCl-CM seems essential to induce the polarization of naïve T cells into IL-10 and IL-17 producing lymphocytes. *Freshly isolated CD14$^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF ± MSCl-CM for 4 days. On the fourth day of the differentiation process, monocyte-derived cells were incubated in the presence of anti-CTLA-4 neutralizing antibody and were co-cultured with allogeneic naive T-lymphocytes for a further 3 (IFNγ), 5 (IL-4), or 9 (IL-17, IL-10) days at a moDC : T-cell ratio of 1 : 10. ELISPOT assays were used to determine the number of (**A**) IFNγ, IL-4, (**B**) IL-17, and **(C)** IL-10 producing T cells. **(D)** To check the effect of isotype control antibody on T cell polarizing activity of monocyte-derived cells, moDCs were incubated with IgG1 isotype control antibodies and co-cultured with T cells for nine days at a moDC : T-cell ratio of 1 : 10. ELISPOT assays were used to determine the number of IFNγ, IL-4, IL-17, or IL-10 producing T cells. The average values of spot numbers indicating T-lymphocyte responses were counted from 3 micro-wells. Mean values of spot numbers were calculated from 3 independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *P < 0.05.*

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 ***(Figure 23)***. 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 ***(Figure 23 panel A, C)***.

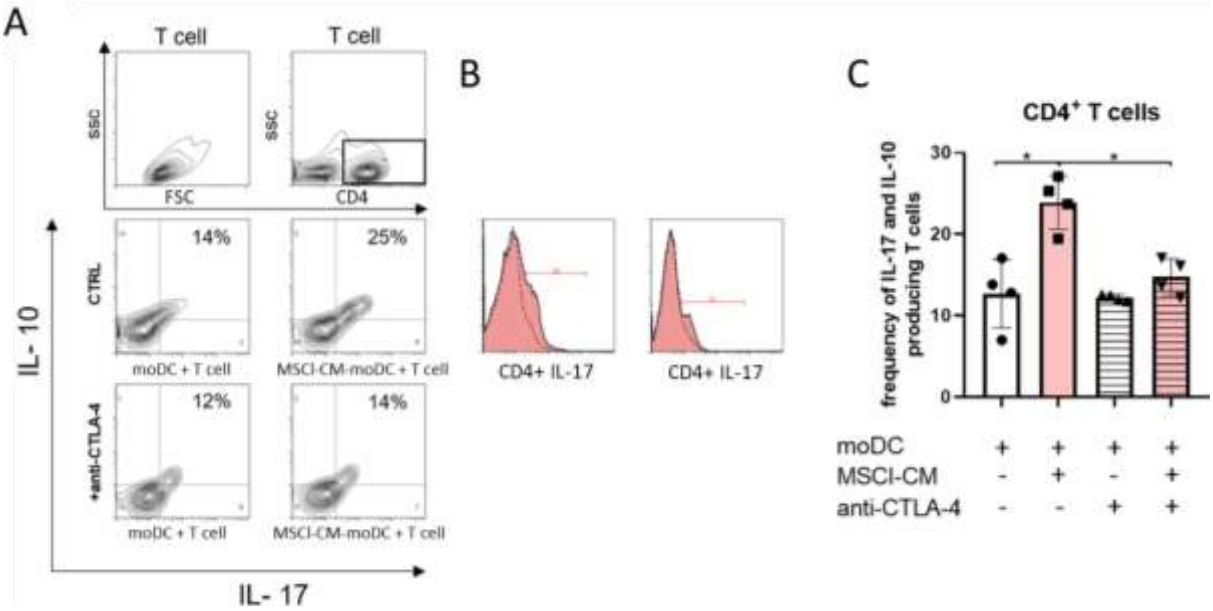


Figure 23. Expression of CTLA-4 on moDCs educated by MSCl-CM seems essential to induce the polarization of naïve T cells into IL-10 and IL-17 (double-positive) producing lymphocytes. *Freshly isolated CD14$^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF ± MSCl-CM for 4 days. On the fourth day of the differentiation process, monocyte-derived cells were incubated in the presence of anti-CTLA-4 neutralizing antibody and were co-cultured with allogeneic naive T-lymphocytes for a further 9 days at a moDC : T-cell ratio of 1 : 10. Mean values IL-10 and IL-17 producing CD4$^+$ T cells were detected by flow cytometry.* ***(A)*** *Contour plots show one of the four independent experiments.* ***(B)*** *Histograms show the fluorescence intensity of IL-17 or IL-10 and the autofluorescence of the cells (dotted lines).* ***(C)*** *Mean values of T cells' ratio positive for the measured cell intracellular cytokines were calculated from four independent experiments. Data are represented as individual data points with the mean ± standard deviation. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *P < 0.05.*

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.

7. 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. MSC exhibits immune homeostatic functions maintained by exposure of the cells to pro-inflammatory cytokines such as IFNγ and TNFα or TLR ligands, as evidenced by enhanced secretion of cytokines, chemokines, and lipid mediators[150,151]. 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[156]. Furthermore, they can drive the differentiation of monocytes or the polarization of the T cell response[157]. 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. As an essential cellular component in all organs and tissues, MSCs could be isolated from almost every vascularized organ. However, MSCs with different origins and organ/tissue localization differ in their morphology and biological features[11], and many experiments suggest variations in the differentiation capacity of MSCs from different tissue sources[12]. MSCs are crucial players in regulating immune responses. If the communication between the stromal and other tissue-resident cells is impaired, it could result in a wide array of structural and functional failures. To demonstrate their role in the maintaining and restoration of homeostasis, the depletion or dysfunction of MSCs leads to the development of pathological conditions, which may be manifested in a wide array of diseases[5,158]. Based on these observations, the MSCs could provide promising alternatives to develop treatments for patients suffering from chronic diseases of inflammatory or degenerative origin. For this purpose, we must attempt to collect and describe the properties of MSCs as clearly as possible.

