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Mechanismy imunomodulačního působení kmenových buněk a jejich využití k léčbě onemocnění oka

Immunomodulatory mechanisms of stem cells and their use for therapy of ocular disorders

DISERTAČNÍ PRÁCE

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne

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Poděkování

Ráda bych poděkovala svému školiteli prof. RNDr. Vladimíru Holáňovi, DrSc. za věnovaný čas a odborné vedení doktorského projektu. Dále bych ráda poděkovala všem kolegům z Oddělení transplantační imunologie za všestrannou pomoc a podporu během doktorského studia. Velké poděkování patří také mému příteli a všem mým blízkým za poskytnutou podporu a pochopení.

Abstrakt

Kmenové buňky představují perspektivu pro léčbu řady doposud neléčitelných onemocnění. V současnosti mezi nejvíce studované kmenové buňky patří mezenchymální kmenové buňky (MSC). Tyto buňky jsou schopné diferenciace v různé buněčné typy, produkovat růstové a trofické faktory a prostřednictvím imunomodulačních molekul regulovat funkce buněk imunitního systému. Při studiu imunomodulačních vlastností MSC jsme se zaměřili na jejich vliv na B buňky a na studium mechanismu působení MSC ovlivněných interferonem-γ (IFN-γ) na produkci interleukinu 10 (IL-10) B buňkami. Prokázali jsme, že MSC ovlivněné IFN-γ inhibují produkci IL-10 aktivovanými B buňkami prostřednictvím dráhy cyklooxygenázy-2.

Vzhledem ke svým regenerativním a imunomodulačním vlastnostem nacházejí MSC uplatnění v léčbě řady onemocnění. V této práci jsme se zabývali možností využití MSC k léčbě onemocnění a poškození oka. Při léčbě poškozeného povrchu oka jsou používány limbální kmenové buňky (LSC), ale jejich izolace je obtížná a nemohou být použity ve všech případech poškození. Vhodným kandidátem v těchto případech mohou být MSC. Proto jsme srovnávali terapeutický potenciál LSC a MSC izolovaných z kostní dřeně a tukové tkáně. Studie ukázala, že MSC izolované z kostní dřeně mají srovnatelný regenerativní vliv na hojení poškozeného povrchu oka jako tkáňově specifické LSC.

Kromě léčby poškozeného povrchu oka mohou být MSC využity také v léčbě degenerativních onemocnění sítnice. Prokázali jsme, že MSC jsou schopné v prostředí simulující zánět v poškozené sítnici diferencovat na buňky exprimující znaky sítnice a ukázali jsme, že klíčovou podpůrnou roli v tomto diferenciačním procesu hraje IFN-γ. Dále jsme ukázali, že MSC produkují neurotrofické faktory a dokážou snížit expresi prozánětlivých cytokinů v sítnici.

Abstract

Stem cell-based therapy represents a perspective approach for the treatment of many so far incurable diseases. Mesenchymal stem cells (MSC) are currently the most studied stem cells. They are able to differentiate into different cell types, to produce growth and trophic factors and can suppress the functions of cells of the immune system. During the study of the immunomodulatory properties of MSC, we focused on their effect on B cells. The mechanism of impact of interferon-γ (IFN-γ) on MSC and their effect on the production of interleukin 10 (IL-10) by B cells was analysed. We have demonstrated that MSC-treated with IFN-γ inhibit production of IL-10 by activated B cells via the cyclooxygenase-2 involving pathway.

Due to their regenerative and immunomodulatory properties, MSC can be for treatment of many diseases. In this study we focused on the disease and damage of the eye. The limbal stem cells (LSC) are used for the treatment of damaged ocular surface, however their isolation is difficult and they can not be used in all cases of damage. Appropriate candidates in these cases are MSC. Therefore we have decided to compare the therapeutic potential of LSC and MSC isolated from bone marrow or adipose tissue. The study have shown that MSC isolated from bone marrow have a similar regenerative effect on healing of the damaged ocular surface of the eye as have LSC.

In addition, MSC can be also used for the treatment of retinal degenerative diseases. We found that MSC are able to differentiate into cells expressing retinal markers in the environment simulating inflammation in the damaged retina and we have shown that IFN-γ plays a key role in the differentiation process. Furthermore, we have shown that MSC produce numerous neurotrophic factors and can reduce expression of proinflammatory cytokines in the retina.

Obsah

1 Seznam zkratek

ABCB5	ATP-binding cassette subfamily 5 protein
ABCG2	ATP-binding cassette transporter group 2 protein
AD-MSC	adipose-derived mesenchymal stem cells, mezenchymální kmenové buňky izolované z tukové tkáně
BDNF	brain-derived neurotrophic factor, mozkový neurotrofický faktor
bFGF	basic fibroblast growth factor, bazický fibroblastový růstový faktor
BM-MSC	bone marrow-derived mesenchymal stem cells, mezenchymální kmenové buňky izolované z kostní dřeně
BRB	blood retinal barrier, hematoretinální bariéra
Breg	regulatory B cells, regulační B lymfocyty
CD	cluster of differentiation, diferenciační antigen
CNTF	ciliary neurotrophic factor, ciliární neurotrofický faktor
COX	cyklooxygenase, cyklooxygenáza
DR	diabetická retinopatie
EGF	epidermální růstový faktor, epidermal growth factor
Fas-L	Fas ligand
GDNF	glial cell line-derived neurotrophic factor, neurotrofický faktor z gliální buněčné linie
HGF	hepatocyte growth factor, růstový faktor hepatocytů
IDO	indolamin-2,3-dioxygenáza
IFN	interferon
IGF	insulin-like growth factor, inzulinu podobný růstový faktor

IL	interleukin
iNOS	inducible nitric oxid synthase, indukovatelná syntáza oxidu dusnatého
K3	keratin 3
LSC	limbal stem cells, limbální kmenové buňky
LSCD	limbal stem cell deficiency, deficience limbálních kmenových buněk
LPS	lipopolysacharid
MSC	mesenchymal stem cells, mezenchymální kmenové buňky
NGF	nerve growth factor, nervový růstový faktor
NO	nitric oxide, oxid dusnatý
NK	natural killers, přirození zabíječi
PCR	polymerase chain reaction, polymerázová řetězcová reakce
PD-L1	programmed death ligand 1, ligand programované buněčné smrti
PDGF	platelet-derived growth factor, destičkový růstový faktor
PGE	prostaglandine E, prostaglandin E
RPE	retinal pigment epithelium, epiteliální pigmentové buňky
TGF	transforming growth factor, transformující růstový faktor
Th	helper T cells, pomocné T lymfocyty
TNF	tumor necrosis factor, faktor nekrotizující nádory
Treg	regulatory T cells, regulační T lymfocyty
TSG-6	TNF-α stimulated gene 6 protein
VEGF	vascular endothelial growth factor, cévní endoteliální růstový faktor
VPMD	věkem podmíněná makulární degenerace

2 Literární přehled

2.1 Úvod

Kmenové buňky se za poslední roky staly středem zájmů biomedicínského výzkumu, protože svými vlastnostmi představují nadějné kandidáty pro léčbu řady doposud neléčitelných poškození a onemocnění. Proto se tato práce se zabývá mezenchymálními kmenovými buňkami (MSC), které v současné době patří mezi nejstudovanější typy kmenových buněk. MSC disponují diferenciačním potenciálem, imunomodulačními vlastnostmi a produkcí trofických a růstových faktorů. Další jejich výhodou je poměrně snadná izolace a kultivace z tkání pacienta a tedy možnost využití pro autologní transplantaci bez použití imunosupresivní léčby.

MSC jsou studovány pro léčbu autoimunitních onemocnění nebo při transplantacích, kde se využívá především jejich schopnosti imunomodulace. MSC působí na buňky adaptivní i přirozené imunity, inhibují zánětlivé reakce a naopak aktivují regulační protizánětlivou imunitní odpověď. Na druhé straně jsou MSC využívány v terapii pro své regenerativní účinky a schopnost diferenciace na mnoho buněčných typů. Diferenciací a produkcí celé řady růstových a trofických faktorů podporují regeneraci poškozené tkáně, například při poškození oka. Při rozsáhlém poškození rohovky i s limbální částí jsou schopné svou funkcí nahradit limbální kmenové buňky (LSC) a zajistit regeneraci poškozeného povrchu oka. Úspěšné výsledky se ukazují také při aplikaci MSC v případě poškození sítnice. Bylo prokázáno, že MSC jsou schopné diferenciace na buňky exprimující znaky sítnice, podporují obnovu poškozených buněk a potlačují zánětlivé procesy v poškozené sítnici.

2.2 Kmenové buňky

Kmenové buňky představují řadu odlišných buněčných populací, pro které stále nebyl definován žádný společný fenotypový znak. Mezi jejich hlavní společné vlastnosti patří neomezená schopnost sebeobnovy a diferenciace na různé typy buněk. Kmenové buňky můžeme rozdělit na embryonální kmenové buňky, indukované pluripotentní kmenové buňky a kmenové buňky z dospělého organismu.

Embryonální kmenové buňky jsou pluripotentní kmenové buňky získávané z blastocysty zárodku. Jejich nevýhodou při terapeutickém použití je tvorba teratomů a etické problémy spojené s jejich izolací (Bobbert 2006; Gong et al. 2014). Dalším typem

jsou indukované kmenové buňky, které jsou uměle připravovány ze somatických buněk vnesením genů potřebných pro reprogramování somatické buňky na buňku s kmenovými vlastnostmi. Reprogramované buňky se mohou diferencovat do všech tří zárodečných linií. Jejich použití negativně ovlivňují problémy spojené s použitím virových vektorů a také neúplným reprogramováním buněk (Yu et al. 2007; Yamanaka et al. 2012).

V současné době se jako nejvhodnější kandidát pro buněčnou terapii ukazují kmenové buňky z dospělého organismu, kam můžeme zařadit hematopoetické kmenové buňky, tkáňově specifické kmenové buňky nebo MSC. Kmenové buňky z dospělého organismu zajišťují obnovu buněk diferenciací. Kromě diferenciace na buňky odpovídající buněčné linie jsou některé z nich schopné i transdiferenciace na buňky jiných zárodečných linií. U MSC byly prokázané imunomodulační vlastnosti, schopnost produkce růstových a trofických faktorů a také možnost transdiferenciace (Guadix et al. 2016).

2.3 MSC

MSC jsou multipotentní kmenové buňky, které byly v roce 1966 popsány Friedensteinem et al. jako populace buněk v kostní dřeni. Pro MSC nebyl doposud stanoven žádný unikátní znak, proto se pro jejich charakterizaci využívá tří kritérií stanovených Mezinárodní společností pro buněčnou terapii. Jedním z kritérií je schopnost MSC *in vitro* diferencovat na adipocyty, chondroblasty a osteoblasty. Kromě toho musí být MSC adherentní k plastovému povrchu a musí exprimovat diferenciační antigeny (CD, cluster of differentiation) CD73, CD90, CD105 a naopak neexprimovat hematopoetické znaky CD11b, CD14, CD19, CD34, CD45, CD79α a molekuly hlavního histokompatibilního komplexu II. třídy (Dominici et al. 2006).

2.3.1 Výskyt a migrace MSC

MSC můžeme nalézt v celé řadě tkání jako je například kostní dřeň, tuková tkáň, pupečníková krev, placenta, zubní dřeň, kosterní sval, synoviální tekutina nebo vlasový folikul (Tuan et al. 2003). Nejčastěji jsou však MSC získávány z kostní dřeně, tukové tkáně nebo pupečníkové krve. Rozdílné vlastnosti MSC izolovaných z různých tkání jsou předmětem řady studií. Na základě jejich původu je nejčastěji srovnáván jejich fenotyp, morfologie, proliferační schopnost, rozdíly v inhibici buněk imunitního

systému a diferenciační potenciál (Kern et al. 2006; Ribeiro et al. 2013; Isobe et al. 2016).

Z místa výskytu jsou MSC schopné migrovat do místa poškození či zánětu a zde zajistit regeneraci poškozené tkáně či potlačit probíhající zánět. Migrace MSC do místa poškození je ovlivněna řadou chemoatraktantů, pro něž mají MSC na svém povrchu receptory. Nárůst koncentrace zánětlivých chemokinů v místě poškození je hlavním faktorem zahájení migrace MSC (Ponte et al. 2007; Li and Jiang 2011).

2.3.2 Diferenciace MSC

Diferenciace je komplexní proces potřebný pro obnovu buněk a tkání. MSC jsou kromě diferenciace na adipocyty, chondrocyty, osteocyty a jiné mezodermální buňky schopné také transdiferenciace na buňky entodermálního a ektodermálního původu. Byla popsána diferenciace MSC na hepatocyty (Zhao et al. 2009), plicní buňky (Rojas et al. 2005), kardiomyocyty (Toma et al. 2002), buňky ledvin (Liu et al. 2013), neurony (Tropel et al. 2006), buňky rohovky (Nieto-Miguel et al. 2013) a další buněčné typy.

2.3.3 Parakrinní působení MSC

MSC produkují řadu cytokinů, chemoatraktantů, růstových a trofických faktorů a imunomodulačních molekul. Růstové a trofické faktory mají anti-apoptotickou, regenerativní, diferenciační a angiogenní funkci. Mezi tyto faktory produkované MSC patří například cévní endoteliální růstový faktor (VEGF, vascular endothelial growth factor), angiopoietin-1, erytropoietin, leukemický inhibiční faktor, destičkový růstový faktor (PDGF, platelet-derived growth factor), bazický fibroblastový růstový faktor (bFGF, basic fibroblast growth factor), inzulinu podobný růstový faktor (IGF, insulin-like growth factor) nebo epidermální růstový faktor (EGF, epidermal growth factor) (Meirelles et al. 2009). Kromě trofických faktorů MSC produkují také anti-fibrotické, imunomodulační faktory a chemoatraktanty (Spees et al. 2016).

Studie z poslední doby ukázaly, že růstové faktory mohou být sekretovány také ve formě extracelulárních váčků (exosomy, mikrovesikly). Váčky kromě růstových faktorů mohou obsahovat různé cytokiny, mRNA, mikroRNA a imunomodulační molekuly. Transport faktorů přes extracelulární váčky chrání molekuly před jejich degradací a umožňuje přenos na delší vzdálenosti. Dalšími výhodami využití extracelulárních váčků v terapii je zamezení rejekce a přenosu poškozených buněk, možnost aplikace větší dávky a snadný průchod vzhledem k malé velikosti váčků. Na druhou stranu nemůže

docházet k diferenciaci MSC a působení růstových a trofických faktorů je omezeno (Nooshabadi et al 2017; Phinney et al. 2017).

2.3.4 Vliv MSC na fibrózu

Bylo prokázáno, že MSC jsou schopné inhibovat fibrotický proces a podpořit regeneraci tkáně. Fibrotickým procesem dochází ke zvyšování poměru vazivové tkáně na úkor tkáně daného orgánu. Fibróza tkání je způsobena nejčastěji poraněním, zánětem, rejekcí nebo oxidačním poškozením (Spees et al. 2016). MSC inhibují diferenciaci epiteliálních buněk na fibroblasty, oxidační poškození buněk a umožňují remodelaci tkáně (Usunier et al. 2014). MSC také svým působením snižují infiltraci lymfocytů a neutrofilů do tkáně a snižují tak produkci zánětlivých a fibrotických faktorů (Ono et al. 2014).

2.3.5 Imunomodulační vlastnosti MSC

Kromě diferenciačního potenciálu disponují MSC také imunomodulačními vlastnostmi, kterými inhibují funkci buněk adaptivní i přirozené imunity. Bylo ukázáno, že MSC jsou schopné snížit proliferaci a funkci T buněk, B buněk, dendritických buněk a NK buněk a naopak indukovat vznik regulačních makrofágů, T regulačních (Treg) buněk a B regulačních (Breg) buněk (Lee and Song 2017). V řadě případů není objasněn přesný mechanismus jejich působení. Na buňky imunitního sytému mohou MSC působit jak buněčným kontaktem, tak prostřednictvím solubilních imunomodulačních molekul, které mohou být produkovány konstitutivně nebo je k jejich aktivaci potřeba stimulace (Zhao et al. 2016). V řadě studií bylo potvrzeno, že pro zvýšení exprese většiny imunomodulačních molekul je potřeba stimulace pomocí interferonu- γ (IFN-γ), faktoru nekrotizujícího nádory α (TNF-α, tumor necrosis faktor), interleukinu (IL) 1 nebo jejich kombinace. V zánětlivém prostředí dochází k aktivaci MSC a expresi vyšší hladiny imunomodulačních molekul, což vede k inhibici nežádoucí zánětlivé reakce a směřování imunitní odpovědi k tolerogenní (Krampera et al. 2006; Dorronsoro et al. 2013; Gao et al. 2016).

Mezi molekuly zprostředkovávající kontaktní inhibici buněk imunitního systému patří například ligand programované buněčné smrti (PD-L1, programmed death ligand 1) a Fas ligand (FasL). Exprese PD-L1 na povrchu MSC je několikanásobně zvýšena po působení IFN-γ a je zodpovědná například za snížení počtu pomocných T buněk 17 v kultuře (Th17, T helper cell) (Sheng et al. 2008; Luz-Crawford et al. 2012). FasL na

povrchu MSC se uplatňuje v indukci apoptotické smrti u aktivovaných buněk imunitního systému a potlačení nežádoucí zánětlivé reakce (Akiyama et al. 2012).

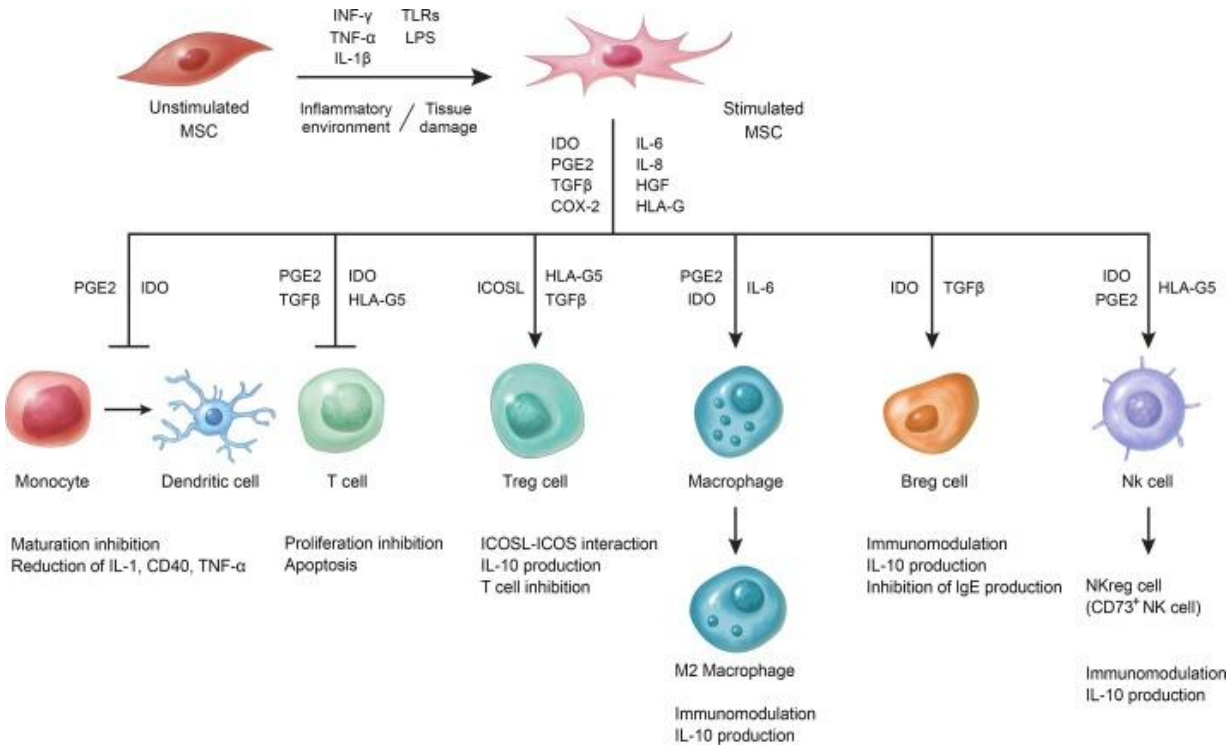
MSC produkují velký počet solubilních imunomodulačních cytokinů a molekul jako například IL-6 (Djouad et al. 2007), transformující růstový faktor β (TGF-β, transforming growth factor β) (English et al. 2009), oxid dusnatý (NO, nitric oxide) produkovaný indukovatelnou syntázou NO (iNOS) (Sato et al. 2007), produkt cyklooxygenázy 2 (COX-2) prostaglandin E2 (PGE2) (English et al. 2009), u lidí lidský leukocytární antigen G5 (Selmani et al. 2008), TSG-6 (TNF-α stimulated gene 6 protein) (Oh et al. 2012), růstový faktor hepatocytů (HGF, hepatocyte growth factor) (Neuss et al. 2004) a také intracelulární enzym katabolizující tryptofan indolamin 2,3-dioxygenáza (IDO) (Meisel et al. 2004).

2.3.5.1 Vliv MSC na buňky imunitního systému

Jak již bylo zmíněno výše, MSC působí na celou řadu buněk přirozené i adaptivní imunity, ovlivňují jejich proliferaci, aktivaci, migraci, expresi povrchových molekul nebo produkci cytokinů (Obr. 1). Imunomodulační efekt MSC je zprostředkován často kombinací několika solubilních faktorů i membránových molekul, ale jejich přesné mechanismy působení jsou stále předmětem studií (Lee and Song 2017).

2.3.5.2 Vliv MSC na T lymfocyty

MSC jsou schopné potlačit aktivaci a proliferaci T buněk a také působí na jejich diferenciaci. Jedním z mechanismů inhibice proliferace T lymfocytů je zastavení jejich buněčného cyklu v G0/G1 fázi (Glennie et al. 2005). MSC mohou na T buňky působit přímo nebo zprostředkovaně přes inhibici dendritických buněk (Spaggiari et al. 2009). Po působení MSC na T buňky dochází k ovlivňování rovnováhy mezi Th1 a Th2 buňkami ve prospěch protizánětlivé odpovědi s Th2 buňkami. MSC potlačují produkci IFN-γ u Th1 buněk a naopak podporují Th2 buňky a jejich produkci IL-4 a IL-10 (Aggarwal and Pittenger 2005). MSC vzhledem k produkci TGF-β a IL-6 také ovlivňují diferenciaci naivních T buněk na Treg nebo Th17 buňky. Pokud MSC produkují pouze TGF-β dochází k vývoji T buněk regulačním směrem, naopak pokud se T buňky vyskytují v prostředí kombinace cytokinů TGF-β a IL-6 diferenciace probíhá na Th17 buňky (Svobodova et al. 2012). Diferenciaci T buněk regulačním směrem mohou MSC ovlivnit i působením přes PGE2 nebo IDO (English et al. 2009; Ghannam et al. 2010).



Obrázek 1. Vliv MSC na buňky přirozené a adaptivní imunity. MSC ovlivňují buňky imunitního systému přímým kontaktem i produkcí parakrinně působících faktorů. Po stimulaci zánětlivým prostředím (IFN-γ, TNF-α, IL-1β) MSC produkují vyšší hladinu imunomodulačních molekul. Dochází k inhibici prozánětlivé imunitní odpovědi a naopak k přesmyku na regulační odpověď (převzato z Lee and Song 2017).

2.3.5.3 Vliv MSC na B lymfocyty

Kromě přímého působení MSC mohou být B lymfocyty ovlivněny i nepřímo přes inhibované T lymfocyty. MSC inhibují proliferaci B buněk zastavením jejich buněčného cyklu v G0/G1 fázi stejně jako u T lymfocytů. Ovlivňují také diferenciaci B buněk na plazmatické buňky, produkci imunoglobulinů a mají vliv na snížení exprese receptorů pro chemokiny (CXCR4, CXCR5 a CCR7), čímž dochází k inhibici migrace B buněk do sekundárních lymfoidních orgánů (Corcione et al. 2006; Franquesa et al. 2012).

Bylo prokázáno, že stejně jako u T lymfocytů a makrofágů i B lymfocyty mají regulační subpopulaci Breg, která produkuje převážně IL-10 (Ma and Chan 2016). V několika studiích bylo potvrzeno, že MSC podporují vznik Breg lymfocytů. Na modelu experimentální autoimunitní encefalomyelitidy po podání MSC došlo k inhibici

Th17 buněk a naopak ke zvýšení aktivity Breg buněk (Guo et al. 2013). Podobné výsledky byly popsány i na myším modelu onemocnění systémového lupus erytematoides, kde po podání MSC došlo také k nárůstu počtu Breg lymfocytů v *in vitro* i *in vivo* pokusech (Park et al. 2015). V současné době není jasné, jakým způsobem se MSC podílejí na aktivaci Breg, zda je to jejich přímým působením nebo prostřednictvím IL-10 produkovaného Treg lymfocyty, dendritickými buňkami nebo makrofágy (Ma and Chan 2016).

2.3.5.4 Vliv MSC na makrofágy

Makrofágy můžeme rozdělit na dvě subpopulace M1 a M2 s odlišným fenotypem. M1 makrofágy mají prozánětlivý charakter s produkcí NO, IL-1, IL-6, IL-12 a TNF-α, naopak M2 populace podporuje protizánětlivou imunitní odpověď a produkuje IL-4 a IL-10. Po působení MSC mění makrofágy svůj profil na protizánětlivý, M2 makrofágy exprimují ve zvýšené míře molekulu CD206 a je u nich zvýšena schopnost fagocytózy (Maggini et al. 2010; Cho et al. 2014).

2.3.5.5 Vliv MSC na dendritické buňky a NK buňky

U dendritických buněk jsou MSC schopné inhibovat jejich diferenciaci z monocytů a následně potlačit expresi molekul sloužících k prezentaci antigenu a kostimulačních molekul. Inhibice těchto molekul vede zprostředkovaně ke snížení funkce T lymfocytů bez přímého působení MSC (Jiang et al. 2005, Chiesa et al. 2011). Bylo prokázáno, že MSC mají supresivní účinky i na NK buňky, inhibují jejich proliferaci, cytotoxicitu a expresi aktivačních receptorů. Inhibiční vliv MSC je větší v případě neaktivovaných NK buněk než aktivovaných NK buněk (Spaggiari et al. 2008).

2.4 Možnosti terapeutického využití MSC

MSC vzhledem ke svým vlastnostem představují perspektivní kandidáty pro léčbu řady onemocnění. Mohou být využity jak v regenerativní medicíně, tak v léčbě autoimunitních onemocnění či při transplantacích. V současné době probíhá přibližně 500 klinických studií zabývajících se MSC a jejich uplatněním v terapii (www.clinicaltrials.gov). V regenerativní medicíně se uplatňuje především schopnost MSC se diferencovat v různé buněčné typy. MSC se mohou diferencovat například na chondrocyty, osteocyty (Pittenger et al. 1999), kardiomyocyty (Toma et al. 2002),

hepatocyty (Zhao et al. 2009), buňky rohovky (Nieto-Miguel et al. 2013) nebo sítnice (Kicic et al. 2003). Další významnou vlastností MSC je produkce růstových, anti-apoptotických, anti-fibrotických, angiogenních faktorů a cytokinů, které podporují diferenciaci buněk a regeneraci poškozené tkáně (Meirelles et al. 2009). V rámci regenerativní uplatnění MSC se jedná především o použití v léčbě poškozených chrupavek (Wakitani et al. 2011), komplikovaných zlomenin (Quarto et al. 2001), osteoartritidy (Soler et al. 2016), genetického onemocnění osteogenesis imperfekta (Horwitz et al. 2002), infarktu myokardu (Orlic et al. 2001), plicní fibrózy (Glassberg et al. 2017), poškození jater (Peng et al. 2011) nebo poranění míchy (Jarocha et al. 2015).

