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Využití imunoregulačních vlastností mezenchymálních kmenových buněk a jejich terapeutický potenciál

The use of immunoregulatory properties of mesenchymal stem cells and their therapeutic potential

Disertační práce

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Prohlašuji, že jsem tuto disertační práci vypracovala 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.

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Abstrakt

Mezenchymální kmenové buňky (mesenchymal stem cells – MSC) mají schopnost diferencovat v různé buněčné typy a zároveň disponují rozsáhlými imunomodulačními vlastnostmi, jejichž prostřednictvím mohou ovlivňovat řadu funkcí různých buněk imunitního systému. Protože imunoregulační vlastnosti MSC mohou být ovlivněny působením cytokinů, porovnávali jsme účinek neovlivněných MSC a MSC stimulovaných interleukinem 1 (interleukin – IL), interferonem-γ (interferon – IFN), transformujícím růstovým faktorem-β (transforming growth factor – TGF) a IL-10 na vývoj regulačních T (regulatory T cells – Treg) a pomocných T17 (helper T cells – Th) lymfocytů *in vitro* a na rozvoj časného zánětu v oku *in vivo*.

MSC mohou produkovat významná množství TGF-β a IL-6. Tyto dva cytokiny představují klíčové faktory, které recipročně regulují vývoj naivních T lymfocytů v Treg nebo Th17 buňky. Nestimulované MSC produkují TGF-β, ale neprodukují IL-6. Produkce TGF-β může být dále zesílena působením IL-10 a TGF-β na MSC. V přítomnosti prozánětlivých cytokinů naopak MSC produkují významná množství IL-6 a zároveň konstitutivně produkují TGF-β. MSC produkující TGF-β indukovaly přednostně expresi Foxp3 a aktivaci Treg lymfocytů, zatímco supernatanty z MSC obsahující TGF-β i IL-6 podporovaly expresi RORγt a vývoj Th17 lymfocytů. Ukázali jsme, že MSC a jimi produkované cytokiny účinně kontrolují vývoj Treg a Th17 lymfocytů v populaci myších slezinných buněk stimulovaných aloantigeny.

Dále jsme studovali účinek systémově podaných MSC na časnou fázi zánětu v oku poškozeném hydroxidem sodným. Zjistili jsme, že intravenózně podané MSC cíleně migrují do poškozeného oka a že MSC stimulované IFN-γ jsou nejúčinnější v potlačení akutní fáze rohovkového zánětu, ve snižování leukocytární infiltrace a v ovlivnění zánětlivého prostředí. Rovněž jsme prokázali, že nanovlákna připravená z polymeru polyamid 6/12 nebo obsahující cyklosporin A představují vhodný nosič pro růst MSC a jejich následný přenos na poškozený povrch oka.

Abstract

Mesenchymal stem cells (MSCs) have the potential to differentiate into various cell types, possess potent immunomodulatory properties and can influence various functions of immune cells. Since the immunomodulatory properties of MSCs can be modified by cytokines, we compered the effect of unstimulated MSCs and MSCs pretreated with interleukin (IL)-1, interferon (IFN)-γ, transforming growth factor (TGF)-β and IL-10 on the development of regulatory T cells (Treg) and T helper 17 (Th17) cells *in vitro* and on the inflammatory environment in the eye.

MSCs can produce significant levels of TGF-β and IL-6. These cytokines represent the key factors that reciprocally regulate the development of naive T cells into Treg and Th17 cells. Unstimulated MSCs produce TGF-β, but not IL-6, and the production of TGF-β can be further enhanced by IL-10 or TGF-β. In the presence of IL-1, MSCs secrete significant levels of IL-6, in addition to spontaneous production of TGF-β. MSC producing TGF-β induced preferentially expression of Foxp3 and activation of Treg lymphocytes, whereas MSCs supernatants containing TGF-β together with IL-6 supported RORγt expression and development of Th17 cells. We demonstrated that MSCs and their products effectively control the development of Tregs and Th17 cells in a population of alloantigen-activated mouse spleen cells.

We also investigated the effects of systemically administered MSCs on the early acute phase of inflammation in the alkali-burned eye. The results show that intravenously injected MSCs specifically migrate to the damage eye and that IFN-γ pretreated MSCs are superior in inhibiting the acute phase of inflammation, decreasing leukocyte infiltration, and attenuating the early inflammatory environment. We also show, that nanofibers prepared from polymer PA6/12 or containing Cyclosporine A represent a conventional scaffold for growth of MSCs and for their transfer to treat ocular surface injuries.

Obsah

1 Seznam zkratek

APC	antigen-presenting cells, buňky prezentující antigen
Breg	regulatory B cells, regulační B lymfocyty
CD	cluster of differentiation, diferenciační antigen
COX	cyclooxigenase, cyklooxigenáza
Cs	cyclosporine, cyklosporin
DC	dendritic cells, dendritické buňky
EAU	experimental autoimmune uveoretinitis, exprimentální autoimunitní uveoretinitida
Foxp3	forkhead box p3
GVHD	graft versus host disease, reakce štěpu proti hostiteli
HGF	hepatocyte growth factor, růstový faktor hepatocytů
HO	heme oxygenase, hemoxygenáza
HSC	hematopoietic stem cells, hematopoetické kmenové buňky
IDO	indoleamine 2,3-dioxygenase, indolamin 2,3-dioxygenáza
IFN	interferon, interferon
Ig	immunoglobulin, imunoglobulin
IL	interleukin, interleukin
LPS	lipopolysaccharide, lipopolysacharid
LSC	limbal stem cells, limbální kmenové buňky
MHC gp	glykoproteiny hlavního histokompatibliního komplexu, major histocompatibility complex glycoprotein
MSC	mesenchymal stem cells, mezenchymální kmenové buňky
NK	natural killers, přirození zabíječi
NO	nitric oxide, oxid dusnatý
NOS	nitric oxide synthase, syntáza oxidu dusnatého
PA	polyamide, polyamid
PGE	prostaglandine E, prostaglandin E
ROR	retinoic acid receptor-related orphan receptor
STAT	signal transducer and activator of transcription
Tc	cytotoxic T cells, cytotoxické T lymfocyty
TGF	transforming growth factor, transformující růstový faktor