The knowledge about the vessel wall-derived MSCs was broadened

The greater reservoir of MSCs is the bone marrow. The vast number of studies focused on investigating bone-marrow-derived MSCs (BM-MSCs)-biology[159,160]. 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). Despite the increasing number of projects focusing on changes after MSCs administration, few puzzle pieces are still missing, and the results are often inconsistent. These divergences in projects' messages could be explained by the differences in the source, culture conditions, phenotype, epigenetic

background, proliferating capacity, and the quality or quantity of MSC-produced mediators. 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[13,161-163]. 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)[164]. 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[165,166].

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[167] 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[168].

SV- and BM-MSCs also have immunomodulatory properties; however, their priming could lead to a different outcome

The immunomodulatory activity of BM-MSCs is well known[5]. 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. Isolated perivascular cells exposed to hypoxic conditions show enhanced proliferation, while if degraded ECM products activate them, their increased migratory capacity is detectable[169]. Additionally, MSCs express a wide array of pattern recognition, cytokine, chemokine, and growth factor receptors. In vessels, "MSC licensing" resulted in increased secretion of factors supporting angiogenesis driven by MSCs[170,171]. The response of MSCs to various stimulatory factors influences the differentiation and functional features of neighboring cells[59,150]. 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; BM-MSCs and SV-MSCs constantly secreted IL-6 under normal conditions. Patterns in the secretion of IL-6 cytokine and IL-8 chemokine were similar in both MSC cultures. However, BM-MSC produces more IL-6 and IL-8 upon any stimuli than SV-MSC. Interestingly, although IFNγ is generally used to stimulate MSCs, it did not induce the secretion of IL-6 and IL-8. Both types of MSCs produced CXCL-10/IP-10 chemokine after TLR- and cytokine receptor ligation. In contrast to IL-6 and IL-8 levels, SV-MSCs produced more CXCL-10/IP-10 in response to stimulation than BM-MSCs. In BM-MSCs cultures, PolyI:C and IFNγ were the most efficient activators of CXCL-10/IP-10 production, while SV-MSCs secreted this chemokine in higher concentrations as a result of any applied stimuli; however, SV-MSCs released more CXCL-10/IP-10 when they were exposed to LPS, PolyI:C or IFNγ. In line with the previous findings[172], although MSCs express CD14 (playing a vital role in TLR4 signaling) at a low level, both BM-MSCs and SV-MSCs could be triggered by LPS. Exposure cells with LPS may slightly upregulate the production of cytokines and chemokines in MSCs without activation of AKT, NF-κB, and P38[172]. It is known that TNFα and IL-1β are potent inducers of MSC[173]; however, these cytokines did not initiate the production of CXCL-10/IP-10 by BM-MSCs at all. Based on our findings, priming induces the activation of MSCs with various origins in diverse ways and may lead to different outcomes.