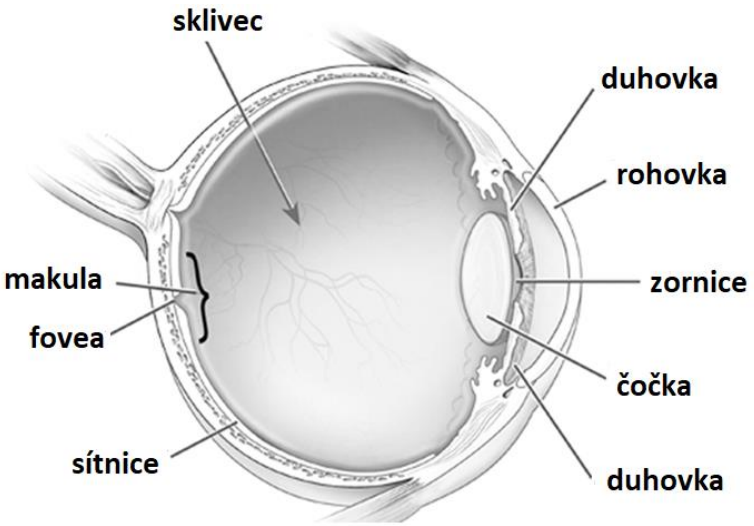
Velký potenciál se skrývá také v imunomodulačních vlastnostech MSC a možnosti regulace adaptivní i přirozené imunity (Guadix et al. 2017; Lee and Song 2017). Těchto vlastností je využíváno při transplantacích k potlačení nežádoucích imunitních reakcí, například při transplantaci ledvin (Mudrabettu et al. 2015), a dále k léčbě chronické a akutní reakce štěpu proti hostiteli (Le Blanc et al. 2008; Kurtzberg et al. 2014). V neposlední řadě jsou MSC používány v léčbě autoimunitních onemocnění, jako je například diabetes mellitus (Cai et al. 2016), roztroušená skleróza (Llufriu et al. 2014), revmatoidní artritida (Liang et al. 2012) nebo Crohnova choroba (Ciccocippo et al. 2015).

V řadě onemocnění je využívána kombinace regenerativních vlastností MSC spolu s jejich imunomodulačními vlastnosti. Příkladem mohou být různá onemocnění a poškození zraku. Poškození zraku nebo jeho ztráta vedou k výraznému snížení kvality života, které se promítá do všech jeho oblastí a omezuje dosavadní činnosti člověka. Mezi nejčastější příčiny ztráty zraku můžeme zařadit poškození rohovky či onemocnění sítnice.

2.4.1 Rohovka

Světlo vstupuje do oka přes rohovku a dopadá přes zornici na čočku, která se díky duhovce roztahuje nebo zužuje a mění tak své zakřivení, pak paprsek dopadá na zadní stěnu oka na sítnici (Obr. 2). Na povrchu rohovky se nachází dlaždicový vrstevnatý rohovkový epitel, jehož odumřelé buňky jsou odplavovány prostřednictvím slz. Dále je rohovka tvořena Bowmanovou membránou tvořenou vlákny kolagenu, nejsilnější částí rohovkovým stroma, descemetskou membránou a nakonec jednovrstevným rohovkovým endotelem. Na zevním okraji rohovky se nachází limbus, který slouží jako

bariéra rozrůstání spojivkového epitelu na rohovku a také se zde nachází populace LSC (Echevarria and Di Girolamo 2011; Zhao et al. 2016).



Obrázek 2. Schéma lidského oka. Lidské oko je z velké části tvořeno sklivcem, dále je složeno z přední části s rohovkou, duhovkou se zornicí, čočkou a zadní části, kde se nachází sítnice. Na sítnici je znázorněno místo nejostřejšího vidění žlutá skvrna neboli makula (převzato a upraveno ze Zheng et al. 2012).

2.4.1.1 Poškození rohovky

Za fyziologických podmínek je rohovka průhledná a nenachází se v ní žádné cévy. Při poškození může dojít k vaskularizaci a ke snížení průhlednosti rohovky, což může mít za následek až ztrátu zraku. Poškození povrchu oka může být způsobeno mechanicky, tepelně, chemicky nebo sekundárně jako následek infekčního onemocnění. Další možností poškození jsou geneticky podmíněné dystrofie rohovky nebo poškození při autoimunitním onemocnění (například oční pemphigoid). Regenerace rohovkového epitelu je zprostředkována LSC a jejich následnou diferenciací. Populace LSC se nachází v bazální vrstvě limbálního epitelu ve struktuře nazývané Vogtovy palisády. Při rozsáhlejším poškození rohovky i s oblastí limbu dochází k deficienci LSC (LSCD) a regenerace rohovkového epitelu není možná. LSCD je charakterizována invazí spojivkového epitelu, zánětlivým prostředím, vaskularizací a ztenčením rohovky (Dua et al. 2009; Ordonez and Di Girolamo 2012).

2.4.1.2 Transplantace rohovky

Možnost léčby povrchu oka závisí na rozsahu poškození, zda je zasažen limbus a také zda se jedná o jednostranné či oboustranné poškození oka. Pokud je poškozena pouze rohovka a limbální tkáň je alespoň částečně zachována, je možné přistoupit k transplantaci rohovky. Vzhledem k imunoprivilegovanosti rohovky je její transplantace ve většině případů úspěšná a patří k jedné z nejčastějších transplantací. Problém může nastat, pokud při poškození rohovky došlo k vaskularizaci a zvýšení počtu buněk imunitního systému v rohovce. V těchto případech je narušena její imunoprivilegovanost a může dojít k odhojení štěpu (Maddula et al. 2011; Zhao et al. 2016).

2.4.1.3 Využití LSC v léčbě poškozené rohovky

LSC jsou schopné nahradit poškozené buňky rohovkového epitelu. Stejně jako pro MSC, ani pro LSC není v současné době známý žádný specifický znak. Pro jejich charakterizaci je využívána exprese membránového transportéru ABCG2 (ATP-binding cassette transporter group 2 protein), transkripčního faktoru p63, ABCB5 (ATP-binding cassette subfamily 5 protein), keratin 19 (K19) a naopak nepřítomnost znaků typických pro buňky rohovky jako například K3 nebo K12 (Krulova et al. 2008; Echevarria and Di Girolamo 2011; Ekici et al. 2015).

V případě poškození zahrnující i limbus dochází k LSCD a rohovka není schopna se regenerovat ani po případné transplantaci. Při jednostranném poškození limbu může být využita transplantace části limbálního epitelu ze zdravého oka do oka poškozeného, nevýhodou je možnost poškození zdravého oka po odběru části limbu (Rama et al. 2010). Další způsob léčby představuje transplantace laboratorně kultivovaných LSC získaných z malého kousku nepoškozeného limbu pacienta (Basu et al. 2012). Již v roce 1997 byla publikována skupinou Pellegrini et al. úspěšná transplantace epiteliální vrstvy buněk kultivované z LSC pacienta. Po autologní transplantaci na chemicky poškozený povrch oka došlo k obnovení rohovkového epitelu (Pellegrini et al. 1997). LSC jsou na povrch oka transplantovány pomocí různých nosičů, využívá se například amniová membrána (Pauklin et al. 2010; Basu et al. 2012), kontaktní čočky (Pellegrini et al. 1997; Brown et al. 2014), nanovlákenné nosiče (Zajicova et al. 2010; Baradan-Rafii et al. 2015) nebo fibrinové lepidlo (Marchini et al. 2011).

U poškození limbu u obou očí není transplantace limbu nebo LSC z druhého nepoškozeného oka pacienta možná. Využívá se alogenní transplantace limbu s následným podáním imunosupresivních léčiv (Dua et al. 1999), případně aplikace kultivovaných alogenních LSC (Pauklin et al. 2010). Oba způsoby terapie jsou spojeny s aktivací imunitního systému, a je nutné podávání silných dávek imunosupresivních léčiv, které mají řadu vedlejších účinků. Vhodnou alternativu pro léčbu poškozeného povrchu oka představují MSC, které se mohou diferencovat na buňky rohovky a zároveň podporovat regeneraci v místě poškození. MSC je možné izolovat z tkání pacienta a získat tak dostatečné množství buněk pro autologní transplantaci.

2.4.1.4 Využití MSC v léčbě poškozené rohovky

MSC jsou perspektivním kandidátem pro léčbu poškozeného povrchu oka. Bylo prokázáno, že mohou jak diferencovat na buňky rohovky, tak potlačit zánětlivou reakci probíhající v místě poškození. Svými vlastnostmi mohou MSC nahradit LSC, které nemohou být použity v řadě případů vážného poškození povrchu oka. MSC mají navíc oproti LSC výhodu snadné izolace a kultivace z tkání pacienta.

2.4.1.4.1 Diferenciace MSC na buňky rohovky

MSC mají řadu podobných vlastností jako LSC, jednou z nich je schopnost diferenciace na buňky epitelu rohovky. Bylo prokázáno, že MSC jsou schopné diferenciace *in vitro* po kultivaci s LSC nebo se supernatantem z kultivovaných LSC, v obou případech došlo u MSC k expresi znaku K3 typického pro rohovku (Gu et al. 2009). Kultivace MSC izolovaných z kostní dřeně (BM-MSC, bone marrow-derived MSC) s kondiciovaným médiem získaným kultivací buněk rohovky byla využita i v jiných experimentech, které diferenciaci MSC na buňky exprimující znaky K3 a K12 potvrdily (Nieto-Miguel et al. 2013). V jiné studii byly MSC kultivovány přímo se stromálními buňkami rohovky, i v tomto případě došlo k diferenciaci BM-MSC na buňky exprimující K12 (Jiang et al. 2010). Diferenciace BM-MSC na buňky rohovky byla zaznamenána i po jejich transplantaci na modelu alkalického poškození oka. MSC byly aplikovány na poškozený povrch oka potkanů prostřednictvím amniové membrány (Jiang et al. 2010, Rohaina et al. 2014) nebo fibrinového gelu (Gu et al. 2009). V obou případech došlo k diferenciaci MSC na buňky exprimující znaky rohovky a k ústupu neovaskularizace a regeneraci rohovky.

2.4.1.4.2 Inhibice zánětlivé reakce

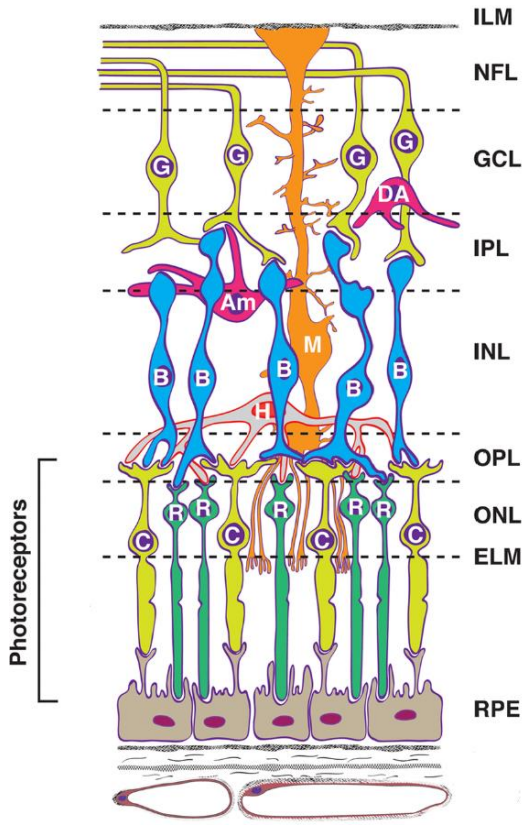
Schopnost diferenciace není jediná vlastnost MSC, která může být využita při léčbě poškození povrchu oka. MSC také mohou potlačit zánětlivé reakce probíhající v poškozeném oku a podpořit regeneraci rohovkového epitelu. Ma et al. prokázali po aplikaci lidských BM-MSC prostřednictvím amniové membrány na chemicky poškozené oko potkana snížení neovaskularizace a zánětu, ale nepozorovali diferenciaci BM-MSC na buňky exprimující znaky rohovky (Ma et al. 2006). Kromě fibrinového gelu a amniové membrány je MSC možné na povrch oka přenášet také prostřednictvím nanovlakenného nosiče. Bylo ukázáno, že i v tomto případě dochází k inhibici lokální zánětlivé reakce na povrchu oka (Zajicova et al. 2010).

Dalším způsobem podání MSC je intravenózní aplikace, po podání jsou MSC schopné migrovat do místa poškození (Javorková et al. 2014) nebo také působit prostřednictvím produkované molekuly TSG-6 (Oh et al. 2012). Po intravenózním podání BM-MSC u myší byla pozorována inhibice časné fáze zánětu v chemicky poškozené rohovce, kde došlo ke snížení infiltrace rohovky buňkami imunitního systému a snížení produkce IL-1 a IL-6 (Javorkova et al. 2014). V jiné práci byly lidské MSC po intravenózním podání myším zachyceny v plicích, odkud přes molekulu TSG-6 byly schopné inhibovat zánět a buňky imunitního systému v rohovkovém transplantátu a prodloužit tak přežívání štěpu (Oh et al. 2012). Aplikace BM-MSC po transplantaci alogenní rohovky také prodloužila přežití transplantátu na modelu potkana, kde došlo k inhibici prozánětlivé Th1 odpovědi a naopak ke zvýšení počtu Th2 a Treg buněk (Jia et al. 2012). V současné době probíhá klinická studie věnující se transplantaci MSC izolovaných z tukové tkáně (AD-MSC, adipose-derived mesenchymal stem cells) při onemocnění rohovky. Studie je rozdělena na 3 skupiny, kdy jedné skupině jsou transplantovány pouze AD-MSC, druhé samotný nosič a třetí nosič s AD-MSC. Účelem studie je zhodnotit bezpečnost a předběžnou účinnost transplantace AD-MSC (www.clinicaltrials.gov).

2.4.2 Sítnice

Sítnice se nachází na vnitřní straně oka a slouží ke zpracování světelných signálů. Světelný signál dopadá do oka skrz rohovku a dále pokračuje přes čočku až na sítnici. V sítnici se nachází deset rozdílných buněčných vrstev (Obr. 3). Jednou z nich je vrstva fotoreceptorů, které jsou zodpovědné za přeměnu světelné energie na nervový vzruch.

Tato informace je pomocí optického nervu předána dále do mozku. Jednotlivé vrstvy sítnice si mezi sebou předávají signály a vzájemně spolu komunikují. První nejvzdálenější vrstvou od sklivce jsou epiteliální pigmentové buňky (RPE), pak směrem ke sklivci následuje vrstva fotoreceptorů – tyčinek a čípků, zevní ohraničující membrána, zevní jádrová vrstva, zevní plexiformní vrstva, vnitřní vrstva jádrová s bipolárními, amakrinními a horizontálními buňkami, dále vnitřní plexiformní vrstva, vrstva gangliových buněk, vrstva nervových vláken a nakonec vnitřní ohraničující membrána (Alonso-Alonso and Srivastava 2015). Na povrchu sítnice můžeme nalézt slepou skvrnu neboli optický disk, což je místo, kde sítnici opouští optický nerv a naopak sem vstupuje cévní zásobení. Dalším důležitým místem sítnice je žlutá skvrna neboli makula, která je místem nejostřejšího vidění. Na udržení homeostáze sítnice a její imunologické privilegovanosti se podílí hematoretinální bariéra (BRB, blood retinal barrier), která je tvořena těsnými spoji mezi epiteliálními buňkami (Klassen et al. 2013).



Obrázek 3. Schéma uspořádání vrstev buněk sítnice. Sítnice je složena z deseti vrstev buněk - první vrstvou jsou epiteliální pigmentové buňky (RPE), poté následuje vrstva fotoreceptorů – tyčinek (R) a čípků (C), zevní ohraničující membrána (ELM), zevní jádrová vrstva (ONL), zevní plexiformní vrstva (OPL), vnitřní vrstva jádrová (INL) s bipolárními (B), amakrinními (Am, DA), Müllerovými (M) a horizontálními (H) buňkami, dále vnitřní plexiformní vrstva (IPL), vrstva gangliových (G) buněk (GCL), vrstva nervových vláken (NFL) a nakonec vnitřní ohraničující membrána (ILM) (převzato a upraveno ze Zheng et al. 2012).

2.4.2.1 Onemocnění sítnice

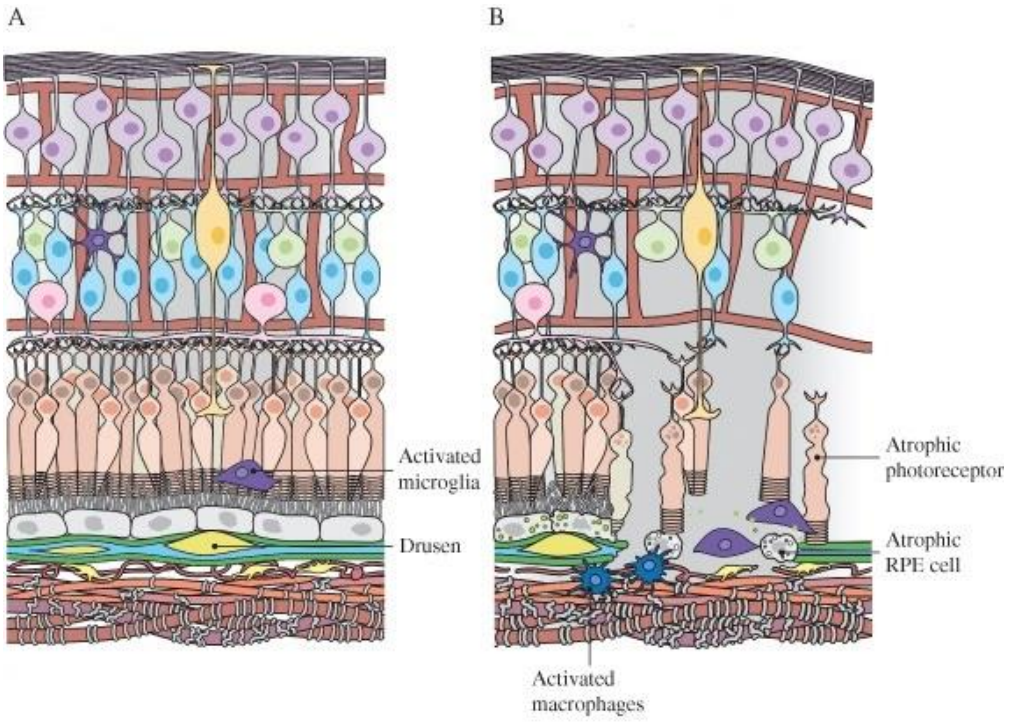
Jednotlivé vrstvy buněk sítnice jsou navzájem propojeny a komunikují spolu. Poškození v jedné vrstvě buněk vede ke ztrátě propojení a následně ke ztrátě funkce sítnice. Onemocnění sítnice patří k závažným onemocněním, u kterých je důležitá včasná diagnóza. Postup degenerace sítnice je u některých onemocnění velmi rychlý a ztráta zraku je nevratná. Mezi nejčastější onemocnění můžeme zařadit diabetickou retinopatii, věkem podmíněnou makulární degeneraci, glaukom, odchlípení sítnice a z dědičných onemocnění například retinitis pigmentosa (Park et al. 2017).

Diabetická retinopatie (DR) je onemocnění sítnice, při kterém je poškozeno její cévní zásobení a v pokročilém stádiu může vést až k nevratné ztrátě zraku, DR postihuje v pokročilé fázi cukrovky vysoké procento pacientů (Resnikoff et al. 2004). V počáteční fázi dochází k nepříznivým biochemickým procesům vedoucím ke změnám tlaku v oku a zeslabování stěn cév s tvorbou mikroaneurizmat v jejich stěnách. Dochází k nedostatečnému okysličování, což způsobuje odumírání buněk zajišťujících kontraktibilitu cév a také zvýšení propustnosti cévní stěny (Lorenzi and Gerhardinger 2001). Následně vzniká vaskulární okluze, která zabraňuje dostatečnému vyživování a v důsledku toho zde dochází k hypoxii až ischemii. Tento proces je doprovázen produkcí angiogenního faktoru a tvorbou nových cév. U nově vzniklých cév dochází k rupturám a krvácení do sklivce, což společně s tvorbou fibrózních jizev vede k oslepnutí (Cheung et al. 2010).

Věkem podmíněná makulární degenerace (VPMD) je multifaktoriální onemocnění, které je nejčastější příčinou ztráty zraku ve vyspělých zemích. Dochází k porušení žluté skvrny neboli makuly, která je zodpovědná za centrální a barevné vidění. VPMD se vyskytuje převážně u pacientů starších 50 let, kromě věku je rizikovým faktorem také nesprávná životospráva a genetická predispozice. Postupně s pokročilým věkem dochází k poklesu aktivity RPE buněk, fotoreceptory nejsou dostatečně vyživovány a dochází k hromadění odpadních látek v sítnici. Hromadění deposit v sítnici vede k odumření RPE buněk i fotoreceptorů (Ng et al. 2014; Nazari et al. 2015) (Obr. 4).

Dalším onemocněním sítnice je glaukom, při kterém je jedním z rizikových faktorů vysoký nitrooční tlak. Během onemocnění dochází k postupnému odumírání gangliových buněk a jejich axonů tvořících optický nerv (Bull and Martin 2011; Cohen et al. 2014). Kromě výše zmíněných onemocnění jsou i dědičná degenerativní poškození

sítnice. Do této skupiny můžeme zařadit například onemocnění retinitis pigmentosa. Existuje celá řada forem tohoto onemocnění způsobená různými mutacemi, nejčastější je mutace způsobující poruchu tvorby rodopsinu. Nejdříve dochází k odumírání tyčinek na vnějším okraji sítnice, což způsobuje zhoršené vidění za šera. Později jsou ale zasaženy i čípky v centrální oblasti sítnice a postupně pomalu dochází k úplné ztrátě zraku. Účinná léčba tohoto onemocnění nyní neexistuje (Ng et al. 2014; Nash et al. 2015).



Obrázek 4. Poškození sítnice při VPMD. Při VPMD dochází k porušení struktury sítnice v místě makuly. Následkem hromadění depozit v sítnici (drusen) dochází k postupnému odumírání RPE buněk, fotoreceptorů a dalších buněk sítnice. V místě poškození jsou aktivovány makrofágy a integrita sítnice je narušena. Na obrázku A je znázorněn stav v počátečním stádiu VPMD, na obrázku B pak pokročilé stádium VPMD (převzato a upraveno z van Lookeren Campagne et al. 2014).

2.4.2.2 Využití MSC v léčbě onemocnění sítnice

V současné době pro řadu degenerativních onemocnění sítnice stále neexistuje účinná léčba nebo je léčba úspěšná pouze v počátečních stádiích onemocnění. Často je využíváno invazivních metod, kdy při jejich opakovaném použití může dojít

k poškození i zdravé tkáně sítnice. Perspektivu pro tato onemocnění představuje buněčná terapie založená na aplikaci kmenových buněk. MSC mají řadu vlastností, které je možné využít v léčbě degenerativních onemocnění sítnice. MSC jsou schopné diferenciace na buňky sítnice, produkují růstové a trofické faktory podporující regeneraci a v neposlední řadě jsou schopné inhibovat probíhající zánětlivou reakci v sítnici (Ding et al. 2017).

2.4.2.2.1 Diferenciace MSC na buňky sítnice

Sítnice je složena z deseti vrstev buněk, při poškození či odumření jednoho typu buněk dochází k porušení jejich propojení a následně ztrátě funkce. Bylo zjištěno, že MSC jsou schopné diferenciace na různé buňky sítnice – fotoreceptory (Kicic et al. 2003; Castanheira et al. 2008; Yang et al. 2010; Huo et al. 2010; Nadri et al. 2013), RPE buňky (Vossmerbaeumer et al. 2009; Huo et al. 2010; Guan et al. 2013), bipolární buňky (Castanheira et al. 2008; Nadri et al. 2013) a další buněčné typy. MSC představují perspektivní buněčný typ vhodný pro nahrazení a regeneraci poškozených buněk sítnice.

Diferenciace MSC na buňky sítnice *in vitro* je možná za použití synteticky připravených látek nebo společnou kultivací s buňkami sítnice. MSC izolované ze spojivky potkanů po kultivaci na nanovlákenných nosičích v přítomnosti taurinu exprimovaly znaky charakteristické pro fotoreceptory a bipolární buňky (Nadri et al. 2013). Taurin spolu s aktivinem A a EGF byl použit k diferenciaci BM-MSC i v jiné studii. Buňky exprimovaly po 7 dnech kultivace v diferenciačním médiu znaky fotoreceptorů. Ve stejné studii bylo ukázáno, že BM-MSC jsou schopné také diferenciace *in vivo*. Po aplikaci BM-MSC do subretinálního prostoru došlo k integraci BM-MSC do sítnice a k expresi znaků typických pro fotoreceptory (Kicic et al. 2003). U laserem poškozené sítnice potkanů byla po transplantaci BM-MSC do sklivce pozorována diferenciace na buňky exprimující rodopsin jako znak fotoreceptorů a parvalbumin, který je typický pro bipolární a amakrinní buňky (Castanheira et al. 2008). Na potkaním modelu DR došlo po 4 týdnech od intravenózního podání lidských AD-MSC ke snížení hladiny glukózy v krvi. Dále byla pozorována diferenciace AD-MSC na buňky exprimující znaky fotoreceptorů a astrocytů a již po 1 týdnu bylo zaznamenáno snížení poškození BRB oproti neléčeným jedincům (Yang et al. 2010). Na jiném modelu DR došlo po intravitreálním podání BM-MSC k integraci buněk do vnitřní části sítnice a diferenciaci na gliové buňky (Cerman et al. 2016).

Podobné výsledky byly ukázány i pro RPE buňky. Po destrukci RPE buněk a fotoreceptorů pomocí jodičnanu sodného v potkaním modelu retinitis pigmentosa se subretinálně aplikované BM-MSC diferencovaly na buňky exprimující znaky jak fotoreceptorů, tak RPE buněk (Huo et al. 2010). V jiné studii byly lidské BM-MSC *in vitro* kultivovány s lidskými RPE buňkami. Po 1 týdnu byly diferencované BM-MSC pozitivní na znaky typické pro fotoreceptory (Chiou et al. 2005). Dalším onemocněním s poškozením RPE buněk je VPMD. U potkanů s chemicky navozeným poškozením sítnice po subretinální aplikaci BM-MSC došlo k integraci MSC do sítnice a nahrazení poškozených RPE buněk (Guan et al. 2013).

2.4.2.2.2 Parakrinní efekt MSC

MSC produkují růstové a trofické faktory, z nichž některé mají neuroprotektivní charakter. Bylo ukázáno, že MSC jsou schopné snížit a regenerovat poškození sítnice také svým parakrinním působením, například přes produkci nervového růstového faktoru (NGF, nerve growth factor), mozkového neurotrofického faktoru (BDNF, brain-derived neurotrophic factor), ciliárního neurotrofického faktoru (CNTF, ciliary neurotrophic factor), neurotrofického faktoru z gliální buněčné linie (GDNF, glial cell line derived neurotrophic factor), bFGF, IGF, PDGF, HGF a dalších (Meirelles et al. 2009; Kolomeyer a Zarbin 2011; Park et al. 2017). MSC po aplikaci do oka podporují přežívání fotoreceptorů (Arnhold et al. 2007; Inoue et al. 2007), gangliových buněk (Na et al. 2009; Mead et al. 2016) a celkovou funkci sítnice.