Th	helper T cells, pomocné T lymfoycyty
TLR	Toll-like receptors, receptory ze skupiny Toll
TNF	tumor necrosis factor, faktor nekrotizující nádory
Treg	regulatory T cells, regulační T lymfocyty
TSG	TNF-α stimulated gene 6 protein

2 Literární přehled

2.1 Úvod

Kmenové buňky se dostaly během posledních dvou desetiletí do centra zájmu velkého množství vědeckých skupin na celém světě. Počet publikací týkajících se kmenových buněk každoročně stoupá a podle údajů v databázi PubMed dosáhl v minulém roce téměř 20 000. Tato práce je věnovaná především mezenchymálním kmenovým buňkám (mesenchymal stem cells – MSC) a jejich imunoregulačnímu působení. MSC patří v současné době díky svému širokému diferenciačnímu potenciálu a rozsáhlým imunomodulačním schopnostem k nejstudovanějšímu typu kmenových buněk. Jejich výzkumem se v posledních letech zabývala přibližně čtvrtina všech publikací týkajících se kmenových buněk. Studium MSC přineslo velké množství poznatků, které jsou nyní využívány při jejich použití v klinické praxi při léčbě řady závažných onemocnění.

Dalším důležitým typem kmenových buněk popsaným v této práci jsou limbální kmenové buňky (limbal stem cells – LSC), které zajišťují nepřetržitou obnovu rohovkového epitelu. Přibližně 4/5 všech informací o svém okolí získáváme prostřednictvím zraku. Rohovka zprostředkovává přenos světelných paprsků na sítnici a snížení její průhlednosti v důsledku poškození nebo onemocnění může vést v krajním případě až ke slepotě. LSC sídlí v bazální vrstvě limbu. Pokud je limbus poškozený, nebo nefunkční, může dojít k deficitu LSC a narušení procesu obnovy rohovkového epitelu. Studium LSC vedoucí k nalezení nových způsobů kultivace a přenosu těchto buněk na poškozené oko tedy představuje velmi důležité odvětví výzkumu kmenových buněk.

2.2 Kmenové buňky

Mezi základní vlastnosti definující kmenovou buňku patří neomezená nebo alespoň dlouhodobá schopnost sebeobnovy a schopnost diferenciace v nejméně jeden typ vysoce specializovaných dceřiných buněk (Lajha et al., 1979). Kmenové buňky lze rozdělit na embryonální kmenové buňky a kmenové buňky z dospělého organizmu.

Samostatné kategorie pak tvoří kmenové buňky z pupečníkové krve novorozence a indukované pluripotentní kmenové buňky.

Embryonální kmenové buňky jsou odvozeny z vnitřní vrstvy blastocysty (Evans et al., 1981). Jedná se o pluripotentní kmenové buňky, které jsou za určitých podmínek schopné diferencovat v jakýkoliv buněčný typ. Naproti tomu kmenové buňky z dospělého organizmu jsou přirozeně přítomny v relativně malých populacích ve svých tkáňových nikách, kde zabezpečují obnovu dané tkáně a nahrazují buňky ztracené v důsledku opotřebení nebo poškození (Hall et al., 1989). Diferenciační potenciál některých kmenových buněk je omezen pouze na buněčnou linii odpovídající tkáni, ke které přísluší. Na druhém konci spektra pak stojí multipotentní kmenové buňky, jejichž diferenciační potenciál je mnohem širší. Například MSC jsou schopny diferencovat v buňky mezodermální linie, ale rovněž transdiferencovat v buňky patřící k vývojově nepříbuzné ektodermální či entodermální zárodečné linii (Pittenger et al., 1999).

2.3 **MSC**

MSC se díky svým imunomodulačním schopnostem a širokému diferenciačnímu potenciálu staly v posledním desetiletí předmětem studia řady předních výzkumných pracovišť po celém světě. V současné době běží na základě poznatků získaných v *in vitro* systémech a preklinických testech více než 300 klinických studií využívajících MSC v regenerativní medicíně nebo při terapii autoimunitních onemocnění, těžké reakce štěpu proti hostiteli (graft versus host disease – GVHD) a mnoha dalších (čerpáno z www.clinicaltrials.gov).

MSC byly poprvé popsány v práci Friedenstein et al. (1966), kteří jako první poukázali na přítomnost buněk s osteogenním potenciálem v suspenzi získané z kostní dřeně. Roku 1991 pak vyšla další klíčová studie přinášející teorii, že MSC v organizmu zajišťují přirozenou obměnu a regeneraci mezenchymálních tkání (Caplan et al., 1991).

