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Immunomodulatory and differentiation properties of MSCs in a mouse model of the injured cornea and retina

Imunomodulační a diferenciační vlastnosti MSC v myším modelu poškozené rohovky a sítnice

Ph.D. Thesis

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Declaration

I hereby declare that my dissertation thesis is a presentation of my original research work and that I have listed all information sources and citations used. This work nor its substantial part hasn't been used to get any academic degree or diploma.

Prague, 20. 9. 2022

Prohlášení

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Chtěl bych poděkovat mému školiteli prof. RNDr. Vladimíru Holáňovi, DrSc. Za jeho čas, trpělivost a cenné rady během mého doktorského studia a také za roky předtím. Dále bych rád poděkoval svým kolegům z Oddělení transplantační imunologie a z Laboratoře imunoregulací za pomoc a za vytvoření skvělých a přátelských podmínek v laboratoři a na fakultě. Nakonec bych chtěl velmi poděkovat své rodině a přítelkyni za podporu během posledních několika let ve všem, co dělám.

ABSTRAKT

Kmenové buňky obecně představují potenciál pro léčbu řady onemocnění a poruch, které jsou v dnešní době léčitelné pouze obtížně nebo s řadou vedlejších účinků. Mezi dnes velmi zkoumané kmenové buňky patří námi použité mezenchymální kmenové buňky (MSCs, mesenchymal stem cells). MSCs mají značný imunomodulační a regenerační potenciál pro terapii degenerativních poruch a závažných poškození různých částí oka nebo i dalších orgánů. Stejně tak by jejich aplikace mohla sloužit jako podpůrná léčba při transplantacích rohovky a jiných zánětlivých stavech oka. Při studiu těchto imunomodulačních vlastnosti MSCs jsme se zaměřili především na jejich schopnost diferenciace v buňky různých tkání (v našem případě rohovkového epitelu a sítnice), produkci imunomodulačních molekul v zánětlivém prostředí, schopnosti migrace do místa poškození a jejich lokální protizánětlivé, regenerační a antiapoptotické působení. Terapeutické účinky MSCs jsme testovali na myším modelu poškození povrchu oka, modelu degenerace sítnice a mechanismus tohoto účinku jsme testovali v *in vitro* modelech s explantáty těchto tkání.

Při léčbě závažných poškození rohovky je již používána terapie pomocí limbálních kmenových buněk. Tato léčba je však vhodná pouze pro určité malé procento pacientů, u kterého je potřeba tyto kmenové buňky získat ze zdravého druhého oka nebo případně od dárce, kdy je nutné podávat imunosupresivních léků. V takovém případě by mohly být aplikovány autologní MSCs, získané např. z kostní dřeně pacienta. Ukázali jsme, že MSCs jsou schopny se diferencovat v buňky exprimující znaky rohovkového epitelu v prostředí rohovky pod vlivem růstového faktoru podobného inzulinu. Dále jsme ukázali, že MSCs, po transplantaci na chemicky poškozenou rohovku pomocí nanovlákenného nosiče, migrují do místa zánětu a zde snižují expresi proapoptotických genů pro BAX a p53, zvyšují expresi antiapoptotického genu BCL-2 a snižují procento apoptotických buněk v rohovce pomocí parakrinního mechanismu.

MSCs mohou být také použity pro léčbu degenerativních onemocnění sítnice. Ukázali jsme, že tyto buňky jsou schopny v prostředí zánětu, a pod vlivem interferonu gama, diferencovat se v buňky exprimující znaky typické pro sítnici. Dále jsme prokázali, že MSC produkují v zánětlivém prostředí různé růstové a neurotrofické faktory a jejich aplikace snižuje expresi prozánětlivých molekul v sítnici.

ABSTRACT

Stem cells, in general, represent the potential for treating many diseases and disorders that are currently difficult to treat or the therapy has many side effects. One of the stem cells widely investigated these days are mesenchymal stem cells (MSCs). MSCs have the considerable immunomodulatory and regenerative potential for treating degenerative disorders and severe damage to various parts of the eye or other organs. Likewise, their application could serve as supportive therapy in corneal transplantation and other eye inflammatory conditions. In this study of immunomodulatory properties of MSCs, we have focused mainly on their ability to differentiate into cells of different tissue types (in our case, corneal epithelium and retina), their production of immunomodulatory molecules in the inflammatory environment, their ability to migrate to the site of the injury, and their local anti-inflammatory, regenerative, and anti-apoptotic effects. In addition, we tested the therapeutic effects of MSCs in a mouse model of ocular surface injury and a model of retinal degeneration. Finally, we investigated the mechanism of this effect in *in vitro* models with explants of these tissues.

Limbal stem cells (LSCs) are already used to treat severe corneal damage as limbal stem cell deficiency. However, this treatment is only suitable for a small percentage of patients in whom these stem cells need to be isolated from a healthy second eye or possibly from a donor, and immunosuppressive drugs are required. In this case, autologous MSCs, derived from the patient's bone marrow could be used. We have shown that MSCs are able to differentiate into cells expressing corneal epithelium markers in the corneal environment under the influence of insulin-like growth factor I. We further showed that MSCs, when transplanted onto chemically damaged cornea using a nanofibrous scaffold, migrate to the site of inflammation and there inhibit the expression of the pro-apoptotic genes for BCL-2-associated X protein and p53, increasing the expression of the anti-apoptotic gene B-cell lymphoma 2, and decrease the percentage of apoptotic cells in the cornea via a paracrine mechanism.

MSCs can also be used for the treatment of retinal degenerative diseases. In our work, we have shown that these cells can differentiate into cells expressing retinal-specific markers in the environment of inflammation, especially under the influence of interferon-γ. Furthermore, we have demonstrated that MSCs produce various growth and neurotrophic

factors in an inflammatory environment, and their application reduces the expression of proinflammatory molecules in the retina.

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1. LIST OF USED ABBREVIATIONS

ACAID	Anterior chamber-associated immune deviation
AD-MSCs	Adipose tissue-derived MSCs
α-MSH	Alpha-melanocyte-stimulating hormone
AM	Amniotic membrane
AMD	Age-related macular degeneration
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BM-MSCs	Bone marrow-derived MSCs
CCL	Chemokine C-C motif ligand
CCR	Chemokine C-C motif receptor
CD	Cluster of differentiation
CNTF	Ciliary neurotrophic factor
COVID-19	Coronavirus disease 2019
COX-2	Cyclooxygenase 2
CXCL	Chemokine C-X-C motif ligand
CXCR	Chemokine C-X-C motif receptor
DCs	Dendritic cells
EGF	Epidermal growth factor
EPO	Erythropoietin
ER	Endoplasmic reticulum
EVs	Extracellular vesicles

Fas	Fas receptor
FasL	Fas ligand
GDNF	Glial cell lineage neurotrophic factor
GvHD	Graft versus host disease
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1 alpha
HLA-DR	Human leukocyte antigen – DR isotype
i.a.	Intraarterial (injection)
i.v.	Intravenous (injection)
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IgG/IgM	Immunoglobulin G/M
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LCs	Langerhans's cells
LSCs	Limbal stem cells
LSCD	LSC deficiency
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
miRNA	Micro RNA
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
NK	Natural killer (cells)

NO	Nitric oxide
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
PD-L1/L2	Programmed death ligand 1 and 2
PGE2	Prostaglandin E2
RPE	Retinal pigment epithelium
RT-PCR	Reverse transcription polymerase chain reaction
SDF-1α	Stromal cell-derived factor 1 alpha (CXCL12)
SOD3	Superoxide dismutase 3
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
Th	Helper T (cell)
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T (cell)
TSG-6	TNF-α stimulated gene 6
TSP	Thrombospondin
VCAM-1	Vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR-1	VEGF receptor 1
VIP	vasoactive intestinal peptide
VLA-4	α4β1 integrin very late antigen-4

2. LITERATURE OVERVIEW

2.1 Introduction

Stem cell therapy is one of the most studied approaches for treating various injuries and diseases worldwide. This thesis focuses on using mesenchymal stem cells (MSCs) in the treatment of injured cornea or retina because of their differential, migratory, immunomodulatory, and anti-apoptotic properties. Moreover, MSCs can be relatively easily obtained from most of the human body's tissues. Therefore, the possibility of treating patients with their autologous cells means decreasing doses even to zero of immunosuppressive drugs in cases of transplantations and lowering the burden on the organism. Furthermore, many results in clinical trials and research on *in vitro* and *in vivo* models present MSCs as suitable for using them in regenerative medicine. However, the ability of MSCs to adjust processes in inflammation and regeneration is well known and described, but their mechanism of action in various conditions and environments is still unknown. In this thesis, we present the possibility of using MSCs to treat multiple eye injuries and diseases and how these cells are affected in these conditions.

In case of severe damage to the cornea and its limbal area, MSCs could help with the renewal of corneal regeneration by producing multiple immunomodulatory molecules and trophic factors or even replacing the role of limbal stem cells (LSCs) after their possible differentiation. MSCs can migrate onto the injured ocular surface from the suitable scaffold and survive in the cornea for sufficient time to act. We have shown that MSCs are able to differentiate in the corneal environment to corneal-like cells and that they can inhibit apoptosis of corneal cells after their transplantation onto the injured cornea.

There are promising results in applying MSCs in case of damage to the retina as well. For example, we and others have shown that MSCs can differentiate into the cells expressing retinal markers and inhibit the local inflammatory reaction *in vitro* and *in vivo*.

These results could lead to a better understanding of the mechanisms of MSC actions in the site of ocular injury and inflammation and to plan more effective strategies to treat these conditions. General knowledge of MSC behavior in tissue-specific environments could help us better understand how to work with these unique types of stem cells. How to pre-treat them, what medicaments are suitable for combination with stem cell therapy, and how the

cell functions will differ in various cases. Also, it is necessary to expose all possible shortcomings and problems associated with the treatment by MSCs.

2.2 Stem cells

Stem cells are a small population of cells present in every tissue of the organism. They are represented by many cell populations without common markers, although their characteristics depend on the tissue of origin. Various functional properties can also distinguish stem cells. The typical primary signature of stem cells is their ability to self-renewal, slow cell cycle, and differentiation into different cell types. We can divide them into pluripotent embryonal stem cells and artificial induced pluripotent stem cells or multipotent stem cells of an adult organism.

Embryonal stem cells are pluripotent cells that can be obtained from the inner cell mass of the blastocyst. Their main advantage is the ability of unlimited cell division and that they can potentially differentiate into every cell type of the body. However, apart from ethical reasons, the main disadvantage of their use is the formation of teratomas (Martello and Smith, 2014). A different kind of pluripotent stem cells is induced pluripotent stem cells.

Induced pluripotent stem cells are artificially prepared from adult somatic cell types by the induced expression of four transcription factors OCT4, SOX2, c-MYC, and KLF4. However, their main disadvantage was the transfection of the genes by retroviral vectors, which meant the integration in the cell genome, newer approaches with mRNA transfection or molecular transfer promise safer and more promising results (Yamanaka, 2012).

Adult **multipotent stem cells** represent the most promising types of cells for therapeutic use nowadays. They are naturally present in most of the tissues in the body, and their primary function is the regeneration and renewal of cells by differentiation. In addition, some of them can transdifferentiate in other tissue cell types under certain conditions. MSCs are typical representatives of multipotent stem cells. Moreover, their ability to differentiate into many cell types, produce multiple growth factors and their immunomodulatory, migratory, and anti-apoptotic properties make them one of the most suitable candidates for stem cell therapy (Sobhani et al., 2017).

2.3 MSCs

MSCs are multipotent fibroblast-like stem cells with self-renewal capacity which can differentiate into various cell types. They possess immunomodulatory, anti-apoptotic, and cytoprotective properties. They are studied as the potential for cell therapy for multiple diseases and defects. In past decades there was a significant number of research to understand the mechanisms of their actions.

MSCs were characterized for the first time by Friedenstein et al. in 1966 and were further recognized as stem cells helping with mesenchymal tissue maintenance and regeneration (Caplan, 1991). As the number of studies about MSCs from various tissues increased every year and the approach to their isolation was very different, there was a need to establish basic rules for recognizing these cells. In 2005, the International Society for Cellular Therapy committee established three basic criteria for defining MSCs (Dominici et al., 2006; Horwitz et al., 2005). MSCs must be plastic-adherent; second, they must be able to differentiate into adipocytes, chondroblasts, and osteoblasts under specific culturing conditions; and third, human MSCs must express cluster of differentiation 73 (CD73, 5′-nucleotidase), CD90 (thymocyte differentiation antigen 1) and CD105 (endoglin) on their surface. On the contrary, MSCs cannot express CD11b, CD14, CD19, CD34, CD45, or CD79α, and they don't express the HLA-DR molecule. In the following years, more surface molecules were added to the MSC markers based on tissue origin or species. For example, in our mouse models, we use CD44 as defining MSC marker (alongside CD73 and CD105) expressed on the surface of *in vitro* expanded MSCs after cultivation.

2.3.1 Sources of MSCs

MSCs can be found in multiple tissues of an adult organism like adipose tissue, bone marrow, skin, dental pulp, muscles, peripheral blood, and from birth-derived tissues like umbilical cord blood, Wharton's jelly, amniotic fluid, or placenta (Berebichez-Fridman and Montero-Olvera, 2018). The most used tissues in MSC isolation are represented by the bone marrow, adipose tissue, and umbilical cord blood. The differences between MSCs from different tissue sources can be observed, especially in different surface marker expressions, the intensity of immunomodulatory properties, morphology, and ability to differentiate to specific cell types. MSCs can also be found in the stroma of the cornea. Corneal stroma-derived MSCs are mainly allocated in the stroma near the corneal limbus. They

express typical markers of MSCs and play a role in cornea regeneration, supporting LSCs, especially by their paracrine action (Hashmani et al., 2013; Liu et al., 2021).

2.3.2 Differentiation abilities of MSCs

MSCs must be able to differentiate into adipocytes, chondroblasts, and osteoblasts. However, they can also transdifferentiate into cells of ectodermal and endodermal origin under certain conditions. For example, MSCs were transdifferentiated into neuron-like cells under hypoxic conditions *in vitro* and survived in the tissue after transplantation in a rat model of Parkinson's disease (Wang et al., 2013). As well as, MSCs isolated from multiple tissues were differentiated *in vitro* into neural-like cells with the expression of neural markers (Urrutia et al., 2019).

MSCs from various human and animal sources were differentiated in hepatocytes while exposed to growth factors like hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) (Lange et al., 2005; Sgodda et al., 2007). In cases like severe volumetric injuries of muscles, MSCs could be differentiated into myogenic lineage expressing typical markers of these cells. They can be used for muscle loss therapy in the future (Hodgson et al., 2018; Witt et al., 2017) and for differentiating to smooth muscle cells in the possible treatment of vascular diseases (Gu et al., 2018). MSCs express cardiomyocytic markers after injection into the adult murine heart. They can be directly differentiated into cardiomyocytes by stimulating them with growth factors and an electric field or by treating them with 5-Azacytidine (Guo et al., 2018; Toma et al., 2002).

MSCs are considered as suitable cells for the cell therapy of various injuries and damage to the cornea or retina because of their ability to differentiate in cells expressing markers of cells of the eye. At the same time, they can still retain their immunomodulatory properties (F. Li, 2014; Beeken et al., 2021; Reboussin et al., 2022).

2.3.3 Migration abilities of MSCs to the site of the injury

One of the most important topics about the therapy of different diseases and injuries is the approach to the site of the damage. How to transfer medicaments, immunosuppressive drugs, or others in the place of action. MSCs migrate from their niche to the inflammatory environment in a physiological process as the mechanism for regenerating tissues. Nevertheless, the exact mechanisms of MSCs migratory properties are still not known. After the injury of the tissue, MSCs start to proliferate and leave their niche in the bloodstream, where they come into contact with vascular endothelium by tethering, rolling, and adhesion following diapedesis. The increased level of pro-inflammatory cytokines after the tissue

damage triggers the expression of vascular cell adhesion molecule 1 (VCAM-1) by vascular endothelium and, at the same time, the expression of various integrins as α4β1 integrin very late antigen-4 (VLA-4) by MSCs, which is typical for migrating MSCs (Cui et al., 2017). The injury and inflammation also trigger chemokine and growth factor secretions such as the chemokine (C-X-C motif) ligand 12 (CXCL12), which interacts with chemokine (C-X-C motif) receptor 4 (CXCR4) expressed on the surface of MSCs. Stimulated MSCs to start to express matrix metalloproteinase 2 (MMP-2) and membrane type of matrix metalloproteinase 1 (MMP-1) to transmigrate through the extracellular basement membrane. This diapedesis of MSCs takes about two hours and is similar to typical lymphocyte extravasation, as was shown in *in vitro* models (Semon et al., 2010; Teo et al., 2015). Homing to the injured tissues also depends on CD44, one of the MSC markers associated with cell-cell interactions and migration. In addition, chemokine (C-C motif) receptor 2 (CCR-2), HGF, and monocyte chemoattractant protein-1 (MCP-1, CCL2) can mobilize MSCs *in vivo* (Ishikawa et al., 2014; Zhang et al., 2014). MSCs migrate toward a higher concentration of CXCL12 (Park et al., 2017a).

MSCs also express other chemokine receptors, such as CXCR5, CXCR6, CCR1, CCR7, and CCR9. The expression of CCR7 directs MSCs into the local secondary lymphoid organs, which could be important in the immunomodulatory effect mechanisms of MSCs (Honczarenko et al., 2006; Li et al., 2014a). The number of circulating endogenous MSCs in the blood is significantly elevated at high substance P levels. Such migration and mobilization of bone marrow-derived MSCs (BM-MSCs) are induced by transforming growth factor beta (TGF-β) (Dubon et al., 2018).

The migratory ability of MSCs can be affected by various drugs administered, especially immunosuppressants and painkillers (Lightner et al., 2019). For example, the administration of morphine to mice affected the migratory ability of injected MSCs to the site of inflammation after transplantation of the skin grafts due to the decreased expression of typical MSC molecules CD44, CD54, and CD106 involved in their migratory mechanism (Holan et al., 2021).

2.3.4 Paracrine action of MSCs

The replacement and renewal of whole tissues is a much more complex process, and just transplantation of MSCs could not be the only solution. There has been a paradigm shift in MSC therapy in recent years. From the point of view of cell replacement to the supporter of regeneration, immunosuppression, and healing by their paracrine actions and possible

cell-cell contact. The pre-differentiation of MSC or their differentiation in target tissue markers expressing cells can still play a role in their survival in the environment while retaining their regenerative and immunomodulatory functions, as shown in our research.

MSCs produce multiple cytokines, chemokines, growth factors, and other immunomodulatory molecules. Growth factors especially play a role in these regenerative actions and the anti-apoptotic effects of MSCs. They produce angiogenetic factors such as vascular endothelium growth factor (VEGF), angiopoietin-1, erythropoietin (EPO), and platelet-derived growth factor (PDGF) that can help to regenerate and vascularize a newly formed and repaired tissue (Watt et al., 2013). On the other hand, in corneal repair, such action of MSCs is unwanted and depends on the environment in which MSCs are transplanted. In the case of the therapy of corneal injury by MSCs, they can actively prevent neovascularization by producing pigment epithelium-derived factor (PEDF) or thrombospondin-1 (TSP-1), inhibiting VEGF production (Eslani et al., 2017; Oh et al., 2008). Other growth factors such as EGF, basic FGF (bFGF), HGF, and insulin-like growth factor (IGF) could help in various tissues to expand the numbers of MSCs themselves, reepithelization, anti-fibrotic action, or activation proliferation of tissue-resident stem cells (Trosan et al., 2012; Wu et al., 2013). Especially HGF seems as a crucial factor of MSC-protective effects as it prevents epithelial cells from apoptosis, and it plays a role in cell mobilization, tissue repair, and wound healing, for example, in the cornea (Miyagi et al., 2018a; Xiao et al., 2001).

Stimulated MSCs can also modulate inflammation by expressing tumor necrosis factor-alpha (TNF-α) stimulated gene 6 (TSG-6). The production of this protein occurs after stimulation by pro-inflammatory cytokines, mainly interleukin 1 (IL-1) and TNF-α. Increased levels of TSG-6 protein have been found in patients with various diseases ranging from bacterial sepsis to autoimmune defects. After administration of TSG-6 protein alone in a model of the damaged cornea, there was a significant improvement in corneal transparency, the inhibition of neutrophil infiltration, and neovascularization (Oh et al., 2010). TSG-6 also improves wound healing by inhibiting macrophage activation. Thus, it suppresses inflammation and fibrosis at the injury site (Qi et al., 2014).

MSCs also produce growth factors, which have neuroprotective characteristics. MSCs can reduce and regenerate neuronal or retinal damage also through their paracrine action, for example, through the production of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial cell lineage neurotrophic factor (GDNF) (Meirelles et al., 2009; Park et al., 2017).

Later findings have shown another possible mechanism of therapeutic action of MSCs. Extracellular vesicles (EVs, exosomes, microvesicles) are products of various cell types, including MSCs in body fluids. They can be observed as particles with plasmatic membranes, and depending on their origins, they can be detected by flow cytometry (Lötvall et al., 2014). EVs encapsulate a lot of bioactive molecules, including cytokines, growth factors, mRNA, micro-RNA, enzymes, and other immunomodulatory molecules. These factors are secured in the vesicle against degradation. The supernatants containing EVs could be used to transport these molecules for longer distances (Jafarinia et al., 2020).

Another mechanism that includes close contact between MSCs and damaged cells is represented by mitochondrial transfer. It was shown that in the environment of cells with damaged mitochondria is a high concentration of danger-associated molecular patterns such as mitochondrial DNA and other molecules associated with energetic metabolism, which can trigger the cytoprotective transfer of mitochondria from MSCs to damaged cells (Nakahira et al., 2015). Several mechanisms of this transfer were described, such as gap junctions, tube formations, microvesicle transport, and cell fusion (Paliwal et al., 2018). Lately, it has been found that mitochondrial transfer could be responsible for an inhibition of interleukin 17 (IL-17) production by type 17 helper T cells (Th17 cells) and their differentiation into regulatory cell types (Luz-Crawford et al., 2019).

2.3.5 Immunomodulatory properties of MSCs

In the past, stem cell therapy and MSC therapy research were targeted more on their differentiation properties in the hope that they could replace original cells in damaged tissue and retake their functions. It was shown that the most therapeutic function of MSCs is their immunomodulatory ability, as shown in Figure 1. As mentioned before, MSCs can migrate directly to the injury site, and there they inhibit local inflammatory reactions by paracrine actions or by direct contact with immune cells. The mechanism of their action is not, in most cases, well known.

MSCs can inhibit the proliferation and functions of B cells, T cells, dendritic cells (DCs), and natural killer (NK) cells. Conversely, they can support the differentiation of regulatory immune cells like M2 macrophages, regulatory B cells, and T cells with a final immunosuppressive effect (Wu et al., 2020).

The production of immunomodulatory molecules or growth factors can be spontaneous or elevated after the stimulation or in hypoxic conditions (Noronha et al., 2019; Zhao et al., 2016). It was shown that typical pro-inflammatory molecules like IL-1, TNF-α, and

interferon-γ (IFN-γ) are a crucible for stimulating MSCs to express and produce most of the immunomodulatory molecules separately and in various combinations (Gao et al., 2016; Krampera et al., 2006). MSCs can also be stimulated by activation through toll-like receptors (TLR), which are expressed on RNA and protein levels. Their pro- or anti-inflammatory effect after TLR activation depends on the microenvironmental context. A high gene expression level of TLR 1, 2, 3, 4, 5, and 6 was detected by PCR. TLR 2, 3, 4, 7, and 9 were detected by flow cytometry (Dazzi and Krampera, 2011).

MSCs produce high amounts of immunomodulatory molecules such as IL-6, TGF-β, prostaglandin E2 (PGE2), and other products of cyclooxygenase 2 (COX-2), nitric oxide (NO), IL-1 receptor agonist (IL-1Ra), TSG-6, HGF, and indoleamine-2,3-dioxygenase (IDO).

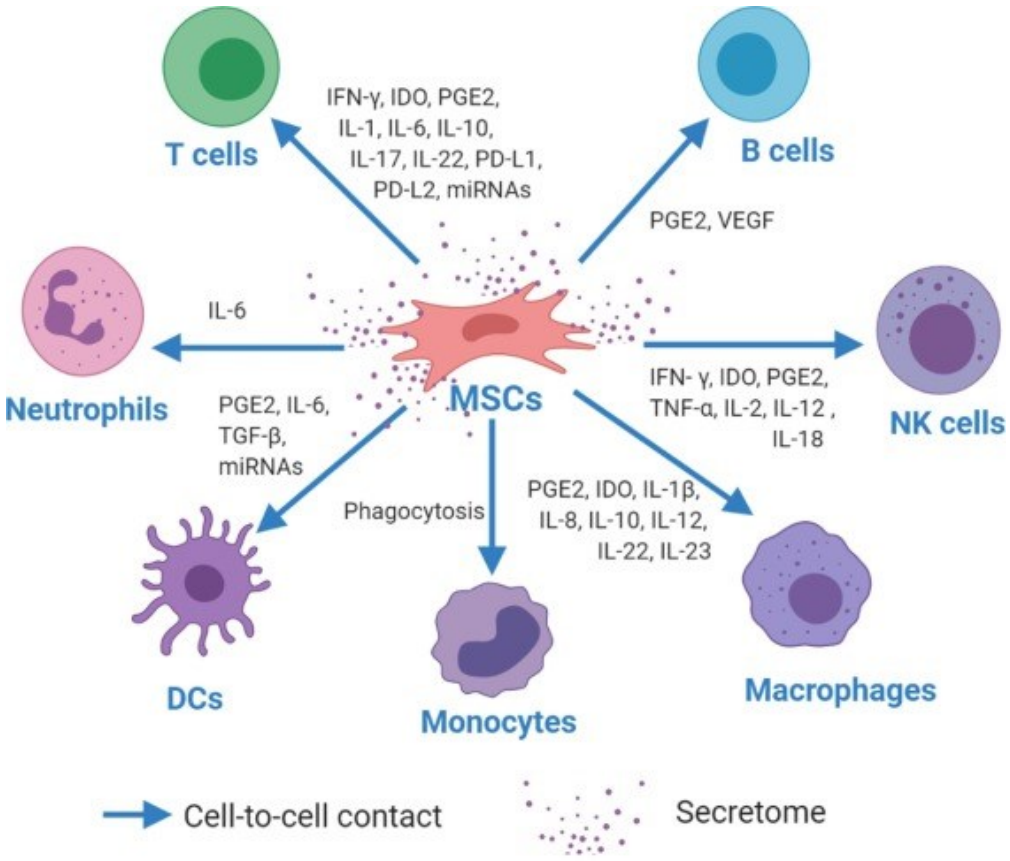

Figure 1. Immunomodulatory properties of MSCs. *MSCs modulate immune reactions via interactions with immune cells such as T cells, B cells, NK cells, macrophages, monocytes, dendritic cells (DCs), and neutrophils through paracrine secretion of molecules or extracellular vesicles (dots), or cell-cell contact (blue arrows). Most of the immunomodulatory properties of MSCs are triggered by pro-inflammatory cytokines.* From Song et al., 2018

MSCs also exert an immune tolerant type by expressing low levels of major histocompatibility complex class I (MHC class I) surface antigens. They do not express MHC class II until stimulated by an inflammatory environment (Aggarwal, 2005; Pittenger et al., 2019), as well as Fas ligand (FasL) or other co-stimulatory molecules like CD40/CD40L and CD80/86 (B7-1/7-2). It is discussed that MSCs could be used in allotransplantation without possible rejection of MSCs or the use of immunosuppressive drugs (Le Blanc and Davies, 2015).

MSCs are also able to inhibit immune cells by cell-cell contact. On their surface, they express molecules like FasL and programmed death-ligand 1 and 2 (PD-L1 and PD-L2). Significant PD-L1 expression is highly elevated after IFN-γ and TNF-α stimulation, and it suppresses the activation of $CD4^+$ T cells and downregulates IL-2 production. PD-L1 also inhibits the proliferation of Th17 cells in the culture (Davies et al., 2017; Luz-Crawford et al., 2012). FasL induces apoptotic cell death in activated T cells because of the high expression of the Fas receptor (Fas), which could be used in treating autoimmune diseases by MSCs (Akiyama et al., 2012).

2.3.5.1 Interaction of MSC with B cells

MSCs can inhibit the expression of chemokine receptors in B cells like C-X-C chemokine receptors CXCR4, CXCR5, and CCR7 which inhibits the migration abilities of B cells into secondary lymphoid organs and the ability to mature, as well as IgM and IgG production (Asari et al., 2009; Cvija et al., 2012). Another mechanism inhibiting B cell proliferation stops them in the G0/G1 phase of the cell cycle (Krampera et al., 2006). The continuous output of TGF-β by MSCs and the expression of PD-L1 and 2 after stimulation are involved in these effects on B cells. Recent studies have shown that MSCs can support the differentiation of B cells towards regulatory phenotype through their multiple modulatory mechanisms, including cell-cell contact, soluble factors, and EVs (Liu et al., 2020). Another mechanism could be the ability of MSCs to induce regulatory T cells (Tregs) and the production of IL-10, which can cause differentiation of regulatory B cells by a positive feedback loop (Ma and Chan, 2016).

2.3.5.2 Interaction of MSCs with T cells

As well as in the case of MSCs interacting with B cells, MSCs can inhibit the activation and proliferation of T cells by stopping them in the G0/G1 phase of the cell cycle (Glennie, 2005). The effect of MSCs may be driven by direct cell-cell contact and the expression of surface and soluble molecules like PD-L1, PD-L2, and FasL (Davies et al., 2017), NO

produced by inducible NO synthase (iNOS) (Ren et al., 2008), IDO (Krampera et al., 2006), PGE2 as the product of COX-2 (Bouffi et al., 2010), and HGF with TGF-β (Li et al., 2014; Yanai et al., 2006). MSCs regulate the balance between type 1 and type 2 helper T cells (Th1 and Th2 cells) immune response. MSCs suppress the production of IFN-γ by Th1 cells and induce the production of IL-4 and Il-10 by Th2 cells, leading to regulatory phenotypes (Aggarwal, 2005; Ma and Chan, 2016). MSCs also produce TGF-β and IL-6. This combination induces the differentiation of naïve T cells into Tregs or Th17 cells. Production of TGF-β leads to preferably into Tregs phenotype, in combination with IL-6 naïve T cells preferentially develops in Th17 cells (Alawad et al., 2015; Svobodova et al., 2012). Moreover, MSCs support differentiation in Tregs regulatory phenotype by expressing PGE2 (Hsu et al., 2013), IDO (Ge et al., 2010), or production of HGF (Chen et al., 2020).

2.3.5.3 Interaction of MSCs with macrophages

In general, two types of macrophages are recognized: type M1, which produces pro-inflammatory cytokines IL-1, IL-6, IL-12, TNF-α, and NO; and anti-inflammatory M2, with the production of IL-4 and IL-10 (Ley, 2017). MSCs can change macrophage phenotype to M2 type, which is associated with the expression of CD206. MSCs can modulate this process by direct cell contact, by the production of IL-6 or PGE2, which inhibits the production of TNF-α, IL-6, and NO by activated macrophages. These induced M2 macrophages produce more IL-4 and IL-10, and their phagocytic ability of apoptotic cells is enhanced, which helps in the regeneration of damaged tissues (Kim and Hematti, 2009; Maggini et al., 2010).

2.3.5.4 Interaction of MSCs with DCs

The inhibition of maturation of monocytes into DCs is one of the mechanisms of how MSCs are suppressing T cell reactions. MSCs inhibit the expression of costimulatory molecules and CCR7 on the surface of DCs, stopping them from migrating to secondary lymphoid organs and presenting antigens to T cells (Aggarwal, 2005; Chiesa et al., 2011). The effect is provided by direct contact or by the production of soluble factors such as PGE2, which plays a role in the inhibition of the maturation of monocytes into DCs (Spaggiari et al., 2009).

2.3.5.5 Interaction of MSCs with NK cells

It was shown that MSCs could inhibit NK cell proliferation by lowering IFN-γ concentration in their environment and inhibiting their cytotoxicity against target cells. Also,

MSCs can escape the NK cell cytotoxic action by suppressing the production of IL-2 and IL-15 by T cells, which are needed to activate NK cells. The inhibition effect is mainly caused by MSC expression of IDO, PGE2, and TGF-β (Spaggiari et al., 2008; Le Blanc and Davies, 2015).