Na modelu glaukomu u potkanů bylo zjištěno, že MSC se po intravitreálním podání nediferencují v gangliové buňky, ale podporují jejich přežívání a to především produkcí řady trofických faktorů jako BDNF, GDNF, CNTF, HGF, bFGF (Yu et al. 2006). Jiné výsledky ukázaly, že po aplikaci do oka se zvýšeným nitroočním tlakem pouze minimum injikovaných MSC migrovalo do gangliové vrstvy. Přesto ve srovnání s potkany, kterým nebyly aplikovány MSC, přežívalo v sítnici po injekci MSC více gangliových buněk. U injikovaných MSC byla zjištěna produkce bFGF, BDNF, CNTF a dalších neurotrofických faktorů, které podporovaly přežívání a regeneraci gangliových buněk v sítnici (Na et al. 2009). Na modelu glaukomu u potkanů byl také porovnán regenerativní efekt MSC izolovaných ze zubní dřeně, AD-MSC a BM-MSC. U očí léčených BM-MSC a MSC izolovaných ze zubní dřeně bylo zjištěno zvýšené přežívání gangliových buněk a obnova fungování sítnice na základě produkce PDGF a NGF (Mead et al. 2016). V *in vitro* pokusech se ukázalo, že BM-MSC kultivované

s gangliovými buňkami v hypoxickém prostředí jsou schopné potlačit jejich apoptózu (Yuan et al. 2016).

Na myším modelu DR došlo po intravitreální aplikaci AD-MSC k zabránění ztráty gangliových buněk. Nebyla zde prokázána diferenciace MSC, ale došlo k nárůstu produkce NGF, GDNF a bFGF a potlačení oxidativního poškození (Ezquer et al. 2016). K poškození sítnice může dojít také prostřednictvím laseru nebo vystavení silnému světelnému zdroji. Po transplantaci BM-MSC do takto poškozené sítnice byla inhibována apoptóza fotoreceptorů a podpořena regenerace poničené sítnice, u MSC byla prokázána produkce BDNF (Zhang and Wang 2010). V jiným studiích bylo do poškozeného oka aplikováno pouze kondiciované médium získané po kultivaci BM-MSC v normálních podmínkách (Dreixler et al. 2014) nebo v hypoxickém prostředí (Roth et al 2016). V obou případech došlo ke zlepšení funkce ischemicky poškozené sítnice a k poklesu apoptózy sítnicových buněk. Neuroprotektivní efekt MSC byl pozorován také po aplikaci AD-MSC do oka s poškozenou sítnicí pomocí světla. Po studiu kondiciovaného média *in vitro* bylo zjištěno, že oproti kontrole obsahuje zvýšené množství progranulinu, který má neuroprotektivní efekt (Tsuruma et al. 2014). Také *in vitro* pokusy ukázaly, že kondiciované médium získané po kultivaci BM-MSC snížilo apoptózu fotoreceptorů (Inoue et al. 2007).

2.4.2.2.3 Inhibice zánětlivé reakce

Nežádoucí imunitní reakce v sítnici jsou potlačeny několika způsoby. Migraci buněk imunitního systému zabraňuje BRB a na buňkách sítnice jsou exprimovány inhibiční molekuly jako například cytotoxický T lymfocytární antigen 4 nebo PD-L1. K narušení imunitní rovnováhy uvnitř oka dochází při patologických stavech, příkladem může být glaukom, VPMD nebo DR. Ve všech případech dochází k produkci prozánětlivých cytokinů, chemokinů, narušení BRB a infiltraci buněk imunitního systému (Klassen et al. 2013; Perez and Caspi 2015). MSC jsou vzhledem ke svým imunomodulačním vlastnostem schopné inhibovat nežádoucí imunitní reakce probíhající v oku a podpořit tak regeneraci v poškozené sítnici.

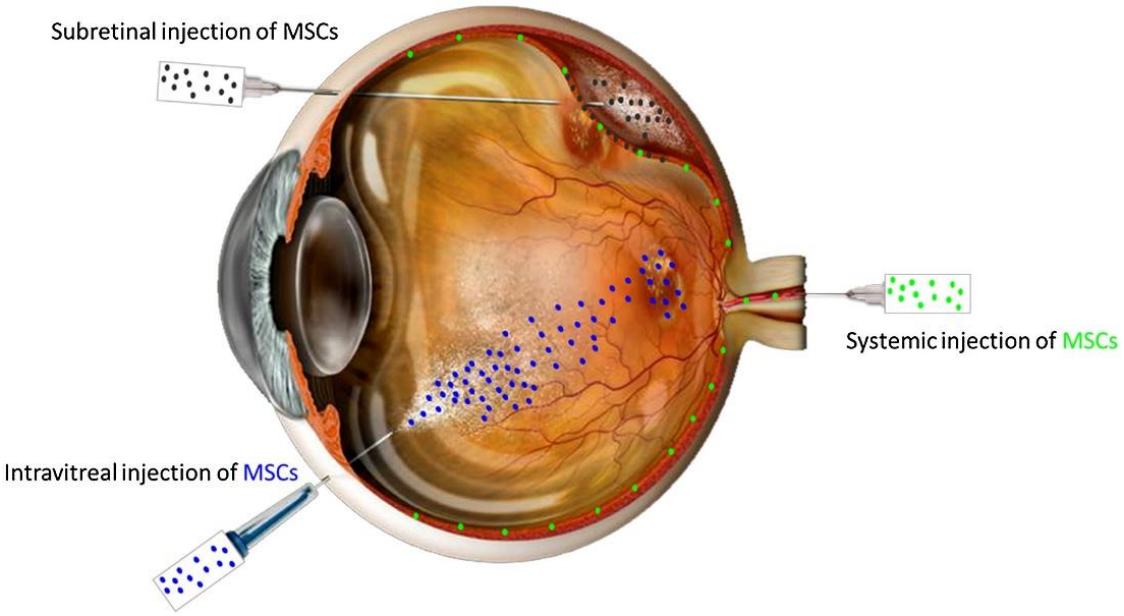
V *in vitro* pokusech bylo zjištěno, že při kultivaci BM-MSC a gangliových buněk poškozených peroxidem vodíku byla snížena produkce prozánětlivých cytokinů jako IL-1β a TNF-α produkovaných oxidativně poškozenými gangliovými buňkami a naopak byla zvýšena produkce neurotrofických faktorů jako BDNF a CNTF (Cui et al. 2016). Podobné výsledky *in vivo* byly ukázány jinou skupinou u potkanů se

světlem poškozenou sítnicí. Po aplikaci BM-MSC byla v sítnicích snížena produkce IL-1β a TNF-α oproti neléčeným poškozeným sítnicím. Transplantované MSC produkovaly neurotrofické faktory a inhibovaly apoptózu sítnicových buněk (Huang et al. 2013). Pokles produkce prozánětlivých cytokinů IL-1β, IL-6 a TNF-α byl prokázán i na modelu ischemie sítnice u potkanů po intravitreálním podání BM-MSC (Mathew et al. 2017). Po intravenózní aplikaci MSC potkanům s degenerací sítnice byla celkově zlepšena funkce sítnice a bylo zaznamenáno snížení počtu lymfocytů a monocytů v krvi (Bakondi et al. 2016).

2.4.2.2.4 Způsoby aplikace MSC

Při použití MSC pro terapeutické účely je potřeba vzít v úvahu také případné zamrazování MSC a jeho vliv na terapeutické vlastnosti MSC. Gramlich et al. ukázali, že kryoprezervace lidských MSC neměla vliv na imunomodulační vlastnosti, životnost a produkci růstových faktorů MSC. Po intraokulární aplikaci na myším modelu ischemického poškození sítnice došlo při použití zamrazených MSC ke zlepšení přežívání gangliových buněk (Gramlich et al. 2016).

V preklinických studiích dochází nejčastěji k aplikaci MSC intravitreálně (Yang et al. 2010; Ezquer et al. 2016), subretinálně (Kicic et al. 2003; Inoue et al. 2007) nebo případně intravenózně (Yang et al. 2010; Bakondi et al. 2016) (Obr. 5). Mezi méně obvyklé cesty transplantace MSC patří retrobulbární aplikace nebo aplikace pod vazivový obal oka (Weiss et al. 2015). Byla také provedena transplantace MSC do tenké epiretinální vrstvy kolem sklivce, transplantované BM-MSC zajistily zvýšené přežívání fotoreceptorů a zachování funkce sítnice u potkanů s degenerativním poškozením sítnice (Tzameret et al. 2015).



Obrázek 5. Možnosti aplikace MSC do oka. MSC mohou být aplikovány do oka několika cestami, mezi nejčastější způsoby aplikace patří intravitreální, subretinální nebo intravenózní systémové podání MSC (převzato z Salehi et al. 2016).

2.4.2.2.5 Přežívání a migrace MSC po aplikaci

Otázkou zůstává, jak dlouho jsou MSC schopné v sítnici přežívat a zda zůstávají pouze v místě poškození nebo migrují i do dalších tkání. Lidské AD-MSC se po intravitreální aplikaci integrovaly do různých vrstev sítnice potkana a přežívaly tam až po dobu 6 měsíců, některé fluorescenčně označené MSC ale překročily BRB a byly nalezeny například ve slezině (Haddad-Mashadrizeh et al. 2013). Na druhé straně v jiných studiích nebyl průnik MSC přes BRB po intravitreální aplikaci do oka pozorován (Velandia et al. 2017). Na modelu glaukomu u potkana byla porovnávána intravitreální a intravenózní aplikace BM-MSC. Po intravitreálním podání BM-MSC přežívaly v oku minimálně po dobu 5 týdnů, po této době byla většina MSC nalezena ve sklivci a malá část v sítnici. Naopak po intravenózním podání BM-MSC do poškozeného oka nemigrovaly a na progresi glaukomu neměly žádný pozitivní vliv (Johnson et al. 2010). Po intravitreální aplikaci lidských AD-MSC myším s DR byla po 7 dnech od aplikace většina AD-MSC nalezena v těsné blízkosti cévního zásobení sítnice, u myší bez indukované DR byl výskyt AD-MSC v oblasti kapilár menší (Rajashekhar et al. 2014).

2.4.2.2.6 Klinické studie

V jedné z klinických studií zaměřených na léčbu pacientů s pokročilým onemocněním retinitis pigmentosa bylo intravitreálně aplikováno 10^6 BM-MSC v 0,1 ml. U dvou ze tří pacienů po aplikaci bylo dočasně zlepšeno vnímání světla, u třetího pacienta došlo po transplantaci MSC k fibróze, odchlípení sítnice a neovaskularizaci duhovky (Satarian et al. 2017). U další klinické studie zaměřené na pacienty s VPMD bylo popsáno, že u obou pacientů došlo po aplikaci BM-MSC k zlepšení zrakové ostrosti, ale po 6 měsících se u jednoho z nich zraková ostrost zhoršila zpět na původní hodnoty (Park et al. 2015). Na druhé straně výsledky jiné klinické studie ukázaly, že po aplikaci MSC u pacienta s optickou neuropatií a poškozením zraku došlo k zlepšení zrakové ostrosti u obou očí. BM-MSC byly aplikovány do jednoho oka spolu s vitreoktomií a do druhého oka byly injikovány retrobulbárně, intravitreálně a pod vazivové pouzdro oka (Weiss et al. 2015).

V současné době probíhá 5 klinických studií věnujících se léčbě poškozené sítnice, 3 na aplikaci BM-MSC, 1 na aplikaci AD-MSC a 1 na aplikaci exosomů z MSC izolovaných z pupečníku. Všechny studie jsou ve fázi 1-2, kdy se jedná o studium bezpečnosti použití MSC. Studie jsou zaměřeny na pacienty s ischemickým poškozením sítnice, retinitis pigmentosa, VPMD, DR a další poškození sítnice. Studie zabývající se aplikací exosomů MSC izolovaných z pupečníku je zaměřena na léčbu makulárních děr. Ve všech případech kromě aplikace exosomů jsou používány autologní MSC, které jsou intravitreálně aplikovány do oka pacienta, jen v případě jedné studie jsou MSC aplikovány kombinací několika metod (www.clinicaltrials.gov).

3 Cíle práce

Cílem projektu je studium imunomodulačních, trofických a diferenciačních vlastností a mechanismů MSC v zánětlivém prostředí a jejich využití v léčbě poškozeného povrchu oka a degenerativních onemocnění sítnice.

- **Analyzovat mechanismus imunomodulačního působení MSC na B lymfocyty.**

MSC jsou schopné potlačit proliferaci a funkci B buněk a naopak podporují vývoj imunitních buněk regulačním směrem. Bude studován vliv MSC na produkci IL-10 stimulovanými B buňkami a stanoven mechanismus imunomodulačního působení MSC.

- **Stanovit vliv cytokinů na imunomodulační a diferenciační vlastnosti MSC.**

Pro aktivaci MSC je potřeba stimul v podobě zánětlivých cytokinů. Bude studován vliv cytokinů na expresi imunomodulačních molekul a trofických faktorů a na proces diferenciace MSC na buňky exprimující znaky sítnice.

- **Studovat možnosti diferenciace MSC na buňky exprimující znaky sítnice.**

MSC se mohou diferencovat na řadu buněčných typů. Bude analyzována schopnost MSC diferencovat se na buňky exprimující znaky sítnice. MSC budou kultivovány *in vitro* spolu s extraktem ze sítnic a supernatantem ze stimulovaných splenocytů simulujících zánětlivé prostředí poškozené sítnice.

- **Porovnat terapeutický potenciál MSC a LSC při léčbě poškození povrchu oka.**

MSC mají řadu podobných vlastností jako LSC, nabízí se tedy možnost jejich využití v případech, kdy nemohou být použity LSC. Terapeutický potenciál MSC z kostní dřeně a tukové tkáně bude porovnán s LSC na modelu chemicky poškozeného povrchu oka. Po aplikaci buněk na nanovlákenných nosičích bude sledován proces reepitelizace, neovaskularizace a potlačení lokální zánětlivé reakce.

- **Studovat schopnost MSC produkovat trofické a růstové faktory a inhibovat zánětlivé reakce v sítnici.**

Při regeneraci poškozené sítnice hrají důležitou roli trofické a růstové faktory. Bude sledována jejich exprese v prostředí simulující zánětlivé prostředí v poškozené sítnici. Dále bude studován vliv MSC na produkci prozánětlivých cytokinů v kultuře s explantáty sítnic.

4 Seznam vlastních publikací

4.1 Seznam použitých publikací

Holan V, Trosan P, Cejka C, Javorkova E, Zajicova A, **Hermankova B**, Chudickova M, Cejkova J. A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction. Stem Cells Transl Med. 2015;4:1052-1063.

Hermankova B, Zajicova A, Javorkova E, Chudickova M, Trosan P, Hajkova M, Krulova M, Holan V. Suppression of IL-10 production by activated B cells via a cell contact-dependent cyclooxygenase-2 pathway upregulated in IFN-γ-treated mesenchymal stem cells. Immunobiology. 2016;221:129-136.

Holan V, **Hermankova B**, Kossl J. Perspectives of stem cell-based therapy for age-related retinal degenerative diseases. Cell Transplant. 2017;26:1538-1541.

Hermankova B, Kossl J, Javorkova E, Bohacova P, Hajkova M, Zajicova A, Krulova M, Holan V. The identification of interferon-γ as a key supportive factor for retinal differentiation of murine mesenchymal stem cells. Stem Cells Dev. 2017;26:1399-1408.

4.2 Seznam ostatních publikací

4.2.1 Impaktované publikace

Trosan P, Javorkova E, Zajicova A, Hajkova M, **Hermankova B**, Kossl J, Krulova M, Holan V. The supportive role of insulin-like growth factor-I in the differentiation of murine mesenchymal stem cells into corneal-like cells. Stem Cells Dev. 2016;25:874-881.

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5 Výsledky

5.1 Inhibice produkce IL-10 aktivovanými B buňkami pomocí IFN-γ stimulovaných mezenchymálních kmenových buněk přes dráhu cyklooxygenázy-2 závislou na buněčném kontaktu

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Imunoregulační vlastnosti MSC byly popsány v různých modelech *in vitro* a *in vivo*. Kromě toho byla nedávno popsána populace Breg buněk, které produkují relativně vysoké koncentrace IL-10. V této práci jsme studovali vztah mezi MSC a Breg buňkami, analyzovali jsme účinky MSC na produkci IL-10 myšími B buňkami aktivovanými lipopolysacaridem (LPS). Produkce IL-10 B buňkami zůstala zachována v přítomnosti MSC a byla dokonce významně zvýšena působením IFN-γ. Produkce IL-10 však byla silně inhibována v kulturách obsahujících MSC a IFN-γ dohromady. Preinkubace MSC, ale ne B buněk, s IFN-γ indukovala potlačení sekrece IL-10 v kulturách obsahujících MSC a B buňky. Supernatanty z MSC stimulovaných IFN-γ neměly žádný inhibiční účinek a při oddělení buněk semipermeabilní membránou také nebylo pozorováno potlačení produkce IL-10. Analýza genové exprese MSC ovlivněných IFN-γ nebo IFN-γ a LPS odhalila silné zvýšení exprese genů pro IDO, COX-2 a PD-L1. V případě inhibice aktivity IDO nebo neutralizační monoklonální protilátky anti-PD-L1 nedošlo ke zrušení suprese IL-10. Na druhé straně indometacin, inhibitor COX-2, zcela inhiboval pokles produkce IL-10 zprostředkované MSC. Pro potvrzení byla produkce IL-10 B buňkami snížena také exogenním PGE2. Výsledky ukazují, že MSC ovlivněné IFN-γ inhibují produkci IL-10 aktivovanými B buňkami mechanizmem, který je závislý na buněčném kontaktu a je zprostředkován přes dráhu COX-2.

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Suppression of IL-10 production by activated B cells via a cell contact-dependent cyclooxygenase-2 pathway upregulated in IFN-γ-treated mesenchymal stem cells

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ABSTRACT

The immunoregulatory properties of mesenchymal stem cells (MSCs) have been well documented in various models in vitro and in vivo. Furthermore, a population of regulatory B cells (Bregs) that produce relatively high concentrations of IL-10 has been recently described. To study the relationship between MSCs and Bregs, we analyzed the effects of MSCs on IL-10 production by lipopolysaccharide (LPS)-activated mouse B cells. The production of IL-10 by B cells remained preserved in the presence of MSCs and was even significantly enhanced by IFN-γ. However, the production of IL-10 was strongly suppressed in cultures containing MSCs and IFN-γ. Preincubation of MSCs, but not of B cells, with IFN-γ induced the suppression of IL-10 secretion in cultures containing MSCs and B cells. The supernatants from IFN-γ-treated MSCs had no inhibitory effect, and the suppression of IL-10 production was abrogated if the MSCs and B cells were separated in a transwell system. Analysis of the gene expression of IFN-γ- or IFN-γ and LPS-treated MSCs revealed a strong upregulation of genes for indoleamine-2,3-dioxygenase (IDO), cyclooxygenase-2 (Cox-2) and programmed cell death-ligand 1 (PD-L1). While the inhibition of IDO activity or the inclusion of the neutralization monoclonal antibody anti-PD-L1 did not abrogate the suppression, indomethacin, an inhibitor of Cox-2, completely inhibited the MSC-mediated suppression of IL-10 production. Accordingly, the production of IL-10 by B cells was inhibited by exogenous prostaglandin E_2. The results thus suggest that IFN-γ-treated MSCs strongly inhibit IL-10 production by activated B cells by a mechanism requiring cell contact and involving the Cox-2 pathway.

1. Introduction

Bone marrow-derived mesenchymal stromal/stem cells (MSCs) represent a population of nonhaematopoietic stem cells that primarily preserve a stem cell niche in the bone marrow, but can also differentiate into other cell populations within the mesodermal lineage and thus contribute to tissue regeneration and healing (Le Blanc, 2006; Pittenger et al., 1999). In addition to these properties, MSCs have potent immunoregulatory properties. It has been well documented that MSCs inhibit the proliferation of mitogen- or antigen-stimulated lymphoid cells, suppress the production of proinflammatory cytokines and inhibit the development of cytotoxic T lymphocytes in vitro (Aggarwal and Pittenger, 2005; Bartholomew et al., 2002; Di Nicola et al., 2012; Le Blanc and Ringdén, 2007) as well as suppress inflammatory, autoimmune and transplantation reactions in vivo (Abumaree et al., 2012; Bartholomew et al., 2002). To date, multiple mechanisms of the MSC-mediated suppression of T-cell-dependent immune reactions have been demonstrated. The production of various suppressive factors, the expression of inhibitory membrane molecules, negative effects on antigen-presenting cells and the activation of regulatory T cells, have all been proposed as possible mechanisms of MSC-mediated immunosuppression (Di Nicola et al., 2012; Ghannnam et al., 2010; Maccario et al., 2005; Najar et al., 2012). Although extensive data have been published on the effects of MSCs on T cells (Abumaree et al., 2012; Aggarwal and Pittenger, 2005; Bartholomew et al., 2002; Di Nicola et al., 2012; Le Blanc and

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Ringdén, 2007), only limited knowledge is available on the effect of MSCs on B lymphocytes (Corcione et al., 2006; Franquesa et al., 2012; Glennie et al., 2005; Rasmusson et al., 2007).

The primary function of B cells is the production of antibodies. However, a population of B cells that regulate immune responses independently of antibody production has been described (Bouaziz et al., 2012; Matsushita and Tedder, 2011; Mauri and Bosma, 2012). These B cells inhibit immune reactions mainly by the production of IL-10 and are termed regulatory B cells (Bregs) or B10 cells (Bouaziz et al., 2012; DiLillo et al., 2010). Bregs inhibit autoimmune, tumour and transplantation reactions mainly through IL-10 secretion, but the suppressive mechanisms attributed to other types of regulatory cells can also be found among B cells (Klinker and Lundy, 2012). Although both MSCs and Bregs regulate immune responses there are no data on the mutual regulation of their immunomodulatory functions.

Since MSCs produce a number of molecules with immunomodulatory properties (Oh et al., 2009; Roddy et al., 2011; Svobodova et al., 2012), express membrane-bound regulatory molecules (Akiyama et al., 2012; Luz-Crawford et al., 2012), enhance the development of regulatory T cells (Ge et al., 2010), and regulate the activation of dendritic cells and macrophages (Abumaree et al., 2013; Spaggiari et al., 2009), we studied the effects of MSCs on the development of IL-10 producing B cells. We have recently prepared highly purified MSCs from the mouse bone marrow and described their ability to inhibit T cell functions (Javorkova et al., 2014; Svobodova et al., 2012). We also described the activation of IL-10-producing cells in a purified B-cell population and demonstrated the effects of cytokines on Breg development and IL-10 production (Holan et al., 2014). Here we show that highly purified untreated MSCs do not influence the development of Bregs and IL-10 production. However, the production of IL-10 by B cells was strongly suppressed if the B cells were stimulated in the presence of MSCs pretreated with IFN-γ. This suppression depended on contact between the MSCs and B cells and involved the cyclooxygenase-2 (Cox-2) pathway, which was highly upregulated in IFN-γ-treated MSCs.

2. Materials and methods

2.1. Mice

Mice of both sexes of the inbred strain BALB/c were used in the experiments at the age of 7–9 weeks. The animals were purchased from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague. The use of the animals was approved by the Local Ethical Comittee of the Institute of Experimental Medicine.

2.2. MSCs and B-cells enrichement procedure

MSCs were prepared from bone marrow isolated from the femurs and tibias of female BALB/c mice. The bone marrow was flushed out, and a single-cell suspension was seeded at a concentration of 4×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) containing 10% fetal calf serum (FCS, Gibco BRL, Grand Island, NY, USA), antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin) and 10 mM HEPES buffer (hereafter referred to as complete DMEM) in 75-cm^2 tissue culture flasks (Techno Plastic Products, Trasadingen, Switzerland). Non-adherent cells were washed out after 48 h of cultivation, and the remaining adherent cells were cultured for an additional 3 weeks (2–3 passages) at 37 °C in an atmosphere of 5% CO_2. Plastic adherent cells were harvested by a short trypsinization and subsequent gentle scraping. The resulting cell suspension was incubated 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 (AutoMACS, Miltenyi Biotec). The remaining $CD11b^-$ and $CD45^-$ cells were characterized in terms of their purity and differentiation potential.

For the preparation of B cells, a single cell suspensions of spleen cells were prepared in RPMI-1640 medium (Sigma) containing 10% FCS (Sigma), antibiotics (penicillin, streptomycin), 10 mM Hepes buffer and 5×10^{-5} M 2-mercaptoethanol. The B cells were isolated by positive selection using a CD19 MicroBeads isolation kit (Miltenyi Biotec). Flow cytometry analysis showed that this procedure yielded a cell population containing less than 3% of $CD3^+$ cells, and more than 90% of the cells were $CD19^+$.

2.3. Characterization of isolated B cell and MSC populations

The purity and phenotype of the enriched B cell population were characterized by flow cytometry using the following monoclonal antibodies (mAb): FITC-labelled anti-CD19 (clone 6D5), Alexa Flour 647 labelled anti-CD5 (clone 53-73), Alexa Flour 647 labelled anti-CD22 clone (OX-97), PE-labelled anti-CD14 (clone Sa14-2) and APC-labelled anti-CD3 (clone 17A2). All antibodies were purchased from BioLegend (San Diego, CA, USA).

To characterize the phenotype of the MSCs, the cells were incubated for 30 min with the following anti-mouse mAb: allophycocyanine (APC)-labelled anti-CD44 (clone IM7, BD PharMingen, San Jose, CA, USA), phycoerythrin (PE)-labelled anti-CD73 (cloneTY/11.8, eBioscience, San Diego, CA, USA), APC-labelled anti-CD11b (clone M1/70, BioLegend) or fluorescein isothiocyanate (FITC)-labelled anti-CD45 (clone 30-F11, BioLegend). Cells stained with PE-labelled rat IgG2a (clone RTK2758, BioLegend), APC-labelled rat IgG2b (clone RTK4530, BioLegend) or FITC-labelled rat IgG2b (clone RTK4530, BioLegend) were used as negative controls. Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA, USA) added to the samples 15 min before flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The morphological characteristics and differentiation potential of purified MSCs have been described in detail elsewhere (Javorkova et al., 2014; Svobodova et al., 2012).

2.4. Production and detection of IL-10

B cells at a concentration 0.9×10^6 cells/ml were incubated in 48-well tissue culture plates (Corning Inc., Corning, NY, USA) in a final volume of 0.6 ml of complete RPMI-1640 medium unstimulated or stimulated with LPS (5 μg/ml, Difco Laboratories, Detroit, MI, USA). After a 72-h incubation, the supernatants were harvested and tested for the presence of IL-10 by ELISA. To test the effects of MSCs and IFN-γ on IL-10 production by B cells, MSCs at the ratios 1: 4 to 1: 64 or recombinant mouse IFN-γ (10 ng/ml, PeproTech, Rocky Hill, NJ, USA) were added to the cultures of LPS-activated B cells and the production of IL-10 was determined.

The production of IL-10 was determined by ELISA using capture and detection mAb anti-IL-10 purchased from R and D Systems (Minneapolis, MN, USA) and following the instructions of the manufacturer. The reactions were quantified by spectrophotometry using a Sunrise Remote ELISA Reader (Gröding, Austria).

2.5. Effects of the separation of B cells and MSCs on IL-10 production

To test the effect of the separation of MSCs from B cells on IL-10 production, MSCs were separated from B cells by cell culture inserts

(Nunc, Roskilde, Denmark) and cultured for 72 h in the presence of 5 μg/ml of LPS. The production of IL-10 was determined in cultures of LPS-stimulated B cells alone, B cells mixed with MSCs or B cells separated from MSCs by a semipermeabile membrane.

In the other set of experiments, supernatants were prepared by a 48-h incubation of MSCs alone, with IFN-γ (10 ng/ml), LPS (5 μg/ml) or both IFN-γ and LPS. The supernatants were added to the cultures of B cells stimulated with LPS to achieve final concentrations of 50% of the cell culture volume, and the production of IL-10 by the cells was determined after a 72-h incubation.