2.3.1 Zdroje MSC a jejich role v organizmu

Přítomnost MSC byla prokázána v celé řadě orgánů a tkání dospělého organizmu včetně zubní dřeně, krve, svalstva, slinivky břišní, kloubní výstelky, škáry, okostice, plic a mnoha dalších (Tuan et al., 2003; Seeberger et al., 2006). Mezi nejčastěji používané zdroje těchto buněk však stále patří kostní dřeň spolu s tukovou tkání a pupečníkovou krví. Během zárodečného vývoje pak byly MSC detekovány ve fetální krvi, játrech a kostní dřeni (Campagnoli et al., 2001).

Populace MSC je do značné míry heterogenní, jednotlivé buňky se liší svými biologickými funkcemi i diferenciačním potenciálem. Transkriptom MSC vykazuje vysokou míru komplexity a kóduje velké množství proteinů souvisejících s různými vývojovými liniemi a biologickými procesy včetně buněčného pohybu a komunikace, angiogenze a regulace hematopoezy, imunitních reakcí a funkcí nervové soustavy (Phinney et al., 2006; Anam et al., 2013).

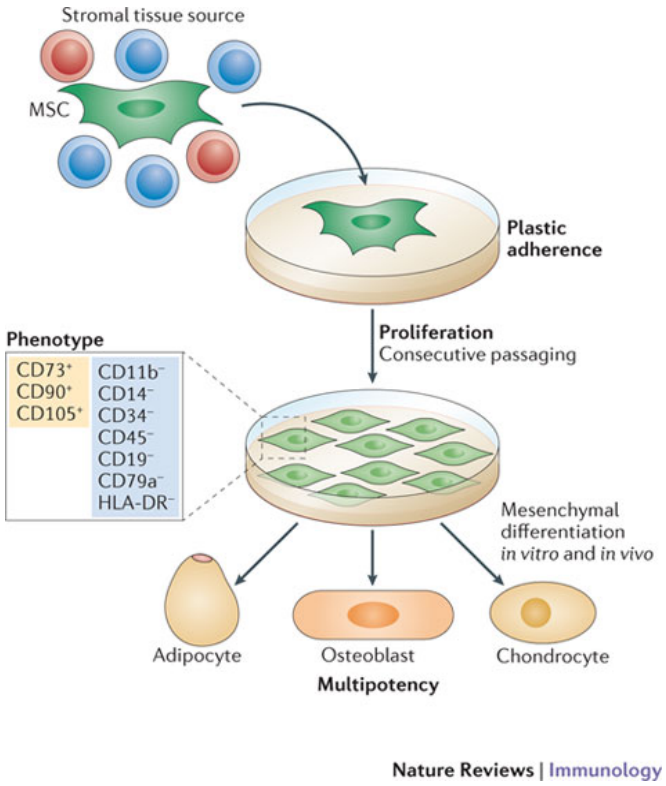
2.3.2 Charakterizace a multipotence MSC

Populace MSC disponuje, stejně jako jiné typy kmenových buněk, schopností sebeobnovy. Při kultivaci *in vitro* MSC adherují k podkladu a mají vřetenovitý tvar (Soleimani et al., 2009).

Doposud nebyl nalezen znak, který by byl unikátní pouze pro populaci MSC. Bez ohledu na to však mohou být MSC charakterizovány kombinací exprese pozitivních znaků CD (cluster of differentiation) 29, CD44, CD73, CD90 a CD105 a naopak nepřítomností leukocytárních znaků CD11b, CD31, CD34, CD45 a CD135 (Wagner et al., 2005; Soleimani et al., 2009). Úroveň exprese těchto znaků však záleží na řadě faktorů. Důležité jsou především tkáň a živočišný druh, ze kterých byly MSC získány, velký význam mají v tomto ohledu i kultivační podmínky. Značné rozdíly v metodách izolace a charakterizace lidských MSC používaných ve studiích prakticky znemožnily srovnání takových studií i jejich výsledků. Proto roku 2006 Mezinárodní společnost pro buněčnou terapii vydala soubor kritérií definujících lidské MSC (Dominici et al., 2006):

- adherence k plastu za standardních kultivačních podmínek

- povrchová exprese CD73, CD90 a CD105 a současná absence CD11b, CD14, CD19, CD34, CD45, CD79α a HLA-DR (human leukocyte antigen)
- diferenciace v osteoblasty, adipocyty a chondrocyty *in vitro* (Obr. 1).



Obr. 1: Kritéria definující lidské MSC. MSC představují populaci prekurzorových buněk, které mohou být během kultivace odlišeny od ostatních buněčných typů z kostní dřeně na základě adherence k plastu. Pro MSC je charakteristický vřetenovitý tvar a jako důkaz jejich multipotence se standardně využívá potvrzení schopnosti diferenciace v adipocyty, osteoblasty a chondrocyty. Zároveň by u MSC měla být prokázána povrchová exprese CD73, CD90 a CD105 a současná absence leukocytárních, hematopoetických a endoteliálních znaků CD11b, CD14, CD34, CD45, CD19, CD79α a HLA-DR (human leukocyte antigen). Převzato z Le Blanc et al., 2012.