2.3.5.6 Interaction of MSCs with neutrophils

Neutrophils are one of the first respondents at the beginning of the inflammation. They are critical effector cells in innate immunity and play a vital role in phagocytosis and microbial killing. MSCs prevent tissue damage by suppressing hydrogen peroxide production in activated neutrophils *in vitro* by upregulation of the expression of superoxide dismutase 3. It was also shown that MSCs could phagocyte neutrophils (Jiang, Muschhammer, et al., 2016a). It was also demonstrated that MSCs could prevent apoptosis in resting and IL-8-activated neutrophils and inhibit oxidative burst. The mechanism is not cell-cell contact-dependent, as the effect was preserved in transwell experiments. It was shown that this effect is mediated mainly by IL-6 produced by MSCs. However, the migration ability of neutrophils is unchanged (Mittal et al., 2018). On the contrary, it was shown that the effect of MSCs on neutrophils in the injured cornea is mainly due to cell-cell contact. MSCs prevented the expression of tissue-damaging enzymes myeloperoxidase and N-elastase by neutrophils (Raffaghello et al., 2008).

2.3.6 Anti-apoptotic properties of MSCs

Anti-apoptotic effect of MSCs on other cells is well known from many reports and may be one of the essential parts of MSC actions in regenerative therapy. Still, the exact mechanism remains unclear. The differentiation of MSCs can prevent apoptosis and cell death in the target tissue cell type. MSCs produce many trophic and growth factors that actively interact with target cells, helping them with survival and proliferation. According to reports, HGF plays one of the leading roles in the anti-apoptotic mechanisms of MSCs. It prevents epithelial cells from apoptosis, and it stops fibrotic processes (Cahill et al., 2016). In combination with IGF-I, it prevents apoptosis induced by hypoxia in the model of myocardial infarction (Zhang et al., 2015). In experimental diabetic retinopathy, HGF can prevent apoptosis of pericytes and retinal ganglions (Tönges et al., 2011; Yun, 2021). HGF also inhibits apoptosis in corneal epithelium cells (Miyagi et al., 2018a).

Later it was shown that the anti-apoptotic properties of MSCs could depend on microvesicle secretion and transportation of micro-RNA (miRNA), growth factors, and cytokines directly to target cells. This exosomal secretion of miRNA prevented apoptosis in

myocardial infarction, or MSC-derived exosomes prevented ischemia-induced apoptosis in the retina (Mathew et al., 2019; Zhu et al., 2018). Also, mitochondrial transfer from MSCs reduces inflammation and inhibits apoptosis. It was shown that transfer of mitochondria via tunneling nanotubes rescued corneal epithelium cells from oxidative stress and apoptosis (Jiang et al., 2016; Paliwal et al., 2018).

2.3.7 Administration of MSCs and the possibility of using MSC transplantation for therapy

MSCs, used in clinical or experimental applications, can be administered systemically by intravenous (i.v.) or intraarterial (i.a.) injection or locally by intravitreal and intraarticular injections (McIntyre et al., 2017; Satarian et al., 2017). For example, cells are migrating, preferably in the injured cornea after i.v. administration to the tail in a mouse model (Javorkova et al., 2014). In the case of surface injury, MSCs can be transplanted directly into the wound using different scaffolds to inhibit inflammation and support regeneration of chemically burned rabbit cornea (Holan et al., 2015). However, several problems occur when systemic administration is used. After i.v. injection MSCs are trapped in the lungs. After 24 hours, MSCs move to other organs like the kidneys, spleen, and liver. Although i.a. injection should resolve the problem, there is another risk of forming thrombosis in a microvasculature by injected MSCs (De Becker and Riet, 2016).

The migration mechanisms of injected MSCs are the same mechanisms present in endogenous stem cells. But *in vitro* cultivation of MSCs affects their phenotype. Small round-shaped cells change into approximately 20 μm large spindle-shaped cells, which can be easily trapped in the lungs or other organs. Furthermore, cultivated MSCs can lose homing molecules from their surface, such as CXCR4, during *in vitro* expansion (Honczarenko et al., 2006). Pre-treatment of MSCs by pro-inflammatory cytokines like IL-1, IFN-γ, and IL-6 or growth factors like IGF-I and HGF during *in vitro* cultivation can renew the expression of CXCR4 (De Becker and Riet, 2016). Endogenous MSCs can also be mobilized under hypoxic conditions, and such cells express more CXCR4 upregulated by hypoxia-inducible factor 1 alpha (HIF-1α) (Ciria et al., 2017). However, hypoxic *in vitro* conditions can cause unpreferred changes that lead to increased adipogenic and osteogenic differentiation or even cancerous changes in later passages (Ejtehadifar et al., 2015). CCR7 gene modification of MSCs can improve their immunomodulatory effect when used as therapy for graft versus host disease (GvHD) (Li et al., 2014). MSCs seeded on a suitable scaffold and transplanted on the injured surface migrate to the site of inflammation.

Therefore, such transplanted MSCs are not lost during migration through the body and should not be affected by a loss of homing molecules after *in vitro* cultivation.

To this day, 340 clinical trials of MSCs used in therapy have been finished, and approximately the same number of studies are in various phases and stages of progress. The most frequently studied are musculoskeletal injuries, including different arthritic conditions, respiratory tract diseases including COVID-19, GvHD, immune system dysfunction, digestive tract diseases, neurodegenerative diseases, and retinal and corneal disorders and injuries (clinicaltrials.gov).

2.4 Retina

The retina is a light-sensitive layer in the eye and one of the most critical tissues for normal and healthy vision. As shown in Figure 2, the retina consists of ten different layers. The inner limiting membrane, separating vitreous from other retinal layers, is a basal membrane formed by Müller cells, nerve fiber layer formed by axons of ganglion cell nuclei (multipolar neurons), ganglion cell layer, an inner plexiform layer consisting of synapses between ganglion cells and bipolar neurons, internal nuclei layer of bipolar cells, amacrine cells and horizontal cells, an outer plexiform layer which is a place of synapses between photoreceptors (rods and cones) and bipolar cells, outer nuclei layer consisting of cell bodies of rods and cones, an outer limiting membrane separates cell nucleus of photosensitive cells and their inner segments, the layer of rod cells and cone cells and retinal pigment epithelium (RPE). Any damage in only one of these layers leads to decreased retinal function and causes impaired vision or even blindness. The retinal macula is a disc-shaped pigmented area in the center of the retinal surface. It is responsible for high acuity vision thanks to the high density of cones (Alonso-Alonso, 2015).

2.4.1 Retinal degeneration and available therapy

Several mechanisms cause retinal degeneration, which induces the loss of cells in the central retina. In addition, damage to the single one of the retinal layers causes reduced quality or total loss of vision due to the connection and chemical communication between the separate layers. The mechanisms of degenerations and injuries of the retina could be mechanical, chemical, light, hereditary, ischemic, and age-related.

Retinal detachment can be caused by vitreous fluid leaking behind the retina through tears after the injury of the eye, by retinal inflammation and secretion of fluid behind the retina, or by the traction of fibrous or fibrovascular tissue formed after the injury or inflammation.

This traction pulls inner retinal layers from the RPE. The current treatment consists of photocoagulation, cryotherapy, or vitrectomy. These therapies stop further retinal degeneration but will not provide retinal reconstruction and improvement of vision quality. The retina can also be damaged by light. For example, direct UV irradiation causes photoreceptors' damage and apoptosis, similar to age-related macular degeneration (AMD) (Zhang and Wang, 2010).

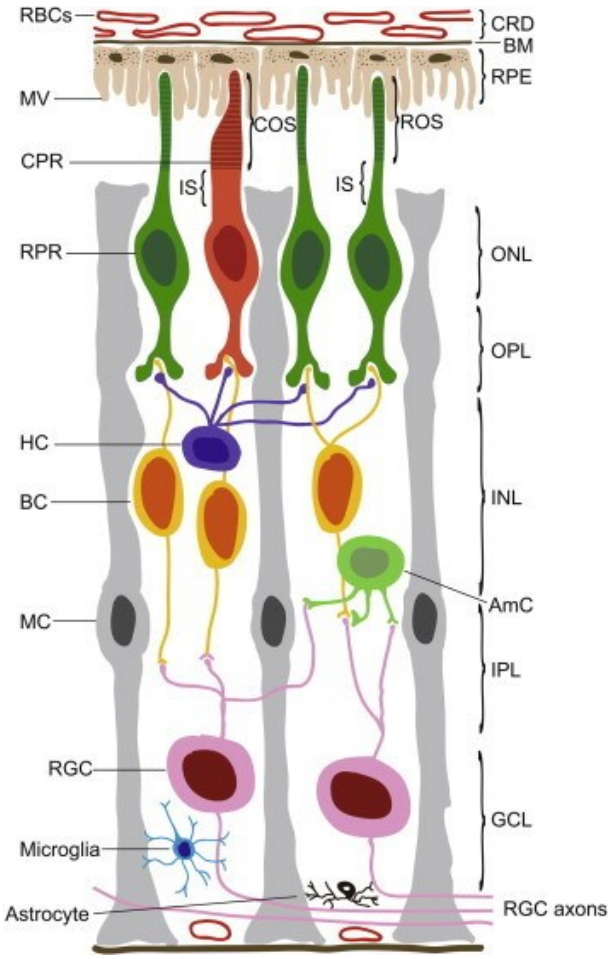

Figure 2. Layers of the retina. *The choroid layer (CRD) is located at the back of the eye bulb containing red blood cells (RBCs), and it is separated from the RPE layer by Bruch's membrane (BM). RPE interacts with cone and rod photoreceptors (CPR/RPR) by microvilli (MV)***.** *CPR and RPR consist of cone and rod outer segments (COS/ROS) and inner segments (IS). The outer nuclear layer (ONL) and outer plexiform layer (OPL) contain synapsis of PR, horizontal cells (HC), and bipolar cells (BP). The inner nuclear layer (INL) contains the bodies of HC, BP, and amacrine cells (AmC). The inner plexiform layer (IPL) is formed of the synapsis between BC, AmC, and retinal ganglion cells (RGC) located in the ganglion cell layer (GCL).* From Athanasiou et al., 2013

AMD occurs in patients above 50 years of age, affecting photoreceptors and RPE while inner structures of the retina remain intact. This condition is ideal for photoreceptor replacement therapy. AMD causes macular degeneration and leads to significant loss of vision. AMD occurs in two forms (compared in Figure 3); the dry form of AMD is a condition in which the retina accumulates amorphous deposits that leads to RPE degeneration. The wet form is rare and severe because of rapid neovascularization in the

sub-pigmental epithelium and sub-retinal space. New blood vessels are leaky, which leads to edema. While the loss of rods in the dry form of AMD is diffuse and the ganglion layer is more or less intact, in the wet state, the loss of photoreceptors is much more severe, and nearly 47 % of ganglion cells are lost in the terminal phase of the disease (Garcia, 2015; Medeiros and Curcio, 2001). The current treatment for the wet form of AMD is photodynamic therapy, surgical excisions, and anti-VEGF therapy. There is currently no treatment for the dry form (Rubner et al., 2022).

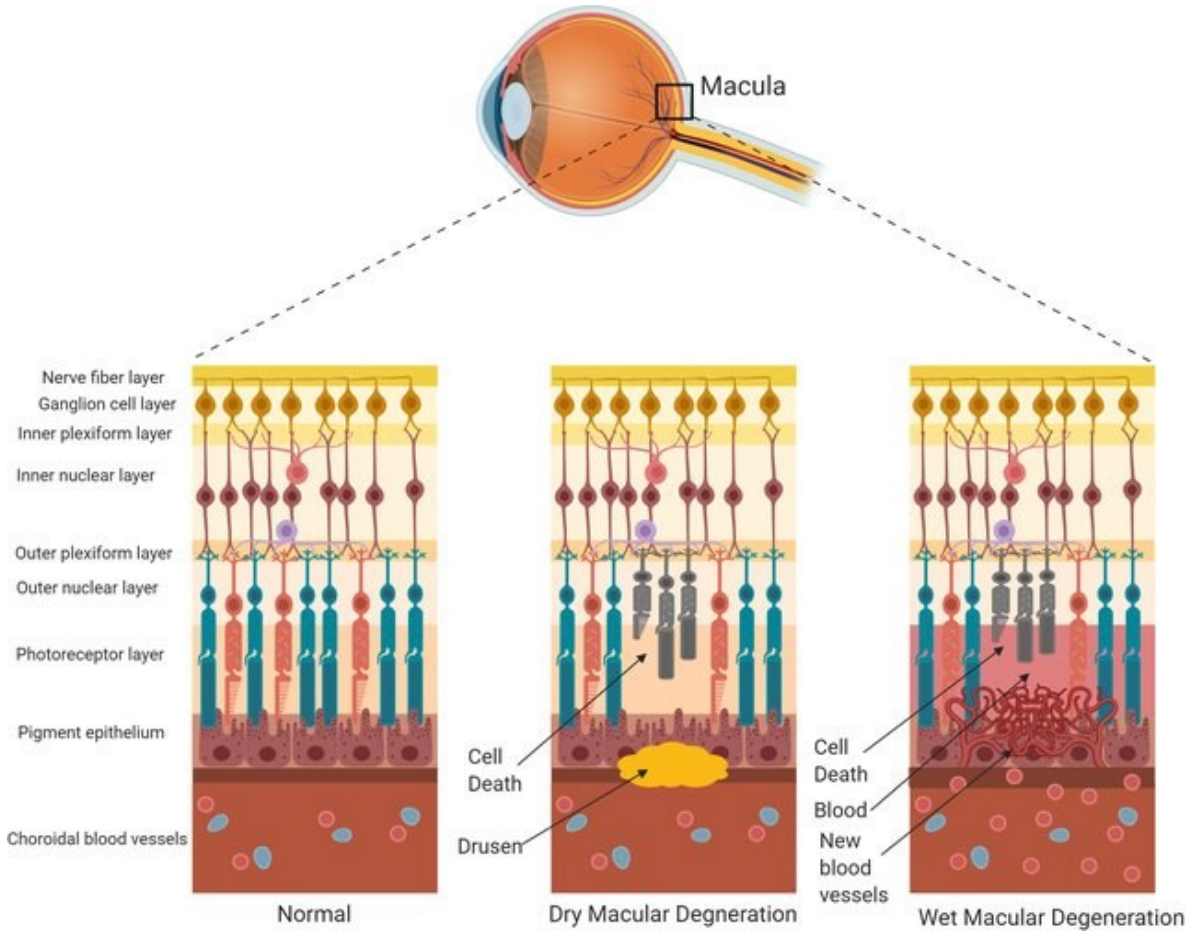

Figure 3. **Schematic diagram of dry and wet macular degeneration.** *In dry AMD, the structure of the retina at the macula is damaged because of the accumulation of deposits in the retina (drusen). RPE cells, photoreceptors, and other retinal cells gradually die. The wet form of AMD is caused by rapid neovascularization, leakage, and edema with similar or more severe effects.* From Nayyar et al., 2020

Glaucoma is another age-related disease that causes progressive degeneration of retinal ganglion cells. The mechanism is possibly related to increased eye pressure. Current therapy focuses on lowering eye pressure and stimulating ganglion cell metabolism by intravitreal injections of neurotrophic factors such as BDNF, CNTF, or GDNF. Unfortunately, these factors must be injected repeatedly to achieve therapeutic effects (Athanasiou et al., 2013; Johnson et al., 2011).

Retinitis pigmentosa is a hereditary disease that causes total blindness due to rods, cones, and RPE degeneration. Stargardt disease is another congenital retinal disease that occurs mainly in childhood. It´s caused by a protein transport defect that leads to the accumulation of metabolic products in photoreceptor cells and RPE, characterized by loss of central vision (Garcia, 2015). No treatment is available for hereditary retinal diseases; only supplements like vitamin A are used to slow the degeneration.

Diabetic retinopathy is an ischemic retinal disease that induces vasodegeneration, hypoxia, or ischemia and releases VEGF. This causes neovascularization and consequently blood leakage into the vitreous and induces retinal edema due to degeneration of overgrown blood vessels. These symptoms with fibrovascular proliferation can cause retinal detachment and loss of retinal endothelium and pericytes, which lead to impaired vision or blindness. Current clinical treatment involves the intravitreal application of corticosteroids or anti-VEGF antibodies and laser photocoagulation which can slow down further degeneration. However, this treatment is invasive and has various side effects (Bavinger et al., 2016; Bull and Martin, 2011; Stitt et al., 2011).

2.4.2 MSC treatment of retinal degeneration

The ability of MSCs to suppress inflammatory reactions and differentiate into adipose, bone, and cartilage tissue cells is well known. Moreover, they can differentiate into cells expressing retinal cell markers. For example, MSCs isolated from rat conjunctiva differentiate into cells expressing typical markers for photoreceptors and bipolar cells after cultivation on a nanofiber scaffold with the addition of taurine (Nadri et al., 2013). Alternatively, they differentiated into cells expressing photoreceptor markers after *in vitro* cultivation with activin A, EGF, and taurine (Kicic et al., 2003). Human bone marrow-derived MSCs (BM-MSCs) were also differentiated to RPE after co-cultivation of BM-MSCs with isolated human RPE. Differentiated cells expressed the typical RPE marker RPE65 (Mathivanan et al., 2015). Similarly, rat MSCs were differentiated to RPE marker

expressing cells under the retinal environment simulation using an RPE cell-conditioned medium and co-cultivation with outer segments of photoreceptors (Huang et al., 2012).

Subretinal injection of rat BM-MSCs was used in an experimental rat model of chemically induced retinitis pigmentosa. These stem cells migrated into the outer layers of the retina and expressed typical markers of photoreceptors and RPE, but the functionality remained unknown (Huo et al., 2010). The injection of MSCs prolonged the survival of photoreceptor cells in the murine rhodopsin knock-out model. In the rat model of diabetic retinopathy, there were i.v. injected human adipose-derived MSCs (AD-MSCs). One week after the transplantation, the blood glucose level was significantly lower, and the damaged blood-retinal barrier was improved. MSCs migrated to the retina and expressed specific photoreceptor and glial markers (Yang et al., 2010). In another study, human AD-MSCs were injected into the vitreous in a diabetic rat model. Retinal histopathological evaluation revealed decreased blood leakage and significantly longer cell survival around retinal vessels (Rajashekhar et al., 2014). The intravitreal application of MSCs in the rat eyes also inhibited the production of IL-1β, TNF-α, and IL-6 in an ischemic retinal injury model (Mathew et al., 2017).

In a rat glaucoma model, it was found that MSCs do not differentiate into ganglion cells after intravitreal administration but promote their survival, mainly by producing several trophic factors such as BDNF, GDNF, CNTF, HGF, bFGF (Yu et al., 2006). In another glaucoma rat model, intravitreally administered MSCs produced missing neurotrophic factors, and the survival of retinal ganglion cell axons was significantly higher. On the other side, i.v. injection of MSCs did not have any therapeutic effect (Johnson et al., 2010). A rat glaucoma model also compared the regenerative impact of MSCs isolated from dental marrow, AD, and BM. In eyes treated with BM-MSCs and MSCs isolated from dental pulp, increased survival of ganglion cells and restoration of retinal function based on PDGF and NGF production were found (Mead et al., 2016). Experimental AMD was induced by sodium iodate in a rat model. MSCs were injected into the subretinal space; they replaced PBE and stabilized other retinal layers.

Furthermore, such effects were enhanced by erythropoietin gene modification of used MSCs (Guan et al., 2013). In other studies, only a conditioned medium was injected into the rat eye to treat damaged retina in normal conditions or a hypoxic environment (Dreixler et al., 2014; Roth et al., 2016). Both studies showed a significant improvement in functions in ischemic damaged retina and inhibition of apoptosis.

The application of MSC-derived exosomes provides another therapeutic approach to treating retinal disorders. For example, it has been shown that the administration of the exosomes secreted by BM-MSCs promoted the survival of retinal ganglion cells and improved the retinal structure in the eye of rats after optic nerve crush injury through the mechanism of miRNA transfer (Mead and Tomarev, 2017). In addition, exosomes from umbilical cord-derived MSCs had beneficial effects on blue light-induced retinal laser injury (He et al., 2018). A mitochondrial transfer could also be a therapeutical mechanism of MSCs in treating retinal damage, as many retinal disorders are associated with mitochondria dysfunction in the neural retina (Eells, 2019).

2.4.2.1 Clinical trials of MSC therapy in retinal degeneration

One clinical trial in phase three of MSCs isolated from Wharton's jelly has been finished to treat retinitis pigmentosa with good safety results and promising outcomes and improvement of the sight (Özmert and Arslan, 2020). Patients evaluated their condition of vision better than before the application of stem cells in the third month after the intravitreal injection of autologous BM-MSCs. Unfortunately, this improvement was lost twelve months after the BM-MSCs application (Siqueira et al., 2015). Further studies are conducted to test the safety of different approaches to injecting MSCs into the eye, with positive (Gu et al., 2018) and adverse outcomes (Mangunsong et al., 2019). Patients with the condition of refractory macular holes were treated with MSCs and MSC-derived exosomes with promising results of functional and tissue recovery.

Moreover, MSC-derived exosomes may help improve visual outcomes after macular hole surgery (Zhang et al., 2018). There are six more clinical studies in various stages of progress. Only one is currently active – The treatment of macular holes by MSC-derived exosomes; the others are in recruiting state (clinicaltrials.org).

2.5 Cornea

The cornea is avascular transparent tissue forming 20 % of the anterior surface of ocular bulb and it is an essential component of the visual apparatus. Its structure is vital for standard light transmission to the retina and healthy vision. It comprises several layers of stratified corneal epithelium, with Bowman's membrane underneath, made up of collagen fibers. The membrane is followed by the thickest layer of the cornea - the stroma. Its unique 3D structure of transverse-oriented collagen fibers creates a perfectly transparent environment. Between

the collagen fibers of the stroma are keratocytes. Behind the corneal stroma is the collagen-formed Descemet's membrane, which is the basal membrane of the corneal endothelium. It is composed of a single-layered flattened epithelium with the presence of many mitochondria and high proteosynthesis. The corneal limbus is located at the periphery. Unlike the cornea, the limbus is highly vasculated, creating a suitable environment for LSCs regenerating the cornea. The basic corneal stratification and the location of limbal area is shown in Figure 4.

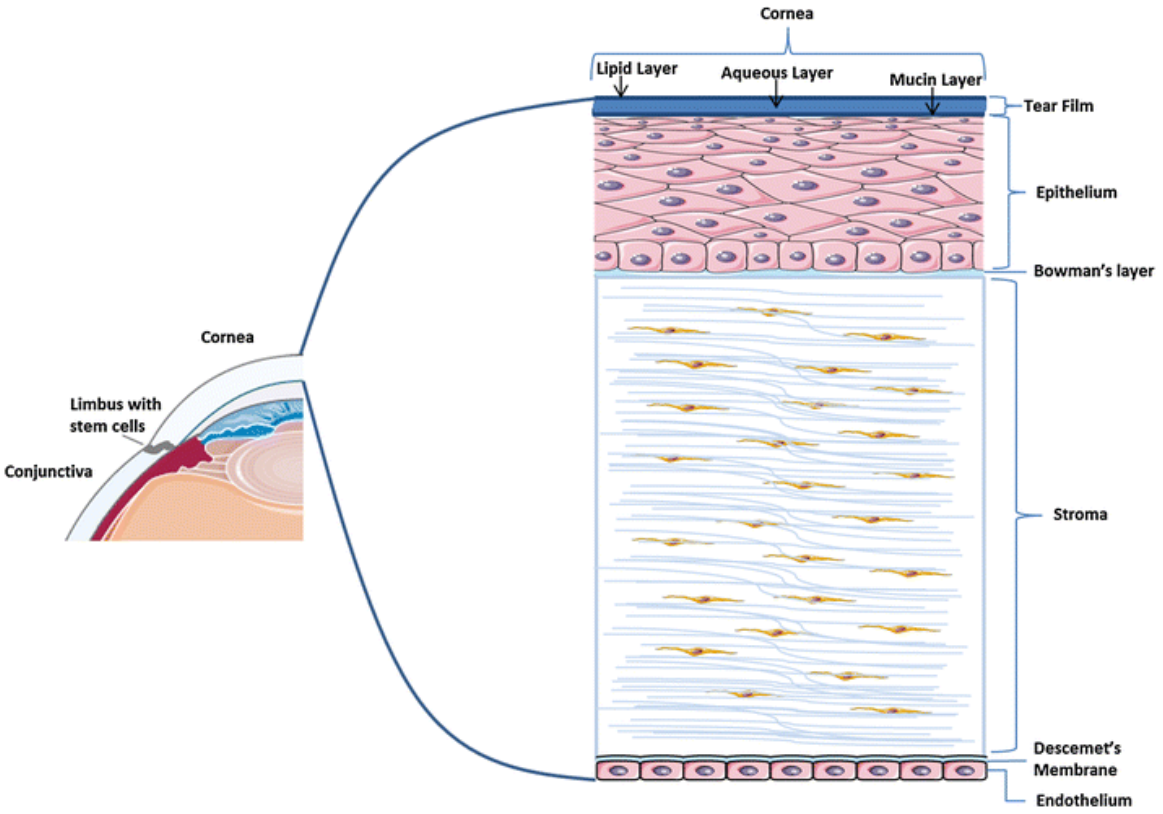

Figure 4: **Structure of the cornea.** *Schematic of layers of the cornea. The transition between conjunctiva and cornea on the left with the location of the limbus.* From Rowsey and Karamichos, 2017

2.5.1 Immune privilege of the cornea

The cornea is well known for its immune privilege, which is the reason for its high success rate of transplantation. Three significant mechanisms provide the immune privilege of the cornea – molecular and cellular barriers; tolerance established by anterior chamber anterior chamber-associated immune deviation (ACAID), preferred development of Tregs, and overall immunosuppressive intraocular environment. There is no vascular or lymphatic drainage, which inhibits the transportation of antigens and antigen-presenting cells to secondary lymphoid organs and the start of immune response ending with possible migration

of immune cells in the cornea. One of the mechanisms to prevent neoplasia of blood and lymphatic vessels is the collagen composition of the cornea itself. The presence of angiostatic molecules such as endostatin, TSP, and soluble vascular endothelium growth factor 1 receptor (VEGFR 1) and 3 in the cornea prevents the neovascularization of healthy cornea. Cells in all layers of the cornea express only a minimum of MHC class II molecules (Cursiefen et al., 2011; Gabison et al., 2004).

There is also a continuous production of PD-L1, IL-1R, FasL, and TNF-related apoptosis-inducing ligand (TRAIL) directly by corneal cells or by their presence in the anterior chamber of the eye. Moreover, TGF-β (Tregs differentiation, immunosuppression), TSP-1 (activation of inactive TGF-β), alpha-melanocyte-stimulating hormone (α-MSH; suppresses inflammation and induces regulatory and tolerogenic immunity), and vasoactive intestinal peptide (VIP; reduces pro-inflammatory cytokine production, suppresses cell-mediated immunity, and inhibits macrophage and T cell proliferation) are present in the aqueous humor and are responsible for ACAID as shown in Figure 5 (Hori et al., 2006; Taylor and Ng, 2018; Hori et al., 2019).

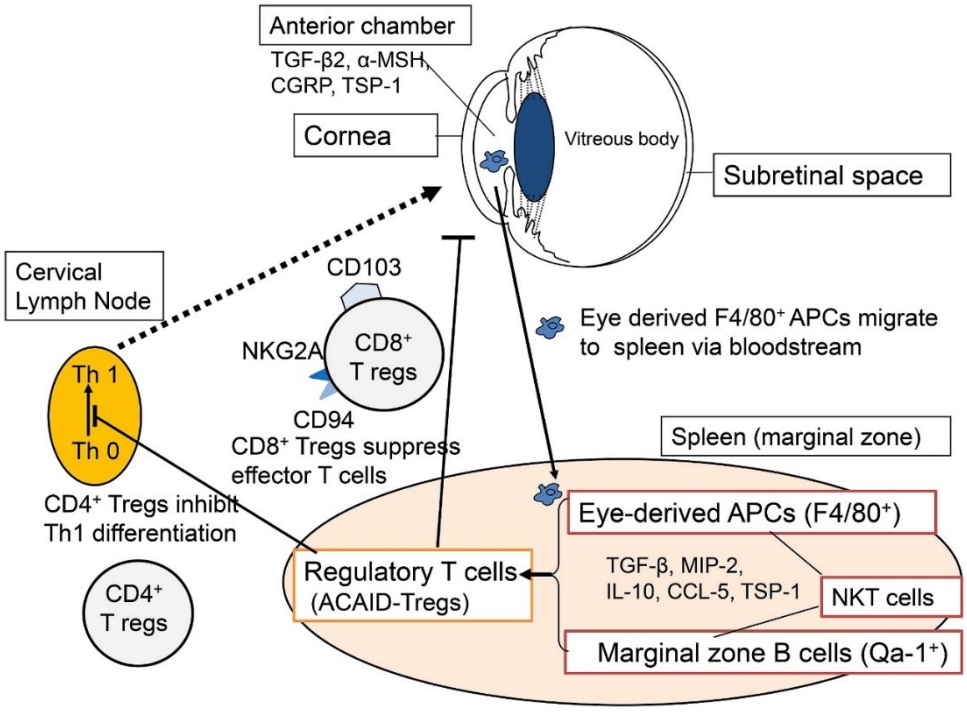

Figure 5. Induction of ACAID. *Eye-derived F4/80+ antigen-presenting cells (APCs) migrate from the anterior chamber (containing TGF-β, α-MSH, TSP-1, etc.) via the bloodstream into the marginal zone of the spleen, producing TGF- β, MIP-2, and TSP-1. APCs attract NKT cells and contact them cell-cell via CD1d. NKT cells produce IL-10, TSP-1, and CCL5, which attract Qa-1⁺ B cells. These three cell types form clusters creating a strong anti-inflammatory, pro-regulatory environment and*

inducing differentiation of two Tregs types. CD4+ ACAID Tregs locally inhibit differentiation of Th1 cells in lymph nodes, and CD8+ ACAID Tregs inhibit the function of effector Th1 and Th2 cells in the eye. (Hori et al., 2019)

2.5.2 Resident immune cells of the cornea

Despite the immune privilege of the cornea, there are present various types of immune cells. Until 20 years ago, the cornea was considered bone marrow-derived antigen-presenting cells (APCs) free tissue. In 2002 and 2003, two reports showed a network of $CD11b^+$ macrophages in the periphery of stroma and $CD11c^+$ DC in the epithelium of healthy murine corneas (Brissette-Storkus et al., 2002; Hamrah et al., 2003). In 2020 there was a report on residential plasmacytoid DC in the cornea, which play a role in barrier functions (Jamali et al., 2020). It was shown that two subtypes of macrophages are present in the cornea. $CCR2^+$ macrophages express genes as M1-subtype (IL-1, TNF-α). They are continuously repopulated by monocytes and play a role in the early stages of corneal inflammation after the injury. CCR^- macrophages express M2-subtype genes like IL-10, similar to macrophages originating from the yolk sac. This type of macrophages has self-proliferative capacity in the cornea, and monocytes replace them only after extensive injury (Liu et al., 2017).

Langerhans cells (LCs) are one of the well-known APCs in the cornea. LCs are MHC class II expressing cells located mainly in the healthy peripheral cornea (Machetta et al., 2014). Previously described as DCs, but lately, it was shown that LCs are subtypes of specialized macrophages. However, unlike macrophages in other tissues, LCs continue to migrate to the lymph nodes under physiologic conditions to perform antigen presentation (Doebel et al., 2017). LCs originate from primitive macrophage progenitors developing in the yolk sac and fetal liver (Hoeffel et al., 2012). Their primary function seems to be migration to the secondary lymphoid organs and presenting antigen to naïve T cells to induce tolerance as they play an immunosuppressive role in various immunologic disease models (Price et al., 2015). The innate immune cells are represented mainly by neutrophils undergoing diapedesis from the limbal region to the peripheral cornea. Their role in the cornea is primarily in phagocytosis, epithelium recovery, and regeneration of sensory neurons (Liu et al., 2012). Excessive neutrophile infiltration occurs in corneal injuries like chemical burns (Eslani et al., 2014).

Recently, it was shown that the recruitment of $CD8^+$ T cells after viral infection of the cornea could lead to the development of residential memory T cells, which start to patrol in

ocular surface and respond rapidly in the case of another infection without further damage to the corneal tissue (Loi et al., 2022).

2.5.3 Corneal injury

A total of 286 million patients worldwide suffer from impaired vision, 39 million are considered blind, and 25 % of such cases are caused by ocular surface injury (Pascolini and Mariotti, 2012). The ocular surface can be damaged mechanically, chemically, or indirectly because of infection, genetic disorder, or autoimmune disease. The cornea has the self-renewal ability. LSCs continuously migrate from the limbal area to the center of the cornea differentiating into corneal epithelium cells. It has been shown that after the corneal injury, LSC migration and differentiation are enhanced by the production of cytokines and growth factors like EGF, FGF, HGF, IGF-I, and TGF-β (Li and Tseng, 1996; Imanishi et al., 2000; Trosan et al., 2012).