2.6. Pretreatment of MSCs and B cells with IFN-γ

MSCs (10^5 cells/well) were cultured in a volume of 1 ml in 24-well tissue culture plates (Techno Plastic Products) untreated or in the presence of 10 ng/ml of IFN-γ. After a 24-h incubation, the adherent MSCs were washed with an excess of culture medium. B cells, preincubated for 24 h alone or with IFN-γ (10 ng/ml) and then thoroughly washed, were added (0.9×10^6 cells /ml) to the cultures of MSCs and were stimulated with LPS (5 μg/ml). The supernatants were harvested after a 72-h incubation and the concentrations of IL-10 determined by ELISA.

2.7. Inhibition of the MSC-mediated suppression of IL-10 production

Purified B cells were stimulated with LPS in the presence of MSCs and IFN-γ, and 1-methyl-D-tryptophane (1-MT, Sigma), a selective inhibitor of indoleamine-2,3-dioxygenase (IDO), or indomethacin (Sigma), an inhibitor of Cox-2 and prostaglandin E_2 (PGE_2) synthesis, was added to the cultures to achieve a final concentration 10^{-3} M or 10^{-8} M, respectively. To block the progammed cell death-ligand 1 (PD-L1) (CD274)—PD-1 (CD279) pathway, the inhibitory mAb anti-PD-L1 (clone MIH5, eBioscience) was added to the cultures at a concentration of 5 μg/ml.

To test the effects of PGE_2 on IL-10 production by B cells, the cells were stimulated with LPS (5 μg/ml), and synthetic PGE_2 (Sigma) was added to these cultures to concentrations ranging from 200 μM to 2 μM. The production of IL-10 was determined after a 72-h incubation by ELISA.

2.8. Detection of gene expression

The expression of genes for IDO, Cox-2, trasnsforming growth factor-β (TGF-β), IL-6, hepatocyte growth factor (HGF), PD-L1, Fas and Fas-L was detected using real-time PCR, as we have described previously (Trosan et al., 2012). In brief, MSCs were cultured for 24 h unstimulated or stimulated with IFN-γ (10 ng/ml), LPS (5 μg/ml) or IFN-γ and LPS, and total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. One μg of total RNA was treated with deoxyribonuclease I (Promega, Madison, WI, USA) 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, USA) as previously described (Trosan et al., 2012). The sequences of the primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-6, TGF-β, IDO, HGF, Cox-2, PD-L1, Fas and FasL 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 an elongation step at 80 °C for 5 s and were analyzed using StepOne Software version 2.2.2 (Applied Biosystems).

Table 1
Murine primer sequences used for real-time PCR.

Gene	Sense primer	Antisense primer
GAPDH	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
IDO	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
Cox-2	AGCCCACCCCAAACACAGT	AAATATGATCTGGATGTCAGCACATATT
TGF-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
HGF	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
PD-L1	CTACGGTGGTGCCGGACTACA	CATGCTCAGAAGTGGCTGCAT
PD-1	CGTCCCTCAGTCAAGAGGAG	GTCCCTAGAAGTGCCCAACA
FasL	TGGGTAGACAGCAGTGCCAC	GCCCACAAGATGGACAGGG
Fas	GGCATCATTGGGCACTCCTT	GCTGCAAGCACAGCCTCTCT

2.9. Statistical analysis

The results are expressed as the mean ± SD. Comparisons between 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.

3. Results

3.1. Phenotypic characterization of purified B-cell and MSC populations

Purified B-cell and MSC populations were phenotypically characterized by flow cytometry. As demonstrated in Fig. 1, the isolated B-cell population contained 93.8 ± 1.5 % $CD19^+$ cells, which included about 92.2 ± 1.9 % $CD19^+CD22^+$ cells, 7.9 ± 1.0 % $CD19^+CD5^+$ cells, 2.8 ± 0.8 % $CD3^+$ cells and 1.4 ± 0.7 % $CD14^+$. After the MACS-separation, 0.7 ± 0.2 % of the MSCs were $CD11b^+$ and 2.8 ± 0.4 % were $CD45^+$. The cells were positive with corresponding intensity for CD44 and CD73, which are markers attributed to murine MSCs (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, when cultured in specific differentiation mediums, as we have described (Svobodova et al., 2012).

3.2. IL-10 production by LPS-stimulated B cells in the presence of MSCs and IFN-γ

Purified B cells stimulated with LPS produced a substantial level of IL-10 (Fig. 2). The production of IL-10 by B cells remained preserved or was slightly decreased in the presence of MSCs and was significantly enhanced in B cell cultures supplemented with IFN-γ. However, the production of IL-10 was strongly suppressed in the cultures containing both MSCs and IFN-γ (Fig. 2A). The suppression was MSC dose-dependent and disappered at low doses of MSCs (at ratio 1:64 or less, data not shown). To test whether the suppression of IL-10 production is mediated by cell–cell contact or by a soluble factor(s), supernatants were prepared by the incubation of MSCs with IFN-γ, LPS, or IFN-γ and LPS together and were added to cultures of LPS-stimulated B cells. As demonstrated in Fig. 2B, none of these supernatants inhibited IL-10 production. Moreover, B cells and MSCs were separated in a transwell system. As shown in Fig. 2C, the suppression of IL-10 production was not observed when B cells and MSCs were separated by a semipermeabile membrane.

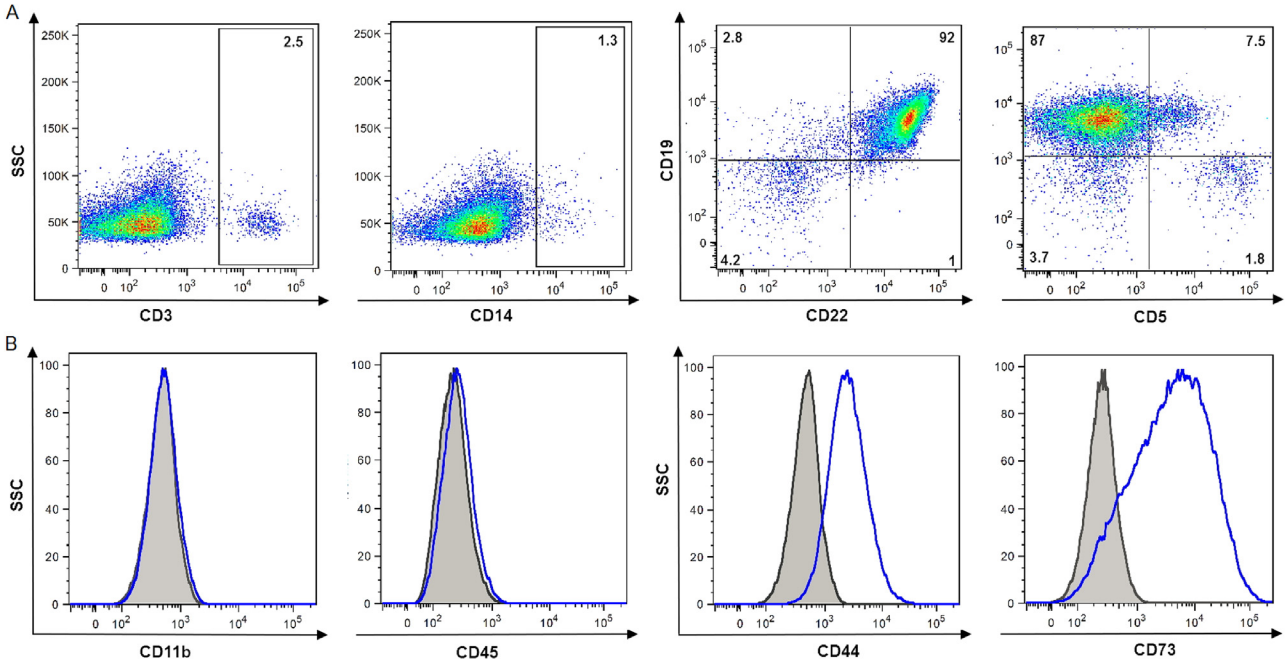

Fig. 1. Phenotypic characterization of enriched B-cell (A) and MSC (B) populations. The cells were separated on MACS and characterized using flow cytometry. Representative dot plots indicate the percentage of $CD19^+$ $CD5^+$, $CD19^+$ $CD22^+$, $CD3^+$ and $CD14^+$ cells among the B-cell population, and the percentage of $CD44^+$, $CD73^+$, $CD11b^+$ and $CD45^+$ cells among the MSCs. One of three similar experiments is shown.

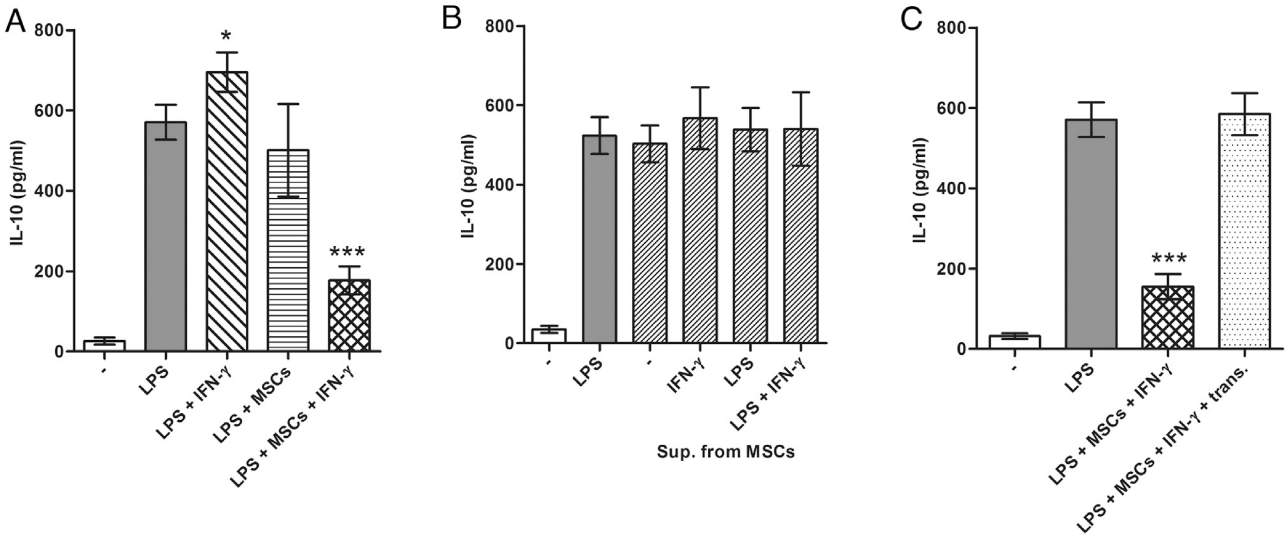

Fig. 2. Inhibition of IL-10 production by LPS-stimulated B cells in the presence of MSCs and IFN-γ. (A) IL-10 production was determined in unstimulated B cells or in B cells stimulated with LPS in the presence of MSCs and IFN-γ. (B) Production of IL-10 in cultures of B cells stimulated with LPS, IFN-γ and MSCs, in which the B cells were separated from MSCs by a transwell system. (C) Production of IL-10 by LPS-activated B cells in the presence of supernatants (50% of culture volume) from MSCs that were either unstimulated or stimulated with LPS, IFN-γ or LPS plus IFN-γ. The concentrations of IL-10 were determined by ELISA. Each bar represents the mean ± SD from 4 independent determinations. Values with asterisks are significantly different ($^*P < 0.05$, $^{***}P < 0.001$) from the control (B cells stimulated with LPS only).

3.3. The effect of preincubation of MSCs or B cells with IFN-γ on the suppression of IL-10 production

Purified B cells were preincubated for 24 h in culture medium or in medium with IFN-γ, then thoroughly washed with an excess of medium, and added to cultures of MSCs that had been preincubated in medium alone or in medium with IFN-γ. The cultures were stimulated with LPS and the production of IL-10 was determined. The suppression of IL-10 production was observed only in those cultures in which the MSCs were preincubated with IFN-γ (Fig. 3). The suppression of IL-10 production did not depend on the preincubation of B cells with IFN-γ.

3.4. The expression of genes for immunomodulatory molecules by IFN-γ–activated MSCs

To identify the molecule(s) responsible for the IFN-γ-activated MSC-mediated suppression of IL-10 production, MSCs were cultured for 24 h unstimulated or in the presence of IFN-γ, LPS or IFN-γ and LPS, and the expression of genes for immunoregulatory molecules (IDO, Cox-2, TGF-β, HGF, PD-L1, Fas and Fas-L) was determined by real-time PCR. We identified 3 genes (IDO, Cox-2 and PD-L1) that were weakly expressed or not expressed in control unstimulated MSCs but which were significantly upregulated in the presence of IFN-γ (Fig. 4).

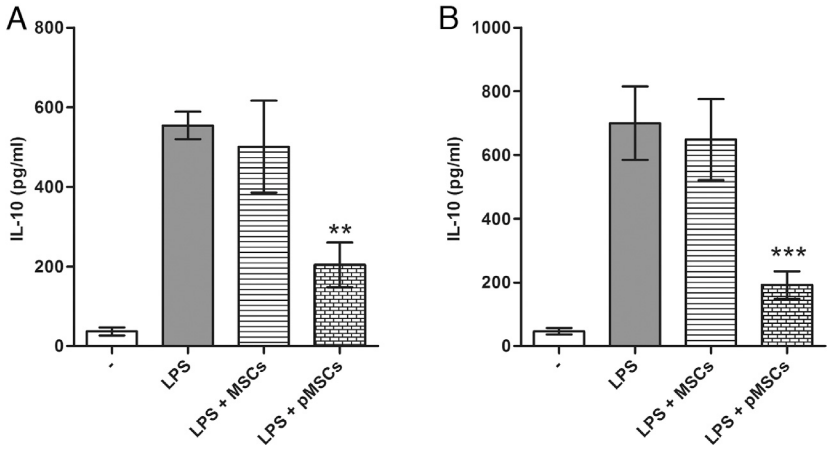

Fig. 3. The effect of the preincubation of MSCs or B cells with IFN-γ on the suppression of IL-10 production. MSCs were preincubated for 24 h alone or in the presence of 10 ng/ml of IFN-γ. B cells that were preincubated for 24 h alone (A) or with IFN-γ (B) were added to the cultures of MSCs and stimulated with LPS. The production of IL-10 was determined by ELISA. Each bar represents the mean ± SD from 3 independent determinations. Values with asterisks are significantly different (**$P < 0.01$, ***$P < 0.001$) from the control (B cells stimulated with LPS in the absence of MSCs).

3.5. MSC-mediated suppression of IL-10 production by B cells depends on a pathway involving Cox-2

Purified B cells were stimulated with LPS alone or in the presence of MSCs and IFN-γ to induce the suppression of IL-10 production. Indomethacin (an inhibitor of Cox-2 and PGE_2 synthesis), 1-MT (a selective inhibitor of IDO), or inhibitory mAb anti-PD-L1 was added to cultures containing MSCs and IFN-γ, and the production of IL-10 was determined after a 72-h incubation period. As shown in Fig. 5, 1-MT and the mAb anti-PD-L1 did not reverse the inhibition, while indomethacin (an inhibitor of Cox-2) completely abrogated the MSC-mediated suppression of IL-10 production.

3.6. Suppression of IL-10 production by PGE_2

To demonstrate that PGE_2, as the final effector molecule in the Cox-2-dependent pathway, could be responsible for the suppression of IL-10 production, B cells were stimulated with LPS, and synthetic PGE_2 was added to these cultures. As demonstrated in Fig. 6, PGE_2 inhibited IL-10 production by B cells in a dose-dependent manner.

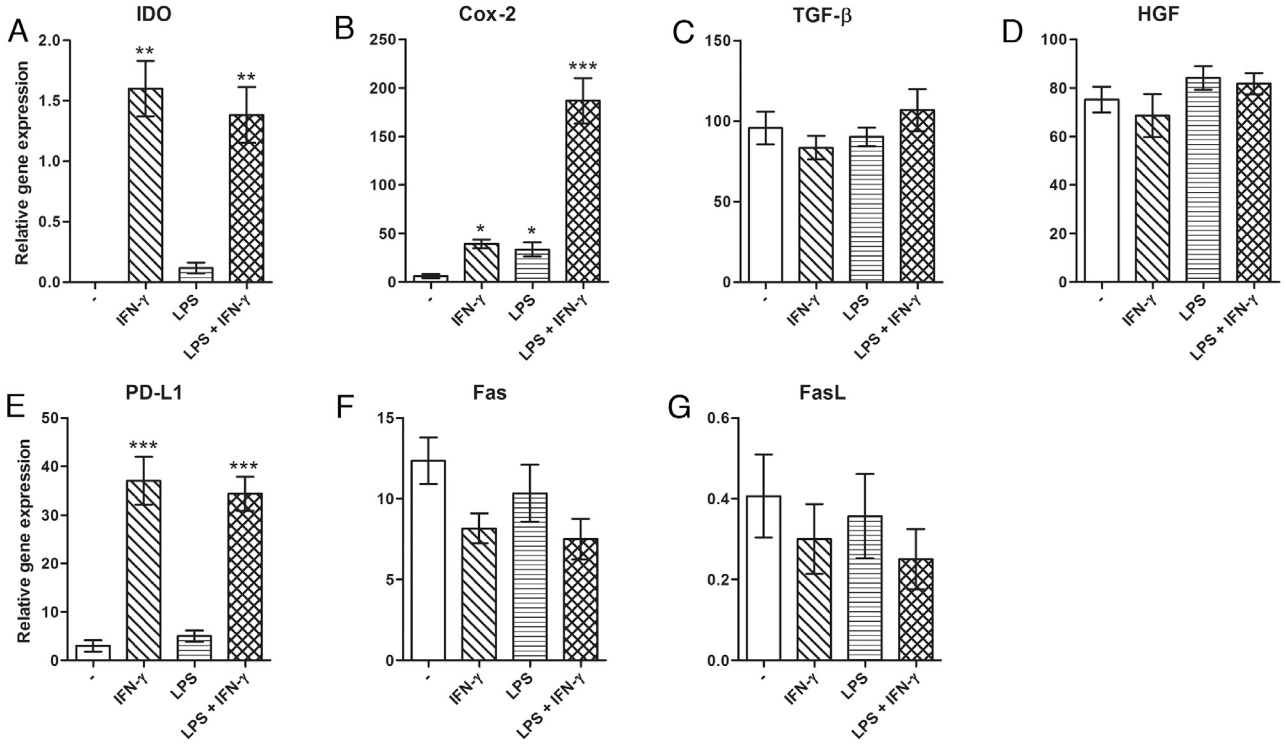

Fig. 4. The expression of genes for immunomodulatory molecules in control and IFN-γ-, LPS- or IFN-γ and LPS-stimulated MSCs. MSCs were cultured for 24 h unstimulated or stimulated with IFN-γ (10 ng/ml), LPS (5 μg/ml) or IFN-γ and LPS, and the expression of the genes for IDO (A), Cox-2 (B), TGF-β (C), HGF (D), PD-L1 (E), Fas (F) and Fas-L (G) was determined by real time PCR. Each bar represents the mean ± SD from 3 independent determinations. Values with asterisks are significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from those of untreated MSCs.

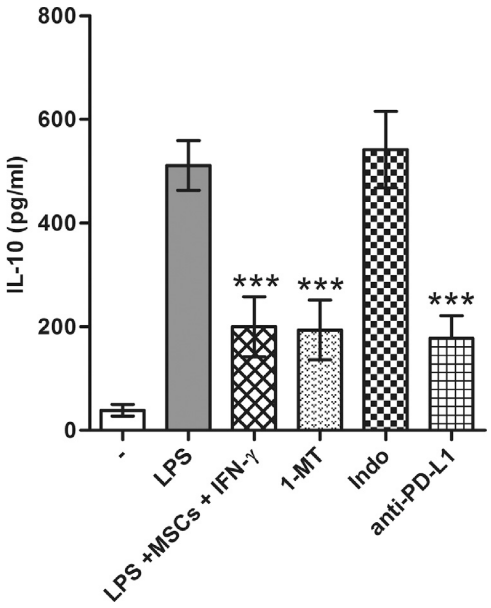

Fig. 5. The effects of IDO and Cox-2 inhibitors or the inhibitory mAb anti-PD-L1 on IL-10 production by B cells stimulated with LPS in the presence of MSCs and IFN-γ. Purified B cells were stimulated with LPS in the presence of MSCs and IFN-γ, and 1-MT (a selective inhibitor of IDO), indomethacin (an inhibitor of Cox-2) or the mAb anti-PD-L1 was added to the cultures containing MSCs and IFN-γ. The production of IL-10 was determined after a 72-h incubation by ELISA. Each bar represents the mean ± SD from 3 independent determinations. Values with asterisks are significantly different (***$P < 0.001$) from the control (B cells stimulated with LPS in the presence of MSCs and IFN-γ).

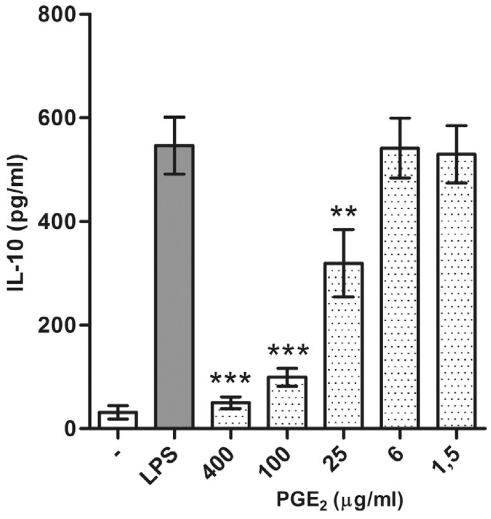

Fig. 6. The effect of exogenous PGE_2 on IL-10 production by activated B cells. The cells were stimulated with LPS, and PGE_2 was added to the cultures at the indicated concentrations. The production of IL-10 was determined after a 72-h incubation. Each bar represents the mean ± SD from 3 independent determinations. Values with asterisks demonstrate a statistically significant (**$P < 0.01$, ***$P < 0.001$) inhibition of IL-10 production.

4. Discussion

A number of different cell populations are involved in regulating the immune response, and thus they direct the development and final manifestation of the immune reaction. One of these cell populations are Bregs, which are characterized by their ability to inhibit, in an antibody-independent fashion, immune reactions in vitro and in vivo (Bouaziz et al., 2012; Corcione et al., 2006; Mauri and Bosma, 2012). The main mechanism of Breg action involves the production of IL-10. It has been shown that the development of Bregs and their activity are strictly regulated by cytokines. In this respect, IFN-γ and IL-12 were shown to enhance Breg development and IL-10 secretion, while IL-21 and TGF-β are rather inhibitory for Breg development (Holan et al., 2014; Yoshizaki et al., 2012). The effects of regulatory cells on the development and function of Bregs are not known.

Another recently intensively studied cell population with immunomodulatory actions and clinical potential are MSCs. These cells possess potent suppressive properties and spontaneously produce the inhibitory cytokine TGF-β. However, in an inflammatory environment or in the presence of proinflammatory cytokines MSCs produce, in addition to TGF-β, also IL-6. These two cytokines (i.e., TGF-β and IL-6) drive rather the development of proinflammatory Th17 cells and stimulate IL-17 production (Bettelli et al., 2006; Svobodova et al., 2012). Since there is a large number of publications on the effects of MSCs on different T-cell populations, but considerably less on B cells, we studied the effect of MSCs on the development of IL-10-producing B cells. We found that untreated MSCs did not significantly modify IL-10 production by LPS-activated B cells. In accordance with the published data (Holan et al., 2014; Yoshizaki et al., 2012), the production of IL-10 by B cells was significantly enhanced in the presence of IFN-γ. However, the simultaneous addition of MSCs and IFN-γ into cultures of LPS-stimulated B cells resulted in a profound inhibition of IL-10 production. We also observed that preincubation of MSCs with IFN-γ, but not of B cells, induced the suppression of IL-10 production. This finding suggests that IFN-γ acts through MSCs and not via an effect on B cells. The supernatants from IFN-γ-treated MSCs did not have a suppressive effect, and the separation of MSCs and B cells in a transwell system abrogated the suppression. These observations demonstrated that cell–cell contact between IFN-γ-activated MSCs and B cells is required for the suppression of IL-10 production by the B cells.

To search for the molecule that is expressed by IFN-γ-activated MSCs and which could be responsible for the suppression of IL-10 production, we cultured MSCs untreated or in the presence of IFN-γ alone or IFN-γ and LPS together. The expression of genes coding potentially immunoregulatory molecules was evaluated using real-time PCR. We identified three genes that were weakly expressed or not expressed in untreated MSCs but which were strongly upregulated after stimulation with IFN-γ or IFN-γ and LPS. One of these genes codes the membrane molecule PD-L1, which has been shown to be involved in MSC-mediated immunosuppression in other models (Luz-Crawford et al., 2012). Other molecules strongly upregulated in IFN-γ-treated MSCs were Cox-2, which is involved in the biosynthesis of PGE_2 and in immunosuppression (Duffy et al., 2011; English et al., 2009; Spaggiari et al., 2008), and IDO, which interferes with the bioactivity of tryptophane (Ge et al., 2010). Experiments utilizing selective inhibitors of IDO or Cox-2, or the use of the blocking mAb anti-PD-L1, showed that the MSC-mediated suppression of IL-10 production was completely abrogated by indomethacin, an inhibitor of Cox-2, but not by the IDO inhibitor 1-MT or by anti-PD-L1 antibody. Furthermore, we showed that the production of IL-10 by B cells can be inhibited by PGE_2, a molecule synthetized in the Cox-2 pathway. The involvement of PGE_2 in MSC-mediated immunosuppresion has been suggested (Duffy et al., 2011; Spaggiari et al., 2008).

To date, a number of distinct mechanisms have been proposed to be responsible for MSC-mediated immunosuppression. Rasmusson et al. (2005) showed that different mechanisms are involved in the suppression depending on the signal activating lymphocytes. Among the mechanisms based on contact between MSCs and lymphocytes, in addition to Cox-2, IDO and PD-L1, the Fas - FasL pathway has been proposed (Akiyama et al., 2012). In our model, neither Fas-L nor Fas were upregulated in IFN-γ-treated MSCs.

There is no published data on the MSC-mediated suppression of IL-10 production by B cells. Our results show that the suppression entirely depended on the Cox-2 pathway. Since the final effector molecule of this pathway is PGE_2, it is of interest that the suppression requires contact between IFN-γ-activated MSCs and B lymphocytes. In fact, the dependence of immunosuppression on cell contact has also been observed in other models in which the mechanism of suppression was Cox-2-dependent (Duffy et al., 2011; English et al., 2009). This might be due to a requirement for higher concentrations of PGE_2, which are achieved only at the site of cell–cell contact. This hypothesis is also supported by our observation that relatively high concentrations of PGE_2 (μM) are required to achieve a significant suppression of IL-10 production by B cells. It has been shown that MSCs spontaneously produce low levels of PGE_2, and we showed that the expression of the Cox-2 gene in MSCs is significantly increased in the presence of IFN-γ (and even more in the presence of IFN-γ and LPS). In accord with this observation, untreated MSCs did not significantly inhibit IL-10 production while in the presence of IFN-γ the suppression was highly significant. A requirement for priming of MSCs with IFN-γ has been shown in some other models of MSC-mediated suppression (Chinnadurai et al., 2014; Duijvestein et al., 2011; Ge et al., 2010).

In summary, we have shown that IFN-γ upregulates the expression of the Cox-2 gene in highly purified mouse MSCs. Furthermore, we have demonstrated for the first time that IFN-γ-pretreated MSCs strongly inhibit the development and function of IL-10-producing (regulatory) B lymphocytes and that this suppression is cell contact-dependent and involves the Cox-2 pathway.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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5.2 Srovnání terapeutického potenciálu mezenchymálních kmenových buněk a limbálních kmenových buněk pro regeneraci poškozeného povrchu oka.