Diferenciace MSC ve zralé specializované buňky tvořící organizmus je komplikovaný a přísně regulovaný děj, v němž je zapojena řada růstových faktorů, cytokinů a složek extracelulární matrix. MSC mají schopnost diferencovat v buňky tvořící mezodermální tkáně, ale rovněž transdiferencovat v buňky tvořící ektodermální a entodermální tkáně. Doposud byla prokázána schopnost MSC diferencovat za specifických podmínek v adipocyty, osteocyty a chondrocyty (Soleimani et al., 2009), neurony a astrocyty (Kopen et al., 1999), kardiomyocyty a buňky kosterního svalstva (Toma et al., 2002; Shiota et al., 2007), hepatocyty (Lee et al., 2004), buňky sítnice

(Kicic et al., 2003), epiteliální buňky kůže (Nakagawa et al., 2005; Sasaki et al., 2008), plicní epiteliální buňky (Rojas et al., 2005), buňky produkující inzulin (Xie et al., 2013), tubulární epiteliální buňky ledviny (Liu et al., 2013) a mnoho jiných.

2.3.3 Imunomodulační vlastnosti MSC

Výzkum MSC byl zpočátku zaměřen spíše na jejich široký diferenciační potenciál a z toho plynoucí využití v regenerativní medicíně. Postupně se však objevovaly práce poukazující na jejich rozsáhlé imunomodulační schopnosti, čímž se rozšířila oblast možného využití MSC o léčbu především autoimunitních onemocnění. V současné době se výzkum MSC ubírá velice úspěšně oběma směry a v řadě případů je dokonce využito kombinovaného efektu podaných MSC, které mohou v místě poškození diferencovat v potřebný buněčný typ a zároveň prostřednictvím imunomodulační aktivity potlačit nežádoucí zánětlivou reakci.

Imunomodulační schopnosti MSC jsou rozsáhlé a ovlivňují přirozenou i adaptivní složku imunitního systému. MSC působí na řadu komponent přirozené imunity včetně produkce komplementu (Tu et al., 2010), dendritických buněk (dendritic cells – DC) (English et al., 2008), monocytů a makrofágů (Kim et al., 2009; Cutler et al., 2010), granulocytů (Brandau et al., 2010) a NK buněk (natural killers – NK) (Spaggiari et al., 2008). Zároveň ovlivňují i adaptivní imunitní odpověď a regulují aktivitu a funkce T lymfocytů (Di Nicola et al., 2002) a B lymfocytů (Corcione et al., 2006).

Efekt MSC je dán jak přímým kontaktem s cílovými buňkami, tak produkcí řady cytokinů a imunologicky aktivních molekul. Tyto faktory mohou být uvolňovány konstitutivně, nebo až jako reakce na setkání s cílovou buňkou, či vystavení zánětlivým stimulům prostředí.

2.3.3.1 Molekuly a mechanizmy klíčové pro imunomodulační působení MSC

Imunomodulační účinky MSC jsou zprostředkovány celou řadou faktorů, imunomodulačních molekul a enzymů, mezi něž patří růstový faktor hepatocytů (hepatocyte growth factor – HGF), transformující růstový faktor-β (transforming growth factor – TGF), interleukin-6 (interleukin – IL), TSG-6 (TNF-α stimulated

gene 6 protein), inducibilní syntáza oxidu dusnatého (inducible nitric oxide synthase – iNOS), indolamin 2,3-dioxygenáza (indoleamine 2,3-dioxygenase – IDO), cyklooxigenáza-2 (cyclooxigenase – COX) a hemoxygenáza-1 (heme oxygenase – HO). Některé z nich jsou produkovány konstitutivně, jiné jsou inducibilní a jejich produkce může být navozena stimulací MSC prozánětlivými cytokiny.

Efekt MSC může být dán i přímým kontaktem s cílovými buňkami, důležitou molekulou exprimovanou MSC je například PD-L1 (English et al., 2007).

IDO

IDO je intracelulární enzym obsahující hemovou skupinu a katalyzující přeměnu tryptofanu na kynurenin. Jelikož tryptofan představuje esenciální aminokyselinu vyžadovanou pro proliferaci lymfocytů, může jeho vyčerpání z prostředí a hromadění jeho metabolitů významně ovlivnit průběh imunitní odpovědi. Tento enzym není MSC produkován konstitutivně, jeho expresi však lze vyvolat stimulací interferonem-γ (interferon – IFN) (Ryan et al., 2007) nebo ligandy receptorů ze skupiny Toll 3 (Toll-like receptors – TLR) (Waterman et al., 2010).

Aktivita IDO ovlivňuje řadu aspektů imunitní reakce včetně indukce protizánětlivých M2 makrofágů, tolerogenních DC a regulačních T lymfocytů (regulatory T cells – Treg) (Ge et al., 2010; Francois et al., 2012). IDO rovněž inhibuje proliferaci T lymfocytů a NK buněk a potlačuje vývoj pomocných T lymfocytů 17 (helper T cells – Th) (Ryan et al., 2007; Tatara et al., 2011, Li et al., 2014).

Přidání IDO inhibitoru nebo použití MSC z $IDO^{-/-}$ myší částečně obnovilo zablokovanou proliferaci a funkce leukocytů a potvrdilo tak důležitou, avšak ne nezastupitelnou roli tohoto imunomodulačního enzymu v působení MSC. Zdá se, že podstatou působení IDO je spíše efekt lokální akumulace metabolitů tryptofanu než vyčerpání tryptofanu samo o sobě (Ryan et al., 2007).

Prostaglandin E2

Prostaglandin E2 (prostaglandine E – PGE) je krátkodobě působící malá lipidová molekula, jejíž syntéza z kyseliny arachidonové zahrnuje aktivitu enzymů COX a prostaglandin syntázy. COX se v organizmu vyskytuje ve dvou formách jako COX-1 a COX-2. MSC konstitutivně produkují obě formy, expresi a aktivitu COX-2 lze navíc posílit stimulací MSC pomocí faktoru nekrotizujícího nádory-α (tumor

necrosis factor – TNF) a IFN-γ (English et al., 2007) nebo ligandů TLR3 (Waterman et al., 2010).