However, the distinct molecular mechanisms behind the MSC-mediated regulation have yet to be revealed.

One of the strategies to overcome limitations and inconsistent results about MSCs-derived from different tissue sources is the use of MSCl cells

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[174]. In our previous studies, we described the characterization of a human MSCI cell line generated from pluripotent HUES9 embryonic stem cells[147] and their capacity to modulate the responses moDCs to RIG-I receptor-mediated stimulation[156]. Based on our previous findings, the MSCl cell-derived direct signals efficiently suppressed the activation of moDCs. 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.

Direct or indirect presence of MSCl cells together with IL-4 and GM-CSF during the differentiation of monocyte-derived cells alter their phenotype and functions

Human DCs represent one of the most heterogeneous leukocyte-population, including well distinguishable and characterized subsets associated with regulatory or inflammatory functions and significant phenotypic and functional plasticity[175].

MSCs greatly suppress the differentiation and activation of moDCs, which is dependent on the cell ratio. Characteristics of these MSC-educated moDCs were the inhibited expression of CD1a and the preserved expression of CD14. However, the results are inconsistent about the direct or indirect effect of MSCs on the expression of co-stimulatory and MHC molecules on the surface of monocyte-derived cells differentiated in the presence of IL-4 and GM-CSF[68,141,145].

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[141]. 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[177]. 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. These cells could be characterized by up-regulated expression of PD-L1 and secretion of IL-10[70]. Additionally, Notch ligands could alter the function of monocytes or macrophages, and they can determine the direction of macrophage polarization[178]. Since Notch-mediated signals play a role in the protection against inflammation by MSCs[179-181], it is possible that the Notch pathway becomes stimulated during the direct cell-cell contact between MSCl cells and monocytes. Additionally, the literature highlights the importance of adhesive interactions between VCAM-1 (on MSCs) and VLA-4 (on monocytes), which may participate in the maintenance of monocytes in a co-culture system[182,183]. Based on the findings of Ammon et al, monocytes and monocyte-derived macrophages or DCs are different from each other based on the adhesion molecule expression profile involved in the regulation of the differentiation process. They observed that macrophages express VLA-3, VLA-4, and VLA-6 at a higher level than the other cell types[184]. These $CD14^{+}$, VLA-4 expressing monocytes tend to differentiate into M2 macrophages in the presence of IL-4. Additionally, elevated levels of IL-6 and IL-10 strongly immunomodulatory cytokines in our experiments could have a pivotal role in the induction of the M2 cell generation[176].

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[146]. 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.

MSCl-CM-educated moDCs able to polarize the T cells into IL-10 and IL-17 producing subtype in a CTLA-4-dependent manner

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[185-190]. 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[146]. 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. Although we could show the presence of IL-10 and IL-17 producing double-positive cells, the factors inducing IL-10 production in Th17 cells are still largely unknown[191,192].

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[111,113]. CTLA-4 expressing DCs was detected in patients suffering from certain carcinomas, highlighting this molecule's importance during tumor progression[193]. 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[194]. 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.

MSCl cell-derived RARα ligands, e.g., ATRA supports the generation of CTLA-4-expressing moDCs

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[197]. 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. Results of Part II are summarized in ***Figure 24***.