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Stem Cells Trans Med. 2015;4,1052-1063

Léčba založená na kmenových buňkách je slibným prostředkem pro léčbu těžkých poranění nebo dosud neléčitelných onemocnění. Použití kmenových buněk je však často omezeno nedostatkem dostupných tkáňově specifických kmenových buněk, proto se hledají a testují další možné zdroje kmenových buněk. V tomto ohledu se ukázalo, že MSC jsou slibným kandidátem. V této studii jsme připravili BM-MSC, AD-MSC a LSC a porovnávali jsme jejich růst, diferenciaci a sekreční vlastnosti. Buňky byly pěstovány na nanovlákenných nosičích a přeneseny na chemicky poškozené oko králíků, kde byl studován jejich terapeutický potenciál. Zjistili jsme, že BM-MSC a tkáňově specifické LSC měly podobné terapeutické účinky. Proces hojení, hodnocení tloušťky rohovky, reepitelizace, neovaskularizace a inhibice lokální zánětlivé reakce byly srovnatelné u očí ošetřených BM-MSC a LSC. Výsledky byly významně lepší u očí s aplikovanými BM-MSC nebo LSC než u neléčených očí nebo očí ošetřených jen samotným nanovlákenným nosičem nebo nanovlákenným nosičem s AD-MSC. Výsledky ukazují, že terapeutický účinek BM-MSC na hojení poškozeného povrchu rohovky je srovnatelný s terapeutickým účinkem tkáňově specifických LSC. Z toho vyplývá, že BM-MSCs by mohly být použity pro regeneraci očního povrchu v případech, kdy autologní LSC chybí nebo je obtížné je získat.

Můj podíl na publikaci:
experimentální část (separace a kultivace MSC a LSC, real-time PCR), analýza výsledků

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A Comparative Study of the Therapeutic Potential of Mesenchymal Stem Cells and Limbal Epithelial Stem Cells for Ocular Surface Reconstruction

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Key Words. Limbal stem cells • Mesenchymal stem cells • Alkali-injured ocular surface • Corneal regeneration • Stem cell-based therapy

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ABSTRACT

Stem cell-based therapy has become an attractive and promising approach for the treatment of severe injuries or thus-far incurable diseases. However, the use of stem cells is often limited by a shortage of available tissue-specific stem cells; therefore, other sources of stem cells are being investigated and tested. In this respect, mesenchymal stromal/stem cells (MSCs) have proven to be a promising stem cell type. In the present study, we prepared MSCs from bone marrow (BM-MSCs) or adipose tissue (Ad-MSCs) as well as limbal epithelial stem cells (LSCs), and their growth, differentiation, and secretory properties were compared. The cells were grown on nanofiber scaffolds and transferred onto the alkali-injured eye in a rabbit model, and their therapeutic potential was characterized. We found that BM-MSCs and tissue-specific LSCs had similar therapeutic effects. Clinical characterization of the healing process, as well as the evaluation of corneal thickness, re-epithelialization, neovascularization, and the suppression of a local inflammatory reaction, were comparable in the BM-MSC- and LSC-treated eyes, but results were significantly better than in injured, untreated eyes or in eyes treated with a nanofiber scaffold alone or with a nanofiber scaffold seeded with Ad-MSCs. Taken together, the results show that BM-MSCs' therapeutic effect on healing of injured corneal surface is comparable to that of tissue-specific LSCs. We suggest that BM-MSCs can be used for ocular surface regeneration in cases when autologous LSCs are absent or difficult to obtain. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:1052–1063*

SIGNIFICANCE

Damage of ocular surface represents one of the most common causes of impaired vision or even blindness. Cell therapy, based on transplantation of stem cells, is an optimal treatment. However, if limbal stem cells (LSCs) are not available, other sources of stem cells are tested. Mesenchymal stem cells (MSCs) are a convenient type of cell for stem cell therapy. The therapeutic potential of LSCs and MSCs was compared in an experimental model of corneal injury, and healing was observed following chemical injury. MSCs and tissue-specific LSCs had similar therapeutic effects. The results suggest that bone marrow-derived MSCs can be used for ocular surface regeneration in cases when autologous LSCs are absent or difficult to obtain.

INTRODUCTION

Severe injuries or defects of the cornea represent are among the most common causes of decreased quality of vision or even blindness. In many cases, penetrating keratoplasty is performed as the first treatment option. However, if the corneal damage is more extensive and the limbal region is involved, the defect can lead to limbal stem cell deficiency (LSCD). In such cases, corneal transplantation alone is not a sufficient treatment method. The only effective way to treat LSCD is by the transplantation of whole limbal tissue or the transfer of limbal epithelial stem cells (LSCs). Although beneficial effects of limbal transplantation have been reported [1–3], the shortage of limbal tissue and a strong immune response to a limbal allograft are the main obstacles to such treatment protocols. Therefore, a more promising treatment method is offered by LSC transplantation. The first encouraging results from LSC transplantation have been published [4–6]. Since LSCs represent a relatively small population of limbal cells that are difficult to isolate and prepare in sufficient quantities, other stem cell sources are being explored and tested to treat LSCD.

An alternative source of stem cells for ocular surface regeneration and the treatment of LSCD is mesenchymal stem cells (MSCs). These cells can

be obtained relatively easily in a sufficient amount from various types of tissues (e.g., bone marrow, adipose tissue) and expanded in vitro for autologous application. It has been shown that MSCs retain their differentiation potential during in vitro expansion and that they can differentiate into various cell types [7], including cells expressing corneal epithelial cell markers [8, 9]. The first results of using MSCs for ocular surface healing in small animal models have been published [9, 10]. We have shown in mice [11] and rabbits [12] that MSCs grown on a nanofiber scaffold and transferred onto the damaged ocular surface significantly inhibit the local inflammatory reaction and support the healing process.

Although LSCs and MSCs have different origins, they share comparable immunoregulatory properties in vitro [13]. Similarly, numerous common properties have been described for tissue-specific stem cells isolated from different organs [14]. Comparative studies on MSCs prepared from bone marrow and other sources have shown many similarities but also some differences [15, 16]. For the treatment of ocular surface injuries and LSCD, both tissue-specific LSCs and MSCs isolated from the bone marrow (BM-MSCs) or adipose tissue (Ad-MSCs) have been proposed and tested. In these studies, MSCs proved to be a promising cell type to support the healing of the damaged ocular surface [9–12, 17–19]. However, so far, there is no direct evidence that MSCs can support the healing and regeneration of damaged corneal tissue as effectively as the tissue-specific LSCs. Therefore, in the present study, we used a well-established model of the alkali-damaged ocular surface in rabbits and directly compared the regenerative and reparative potential of tissue-specific LSCs and MSCs derived from bone marrow or adipose tissue. On the basis of several evaluated parameters, we show the therapeutic potential of BM-MSCs for the treatment of damaged ocular surface is comparable to that of tissue-specific LSCs, and, thus, BM-MSCs can be used therapeutically as a convenient source of stem cells to support healing of the wounded cornea.

Materials and Methods

Animals and Alkali-Induced Corneal Damage

Adult, female New Zealand white rabbits (2.5–3.0 kg) obtained from Velaz Ltd. (Prague, Czech Republic, http://www.velaz.cz) were used in the experiments. Rabbits were anesthetized by an intramuscular injection of a 1:1 mixture of xylazinum hydrochloridum 2% (0.2 ml/kg body weight; Rometar; Spofa, Prague, Czech Republic, http://www.spofa.cz) and ketaminum hydrochloridum 5% (1 ml/kg body weight; Narkamon; Spofa). The right corneas of anesthetized rabbits were injured by dropping 0.25N sodium hydroxide (NaOH) on the corneal surface (10 drops during 1 minute, alkali injuring the whole cornea, including the limbal region), then the eyes were immediately rinsed with an excess of tap water. After the alkali injury and awakening from the anesthesia, the rabbits were treated with analgesia (ketoprofen, 1.0 mg/kg i.m.) 2 times daily for 5 days. All experiments were conducted according to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the local ethics committee.

Isolation of LSCs and MSCs

LSCs were obtained by the enzymatic digestion of limbal tissue, as we have described in a mouse model [20]. In brief, limbal tissue was cut with scissors and subjected to 10 short (10 minutes each) trypsinization cycles. The released cells were harvested after each cycle, centrifuged (8 minutes at 250*g*), and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Corp., St. Louis, MO, http://www.sigmaaldrich.com) containing 10% fetal calf serum (FCS; Sigma-Aldrich), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 10 mM HEPES buffer. The cells were seeded in 25-cm^2 tissue culture flasks (Corning Inc., Schiphol-Rijk, The Netherlands, http://www.corning.com). For characterization of the cells and for their transfer onto a nanofiber scaffold, cells grown in vitro for 2–3 weeks (third passage) were used.

BM-MSCs were isolated from the femurs of rabbits. The bone marrow was flushed out, a single-cell suspension was prepared by homogenization, and the cells were seeded at a concentration of 4×10^6 cells per milliliter in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% FCS, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin), and 10 mM HEPES buffer in 25-cm^2 tissue culture flasks (Corning). After a 48-hour incubation, the nonadherent cells were washed out and the adherent cells were cultured with a regular exchange of the medium and passaging of the cells to maintain their optimal concentration. The cells were characterized and used at the third passage.

Ad-MSCs were isolated from subcutaneous adipose tissue. The tissue was cut into small pieces with scissors and incubated in 1 ml of Hanks' balanced salt solution containing 10 mg/ml collagenase type I (Sigma-Aldrich) for 60 minutes at 37°C with gentle agitation. Then the collagenase was diluted with complete DMEM. The cells were filtered and centrifuged at 250*g* for 8 minutes. The upper adipose layer was removed, the cells were centrifuged, resuspended in 6 ml complete DMEM (4×10^6 cells per milliliter), and seeded in 25-cm^2 tissue culture flasks (Corning). After incubation for 48 hours, the cells were washed with medium to remove nonadherent cells and cell debris, and cultured under standard conditions. Ad-MSCs were used in passages 3 and 4.

Stem Cell Growth, Differentiation, and Gene Expression

To show the morphology of MSCs and LSCs, the cells were grown on glass cover slips, fixed with paraformaldehyde, and incubated with Alexa Fluor 568 phalloidin (Invitrogen/Thermo Fisher Scientific Inc., Paisley, U.K., http://www.thermoscientific.com) to label F actin. The nuclei were visualized by using 4′,6-diamidino-2-phenylindole (DAPI) fluorescent dye (Invitrogen). Images were taken by a laser scanning confocal microscope (Zeiss International, Jena, Germany, http://www.zeiss.com). For characterization of their growth properties, cells were seeded (1×10^4 cells per well) in 500 μl of complete DMEM in 48-well tissue culture plates (Nunc/Thermo Fisher Scientific Inc., Roskilde, The Netherlands, http://www.thermoscientific.com), and the growth of the cells was determined after 3-, 24-, and 48-hour cultivation using the WST assay, as we have described [21]. In brief, WST-1 reagent (Roche Diagnostics, Mannheim, Germany, http://www.roche.de) was added to each well to form formazan. The plates were then incubated for another 4 hours, and the absorbance was measured by spectrophotometry. The assay is based on the ability of living cells to use mitochondrial dehydrogenases to cleave tetrazolium salts into water-soluble formazan, which is then measured by spectrophotometry. To compare the growth

of stem cells on plastic or on a nanofiber scaffold, MSCs and LSCs were seeded (4×10^4 cells per well) in 700 μl DMEM in 24-well tissue culture plates (Corning) directly into wells or onto a nanofiber scaffold fixed into CellCrown TM24 inserts (Scaffdex Ltd., Tampere, Finland, http://www.scaffdex.com). The growth of cells was determined after 48 hours by the WST assay.

The ability of stem cells to differentiate into adipocytes was determined using specific adipogenic medium containing 0.1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 mM indomethacine, and 0.5 μg/ml insulin, as we described previously [22]. The differentiation of the cells was confirmed by staining with Oil Red O and by quantifying the expression of the adipocyte-specific genes for adiponectin (*ADPC*) and peroxisome proliferator-activated receptor γ (*PPAR*γ) using real-time polymerase chain reaction (PCR).

The expression of genes for the immunoregulatory molecules indoleamine-2,3-dioxygenase (IDO-2), cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS), and for hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) was determined in unstimulated and lipopolysaccharide (LPS)-stimulated MSCs and LSCs. In these experiments, the cells (4×10^4 cells per well) were cultured in 700 μl of DMEM for 48 hours in 24-well tissue culture plates (Corning) with or without 5 μg/ml LPS, and the expression of the genes was determined by real-time PCR, as described below.

Nanofiber Scaffolds

Nanofiber scaffolds were prepared from the biocompatible polymer poly(L-lactic) acid (PLA) by a needleless electrospinning procedure, as we have described [23]. In brief, PLA polymer was dissolved in chloroform and two other solvents, 1,2-dichlorethane and ethyl acetate, were added to this solution. The mixture was stirred until a homogenous polymer solution was obtained. The modified needleless Nanospider technology (Elmarco s.r.o., Liberec, Czech Republic, http://www.elmarco.com), in which polymeric jets are spontaneously formed from liquid surfaces on a rotating spinning electrode, was used for the preparation of the nanofibers. In this study, nanofiber material with a mass per unit area of 10 g/m^2 and with a nanofiber diameter ranging from 290 to 539 nm was used. The morphology of the nanofibers and their nanofibrous architecture were analyzed using scanning electron microscopy and shown previously [11, 21].

Stem Cell Growth on Nanofiber Scaffold and Cell Transfer

Nanofiber scaffolds were cut into squares (approximately 1.5 $\times$ 1.5 cm) and fixed into CellCrown TM24 inserts (Scaffdex). The inserts with nanofibers were transferred into 24-well tissue culture plates (Corning). Stem cells (3×10^5) in 700 μl of complete DMEM were transferred into each well. The plates were incubated for 24 hours to allow the cells to adhere to the scaffold.

For stem cell transfer, nanofiber scaffolds seeded with stem cells were transferred within 1 hour after the injury with the cell side facing down on the damaged ocular surface. The scaffolds were sutured to the conjunctiva with four interrupted sutures using 11.0 Ethilon (Ethicon, Johnson & Johnson, Livingston, U.K., http://www.ethiconproducts.co.uk). The eyelids were closed by tarsorrhaphy using 1 suture of Resolon 7.0 (Resorba Medical GmbH, Nuremberg, Germany, http://www.resorba.com) for 72 hours. An ophthalmic ointment compound containing bacitracin and neomycin (Ophthalmo-Framykoin; Zentiva Group, Prague, Czech Republic, http://www.zentiva.com) was applied on the ocular surface for 3 days. The nanofiber scaffolds were removed from the ocular surface on day 3 after the operation. The animals were sacrificed following an i.v. injection of thiopental anesthesia (30 mg/kg thiopental; Spofa) after premedication with an intramuscular injection of xylazinum hydrochloridum/ketaminum hydrochloridum. Each experimental group involved six rabbits (i.e., six experimental eyes). In all experiments with alkali injury, the corneas of healthy rabbit eyes served as controls.

Immunohistochemistry

After sacrificing the animals, the eyes were enucleated and the anterior eye segment dissected out and quenched in light petroleum chilled with an acetone-dry ice mixture. Sections were cut on a cryostat and transferred onto glass slides. Subsequently, the cryostat sections were fixed in acetone at 4°C for 5 minutes. For the immunohistochemical detection of cells staining for CD3, iNOS, VEGF, or the cytokeratins K3 and K12 (K3/12), the following primary monoclonal antibodies (mAbs) were used: anti-CD3 (Abcam, Cambridge, U.K., http://www.abcam.com), anti-iNOS (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com), anti-caspase-3 (Abcam), anti-K3/12 (Abcam), and anti-VEGF (Abcam). The binding of the primary antibodies was demonstrated using the horseradish peroxidase/3,3'-diaminobenzidine (HRP/DAB) Ultra Vision detection system (Thermo Fisher Scientific) following the instructions of the manufacturer. Individual steps involved the following: hydrogen peroxide block (15 minutes), ultra V block (5 minutes), incubation with the primary antibody (60 minutes), incubation (10 minutes) with biotinylated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific) and peroxidase-labeled streptavidin incubation (10 minutes). Visualization was performed using a freshly prepared DAB substrate-chromogen solution. Cryostat sections in which the primary antibodies were omitted from the incubation media served as negative controls. Some sections were counterstained with Mayer's hematoxylin.

The counting of cells positive for CD3 and iNOS in the corneal stroma and for caspase-3 in the corneal epithelium was performed by an examiner without prior knowledge of the experimental procedure. Three randomly chosen fields of corneal sections (of the same field size and the same microscope magnification) from six corneas of each experimental animal group were used. For each cornea, the mean value from the three fields was calculated.

Determination of Corneal Thickness

Changes of corneal transparency after the injury and during healing were examined according to the measurement of the central corneal thickness (taken as an index of corneal hydration). The central corneal thickness was measured in anesthetized animals using an ultrasonic pachymeter SP-100 (Tomey Corp., Nagoya, Japan, http://www.tomey.com) in the corneal center. The corneal thickness was measured in the same corneas before alkali injury (corneas of healthy eyes) and on days 5 and 12 after the injury (all experimental groups). Each cornea was measured four times and the mean value of the thickness (in μm) was computed.

Determination of Corneal Neovascularization and Re-epithelialization

For the evaluation of corneal neovascularization, the number of vessels was counted in each 60° sector of the corneal surface. The mean value and standard deviation were determined from six eyes in each group.

To characterize corneal re-epithelialization, postfixed cryostat sections of the corneas were stained with a mAb directed against the corneal epithelial cell-associated cytokeratins K3 and K12, using hematoxylin and eosin stain for counterstaining. The images were evaluated microscopically. For the quantification of re-epithelialization, the expression of genes for K3 and K12 was determined by real-time PCR in healthy, injured and treated corneas.

Detection of Gene Expression by Real-Time PCR

The expression of genes in cultured cells or in control and treated corneas was determined by quantitative real-time PCR. The corneas or cultured stem cells were transferred into Eppendorf tubes containing 500 μl TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, http://mrcgene.com). The details of RNA isolation, transcription, and the PCR parameters have been described previously [24]. In brief, total RNA was extracted using TRI Reagent according to the manufacturer's instructions. Total RNA (1 μg) was treated using deoxyribonuclease I (Promega Corp., Madison, WI, http://www.promega.com) and subsequently used for reverse transcription. The first-strand cDNA was synthesized using random primers (Promega) in a total reaction volume of 25 μl using Moloney murine leukemia virus reverse transcriptase (Promega).

Quantitative real-time PCR was performed using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The relative quantification model with efficiency correction was applied to calculate the expression of the target gene in comparison with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as the housekeeping gene. The following primers were used for amplification: GAPDH: 5′-CCCAACGTGTCTGTCGTG (sense), 5′-CCGACCCAGACGTACAGC (antisense); K3: 5′-GAACAAGGTCCTGGAGACCA (sense), 5′-TTGAAGTCCTCC ACCAGGTC (antisense); K12: 5′-AGGAGGTGGTGAATGGTGAG (sense), 5′-GTTGTTTCCCAGGAGCAAAA (antisense); ADPC: 5′-ACCAGGACAAGAACGTGGAC (sense), 5′-TGGAGATGGAATCGTTGACA (antisense); PPARγ: 5′-AGTCGCCATCC GCATCTT (sense), 5′-ATCTCATGGACGCCGTACTTG (antisense); IDO-2: 5′-GTTTC CTTGGCTCGTTGG (sense), 5′-CCTTTTCTGAAAGGATAAACTCTCG (antisense); iNOS: 5′-AGGGAGTGTTGTTCCAGGTG (sense), 5′-TCCTCAACCTGCTCCTCACT (antisense); Cox-2: 5′-ACATCGTCAATAGCATTC (sense), 5′-TAGTAGGAGAGGTT GAGA (antisense); TGF-β1: 5′-GCCTGCAAGTGCTCAAGTTAC (sense), 5′-TGCTG CATTTCTGGTACAGC (antisense); HGF: 5′-AGGCAGCTATAAGGGAACAGTG (sense), 5′-ATGGAACTCCAGGGCTGAC (antisense); and VEGF: 5′-CGAGACCTTGGTG GACATCT (sense), 5′-ATCTGCATGGTGACGTTGAA (antisense). The PCR parameters included denaturation at 95°C for 3 minutes, then 40 cycles at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 seconds and were analyzed using StepOne Software, version 2.2.2 (Thermo Fisher Scientific). Each individual experiment was performed in triplicate.

Statistical Analysis

Analysis of the data showed a normal distribution and the results are expressed as mean ± SD. Comparisons between two groups were made using Student *t* test, and multiple comparisons were analyzed by analysis of variance. A value of $p < .05$ was considered statistically significant.

Results

Growth, Differentiation, and Gene Expression of Rabbit MSCs and LSCs

The morphology of BM-MSCs, Ad-MSCs, and LSCs growing on glass cover slips in vitro is shown in Figure 1A. All three cell types adhered to plastic and glass surfaces and had a typical fibrocyte-like shape. The cells had similar growth characteristics when cultured on plastic (Fig. 1B) and proliferated comparably on a nanofiber scaffold (Fig. 1C). When all three cell types were cultured in a specific adipogenic differentiation medium, the highest differentiation potential was recorded in BM-MSCs, as demonstrated microscopically (Fig. 1D) and also according to the expression of genes for the adipocyte markers ADPC and PPARγ determined by real-time PCR (Fig. 1E).

To test the ability of BM-MSCs, Ad-MSCs, and LSCs to produce basic immunoregulatory molecules and growth factors, the cells were cultured for 48 hours unstimulated or stimulated with LPS, and the expression of genes for IDO-2, Cox-2, iNOS, TGF-β, HGF, and VEGF was determined by real-time PCR. As shown in Figure 2, some of these genes were expressed spontaneously and comparably in all cell types, while other factors were produced preferentially in only some cell populations or only after stimulation with LPS.

Immunohistochemical Detection of $CD3^+$, $iNOS^+$, and Caspase-3^+ Cells in Alkali-Injured and Stem Cell-Treated Corneas

The presence of $CD3^+$ cells (Fig. 3A) or cells expressing iNOS (Fig. 3B) or caspase-3 (Fig. 3C) was very low or absent in healthy control corneas. After alkali injury, the corneas were strongly infiltrated with $CD3^+$ cells (Fig. 3D) and the expression of iNOS (Fig. 3E) was high. Similarly, the number of apoptotic caspase-3^+ cells was high in the remaining islands of the corneal epithelium (Fig. 3F). The infiltration of corneas with $CD3^+$ cells (Fig. 3G) or the presence of $iNOS^+$ (Fig. 3H) or caspase-3^+ (Fig. 3I) cells was slightly decreased in injured corneas treated with cell-free nanofiber scaffolds. However, after the treatment of injured corneas with stem cell-seeded nanofibers (Fig. 3J–3R), the numbers of $CD3^+$, $iNOS^+$, or caspase-3^+ cells were significantly decreased. The expression of caspase-3 in apoptotic cells, which was high in the remaining islands of the corneal epithelium in untreated injured corneas (Fig. 3F, arrow), was only weakly expressed in the epithelium of corneas treated with a nanofiber scaffold seeded with BM-MSCs (Fig. 3L, arrow) or LSCs (Fig. 3R, arrow). The number of $CD3^+$ cells (Fig. 3V) as well as cells expressing iNOS (Fig. 3W) or caspase-3 (Fig. 3X) was counted in defined fields of corneal sections for each experimental group. The graphs show that the numbers of $CD3^+$, $iNOS^+$, or caspase-3^+ cells, which were high in untreated injured corneas or corneas treated with cell-free nanofiber scaffolds, were significantly decreased in the groups treated with stem cell-seeded nanofibers.

Corneal Thickness After Alkali-Injury and Treatment With MSCs and LSCs

The central corneal thickness of healthy corneas was about 380 μm (Fig. 4). Shortly after alkali injury, the corneal thickness increased (as a result of hydration) more than twofold and remained high on day 5

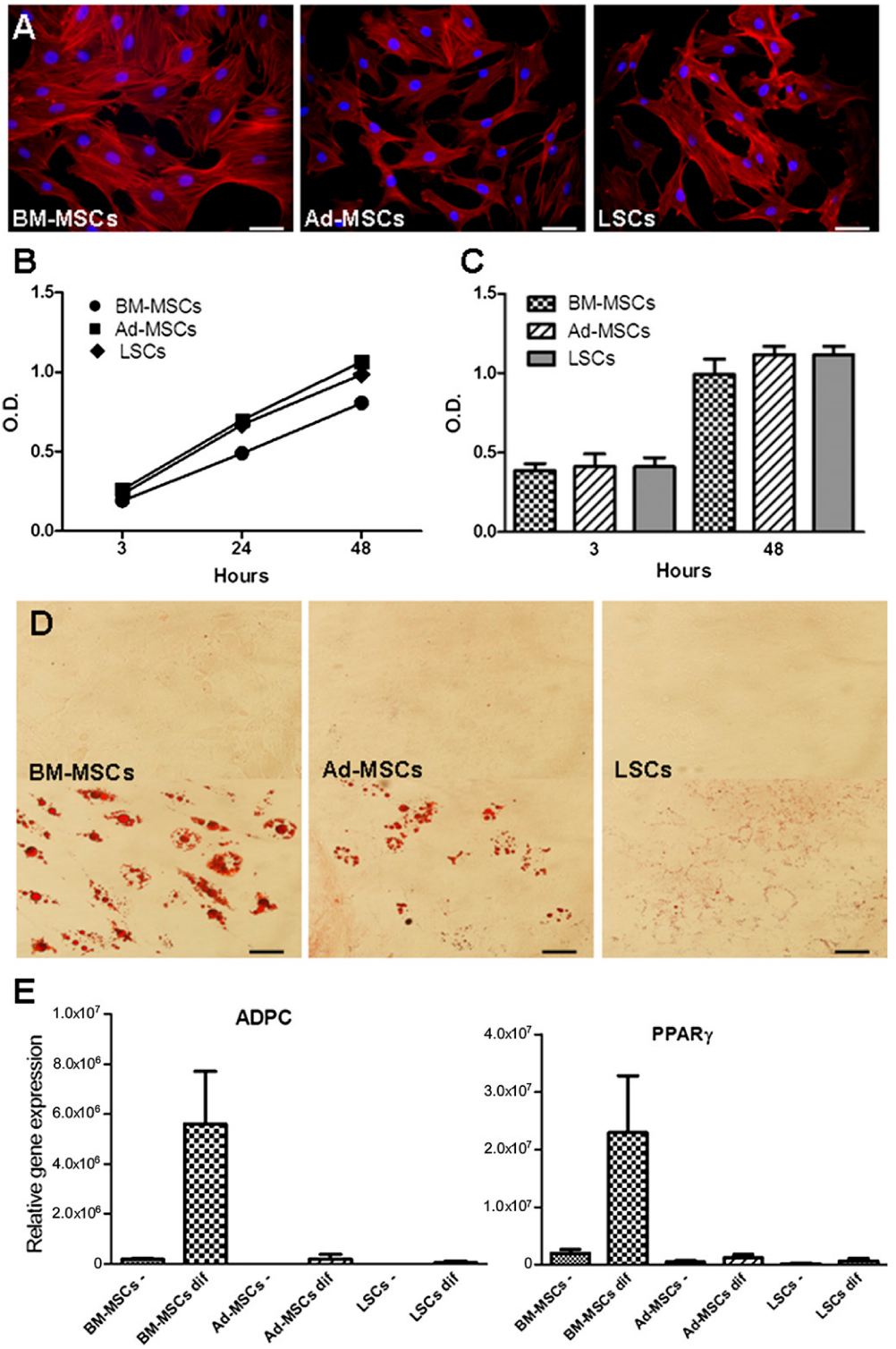

Figure 1. Characterization of BM-MSCs, Ad-MSCs, and LSCs. **(A):** The morphology of the cells is shown by staining for F actin with phalloidin (red filaments). The nuclei are blue (4′,6-diamidino-2-phenylindole [DAPI] staining). Scale bars = 200 μm. **(B–E):** The growth of cells on plastic **(B)** or on a nanofiber scaffold **(C)** was determined by the WST assay. The ability of cells to differentiate into adipocytes was characterized microscopically (**D**, upper: undifferentiated cells; lower: cells in differentiation medium) or according to the expression of the *ADPC* and *PPARγ* genes detected by real-time polymerase chain reaction **(E)**. Each bar represents the mean ± SD from three determinations. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; *ADPC*, adiponectin gene; BM-MSC, bone marrow-derived mesenchymal stem cell; dif, differentiation medium; LSC, limbal epithelial stem cell; o.d., optical density (absorbance); *PPARγ*, peroxisomeproliferator-activated receptor γ.