In the case of extensive injury, destruction of the limbus, and therefore LSCs, regeneration of the corneal epithelium is abrupted**. Limbal stem cell deficiency (LSCD)** is associated with the break of immune privilege of the cornea, a harmful local inflammatory reaction, and a loss of corneal epithelium cells caused by damage after the injury and by apoptosis in a later phase of local inflammation (Ordonez and Di Girolamo, 2012). Corneal epithelium starts producing pro-inflammatory cytokines, including IL-1α, IL-1β, and TNF-α, immediately after the injury (Ambrósio et al., 2009; Horwitz et al., 2018). IL-1 and TNF-α are responsible for further development of apoptosis in the epithelium and deeper layers of the cornea, causing the death of keratocytes in the stroma. The remaining cells are differentiating to fibroblast migrating to the wound. In persistent chronic inflammation, fibroblasts are determined to be myofibroblasts, and scars with corneal opacities are formed (Wilson et al., 1999; Bernstein et al., 2007).

When the corneal immune privilege is lost due to chronic inflammation, the conjunctival epithelium migrates over the damaged cornea and creates an atypical environment. Neovascularization occurs, and the immune privilege of the cornea is lost. Chronic inflammatory reaction results in stromal tissue scarring and increased opacity of the ocular surface leading to blindness (Dua et al., 2009). Corneal transparency gradually decreases, and this process leads to visual impairment or even blindness. Chronic inflammation is also associated with endoplasmic reticulum (ER) stress (Woodward et al., 2020a).

2.5.4 Corneal transplantation

Corneal transplantation is the most successful transplantation of a solid tissue because of its immune privilege. However, in the case of LSCD, transplantation alone is not successful. A healthy limbus with sufficient LSCs is needed for further corneal regeneration. Transplantation of the limbal (or keratolimbal) tissue or cultured LSCs from a patient's or related donor's healthy eye represents the only treatment of unilateral LSCD. Clinical studies have shown the therapeutic efficacy of refined LSC transplantation onto the ocular surface. The use of LSCs is the only approved stem cell treatment for corneal injury in the European Union (Rama et al., 2010; Pellegrini et al., 2014).

In the case of bilateral LSCD, it is necessary to use the allogeneic limbal tissue or LSCs. The limbus is highly vascularized tissue, and such allogeneic transplantation requires an administration of high doses of immunosuppressive medication with various side effects (Serna-Ojeda et al., 2020). Transplantation of autologous LSCs has better outcomes than allogeneic LSC transplantation (Pauklin et al., 2010). Although it is possible to obtain larger grafts of the limbal tissue from cadaver donors for allogeneic transplantation, using limbal grafts from healthy donors has better results (Titiyal et al., 2015). However, such treatment is associated with a risk of damaging the healthy eye (Chen and Tseng, 1991). In a murine experimental model of allogeneic limbal transplantation, healing occurred with Th1 lymphocytes playing an important role. The expression of genes for iNOS and Th1 cytokines, such as IL-2 and IFN-γ, and minimal expression of genes for Th2 cytokines (IL-4, IL-10) were detected in the healed tissue (Lenčová et al., 2011). Previously, it was shown in an experimental rabbit model that excision in a healthy eye could lead to irreversible changes in the regeneration of the donor cornea and partial invasion of the conjunctival epithelium (Chen and Tseng, 1991).

2.5.5 The use of MSCs in the treatment of the corneal injury

MSCs represent a suitable autologous cell substitution for the cell therapy of the damaged surface of the eye due to their immunomodulatory properties. They can differentiate into many cell types, migrate to the injury site, and inhibit inflammation or apoptosis (Khubutiya et al., 2014; Ren et al., 2008).

In the murine experimental model of corneal injury increased amount of circulating endogenous MSCs was observed after 48 hours. It was accompanied by increased levels of previously described chemoattractant substance P and SDF-1α in the injured cornea and

peripheral blood (Lan et al., 2012). MSCs can differentiate into corneal epithelium both *in vitro* and *in vivo*. After culturing rabbit MSCs with LSCs, in the medium after limbal epithelium culture or after transplantation onto the damaged cornea, these cells expressed the corneal cytokeratin K3 marker (Gu et al., 2009; Rohaina et al., 2014). Mouse MSCs cocultured with corneal stromal cells expressed cytokeratin K12. After transplantation onto the injured eye surface, corneal transparency was renewed, and neovascularization receded (Jiang et al. 2010). Mouse BM-MSCs survived in the corneal stroma without obvious pathology and acquired a keratocyte phenotype in keratocan knock-out mice. MSCs were not detected four weeks after transplantation (Liu et al., 2012).

MSCs need to be transplanted on the cornea using a scaffold; otherwise, they would be washed away by continuous tear production and blinking. After that, MSCs can migrate from the scaffold onto the injured ocular surface. Multiple materials have been tested as scaffolds, like an amniotic membrane (AM), contact lens, synthetic hydrogels, or three-dimensional electrospunned nanofiber scaffolds. Another strategy would be the injection of MSCs in the surrounding tissues, like in the supraorbital space, into the corneal stroma, subconjunctivally or i.v. (Beeken et al., 2021). Subconjunctival injection of human MSCs inhibited inflammatory reaction and prevented rejection in an animal model of GvHD (Martíne-Carrasco et al., 2019).

In 2006, human MSCs were applied to the damaged surface of the rat eye using an AM. Corneal regeneration was observed, but this was demonstrated by inhibition of inflammation and angiogenesis rather than trans-differentiation of MSCs into the corneal epithelium (Ma et al., 2006). Similar changes were observed after the same application of rat MSCs cultured with corneal stromal cells (Jiang et al., 2010). Syngeneic MSCs were transplanted onto the damaged surface of the mouse eye using a nanofiber carrier (Zajicova et al., 2010). They were also injected under the previously transplanted AM in the rabbit eye (Reinshagen et al., 2011). Finally, MSCs from human bone marrow were cultured in the limbus-conditioned medium for ten days and transferred to the injured cornea with LSCD (Rohaina et al., 2014). Corneal regeneration, inflammation attenuation, and neovascularization reduction were observed in all these experiments. While treating ocular surface damage in the rabbit, these therapeutic effects of MSCs were comparable to LSCs (Holan et al., 2015).

Another route of administration of MSCs is represented by systemic i.v. injection. After i.v. administration MSCs can migrate to the site of damage in the eye, inhibit the infiltration of immune cells and suppress the production of IL-1 and IL-6 (Javorkova et al., 2014) or act

via the production of molecule TSG-6 and inhibit the infiltration of neutrophils and other immune cells in the transplanted cornea, reduce the inflammatory reaction, and prolong graft survival (Oh et al. 2010).

Paracrine secretion, such as the production of various growth factors and cytokines and immunomodulatory properties, is primarily responsible for the therapeutic action of MSCs transplanted or migrated into the cornea. Lately, it has been shown that EVs originating from MSCs could participate in the paracrine effect of these cells. MSCs-derived EVs significantly suppressed ER stress and apoptosis, in corneal endothelium, possibly by miRNA transfer (Buono et al., 2021). Exosomes isolated from corneal MSCs accelerated corneal epithelium healing *in vitro* and *in vivo*. Injured corneas were treated topically by exosome suspension (Samaeekia et al., 2018). EVs secreted by corneal MSCs fused with corneal epithelial and stromal cells *in vitro*. *In vivo*, exosomes reduced corneal scarring in a murine model, inhibited pro-fibrotic genes, and blocked neutrophil infiltration. (Shojaati et al., 2019). The close interaction between cells could also be needed as only direct co-culturing of MSCs with corneal epithelium cells showed that mitochondrial transfer through tunneling nanotubes protects corneal cells against mitochondrial damage caused by oxidative stress. The effect was not observed in cultures with inserts (Jiang et al., 2016).

2.5.5.1 Clinical trials of MSC therapy in injured cornea

There are only two clinical trials that were completed. Allogeneic BM-MSCs were used to treat LSCD and were as effective as cultivated allogeneic LSC transplants (Calonge et al., 2019). The second one does not have any published outcome yet (NCT04484402). The other six clinical trials are still active or recruiting. One phase I clinical trial of locally transplanted allogeneic MSCs has early results supporting the safety of their transfer onto the injured ocular surface (clinicaltrials.gov). The various positive effects of the healing of corneal epithelium were observed in all three patients (Margolis et al., 2022).

3. THESIS AIMS

The main aim of the thesis is to investigate the possibility of using MSCs to treat severely damaged cornea and degenerative retinal diseases. Also, we want to describe how MSCs act on the damaged tissue cells and how the injured tissue's pro-inflammatory environment influences MSCs.

- **To analyze how MSCs can differentiate into cornea-like cells and what is the main factor in their differentiation**

  MSCs are well known for their differentiation and immunosuppressive abilities. The impact of the corneal environment and IGF-I, one of the main growth factors responsible for corneal regeneration on properties of MSCs, will be analyzed.

- **To study the ability of MSCs to differentiate into the cells expressing retinal markers**

  MSCs are able to differentiate into many cell types. We will culture MSCs *in vitro* with retinal extracts and stimulated splenocytes, lymphocytes or supernatants to simulate the inflammatory environment of the injured retina.

- **To study of immunomodulatory potential of MSCs and their ability to suppress inflammation in the injured retina**

  Production of various growth factors is crucial for retinal regeneration. We will study the ability of MSCs to produce them in the simulated inflammatory environment of the retina and their influence on cytokine production by retinal explants *in vitro*. *In vivo* model of retinal inflammation will be used to observe the effect of MSCs on the production of cytokines and immune cell migration.

- **Describe anti-apoptotic properties of MSCs transplanted on the injured cornea**

  Apoptosis of corneal epithelial cells is one of the factors influencing the outcome of treatment of the damaged ocular surface. We will investigate how MSCs affect apoptotic cells in the cornea in vivo and in vitro and the character of their action. We will also investigate how MSCs are affected by the pro-inflammatory environment of the injured cornea.

4. LIST OF PUBLICATIONS

4.1 List of publications used to prepare this thesis

Trosan, P., Javorkova, E., Zajicova, A., Hajkova, M., Hermankova, B., **Kossl, J.**, Krulova, M., Holan, V., 2016. The Supportive Role of Insulin-Like Growth Factor-I in the Differentiation of Murine Mesenchymal Stem Cells into Corneal-Like Cells. Stem Cells Dev. 25, 874-881.

Hermankova, B., **Kossl, J.**, Javorkova, E., Bohacova, P., Hajkova, M., Zajicova, A., Krulova, M., Holan, V., 2017. The Identification of Interferon-γ as a Key Supportive Factor for Retinal Differentiation of Murine Mesenchymal Stem Cells. Stem Cells Dev. 26, 1399-1408.

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4.2 List of other impacted publications

Reinstein Merjava, S., **Kossl, J.**, Neuwirth, A., Skalicka, P., Hlinomazova, Z., Holan, V., and Jirsova, K. (2022). Presence of Protease Inhibitor 9 and Granzyme B in Healthy and Pathological Human Corneas. Biology, 11(5), 793.

Holan, V., Echalar, B., Palacka, K., **Kossl, J.**, Bohacova, P., Porubska, B., Krulova, M., Javorkova, E., and Zajicova, A., 2022. The Inability of Ex Vivo Expanded Mesenchymal Stem/Stromal Cells to Survive in Newborn Mice and to Induce Transplantation Tolerance. Stem Cell Rev and Rep. 18, 2365–2375.

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Hermankova, B., **Kossl, J.**, Bohacova, P., Javorkova, E., Hajkova, M., Krulova, M., Zajicova, A., Holan, V., 2019. The Immunomodulatory Potential of Mesenchymal Stem Cells in a Retinal Inflammatory Environment. Stem Cell Rev. Rep. 15, 880-891.

Bohacova, P., **Kossl, J.**, Hajkova, M., Hermankova, B., Javorkova, E., Zajicova, A., Krulova, M., Holan, V., 2020. Interleukin-10 production by B cells is regulated by cytokines but independently of GATA-3 or FoxP3 expression. Cell. Immunol. 347, 103987.

Javorkova, E., Matejckova, N., Zajicova, A., Hermankova, B., Hajkova, M., Bohacova, P., **Kossl, J.**, Krulova, M., Holan, V., 2019. Immunomodulatory Properties of Bone Marrow Mesenchymal Stem Cells from Patients with Amyotrophic Lateral Sclerosis and Healthy Donors. J. Neuroimmune Pharmacol. 14, 215-225.

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4.3 Non-impact publications:

Kossl, J., Zajicova, A., Hermankova, B., Javorkova E., Bohacova, P., Holan, V., 2018. Use of the Nanofiber Scaffold for Transfer of Stem Cells onto the Injured Ocular Surface in

Mouse Experimental Model. 9th International Conference on Nanomaterials - Research and Application (NANOCON 2017), pp. 598-604, ISBN 978-80-87294-81-9.

4.4 Chapters in books

Kossl, J., Zajicova, A., Holan, V., 2018. Mechanisms of Mesenchymal Stem Cell Migration after Their Therapeutic Application. Nanostructures in Biomedicine: Their Regenerative and Toxic Properties, pp. 82-86, ISBN 978-80-906655-7-6.

Javorkova, E., Zajicova, A., **Kossl, J.**, 2017. Comparison of Phenotype and Secretory Functions of MSC Isolated from Patients Suffering from ALS and Health Donors. Nanomaterials in biomedical research, pp. 129-133. ISBN 978-80-906655-5-2.

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Kossl, J., Hermankova, B., Holan, V., 2016. A Potential of Stem Cells for the Treatment of Retinal Degenerative Diseases. Novel Biomaterials in regenarative medicine, pp. 141-151. ISBN 978-80-88113-69-0.

Holan, V., Bohacova, P., **Kossl, J.**, Javorkova, E. 2015. Immunosuppressive Drug-Loaded Nanofibers for the Local Inhibition of Harmful Inflammatory Reactions. From Functionalized Nanostructures towards Engineered Macrostructures, pp. 17-23. ISBN-978-80-88113-20-1.

I confirm that the author of this thesis, Jan Kössl, has contributed to all publications included in this thesis as described in Aspirant´s contribution section in the Results chapter. He performed most of the experimental work and prepared the manuscript in case of his first author publication.

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prof. RNDr. Vladimír Holáň, DrSc.

5. RESULTS

5.1 The Supportive Role of Insulin-Like Growth Factor-I in the Differentiation of Murine Mesenchymal Stem Cells into Corneal-Like Cells

Peter Trosan, Eliska Javorkova, Alena Zajicova, Michaela Hajkova, Barbora Hermankova, **Jan Kossl**, Magdalena Krulova and Vladimir Holan

Stem Cells Dev. 2016; 25(11): 874-81.

IGF-1 is crucial for the renewal of the corneal epithelium after the injury. IGF-1 is highly expressed in the damaged cornea, and corneal limbus IGF-I is responsible for starting the limbal stem cell migration and differentiation process. MSCs are known for their immunomodulatory, migration, and differentiation properties. Therefore, MSCs are considered one of the candidates for the cell therapy of severely injured ocular surface.

This paper focused on differentiating BM- MSCs into corneal-like cells. Murine MSCs were isolated from bone marrow, cultured for three weeks, and purified using a magnetic cell sorter. Purified MSCs were co-cultivated with extracts prepared from excised murine corneas alone or stimulated by IGF-I. We used a real-time quantitative polymerase chain reaction to detect the relative expression of typical corneal markers such as cytokeratin K12, keratocan, and lumican.

All the relative gene expressions were elevated after three days of cultivation and were further increased gradually during the 10-day cultivation with corneal extracts and stimulation by IGF-I. The expression of genes was significantly higher in MSCs cultivated with corneal extracts and stimulated by IGF-I than in MSCs cultivated only with extracts on day 10. We observed the differentiation of these cells in corneal-like cells by immunostaining for cytokeratin K12. However, their morphology and the expression of surface markers typical for MSCs were comparable between them and undifferentiated controls. We compared their proliferative abilities, which were even higher in differentiated cells. The immunosuppressive properties of untreated and differentiated MSCs were tested by co-cultivating with Concanavalin-A stimulated splenocytes. The production of IL-2 and interferon-gamma (IFN-γ) was determined by ELISA after 24 (IL-2) and 48-hour (IFN-γ) in

supernatants, and both treated and untreated cells were able to inhibit the production of these cytokines.

Aspirant's contribution: cell cultures preparation, participation in qPCR, data analysis, and interpretation.

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The Supportive Role of Insulin-Like Growth Factor-I in the Differentiation of Murine Mesenchymal Stem Cells into Corneal-Like Cells

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This study was focused on characterizing the differentiation of bone marrow-derived mesenchymal stem cells (MSCs) into corneal-like cells. Mouse MSCs were isolated from the bone marrow, grown in cell culture for 3 weeks, and purified using a magnetic activated cell sorter. Purified MSCs were cultured with an extract prepared from excised corneas and in the presence or absence of insulin-like growth factor-I (IGF-I). Analysis by quantitative real-time polymerase chain reaction showed that the expression of corneal specific markers, such as cytokeratin 12 (K12), keratocan, and lumican, was already induced after a 3-day cultivation and gradually increased during the 10-day incubation of MSCs with the extract. The presence of IGF-I significantly increased differentiation. Immunofluorescence analysis of differentiated MSCs showed positive results for the K12 protein. The morphology of the differentiated cells and the expression of cell surface markers CD45, CD11b, CD73, CD44, and CD105 were comparable in the control and differentiated MSCs. Proliferative activity was even higher in differentiated cells than in untreated MSCs. Both untreated and differentiated MSCs inhibited the production of interleukin-2 and interferon-γ in spleen cells stimulated with Concanavalin A. The results thus show that MSCs cultured in the presence of corneal extract and IGF-I efficiently differentiate into corneal-like cells. The differentiated cells possess characteristics of corneal epithelial cells and keratocytes, while at the same time maintaining MSC properties.

Introduction

SEVERE INJURIES OR DAMAGE of the ocular surface can lead to limbal stem cell deficiency (LSCD). In such cases, as the cornea cannot heal properly, corneal transparency is decreased and the defect cumulates in a loss of vision. The only way to treat such defects is transplantation of limbal stem cells (LSCs) from the healthy eye [1–3] or from an unrelated donor. If the LSCD is bilateral, autologous LSCs are not available and allogeneic LSCs have to be used. However, the use of allogeneic cells requires strong immunosuppression, and treatment results are not always satisfactory [4,5]. To overcome these problems, various other types of autologous stem cells have been proposed and tested [6–8]. Among them, mesenchymal stem cells (MSCs) are the most promising and prospective cell type.

MSCs represent a population of multipotent stem cells that can be obtained relatively easily from various sources. They can be isolated in a sufficient amount from bone marrow or adipose tissue and are able to differentiate into a number of various cell types, including those that form bone, cartilage, muscle, fat, and other connective tissues [9], or can even transdifferentiate into other cell types, including corneal epithelial cells [7,10]. Furthermore, MSCs possess potent immunosuppressive and immunoregulatory properties [11,12] and are a source of numerous growth and trophic factors [13,14]. All these properties contribute to their therapeutic potential and make them promising candidate for cell populations for ocular surface regeneration. Indeed, numerous studies have demonstrated the ability of MSCs to treat damaged ocular surface and LSCD [7,15,16].

Although the ability of MSCs to differentiate into corneal cells is still a matter of debate [17], many authors clearly demonstrated the expression of markers of corneal epithelial cells or keratocytes in differentiated MSCs under selective conditions. For example, Du *et al.* [18] used reduced-serum medium supplemented with ascorbate and insulin for differentiation, Park *et al.* [19] cultured MSCs in keratocyte-

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conditioned medium, and the medium from LSC cultures was used by Gu *et al.* [10]. In other studies, the coculture of MSCs with corneal epithelial cells or with corneal stromal cells induced the expression of corneal epithelial cell-associated markers [20,21].

In our previous study, we found that insulin-like growth factor-I (IGF-I) supports the differentiation of LSCs into corneal-like cells [22]. In the present study, we tested whether mouse bone marrow-derived MSCs have the potential to differentiate into corneal epithelial cells using the extract from the cornea, and whether the differentiation process is increased in the presence of IGF-I. We also evaluated the characteristics of MSCs differentiated with corneal extract and IGF-I and compared them to the untreated MSCs.

Materials and Methods

Animals

Mice of both sexes of the inbred strain BALB/c at the age of 2–4 months were used in the experiments. The animals were obtained from the breeding unit of the Institute of Molecular Genetics, Prague. The use of animals was approved by the local Animal Ethics Committee of the Institute of Experimental Medicine, Prague. The animals were treated in accordance with the Principles of Laboratory Animal Care.

Isolation, culture, and purification of MSCs

MSCs were isolated from the bone marrow of BALB/c mice. The bone marrow was flushed out from the femurs and tibias, and a single-cell suspension was prepared with a tissue homogenizer. The cells were seeded at a concentration of 4×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal calf serum (Gibco BRL), antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), and 10 mM HEPES buffer (after this referred to as complete DMEM) in 75-cm^2 tissue culture flasks (Trasadingen). After 72-h cultivation, the nonadherent cells were removed by washing and the remaining adherent cells were cultured with a regular exchange of medium and held to optimal cell concentration for an additional 2–3 weeks at 37°C in an atmosphere of 5% CO_2. The adherent cells were harvested by 5-min incubation with 1 mL of 0.5% trypsin and gently scrapped. The cell suspension was incubated for 15 min with CD11b MicroBeads and CD45 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions and immunodepleted $CD11b^+$ and $CD45^+$ cells using a magnetic activated cell sorter (AutoMACS; Miltenyi Biotec). The remaining $CD11b^-$ and $CD45^-$ cells were evaluated in terms of their purity and differentiation potential.

Phenotypic characterization of MSCs by flow cytometry

Untreated and differentiated MSCs were washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and incubated for 30 min on ice with the following anti-mouse monoclonal antibodies (mAbs): allophycocyanine (APC)-labeled anti-CD44 (clone IM7; BD PharMingen), phycoerythrin (PE)-labeled anti-CD105 (clone MJ7/18; eBioscience), APC-labeled anti-CD11b (clone M1/70; BioLegend), fluorescein isothiocyanate-labeled anti-CD45 (clone 30-F11; BioLegend), or PE-labeled anti-CD73 (clone: TY/11.8; eBioscience). Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen) added to the samples 10 min before flow cytometry analysis. Data were collected using an LSR II cytometer (BD Biosciences) and analyzed using a FlowJo software (Tree Star).

Differentiation of MSCs to adipocytes and osteoblasts

MSCs were cultured for 2–3 weeks and separated by magnetic cell sorting. The cells were cultured in a complete DMEM supplemented with specific adipogenic (containing 0.1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methyl xanthine, 0.1 mM indomethacine, and 0.5 mg/mL of insulin) or osteogenic (0.1 mM dexamethasone, 0.1 mM L-ascorbic acid, and 10 mM β-glycerophosphate disodium salt pentahydrate) reagents [23]. Cell differentiation was confirmed by staining with Oil Red O or Alizarin Red S.

Preparing the corneal extract

The corneas were harvested and cut into small pieces in serum-free DMEM (one cornea/125 μL of medium) postmortem. The samples were frozen at −80°C and thawed/frozen in three cycles for 10 min each. The extracts were filtered through a 0.22 μm filter and stored at −80°C until used.

Differentiation of MSCs

MSCs were cultured for 3, 7, or 10 days in complete DMEM with extract from the corneas and in the absence or presence of IGF-I (20 ng/mL; PeproTech). The concentration of the extract in the culture medium was 20% and increased to 40% during the culturing and exchange of the medium. The culture medium was exchanged every 2–3 days.

Detecting gene expression

The expression of genes for K12, keratocan, and lumican in cultured MSCs was detected using a quantitative real-time polymerase chain reaction (qPCR). The following primers were used for amplification: K12 (sense: GTGAGTCCGC TGGTGGTAAC, antisense: CATCAGCACAGCAGGAA GTG), keratocan (sense: TCCCCCATCAACTTATTTTAGC, antisense: AGTTTGGGGTTGCCATTACA), lumican (sense: GGATGGCAATCCTCTCACTC, antisense: TCATTTGCT ACACGTAGACACTCAT), and GAPDH (sense: AGAACA TCATCCCTGCATCC, antisense: ACATTGGGGGTAGG AACAC). Untreated or differentiated cells were transferred into Eppendorf tubes containing 500 μL of TRI Reagent (Molecular Research Center), and the total RNA was extracted according to the manufacturer's instructions. One microgram of RNA was treated with deoxyribonuclease I (Promega) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 μL using M-MLV Reverse Transcriptase (Promega). qPCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems) as previously described [22]. The PCR parameters included denaturation at 95°C for 3 min, 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80°C for

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5 s and were analyzed using StepOne Software version 2.2.3 (Applied Biosystems). Each individual experiment was done in triplicate. A relative quantification model was applied to calculate the expression of the target gene in comparison to GAPDH used as an endogenous control.

Determining metabolic cell activity

The metabolic activity of living cells was determined by the WST-1 assay. The assay is based on the ability of living cells to cleave tetrazolium salts by mitochondrial dehydrogenases into water soluble formazan, which is then measured by spectrophotometry. MSCs (2×10^5 cells/mL) were cultured in complete DMEM with or without extract from the corneas and IGF-I in 24-well tissue culture plates (Corning) for 7 days at 37°C in an atmosphere of 5% CO_2. WST-1 reagent (Roche) (10 µL/100 µL of the medium) was added to each well and the plates were incubated for another 4 h to form formazan [24]. Formazan-containing medium (100 µL) was transferred from each well into the 96-well tissue culture plates (Corning) and the absorbance was measured using a Sunrise Remote ELISA Reader (Grődig) at a wavelength of 450 nm.

Immunostaining with anti-K12 antibody

Corneal cells (prepared by trypsinization of corneal tissue) and untreated or differentiated MSCs (3.7×10^5 cells/mL) were fixed for 20 min with 4% paraformaldehyde and permeabilized for 10 min with 0.1% Triton X-100. The samples were incubated with goat polyclonal anti-K12 antibody (Santa Cruz Biotechnology) for 1 h at room temperature and then with a secondary donkey anti-goat IgG antibody conjugated with Alexa Flour 594 (Invitrogen). The cells were rinsed with PBS containing 0.05% TWEEN and fixed on glass slides with Mowiol 4-88 (Calbiochem) in the presence of the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI). Visualization of the fluorescent label was performed using a fluorescent microscope (Leica).

Immunostaining with phalloidin

Untreated or differentiated MSCs (2.5×10^5 cells/mL) were fixed for 20 min with 4% paraformaldehyde and permeabilized for 10 min with 0.1% Triton X-100. The samples were then incubated with Phalloidin conjugated with Alexa Fluor 568 (Invitrogen) for 1 h at room temperature. Cell nuclei were stained with DAPI for 1 min and samples were mounted with VECTASHIELD. Visualization of the fluorescent label was performed using a fluorescent microscope (Leica).

Comparing the immunosuppressive properties of untreated and differentiated MSCs

Spleen cells (0.6×10^6/mL) from BALB/c mice were stimulated with Concanavalin A (ConA; Sigma-Aldrich), as described previously [25]. Cells were cultured in a volume of

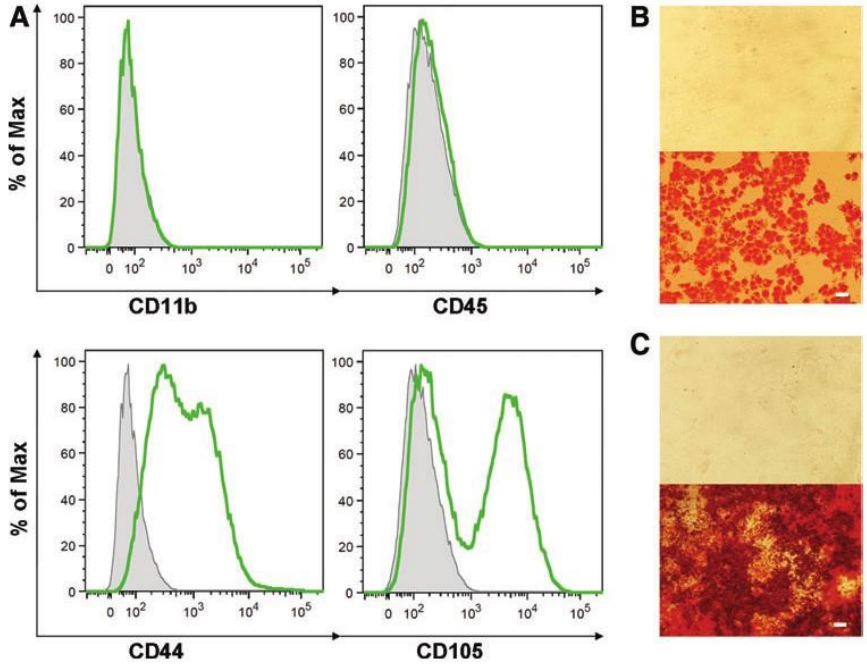

FIG. 1. Characterization of untreated bone marrow-derived MSCs. **(A)** Flow cytometry analysis of CD11b, CD45, CD44, and CD105 markers are expressed by MSCs (*green curve*) in comparison with unlabeled MSCs (*gray-tinted curve*). One of three similar experiments is shown. **(B)** The ability of MSCs to undergo adipogenic differentiation. The cultures without (*upper panel*) or with the addition of differentiation agents (*lower panel*) were stained with Oil Red O (scale bar represents 50 µm, original magnification: 200×). **(C)** The ability of MSCs to undergo osteogenic differentiation. The cultures without (*upper panel*) or with the addition of differentiation agents (*lower panel*) were stained with Alizarin Red S (scale bar represents 250 µm, original magnification: 40×). MSCs, mesenchymal stem cells. Color images available online at www.liebertpub.com/scd

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0.4 mL of complete DMEM in 48-well tissue culture plates (Corning) alone or were stimulated with 1 μg/mL of ConA. Untreated or differentiated MSCs were added to these cultures at a lymphocyte/MSC ratio of 8:1. Supernatants were harvested after a 24-h incubation for interleukin-2 (IL-2) determination and after a 48-h incubation period for interferon-γ (IFN-γ) determination. The concentrations of cytokines in the supernatants were determined by ELISA using cytokine-specific capture and detection mAbs purchased from BD Pharmingen and following the manufacturer's instructions.

Statistical analysis

The statistical significance of differences between individual groups was calculated using the Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of MSCs

The purified cells had a uniform spindle-shaped morphology. The purity and phenotypic markers of MACS-separated MSCs were evaluated by flow cytometry. The results showed that MSCs were positive for CD44 and CD105, but negative for CD11b and CD45 (Fig. 1A). In addition, the MSCs were characterized by their ability to undergo specific adipogenic (Fig. 1B) and osteogenic differentiation (Fig. 1C). These observations showed that the adherent MACS-separated bone marrow-derived cells possess the phenotype and differentiation characteristics of MSCs.

Differentiation of MSCs

The MSCs were cultured in the absence or presence of the corneal extract and with or without recombinant IGF-I (20 ng/mL) for 3, 7, or 10 days. The expression of genes for cornea-associated markers was determined by qPCR. Figure 2 shows that the expression of genes for K12, keratocan, and lumican was already upregulated 3 days after the culture with the extract. Adding IGF-I to the culture medium significantly increased the expression of the tested genes.

The differentiation potential of the MSCs was confirmed by immunostaining for the K12 protein using anti-K12 antibody. As demonstrated in Fig. 3, the K12 protein was clearly detected in the MSCs differentiated with the extract (Fig. 3B) and with the extract and IGF-I (Fig. 3C). Untreated MSCs were used as a negative control for K12 expression (Fig. 3D), while isolated corneal epithelial cells served as a positive control (Fig. 3A).

Morphology, growth, and gene expression of differentiated MSCs

The morphology of the untreated and differentiated MSCs is shown in Fig. 4. Both cell types had a typical fibroblast-like shape and adhered to plastic and glass surfaces. The expression of cell surface markers CD45, CD11b, CD73, CD44, and CD105 was determined by flow cytometry. The analysis revealed that both cell types had a similar expression profile (Fig. 5). Results from the WST-1 assay showed that differentiated MSCs have rather better proliferation activity than untreated cells (Fig. 6).