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.

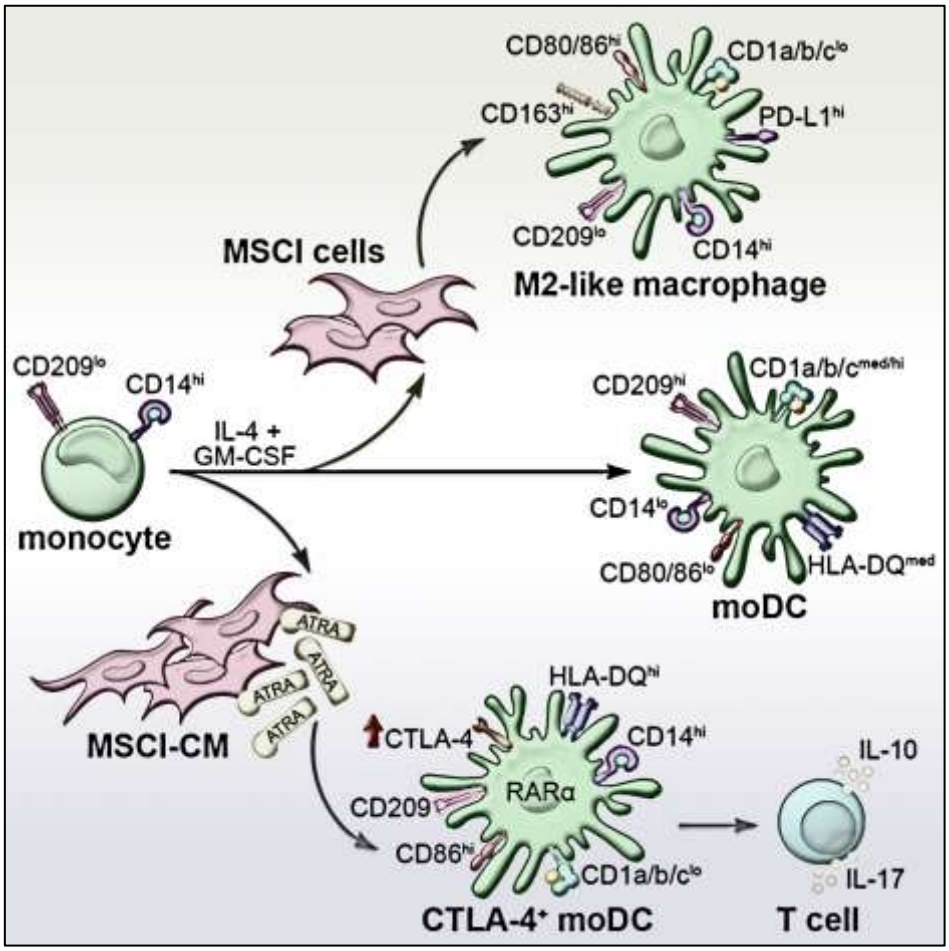


Figure 24. The model is presenting the regulation of moDC differentiation modulated by MSCls. *We propose that MSCls show immunomodulatory properties by the modulation of moDC phenotype and functions. MSCl interacts with the surrounding cells by direct cell-cell contacts and by releasing immunomodulatory molecules, including ATRA. MSCls elevate the cell surface expression level of the co-stimulatory B7 and co-inhibitory PD-L1 molecules while the class I CD1 lipid antigen-presenting receptors are down-modulated. MSCls produce ATRA targeting nuclear hormone receptors in moDC. The presence of ATRA can increase the cell surface expression level of antigen-presenting molecule HLA-DQ and co-inhibitory molecules CTLA-4 and PD-L1 in moDCs expressing CD14 and DC-SIGN. The inhibition of ATRA synthesis in MSCl and consequently RARα function in moDCs in the presence of MSCl-CM can reverse the differentiation of moDC partly to the gold-standard phenotype. MoDCs differentiated in the presence of MSCI-CM can elevate the number of IL-10 and IL-17 producing autologous T cells in a CTLA-4 dependent manner.*

8. 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.