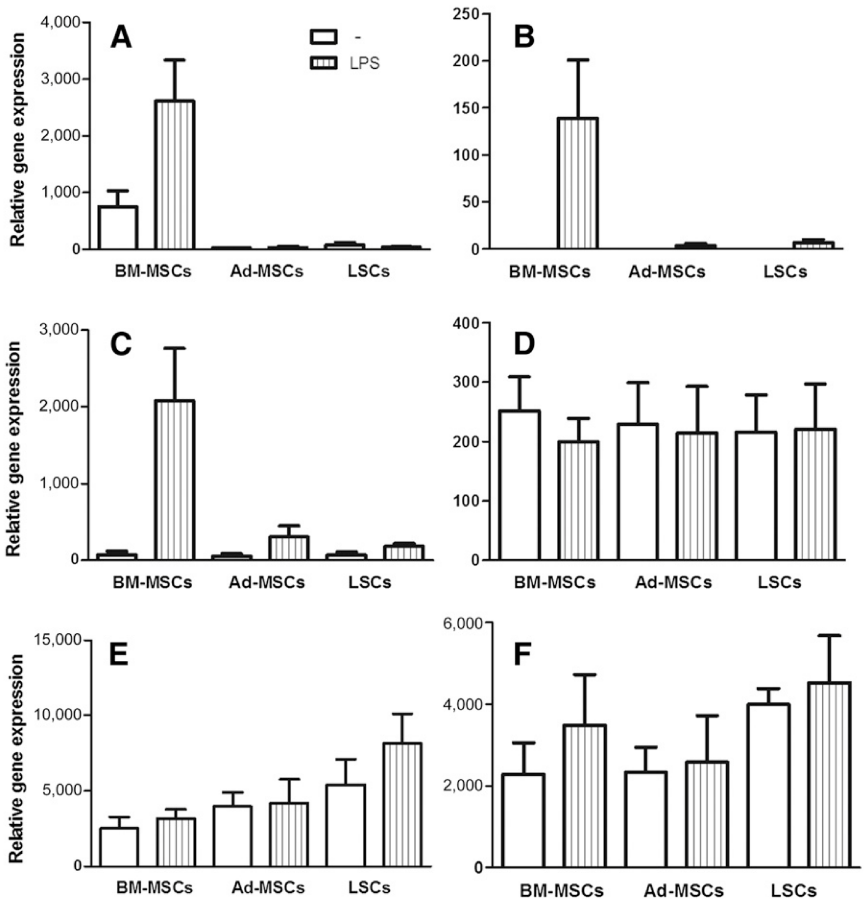

Figure 2. Expression of genes for immunoregulatory molecules and growth factors by BM-MSCs, Ad-MSCs, and LSCs. **(A–F):** The cells were cultured for 48 hours unstimulated or stimulated with LPS and the expression of genes for indoleamine-2,3-dioxygenase **(A)**, cyclooxygenase-2 **(B)**, inducible nitric oxide synthase **(C)**, transforming growth factor-β **(D)**, hepatocyte growth factor **(E)**, and vascular endothelial growth factor **(F)** was determined by real-time polymerase chain reaction. Each bar represents the mean $\pm$ SD from four determinations. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LPS, lipopolysaccharide; LSC, limbal epithelial stem cell.

in untreated corneas and in corneas treated with cell-free nanofiber scaffolds. In corneas treated with Ad-MSC-seeded nanofiber scaffolds, the corneal thickness slightly decreased, but it was significantly reduced already on day 5 in corneas treated with nanofiber scaffolds seeded with BM-MSCs or LSCs (Fig. 4). On day 12 after injury, the corneal thickness remained enhanced in untreated injured corneas but was significantly decreased in corneas treated with cell-free or Ad-MSC-seeded nanofiber scaffolds. In corneas treated with nanofiber scaffolds seeded with BM-MSCs or LSCs, the corneal thickness returned to the values observed before injury (Fig. 4).

Expression of VEGF and Neovascularization in Injured and Stem Cell-Treated Corneas

The expression of VEGF was very low in healthy control corneas (Fig. 5A). On day 12 after the injury, the expression of VEGF was high in untreated corneas (Fig. 5B) and was only slightly decreased in corneas treated with cell-free nanofiber scaffolds (Fig. 5C). The treatment of injured corneas with Ad-MSC-seeded nanofiber scaffolds apparently reduced VEGF expression (Fig. 5E), but the greatest reduction in VEGF expression was seen in corneas treated with nanofiber scaffolds seeded with BM-MSCs (Fig. 5D) or LSCs (Fig. 5F). The quantification of corneal neovascularization is summarized in Figure 5H. The number of vessels was high in untreated injured corneas and was partially reduced in injured corneas treated with cell-free nanofibers. The treatment of injured corneas with nanofiber scaffolds seeded with all three types of stem cells significantly decreased neovascularization. The greatest decrease was found in injured corneas treated with nanofiber scaffolds seeded with BM-MSCs or LSCs.

Corneal Re-epithelialization After Treatment of Injured Eyes With MSCs or LSCs

The extent of corneal re-epithelialization was evaluated on day 12 after alkali injury by examining corneal sections stained with mAb anti-K3/K12. A typical image of a normal healthy cornea is shown in Figure 6A. In contrast, only rare and isolated islands of the epithelium were detected in untreated injured corneas (Fig. 6B). Covering the injured ocular surface with a cell-free nanofiber scaffold improved re-epithelialization and islands with epithelium covered about 20%–40% of the corneal surface (Fig. 6C, 6D). The transfer of stem cell-seeded nanofiber scaffolds onto the damaged ocular surface (Figs. 6E–6G) significantly improved corneal healing, with the best re-epithelialization observed in corneas treated with nanofiber scaffolds seeded with BM-MSCs (Fig. 6E) or LSCs (Fig. 6G). The expression of genes for the cytokeratins K3 and K12 in healthy, injured and treated corneas was

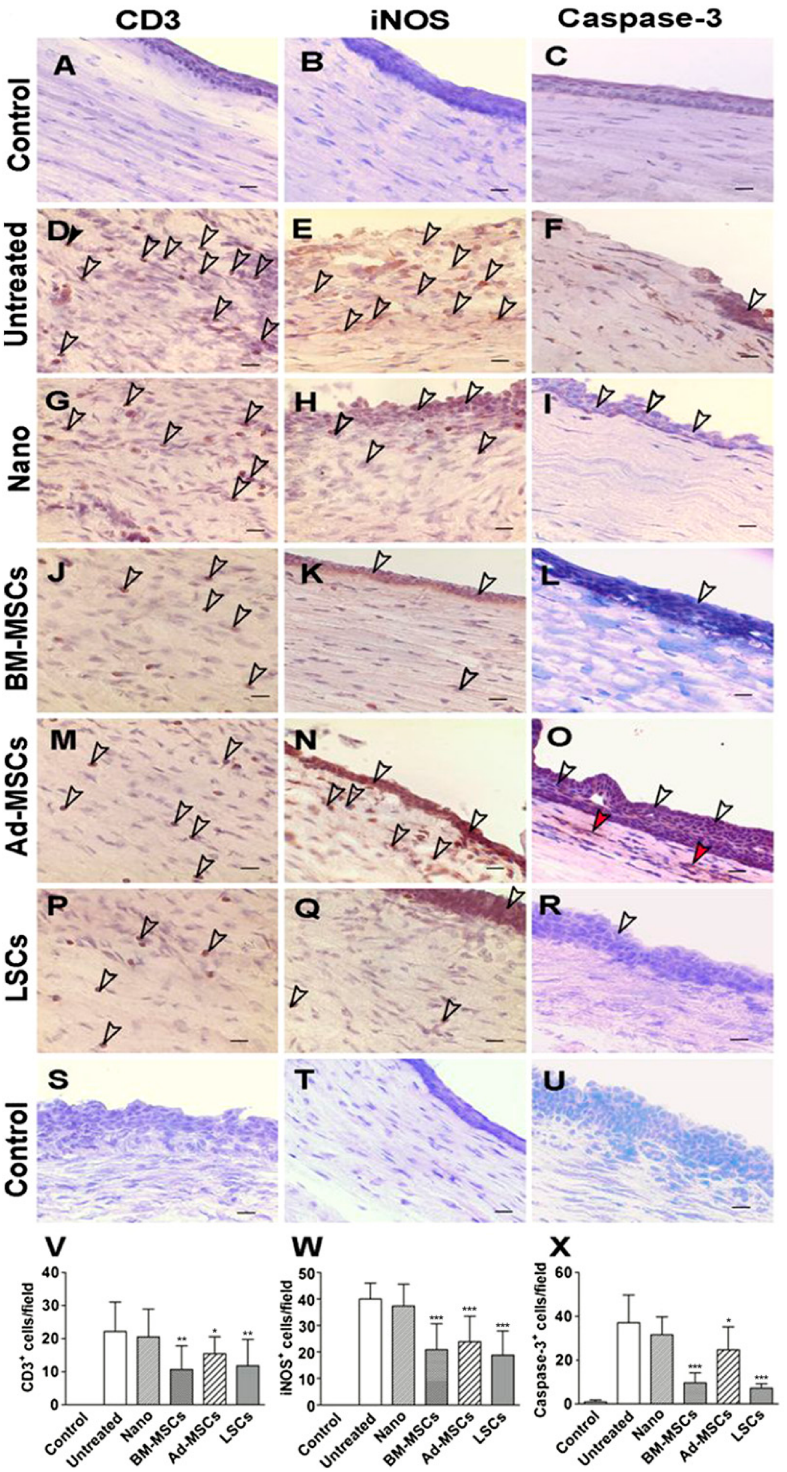

Figure 3. The immunohistochemical detection of $CD3^+$, $iNOS^+$, and caspase-3^+ cells in healthy, injured and stem cell-treated corneas. **(A–C):** Undetectable or very low numbers of $CD3^+$ **(A)**, $iNOS^+$ **(B)**, or caspase-3^+ **(C)** cells were found in healthy corneas. **(D–I):** On day 12 after alkali injury, the number of $CD3^+$ **(D)**, $iNOS^+$ **(E)**, and apoptotic caspase-3^+ **(F)** cells was significantly increased and remained high in corneas treated with a cell-free nanofiber scaffold **(G–I)**. **(J, M, P):** The presence of $CD3^+$ cells was clearly decreased in corneas treated with nanofiber scaffolds seeded with BM-MSCs **(J)**, Ad-MSCs **(M)**, or LSCs **(P)**. **(K, N, Q):** Similarly, the number of $iNOS^+$ cells was decreased in corneas treated with BM-MSCs **(K)**, Ad-MSCs **(N)**, or LSCs **(Q)**. **(L, Q, R):** The presence of caspase-3^+ cells, which were numerous in the remaining islands of the corneal epithelium in untreated injured corneas, was decreased in the corneas treated with BM-MSCs **(L)**, Ad-MSCs **(O)**, or LSCs **(R)**. **(S–U):** Cells expressing CD3 **(S)**, iNOS **(T)**, or caspase-3 **(U)** were absent in corneal sections stained only with counterstaining, where the primary antibody was omitted from the incubation medium (negative control). Scale bars = 50 μm. **(V–X):** The numbers of $CD3^+$ **(V)**, $iNOS^+$ **(W)**, and caspase-3^+ **(X)** cells counted in comparable fields of corneal sections were determined from six corneas in the individual experimental groups. The values with asterisks are significantly different (*, $p < .05$; **, $p < .01$; ***, $p < .001$) from those of untreated injured corneas. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; iNOS, inducible nitric oxide synthase; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

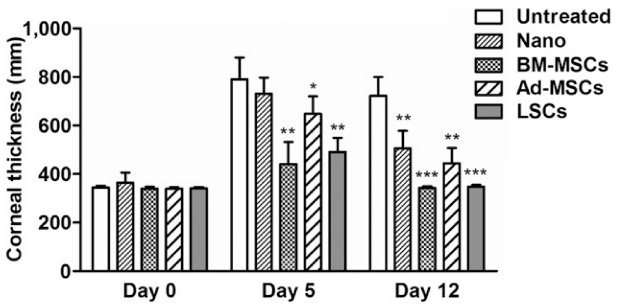

Figure 4. Central corneal thickness of healthy, alkali-injured and stem cell-treated corneas. The corneas were injured with alkali and then left untreated, treated with a nanofiber scaffold alone or treated with nanofiber scaffolds seeded with BM-MSCs, Ad-MSCs, or LSCs. The central corneal thickness was measured in the same rabbit before injury (day 0) and on days 5 and 12 after the injury. Each bar represents the mean ± SD from six corneas. The values with asterisks for day 5 are significantly different from those of untreated injured corneas on day 5; similarly, the values with asterisks for day 12 are significantly different from those of untreated injured corneas on day 12 (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

quantified by real-time PCR (Fig. 6I, 6J). Cytokeratin gene expression was apparent in healthy corneas but was absent or very low in untreated injured corneas and in corneas treated with cell-free nanofiber scaffolds. In accordance with the immunohistochemical results, the treatment of injured corneas with BM-MSC- or LSC-seeded nanofiber scaffolds significantly enhanced the expression of both cytokeratin genes.

Corneal Opacity of Alkali-Injured and Stem Cell-Treated Eyes

Representative photographs of healthy, injured and treated eyes are shown in Figure 7. In comparison with healthy control eyes (Fig. 7A), the corneas of injured eyes became opalescent shortly after the injury and remained opalescent and highly vascularized on day 12 after injury (Fig. 7C). An eye on day 2 after injury and covered with a nanofiber scaffold is shown in Figure 7B. Only a weak improvement in the appearance of the corneas was observed on day 12 in the eyes treated with cell-free nanofiber scaffolds (Fig. 7D). In the eyes treated with stem cell-seeded nanofibers, corneal opacity was decreased and corneal neovascularization was less apparent (Fig. 7E–7G), with the best therapeutic effects seen with nanofiber scaffolds seeded with BM-MSCs (Fig. 7E) or LSCs (Fig. 7G).

Discussion

Stem cell-based therapy holds great promise for the treatment of severe injuries as well as a number of thus-far incurable diseases. The best source of stem cells for tissue therapy is tissue-specific stem cells, but these cells are often rare in the body, difficult to isolate, and not easily handled in vitro. Therefore, research is focused on the search for alternative cell sources that could effectively replace tissue-specific stem cells.

One possibility has been offered by embryonic stem cells (ESCs), but the use of ESCs is limited by their uncontrolled growth, the risk of teratoma formation, and ethical problems associated with their isolation and use [25, 26]. Induced pluripotent stem cells, which could be used as autologous cells, initially appeared to offer great promise, but these cells turned out to be immunogenic even in syngeneic hosts [27] and frequently form teratomas after in vivo application [28]. MSCs represent a convenient type of stem cells with a wide spectrum of potential applications. These cells can be obtained in relatively sufficient numbers from an individual patient, can be easily propagated in vitro, and can then be used as autologous cells without requiring immunosuppression after their transplantation.

To treat ocular surface injuries or various types of LSCD, LSCs represent the optimal cell source, and LSC transplantation has resulted in the recovery of vision in blind patients [4, 6, 29]. However, the use of LSCs is limited by the absence of autologous LSCs in the case of bilateral LSCD and by a requirement for strong immunosuppression if allogeneic LSCs are used. To overcome these limitations, attempts have been made to use other cell sources for ocular surface regeneration, and the results of experimental studies using various cell types to treat LSCD have been published [30–32], but the majority of these studies have used MSCs [12, 17–19]. The rationale for the use of MSCs is based on their ability to differentiate into various cell types even apart from the mesodermal lineage from which they originate [7, 9], to produce numerous growth and trophic factors [33], and to inhibit harmful inflammatory reactions [12, 17]. Although an apparent improvement of corneal healing after the application of MSCs has been observed in various models, a direct comparison of the therapeutic effects of MSCs and LSCs has not previously been made.

In this study, we prepared BM-MSCs, Ad-MSCs, and LSCs always from the same rabbit and we compared their growth, differentiation properties, and ability to produce immunoregulatory and growth factors and to support the healing of the damaged ocular surface. All of these cell types have similar fibrocytic morphology and comparable growth characteristics. After cultivation in adipogenic differentiation medium, the highest differentiation potential was observed in BM-MSCs. In accordance with the literature data [33–35], all three types of stem cells spontaneously, or after stimulation with LPS, expressed genes for a number of immunoregulatory and growth factors, but the secretion profiles were different among the individual cell types. Though we were not able to characterize the stem cell populations phenotypically because of the lack of species-specific antibodies for the rabbit model, our MSCs fulfilled other criteria for MSCs: their morphology, adherence to plastic, differentiation, and factor production [36]. Similarly, the population of LSCs was prepared by a standard method described for the preparation of mouse [20], rat [9], rabbit [37, 38], or human [5, 6] LSCs. We are aware that the LSC population contains a significant proportion of descendants of LSCs, such as transient corneal epithelial cells and corneal epithelial cells, in addition to LSCs. Indeed, we observed a gradual increase in CK3/CK12 expression during culture of rabbit LSCs (unpublished observation). Some reports suggest that MSCs can be expanded from the limbal tissue in vitro rather than corneal epithelial cells [39, 40]. For example, Basu et al. [40] described the expansion of mesenchymal stromal cells derived from human limbal biopsies and their use for the treatment of mouse corneal wounds. These authors also showed that the type of enzymatic digestion influences the preferential growth of cells with epithelial or mesenchymal morphologies. Thus, there may be species-specific and cell culture-dependent differences that support preferential growth of limbal MSCs or corneal epithelial cells.

We have shown previously in the mouse model that MSCs transferred onto the damaged ocular surface, using a nanofiber scaffold, migrate from the scaffold onto the ocular surface and inhibit the local inflammatory reaction [21]. In the present study, we used a model

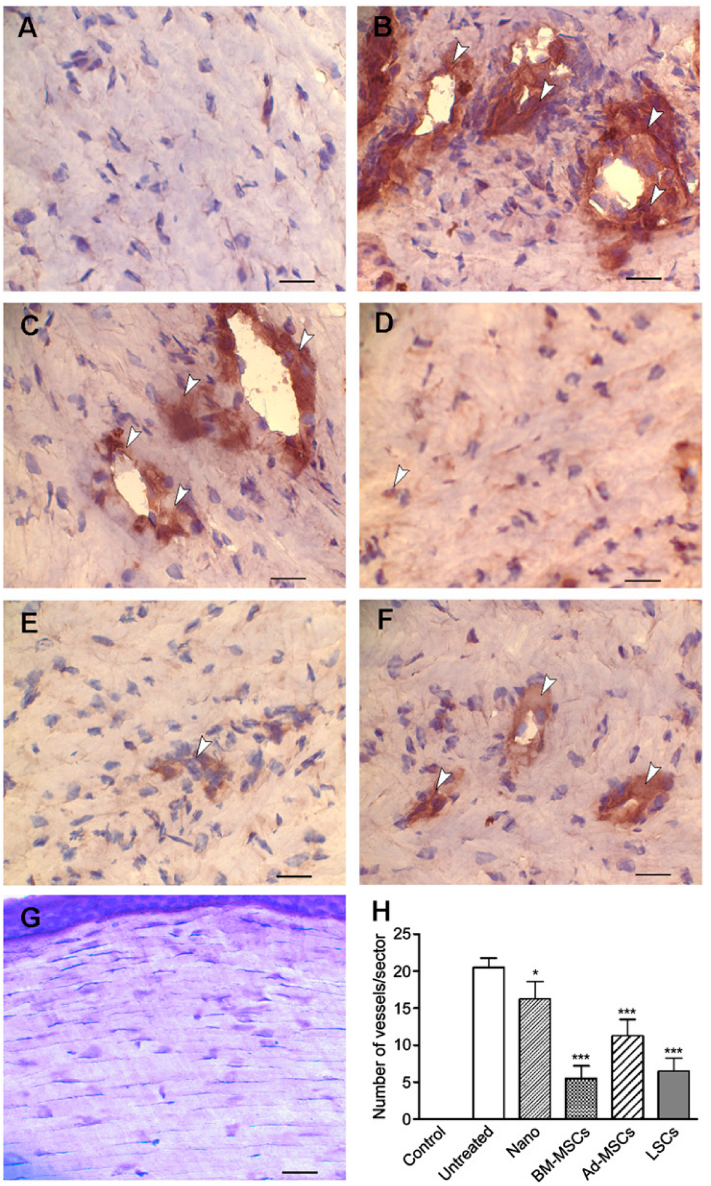

Figure 5. The expression of vascular endothelial growth factor (VEGF) and corneal neovascularization after alkali injury and treatment with stem cell-seeded nanofiber scaffolds. **(A–F):** The expression of VEGF was determined by immunohistochemistry in healthy corneas **(A)** and in alkali-injured corneas on day 12 that were untreated **(B)**, treated with cell-free nanofibers **(C);** or treated with nanofiber scaffolds seeded with BM-MSCs **(D)**, Ad-MSCs **(E)**, or LSCs **(F)**. **(G):** In the negative control, the sections were stained only with counterstaining (hematoxylin). Scale bars = 10 μm. **(H):** The quantification of corneal neovascularization was performed by counting the number of vessels in defined corneal sectors. Each bar represents the mean $\pm$ SD from six corneas. The values with asterisks are significantly different ($*$, $p < .05$; $***$, $p < .001$) from those determined in untreated injured corneas. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

of the alkali-injured ocular surface in rabbits and compared the therapeutic potential of two types of MSCs and tissue-specific LSCs. The injury of the cornea with 0.25N NaOH induced damage of the corneal epithelium, an increase in corneal thickness, a strong infiltration with cells of adaptive (T lymphocytes) and innate (iNOS-expressing cells) immunity, an increase in the presence of apoptotic cells (caspase-3^+ cells), neovascularization, and corneal opacity associated with decreased corneal transparency. All these parameters characterizing the ocular injury were decreased in the treated eyes. The nanofiber scaffold itself slightly supported healing and decreased the harmful impacts of injury, similarly to what has been described after the treatment of a skin wound [41] or corneal injury [12]. The treatment of injured eyes with a nanofiber scaffold seeded with stem cells significantly decreased all of the harmful manifestations of the injury. The alkali injury strongly damaged the corneal epithelium (as demonstrated by immunohistochemistry and real-time PCR for K3 and K12), and treatment with a stem cell-seeded nanofiber scaffold improved re-epithelialization. The less-pronounced therapeutic effects of Ad-MSCs in comparison with

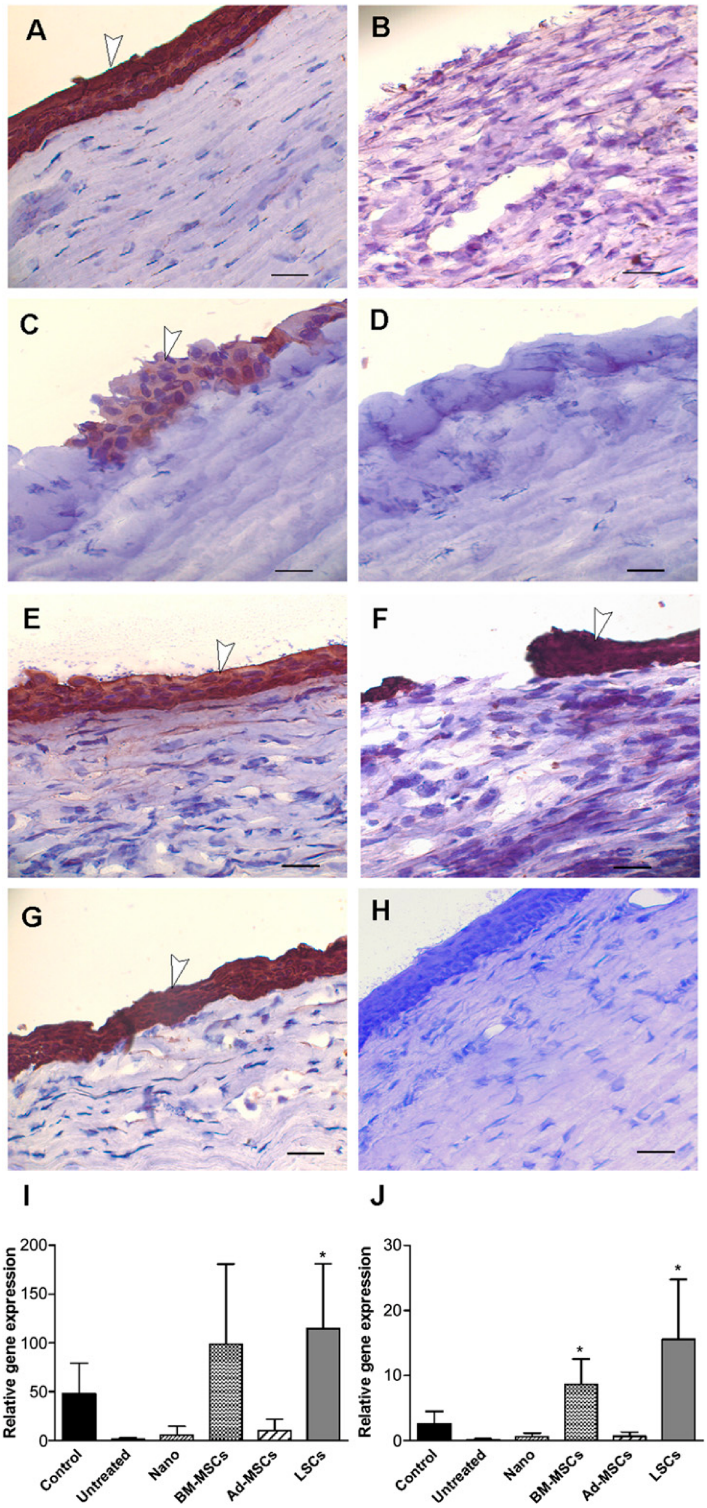

Figure 6. Corneal re-epithelialization in alkali-injured and stem cell-treated corneas. **(A–G):** The individual photographs show representative images of an anti-K3/12 stained healthy cornea **(A)**, an untreated injured cornea **(B)**, or injured corneas treated with cell-free nanofibers **(C, D)**, nanofibers seeded with BM-MSCs **(E)**, Ad-MSCs **(F)**, or LSCs **(G)**. All injured corneas are shown on day 12 after injury. **(H):** The staining for the cytokeratins K3/12 was negative in corneal sections stained only with counterstaining, where the primary antibody was omitted from the incubation medium. Scale bars = 50 μm. **(I, J):** The expression of genes for K3 **(I)** and K12 **(J)** in individual experimental groups on day 12 after injury was determined by real-time polymerase chain reaction. Each bar represents the mean $\pm$ SD from six individual corneas. The values with an asterisk represent a statistically significant ($p < .05$) difference from the values determined in untreated injured corneas. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

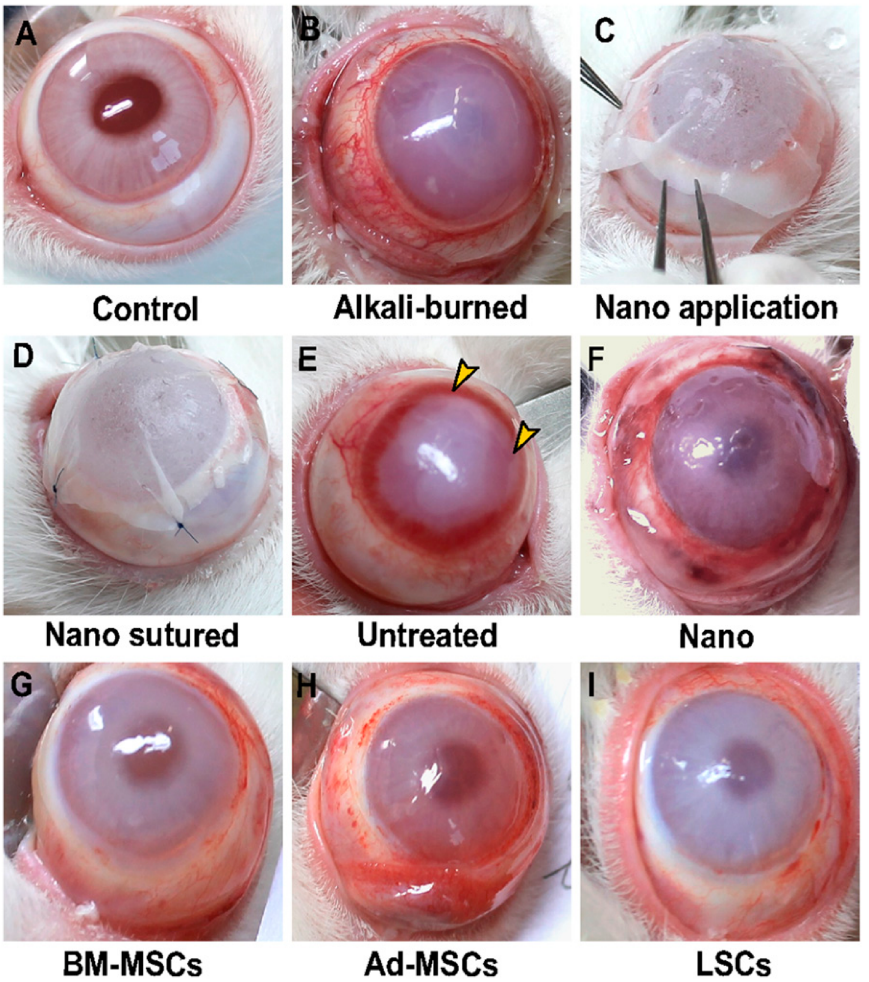

Figure 7. Corneal opacity of alkali-injured and stem cell-treated eyes. Representative photographs show a healthy control eye **(A)**, an alkali-injured eye (immediately after the injury) **(B)**, an injured eye with nanofiber application **(C)**, and a sutured nanofiber scaffold (immediately after the injury) **(D)**; and injured eyes on day 12 that were either untreated **(E)**, treated with a cell-free nanofiber scaffold **(F)**, or treated with nanofiber scaffolds seeded with BM-MSCs **(G)**, Ad-MSCs **(H)**, or LSCs **(I)**. **(E, G, I):** Corneal neovascularization was clearly visible expressed in untreated injured corneas (**E**, arrows) and strongly suppressed in corneas treated with nanofiber scaffolds seeded with BM-MSCs **(G)** or LSCs **(I)**. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

BM-MSCs or LSCs could be due to the lower differentiation potential of Ad-MSCs and to the different spectrum of growth and immunoregulatory factors produced by these cells.