PGE-2 produkovaný MSC ovlivňuje aktivaci a proliferace T lymfocytů (English et al., 2007), makrofágů (Maggini et al., 2010), DC (Spaggiari et al., 2009), žírných buněk (Brown et al., 2011), NK buněk (Spaggiari et al., 2008), B lymfocytů (Ji et al., 2012) a inhibuje diferenciaci v Th17 lymfocyty (Duffy et al., 2011).

TGF-β

TGF-β je cytokin ovlivňující proliferaci, diferenciaci i jiné děje u řady buněk imunitního sytému i mimo něj. Zároveň TGF-β hraje klíčovou roli v diferenci $CD4^+$ T lymfocytů v Treg lymfocyty (Zhou et al., 2008). TGF-β patří k faktorům, které jsou MSC produkovány konstitutivně, a zesílení jeho produkce lze docílit působením exogenního TGF-β nebo IL-10.

Nedávno bylo zjištěno, že MSC mohou podporovat vývoj Treg lymfocytů dvěma různými dráhami. První z nich představuje přímý efekt TGF-β produkovaného MSC, druhá pak působí přes indukci diferenciace monocytů v M2 makrofágy, které produkují CC-chemokinový ligand 18 a ten indukuje diferenciaci Treg lymfocytů (Melief et al., 2013). MSC také přes kombinované působení PGE-2, IDO a TGF-β snižují expresi aktivačního receptoru NKG2D (natural killer group 2, member D) u Tc lymfocytů, což vede k utlumení jejich proliferace (Li et al., 2014).

TSG-6

TSG-6 je multifunkční, systémově působící protein s protizánětlivými účinky, jehož produkci lze výrazně zesílit, pokud jsou MSC vystaveny zánětlivému prostředí. TSG-6 umožňuje systémově podaným MSC působit i na dálku, bez nutnosti migrace přímo do poškozené oblasti.

Systémové působení TSG-6 produkovaného MSC tlumilo zánět u modelu poškozené rohovky a těžkých popálenin kůže (Roddy et al., 2011; Liu et al., 2014). Podobně tomu bylo i u modelu alogenní transplantace rohovky, kde intravenózně podané MSC zachycené v plicích působily přes produkci TSG-6 potlačení časného zánětu a snižovaly aktivaci buněk prezentujících antigeny (antigen-presenting cells – APC) v transplantované rohovce a spádových lymfatických uzlinách, což vedlo k oddálení rejekce a prodlouženému přežití transplantátu (Oh et al., 2012).

Oxid dusnatý

Oxid dusnatý (nitric oxide – NO) je rychle difundující bioaktivní molekula, která může interagovat s různými enzymy, iontovými kanály a receptory. NO je produkován NOS, které se vyskytují v organizmu ve 3 formách a jsou v lidském i myším genomu kódovány 3 různými geny. Jedná se o neuronální nNOS, endoteliální eNOS a inducibilní iNOS, která je důležitá především z imunologického hlediska. iNOS je spojena s funkcí makrofágů a bylo prokázáno, že produkované NO může ovlivňovat signalizaci přes receptor T lymfocytů a expresi receptorů pro cytokiny (Niedbala et al., 2006).

Na molekulární úrovni dochází pod vlivem NO k potlačení fosforylace STAT5 (signal transducer and activator of transcription), což je transkripční faktor rozhodující o aktivaci a proliferaci T lymfocytů. Použití inhibitoru iNOS nebo prostaglandin syntázy vedlo k obnově proliferace T lymfocytů, což svědčí o důležité roli NO v imunomodulačním působení MSC (Sato et al., 2007).

Stimulace pomocí IFN-γ a TNF-α nebo IL-1 vyvolává u MSC expresi iNOS a různých chemokinů. Je pravděpodobné, že úkolem produkovaných chemokinů je řídit migraci T lymfocytů do blízkosti MSC, což následně umožní, aby nestabilní a pouze lokálně působící NO ovlivnilo T lymfocyty a inhibovalo jejich proliferaci, případně u nich navodilo apoptózu (Ren et al., 2008). Podle nejnovějších výsledků může být navíc exprese iNOS vyvolaná u MSC působením IFN-γ a TNF-α výrazně posílena přítomností IL-17 (Han et al., 2014).

HO-1

HO jsou intracelulární enzymy schopné odbourávat hem na biliverdin a oxid uhelnatý. Existují 3 izomery HO, avšak pouze HO-1 není exprimována konstitutivně, ale musí být indukována působením prozánětlivých cytokinů nebo lipopolysacharidu (lipopolysaccharide - LPS). (Pae et al., 2004).

Ukázalo se, že tento účinný imunosupresivní enzym se zřejmě spolu s dalšími faktory podílí na imunomodulačním působení MSC. Bylo zjištěno, že MSC schopné inhibovat proliferaci T lymfocytů exprimují HO-1 a iNOS a pouze současná inhibice obou těchto enzymů měla za následek zrušení efektu MSC a obnovení lymfocytární proliferace. U modelu alogenní transplantace srdce podání MSC oddálilo rejekci

transplantátu a inhibice iNOS nebo HO-1 naopak navozovala rejekci (Chabannes et al., 2007).