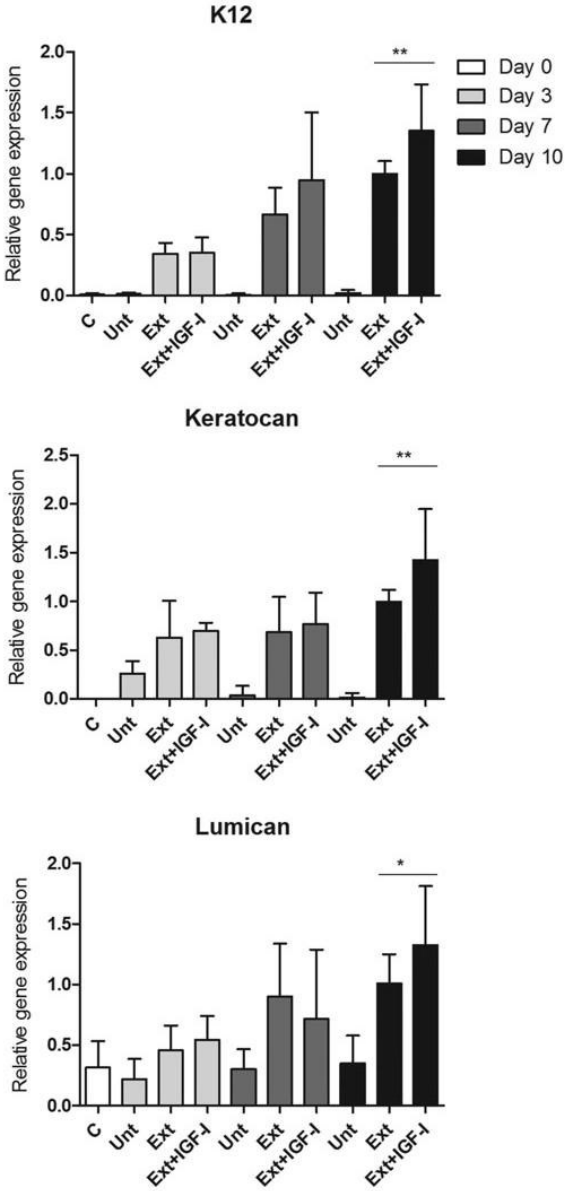

FIG. 2. The expression of genes for K12, keratocan, and lumican in untreated and differentiated MSCs was determined by qPCR. The cells were cultured for 3, 7, or 10 days untreated (Unt), with the extract from the corneas (Ext) and in the presence of the extract and IGF-I (Ext+IGF-I). Each bar represents mean ± SD from four to five determinations. The *asterisks* represent statistically significant ($*P < 0.05$, $**P < 0.01$) difference in the gene expression between MSCs treated only with the extract or with the extract and IGF-I. Freshly purified MSCs are marked as a control (C). IGF-I, insulin-like growth factor-I; qPCR, quantitative real-time polymerase chain reaction.

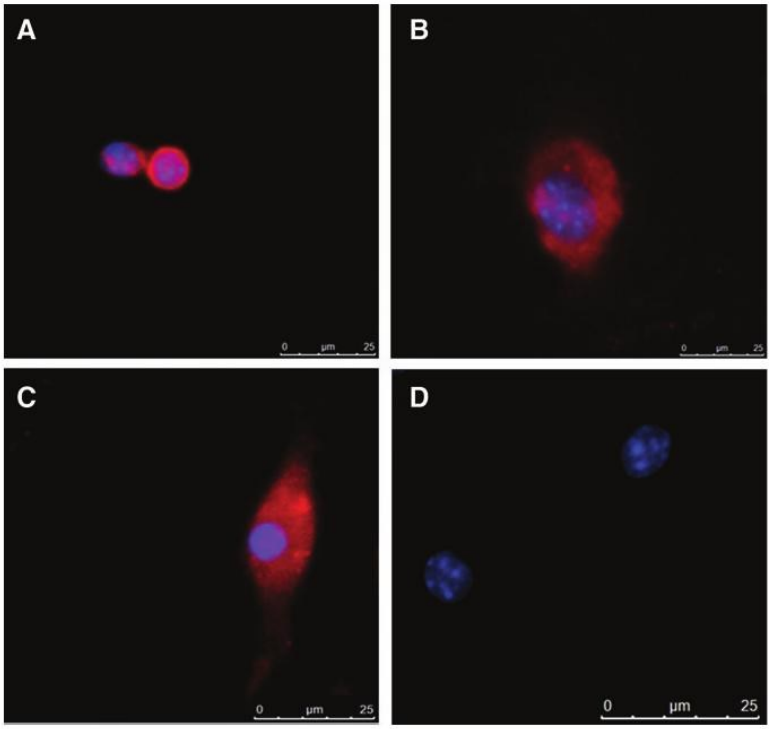

FIG. 3. Immunostaining for K12 protein in corneal cells and untreated or differentiated MSCs. Single cell suspensions of corneal cells **(A)**, MSCs cultured with the extract **(B),** or with the extract and IGF-I **(C),** or untreated MSCs **(D)** were stained with a goat antibody against mouse K12. The nuclei were stained with DAPI (*blue*). One representative experiment of four similar ones is shown. DAPI, 4′,6-diamidino-2-phenylindole. Color images available online at www.liebertpub.com/scd

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Immunosuppressive properties of untreated and differentiated MSCs

Spleen cells were stimulated with T-cell mitogen ConA in the absence or presence of untreated or differentiated MSCs (the ratio of lymphocytes to MSCs was 8:1). The production of IL-2 and IFN-γ was determined in the supernatants by ELISA. As demonstrated in Fig. 7, both cell types significantly inhibited production of tested pro-inflammatory cytokines.

Discussion

The integrity of the cornea is ensured by a population of stem cells that reside in the limbus. When the cornea is

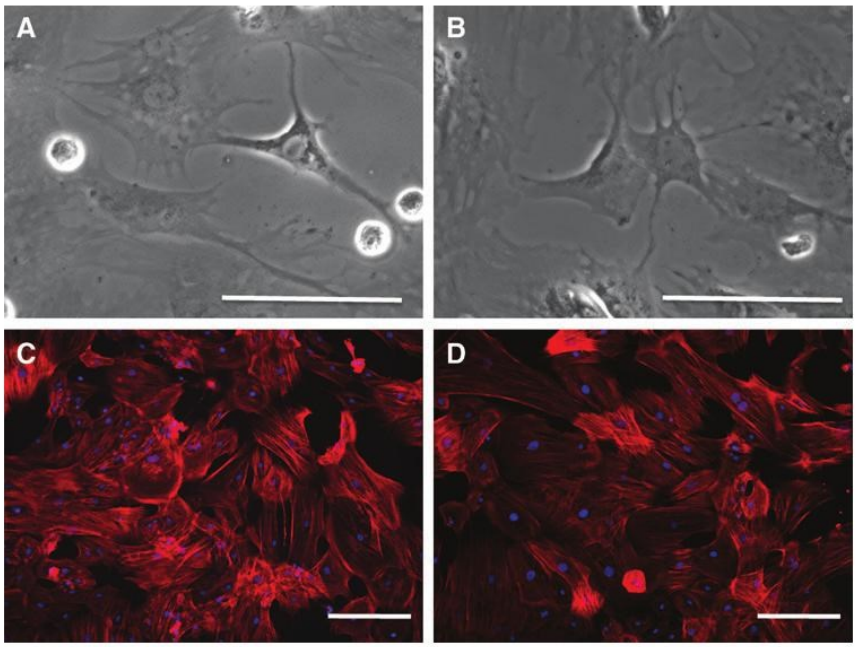

FIG. 4. Comparison of morphology of untreated and differentiated MSCs. The growing untreated MSCs **(A, C)** or MSCs treated for 10 days with the extract and IGF-I **(B, D)** are shown. The cells for the light microscopy analysis **(A, B)** remained unstained, the cells for the immunofluorescence analysis were stained with phalloidin for F-actin (*red filaments*) **(C, D)**. The nuclei were stained with DAPI (*blue*). One representative experiment of three similar ones is shown. Scale bars represent 25 µm. Color images available online at www.liebertpub.com/scd

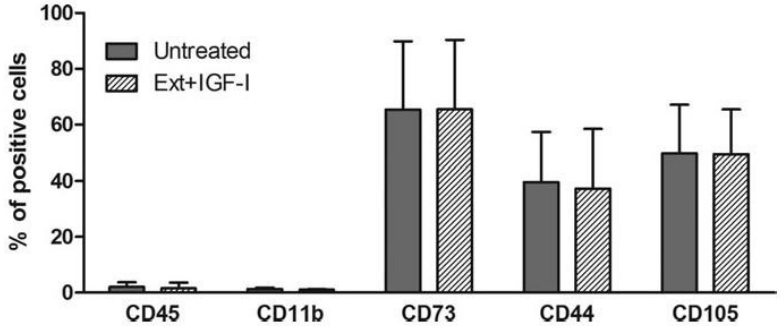

FIG. 5. Comparison of the expression of cell surface markers in untreated and differentiated MSCs. Flow cytometry analysis of CD45, CD11b, CD73, CD44, and CD105 markers expressed in untreated (Unt) or differentiated (Ext+IGF-I) MSCs is demonstrated. Each bar represents mean ± SD from three determinations.

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injured, LSCs start to proliferate, differentiate, and migrate to the site of injury. To treat corneal defects in patients with unilateral LSCD, LSCs can be isolated from healthy eyes, propagated in vitro, and using an appropriate scaffold transferred to treat the damaged cornea [2,26]. However, LSC therapy is limited by the low number of cells available and harmful immunological rejection if the cells are transplanted from a genetically unrelated donor. Therefore, other sources of autologous stem cells have been tested. These include conjunctival epithelial stem cells [8], oral mucosal cells [6], dental pulp stem cells [27], hair follicle stem cells [28], or various types of MSCs [7,15,29].

One of the properties required for stem cells used in the treatment of LSCD is their capability to differentiate into corneal cells. Therefore, in the present study, we characterized mouse bone marrow-derived MSCs and tested their ability to differentiate into cells expressing cornea-associated markers, which were not detected in untreated MSCs.

MSCs isolated by a negative sorting from the population of adherent bone marrow cells were positive for CD44 and CD105 and negative for CD11b and CD45, as described earlier [30]. In addition, these cells effectively differentiated into adipocytes and osteoblasts, thus fulfilling the basic criteria for definition of MSCs [31]. Based on screening the gene expression in untreated MSCs, we identified three genes, which were not (*K12*, keratocan) or only weakly (lumican) expressed in unstimulated MSCs.

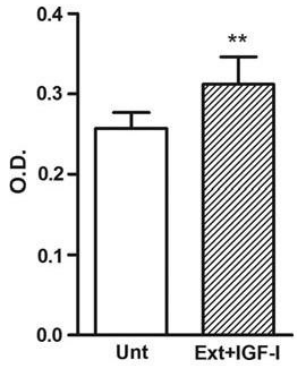

FIG. 6. Comparison of the metabolic activity of the untreated (Unt) and differentiated (Ext+IGF-I) MSCs. WST-1 reagent was added to the cell cultures for 4 h to form formazan. The absorbance was measured using a Sunrise Remote ELISA Reader at a wavelength of 450 nm. Each bar represents mean ± SD from three determinations (**$P < 0.01$).

Previous studies have demonstrated the effects of the coculture of MSCs with corneal cells in limbal or corneal cell-conditioned medium on the differentiation of MSCs into keratocytes [18,19] or cells with markers and characteristics of corneal epithelial cells [10,20,21,32]. In the present study, we used the extract from corneas for differentiation of MSCs. We observed that already after a 3-day culture of the MSCs in the presence of the extract, the cells started to express corneal markers and their expression gradually increased. This observation is in accordance with the above studies showing the expression of corneal markers in MSCs cultured in the presence of corneal cells or in corneal cell-conditioned medium. In our previous study, we found that IGF-I plays an important role in the differentiation of LSCs into corneal epithelial cells. IGF-I, which is highly expressed in the cornea after the injury, migrates to the limbus where it binds to its receptor and triggers the differentiation process of LSCs [22]. Huang *et al.* [33] demonstrated that IGF-I can dose-dependently stimulate the proliferation of MSCs, upregulate the expression of CXCR4, and accelerate their migration. It has been also observed that IGF-I is secreted by MSCs after their therapeutic administration [34–36]. Therefore, we tested whether IGF-I could also play a role in the differentiation of MSCs into corneal-like cells. Adding IGF-I into MSC cultures with extract from the corneas significantly increased the expression of genes for K12, keratocan, and lumican. IGF-I alone had no effect on the expression of these genes (data not shown).

Purified bone marrow MSCs have fibroblastic morphology. Differentiated MSCs did not change their morphology and remained in fibroblastic shape, which is comparable to previous results [20,32]. Both untreated and differentiated MSCs adhered to plastic and glass surfaces. Comparing the expression of cell surface markers did not reveal differences between untreated and differentiated cells. A similar conclusion was reached in the study where unstimulated MSCs and MSCs stimulated with a cocktail of pro-inflammatory cytokines were tested for the expression of endothelial, stromal, and adhesive markers [37]. In accordance with other studies on the proliferative and metabolic activity of differentiated cells [33,38,39], we found that MSCs differentiated with corneal extract and IGF-I have comparable or even slightly enhanced metabolic activity to untreated cells.

MSCs possess potent immunosuppressive properties and inhibit the production of various pro-inflammatory cytokines

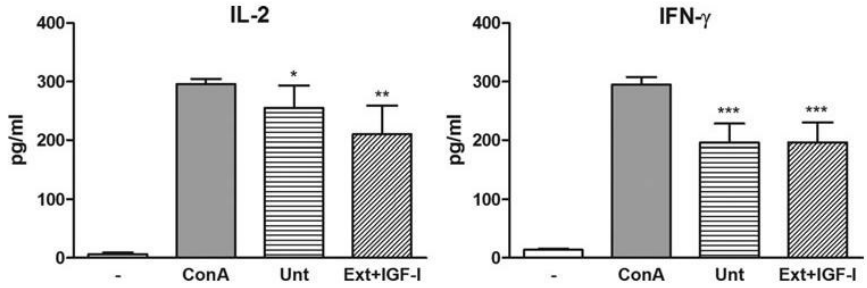

FIG. 7. Comparison of the immunosuppressive properties of untreated and differentiated MSCs. Spleen cells were cultured unstimulated or were stimulated with ConA in the presence of untreated (Unt) or differentiated (Ext+IGF-I) MSCs. The production of IL-2 and IFN-γ was determined in the supernatants after a 24 h (IL-2) or 48 h (IFN-γ) incubation period by ELISA. Each bar represents mean ± SD from three determinations (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). ConA, concanavalin A; IL-2, interleukin-2; IFN-γ, interferon-γ.

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[24,40]. In the present study, we confirmed the suppressive potential of unstimulated MSCs and we showed that differentiated MSCs inhibit the production of IFN-γ and IL-2, similar to untreated MSCs.

In conclusion, we showed that IGF-I supports differentiation of mouse bone marrow-derived MSCs into cells expressing markers of corneal cells. Differentiated MSCs expressed markers of both corneal epithelial cells and keratocytes. This observation makes them a promising source of stem cells for the regeneration of damaged or diseased cornea. In addition, the differentiated cells maintain characteristics of unstimulated MSCs and suppress the production of pro-inflammatory cytokines by activated T lymphocytes.

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Author Disclosure Statement

No competing financial interests exist.

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5.2 The Identification of Interferon-γ as a Supportive Key Factor for Retinal Differentiation of Murine Mesenchymal Stem Cells

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Stem Cells Dev. 2017; 26(19): 1399-1408.

Destruction and injury of the retina are among the leading causes of decreased quality of sight or loss of vision. MSCs can migrate to the injury site, immunosuppress local inflammatory reactions, and differentiate into multiple cell types, including retina-like cells, which express markers typical for this tissue.

In this study, we used MSCs derived from murine bone marrow; cultured them for three weeks and separated them by immunodepleting $CD11b^+$ and $CD45^+$ cells using magnetic cell sorting. We confirmed the purity of isolated and cultured MSC by flow cytometry by the presence of typical MSC markers CD44, CD73, CD105, and the absence of CD11b, CD31, and CD45. MSCs were cultured untreated, with extracts prepared from the posterior of murine eye bulbs or with extracts and supernatants from stimulated splenocytes. The expression of typical molecules for the retina like rhodopsin, S antigen, recoverin, retinaldehyde-binding protein, retinal pigment epithelium-specific protein 65, and calbindin was measured after seven days of culturing using real-time quantitative. All the expressions were significantly increased in MSCs cultured with retinal extracts and supernatants. MSCs were cultured with supernatants from multiple immune cell types, and the effect was observed in MSCs treated with T-cell supernatants. Further, we observed that this effect is present after stimulation by IFN-γ, which was confirmed using an anti-IFN-γ antibody.

We have also shown enhanced gene expression of neurotrophic factors like NGF and GDNF or IL-6 in differentiated MSCs. These properties could point to further use of pre-stimulated or pre-differentiated MSCs in the stem cell-based therapy of retinal degenerative diseases.

Aspirant's contribution: retinal/posterior segment of the eye preparation, qPCR method procedure, and analysis.

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The Identification of Interferon-γ as a Key Supportive Factor for Retinal Differentiation of Murine Mesenchymal Stem Cells

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Retinal disorders represent the main cause of decreased quality of vision and even blindness worldwide. The loss of retinal cells causes irreversible damage of the retina, and there are currently no effective treatment protocols for most retinal degenerative diseases. A promising approach for the treatment of retinal disorders is represented by stem cell-based therapy. The perspective candidates are mesenchymal stem cells (MSCs), which can differentiate into multiple cell types and produce a number of trophic and growth factors. In this study, we show the potential of murine bone marrow-derived MSCs to differentiate into cells expressing retinal markers and we identify the key supportive role of interferon-γ (IFN-γ) in the differentiation process. MSCs were cultured for 7 days with retinal extract and supernatant from T-cell mitogen concanavalin A-stimulated splenocytes, simulating the inflammatory site of retinal damage. MSCs cultured in such conditions differentiated to the cells expressing retinal cell markers such as rhodopsin, S antigen, retinaldehyde-binding protein, calbindin 2, recoverin, and retinal pigment epithelium 65. To identify a supportive molecule in the supernatants from activated spleen cells, MSCs were cultured with retinal extract in the presence of various T-cell cytokines. The expression of retinal markers was enhanced only in the presence of IFN-γ, and the supportive role of spleen cell supernatants was abrogated with the neutralization antibody anti-IFN-γ. In addition, differentiated MSCs were able to express a number of neurotrophic factors, which are important for retinal regeneration. Taken together, the results show that MSCs can differentiate into cells expressing retinal markers and that this differentiation process is supported by IFN-γ.

Keywords: mesenchymal stem cell, differentiation, retina, rhodopsin, neurotrophic factor, interferon-γ

Introduction

THE RETINA IS THE INNER PART of the eye consisting of ten different, mutually connected, and interacting cell layers. Damage in any of these layers can result in a loss of function and homeostasis of the whole retina. Age-related macular degeneration, retinitis pigmentosa, and diabetic retinopathy belong among the most common retinal degenerative diseases, which are connected with a loss of retinal cells. The damage of retinal pigment epithelium (RPE) cells or rupture of tiny blood vessels in the retina can cause degeneration of photoreceptors followed by a visual impairment [1,2]. At present, there are no effective treatment protocols that can prevent, stop, or even cure the retinal degeneration. In most cases, only supportive therapy is indicated and applied. Stem cells hold great promise in regenerative medicine, and they also offer a perspective approach for the treatment of retinal disorders. Among various stem cell types, mesenchymal stem cells (MSCs) are a promising candidate for stem cell-based therapy [2,3].

MSCs are adult stem cells that can be isolated from most tissues of the organism and used as autologous cells. The main sources of MSCs are bone marrow and adipose tissue [4]. MSCs are able to migrate to the site of injury and differentiate into multiple cell types, including adipose, cartilage, and bone cells [5], or even transdifferentiate into neuronal [6,7], corneal [8], retinal cells [9], and other cell types. Moreover MSCs can suppress an inflammatory response by production of soluble immunomodulatory molecules or cell to cell contact [10]. Most of these regulatory molecules are produced at a higher level after the activation of MSCs by inflammation stimuli [11,12]. MSCs also produce a number of growth or trophic factors, which play an important role in the regeneration at the site of tissue injury

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[13]. Considering all these properties, MSCs are a promising candidate for stem cell-based therapy of retinal degenerative diseases.

In our previous studies, we have shown that bone marrow-derived MSCs are able to differentiate into corneal epithelial cells [14] and adipose tissue-derived MSCs were differentiated into neuron-like cells [15]. We also observed that mouse MSCs inhibit the acute phase of inflammation in an alkali-injured eye [16] and support the regeneration and healing of the ocular surface after alkali burn [17]. In the present study, we have characterized the potential of bone marrow-derived MSCs to differentiate into cells expressing retinal markers in cultures simulating the environment of diseased retina, and we have identified the key supportive role of interferon-γ (IFN-γ) in this differentiation process.

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Materials and Methods

Mice

Female BALB/c mice (aged 8–14 weeks) were used in the experiments. The mice were obtained from the breeding unit of the Institute of Molecular Genetics of the Czech Academy of Sciences, Prague. The use of animals was approved by the local Animal Ethics Committee of the Institute of Experimental Medicine, Prague.

Isolation, culture, and purification of MSCs

MSCs were isolated from the bone marrow of female BALB/c mice. The bone marrow was flushed out from the femurs and tibias, and a single-cell suspension was prepared using tissue homogenizer. The cells were seeded at a concentration of 4×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin), and 10 mM HEPES buffer (referred as a complete DMEM) in 75-cm^2 tissue culture flasks (Techno Plastic Products, Trasadingen, Switzerland). After a 48-h incubation at 37°C in an atmosphere of 5% CO_2, the nonadherent cells were washed out and the remaining adherent cells were cultured for an additional 2 weeks at the same conditions. The adherent cells were harvested by incubation with 1 mL of 0.5% trypsin for 5 min and then gently scraped. The resulting cell suspension was cultured for 15 min with CD11b MicroBeads and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cell suspension was then immunodepleted of $CD11b^+$ and $CD45^+$ contaminating cells using a magnetic activated cell sorter (MACS; Miltenyi Biotec). The purity and differentiation potential of MSCs were then characterized.

Phenotypic characterization of MSCs by flow cytometry

The cells were washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and were incubated for 30 min with the following anti-mouse monoclonal antibodies (mAb): allophycocyanine (APC)-labeled anti-CD44 (clone IM7; BD PharMingen, San Jose, CA), phycoerythrin (PE)-labeled anti-CD73 (cloneTY/11.8; eBioscience, San Diego, CA), PE-labeled anti-CD105 (clone TY/11.8; eBioscience), APC-labeled anti-CD11b (clone M1/70; BioLegend, San Diego, CA), fluorescein isothiocyanate (FITC)-labeled anti-CD45 (clone 30-F11; BioLegend), or PE-labeled anti-CD31 (clone MEC 13.3; BD PharMingen). Cells stained with PE-labeled rat IgG2a (clone RTK2758; BioLegend), APC-labeled rat IgG2b (clone RTK4530; BioLegend), or FITC-labeled rat IgG2b (clone RTK4530; BioLegend) were used as negative controls. Dead cells were stained with Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA) added to the samples 10 min before flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). The morphological characteristics and differentiation potential of purified MSCs have been described in detail elsewhere [16,18].

Preparation of tissue extracts

The posterior segments of the mouse eyes (containing retina) were harvested in serum-free DMEM on ice (1 segment/100 μL of DMEM). The posterior segments were used because of a small size of the mouse eye and difficulty to prepare pure retinal tissue in a sufficient quantity. We always tried to minimize the presence of nonretinal tissue. Similarly, small pieces (corresponding in size to the samples of the posterior eye tissue) were collected from the muscle, heart, or lung tissue. The samples were thoroughly homogenized and frozen at −80°C. The homogenate was thawed and frozen three times and centrifuged at 425 *g* for 10 min. The supernatant was filtered through a 0.22 μm filter (Millipore, Billerica, MA) and stored in aliquots at −80°C.

Preparation of supernatants from stimulated splenocytes

Mouse spleen was homogenized to a single-cell suspension and the cells were adjusted to a concentration 1.3×10^6 cells/mL. The cells were stimulated with 1 μg/mL of Concanavalin A (Con A; Sigma-Aldrich) in RPMI-1640 medium (Sigma-Aldrich), containing 10% of FBS, antibiotics, 5×10^{-5} M 2-mercaptoethanol, and 10 mM HEPES buffer (referred as a complete RPMI-1640 medium) at 37°C. The supernatants were harvested after a 48-h incubation, centrifugated, filtered through a 0.22 μm filter, and stored in aliquots at −80°C.

Preparation of supernatants from T cells, B cells, and macrophages

The single-cell suspensions of spleen cells were prepared in a complete RPMI-1640 medium. The B cells were isolated by positive selection using a CD19 MicroBead Isolation Kit (Miltenyi Biotec), and T cells were isolated by negative selection using a Pan T cell Isolation Kit (Miltenyi Biotec). The macrophages were obtained by flushing the peritoneal cavity and washing out the nonadherent cells. Purified T cells (cultured in the presence of macrophages as a source of antigen-presenting cells at a ratio of 20:1) were stimulated with Con A (1 μg/mL). Purified B cells or macrophages were stimulated with 5 μg/mL of lipopolysaccharide (LPS; Sigma-Aldrich). The supernatants were harvested after a 48-h incubation, centrifugated, filtered through 0.22 μm filter, and stored at −80°C.

TABLE 1. MURINE PRIMER SEQUENCES USED FOR REAL-TIME POLYMERASE CHAIN REACTION

Genes	*Sense primer*	*Antisense primer*
Gapdh	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
Rho	ACCTGGATCATGGCGTTG	TGCCCTCAGGGATGTACC
Sag	AAGCATGAGGACACAAACCTG	CACCAGGATCCCCATGAC
Rcvr	AGATCTGGGCATTCTTTGGA	AGGGTCCCCTCGATGAAT
Rlbp	CCCCTCGGATCTCAAGAAG	TTTGAACCTGGCTGGGAAT
Calb2	CGAAGAGAATTTCCTTTTGTGC	TGTGTCATACTTCCGCCAAG
Rpe65	TCAGGAGATATGTACTTCCTTTGACA	TTGTATGGGGCAGTGTGACT
Tgf-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
Pedf	GGACTCTGATCTCAACTGCAAG	AAGTTCTGGGTCACGGTCAG
Nes	TCCCTTAGTCTGGAAGTGGCTA	GGTGTCTGCAAGCGAGAGTT
Ngf	TGGACTGCACGACCACAG	AAATTAGGCTCCCTGGAGGT
Gdnf	GACATCCCATAACTTCATCTTAGAGTC	TCCAACTGGGGGTCTACG
Il-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA

Calb2, calbindin 2; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gdnf, glial cell-derived neurotrophic factor; Il-6, interleukin 6; Nes, nestin; Ngf, nerve growth factor; Pedf, pigment epithelium-derived factor; Rcvr, recoverin; Rho, rhodopsin; Rlbp, retinaldehyde-binding protein; Rpe65, retinal pigment epithelium 65; Sag, S antigen; Tgf-β, transforming growth factor beta.

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Differentiation of MSCs

MSCs were seeded at a concentration of 7×10^4 cells/mL in a 12-well tissue culture plate (Nunc, Roskilde, Denmark) and were cultured for 2, 4, or 7 days in 1 mL of complete DMEM together with retinal extract (30% of the volume), supernatants from Con A-stimulated splenocytes (30% of the volume) or with a combination of the extract and supernatant. Half of the culture medium was exchanged after 3 days of differentiation with a fresh DMEM containing 30% of extract and 30% of supernatant, thus the final composition of the medium remained the same as at the beginning of differentiation process.

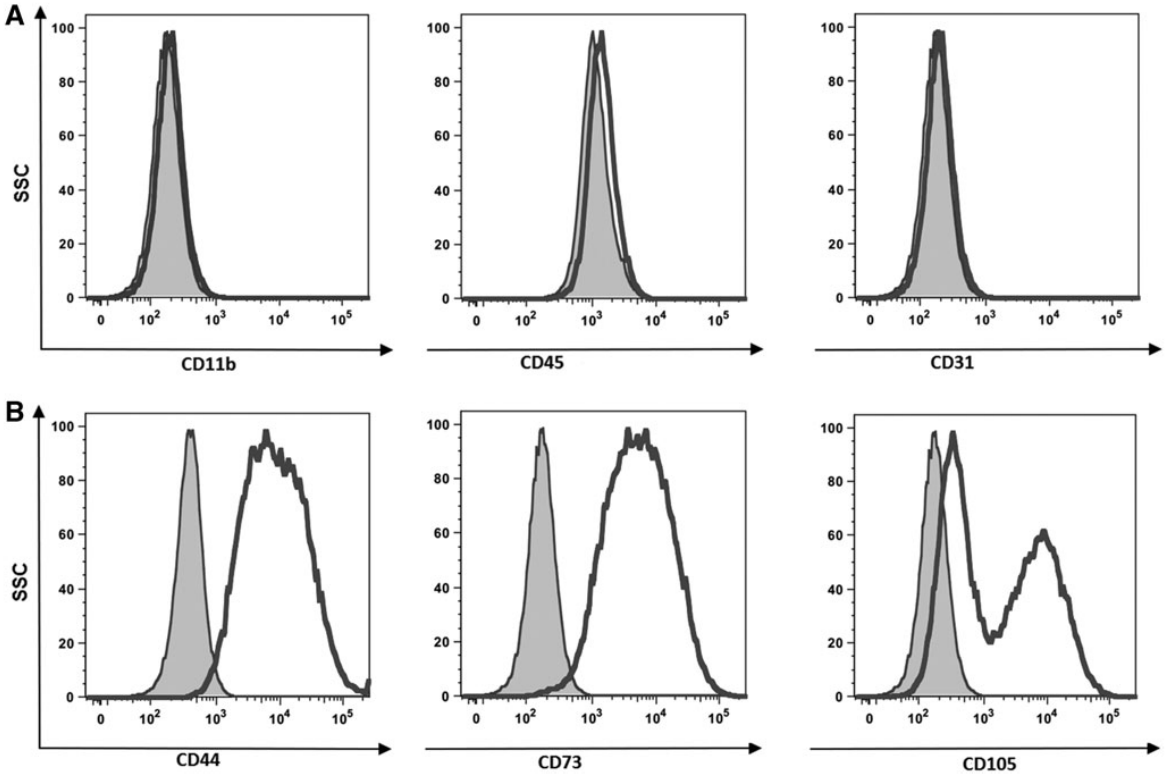

FIG. 1. Phenotypic characterization of MSCs. Representative histograms show the flow cytometry analysis of CD11b, CD45, CD31 **(A)** and CD44, CD73, CD105 **(B)** markers expressed by MSCs in comparison with control unlabeled MSCs. One of three similar experiments is shown. MSC, mesenchymal stem cell.

To identify the supportive molecule in the supernatants, MSCs were cultured with retinal extract, and the supernatant from Con A-stimulated spleen cells was replaced by DMEM containing IL-2, IL-6, IL-10, IL-17, IFN-γ, or TGF-β (all cytokines were purchased from PeproTech, Rocky Hill, NJ). The final concentration of cytokines in cultures was 20 ng/mL.

Detection of gene expression by real-time polymerase chain reaction

The expression of genes for retinal markers and growth factors was detected using real-time polymerase chain reaction (PCR). MSCs were cultured for 2, 4, or 7 days, untreated or in the presence of retinal extract, supernatant from stimulated splenocytes or both together. The total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. One microgram of RNA was treated with deoxyribonuclease I (Promega, Madison, WI) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 μL using M-MLV Reverse Transcriptase (Promega).

Quantitative real-time PCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green (Applied Biosystems) as previously described [14]. The sequences of the primers for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), rhodopsin (*Rho*), S antigen (*Sag*), recoverin (*Rcvr*), retinaldehyde binding protein (*Rlbp*), calbindin 2 (*Calb2*), retinal pigment epithelium 65 (*Rpe65*), pigment epithelium-derived factor (*Pedf*), nestin (*Nes*), transforming growth factor beta (*Tgf-β*), interleukin 6 (*Il-6*), nerve growth factor (*Ngf*), and glial cell-derived neurotrophic factor (*Gdnf*) genes used for amplification are presented in Table 1.

The relative gene expression was normalized by the endogenous control *Gapdh*. The PCR parameters included denaturation at 95°C for 3 min followed by 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 s and were analyzed using StepOne Software version 2.2.2 (Applied Biosystems). The possibility of the presence of RNA in retinal extract was excluded by a negative gel electrophoresis (data not shown), and the extracts were also tested as control for PCR.