Conclusion

Taken together, our results show that BM-MSCs have comparable therapeutic effects to those of tissue-specific LSCs on the healing of corneal injury. Even though there are data on the direct differentiation of MSCs into corneal epithelial cells [8, 18], this transdifferentiation is probably not the main mechanism of the healing effect of MSCs [42]. We suggest that a more important role is represented by the production of numerous trophic and growth factors that can support the growth of residual corneal epithelial cells and LSCs [33–35], and by the ability of MSCs to suppress the local inflammatory reaction that could impede the healing process [12, 43]. All of these properties make BM-MSCs a promising candidate cell population for improving ocular surface healing in situations when autologous LSCs are difficult to obtain or are absent.

Acknowledgments

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Author Contributions

V.H.: conception and design, financial support, manuscript writing; P.T., E.J., B.H., and M.C.: collection and/or assembly of data; C.C. and A.Z.: collection of data, data analysis and interpretation; J.C.: conception and design, collection and/or assembly of data, manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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5.3 Perspektivy využití kmenových buněk pro léčbu věkem podmíněných degenerativních onemocnění sítnice.

Vladimir Holáň, **Barbora Heřmánková** a Jan Kossl

Cell Transplant. 2017;26,1538-1541

Degenerativní onemocnění sítnice, například věkem podmíněná makulární degenerace, retinitis pigmentosa, diabetická retinopatie nebo glaukom, postihují převážně starší populaci a jsou nejčastější příčinou snížené kvality zraku nebo dokonce slepoty. V současné době neexistuje žádná úspěšná metoda, která by pomohla k prevenci, zastavení nebo léčbě těchto poruch. Velkou naději pro pacienty trpícími onemocněním sítnice je terapie založená na kmenových buňkách, která může nahradit nemocné nebo chybějící buňky sítnice a podporovat regeneraci. V tomto ohledu se ukázalo, že MSC jsou pro léčbu slibným typem kmenových buněk. Je možné je získat od konkrétního pacienta a použít jako autologní buňky. Zde ukazujeme, že MSC se mohou diferencovat na buňky exprimující znaky buněk sítnice, inhibovat produkci prozánětlivých cytokinů a produkovat řadu růstových a neuroprotektivních faktorů důležitých pro regeneraci sítnice. Všechny tyto vlastnosti činí MSC perspektivním kandidátem pro buněčnou terapii věkem podmíněných degenerativních onemocnění sítnice.

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Perspectives of Stem Cell–Based Therapy for Age-Related Retinal Degenerative Diseases

Vladimir Holan[1,2], Barbora Hermankova[1,2], and Jan Kossl[1,2]

Abstract

Retinal degenerative diseases, which include age-related macular degeneration, retinitis pigmentosa, diabetic retinopathy, and glaucoma, mostly affect the elderly population and are the most common cause of decreased quality of vision or even blindness. So far, there is no satisfactory treatment protocol to prevent, stop, or cure these disorders. A great hope and promise for patients suffering from retinal diseases is represented by stem cell–based therapy that could replace diseased or missing retinal cells and support regeneration. In this respect, mesenchymal stem cells (MSCs) that can be obtained from the particular patient and used as autologous cells have turned out to be a promising stem cell type for treatment. Here we show that MSCs can differentiate into cells expressing markers of retinal cells, inhibit production of pro-inflammatory cytokines by retinal tissue, and produce a number of growth and neuroprotective factors for retinal regeneration. All of these properties make MSCs a prospective cell type for cell-based therapy of age-related retinal degenerative diseases.

Keywords

age-related retinal degenerative diseases, mesenchymal stem cells, stem cell therapy

Introduction

Retinal degenerative diseases, such as age-related macular degeneration, retinitis pigmentosa, diabetic retinopathy, or glaucoma, represent the leading cause of a decreased quality of vision or even blindness among the elderly population worldwide. Irrespective of the primary cause and etiology, cumulative damage and loss of retinal pigment epithelium (RPE), choriocapillaris, and degeneration of photoreceptors or ganglion cells cause consequential visual impairment leading to a total loss of vision. The current treatment regimens are based on surgical and medical interventions to slow down the disease progression. Since the main cause of retinal degenerative diseases is an impairment and loss of specialized retinal cells, their support or replacement would represent a prospective treatment option. In this respect, stem cell–based therapy holds great promise.[1,2]

Among various stem cell types that have been suggested or already tested for treatment of retinal diseases, the mesenchymal stem cells (MSCs) turned out to be the most promising cells. These cells can be obtained relatively easily from bone marrow or adipose tissue, multiplied ex vivo, and used as autologous (patient's own) stem cells. It has been shown that MSCs possess a number of useful properties[3–6] that make them a promising candidate cell population for stem cell–based therapy of retinal degenerative diseases.

In this communication, we provide support for the above suggestion. Using highly purified mouse MSCs, we show that these cells are a potent source of various growth factors, inhibit expression of genes for pro-inflammatory molecules in stimulated retinal cells, and can differentiate into cells expressing markers of different retinal cell types.

Materials and Methods

Preparation of MSCs

The female mice of the inbred strain BALB/c at the age of 7 to 9 wk (20–25 g of weight) were purchased from the breeding unit of the Institute of Molecular Genetics, Prague. MSCs were prepared from the bone marrow as we have

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described previously.[7] In brief, adherent bone marrow cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), antibiotics, and 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer for 3 wk, and MSCs were purified by magnetic cell sorting to eliminate contaminating cells. The separated MSCs adhered to plastic, had a typical fibrocyte-like morphology, were positive for CD44 and CD73 markers and negative for CD11b and CD45, and were able to undergo adipogenic and osteogenic differentiation.[7] The use of the animals was approved by the local Ethical Committee of the Institute of Experimental Medicine.

Targeted Differentiation of MSCs into Cells Expressing Markers of Retinal Cells

To differentiate MSCs into cells expressing markers of retinal cells, we attempted to mimic the inflammatory environment of the diseased retina. For this purpose, we prepared tissue extracts from the posterior segment of the mouse eye (100 μL of serum-free medium per eye). Control tissue extracts were prepared from the heart, muscle, or lung tissue. To further mimic the environment of the inflammatory site in diseased tissue, we prepared supernatants after a 48-h stimulation of mouse spleen cells with T-cell mitogen Concanavalin A (1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 48 h. The preparation of tissue extracts and cytokine-containing supernatants has been described elsewhere.[8] Purified MSCs (6×10^4 cells in 1 mL of DMEM in 12-well tissue culture plates) were cultured for 7 d with the extract (30% of the culture volume) and supernatant (30% of the volume), and the expression of genes for retinal cell markers rhodopsin, S-antigen, retinaldehyde-binding protein (Rlbp), and calbindin 2 (Calb2; which are not, or only very weakly expressed in MSCs) was determined by real-time polymerrase chain reaction (PCR). The conditions of cell differentiation, RNA extraction, and real-time PCR are described in detail elsewhere.[8] In brief, the total RNA was extracted using TRI Reagent and the first-strand cDNA was synthesized using random hexamers. Quantitative real-time PCR was performed in a StepOnePlus system. The PCR parameters and fluorescence data analysis have been described previously.[8,9]

Anti-Inflammatory Effects of MSCs

Small pieces (1 × 1 mm) of the posterior segment of the mouse eye bulb (containing the retina) were cultured in 500 μL of Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich), containing 10% FCS, antibiotics, and 10 mM HEPES buffer in 48-well tissue culture plates (Nunc, Roskilde, the Netherlands) alone, in the presence of pro-inflammatory cytokines (10 ng/mL of interleukin [IL]-17 and 10 ng/mL of interferon [IFN]-γ), or in the presence of cytokines in wells containing 2×10^4 adherent MSCs. After a 48-h incubation period, the pieces of the eye tissue samples were harvested from the wells, and the expression of genes for pro-inflammatory molecules IL-1α, IL-6, tumor necrosis factor-α, and inducible nitric oxide synthase (iNOS) was determined by real-time PCR.

Production of Growth and Differentiation Factors by MSCs

MSCs (4×10^4 cells in 1 mL of culture medium) were cultured for 48 h in 48-well tissue culture plates (Nunc) unstimulated or in the presence of pro-inflammatory cytokines (10 ng/mL of IL-17 and 10 ng/mL of IFN-γ). The expression of genes for a panel of cytokines and growth factor (including IL-6, transforming growth factor-β [TGF-β], insulin-like growth factor-1 [IGF-1], insulin-like growth factor-2 [IGF-2], nerve growth factor [NGF], hepatocyte growth factor [HGF], plateled-derived growth factor [PEDF], and glial cell line-derived neurotrophic factor [GDNF]) was determined by real-time PCR.[8]

Statistical Analysis

The results are expressed as the mean (SD). Comparisons between the 2 groups were analyzed using Student's *t*-test. A P value of <0.05 was considered statistically significant.

Results

Differentiation Potential, Immunosuppressive Properties, and Secretory Activity of MSCs

Purified MSCs were cultured for 7 d in a standard culture medium or in a medium containing retinal tissue extract, supernatant from activated lymphocytes or extract, and supernatant together (differentiation medium). The expression of genes for the retina-associated markers rhodopsin, S-antigen, Rlbp, and Calb2 was determined by real-time PCR. As demonstrated in Fig. 1A for rhodopsin and S-antigen, a very low expression of these genes was detected in undifferentiated MSCs, but a significant expression was induced in cells cultured in the differentiation medium. Similar effects of differentiation medium were observed on the expression of *Rlbp* and *Calb2* genes (data not shown). No significant expression of retinal markers was found in MSC cultures containing supernatants from activated spleen cells and control tissue extracts (lung, liver, and muscle; data not shown).

As demonstrated in Fig. 1B, organotypic tissue cultures of the posterior segment of the eye expressed very low levels of genes for pro-inflammatory molecules (such as IL-6 or iNOS). However, in the presence of pro-inflammatory cytokines IFN-γ and IL-17, a significant expression of genes for pro-inflammatory molecules was detected. This expression was significantly suppressed if the explants were stimulated with cytokines in the presence of MSCs (Fig. 1B).

To demonstrate the secretory activity of MSCs, the cells were cultured unstimulated or in the presence of IFN-γ and IL-17, and the expression of genes for a panel of cytokines

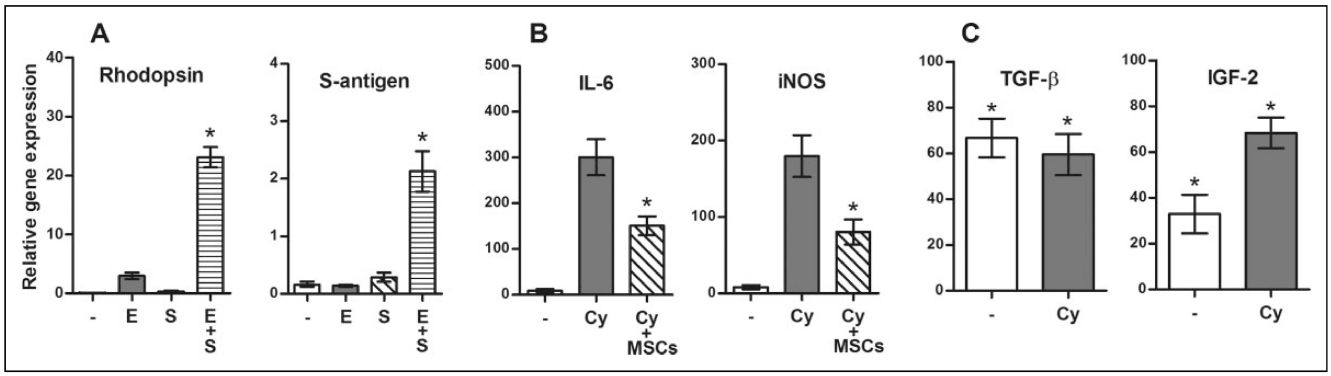

Fig. 1. The ability of mesenchymal stem cells (MSCs) to differentiate into cells expressing markers of retinal cells, to inhibit expression of genes for pro-inflammatory molecules, and to produce growth and differentiation factors. (A) MSCs were cultured for 7 d alone (-) or in the presence of retinal extract (E), in the presence of supernatant from activated T lymphocytes (S), or in the presence of E and S. The expression of genes for rhodopsin and S-antigen was determined by real-time PCR. The explants of the posterior segment of the eye were cultured for 48 h alone (-), with interleukin (IL)-17 and interferon (IFN)-γ (Cy), or were stimulated with cytokines in the presence of MSCs. The expression of genes for pro-inflammatory molecules IL-1β and inducible nitric oxide synthase was determined by PCR. Production of TGF-β and IGF-2 by MSCs. MSCs were cultured for 48 h unstimulated (-) or in the presence of IL-17 and IFN-γ (Cy). The expression of genes for TGF-β and IGF-2 was determined by real-time PCR. Each bar represents the mean (SD) from at least 3 independent determinations. Values with asterisk represent statistical significance ($P < 0.05$; A) gene expression, (B) inhibition of cytokine production, and (C) expression of genes for growth factors.

and growth factors was determined by real-time PCR. As demonstrated in Fig. 1C for TGF-β and IGF-2, MSCs significantly expressed genes for the tested molecules either constitutively (such as TGF-β) or after stimulation with cytokines (such as IGF-2).

Discussion

In spite of great progress in medical research, there are still missing effective therapeutic protocols for the treatment of retinal degenerative diseases, and millions of people worldwide are waiting for a treatment option. In this respect, stem cell–based therapy offers a promising therapeutic approach, which could inhibit degenerative processes or even replace missing retinal cells. Age-related retinal disorders are caused mainly by a degeneration and loss of specialized retinal cells and therefore the support of their survival or even their replacement by descendants of stem cells may offer effective treatment approaches. We observed that MSCs are producers of numerous growth and differentiation factors that can support the survival of the remaining cells in the diseased retina. The damage of the retina is also associated with a local inflammatory reaction that impedes the healing process. We showed that MSCs, by their known immunosuppressive properties,[3,5] inhibit the production of pro-inflammatory cytokines by the cells of the posterior ocular segment. These immunoregulatory properties of MSCs may represent an important mechanism to prevent a harmful local inflammatory reaction and to support the healing process. Finally, it has been shown that MSCs can differentiate into various cell types including cells expressing markers and characteristics of retinal cells. For example, it has been shown that cocultivation of MSCs with RPE cells induced expression of the RPE cell phenotype.[6,10] In our experiments to differentiate MSCs, attempts were made to mimic the inflammatory environment of a diseased retina. We showed that incubation of MSCs with retinal tissue extracts and supernatant from cultures of stimulated spleen cells induced expression of genes for rhodopsin, S-antigen, Rlbp, and Calb2, which are the markers of cells of individual retinal layers.

We have previously shown that bone marrow–derived MSCs have comparable therapeutic properties for ocular surface regeneration as have tissue-specific limbal stem cells.[9] The advantages of MSCs for the therapy of retinal dysfunctions have also been recently discussed by Park et al.[11] Here we showed experimentally that MSCs possess at least 3 different types of properties (immunoregulation ability, secretory activity, and differentiation potential), making them a promising candidate for the cell-based therapy of retinal degenerative diseases. To speed up the transfer of experimental results into clinical practice, numerous MSC-based clinical trials for the treatment of retinal diseases have been initiated.[12] However, further preclinical studies would be desirable.

Ethical Approval

The use of animals was approved by the local Ethical Committee of the Institute of Experimental Medicine.

Statement of Human and Animal Rights

Mice were purchased from the breeding unit of the Institute of Molecular Genetics, Prague. Their treatment was approved by the local Ethical Committee of the Institute of Experimental Medicine.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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5.4 Identifikace interferonu-γ jako klíčového podpůrného faktoru pro diferenciaci myších mezenchymálních kmenových buněk na buňky sítnice.

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Onemocnění sítnice představují hlavní příčinu snížené kvality zraku a slepoty po celém světě. Ztráta buněk sítnice způsobuje její nevratné poškození, pro většinu degenerativních onemocnění sítnice není v současné době k dispozici žádná účinná léčba. Slibným přístupem pro léčbu poruch sítnice je terapie na bázi kmenových buněk. Perspektivními kandidáty jsou MSC, které se mohou diferencovat na řadu buněčných typů a produkovat různé trofické a růstové faktory. V této studii jsme ukázali potenciál myších MSC izolovaných z kostní dřeně diferencovat se na buňky exprimující znaky buněk sítnice a identifikovali jsme klíčovou roli IFN-γ v diferenciačním procesu. MSC byly kultivovány po dobu 7 dní se sítnicovým extraktem a supernatantem ze splenocytů stimulovaných konkanavalinem A, který simuloval zánětlivé prostředí při poškození sítnice. MSC kultivované v takových podmínkách diferencovaly na buňky exprimující znaky buněk sítnice, jako je rodopsin, S antigen, protein vázající retinaldehyd, calbindin 2, rekoverin a RPE-65. Pro identifikaci klíčové molekuly obsažené v supernatantech z aktivovaných buněk sleziny byly MSC kultivovány se sítnicovým extraktem v přítomnosti různých cytokinů T buněk. Exprese znaků sítnice byla zvýšena pouze v přítomnosti IFN-γ a pomocný efekt supernatantu ze slezinných buněk byl zrušen neutralizační protilátkou anti-IFN-γ. Kromě toho byly diferencované MSC schopny exprimovat řadu neurotrofických faktorů, které jsou důležité pro regeneraci sítnice. Výsledky ukazují, že MSC se mohou diferencovat na buňky exprimující znaky buněk sítnice a že tento diferenciační proces je podporován IFN-γ.

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experimentální část (separace a kultivace MSC, příprava diferenciačního protokolu, real-time PCR, cytometrická analýza), analýza a interpretace výsledků, psaní manuskriptu

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ORIGINAL RESEARCH REPORT

AU1 ▶

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

AU2 ▶ Barbora Hermankova,[1,2] Jan Kossl,[1,2] Eliska Javorkova,[1,2] Pavla Bohacova,[1,2] Michaela Hajkova,[1,2] Alena Zajicova,[1] Magdalena Krulova,[1,2] and Vladimir Holan[1,2]

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

AU3 ▶ 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.

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.

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.

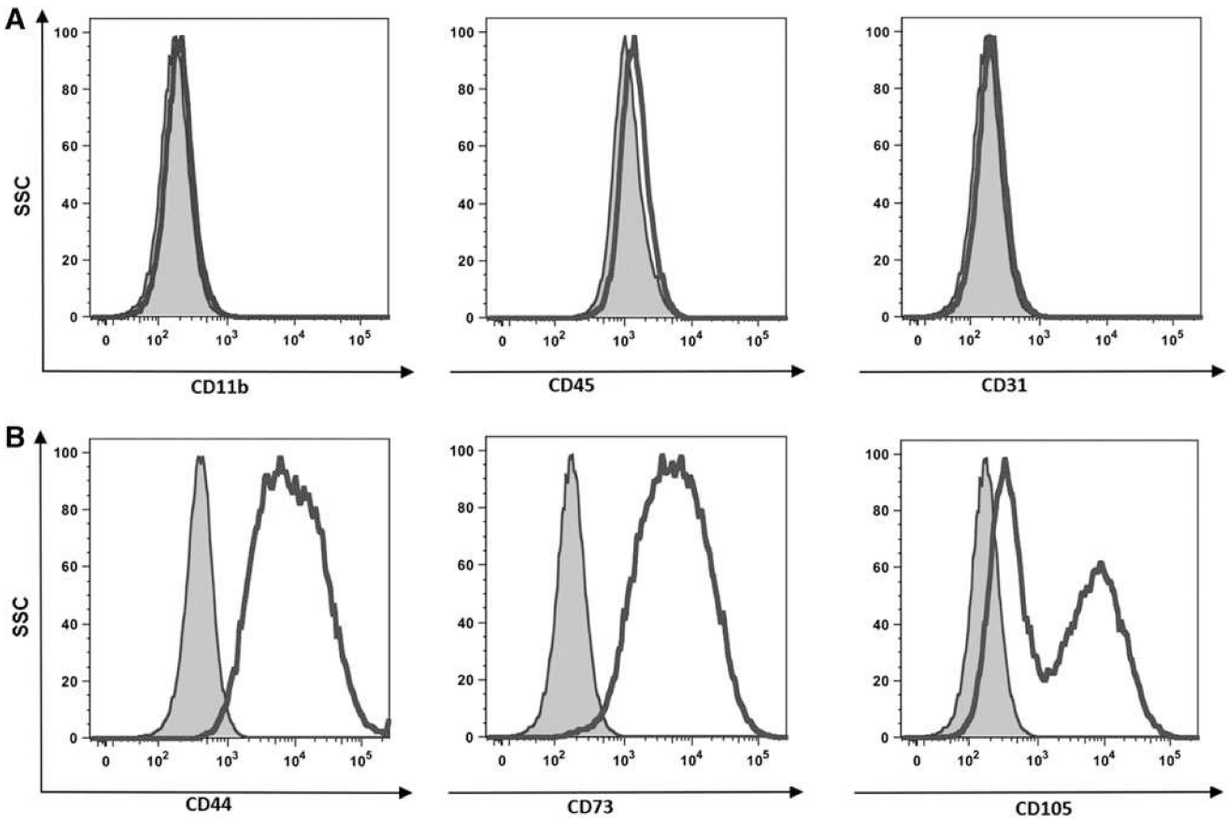


FIG. 1. Phenotypic characterization of MSCs. Representative histograms show the flow cytometry analysis of CD11b, CD45, CD31 **(A)** and CD44, CD73, CD105 **(B)** markers expressed by MSCs in comparison with control unlabeled MSCs. One of three similar experiments is shown. MSC, mesenchymal stem cell.

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.

◀T1

Neutralization of IFN-γ in supernatants from stimulated splenocytes

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

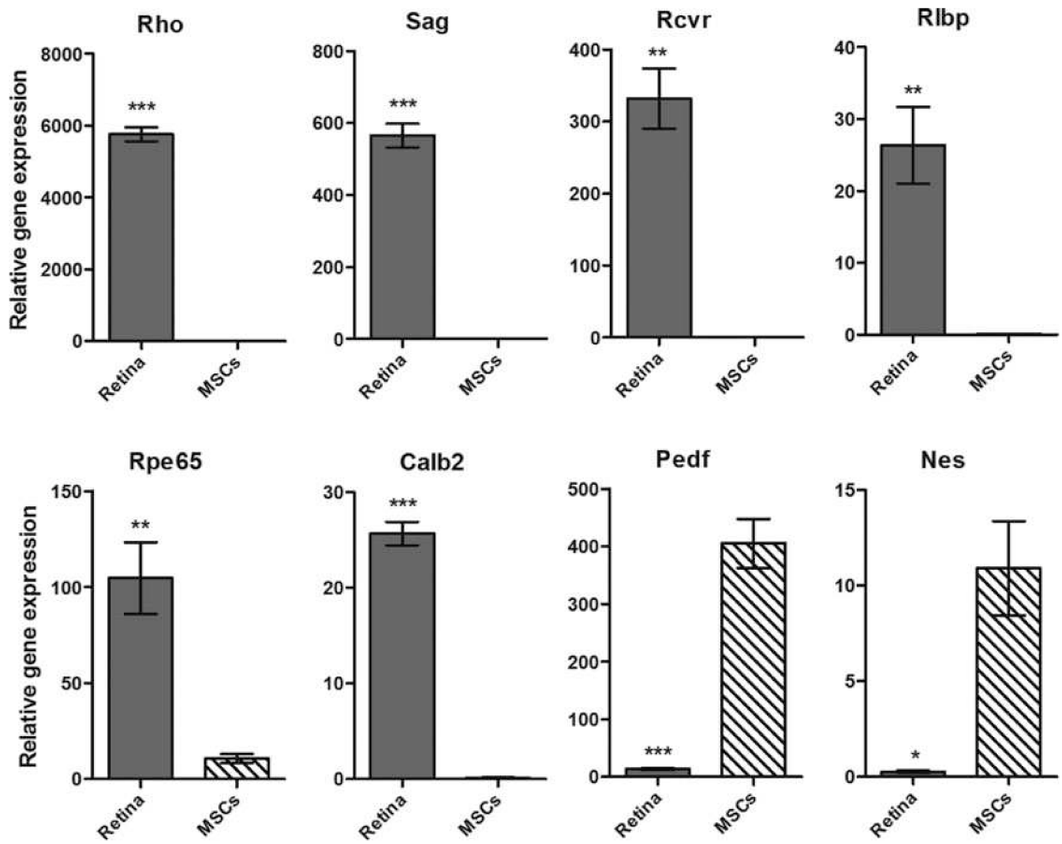


FIG. 2. The expression of genes for retinal markers in MSCs and retina. The expression of rhodopsin (*Rho*), S antigen (*Sag*), recoverin (*Rcvr*), retinaldehyde-binding protein (*Rlbp*), retinal pigment epithelium-specific protein 65 (*Rpe65*), calbindin 2 (*Calb2*), pigment epithelium-derived factor (*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.