IL-6

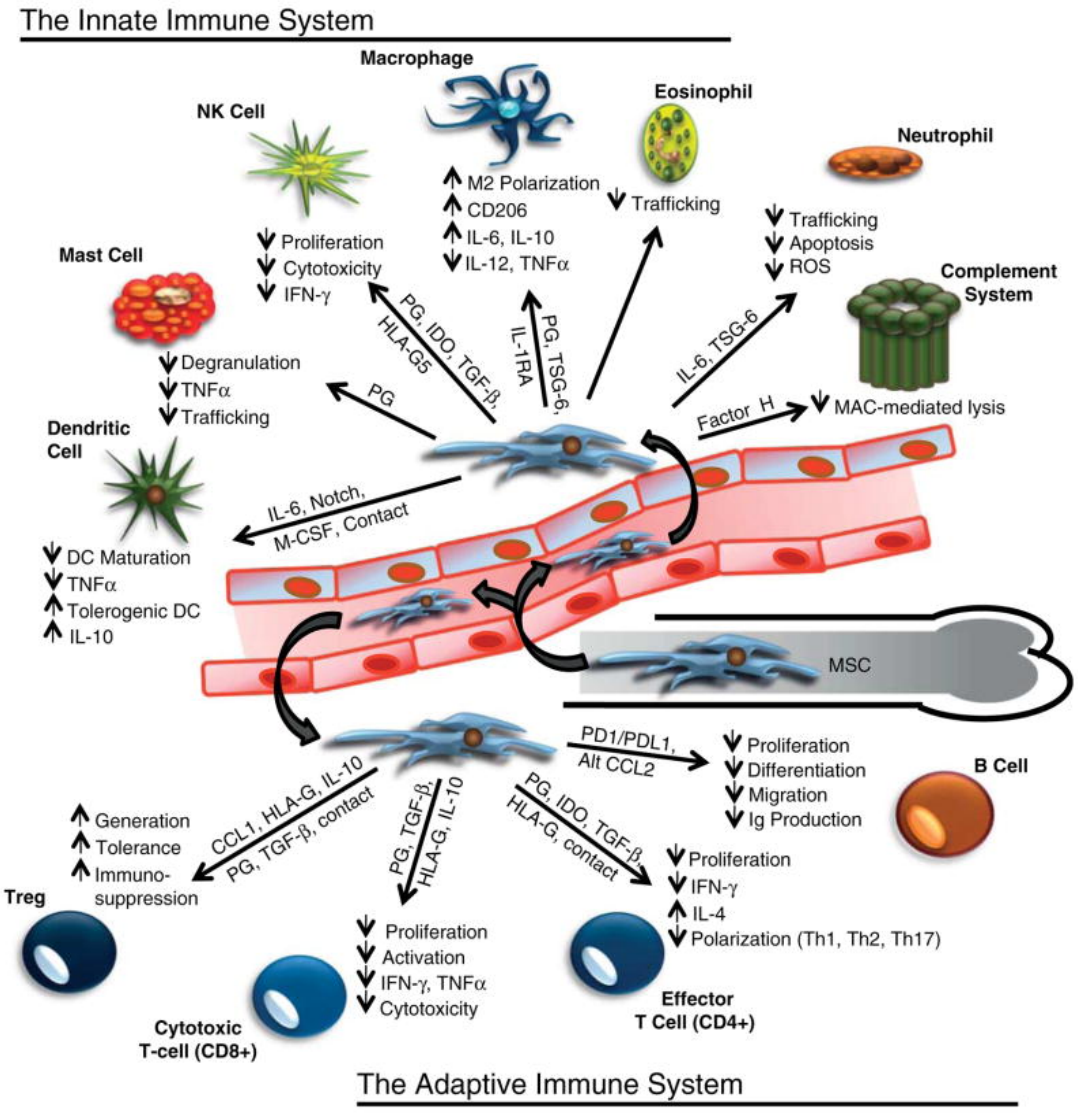
IL-6 je převážně prozánětlivý cytokin zapojený v celé řadě imunitních dějů. Bylo zjištěno, že MSC, které inhibují proliferaci T lymfocytů, produkují vysoké hladiny IL-6. Inhibice působení IL-6 nebo PGE-2 vedla k částečné obnově lymfocytární proliferace. Navíc byla zjištěna snížená exprese glykoproteinů hlavního histokompatibliního komplexu II. třídy (major histocompatibility complex glycoprotein – MHC gp), CD40 a CD86 na zralých DC, což mohlo být rovněž důvodem omezené proliferace T lymfocytů. MSC mohou také prostřednictvím IL-6 částečně inhibovat diferenciaci progenitorových buněk kostní dřeně v DC (Djouad et al., 2007).

HGF

HGF je multifunkční cytokin zapojený v angiogenezi, růstu a pohybu buněk. Je to také účinný imunomodulační faktor, který inhibuje funkce DC a podporuje diferenciaci v Treg lymfocyty na úkor Th17 lymfocytů (Benkhoucha et al., 2010).
MSC mohou prostřednictvím HGF působit na monocyty a navozovat u nich imunomodulační fenotyp, vyznačující se produkcí vysokých hladin IL-10. Tyto monocyty zůstávají nediferencované a jsou schopny potlačovat efektorové funkce T lymfocytů a měnit cytokinový profil T lymfocytů z Th1 na Th2 (Chen et al., 2014).