Neutralization of IFN-γ in supernatants from stimulated splenocytes

MSCs were differentiated in the presence of retinal extract and supernatant from stimulated splenocytes. The

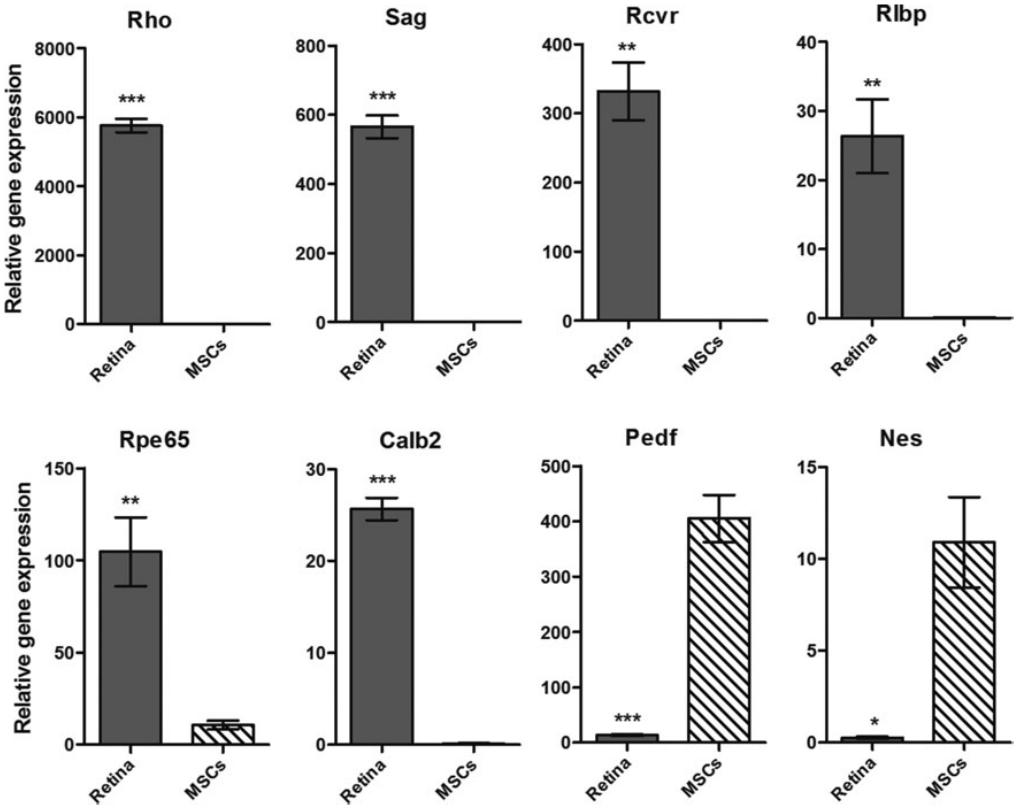

FIG. 2. The expression of genes for retinal markers in MSCs and retina. The expression of rhodopsin (*Rho*), S antigen (*Sag*), recoverin (*Rcvr*), retinaldehyde-binding protein (*Rlbp*), retinal pigment epithelium-specific protein 65 (*Rpe65*), calbindin 2 (*Calb2*), pigment epithelium-derived factor (*Pedf*), and nestin (*Nes*) genes was determined by real-time PCR in untreated MSCs and the retinal tissue. Each bar represents the mean ± SD from three independent determinations. Values with *asterisks* are significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from untreated MSCs. PCR, polymerase chain reaction.

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supernatant was either untreated or preincubated for 10 min with neutralization antibody anti-IFN-γ (PeproTech) at a concentration of 5 μg/mL before being added to the culture. The expression of gene for Rho was determined after a 7-day incubation by real-time PCR.

Immunostaining with antirhodopsin antibody

The untreated or differentiated MSCs were fixed with 4% paraformaldehyde for 1 h and then permeabilized using 0.1% Triton X-100 for 20 min. The samples were incubated with anti-mouse FITC-labeled mAb anti-Rho (clone 4D2; Abcam, Cambridge, United Kingdom) for 2 h. After rinsing with PBS, the cells were fixed on glass slides with Mowiol 4–88 (Calbiochem, San Diego, CA), and nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) dye. The samples were visualized by fluorescent microscope (Leica, Wetzlar, Germany).

Statistical analysis

The results are expressed as the mean ± SD. Comparisons between the two groups were analyzed by Student's *t*-test, and multiple comparisons were performed by ANOVA. A value of $P < 0.05$ was considered statistically significant.

Results

Phenotypic characterization of MSCs

The phenotype of MACS-separated MSCs was characterized by flow cytometry. The cells were positive for CD44, CD73, and CD105, which are markers attributed to murine MSCs, and were negative for leucocyte markers CD11b, CD45, and CD31 (Fig. 1). In addition, the growing MSCs had a typical fibrocyte-like morphology, adhered to plastic and were able to undergo adipogenic and osteogenic differentiation, as we have described previously [18].

Detection of retinal markers in differentiated MSCs

MSCs were cultured with retinal extract and/or supernatant from stimulated splenocytes to simulate the environment of the damaged retina. The expression of genes for retinal markers was detected by real-time PCR. First, we selected six retinal markers Rho, Sag, Rcvr, Rlbp, Rpe65, and Calb2, which were strongly expressed in the retina, but were not or only weakly expressed by MSCs (Fig. 2). Two other tested markers, Pedf and Nes, were expressed in a higher level in MSCs than in the retina and therefore were not used in the next studies.

Untreated MSCs and MSCs cultured with supernatant from Con A-stimulated spleen cells expressed undetectable

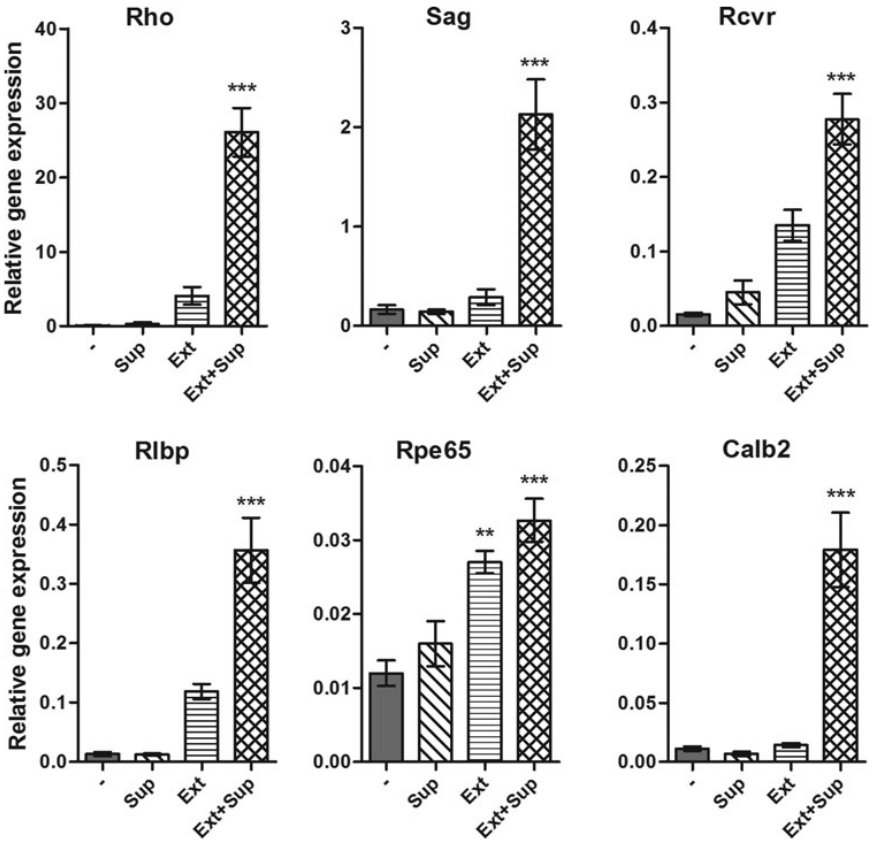

FIG. 3. The expression of retinal markers in differentiated MSCs. MSCs were cultured untreated (-), with supernatant from stimulated splenocytes (Sup), with retinal extract (Ext), or with supernatant and retinal extract together (Ext+Sup). The expression of rhodopsin (*Rho*), S antigen (*Sag*), recoverin (*Rcvr*), retinaldehyde-binding protein (*Rlbp*), retinal pigment epithelium-specific protein 65 (*Rpe65*), and calbindin 2 (*Calb2*) genes was detected after 7 days of differentiation by real-time PCR. Each bar represents the mean ± SD from three independent determinations. Values with *asterisks* are significantly different (**$P < 0.01$, ***$P < 0.001$) from untreated MSCs.

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or only very low levels of retinal markers. The level of expression of retinal markers slightly increased in the presence of retinal extract, but was significantly enhanced after culturing of MSCs with retinal extract and supernatant together. As demonstrated in Fig. 3, MSCs expressed significant levels of genes for markers typical for photoreceptors (*Rho, Sag, Rcvr*), horizontal and bipolar cells (*Calb2*), Muller cells (*Rlbp*), and RPE cells (*Rlbp, Rpe65*). These suggested that our differentiation protocol enabled MSCs to differentiate into cells of multiple retinal layers. For further detailed analysis of the differentiation process, we selected photoreceptor marker Rho, which had the highest level of expression in differentiated MSCs. The number of Rho-positive cells increased with the time of differentiation, and using flow cytometry we detected that 7%–15% of cells expressed rhodopsin protein on day 7 of differentiation (data not shown).

The kinetic of expression of the retinal genes during the differentiation process

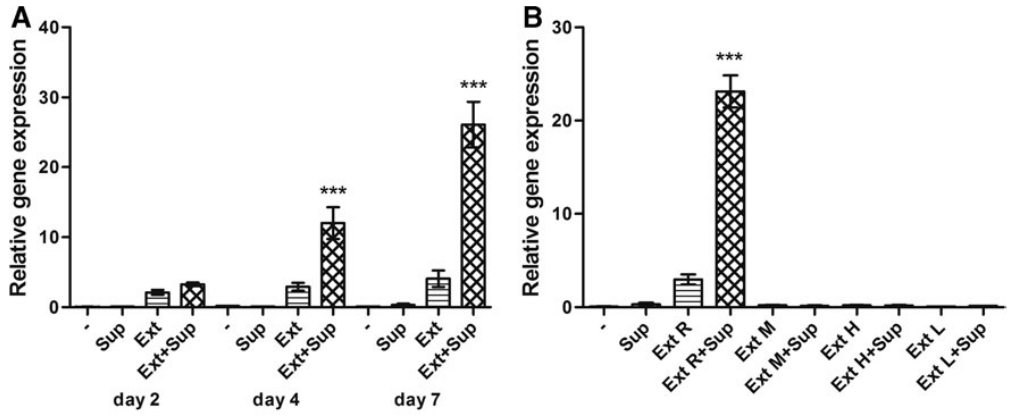
The expression of gene for Rho in untreated MSCs is undetectable and was already upregulated after 2-day cultivation of cells with retinal extract or with extract in combination with supernatant. After 4 and 7 days of differentiation, the expression of the *Rho* gene was gradually increasing, especially in the cultures containing both retinal extract and spleen cell supernatant (Fig. 4A). The expression of other retinal markers displayed a similar trend as the rhodopsin. The fold changes of gene expression of differentiated MSCs (cultivated with retinal extract and supernatant) relative to untreated MSCs are summarized in Fig. 4C.

The specific role of retinal extract in the differentiation process

To demonstrate the tissue specificity of retinal extract in the differentiation process, the extracts from retina, muscle, heart, and lung were compared in differentiation protocol. The significant increase in the *Rho* gene expression was detected only in cultures containing retinal extract and not in MSCs differentiated in the presence of muscle, heart, or lung extract with or without the spleen cell supernatant (Fig. 4B).

The role of IFN-γ in the differentiation process

We observed that the combination of retinal extract and supernatant from Con A-stimulated splenocytes represented



C

Differentiation	*Relative fold increase of gene expression*					
	Rho	*Sag*	*Rcvr*	*Rlbp*	*Rpe65*	*Calb2*
2 days	52,4	5,9	6,9	8,1	0,3	4,5
4 days	75,1	10,2	9,7	13,1	1,3	10,9
7 days	357,4	12,8	17,4	27,5	2,8	16,3

FIG. 4. The kinetics of expression of the retinal genes and the specific role of retinal extract during the differentiation process. MSCs were cultured untreated (-), with supernatant from stimulated splenocytes (Sup), with retinal extract (Ext) or with supernatant and retinal extract together (Ext+Sup). The expression of the *Rho* gene was determined by real-time PCR after 2, 4, and 7 days of incubation **(A)**. To demonstrate the specific role of retinal extract, MSCs were cultured for 7 days with supernatant from Con A-stimulated spleen cells (Sup) and with extract from retina (Ext R), muscle (Ext M), heart (Ext H), or lung (Ext L). The expression of the gene for Rho was detected by real-time PCR after 7 days **(B)**. The kinetics of expression of the retinal genes *Calb2, Rcvr, Rho, Rlbp, Rpe65, Sag* in MSCs cultured in the presence of the retinal extract and supernatant. Values represent relative fold increase of the expression of the particular gene versus untreated **(C)**. Each bar in **(A)** and **(B)** represents the mean ± SD from three independent determinations. Values with *asterisks* are significantly different (***$P < 0.001$) from those of untreated MSCs.

the optimal conditions for retinal differentiation of MSCs. To identify a molecule in the supernatant, which is responsible for the increased expression of retinal markers, the supernatants were prepared from Con A-stimulated spleen cells, from unstimulated or Con A-stimulated T cells, unstimulated or LPS-stimulated B cells, and from unstimulated or LPS-stimulated macrophages. MSCs were cultured for 7 days with these supernatants or with retinal extract and these supernatants. As demonstrated in Fig. 5A, the significant increase in the *Rho* gene expression occurred only in cultures containing retinal extract and supernatant from Con A-stimulated spleen cells or Con A-stimulated purified T cells. The supernatants from unstimulated cells or from mitogen-stimulated B cells or macrophages did not have a supportive effect on MSC differentiation.

These observations indicated that the molecule supporting retinal differentiation is a product of activated T cells. Therefore, we cultured MSCs with retinal extract in the presence of various T-cell cytokines such as IL-2, IL-6, IL-10, IL-17, IFN-γ, and TGF-β. As shown in the Fig. 5B, the enhanced expression of the *Rho* gene occurred only in the presence of IFN-γ, whereas other cytokines were without any supportive effect. Similarly, none of the cytokines from a wider panel of tested cytokines and growth factors (IL-1, 2, 4, 6, 7, 10, 12, 13, 15, 17, TGF-β, TNF-α, IGF-I, EGF, HGF, KGF, LIF, NGF, or FGF) supported the differentiation of MSCs into cells expressing retinal markers (data not shown).

To confirm the supportive role of IFN-γ in a differentiation process, MSCs were cultured with retinal extract and supernatant from Con A-stimulated splenocytes with added neutralization antibody anti-IFN-γ. The anti-IFN-γ antibody completely abrogated the supportive role of the supernatant on the *Rho* gene expression (Fig. 5C). To demonstrate a dose-dependent effect of IFN-γ on MSC differentiation, IFN-γ at the concentrations 2.5–20 ng/mL was added to the cultures of MSCs with retinal extract. As demonstrated in

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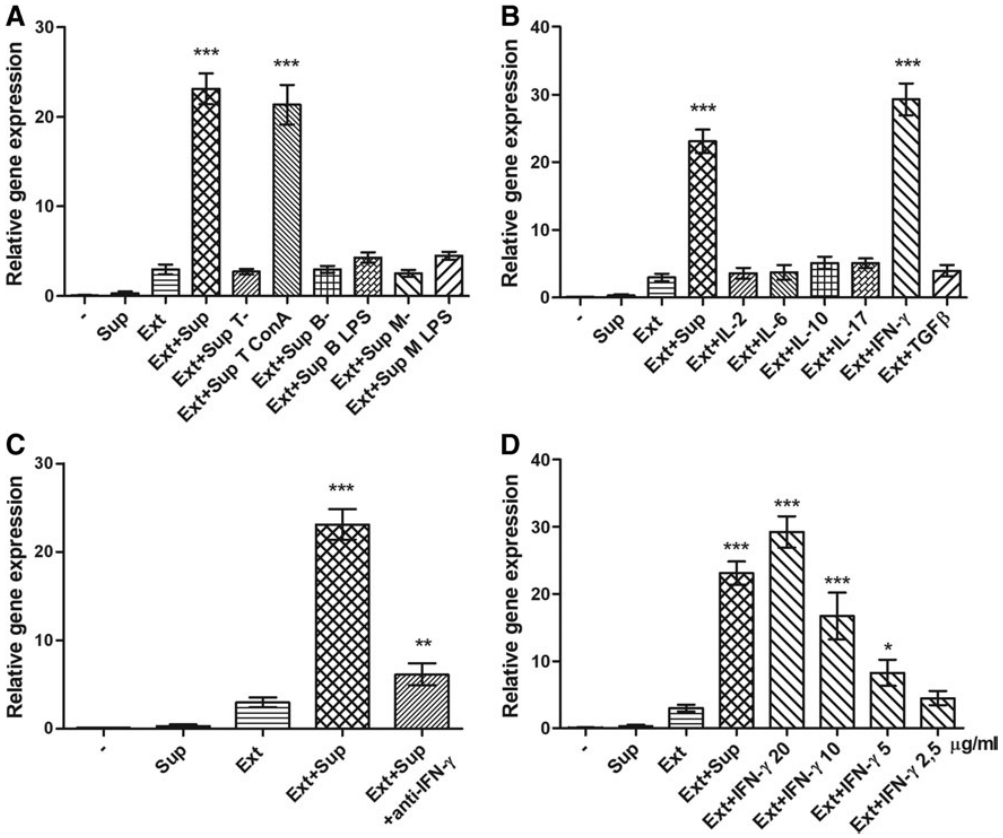

FIG. 5. The effect of IFN-γ in the differentiation process. The effect of supernatant from stimulated splenocytes (Sup) was compared with supernatants from unstimulated T cells (Sup T-), B cells (Sup B-), or macrophages (Sup M-) and Con A-stimulated T cells (Sup T ConA), LPS-stimulated B cells (Sup B LPS), and LPS-stimulated macrophages (Sup M LPS) **(A)**. MSCs were cultured with retinal extract in the presence of selected T-cell cytokines IL-2, IL-6, IL-10, IL-17, IFN-γ, and TGF-β **(B)**. The expression of the *Rho* gene was inhibited by antibody anti-IFN-γ added to cultures with retinal extract and supernatant from stimulated splenocytes **(C)**. The dose-dependent effect of IFN-γ on the *Rho* gene expression. IFN-γ was added to the cultures at the indicated concentrations (2.5–20 ng/mL) **(D)**. The expression of the *Rho* gene was detected by real-time PCR after 7 days of differentiation. Each bar represents the mean ± SD from three independent determinations. Values with *asterisks* are significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from untreated MSCs.

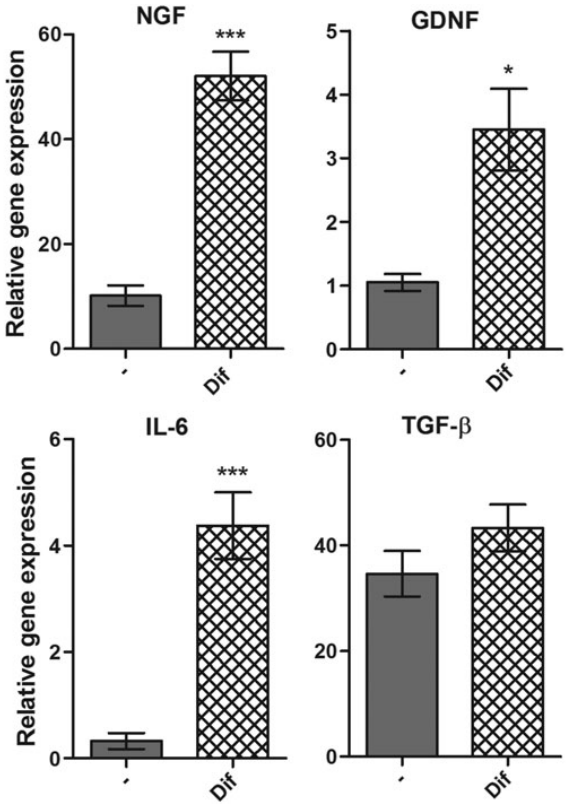

FIG. 6. The expression of genes for growth factors in untreated and differentiated MSCs. The expression of *Ngf*, *Gdnf*, *Il-6*, and *Tgf-β* genes was detected in untreated MSCs (-) and MSCs differentiated with retinal extract and supernatant from splenocytes (dif) by real-time PCR after 7 days. Each bar represents the mean ± SD from three independent determinations. Values with *asterisks* are significantly different (*$P < 0.05$, ***$P < 0.001$) from untreated MSCs.

Fig. 5D, the expression of *Rho* gene was enhanced by IFN-γ in a dose-dependent manner (Fig. 5D).

The expression of genes for growth factors and cytokines in differentiated MSCs

The expression of genes for NGF, GDNF, IL-6, and TGF-β was tested in untreated MSCs, and MSCs differentiated for 7 days with retinal extract and supernatant from activated splenocytes. The level of expression of genes for NGF, GDNF, and IL-6 was significantly increased in differentiated MSCs in comparison with untreated MSCs (Fig. 6). On the contrary, the expression of the *Tgf-β* gene remained on the same level in both untreated and differentiated cells (Fig. 6).

Detection of Rho protein in differentiated cells by immunocytochemistry

The differentiation potential of MSCs was confirmed by immunostaining for the Rho protein. While untreated MSCs were negative for Rho (Fig. 7A), MSCs cultured for 7 days with retinal extract and supernatant from activated spleen cells were clearly positive for the Rho protein (Fig. 7B).

Discussion

There is still an absence of effective treatment protocols for sight-threatening degenerative retinal diseases. For this reason, the potential application of stem cell therapy represents a great promise. MSCs, with their ability to differentiate into multiple cell types are a perspective source of replacement and regeneration of damaged retinal cells.

It has been shown that MSCs are able to differentiate into various retinal cell types [3]. MSCs isolated from rat conjunctiva after culturing in the presence of taurine expressed markers characteristic for photoreceptors and bipolar cells [19]. Taurine, together with activin A and epidermal growth factor, was used in another study to differentiate MSCs to photoreceptors. The cells differentiated for 8–10 days expressed the *Rho* and *Rlbp* genes [9]. The same authors also showed that MSCs injected into the subretinal space are able to

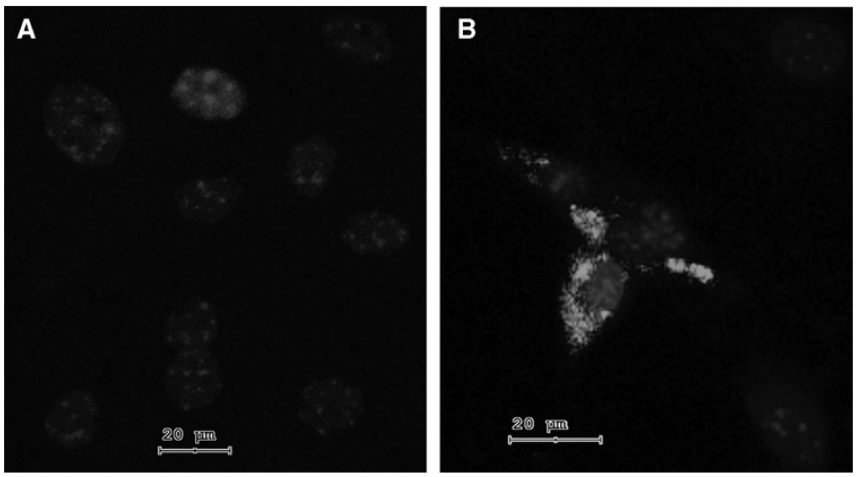

FIG. 7. Demonstration of Rho protein by immunocytochemistry. The expression of Rho protein in untreated MSCs **(A)** and differentiated MSCs **(B)** was detected with antibody anti-mouse Rho. The nuclei were stained with DAPI. One representative experiment of three similar is shown.

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integrate into the retina and express markers specific for photoreceptors. Other studies have demonstrated that transplantation of MSCs into the damaged retina induced expression of markers typical for photoreceptors, bipolar cells, and amacrine cells [20–22] in grafted MSCs. There are also several studies showing the differentiation of MSCs into RPE cells [21,23], which are important for the nourishment of photoreceptors, and disorders of RPE cells result in photoreceptor dysfunction. Human MSCs were also differentiated into RPE-like cell types after culturing with RPE cells in vitro [24].

In our study, to differentiate MSCs, we simulated the environment of diseased or injured retina. The retinal extract mimicked the environment of the damaged retinal tissue, and the supernatant from stimulated lymphocytes simulated the inflammation at the site of injury. MSCs cultured in the presence of retinal extract and supernatant from Con A-stimulated spleen cells expressed markers characteristic not only for one type of retinal cells but also for several types of retinal cells (photoreceptors, bipolar, and horizontal cells, Müller cells and RPE cells). This could be an advantage since retinal degenerative diseases often affect multiple retinal layers and various cell types. In our protocol, the retinal extract ensures the specificity of differentiation. The induction (or increase) of retinal gene expression in MSCs occurred only in the presence of retinal extract. As specificity control, similarly prepared extracts from muscle tissue, heart, or lung did not induce the expression of genes for retinal markers, even in the presence of supernatants.

We observed that MSCs cultured in the presence of retinal extract and supernatant expressed a significantly higher level of photoreceptor marker Rho than MSCs differentiated only with the extract. In this respect, it has been shown that MSCs need, for activation and higher production of inducible molecules, stimulation with proinflammatory factors [12,16]. To search for a molecule responsible for the increased expression of photoreceptor marker Rho, we cultured MSCs with retinal extract and supernatant from unstimulated or mitogen-stimulated T cells, B cells, or macrophages. The level of the *Rho* gene expression was enhanced only in the culture containing retinal extract and supernatant from Con A-stimulated spleen cells or T cells. This finding indicated that the molecule supporting MSC differentiation is a T-cell product. For further characterization of putative molecule supporting MSC differentiation, we cultured MSCs with retinal extract and a panel of T-cell cytokines. The enhanced levels of the *Rho* gene expression were detected only in cultures with retinal extract and IFN-γ.

The key role of IFN-γ in the differentiation of MSCs into cells expressing the *Rho* gene was verified by neutralization antibody anti-IFN-γ. The differentiation of MSCs in cultures containing retinal extract and supernatant from Con A-stimulated spleen cells was completely abrogated by the adding of the neutralization anti-IFN-γ antibody. These results identify IFN-γ as a molecule playing a key supportive role in the differentiation of MSCs into cells expressing retinal markers. The role of IFN-γ in differentiation process has been indicated in some other models. For example, Croitoru-Lamoury et al. [25] demonstrated that IFN-γ inhibited adipogenic and osteogenic differentiation of MSCs, but increased the expression of neural markers in differentiated cells [25]. The ability of IFN-γ to support neuronal differentiation of neural stem cells was observed by Wong et al. [26].

In accordance with the published data [27–29], we have shown that MSCs expressed genes for neurotrophic factors NGF and GDNF and for cytokines IL-6 and TGF-β. The level of expression of genes *Ngf*, *Gdnf*, and *Il-6* was significantly enhanced in differentiated MSCs, which suggests their higher potential for regeneration of retinal cells. It was demonstrated that the supernatants from light-injured retina significantly promote secretion of neurotrophic factors by MSCs and slow down the process of apoptosis in damaged retinal cells [30]. Another study showed that secretion of neurotrophic factors by MSCs promoted viability of photoreceptors in vitro, and also supported their survival after subretinal transplantation of MSCs in a retinal degeneration model [31]. Thus, MSCs differentiated according to our protocol have a higher secretory activity than untreated MSCs and may have a better regenerative potential than primary MSCs.

In conclusion, we have demonstrated the key supportive role of IFN-γ in the differentiation of MSCs into the cells expressing retinal markers. Moreover, it was shown that differentiated MSCs are a potent source of neurotrophic factors, which are important for the regeneration of damaged retinal cells. All these properties make MSCs a promising candidate for stem cell-based therapy of retinal degenerative diseases.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

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5.3 The Immunomodulatory Potential of Mesenchymal Stem Cells in a Retinal Inflammatory Environment

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AMD and diabetic retinopathy are the most common retinal degenerative diseases. The inflammation, mediated by elevated pro-inflammatory cytokine and chemokine production, is one of the most typical signs of these retinal disorders. It is accompanied by the migration of immune cells, vascular dysfunction and leakage, and thus disruption of the blood-retinal barrier. Unfortunately, there is no effective treatment for these diseases, and the procedures are made to slow down the progression or to lower the impact on the patient's vision only.

Stem cell-based therapy presents one of the possible approaches to improving these procedures, or it can even be perspective treatment. MSCs are one of the candidates used in the research and are evaluated due to their multiple immunomodulatory, secretory, and differentiation properties.

It has been shown that in patients with diabetic retinopathy, levels of IL-1β, TNF-α, and IFN-γ are elevated. The increased concentration of such cytokines creates an inflammatory environment and damages retinal vessels, edema, and retinal degeneration. In addition, macrophages are the most common immune cells infiltrating this area, producing more pro-inflammatory cytokines and increasing other immune cells' migration in the damaged retina.

In this paper, we tested the effect of pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ on the retina and leucocyte migration in this inflammatory microenvironment, and we characterized the potential of MSCs to inhibit these processes.

MSCs were again obtained from the murine bone marrow, separated, and purified by their typical markers. The inflammatory microenvironment was created in murine retinas by intravitreal injections of pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ. The eyes were untreated, treated only by PBS or with MSCs injected intravitreally. The relative gene expression of classical cytokines and molecules connected with the inflammation was measured in excised retinas by real-time quantitative PCR. Production of these molecules was determined by ELISA (IL-6, TNF-α, and VEGF) or Griess reaction (NO) in supernatants

from retinal explants cultured *in vitro* for 72 hours and stimulated by LPS and concanavalin A. All relative gene expression levels or production were decreased in retinas treated with MSCs.

The flow cytometry was used to detect immune cell infiltration in inflamed retinas, which was decreased in the eyes injected with MSCs.

In vitro model was used to characterize the mechanism of the MSC effect on retinal explants in an inflammatory environment. The explants were cultured untreated, with cytokines (IL-1β, TNF-α, and IFN-γ) alone or with MSCs. We used reverse transcription polymerase chain reaction (RT-PCR) to measure the expression of genes for pro-inflammatory molecules after 48 hours of co-cultivation and observed inhibition of their gene expression after MSC treatment. In addition, the expression of immunomodulatory molecules in stimulated MSCs (COX2, PD-L1, IDO, IL-6, PEDF, NGF, GDNF, TGF-β, and HGF), which could produce this effect, was also measured by RT-PCR.

Aspirant's contribution: a preparation of retinal/posterior segment of the eye preparation, *in vitro* model development, intraocular injections, qPCR method procedure, data analysis, and interpretation.

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The Immunomodulatory Potential of Mesenchymal Stem Cells in a Retinal Inflammatory Environment

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Abstract
Retinal degenerative disorders are characterized by a local upregulation of inflammatory factors, infiltration with cells of the immune system, a vascular dysfunction and by the damage of retinal cells. There is still a lack of treatment protocols for these diseases. Mesenchymal stem cell (MSC)-based therapy using immunoregulatory, regenerative and differentiating properties of MSCs offers a promising treatment option. In this study, we analyzed the immunomodulatory properties of mouse bone marrow-derived MSCs after their intravitreal delivery to the inflammatory environment in the eye, caused by the application of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ. The intravitreal administration of these cytokines induces an increased expression of pro-inflammatory molecules such as IL-1α, IL-6, inducible nitric oxide synthase, TNF-α and vascular endothelial growth factor in the retina. However, a significant decrease in the expression of genes for all these pro-inflammatory molecules was observed after the intravitreal injection of MSCs. We further showed that an increased infiltration of the retina with immune cells, mainly with macrophages, which was observed after pro-inflammatory cytokine application, was significantly reduced after the intravitreal application of MSCs. The similar immunosuppressive effects of MSCs were also demonstrated in vitro in cultures of cytokine-stimulated retinal explants and MSCs. Overall, the results show that intravitreal application of MSCs inhibits the early retinal inflammation caused by pro-inflammatory cytokines, and propose MSCs as a promising candidate for stem cell-based therapy of retinal degenerative diseases.

Keywords Mesenchymal stem cells · Retina · Cytokines · Inflammation · Immunomodulation

Introduction

Retinal disorders represent the main cause of decreased quality of vision and even blindness worldwide. Age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the most common retinal degenerative diseases, which are associated with a loss of retinal cells and an inflammatory reaction in the eye. The main causes of these inflammatory reaction are disruption of homeostasis, cytokine imbalance, damage of blood retinal barrier and infiltration with immune cells. These processes are mediated by a number of various pro-inflammatory cytokines and chemokines that activate endothelial cells and enable the infiltration with cells of the immune system [1].

To date, there is still no effective treatment to cease or even cure these diseases. Perspective therapeutic approaches are proposed using stem cell-based therapy. Among various types of stem cells evaluated to date, mesenchymal stem cells (MSCs) have many properties which are beneficial for the treatment of degenerative diseases [2, 3]. MSCs can be isolated from most tissues of the organism and used as autologous cells. They are potent producers of a number of factors with immunomodulatory properties [4, 5], express membrane-bound regulatory molecules [6, 7] and secrete various growth, anti-apoptotic, anti-fibrotic and angiogenic factors [8]. In addition, MSCs can differentiate into other cell types such as cells expressing markers and characteristics of corneal cells [9, 10], neurons [11] and also retinal cells [12, 13].