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]. ◀F1

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. ◀F2

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

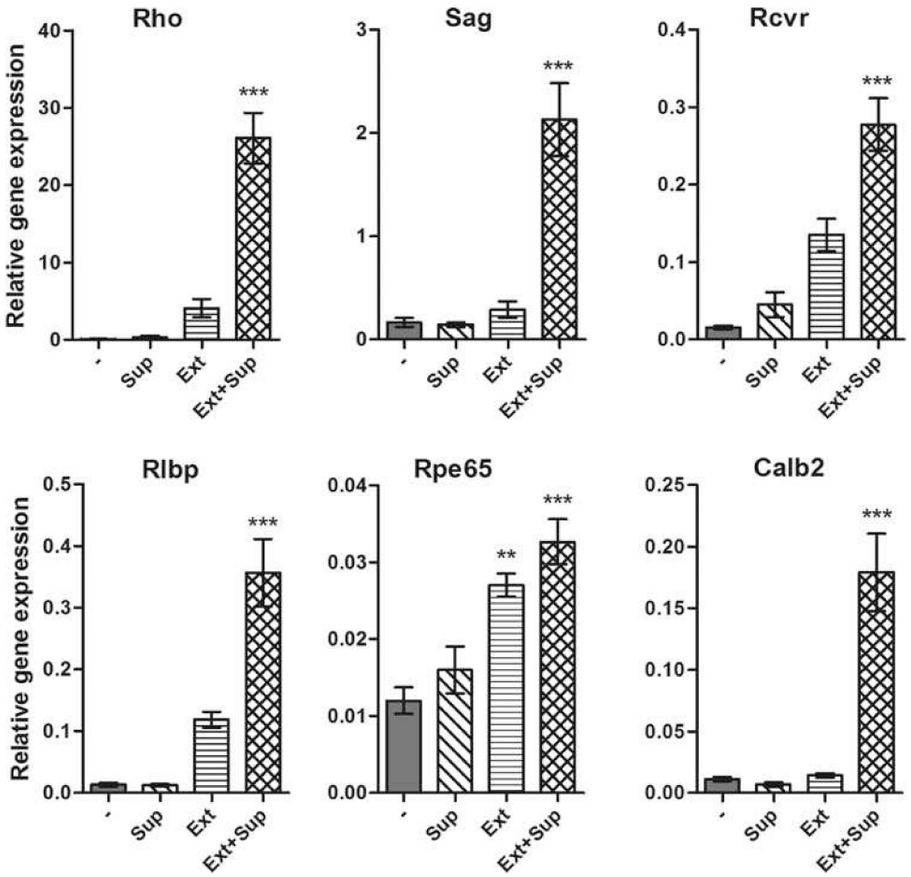


FIG. 3. The expression of retinal markers in differentiated MSCs. MSCs were cultured untreated (-), with supernatant from stimulated splenocytes (Sup), with retinal extract (Ext), or with supernatant and retinal extract together (Ext+Sup). The expression of rhodopsin (*Rho*), S antigen (*Sag*), recoverin (*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 (*$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$) from untreated MSCs. ◀AU4

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

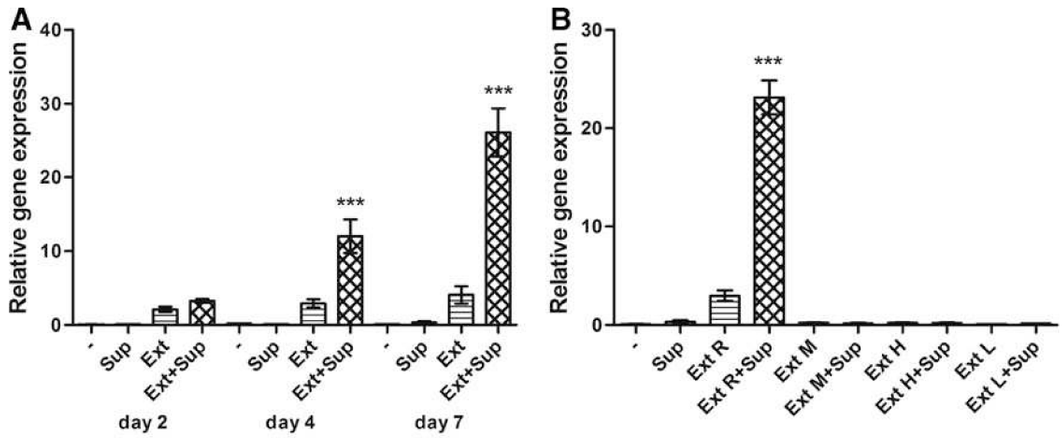
The expression of gene for Rho in untreated MSCs is undetectable and was already upregulated after 2-day cultivation of cells with retinal extract or with extract in combination with supernatant. After 4 and 7 days of differentiation, the expression of the *Rho* gene was gradually increasing, especially 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 Fig. 5D, the

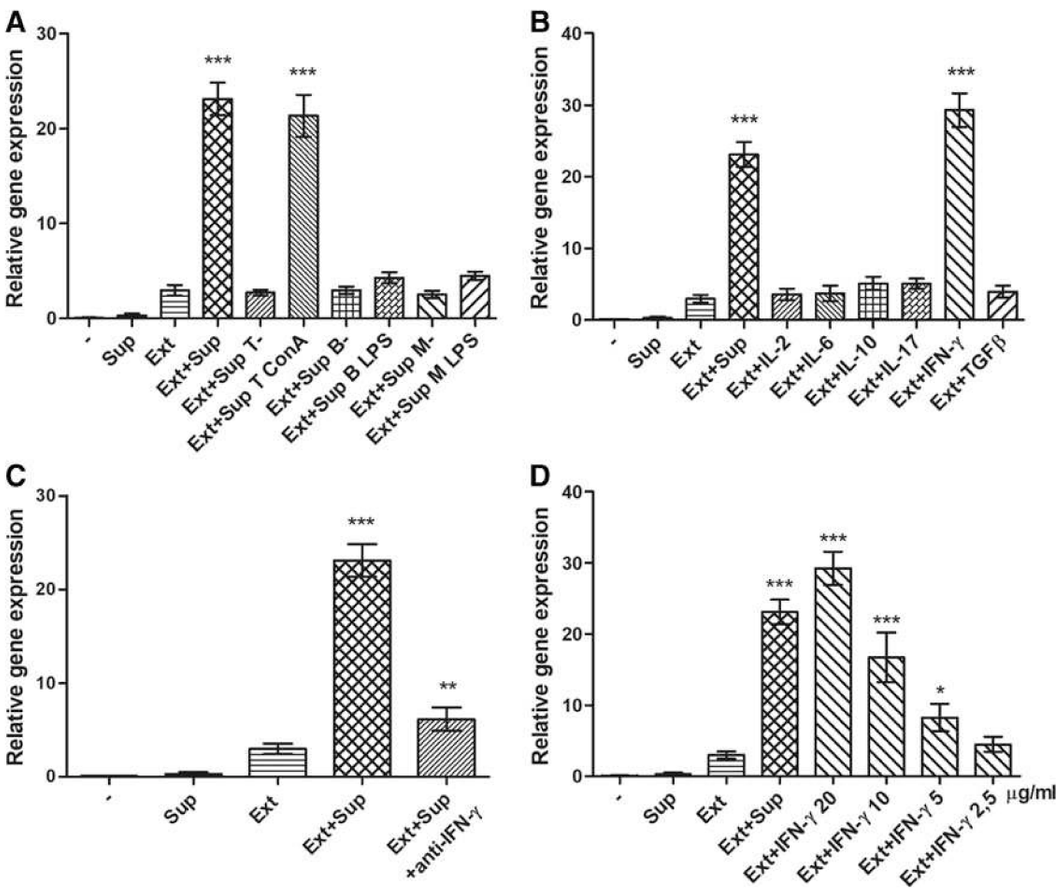


FIG. 5. The effect of IFN-γ in the differentiation process. The effect of supernatant from stimulated splenocytes (Sup) was compared with supernatants from unstimulated T cells (Sup T-), B cells (Sup B-), or macrophages (Sup M-) and Con A-stimulated T cells (Sup T ConA), LPS-stimulated B cells (Sup B 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.

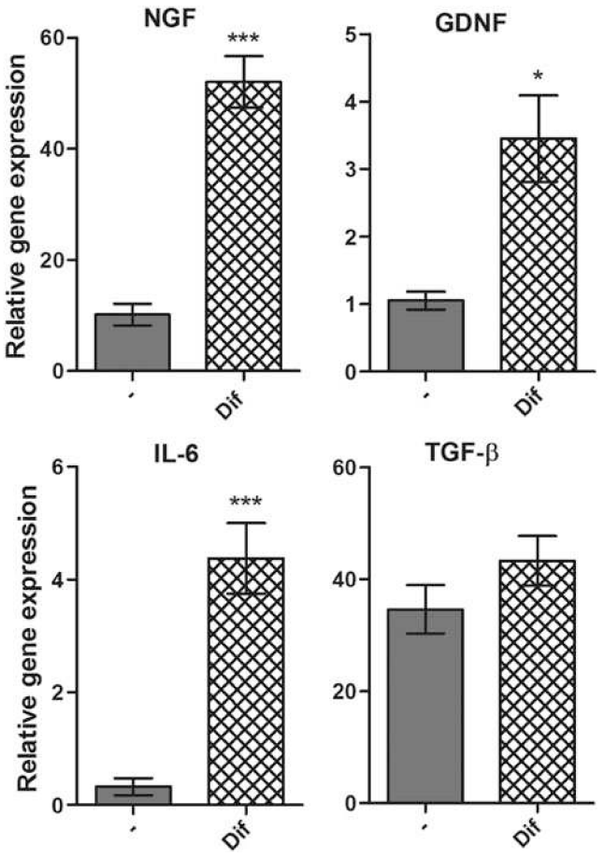


FIG. 6. The expression of genes for growth factors in untreated and differentiated MSCs. The expression of *Ngf*, *Gdnf*, *Il-6*, and *Tgf-β* genes was detected in untreated MSCs (-) and MSCs differentiated with retinal extract and supernatant from splenocytes (dif) by real-time PCR after 7 days. Each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

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). ◀F6

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). ◀F7

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

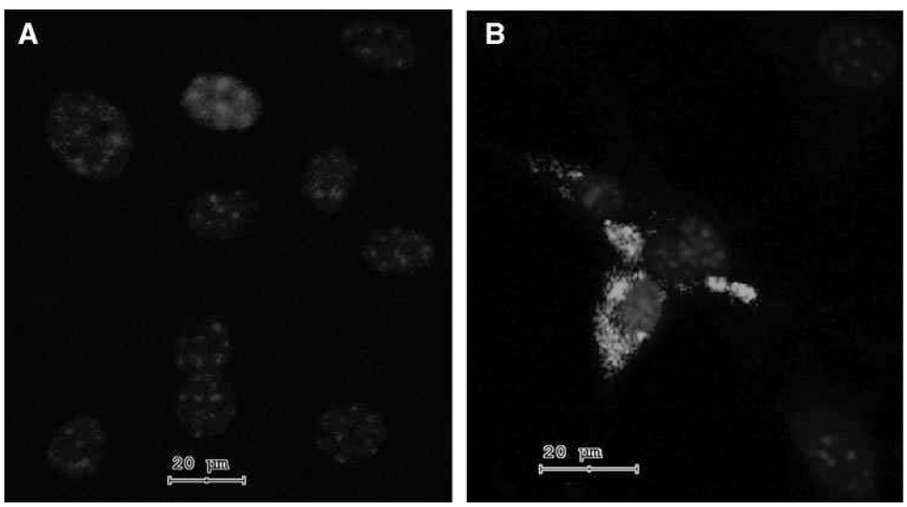


FIG. 7. Demonstration of Rho protein by immunocytochemistry. The expression of Rho protein in untreated MSCs **(A)** and differentiated MSCs **(B)** was detected with antibody anti-mouse Rho (*green*). The nuclei were stained with DAPI (*blue*). One representative experiment of three similar is shown.

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.

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Author Disclosure Statement

No competing financial interests exist.

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6 Diskuze

MSC mohou na základě svých imunomodulačních vlastností inhibovat řadu funkcí buněk imunitního systému. Bylo prokázáno, že MSC podporují diferenciaci buněk imunitního systému na protizánětlivé a regulační populace. MSC produkují TGF-β, který podporuje vývoj Treg buněk. Pokud se T buňky vyskytují v prostředí TGF-β a IL-6, dochází k vývoji Th17 buněk (Bettelli et al. 2006; Svobodova et al. 2012). Další z regulačních populací jsou Breg buňky, které produkují IL-10. Vývoj Breg buněk je ovlivňován cytokinovým prostředím, cytokiny IFN-γ a IL-12 podporují vývoj B buněk regulačním směrem. Naopak IL-21 a TGF-β vývoj Breg buněk inhibují (Yoshizaki et al. 2012; Holan et al. 2014).

V této práci jsme se zaměřili na studium vlivu MSC na Breg buňky a jejich produkci IL-10. Zjistili jsme, že nestimulované MSC nemají na produkci IL-10 B buňkami významný vliv. Pokud byl do kultury k MSC a B buňkám přidán IFN-γ, došlo k výraznému poklesu produkce IL-10 B buňkami, na rozdíl od kultur pouze se stimulovanými B buňkami a IFN-γ. Prokázali jsme, že po preinkubaci MSC s IFN-γ byla produkce IL-10 B buňkami snížena a naopak preinkubace B buněk s IFN-γ neměla na produkci IL-10 žádný vliv. V jiných studiích bylo zjištěno, že MSC působí na buňky imunitního systému jak přímým buněčným kontaktem, tak prostřednictvím solubilních molekul (Lee and Song 2017). K prokázání, zda se v našem modelu jedná o mechanismus založený na buněčném kontaktu nebo na produkci imunomodulačních molekul, byly B buňky kultivovány s MSC oddělené semipermeabilní membránou nebo se supernatantem získaným po kultivaci MSC. Separace B buněk a MSC pomocí semipermeabilní membrány vedla ke ztrátě inhibice produkce IL-10, naopak supernatant získaný po kultivaci MSC s IFN-γ neměl na produkci IL-10 supresivní vliv. Z toho vyplývá, že MSC v tomto případě ke snížení produkce IL-10 B buňkami potřebují buněčný kontakt.

Na základě poznatků o genové expresi u MSC ovlivněných IFN-γ a/nebo LPS jsme vybrali tři molekuly se zvýšenou hladinou exprese, které by mohly v inhibici produkce IL-10 u B buněk hrát roli. První molekulou byl povrchový PD-L1, u kterého bylo prokázáno, že zprostředkovává inhibici aktivace a proliferace T i B buněk (Augello et al. 2005; Davies et al. 2017). Další molekulou byla COX-2, která se spolu se svým produktem PGE2 uplatňuje v potlačení funkce buněk imunitního systému a navození

regulačního fenotypu (English et al. 2009; Duffy et al. 2011). Poslední molekulou se zvýšenou expresí u stimulovaných MSC byla IDO snižující aktivitu buněk imunitního systému na základě rozkladu tryptofanu (Ge et al. 2010). Do kultury stimulovaných B buněk s MSC a IFN-γ byly přidány následující inhibitory – indometacin pro inhibici COX-2, 1-metyl-D-tryptofan jako inhibitor IDO a anti-PD-L1 protilátka pro blokaci PD-L1. Zjistili jsme, že v kultuře s indometacinem nebyla snížena produkce IL-10, v ostatních případech zůstala suprese produkce IL-10 nezměněna. Zapojení dráhy COX-2 do inhibice bylo potvrzeno přidáním PGE2 v různých koncentracích do kultury, v závislosti na použité koncentraci došlo ke snížení produkce IL-10. Podobné výsledky byly prokázány i u jiných buněk imunitního systému, MSC jsou prostřednictvím dráhy COX-2 a PGE2 schopné potlačit diferenciaci T buněk na Th17 (Duffy et al. 2011), naopak indukovat vznik Treg buněk (English et al. 2009), snížit aktivitu CD8+ T buněk (Li et al. 2014), inhibovat aktivitu NK buněk (Spaggiari et al. 2008; Galland et al. 2017). Zároveň bylo zjištěno, že je v tomto případě mezi MSC a buňkami imunitního systému potřebný buněčný kontakt (Duffy et al. 2011; English et al. 2009). To může být způsobeno nezbytností velké koncentrace PGE2, která se nachází pouze v těsné blízkosti MSC. V naší studii jsme ukázali, že k potlačení produkce IL-10 B buňkami dochází až ve vysokých koncentracích PGE2 dodaného do kultury.

V další části projektu jsme se zaměřili na možnost využití imunomodulačních, diferenciačních a regenerativních vlastností MSC v léčbě onemocnění oka. Mezi nejčastější onemocnění oka vedoucí až ke ztrátě zraku patří poškození rohovky nebo onemocnění sítnice. Při poškození povrchu oka je regenerace rohovkového epitelu zprostředkována LSC, které se diferencují na buňky rohovky. Pokud je ovšem zasažen i limbus, mohou být LSC získány ze zdravého oka a transplantovány na poškozený povrch. V několika studiích byla prokázána úspěšná regenerace rohovky po transplantaci LSC na poškozený povrch oka (Zajicova et al. 2010; Marchini et al. 2011; Basu et al. 2012). Transplantace autologních LSC se nedá využít ve všech případech poškození povrchu oka, problém nastává například při oboustranné LSCD. Při využití alogenních LSC je riziko odhojení, proto musí být podávány imunosupresivní látky, jejichž užívání je spojeno s řadou nežádoucích efektů (Dua and Azuara-Blanco 1999; Pauklin et al. 2010).

Vzhledem ke svým vlastnostem představují MSC vhodnou alternativu pro transplantaci na poškozený povrch oka. Proto jsme se rozhodli porovnat na králičím modelu poškozeného povrchu oka terapeutický potenciál a vlastnosti LSC, AD-MSC a

BM-MSC. Povrch oka byl chemicky poleptán a buňky byly na povrch oka transplantovány na nanovlákenném nosiči. V takto poškozené rohovce došlo k infiltraci buněk imunitního systému, zvýšení apoptózy, neovaskularizaci a zhoršení průhlednosti rohovky. Všechny tyto parametry byly u očí léčených kmenovými buňkami zlepšeny, vyšší regenerace rohovky byla pozorována u očí s aplikovanými BM-MSC nebo LSC než u neléčených očí nebo očí se samotným nanovlákenným nosičem nebo nanovlákenným nosičem s AD-MSC.

Z výsledků vyplývá, že BM-MSC mají srovnatelný potenciál v léčbě poškozeného povrchu oka jako tkáňově specifické LSC. Existuje řada studií, které prokázaly diferenciaci MSC na buňky rohovkového epitelu (Gu et al. 2009; Rohaina et al. 2014). Jiné studie poukázaly na produkci růstových a trofických faktorů u MSC hrajících v procesu regenerace rohovky důležitou roli (Ma and Chan 2006; Oh et al. 2009). Kromě toho jsou MSC také schopné potlačit zánětlivé reakce probíhající v poškozené rohovce a tím podpořit proces hojení (Jia et al. 2012; Cejkova et al. 2013; Javorkova et al. 2014).

MSC mají uplatnění i v léčbě poškozené sítnice, v současné době pro některá onemocnění sítnice stále neexistuje účinná léčba. Degenerativní onemocnění sítnice jsou často spojeny se ztrátou specializovaných buněk v sítnici. Bylo ukázáno, že MSC se mohou diferencovat na různé druhy buněk sítnice (Salehi et al. 2017). V *in vitro* pokusech byly MSC diferencovány na buňky sítnice v diferenciačním médiu obsahujícím taurin (Nadri et al. 2013) nebo taurin, aktivin A a EGF (Kicic et al. 2003).

MSC se po transplantaci do oka s onemocněním sítnice nachází v zánětlivém prostředí, kde je velké množství poškozených buněk sítnice. Zajímalo nás, jaký efekt bude mít toto prostředí na diferenciační vlastnosti a produkci trofických faktorů MSC. Zánětlivé prostředí poškozené sítnice jsme simulovali extraktem připraveným ze sítnic a supernatantem získaným po kultivaci stimulovaných lymfocytů. Po 7 dnech kultivace v tomto prostředí MSC exprimovaly markery typické pro různé buňky v sítnici (fotoreceptory, bipolární buňky, horizontální buňky, Müllerovy buňky a RPE buňky). Při degenerativním onemocnění sítnice často dochází k odumření více typů buněk najednou, proto je použití MSC pro jejich náhradu vhodným řešením. V jiných studiích bylo prokázáno, že MSC jsou schopné diferenciace na fotoreceptory (Kicic et al. 2003; Yang et al. 2010; Nadri et al. 2013), bipolární buňky (Castanheira et al. 2008; Nadri et al. 2013), amakrinní buňky (Castanheira et al. 2008), RPE buňky (Vossmerbaeumer et al. 2009) a další. V našem modelu byla největší exprese naměřena pro gen *Rho*, který je typickým znakem fotoreceptorů.

Při studiu diferenciace MSC jsme prokázali, že MSC exprimují více *Rho* v prostředí s extraktem ze sítnic a supernatantem než v kultuře pouze se sítnicovým extraktem. Jak bylo ukázáno dříve, MSC ke své aktivaci a produkci inducibilních molekul potřebují stimul, například IFN-γ, TNF-α nebo IL-1β (English et al. 2007; Javorková et al. 2014; Gao et al. 2016). Zajímalo nás, která konkrétní molekula obsažená v supernatantu je za zvýšení diferenciace zodpovědná. Po diferenciaci MSC s extraktem ze sítnic a supernatanty získanými po kultivaci T buněk, B buněk a makrofágů bylo zjištěno, že ke zvýšené expresi genu *Rho* dochází pouze při použití supernatantu získaného po kultivaci T buněk. Na základě tohoto výsledku jsme diferencovali MSC s extraktem ze sítnic spolu s různými cytokiny produkovanými T buňkami a sítnicovým extraktem. Exprese *Rho* byla zvýšena pouze v kultuře s IFN-γ. Pro potvrzení byla k supernatantu přidána neutralizační protilátka anti-IFN-γ a u MSC diferencovaných v přítomnosti tothoto supernatantu a extraktu ze sítnic byla exprese *Rho* signifikantně snížena. Zjistili jsme, že v procesu diferenciace MSC na buňky exprimující znaky sítnice hraje IFN-γ spolu s extraktem ze sítnic důležitou roli. Podobné výsledky byly zjištěny i v jiných studiích, kde přítomnost IFN-γ podporovala diferenciaci na nervové buňky (Wong et al. 2004; Croitoru-Lamoury et al. 2011), ale naopak inhibovala diferenciaci na adipocyty a osteocyty (Croitoru-Lamoury et al. 2011).

Vedle schopnosti diferenciace MSC také produkují neurotrofické faktory jako například NGF, BDNF, GDNF, CNTF, IGF, bFGF a další (Meirelles et al. 2009; Kolomeyer and Zarbin 2011; Park et al. 2017). Prokázali jsme, že po diferenciaci na buňky exprimující znaky sítnice produkují MSC zvýšené množství NGF, GDNF, TGF-β a IL-6. Podobné výsledky zjistili Xu et al., kdy MSC v prostředí supernatantu získaného po kultivaci světlem poškozených buněk sítnice produkovaly zvýšené množství neurotrofických faktorů (Xu et al. 2013). Produkce neurotrofických faktorů MSC podporuje regeneraci buněk sítnice a potlačuje jejich apoptózu (Yu et al. 2006; Inoue et al. 2007; Na et al. 2009). Vliv neurotrofických faktorů v médiu po kultivaci MSC byl prokázán aplikací tohoto média bez MSC do oka s poškozenou sítnicí. Po aplikaci došlo k potlačení apoptózy a zvýšenému přežívání buněk sítnice (Dreixler et al. 2014; Roth et al. 2016).

V léčbě onemocnění sítnice se mohou MSC uplatňovat také svými imunomodulačními vlastnostmi. V *in vitro* modelu jsme zjistili, že po kultivaci MSC s explantáty sítnic v zánětlivém prostředí došlo ke snížení exprese prozánětlivých molekul. Cui et al. po kultivaci MSC a poškozených gangliových buněk prokázali u

gangliových buněk snížení produkce prozánětlivých cytokinů IL-1β a TNF-α (Cui et al. 2016). Podobné výsledky byly zjištěny *in vivo*, kde po trasnplantaci MSC do oka se světelně poškozenou sítnicí, byla také snížena produkce IL-1β a TNF-α u buněk sítnice (Huang et al. 2013). Snížení prozánětlivých cytokinů po intravitreální aplikaci MSC bylo pozorováno také u ischemicky poškozené sítnice potkanů (Mathew et al. 2017).

MSC vzhledem ke svým vlastnostem představují vhodné kandidáty pro léčbu onemocnění sítnice i poškozené rohovky. Při studiu imunomodulačních vlastností MSC jsme ukázali, že inhibují produkci IL-10 B buňkami a v *in vitro* modelu poškození sítnice snižují produkci prozánětlivých cytokinů v sítnici. MSC jsou schopné diferenciace na buňky exprimující znaky buněk sítnice a také produkují řadu trofických faktorů. V neposlední řadě jsme prokázali, že při léčbě poškozeného povrchu oka mají BM-MSC srovnatelný terapeutický potenciál jako tkáňově specifické LSC.

7 Závěry

- **Mechanismus imunomodulačního působení MSC na produkci IL-10 B buňkami**

  MSC ovlivněné IFN-γ snižují produkci IL-10 B buňkami stimulovanými LPS. Inhibice produkce IL-10 je zprostředkována přes dráhu COX-2 a tento mechanismus je závislý na kontaktu mezi MSC a B buňkami.

- **Vliv IFN-γ na imunomodulační a diferenciační vlastnosti MSC**

  IFN-γ zvyšuje u MSC expresi inducibilních imunomodulačních molekul – IDO, COX-2 a PD-L1 a zvyšuje schopnost diferenciace MSC na buňky exprimující znaky sítnice.

- **Diferenciace MSC na buňky exprimující znaky buněk sítnice**

  MSC diferencují v prostředí simulujícím zánětlivé poškození sítnice na buňky exprimující znaky sítnice. Nejvyšší exprese byla zaznamenána pro gen *Rho*. Jako důležitý podpůrný faktor v procesu diferenciace byl identifikován IFN-γ.

- **Srovnání terapeutického potenciálu MSC a LSC při poškození povrchu oka**

  Regenerace, neovaskularizace a inhibice zánětlivé reakce v poškozené rohovce bylo srovnatelné u očí, které byly ošetřeny LSC nebo BM-MSC. BM-MSC je možné použít při léčbě poškozeného povrchu oka v případech, kdy nemohou být využity LSC.

- **Produkce růstových a trofických faktorů MSC v zánětlivém prostředí sítnice**

  MSC exprimují neurotrofické faktory a cytokiny, které se uplatňují v regeneraci poškozené sítnice. Zvýšená exprese těchto faktorů byla zaznamenána u MSC diferencovaných na buňky exprimující znaky buněk sítnice.

- **Inhibice prozánětlivých cytokinů při poškození sítnice**

  MSC inhibovaly expresi prozánětlivého cytokinu IL-6 a molekuly iNOS v explantátech sítnic kultivovaných spolu s MSC *in vitro*.

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