2.3.3.2 Interakce MSC s jednotlivými složkami imunitního systému

MSC disponují rozsáhlými imunomodulačními schopnostmi a byl popsán jejich možný vliv na prakticky všechny komponenty imunitního systému. Prostřednictvím produkce celé řady cytokinů, faktorů a imunomodulačních molekul jsou schopny ovlivnit aktivaci, migraci, zrání, produkci cytokinů, expresi povrchových molekul, proliferaci, apoptózu a efektorové funkce řady leukocytů. V mnoha případech je efekt MSC zprostředkován kombinovaným působením více faktorů a může být modulována aktivita a funkce více komponent imunitního systému současně (Obr. 2).



Obr. 2: Imunomodulační působení MSC na přirozenou i adaptivní složku imunitního systému. MSC mohou v reakci na zánětlivou stimulaci vycestovat z kostní dřeně a spolu s MSC přítomnými ve tkáni významně ovlivnit celkovou rovnováhu probíhající imunitní odpovědi. Účinek MSC je dán jednak přímým mezibuněčným kontaktem, jednak produkcí množství parakrinně působících faktorů, které ovlivňují aktivitu a funkce řady leukocytů. PG – prostaglandiny, TSG-6 – TNF-α stimulated gene/protein 6, PD1 – programmed death pathway-1. Převzato z Brennen et al., 2013.

Neutrofily

Neutrofily představují důležitou složku přirozené imunity. Na počátku bakteriální infekce jsou rychle mobilizovány a podílí se velmi účinně na likvidaci mikroorganizmů.

Řada tkání obsahuje tkáňově specifické MSC, které jsou pravděpodobně lokalizované převážně v periendoteliální a perivaskulární oblasti. Zajímavá je v této souvislosti teorie, že by tyto rezidentní MSC mohly hrát významnou roli v imunitní reakci na časnou fázi bakteriální infekce, kdy ještě není přítomna klasická imunitní

odpověď. Jako reakci na setkání s mikroorganizmy a stimulaci přes TLR mohou MSC přilákat z periferní krve neutrofily a posilovat jejich antimikrobiální aktivitu. Tuto teorii podporují i zjištění, že MSC stimulované LPS exprimují vyšší hladiny IL-8, chemokinových receptorů a prozánětlivých cytokinů. Atrahované aktivované neutrofily vykazují prodloužený poločas života, zvýšenou expresi zánětlivých chemokinů a posílenou odpovídavost na následnou stimulaci LPS (Brandau et al., 2010).

Jiná studie se zaměřila na vliv MSC na neutrofily v kostní dřeni, která slouží jako rezervoár zralých neproliferujících neutrofilů. MSC pravděpodobně inhibují apoptózu klidových i aktivovaných neutrofilů a tlumí jejich respirační vzplanutí. Antiapoptotická aktivita MSC spočívala v působení IL-6 a signalizaci přes transkripční faktor STAT-3. Naproti tomu MSC neovlivňovaly fagocytózu, chemotaxi ani expresi adhezivních molekul u neutrofilů. MSC tedy v kostní dřeni zřejmě chrání rezidentní populaci neutrofilů před apoptózou a současně zachovávají jejich efektorové funkce a brání nežádoucí aktivaci oxidativního metabolizmu (Raffaghello et al., 2008; Cassatella et al., 2011).

Makrofágy

MSC jsou schopny přes produkci PGE-2 navozovat u makrofágů regulační fenotyp, vyznačující se sníženou produkcí prozánětlivých cytokinů IL-6, TNF-α a IFN-γ a zvýšenou schopností fagocytovat apoptotické buňky. Zároveň MSC potlačují u stimulovaných makrofágů zvýšení exprese CD86 a MHC gp II, což narušuje jejich schopnost aktivovat $CD4^+$ T lymfocyty (Maggini et al., 2010).

Podle nejnovějších studií mohou MSC také řídit diferenciaci monocytů v $CD206^+$ M2 makrofágy, produkující velká množství IL-10 (Melief et al., 2013) a indukovat regulační M2 fenotyp i u mikrogliových buněk z centrální nervové soustavy (Hegyi et al., 2014).

NK buňky

MSC inhibují proliferaci klidových NK buněk stimulovaných pomocí IL-2, zatímco proliferaci aktivovaných NK buněk ovlivňují pouze částečně. Aktivované NK buňky navíc mohou lyzovat autologní i alogenní MSC, ale tato schopnost byla inhibovaná v případě, že byly použity MSC ovlivněné IFN-γ, které na svém povrchu exprimovaly vyšší množství MHC gp I (Spaggiari et al., 2006).

MSC rovněž brání nástupu efektorových funkcí NK buněk včetně jejich cytotoxické aktivity a produkce cytokinů. Imunomodulační efekt MSC na NK buňky spočívá především v aktivitě IDO a produkci PGE-2 (Spaggiari et al., 2008).

Některé studie přinesly důkazy o tom, že MSC mohou mít nežádoucí účinek na protinádorovou imunitu, který je spojen především se sníženou cytotoxickou aktivitou NK buněk a Tc lymfocytů, nižším podílem NK-T lymfocytů a vyšším počtem Treg lymfocytů (Ljujic et al., 2013).

DC

DC jsou nejúčinnější APC a hrají klíčovou roli v zahájení buněčné imunity. MSC u DC inhibují proces dozrávání, potlačují u nich schopnost podporovat proliferaci $CD4^+$ T lymfocytů, schopnost vystavovat peptidy v komplexu s MHC gp II a snižují expresi některých chemokinových receptorů, čímž ovlivňují jejich migraci (English et al., 2008).