During the stem cell therapy of retinal degenerative diseases, MSCs can produce number of neurotrophic factors that

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promote survival and regeneration of damaged retinal cells in the environment of the damaged retina [14, 15]. Due to their immunosuppressive properties, MSCs can attenuate undesirable immune reactions associated with immune cell infiltration, local inflammation and disease progression. One of the main immune cell populations infiltrating the damaged retina are macrophages, which produce pro-inflammatory cytokines and therefore support an inflammatory reaction and the spread of the disease [16, 17]. It has been shown that patients with DR have significantly elevated levels of IL-1β, TNF-α, IFN-γ, IL-6 and IL-17 in vitreous samples [18, 19]. The increased concentrations of IL-1β and TNF-α in the eye cause a vascular dysfunction, retinal degeneration and cellular infiltration, through the endothelial cell barrier [20, 21]. In a mouse model it has been demonstrated that the intravitreal application of IL-1β and TNF-α induced retinal oedema, vessel dilatation beading, and the upregulation of microglia in the retina [22].

In previous studies we have shown that bone marrow-derived MSCs are able to differentiate into cells expressing markers and characteristics of corneal epithelial cells [23] or retinal cells [24]. We also observed that mouse MSCs inhibited the acute phase of inflammation in the eye after alkali burn [25], and supported the regeneration of the ocular surface after chemical damage [26]. In this study, we tested the effect of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ on a local cytokine microenvironment and leukocyte infiltration in the retina, and characterized the immunomodulatory potential of MSCs in this inflammatory environment.

Materials and Methods

Mice

Female BALB/c mice (aged 10–16 weeks) were used in the experiments. The mice were obtained from the breeding unit of the Institute of Molecular Genetics of the Czech Academy of Sciences, Prague. The use of animals was approved by the local Animal Ethics Committee of the Institute of Experimental Medicine, Prague.

Isolation, Culture and Purification of MSCs

The bone marrow was flushed out from the femurs and tibias and a single-cell suspension was prepared using a tissue homogenizer. The cells were seeded at a concentration of 4×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY), antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin) and 10 mM HEPES buffer (referred as a complete DMEM) in 75-cm^2 tissue culture flasks (Techno Plastic Products, Trasadingen, Switzerland). The non-adherent cells were washed out after 48 h of cultivation and the adherent cells were cultured at 37 °C in an atmosphere of 5% CO_2 for an additional 2 weeks with a regular exchange of medium and passaging. The cells were harvested in the 3rd passage by incubation with 1 ml of 0.5% trypsin (Sigma-Aldrich) solution for 5 min and then gently scraped. The cell suspension was cultured for 15 min with CD11b MicroBeads and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Contaminating $CD11b^+$ and $CD45^+$ were immunodepleted, using a magnetic activated cell sorter (MACS, Miltenyi Biotec).

Phenotypic Characterization of MSCs by Flow Cytometry

Purified MSCs were washed in a phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), and were incubated for 30 min with the following anti-mouse monoclonal antibodies (mAb): allophycocyanine (APC)-labeled anti-CD44 (clone IM7, BD PharMingen, San Jose, CA), phycoerythrin (PE)-labeled anti-CD73 (cloneTY/11.8, eBioscience, San Diego, CA), PE-labeled anti-CD105 (clone TY/11.8, eBioscience) APC-labeled anti-CD11b (clone M1/70, BioLegend, San Diego, CA), fluorescein isothiocyanate (FITC)-labeled anti-CD45 (clone 30-F11, BioLegend) or PE-labeled anti-CD31 (clone MEC 13.3, BD PharMingen). Cells stained with PE-labeled rat IgG2a (clone RTK2758, BioLegend), APC-labeled rat IgG2b (clone RTK4530, BioLegend) or FITC-labeled rat IgG2b (clone RTK4530, BioLegend) were used as negative controls. Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA) added to the samples 10 min prior to the flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). The morphological characteristics and differentiation potential of purified MSCs have been described in detail elsewhere [27].

Model of Retinal Inflammation and Application of MSCs

Mice were anesthetized by an intramuscular injection of 0.3 ml of a mixture (1:1) of xylazine (15 mg/kg of body weight, Bioveta, Ivanovice, Czech Republic) and ketamine (70 mg/kg, Bioveta). Pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (all purchased from PeproTech, Rocky Hill, NJ) were injected intravitreally in a volume of 4 μl from a stock solution in PBS, containing each cytokine at a concentration of 10 μg/ml. The mice were injected with PBS (control), cytokines or with cytokines plus MSCs (10^4 MSCs per eye). The intravitreal applications were performed using a

Hamilton syringe with volume 5 μl (Hamilton, Reno, NV) and 33G sharp needle (Hamilton). The mice were injected intravitrealy with cytokines and MSCs simultaneously, only by one injection, keeping a total volume of 4 μl.

Preparation of Retinal Explants

Retinal explants were prepared from the eyes as described by Muller [28] and Valdés et al. [29]. In brief, the eyeballs were enucleated from euthanizated mice, the cornea was removed and the remaining of the eyeball was placed into PBS. The lens and vitreous were then removed and the retina was carefully dissected from the sclera.

Cultivation of Retinal Explants with MSCs In Vitro

Retinal explants were cultured alone or in the presence of MSCs in 48-well tissue culture plates (Techno Plastic Products) in 1 ml of RPMI-1640 medium (Sigma-Aldrich), containing 10% of FBS, antibiotics and 10 mM HEPES buffer (referred as a complete RPMI-1640 medium). MSCs were seeded at a concentration 5×10^4 cells/ml, 3 hours before adding retinal explant to the culture. Retinal explants with or without MSCs were cultured unstimulated or stimulated with pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ at a final concentration of 10 ng/ml.

Cultivation of MSCs with Cytokines In Vitro

MSCs were seeded at a concentration 1.5×10^5 cells/ml in 24-well tissue culture plates (Nunc, Roskilde, Denmark) in 1 ml of complete DMEM and were cultured for 48 h alone or with a combination of pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ). The final concentration of cytokines in cultures was 10 ng/ml.

Preparation of Single Cell Suspension from the Retina

The retina was isolated from the eye and a single retinal cell suspension was prepared for flow cytometry analysis. The retina was gently homogenized and digested for 45 min at 37 °C with 1 mg/ml of collagenase I (Sigma) in Hank's buffer. The digestion was stopped by adding an excess of complete RPMI-1640 medium, and the cell suspension was washed three times by centrifugation in a culture medium.

Labeling of MSCs with PKH26 Fluorescent Dye and the Detection of Stained Cells in the Retina

Purified MSCs were labelled with a fluorescent dye PKH26 (Sigma-Aldrich) to determine the content of PHK26-labeled MSCs in the eye. For staining, a final concentration of fluorescent dye was 2 μM for 1×10^6 MSCs/ml for 5 min of incubation at 37 °C. The stained cells were washed three times and immediately used for intravitreal application. To detect distribution of labeled cells, a single cell suspension of the retina was prepared 72 h after intravitreal injection of MSCs. The cell suspension was incubated with APC-labeled anti-CD44 mAb (BD PharMingen) and FITC-labeled anti-CD45 mAb (BioLegend). Distribution of $CD45^-PHK26^+CD44^+$ cells in the retina was assessed by flow cytometry and data were analyzed by FlowJo software.

Flow Cytometry Detection of Immune Cells in the Retina

Single cell suspensions were prepared from the control, cytokine (IL-1β, TNF-α, and IFN-γ, 10 μg/ml)-treated and cytokine plus MSC-treated retinas, washed in PBS containing 0.5% BSA and incubated for 30 min on ice with the following anti-mouse mAb PE-labeled anti-F4/80 (clone BM8, BioLegend), APC-labeled anti-CD80 (clone 16-10A1, BioLegend), APC-labeled anti-CD3(clone 17A2, BioLegend), FITC-labeled and PE-labeled anti-CD45 (clone 30-F11, BioLegend), FITC-labeled CD54 (clone YN1/1.7.4., BioLegend) and APC-labeled anti-CD11b (clone M1/70, BioLegend). Dead cells were stained by Hoechst 33258 fluorescent dye (Invitrogen) and added to the samples 10 min before measurement. Data were detected by LSRII cytometer and analyzed using FlowJo software. Measured events were gated for live cells and then for $CD45^+$ and particular markers.

Detection of Gene Expression by Real-Time Polymerase Chain Reaction (PCR)

The expression of genes in the retina or in MSCs was detected using real-time PCR. The total RNA was extracted by TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. One μg of RNA was treated with deoxyribonuclease I (Promega, Madison, WI) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 μl using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green (Applied Biosystems) as previously described. The sequences of the primers for genes used for amplification are presented in Table 1. The PCR parameters included denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Fluorescence data were collected at each cycle after

Table 1 Murine primer sequences used for real-time PCR

Gene	Sense Primer	Antisense Primer
Gapdh	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
Il-1α	TTGGTTAAATGACCTGCAACA	GAGCGCTCACGAACAGTTG
Il-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
iNos	CTTTGCCACGGACGAGAC	TCATTGTACTCTGAGGGCTGAC
Tnf-α	GCTCCAGTGAATTCGGAAAG	GATTATGGCTCAGGGTCCAA
Vegf	AAAAACGAAAGCGCAAGAAA	TTTCTCCGCTCTGAACAAGG
Cox-2	AGCCCACCCCAAACACAGT	AAATATGATCTGGATGTCAGCACATATT
Hgf	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
Ido	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
Pd-l1	CTACGGTGGTGCGGACTACA	CATGCTCAGAAGTGGCTGCAT
Tgf-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
Pedf	GGACTCTGATCTCAACTGCAAG	AAGTTCTGGGTCACGGTCAG
Ngf	TGGACTGCACGACCACAG	AAATTAGGCTCCCTGGAGGT
Gdnf	GACATCCCATAACTTCATCTTAGAGTC	TCCAACTGGGGGTCTACG

an elongation step at 80 °C for 5 s, and were analyzed using StepOne Software version 2.2.2 (Applied Biosystems).

Cytokine and Nitric Oxide (NO) Production by the Retina from Control and Treated Eyes

Concentrations of NO, IL-6 and TNF-α were measured in supernatants from the cultures of retinal explants from the control, cytokine (IL-1β, TNF-α, and IFN-γ, 10 μg/ml)-treated and cytokine plus MSC (10 000 cells per eye)-treated eyes. Retinal explants were isolated from the control mice and all mice 72 h after the intravitreal application of cytokines and MSCs, and were stimulated in the culture with 1.5 μg/ml of concanavalin A (ConA, Sigma) and 15 μg/ml of lipopolysaccharide (LPS, Sigma). Supernatants were harvested after 72-h incubation. The production of IL-6, TNF-α and VEGF was quantified by ELISA kits purchased from R&D Systems (Minneapolis, MN). The concentrations of NO in the supernatants were evaluated by Griess reaction. To detect for NO, 100 μl of tested supernatant and 100 μl of 1% sulfanilamide and 0.3% N-1-naphthylethylendiamine dihydrochloride (both in 3% H_3PO_4) were mixed and the reaction was quantified by spectrophotometry at 540 nm, using Sunrise Remote ELISA Reader (Tecan, Switzerland).

Statistical Analysis

The results are expressed as the mean ± SD. Comparisons between the two groups were analyzed by Student's *t* test, and multiple comparisons were performed by ANOVA. A value of $p < 0.05$ was considered statistically significant.

Results

Phenotypic Characterization of MSCs

The phenotype and purity of MACS-separated MSCs was characterized by flow cytometry. The cells were negative for leucocyte markers CD11b, CD45 and CD31 (Fig. 1a) and positive for CD44, CD73 and CD105 (Fig. 1b), which are markers attributed to MSCs. In addition, MSCs were adherent to a plastic surface and were able to undergo differentiation to mesodermal line, as we have previously described [27].

Detection of PKH26-Labeled MSCs in the Retina

MSCs were labeled with fluorescent dye PKH26 and the fluorescent intensity was analyzed before their intravitreal administration (Fig. 1c). The presence of $PKH26^+$ MSCs was determined on cell suspensions prepared from the retina. The flow cytometry analysis showed that 15–25% of intravitreally administered MSCs can be detected in the retina 72 h after their application (Fig. 1d).

Effect of MSCs on the Expression of Inflammatory Molecules in the Retina

Mice were injected intravitreally with pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ alone or in a combination with MSCs. The expression of genes for pro-inflammatory molecules IL-1α, IL-6, TNF-α and inducible nitric oxide synthase (iNOS), and for vascular endothelial growth factor (VEGF) was significantly enhanced in the retina 48 h after application of cytokines in comparison with control retina. This increase in the gene expression was significantly

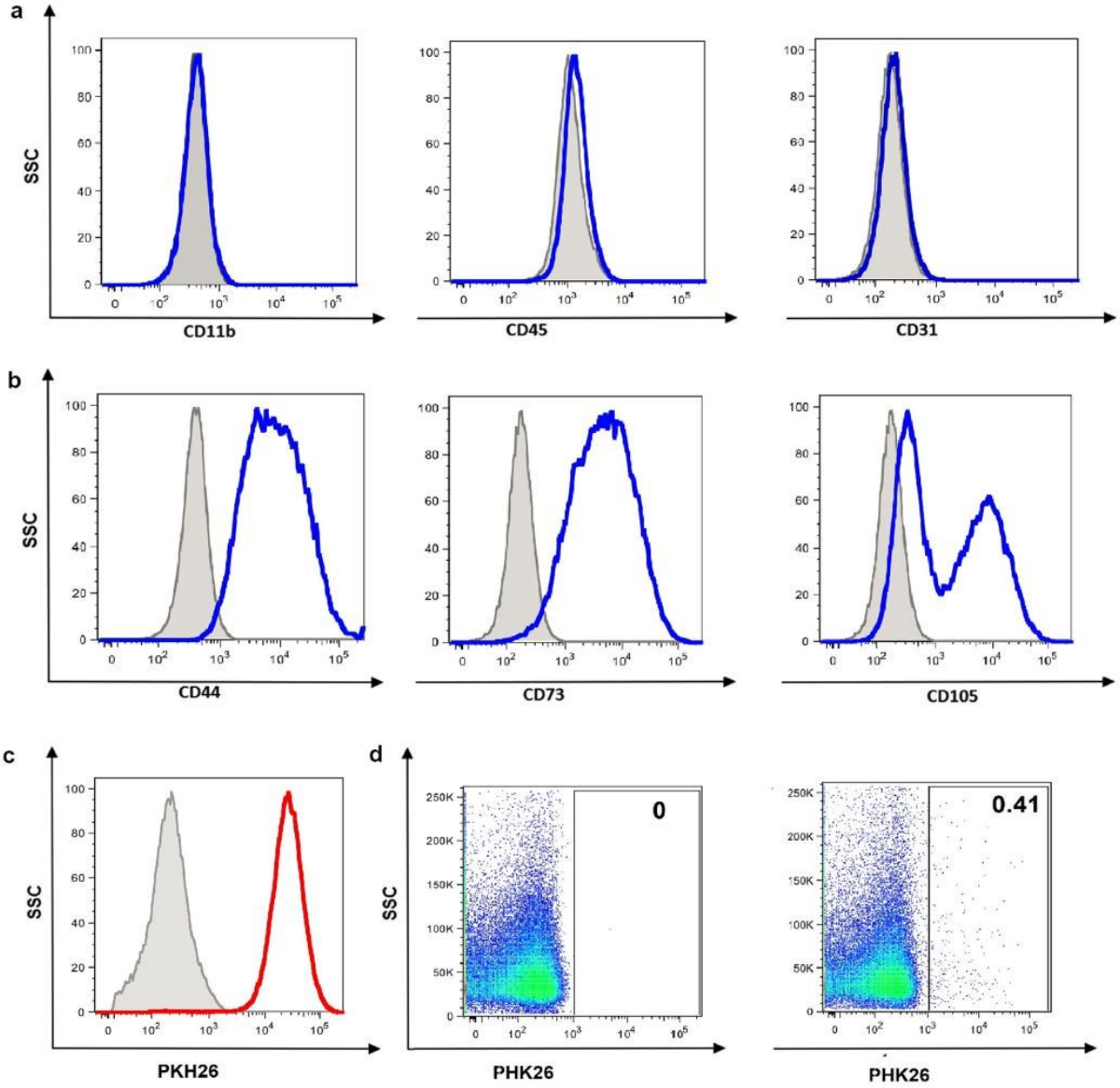

Fig. 1 Phenotypic characterization of MSCs. The cells were separated using MACS and characterized by flow cytometry. Representative histograms indicate the percentage of $CD11b^+$, $CD45^+$, and $CD31^+$ (**a**) or $CD44^+$, $CD73^+$ and $CD105^+$ (**b**) cells among the MSCs. The fluorescence intensity of PKH26-labeled MSCs was analyzed by flow cytometry (red curve) in comparison to unlabelled MSCs (gray-tinted curve) (**c**). Detection of $CD45^-PKH26^+CD44^+$ cells in the retina 72 h after intravitreal application of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (**d**, left) or after application of the cytokines and labelled MSCs (**d**, right). Representative dot plots show the flow cytometry analysis of cell suspension prepared from the retina. One of three similar experiments is shown

attenuated, if the pro-inflammatory cytokines were injected into the eye together with MSCs (Fig. 2).

Production of NO and Cytokines in the Retina after Treatment with MSCs

The concentration of NO, IL-6 and TNF-α were measured in culture supernatants from retinal explants 72 h after intravitreal application of pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ) and MSCs. The production of NO, IL-6 and TNF-α was determined to confirm a decrease in the expression of *iNos*, *Il-6*, *Tnf-α* and *Vegf* genes in the retina after treatment with MSCs. As demonstrated in Fig. 3, the treatment with pro-inflammatory cytokines significantly increased the production of NO, IL-6, TNF-α and VEGF by retinal explants as compared with control retinal explants,

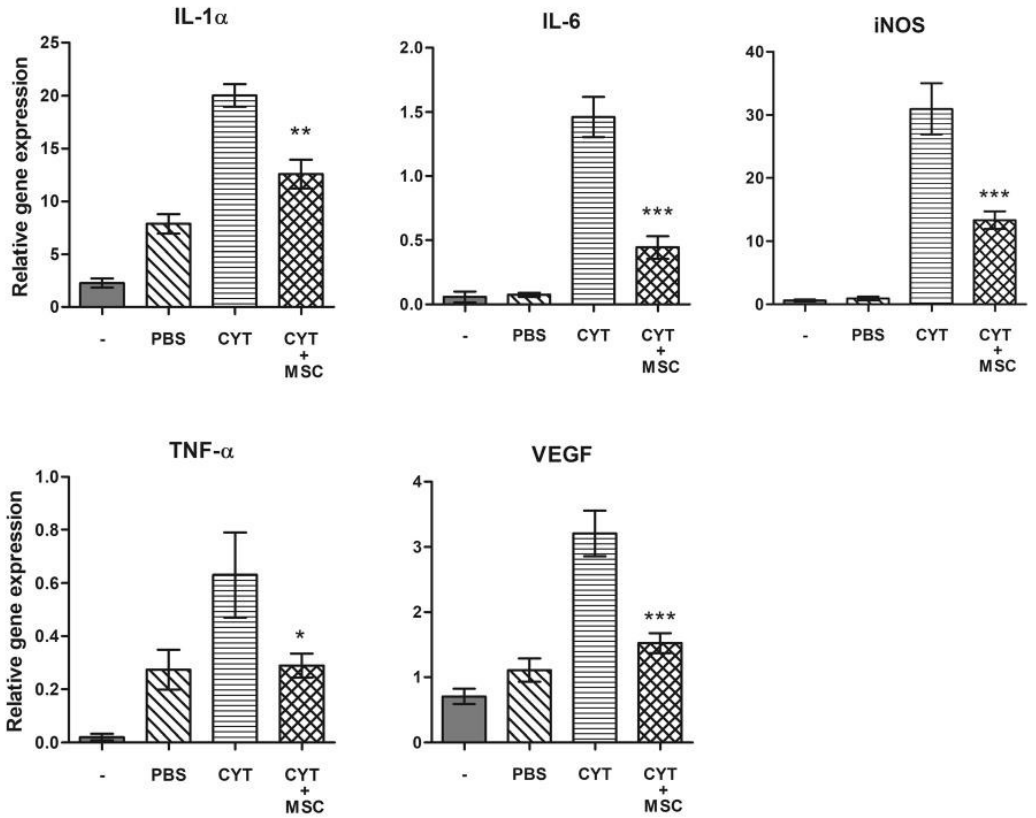

Fig. 2 Effect of MSCs on the expression of inflammatory molecules in the retina. Pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ were injected intravitreally, alone (CYT) or in combination with MSCs (CYT + MSC). The control eyes were without application (−) or were injected with PBS (PBS). The expression of genes for IL-1α, IL-6, TNF-α, iNOS and VEGF was detected 48 h after application of cytokines into the retina (A). Values with asterisks are significantly different ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) from the retina with injected cytokines without MSCs (CYT)

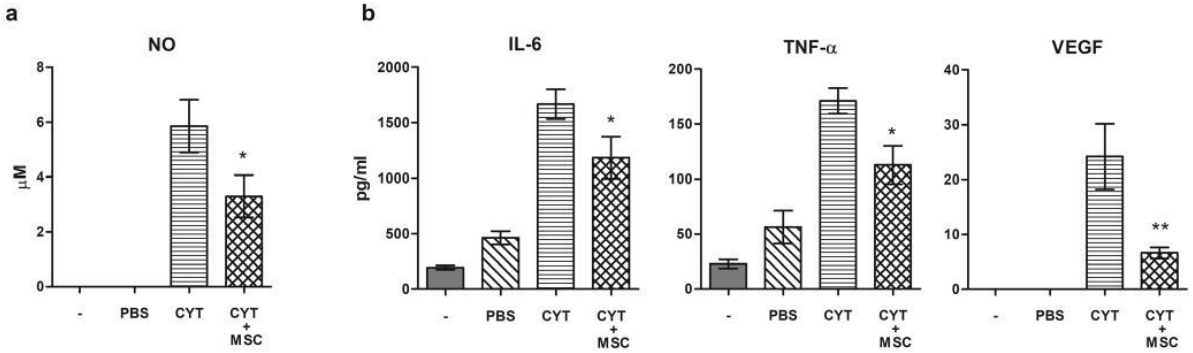

Fig. 3 Production of IL-6, TNF-α, VEGF and NO in the retina treated with cytokines and MSCs. The concentration of IL-6, TNF-α, VEGF and NO were determined in culture supernatants from retinal explants prepared from untreated eyes (−), PBS-treated eyes (PBS), cytokine-treated eyes (CYT) and eyes treated with cytokines and MSCs. Retinal explants were isolated from mice 72 h after the treatment and were stimulated for 72 h with ConA and LPS. The production of IL-6, TNF-α, VEGF and NO was determined by ELISA and Griess reaction, respectively. Each bar represents the mean ± SD from 5 independent determinations. Values with asterisks are significantly different ($*p < 0.05$, $**p < 0.01$) from the retina from the mice injected with cytokines without MSCs (CYT)

and this increase was significantly inhibited in the retina from the eyes treated with MSCs.

Detection of Leukocyte Population in the Retina after Treatment with MSCs

The effect of MSCs on leukocyte population infiltrating the retina was tested by flow cytometry 72 h after intravitreal application of pro-inflammatory cytokines and MSCs. The presence of F4/80$^+$, CD80$^+$, CD11b$^+$, CD3$^+$ and CD54$^+$ was detected in cell suspensions prepared from a cytokine-treated retina and the retina treated with cytokines and MSCs. The percentage of all tested cell types was significantly increased in the retina after the administration of pro-inflammatory cytokines and this increase was significantly decreased after the intravitreal application of MSCs (Fig. 4).

Effect of MSCs on Retinal Explants in the Presence of Pro-inflammatory Cytokines In Vitro

Retinal explants were cultured alone, in the presence of pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ, each at a final concentration 10 ng/ml) or with the cytokines and MSCs. After 48-h cultivation, retinal explants were harvested and the expression of genes for inflammatory molecules was detected by real-time PCR. As demonstrated in Fig. 5, the expression of genes for IL-1α, IL-6, TNF-α and iNOS was significantly increased in the presence of pro-inflammatory cytokines, as compared with control retina, and this increase was significantly inhibited in the presence of MSCs.

The Expression of Genes for Immunomodulatory Molecules and Growth Factors in MSCs

To further characterize the immunomodulatory and secretory potential of MSCs, MSCs were cultured for 48 h unstimulated, or stimulated with a combination of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ. As demonstrated in Fig. 6, the expression of genes for immunomodulatory molecules cyclooxygenase 2 (COX-2), programmed cell death-ligand 1 (PD-L1), indoleamine 2,3-dioxygenase (IDO) and IL-6, and for neurotrophic factors pigment epithelium-derived factor (PEDF), nerve growth factor (NGF) and glial cell derived neurotrophic factor (GDNF)

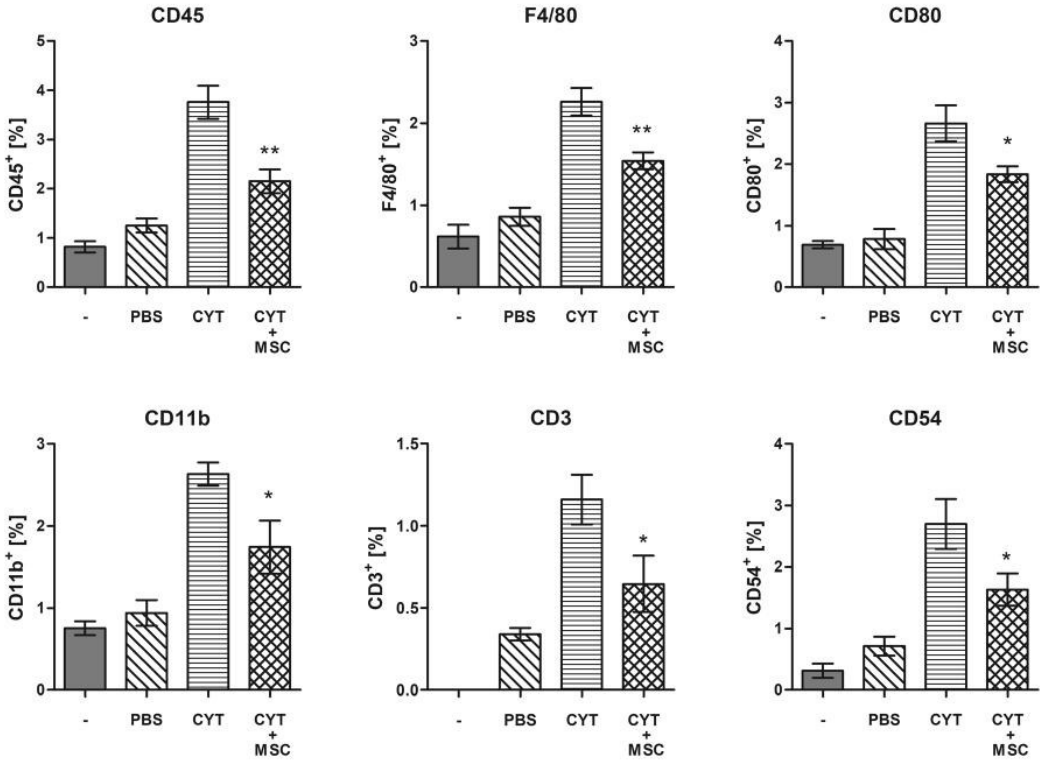

Fig. 4 Flow cytometry analysis of leukocyte populations infiltrating the retina after the intravitreal application of cytokines and MSCs. The effect of MSCs on the leukocyte population was tested 72 h after the intravitreal application of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (CYT) or cytokines in combination with MSCs (CYT + MSC). The control eyes were untreated (−) or were injected with PBS (PBS). The percentage of CD45$^+$, F4/80$^+$, CD80$^+$, CD11b$^+$, CD3$^+$ and CD54$^+$ was analyzed in cell-suspension prepared from the retina after the exclusion of dead cells and gating for CD45$^+$ cells. Each bar represents the mean ± SD from 5 independent determinations. Values with asterisks are significantly different (*$p < 0.05$, **$p < 0.01$) from the retinas with injected cytokines (CYT)

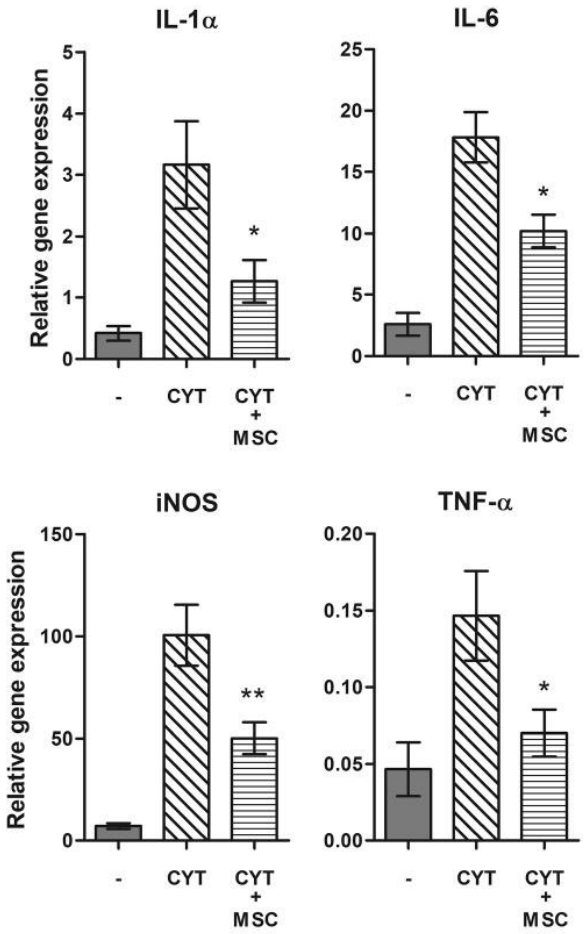

Fig. 5 Immunosuppressive effects of MSCs in vitro. Retinal explants were cultured untreated (−), in the presence of pro-inflammatory cytokines (CYT) or with cytokines and MSCs (CYT + MSC). The expression of genes for pro-inflammatory molecules IL-1α, IL-6, TNF-α and iNOS was detected in the retinal explants by real-time PCR after a 48-h cultivation. Each bar represents the mean ± SD from 5 independent determinations. Values with asterisks are significantly different (*$p < 0.05$, **$p < 0.01$) from the retina cultured with cytokines (CYT)

was enhanced after stimulation of MSCs with cytokines. On the other hand, the expression of genes for TGF-β and HGF was decreased in the stimulated MSCs (Fig. 6).

Discussion

Undesirable immune reactions in the retina are suppressed in a variety of ways, including immunological privilege, production of numerous inhibitory molecules, blood-retinal barrier and by the expression of inhibitory molecules on retinal cells which prevent the infiltration of cells of the immune system. The disruption of this immune balance in the eye occurs in pathological conditions, such as glaucoma, AMD or DR. This process causes the production of pro-inflammatory cytokines, chemokines, penetration of blood-retinal barrier and infiltration with cells of the immune system [30, 31]. Increased levels of pro-inflammatory cytokines IL-1β, TNF-α or IFN-γ have been found in vitreous of patients with DR or AMD [1, 17, 18]. It has been shown that the intravitreal administration of pro-inflammatory cytokines induced pathological changes in the retina resembling processes, occurring during retinal inflammatory disease [22].

In this study, we have characterized the effects of MSCs on immunological processes in the retina in a mouse model of intravitreally applicated pro-inflammatory cytokines. Initially, we found an increased expression of genes for pro-inflammatory molecules IL-1α, IL-6, iNOS, TNF-α and for VEGF, in the retina after the intravitreal administration of pro-inflammatory cytokines. However, a simultaneous injection of MSCs and cytokines into the eye resulted in a significant inhibition of expression of the genes for all of the tested pro-inflammatory molecules. Similarly, Huang et al. [32] have shown that the production of IL-1β and TNF-α in an untreated light-damaged retina was higher than in the retina treated with MSCs. Transplanted MSCs produced neurotrophic factors and inhibited retinal cell apoptosis. A decreased production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α was also demonstrated after

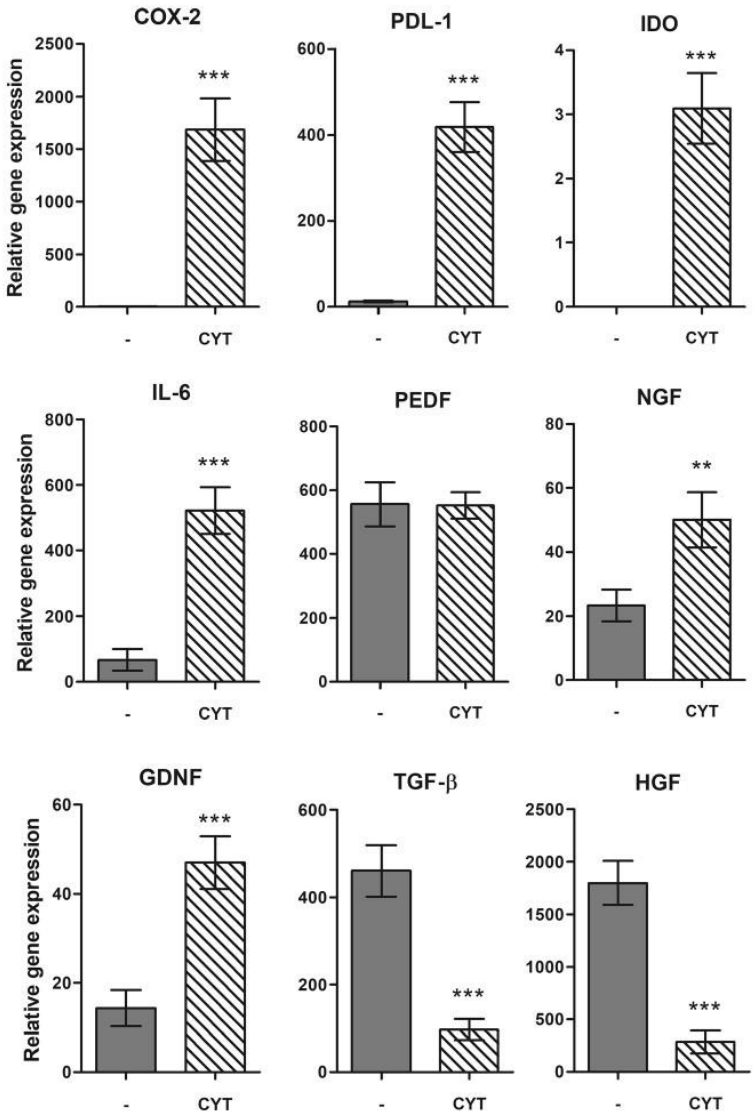

Fig. 6 The expression of genes for immunomodulatory molecules and growth factors by MSCs. MSCs were cultured unstimulated (−) or with a combination of cytokines IL-1β, TNF-α and IFN-γ (CYT). The expression of genes for HGF, IL-6, TGF-β, COX-2, PD-L1, IDO, PEDF, NGF and GDNF. Each bar represents the mean ± SD from 5 independent determinations. Values with asterisks are significantly different (**$p < 0.01$, ***$p < 0.001$) from unstimulated MSCs (−)

the intravitreal administration of MSCs in a rat retinal ischemia model [33].