9. ÖSSZEFOGLALÁS

A mezenchimális őssejtek ígéretes terápiás lehetőséget kínálnak. Ez részben relatíve széles, multipotens differenciálódási képességének, részben pedig jelentős immunmoduláló képeségének köszönhető. Számos kórkép hátterében nem megfelelően szabályozott immunválasz húzódik meg, ami pedig a sejtek között zajló kommunikációs zavar következménye lehet. A szöveti rezidens sejtek, mint az MSC-ek meghatározó szerepet töltenek be a homeosztázis fenntartásában, képesek lehetnek azonban a kialakult patológiás állapotok támogatására is.

Bár legnagyobb számban a csontvelőben fordulnak elő, az MSC-ek számos szövet, így az erek esszenciális sejtes alkotóelemei is. Az MSC-ket nem kezelhetjük egységes populációként, a különböző egyénekből származó sejtek ugyanis különbözhetnek fenotípusukban, epigenetikai hátterükben, életképességükben és a termelt oldott faktorok minőségében. A nagyfokú diverzitás megismerése érdekében célul tűztük ki egy izolálási módszer optimalizálását az érfalakban található MSC-ek (SV-MSC) kinyerésére. Megfigyeléseink alapján az izolálást követően a sejteket karakterizálnunk kell, ugyanis az MSC-ek fenotípusos tulajdonságaikban átfedést mutatnak az erek endothél rétegében és az érfalak subendotheliális régiójában lokalizált egyéb sejttípusokkal. A részletes karakterizálás mellett az SV-MSC-eket összehasonlítottuk a széles körben használt és jól ismert csontvelői MSC-kel (BM-MSC). Az összehasonlító analízis során számos hasonló tulajdonságot találtunk, feltárásra kerültek azonban különbségek is, melyek összességében alátámasztják az eltérő állapotban lévő donorokból nyert, illetve azok különböző szöveteiből izolált MSC-ek alapos karakterizálásának fontosságát.

Az MSC sejtvonalakkal történő kísérletes munka segíthet a frissen izolált MSC-ek diverzitásából fakadó nehézségek kiküszöbölésére. Munkánk során vizsgáltuk az MSCl sejtek direkt és indirekt hatását a monociták dendritikus sejtekké (DS) történő differenciációjára. A DS-ek hídként teremtenek kapcsolatot az öröklött és szerzett immunválasz között. Rendkívül plasztikus sejtekről révén szó, a monociták differenciációja számos ponton és módon befolyásolható, ezáltal biztosítva a monocitákból differenciálódó sejttípusok alkalmazkodását az adott szöveti környezethez. Az MSCl sejtek indirekt módon, ATRA (RARα ligandum) termelés révén elősegítik CTLA-4 molekulát kifejező DS-ek kialakulását, melyek képesek kiváltani az allogén, naiv T-limfociták IL-10 és IL-17 termelő sejtekké történő polarizációját.

Munkánk során új mechanizmusokat sikerült feltárni, melyek szerepet játszanak az MSC-ek által kifejtett immunmoduláció során, és felhívhatják a figyelmet a sejt-mentes őssejtterápiák előnyeire.

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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.
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3. Varga, Z., Rácz, E., **Türk-Mázló, A.**, Korodi, M., Szabó, A., Molnár, T., Szőőr, Á., Veréb, Z., Bácsi, A., Koncz, G.: Cytotoxic activity of human dendritic cells induces RIPK1-dependent cell death.
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12.2. Kulcsszavak

csontvelői-eredetű mezenchymális Ős/Sztróma Sejt, Véna-érfal-eredetű mezenchymális Ős/Sztróma Sejt, dendritikus sejt, mezenchymális Ős/Sztróma Sejt-szerű sejt, monocita, differenciáció, dendritikus sejt, őssejt-vonal, CTLA-4, ATRA, RAR, T-sejt, IL-10, IL-17

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14. APPENDIX

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