The pro-inflammatory molecules can be produced by the damaged retinal cells, endogenous immune cells or by infiltrating exogenous cells of the immune system. It has been shown that increased secretory activity of the endogenous immune cells, such as microglia and Müller cells, is a major source of the majority of factors which play a pivotal role in the initiation and propagation of the neurodegenerative processes [34–36]. Consequently, the retina is infiltrated by cells of the immune system, such as macrophages, monocytes, neutrophils or T cells [1, 16, 17]. We observed the increase in $CD45^+$ cell population in the retina after the intravitreal application of pro-inflammatory cytokines. The main detected population in the retina was macrophages ($F4/80^+$, $CD11b^+$ cells) which can produce pro-inflammatory molecules such as IL-1β, IL-6, TNF-α and iNOS [16]. It has been shown that during degenerative retinal diseases, the activated macrophages are the main infiltrating cell population in the retina and they support the inflammation reaction [16, 37]. After the intravitreal injection of pro-inflammatory cytokines, we also detected a small population of T cells ($CD45^+CD3^+$ cells), enhanced expression of costimulatory molecules CD80 (mainly on macrophages) and an increased expression of ICAM-1 (CD54) molecules, in the retina. ICAM-1 is adhesion molecules enable cell migration and infiltration. Increased levels of this molecule have been found in the eyes of

patients with DR and AMD [38, 39]. We showed that the co-application of pro-inflammatory cytokines and MSCs caused a significant decrease in all tested leukocyte populations and in the expression of ICAM-1 molecule.

To characterize the mechanism of MSC-mediated immunosuppression after its application into the eye, we established a model of the co-cultivation of retinal explants with MSCs in vitro. We observed similar effects as in vivo, the expression of genes for IL-1α, IL-6, TNF-α and iNOS was enhanced after the stimulation of retinal explants with pro-inflammatory cytokines. The expression of genes for all these inflammatory molecules was decreased after the addition of MSCs into the cultures. A similar result has been shown in a model of retinal ganglion cells, damaged in vitro by hydrogen peroxide, where production of pro-inflammatory cytokines was reduced after cultivation with MSCs [40].

It is recognized that MSCs require stimulation with cytokines or other pro-inflammatory signals, for the activation and production of immunomodulatory molecules [41, 42]. To identify molecules that could be responsible for the suppression of production of inflammatory molecules in the retina and for the beneficial effects of MSCs, we cultured MSCs unstimulated or in the presence of IL-1β, TNF-α and IFN-γ. We found that the expression of genes for immunomodulatory molecules IDO, COX-2, PD-L1 and IL-6, and for neurotrophic factors NGF, GDNF, PEDF, was significantly enhanced in cytokine-stimulated MSCs. PD-L1 is an inhibitory molecule that prevents the infiltration of lymphocytes to the retina [43, 44], COX-2 is involved in the biosynthesis of prostaglandin E_2 [45] and IDO molecules interfere with the bioactivity of tryptophan [46]. All these molecules are involved in the inhibition of proliferation and function of T cells and macrophages [41, 47]. The enhanced expression of neurotrophic factors can promote the regeneration of damaged retinal tissue. We detected fluorescent labeled MSCs in the retinas 72 h after their intravitreal application, so they can influence the cells in the diseased retina directly by the production of immunomodulatory molecules and growth factors.

In conclusion, we have shown the immunomodulatory properties of MSCs after their intravitreal application to the inflamatous environment of the eye caused by the administration of pro-inflammatory cytokines. MSCs decrease the number of immune cells infiltrating the retina and inhibit production of pro-inflammatory molecules by retinal cells. These results suggest that MSCs represent a promising choice of cell population for the stem cell-based therapy of retinal degenerative diseases.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable national and institutional guidelines for the care and use of animal were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and was approved by the local Animal Ethics Committee of the Institute of Experimental Medicine.

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5.4 Antiapoptotic Properties of Mesenchymal Stem Cells in a Mouse Model of Corneal Inflammation

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Stem Cells Dev. 2021; 30(8): 418-427

LSCD is one of the most severe injuries in the cornea. The immune privilege of the cornea is lost, immune cells migrate to the cornea, and chronic inflammation occurs. Such processes can lead to neovascularization, tissue scarring, lowering the opacity of the cornea, and, in the end, the loss of vision. Apoptosis is one of the mechanisms causing corneal epithelium cells to die directly by damage from injury or the inflammatory environment. Chronic inflammation is also associated with ER stress, which also promotes apoptosis. In addition, more pro-inflammatory molecules are produced by infiltrating immune cells like T cells and macrophages to the damaged tissue site.

Multiple cell type is being evaluated as appropriate cell therapy for severe corneal injuries like LSCD. In this study, we used murine BM-MSCs because of their known immunomodulatory, migratory, and anti-apoptotic properties and the production of multiple growth factors. Although the mechanism of anti-inflammatory properties of MSCs was well described, the mechanism of their anti-apoptotic effects is still unknown.

MSCs were cultured for three weeks and separated them by immunodepleting of $CD11^+$ and $CD45^+$ cells; their purity was checked by the presence of CD44, CD73, CD105, and absence of CD11b and CD45. We measured the gene expression for immunomodulatory molecules and growth factors such as COX-2, IDO, PD-L1, IL-6, TGF-β, FGF, IGF-I, and HGF, which can play a role in anti-apoptotic effects. The expression of immunomodulatory molecules and IGF-I was elevated after stimulation by pro-inflammatory cytokines (IL-1β, IFN-γ, and TNF-α).

We used our *in vivo* murine model of the severe chemical injury, and its therapy by MSC seeded on a nanofiber scaffold and the gene expression of typical apoptotic markers was measured: downregulated BCL-2 and upregulated BAX and p53. After the injury, the expression of BCL-2 was significantly decreased, and the expression of BAX with p53 increased significantly. After the treatment of the injured ocular surface by MSCs, the

expression of BCL-2 was significantly increased in the treated cornea than in the injured one, and the expression of p53 and BAX genes was decreased.

We used our new *in vitro* model to elucidate the mechanism of these effects of MSCs on the damaged cornea. Excised corneas were cultured alone or with seeded MSCs directly, indirectly through inserts, or with supernatants from stimulated MSCs. These corneas were treated with pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ. Then, the gene expressions of BCL-2, BAX, p53, p21, activating transcription factor 4 (ATF4), and binding immunoglobulin protein (BIP) were measured by RT-PCR. We observed the effect of MSCs on all corneas cultured directly and indirectly with MSCs or treated just by supernatants. Flow cytometry was used to measure the percentage of apoptotic cells in these corneal explants, and we observed the same effect. We showed that the anti-apoptotic effect of MSCs on the injured cornea is paracrine and that it could be one of the mechanisms of their therapeutic action.

Aspirant's contribution: *In vivo* and *in vitro* model setting and development, surgery, samples and data analysis, interpretation, and manuscript writing.

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Antiapoptotic Properties of Mesenchymal Stem Cells in a Mouse Model of Corneal Inflammation

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Mesenchymal stem cells (MSCs) represent a population of adult stem cells that have potent immunoregulatory, anti-inflammatory, and antiapoptotic properties. In addition, they have ability to migrate to the site of inflammation or injury, where they contribute to the regeneration and healing process. For these properties, MSCs have been used as therapeutic cells in several models, including treatment of damages or disorders of the ocular surface. If the damage of the ocular surface is extensive and involves a limbal region where limbal stem cell reside, MSC therapy has been proved as the effective treatment approach. Although the anti-inflammatory properties of MSCs have been well characterized, mechanisms of antiapoptotic action of MSCs are not well recognized. Using a chemically damaged cornea in a mouse model, we showed that the injury decreases expression of the gene for antiapoptotic molecule Bcl-2 and increases the expression of proapoptotic genes *Bax* and *p53*. These changes were attenuated by local transplantation of MSCs after corneal damage. The antiapoptotic effect of MSCs was tested in an in vitro model of co-cultivation of corneal explants with MSCs. The apoptosis of corneal cells in the explants was induced by proinflammatory cytokines and was significantly inhibited in the presence of MSCs. The antiapoptotic effect of MSCs was mediated by paracrine action, as confirmed by separation of the explants in inserts or by supernatants from MSCs. In addition, MSCs decreased the expression of genes for the molecules associated with endoplasmic reticulum stress Atf4, Bip, and p21, which are associated with apoptosis. The results show that MSCs inhibit the expression of proapoptotic genes and decrease the number of apoptotic cells in the damaged corneas, and this action might be one of the mechanisms of the therapeutic action of MSCs.

Keywords: cornea, mesenchymal stem cells, antiapoptotic properties, Bax, Bcl-2, mouse model

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Introduction

Injury of the cornea is associated with a degenerative process in the corneal epithelium and apoptosis of the corneal epithelial cells. Among the most important factors inducing the apoptosis of corneal cells are the activation of endoplasmic reticulum (ER) stress-induced molecules, a local inflammatory reaction, and the production of several proinflammatory cytokines by corneal and immune cells. It has been shown that cells of the damaged cornea produce proinflammatory molecules, such as interleukin-lα (IL-1α), IL-1β, and tumor necrosis factor-α (TNF-α) [1,2]. It has been demonstrated that increased levels of proinflammatory cytokines lead to apoptosis in in vivo and in vitro models [3,4].

Chronic inflammation of the ocular surface is associated with ER stress, which also promotes the induction of apoptosis [5]. Simultaneously, the site of injury is infiltrated with cells of the immune system, which produce various chemokines and cytokines that can potentiate the inflammatory and apoptotic reactions and attract other immune cells to the site of injury. Therefore, the inhibition of a local inflammatory reaction and alleviation of apoptosis might be a promising approach for the treatment of corneal damages and for the support of corneal epithelium regeneration. In this respect, stem cell-based therapy has proven to be a perspective treatment for severe ocular disorders, especially in cases where the limbal region is destroyed and the endogenous limbal stem cells, which are responsible for corneal regeneration, are missing or are nonfunctional.

Among the various stem cell types that have been tested so far, mesenchymal stem cells (MSCs) turned out to be a perspective candidate. These cells have potent immunoregulatory, secretory, differentiation, and antiapoptotic properties [6–8]. Furthermore, MSCs can be obtained relatively easily from the patient, and thus can be used as own (autologous) cells. In addition, MSCs are the producers of numerous growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor

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1 (IGF-1), hepatocyte growth factor (HGF), or transforming growth factor-β (TGF-β), which contribute to corneal healing and regeneration [9–11].

Therefore, due to their anti-inflammatory properties, secretion of growth factors, and antiapoptotic properties, MSCs are a perspective candidate for the stem cell-based therapy of corneal injuries or disorders. Although the MSC-based therapy for corneal injuries or diseases has not been approved for clinical use, the data from experimental models are very encouraging, and MSCs have been successfully used for the treatment of corneal injuries or limbal stem cell deficiencies in various models in mice, rats, and rabbits [12–15].

While the anti-inflammatory properties of MSCs have been well described in various models [6,14], less information is available about the mechanisms of the antiapoptotic action of MSCs. Using an experimental model of a chemically injured cornea, we hereby demonstrated that corneal damage induces changes in the expression of genes associated with apoptosis and the transplantation of MSCs alleviated these changes. To analyze the mechanisms of the antiapoptotic effect of MSCs, we established an in vitro model of co-cultivation of corneal explants in the presence of MSCs. The apoptosis in the explants was induced by proinflammatory cytokines and was decreased in the presence of MSCs. Finally, we demonstrated a paracrine mechanism of the antiapoptotic action of MSCs.

Materials and Methods

Animals

Female BALB/c mice 10–16 weeks of age were used in the experiments. The animals were obtained from the Institute of Molecular Genetics of the Czech Academy of Sciences, Prague. The use of animals was approved by the Local Ethical Committee of the Institute of Experimental Medicine of the Czech Academy of Sciences, Prague.

Isolation and cultivation of MSCs

MSCs were isolated from the femurs and tibias of the mice. The bone marrow was flushed out and a single-cell suspension was prepared by homogenization. The cells were seeded in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum (Gibco BRL, Grand Island, NY), antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Sigma-Aldrich), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma-Aldrich) in 75 cm^2 tissue culture flasks (techno plastic products; Trasadingen, Switzerland). After 48 h of incubation, the nonadherent cells were washed out and the adherent cells were cultured at 37°C in an atmosphere of 5% CO_2 with a regular exchange of the medium and passaging of the cells to maintain their optimal concentration. The cells were harvested after the third passage approximately after 2 weeks of cultivation with 1 mL of 0.5% trypsin (Sigma-Aldrich) for 5 min and by gently scraping.

Purification and characterization of MSCs

The suspension of MSCs was incubated for 15 min with CD11b and CD45 Microbeads (Miltenyi Biotec, Belgisch Gladbach, Germany) according to the manufacturer's instructions. $CD11b^-$ and $CD45^-$ cells were isolated by a magnetic activated cell sorter (AutoMACS; Miltenyi Biotec). Purified cells were washed in phosphate-buffered saline (PBS) containing 0.5% of bovine serum albumin and were incubated for 30 min with anti-mouse monoclonal antibodies: allophycocyanin (APC)-labeled anti-CD44 (clone IM7; BD PharMingen, San Jose, CA), phycoerythrin (PE)-labeled anti-CD73 (cloneTY/11.8; eBioscience, San Diego, CA), PE-labeled anti-CD105 (clone TY/11.8; eBioscience), APC-labeled anti-CD11b (clone M1/70; BioLegend, San Diego, CA), and fluorescein isothiocyanate-labeled anti-CD45 (clone 30-F11; BioLegend). Cells stained with PE-labeled rat IgG2a (clone RTK2758; BioLegend), APC-labeled rat IgG2b (clone RTK4530; BioLegend), or fluorescein isothiocyanate-labeled rat IgG2b (clone RTK4530; BioLegend) were used as negative controls. Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA) added to the samples 10 min before the flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). The ability of MSCs to differentiate to adipocytes and osteoblasts was tested as we described [16].

Preparation of supernatants from stimulated MSCs

Purified MSCs were cultured in a concentration of 2×10^5 cells per well in 24-well tissue culture plates (TPP) in 1 mL of complete DMEM. Cells were stimulated by a mixture of proinflammatory cytokines IL-1β, interferon-γ (IFN-γ), and TNF-α (purchased from Peprotech, Rocky Hill, NJ), each cytokine in a final concentration 10 ng/mL. After a 48-h incubation, MSCs were washed several times with an excess of medium to remove added cytokines and cultured for an additional 48 h in a fresh culture medium without cytokine stimulation. Supernatants were harvested and the cell debris was removed by centrifugation (8 min, 2000 g) and stored at −80°C.

Cultivation of MSCs with cytokines in vitro

Purified MSCs were cultured in 24-well TPP with 1 mL of complete DMEM in a concentration 1×10^5 cells per well. Cells were stimulated by proinflammatory cytokines IL-1β, IFN-γ, and TNF-α (a cocktail of cytokines, each in a final concentration of 10 ng/mL). After a 48-h cultivation, the cells were harvested and tested for the expression of genes for immunoregulatory molecules and growth factors.

In vivo model of chemically induced corneal injury

A model of a chemically impaired ocular surface established in our laboratory [15,17] was used to study the expression of genes associated with apoptosis. In brief, the mice were anesthetized by an intramuscular injection of 1:1 mixture of xylazinum hydrochloridum 2% (0.175 mL; Rometar; Spofa, Prague, Czech Republic) and ketaminum hydrochloridum 5% (0.175 mL; Narkamon; Spofa). The right corneas of the anesthetized mice were treated with 0.25 N sodium hydroxide (NaOH) using 8 μL of NaOH on the cornea-size filter paper, attached for 20 s on the cornea. The eye was rinsed with an excess of PBS. To test the effect of MSCs on gene expression in the damaged cornea, MSCs were transferred onto the

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damaged eye surface using a nanofiber scaffold, as we have described in detail elsewhere [15,17]. The corneas were excised 7 days after corneal injury and immediately transferred into 500 μL of TRI Reagent® [Molecular Research Centre (MRC), Cincinnati, OH] and stored at −80°C.

Induction of apoptosis in corneal explants

The mice were sacrificed, and the central cornea (without limbus) was excised from the enucleated eye. Corneal explants were cultured in 24-well TPP in 1 mL of complete DMEM for 48 h unstimulated or stimulated with IL-1β, IFN-γ, and TNF-α in a concentration 10 ng/mL or with H_2O_2 in a final concentration of 50 μM (multiple concentrations were tested to establish an apoptosis model in stimulated corneas). H_2O_2 represents an apoptosis inducer [18] and was used as a positive standard during establishment of the model.

Cultivation of corneal explants with MSCs or MSC supernatant

MSCs (1×10^5 cells/well) were seeded in 24-well TPP and cultured in 500 μL of complete DMEM to adhere. After a 24-h incubation, the cells were washed with an excess of medium and 1 mL of fresh complete DMEM was added. The corneal explants were cultured unstimulated or stimulated, with proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α) or with previously seeded MSCs directly, in insert (NUNC CC Insert, 0.4 μm PC; Thermo Fisher Scientific Nunc A/S, Roskilde, Denmark) or with MSC supernatant (500 μL of previously prepared supernatant and 500 μL of fresh complete DMEM).

Production of IL-6, IGF-1, and HGF by cytokine-stimulated or cytokine-pretreated MSCs

MSCs were stimulated with IL-1β, IFN-γ, and TNF-α and supernatants were harvested after a 48-h incubation. The cells were washed several times with the excess of medium and the cells were incubated for another 48 h in a fresh medium without cytokines. Supernatants obtained after the first stimulation, and after prolonged cultivation, were tested for the presence of IL-6, HGF, and IGF-1 using enzyme-linked immunosorbent assay kits purchased from R & D Systems (Minneapolis, MN).

Detection of apoptotic corneal cells by flow cytometry

The excised cornea was cultured for 48 h untreated or with proinflammatory cytokines, cut into small pieces, and digested with collagenase II (Sigma-Aldrich) in a concentration of 1 mg/mL in Hank's balanced salt solution for 50 min at 37°C. The digestion was stopped by the addition of an excess of complete DMEM. The cell suspension was centrifuged (8 min, 250 g) and single cells were washed in PBS. The cells were stained for Annexin V using an Annexin V apoptosis detection kit (Apronex, Jesenice, Czech Republic) according to the manufacturer's protocol. Dead cells were discriminated using Hoechst 33258 fluorescent dye (Sigma-Aldrich), added to the samples 15 min before the flow cytometry analysis. Data were collected using an LSRII cytometer (BD) and analyzed using FlowJo software (LLC, Ashland, OR).

Detection of gene expression by real-time polymerase chain reaction

The expression of genes in the cornea and MSCs was detected using real-time polymerase chain reaction (RT-PCR). Total RNA was extracted by TRI reagent (MRC) according to the manufacturer's instructions. The total RNA (1 μg) was treated by deoxyribonuclease I (DNase I; Promega, Madison, WI) in a DNase I buffer (Promega) and used for reverse transcription. The first cDNA strand was synthesized by random primers (Promega) using M-MLV reverse transcriptase (Promega) in a total reaction volume of 25 μL. Quantitative RT-PCR was performed by StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR green (Applied Biosystems). The primers used for amplification are shown in Table 1. The quantitative PCR parameters included denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Fluorescence data were collected at each cycle after the elongation

Table 1. Sequences of Oligonucleotides Used in Real-Time Polymerase Chain Reaction

Gene	*Forward primer*	*Reverse primer*
Atf-4	CGGGTGTCCCTTTCCTCTTC	TGAAGAGCGCCATGGCTTAG
Bax	GTGAGCGGCTGCTTGTCT	GGTCCCGAAGTAGGAGAGGA
Bcl-2	AGTACCTGAACCGGCATCTG	GGGGCCATATAGTTCCACAAA
bFGF	CGGCTCTACTGCAAGAACG	TGCTTGGAGTTGTAGTTTGACG
Bip	GTGTGTGAGACCAGAACCGT	CAGTGAACTTCATCATGCCG
Cox-2	AGCCCACCCCAAACACAGT	AAATATGATCTGGATGTCAGCACATATT
Gapdh	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
HGF	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
IDO	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
IGF-1	TCGGCCTCATAGTACCCACT	ACGACATGATGTGTATCTTTATTGC
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
p21	CTTGCACTCTGGTGTCTG	CTTGGAGTGATAGAAATCTGTCA
p53	GTATTTCACCCTCAAGATCC	TGGGCATCCTTTAACTCTA
PD-L1	CTACGGTGGTGCGGACTACA	CATGCTCAGAAGTGGCTGGAT
TGF-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG

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step at 80°C for 5 s. The relative gene expression was analyzed using StepOne Software 2.3 (Applied Biosystems). A relative quantification model was applied to calculate the expression of the target gene in comparison to glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH).

Statistical analysis

The results are expressed as the mean ± standard deviation. Comparisons between the two groups were analyzed by Student's *t*-test or in the case of multiple comparisons by analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

Results

Phenotypic characterization of MSCs

The phenotype of MACS-separated MSCs was characterized by flow cytometry. The cells were positive for CD44, CD73, and CD105 and negative for hematopoietic markers CD11b and CD45 (Fig. 1). The cells had typical fibrocyte-like morphology, were adherent to a plastic surface, and were able to differentiate to adipocytes and osteoblasts, as we have described elsewhere [19].

The production of immunomodulatory molecules and growth factors by MSCs

To characterize the antiapoptotic potential of MSCs, the cells were cultured for 48 h unstimulated, or in the presence of a mixture of proinflammatory cytokines IL-1β, IFN-γ, and TNF-α, which can be detected in corneal injury. The expression of genes for immunomodulatory molecules cyclooxygenase-2 (COX-2), indolamine 2,3-deoxygenase (IDO), IL-6, and programmed death-ligand 1 (PD-L1), and IGF-1 was significantly increased in the presence of proinflammatory cytokines. On the contrary, the expression of genes for FGF, HGF, and TGF-β was decreased after stimulation with cytokines (Fig. 2).

The concentrations of IL-6, HGF, and IGF-1 proteins were measured in supernatants after a 48-h cultivation of MSCs with proinflammatory cytokines, and after a 48-h consequent cultivation of MSCs in fresh medium without cytokines. After primary stimulation, the concentrations of IL-6 and IGF-1 were significantly increased, while the concentration of HGF was decreased. This pattern of cytokine production was preserved, if the cell cultures were washed and the cells were cultured for another 48 h without cytokines (Fig. 3) for the preparation of supernatants to test the possible paracrine effects of MSCs on the expression of genes associated with apoptosis.

Expression of genes associated with apoptosis in injured corneas and in corneas treated with MSCs

The ocular surface was chemically burned and treated with MSCs on a nanofiber scaffold. The scaffold was removed 3 days after the injury and the corneas were excised on day 7 and analyzed by RT-PCR. As demonstrated in Fig. 4, the expression of gene for Bcl-2 was significantly decreased in injured corneas and this decrease was significantly inhibited after treatment with MSCs. On the contrary, the expression of genes for Bax and p53 was increased in injured corneas and this increase was diminished in corneas treated with MSCs (Fig. 4).

Expression of apoptotic genes in corneal explants cultured in the presence of proinflammatory cytokines

To confirm the published observations that proinflammatory cytokines induce apoptosis in corneal cells [3,4], the corneal explants were cultured with IL-1β, IFN-γ, and TNF-α, or with H_2O_2 as an inductor of apoptosis [18]. After a 48-h cultivation, the expression of genes for antiapoptotic molecule Bcl-1 and for proapoptotic molecules Bax and p53 was determined by RT-PCR. As demonstrated in Fig. 5, the expression of *Bcl-2* gene was significantly inhibited in the presence of proinflammatory cytokines or H_2O_2, while the expression of *Bax* and *p53* genes was increased in the presence of both proinflammatory cytokines and H_2O_2 (Fig. 5).

The effect of MSCs on the expression of genes for molecules associated with apoptosis in the corneal explants

The corneal explants were cultured untreated, with proinflammatory cytokines, with cytokines and MSCs, either directly or in inserts, and with the supernatant from MSCs to test the antiapoptotic properties of MSCs. After a 48-h cocultivation, the corneal explants were transferred to a TRI reagent, and the expression of genes for molecules associated with apoptosis or with ER stress was determined by RT-PCR. The expression of gene for antiapoptotic molecule Bcl-2 was decreased after the stimulation by proinflammatory cytokines in comparison with the untreated explants. This decrease was

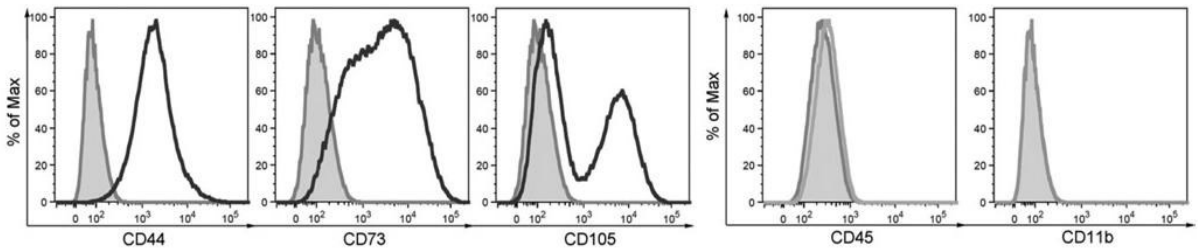

FIG. 1. Phenotypic characterization of purified MSCs. The cells were separated by MACS and the expression of CD44, CD73, CD105, CD11b, and CD45 markers was assessed by flow cytometry. One of three similar experiments is shown. MACS, magnetic activated cell sorter; MSC, mesenchymal stem cell.

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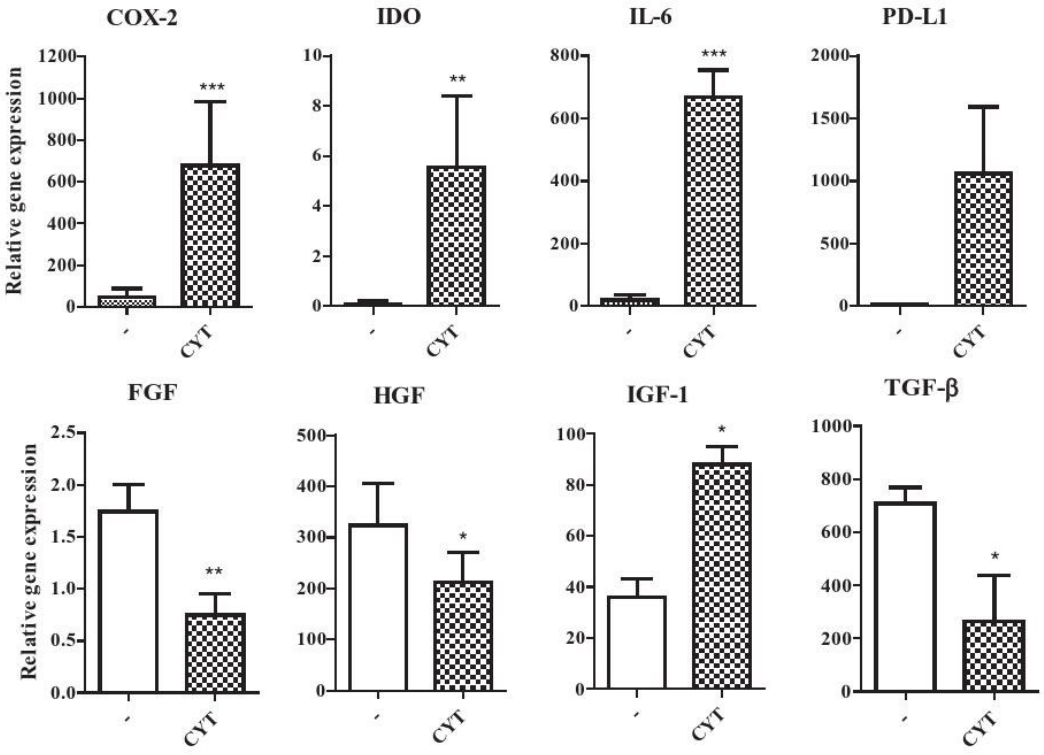

FIG. 2. The expression of genes for immunomodulatory molecules and growth factors by MSCs. MSCs were cultured unstimulated (–) or stimulated with a mixture of proinflammatory CYT IL-1β, TNF-α, and IFN-γ. The expression of genes for COX-2, IDO, IL-6, PD-L1, bFGF, HGF, IGF-1, and TGF-β was determined by RT-PCR. Each bar represents the mean + SD from three independent determinations. Values with *asterisks* are statistically different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from unstimulated MSCs (–). bFGF, basic fibroblast growth factor; COX-2, cyclooxygenase-2; CYT, cytokines; HGF, hepatocyte growth factor; IDO, indolamine 2,3-deoxygenase; IFN-γ, interferon-γ; IGF-1, insulin-like growth factor 1; IL, interleukin; PD-L1, programmed death-ligand 1; RT-PCR, real-time polymerase chain reaction; SD, standard deviation; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

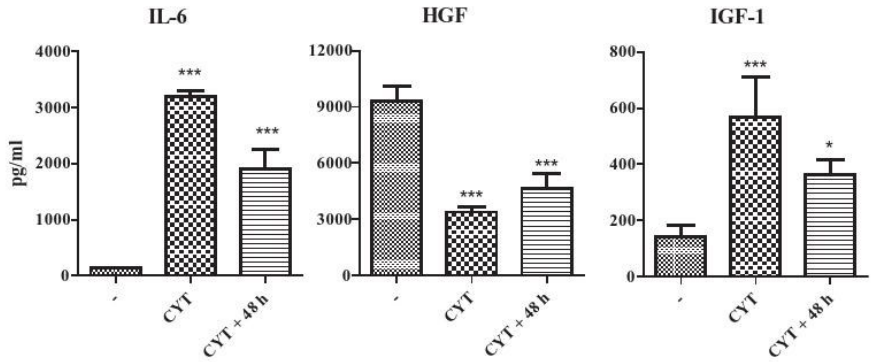

FIG. 3. Production of IL-6, HGF, and IGF-1 by MSCs. MSCs were cultured unstimulated (–), stimulated for 48 h with a mixture of proinflammatory CYT or stimulated with CYT, and then washed and cultured for another 48 h without CYT (CYT +48). The production of IL-6, HGF, and IGF-1 was measured by ELISA. Each bar represents the mean + SD from five independent determinations. Values with *asterisks* are statistically different (*$P < 0.05$, ***$P < 0.001$) from unstimulated MSCs (–). ELISA, enzyme-linked immunosorbent assay.

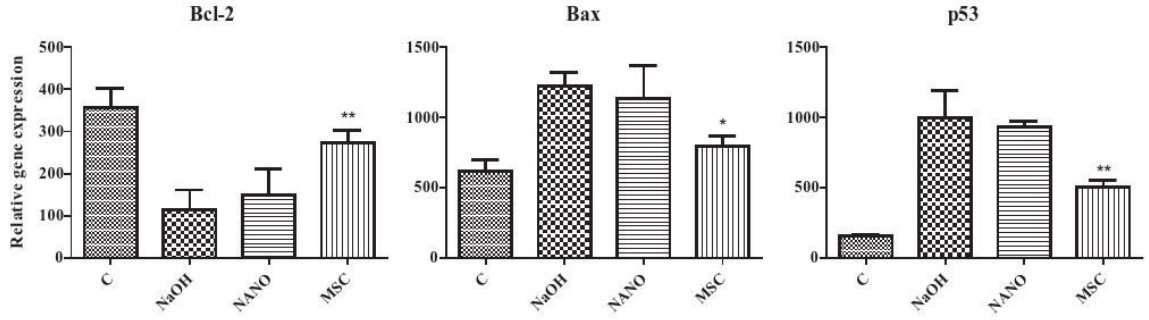

FIG. 4. Antiapoptotic effect of MSCs on chemically burned cornea. Control eyes (C) were left untreated, chemically burned corneas were treated with NaOH for 20 s without additional treatment (NaOH) or were treated by nanofiber scaffold without MSCs (NANO) or with nanofiber scaffold with MSCs (MSC). The expression of genes for Bcl-2, Bax, and p53 was determined by RT-PCR. Each bar represents the mean + SD from six mice. Values with *asterisks* are statistically different ($*P < 0.05$, $**P < 0.01$) from control untreated corneas.

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inhibited, if the explants were stimulated in the presence of MSCs, either directly or in insert, or even in the presence of supernatant from MSCs (Fig. 6A). The expression of proapoptotic *Bax* and *p53* genes was increased in the presence of proinflammatory cytokines (in comparison with the untreated explants) and this enhanced expression was diminished in the presence of MSCs or supernatants from MSCs (Fig. 6B). Furthermore, the expression of genes for molecules associated with ER stress (Atf4, Bip, and, P21) was enhanced after cultivation of the corneal explants with proinflammatory cytokines, and this increase was significantly inhibited by MSCs (Fig. 6C).

The inhibition of apoptosis of corneal cells by MSCs

The corneal explants were cultured for 48 h in the presence of proinflammatory cytokines with or without MSCs (or with supernatants from MSCs) and then homogenized and digested by collagenase II to obtain single-cell suspensions. A flow cytometry analysis was used to detect phosphatidylserine on the surface of cells by annexin V, and to determine the percentage of apoptotic cells. As demonstrated in Fig. 7, the percentage of apoptotic cells was increased after cultivation of the explants with proinflammatory cytokines. The percentage of apoptotic cells was significantly decreased in the presence of MSCs. This effect of MSCs was observed, even when the MSCs were separated from the explant in inserts or if the explants were stimulated with cytokines in the presence of supernatants from MSCs (Fig. 7).

Discussion

Corneal injuries or diseases are one of the main causes of a decreased quality of vision or even blindness. These disorders are associated with a local inflammatory reaction and with a loss of epithelial cells and keratocytes. Any damage of the cornea is accompanied by the production of stress proteins, secretion of proinflammatory cytokines and chemokines, and with infiltration with cells of the immune system. Increased levels of proinflammatory cytokines such as IL-1α, IL-1β, TNF-α, and IFN-γ were found in injured corneas in animal models and in patients [20–22]. The proinflammatory cytokines can be produced by cells of the cornea and by infiltrating immune cells [1,2,23,24].

It has been shown in several in vitro and in vivo models that proinflammatory cytokines induce apoptosis of cells in the site of injury [3,4] and thus negatively influence the healing and regenerative processes. Therefore, a therapeutic strategy should involve the inhibition of an inflammation reaction and the prevention of apoptosis.

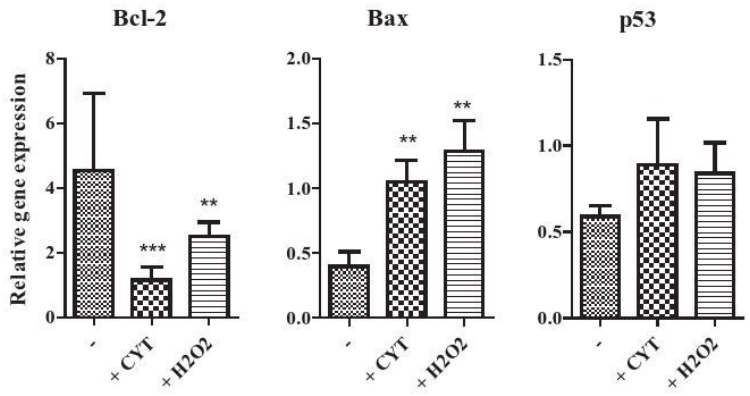

FIG. 5. Expression of genes for proapoptotic and antiapoptotic molecules in the corneas cultured with CYT or H_2O_2. Corneal explants were cultured for 48 h without (–) or with proinflammatory CYT IL-1β, TNF- and IFN-γ (+CYT), or were treated with H_2O_2 (+H_2O_2). The expression of genes for Bcl-2, Bax, and p53 was determined by RT-PCR. Each bar represents the mean + SD from five independent determinations. Values with *asterisks* are statistically different ($**P < 0.01$, $***P < 0.005$) from unstimulated corneas.

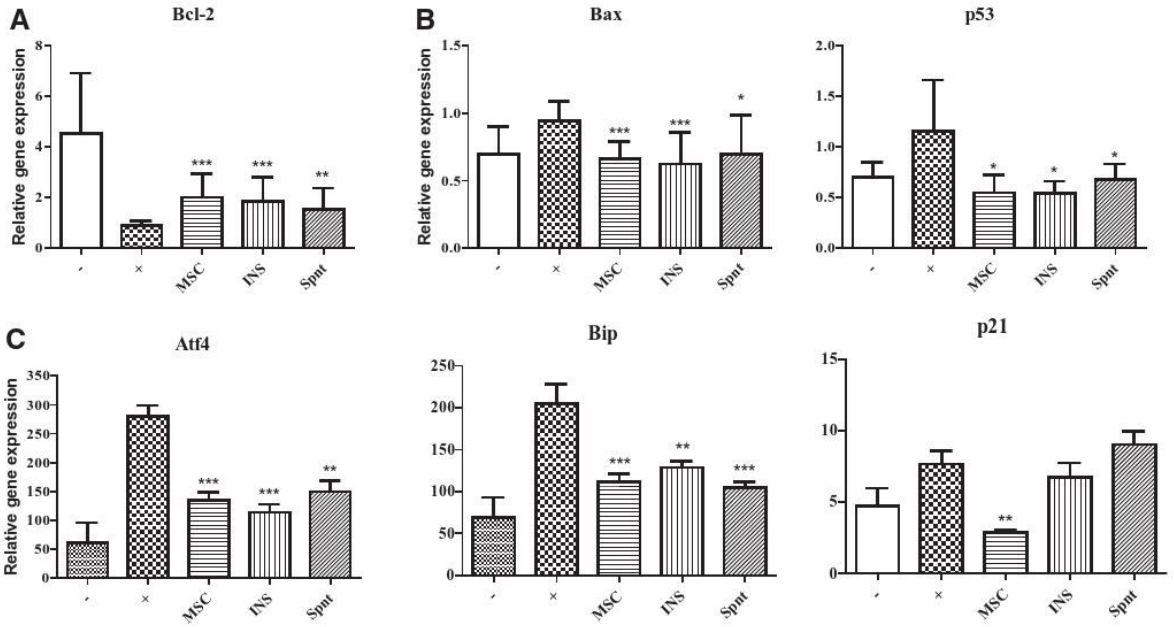

FIG. 6. Antiapoptotic effect of MSCs on stimulated corneal explants in vitro. Corneal explants were cultured for 48 h unstimulated (−), stimulated by IL-1β, TNF-α, and IFN-γ (+) in the presence of MSCs directly (MSC) or in insert (INS), or were stimulated in the presence of supernatant from cultures of MSCs (Spnt). **(A)** The expression of genes for antiapoptotic molecule Bcl-2, **(B)** proapoptotic molecules Bax, p5, and **(C)** molecules associated with endoplasmic reticulum stress Atf4, Bip, and p21 was measured by RT-PCR after a 48-h cultivation. Each bar represents the mean + SD from four independent determinations. Values with *asterisks* are statistically different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from stimulated corneas (+).

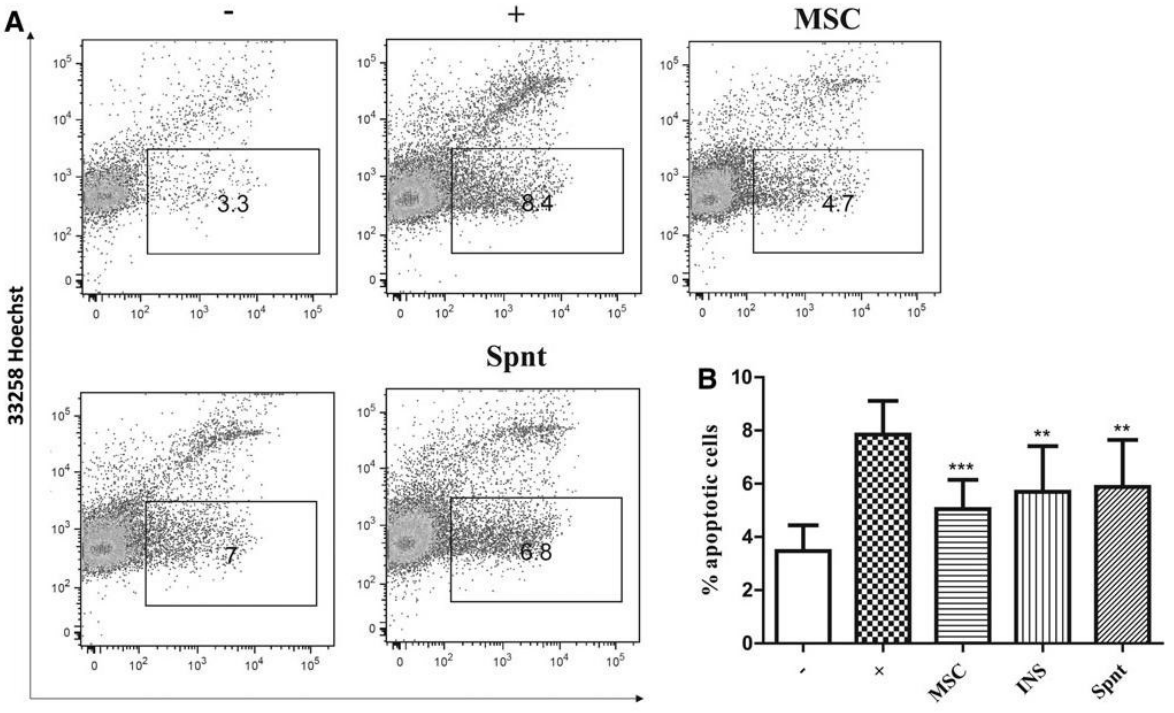

FIG. 7. The inhibition of apoptosis in the cornea by MSCs. Excised corneas were cultured for 48 h unstimulated (−), stimulated with proinflammatory CYT IL-1β, TNF-α, and IFN-γ (+) in the presence of MSCs directly (MSC), or in insert (INS). Corneas were also cultured stimulated and treated by MSC supernatants (Spnt). Single-cell suspensions were prepared by enzymatic digestion from corneas and the percentage of apoptotic cells was measured using flow cytometry by detection of Annexin V. **(A)** Representative *dot* plots indicate the percentage of apoptotic cells in the cornea. One representative experiment of three similar ones is shown. **(B)** The percentage of apoptotic cells measured by flow cytometry. Values with *asterisks* are significantly different (**$P < 0.01$, ***$P < 0.001$) from stimulated corneas (+). Each bar represents the mean + SD from five independent determinations.

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Using a model of corneal damage by alkali burn, we hereby demonstrated that the injury induces an enhanced expression of genes for proapoptotic molecules Bax and p53 and a decrease in the expression of gene for antiapoptotic molecule Bcl-2. These molecules have been used in multiple studies of apoptosis to determine the impacts of antiapoptotic therapy [25,26], and in the study of apoptosis in a model of dry eye in mice [27]. We also showed that the changes in the expression of genes for molecules associated with apoptosis were significantly inhibited by the treatment of injured corneas with MSCs. The ability of MSCs to inhibit a proinflammatory reaction and a harmful inflammation in the damaged cornea has been described [13,14]. Thus, MSCs turned out to be a perspective cell type, not only for the suppression of an inflammatory reaction but also for the attenuation of apoptosis.

To analyze the mechanism of the antiapoptotic effect of MSCs, we established a model of co-cultivation of corneal explants with MSCs in vitro. The apoptosis was induced in the explants by their cultivation for 48 h in the presence of proinflammatory cytokines IL-1β, TNF-α, and IFN-γ. As shown previously [3,4], these cytokines induce changes in the expression of genes associated with apoptosis (*Bcl-2, Bax,* and *p53)* and can induce apoptosis of corneal cells. Furthermore, we showed that the cultivation of corneal explants with proinflammatory cytokines induced the enhanced expression of genes for molecules Atf4, Bip, and p21, which are associated with ER stress. It has been shown that ER stress induced by proinflammatory cytokines enhanced the expression of the Bip gene in human corneas [5]. The level of Atf4 was also elevated in the study of keratoconus in stress conditions, and in the model of induced oxidative stress [28,29]. P21 plays a role in cell survival, but its expression is elevated in induced ER stress, while protecting cells against apoptosis and can be used as an ER stress marker [30,31]. We showed that the expression of genes for all these molecules is enhanced in corneal explants cultivated with proinflammatory cytokines, and that the increase is inhibited in the presence of MSCs.

To characterize the antiapoptotic effect of MSCs in more detail, corneal explants were cultured with proinflammatory cytokines and MSCs either directly or MSCs were separated in the inserts. Furthermore, MSCs were replaced by a supernatant obtained after the stimulation and cultivation of MSCs. The results showed that the antiapoptotic effect of MSCs was preserved if the direct contact of MSCs and explants was prevented, or if the MSCs were replaced by their supernatant. These observations suggest that antiapoptotic effects of MSCs are mediated by the paracrine action of MSCs.

To extend the study on the gene expression level, the corneal explants were cultured with proinflammatory cytokines in the absence or presence of MSCs, and the number of apoptotic corneal cells was determined by flow cytometry. We found that proinflammatory cytokines significantly increased the number of apoptotic cells and that MSCs inhibited this decrease. Again, the effect of MSCs was mediated by paracrine action.

The immunosuppressive properties of MSCs and their ability to modulate immune reactions have been well documented [6–8]. To characterize molecules that could play a role in the antiapoptotic effects of MSCs in the cornea, we cultured MSCs unstimulated or stimulated with proinflammatory cytokines. We observed an elevated expression of genes for IDO, COX-2, PD-L1, and IL-6 after a 48-h stimulation. MSCs also produce several growth factors, such as HGF, TGF-β, basic FGF (bFGF), IGF-1, or EGF, which could be involved in their antiapoptotic and therapeutic effect. The expression of some of these factors (TGF-β, HGF, and bFGF) was decreased after stimulation with proinflammatory cytokines, while the expression of IGF-1 was increased. It has been shown that IDO, COX-2, and PD-L1 play a role in the inhibition of an immune response [32]. This effect might also be responsible for the inhibition of proapoptotic cytokines. IGF-1 plays a role in corneal regeneration and can inhibit apoptosis in corneal cells [33]. IL-6 is a multifunctional molecule, which is involved in both inflammatory and anti-inflammatory reactions, and is able to inhibit apoptosis through signal transducer and activator of transcription 3 activation [34]. IL-6 can also contribute to the inhibition of inflammation by increasing secretion of prostaglandin E2 [35] or by the induction of regulatory T cells in combination with TGF-β [16]. HGF facilitates corneal epithelial cell migration and proliferation, and protects corneal cells against apoptosis [36]. HGF also inhibits a myofibroblast phenotype in the cornea [37], and it has been shown that HGF enhances cell survival under oxidative stress in myocardial infarction [38]. Therefore, numerous factors produced by MSCs spontaneously or in an inflammatory environment can contribute to the antiapoptotic effects of these cells.

Conclusion

In conclusion, we showed that corneal injury or the incubation of corneal explants with proinflammatory cytokines induces the enhanced expression of proapoptotic genes and increases the number of apoptotic corneal cells. These effects can be inhibited by MSCs that attenuate the expression of proapoptotic genes in a paracrine way. We suggest that the inhibition of apoptosis is therefore one of the mechanisms of therapeutic action of MSCs.

Author Disclosure Statement

No competing financial interests exist.

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6. DISCUSSION

MSCs are a perspective source of stem cells for cell-based therapy of various diseases and disorders. Using *in vitro* and *in vivo* models, many reports have shown they have immunomodulatory, anti-apoptotic, and regenerative properties that support their therapeutic use in the future. Furthermore, MSCs can differentiate into other cell types under certain conditions, after the stimulation with pro-inflammatory molecules and after the treatment by various growth factors. In this thesis, we have focused mainly on the abilities of MSCs, which could be used to treat retinal diseases and severe corneal injuries and conditions such as LSCD. I want to discuss on the following pages the suitability and methods of their transplantation into the eye based on our results from the last years and the shift of knowledge about MSCs therapy in the past years.

Corneal damage is one of the most frequent eye injuries leading to vision impairment and blindness. When the surface of the eye is damaged, regeneration of the corneal epithelium is mediated by LSCs that differentiate into corneal epithelial cells. However, if the limbus is also damaged, LSCs can be obtained from the patient's healthy eye and transplanted onto the damaged cornea. Several studies have demonstrated successful corneal regeneration after the transplantation of LSCs to the damaged surface of the eye. The transplantation of LSCs cultivated from the contralateral healthy cornea is the only certified cell therapy method for LSCD (Pellegrini et al., 2014; Rama et al., 2010). This therapy can be used only in the case of unilateral LSCD. Transplantation of allogeneic LSCs from a donor is associated with a risk of graft rejection, and patients must obtain systemic immunosuppressive drugs with many side effects (Serna-Ojeda et al., 2020). Considering the significant differentiation and immunomodulatory properties of MSCs, we concluded that in these cases, they could be used as a replacement for allogeneic cells and thus reduce the risk of rejection of the transplanted cells or the entire transplanted corneal graft. In addition, in such cases, MSCs could differentiate into missing corneal cells and inhibit inflammatory processes at the site of damage.

First, we focused on differentiating BM-MSCs into corneal-like cells and showed the role of IGF-I in the mechanism of differentiation. Murine MSCs were isolated from bone marrow, cultured for three weeks, and purified using a magnetic cell sorter. Purified MSCs

were co-cultivated with extracts prepared from excised murine corneas alone or stimulated by IGF-I. We used a real-time quantitative polymerase chain reaction to detect the relative expression of typical corneal markers such as K12, keratocan, and lumican. We observed the increase of relative gene expression of corneal markers in treated MSCs after ten days of cultivation and stimulation with a significant difference in corneas treated by IGF-I, which plays a role in the corneal regeneration by LSCs (Trosan et al., 2012). The production of one of the typical corneal epithelial cells marker, K12, was also determined by immunostaining. Such treated cells remained immunosuppressive, as we showed by culturing them with stimulated splenocytes. Numerous studies also showed the differentiation capability of MSCs in the corneal-like cells (Gu et al., 2009; Rohaina et al., 2014) while supporting corneal regeneration and the inhibition of neovascularization (Liu et al., 2012).

In recent years, the theory of MSCs differentiating into corneal cells and replacing epithelial cells or even LSCs seems to have been surpassed. Several studies showed that MSCs in the cornea and other tissues do not survive for long periods of time, especially in a pro-inflammatory environment (Liu et al., 2012; Preda et al., 2021). Their most significant benefit in therapy will be the production of immunomodulatory molecules and immunosuppressive action on their surroundings. That will give them sufficient time to produce growth factors, promote healing of the damaged cornea, and help the survival of local stem cells, which can gradually restore the damaged corneal epithelium. As well as suppress inflammation and associated tissue damage. In the case of corneal transplantation, MSCs may increase the chance of transplantation success and help corneal re-epithelialization with original or transplanted LSCs (Li et al., 2014; Martínez-Carrasco et al., 2019). The differentiation of MSCs into cells expressing corneal markers could primarily support the prolonged survival of these cells in the target tissue. In addition, these differentiated MSCs could produce EVs adapted to target cells of injured tissue and provide more effective transport of immunomodulatory molecules and trophic factors (Haque et al., 2015).

MSCs are also used in the treatment of damaged retina, but currently, there is still no effective treatment for some retinal diseases. Degenerative retinal diseases are often associated with the loss of specialized cells in the retina. It was shown that MSCs isolated from various tissues could differentiate into multiple retina cell types. Under *in vitro* conditions, MSCs were differentiated into cells expressing photoreceptor markers after treatment by adding taurine (Nadri et al., 2013). A similar effect *in vitro* was observed after

the stimulation of cells by activin A, EGF, and taurine (Kicic et al., 2003). The possibility of the influence of the retinal environment on the differentiation of MSC was shown by the co-cultivation of human BM-MSCs with isolated RPE. BM-MSCs expressed typical RPE marker RPE65 after differentiation (Mathivanan et al., 2015). Also, transplanted MSCs into the retinal injury environment are influenced by pro-inflammatory molecules and released molecules from damaged retinal cells. In our study, we were interested how this inflammatory environment is affecting the differentiation ability of transplanted cells. Therefore, our *in vitro* model of the inflammatory environment of the damaged retina was created from the extract prepared from excised murine retinas and supernatants from stimulated lymphocytes. After seven days of co-culturing MSCs with retinal extract and lymphocyte supernatant, they expressed markers typical for various retinal cells like bipolar cells, Muller cells (glial cells), photoreceptors, and horizontal retina cells, and RPE cells. This ability to express various retinal markers could be crucial in treating a retinal injury where multiple cell types are damaged. These findings were consistent with previous reports of MSCs differentiation in bipolar cells (Karakaş et al., 2020), photoreceptors (Kicic et al., 2003; Nadri et al., 2013), or RPE cells (Huo et al., 2010; Huang et al., 2012; Mathivanan et al., 2015). We focused mainly on the expression of rhodopsin which was the most significantly increased in our model. Rhodopsin expression was more elevated in the culture with lymphocyte supernatant than in the culture with retinal extract only. As previously described, MSCs are activated by pro-inflammatory cytokines like IL-1β, TNF-α, and IFN-γ (Gao et al., 2016; Krampera et al., 2006).

To elucidate what molecule plays a role in the differentiation of MSCs under these conditions, we focused on stimulating MSCs by supernatants from separated B cells, T cells, or macrophages. We found out that the increased expression of rhodopsin is present only in cells treated with T cell supernatant and retinal extract. Using a panel of different cytokines produced by T cells, we found that rhodopsin expression is preserved only in cells treated with retinal extract and IFN-γ. To confirm this mechanism, we added an anti-IFN-γ antibody to the culture of MSCs with lymphocyte supernatant and retinal extract. The expression of rhodopsin was significantly decreased. The role of IFN-γ was previously shown as responsible for the differentiation abilities of MSCs into neuron-like cells and that the IFN-γ plays a role in neuronal differentiation (Croitoru-Lamoury et al., 2011; Turbic et al., 2011; Wong et al., 2004).

On the contrary, Croitoru-Lamoury et al. have shown that IFN-γ is responsible for the decreased ability of MSCs to differentiate into adipocytes and osteoblasts (Croitoru-Lamoury et al., 2011). Other studies also demonstrated that differentiated MSCs produced an increased amount of GDNF, TGF-β, NGF, and IL-6, typical neurotrophic factors produced by MSCs (Yu et al., 2006; Turbic et al., 2011; Mead et al., 2016). The differentiation, in this case, could play a role more in the prolonged survival of MSCs after the transplantation and in better incorporation to target tissue than in replacing damaged cells. The more significant effect of MSCs would be their paracrine action and support of surviving cells (Hill et al., 2009; Moghadasi et al., 2021). Other studies showed the beneficial effects of injection of conditioned media from cultured MSCs (Dreixler et al., 2014; Roth et al., 2016).

In the following study, we focused on treating the retinal injury with BM-MSCs, using their immunomodulatory properties. For this experiment, we developed a novel *in vitro* model, which we used to study the mechanism of MSCs therapy for the injured retina and cornea. In this model, we have found that pro-inflammatory molecule production by retinal cells are inhibited after co-cultivation of MSCs and excised retinas in an inflammatory environment. We have also shown that MSCs express typical immunomodulatory molecules (IDO, IL-6, COX-2, and PD-L1) and neurotrophic factors (GDNF, NGF, PEDF) in a simulated pro-inflammatory environment. Moreover, we also analyzed the immunomodulatory properties of mouse BM-MSCs after their intravitreal injection into the inflammatory environment of the eye, caused by the application of pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ. The pro-inflammatory stimulation *in vivo* increased gene expression of IL-1α, IL-6, TNF-α, iNOS, and VEGF in murine retinas. Transplanted MSCs significantly decreased the expressions of all of them 48 hours after the transplantation. Also, the production of IL-6, TNF-α, VEGF, and NO in the retina treated with cytokines was significantly inhibited after MSC treatment. As shown before (Cruz-Guilloty et al., 2013), we also detected an increased number of infiltrating immune cells in the inflammatory environment of the retina with the majority of macrophages. In eyes treated with MSCs, the infiltration was significantly reduced. A similar effect was shown in the study of ischemic retinal injury in a rat (Mathew et al., 2017). Lipophilic membrane dye PKH26-stained MSCs were detected in retinas 72 hours after application. However, the survival of MSCs in target tissue remains a question. Many studies show that MSCs can survive after allogeneic or even xenogeneic transplantation for weeks as they are

considered immune-privileged (or immune-evasive) cells because of non-expression of MHC class II and low expression of MHC class I (Ankrum et al., 2014; Pittenger et al., 2019).

On the contrary, other studies show that *in vitro* expanded MSCs cannot survive in the host after i.v. injection for an extended period (Eggenhofer et al., 2012) or even when multiple administrations were compared (Preda et al., 2021). MSCs were undergoing apoptosis in the first days after transplantation. Relatively new regenerative and immunomodulatory mechanism of MSCs could play a role in this case. EVs or microvesicles could be products of these apoptotic cells and prolong the effect of original MSCs (Jafarinia et al., 2020).

Finally, we focused on the anti-apoptotic properties of MSCs transplanted onto the injured ocular surface. *In vivo*, chemically burned corneas have already been used in our laboratory to study the treatment of the inflammation in the damaged cornea with MSCs, i.v. injected into the tail. MSCs not retained in other organs migrated preferentially to the damaged eye (Javorkova et al., 2014). Another study compared the effect of LSCs and MSCs in treating injured cornea in a rabbit model. MSCs and LSCs transplanted onto the surface of the eye using nanofiber scaffold showed comparable therapeutic and regenerative results (Holan et al., 2015). Our preliminary results also showed that BM-MSCs, cultured together with mouse corneal explants in a pro-inflammatory cytokine environment, inhibit the expression of inflammatory cytokines by corneal cells. Thus, this *in vitro* model can be used to study mechanism of MSCs in the treatment of corneal injury as well.

The corneal cells are exposed to chronic inflammation in a severe injury such as LSCD. As previously described, pro-inflammatory cytokines like IL-1β, TNF-α, and IFN-γ can induce cell apoptosis *in vitro*. It was also shown that apoptosis is associated with a shift in the expression ratio of the anti-apoptotic gene BCL-2 and pro-apoptotic genes BAX and p53 (Grunnet et al., 2009; Yang et al., 2019). The damage to cells and apoptosis is preceded by ER stress (Woodward et al., 2020b). We used *in vivo* model of the chemically injured cornea and its treatment by MSC transplantation on a nanofiber scaffold. After the injury, the gene expression of BCL-2 was significantly decreased, and the expression of BAX and p53 increased, which correlated with previous studies of apoptosis in the cornea. After the treatment, BCL-2 expression was significantly elevated, and MSCs also inhibited the expression of pro-apoptotic BAX and p53. To elucidate the anti-apoptotic properties of MSCs in the damaged cornea, we prepared an *in vitro* model of inflammation-induced

apoptosis in corneal cells. Excised murine corneas were cultured alone or with MSCs, unstimulated or stimulated by IL-1β, TNF-α, and IFN-γ. MSCs and corneas were co-cultured directly in the transwell system, or we used just the supernatant from stimulated MSCs. We observed the inhibition of pro-apoptotic molecules and molecules associated with ER stress in corneas treated with MSC directly and indirectly or treated with supernatant alone. This paracrine anti-apoptotic action of MSCs was also confirmed by flow cytometry analysis of cell suspensions from cultured corneas. We also detected the expression of immunomodulatory molecules and growth factors in stimulated MSCs, which could play a role in such anti-apoptotic action. IGF-I plays a role in corneal regeneration and can inhibit apoptosis in corneal cells in combination with HGF (Miyagi et al., 2018b; Yanai et al., 2006). HGF and IGF-I also prevent apoptosis in myocardial infarction (Zhang et al., 2015). MSCs also produce IL-6 after stimulation, which is responsible for inhibiting apoptosis through STAT3 activation (Liu et al., 2010). Other growth factors and molecules produced by MSCs could play a role in regeneration and helping corneal and limbal cells to survive.

Doubts about the survival of MSCs in target tissue may point to another mechanism of their anti-apoptotic effect, which is consistent with our results. The paracrine effect may be caused by dying MSCs affecting their surroundings by the EVs, which could transport cytokines, growth factors, or other immunomodulatory molecules and have anti-apoptotic properties (Mathew et al., 2017; Zhu et al., 2018). MSCs-derived EVs significantly suppressed ER stress and apoptosis in the corneal endothelium (Buono et al., 2021).

Although many issues are still to be resolved before using MSCs in clinical application, the number of clinical studies indicates significant progress in MSC research. However, the numbers are much lower when it comes to the use of MSCs in the treatment of retinal and corneal diseases. Nevertheless, their immunomodulatory, regenerative, and even differentiation properties and the possibility of obtaining them from an autologous source represent a suitable alternative for treating degenerative eye diseases and visual impairment.

MSCs will not be possibly used to replace tissue-resident cells or stem cells. Still, thanks to their properties, they could be used as support in various transplantations to help with possible GvHD, suppress inflammation, inhibit immune response, and immune cell infiltration. Their mechanism of action seems to be more paracrine; in some cases, MSCs can be replaced by a conditioned medium from stimulated MSCs. It contains growth factors, or EVs capable of enhancing tissue regeneration and cell survival or directly impacting

immune cell differentiation to regulatory types and inhibiting the action of effector immune cells. MSC therapy is also suitable for patients without any alternative therapeutic options.

7. CONCLUSIONS

This presented dissertation thesis is based on four articles. Three of them are co-authored, and one is the first-author article. Their results can be summarized as follows:

- **MSCs produce multiple immunomodulatory molecules and trophic factors in the inflammatory environment of the injured eye**

  We have confirmed that MSCs stimulated by pro-inflammatory cytokines like IL-1β, TNF-α, and IFN-γ, which are present in the inflamed tissue, express several immunomodulatory molecules like PD-L1, IL-6, IDO, iNOS, COX-2, TSG-6, and growth/trophic factors like HGF, TGF-β, GDNF, NGF, FGF and more. All of them play a role in immunosuppression, the anti-apoptotic mechanism at the site of the injury, and the faster regeneration of the tissue.

- **Differentiation of MSCs in the cells expressing corneal markers**

  MSCs differentiate in the cells expressing typical corneal markers, including cytokeratin K12, while co-culturing with corneal extracts and one of the main corneal regenerative factors, IGF-I. Differentiated MSCs keep their immunomodulatory abilities.

- **MSCs are able to differentiate into the cells expressing retinal markers**

  MSCs cultivated in the simulated inflammatory environment of injured retina express retinal markers. The most significantly expressed marker was represented by rhodopsin. We have shown that IFN-γ is a crucial factor in the process.

- **MSCs inhibit immune response and inflammation in a model of the injured retina**

  Intravitreally injected MSCs inhibited the production of pro-inflammatory cytokines in the injured retina and suppressed the infiltration of immune cells. MSCs cultured *in vitro* with retinal explants in a simulated inflammatory environment also inhibited the production of pro-inflammatory cytokines in

excised retinas. In addition, MSCs expressed multiple immunomodulatory molecules and trophic factors after stimulation.

- **Anti-apoptotic effect of MSCs in the injured cornea has a paracrine mechanism**

  MSCs were transplanted on a nanofiber scaffold onto the chemically burned ocular surface and significantly lowered the number of apoptotic cells after the treatment. The transplantation also decreased the expression of pro-apoptotic BAX-2 and p53 molecules and increased anti-apoptotic BCL-2 in treated corneas. In our *in vitro* model of corneal inflammation, MSCs had an anti-apoptotic effect even when cultured with corneal explants indirectly through inserts. This result was also present in corneas treated by a conditioned medium from stimulated MSCs. MSCs also decreased the expression of genes associated with ER stress.

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Clinical trials can be found at **https://clinicaltrials.gov/**
For search use: mesenchymal stem/stromal cells retina, mesenchymal stem/stromal cells cornea, MSC cornea, MSC retina