U zralých DC MSC ovlivňují prostřednictvím PGE-2 zejména produkci cytokinů. U myeloidních DC dochází pod vlivem MSC ke snížení produkce prozánětlivého TNF-α, naopak v případě plazmacytoidních DC dochází ke zvýšení produkce protizánětlivého IL-10 (Aggarwal a Pittenger, 2005).

Stejně jako u makrofágů mohou MSC i u DC indukovat přes produkci PGE-2 regulační fenotyp. Tyto regulační DC produkují TGF-β vyvolávající vývoj Treg lymfocytů (Zhang et al., 2014).

Žírné buňky

Žírné buňky hrají klíčovou roli při rozvoji alergického zánětu a rovněž se podílí na patogenezi některých autoimunitních onemocnění včetně revmatoidní artritidy. Bylo zjištěno, že MSC účinně potlačují řadu funkcí žírných buněk *in vitro* i *in vivo* a mohly by se tedy stát základem nové léčebné strategie v terapii onemocnění, v jejichž patogenezi jsou žírné buňky zapojeny. MSC přes aktivitu COX-2 potlačují u žírných buněk degranulaci, produkci prozánětlivého TNF-α i jejich chemotaxi (Brown et al., 2011).

T lymfocyty

MSC mají schopnost ovlivňovat proliferaci i efektorové funkce T lymfocytů včetně produkce cytokinů a aktivity cytotoxických T lymfocytů (cytotoxic T cells – Tc). Efekt MSC však nemusí být ve všech případech výsledkem pouze přímého supresivního působení na T lymfocyty, ale může vycházet například i z inhibičního působení MSC na zrání a aktivaci DC, následkem čehož může být omezena efektivita prezentace antigenů T lymfocytům a jejich následná klonální expanze. Důsledkem snížené aktivace $CD4^+$ T lymfocytů pak může dojít k neúčinné pomoci Th lymfocytů B lymfocytům a k jejich následné snížené proliferaci a omezené produkci protilátek.

Závěry týkající se působení MSC na časnou fázi aktivace T lymfocytů a expresi molekul CD25 a CD69 jsou poněkud rozporuplné. Někteří autoři zjistili, že v přítomnosti MSC dochází ke snížení exprese znaků časné aktivace T lymfocytů (Le Blanc et al., 2004; Liu et al., 2007), jiné práce naopak hovoří o tom, že přítomnost MSC neovlivňuje, nebo dokonce posiluje expresi molekul CD25 a CD69 (Glennie et al., 2005; Ramasamy et al., 2008; Kronsteiner et al., 2011).

Dále bylo zjištěno, že MSC inhibují proliferaci $CD4^+$ i $CD8^+$ T lymfocytů, přičemž tento efekt pravděpodobně nespočívá v indukci apoptózy, ale je důsledkem zastavení buněčného cyklu T lymfocytů v G1 fázi. Po restimulaci a odstranění MSC došlo u T lymfocytů k obnově produkce IFN-γ, ale nikoliv proliferace. Zdá se tedy, že MSC indukují u T lymfocytů stav neodpovídavosti obdobný anergii T lymfocytů *in vivo* (Di Nicola et al., 2002; Glennie et al., 2005; Ramasamy et al., 2008).

Působení MSC na T lymfocyty je zřejmě zprostředkováno mezibuněčným kontaktem i produkcí imunomodulačních molekul včetně PGE-2 (Aggarwal a Pittenger, 2005), TGF-β, HGF (Di Nicola et al., 2002) a aktivity IDO (Li et al., 2014).

Tc lymfocyty

$CD8^+$ Tc lymfocyty rozeznávají antigeny odvozené od cytoplazmatických peptidů v komplexu s MHC gp I, a jsou tedy klíčové pro likvidaci nádorových buněk a buněk infikovaných viry.

Pokud jsou MSC přidány do směsné lymfocytární kultury na jejím počátku, pak jsou schopny potlačit vývoj Tc lymfocytů. Míra lyze cílových buněk však není ovlivněna, pokud jsou MSC přidány do kultury až v cytotoxické fázi. Zdá se tedy, že MSC nejsou lýzovány Tc lymfocyty, jsou schopny prostřednictvím solubilních faktorů

inhibovat vývoj Tc lymfocytů, ale nejsou schopny potlačit jejich cytotoxicitu (Rasmusson et al., 2003).

Podle jiné práce mohou MSC přes indukci tolerogenních monocytů navozovat u Tc lymfocytů supresivní fenotyp spojený se sníženou expresí CD8, CD28 a CD44 a poklesem produkce IFN-γ a granzymu B. Tolerogenní fenotyp u monocytů byl provázený poklesem exprese kostimulačních molekul CD80 a CD86 a byl indukován solubilními faktory produkovanými MSC, zatímco efekt na Tc lymfocyty byl podmíněn jejich přímým kontaktem s tolerogenními monocyty (Hof-Nahor et al., 2012).

Th lymfocyty

MSC mají rovněž schopnost ovlivňovat produkci cytokinů u T lymfocyty a posouvat cytokinovou rovnováhu imunitní reakce od prozánětlivé k protizánětlivé. V přítomnosti MSC dochází ke snížení produkce IFN-γ Th1 lymfocyty a k posunu rovnováhy ve směru vývoje Th2 lymfocytů a zvýšené produkce IL-4 (Aggarwal a Pittenger, 2005).

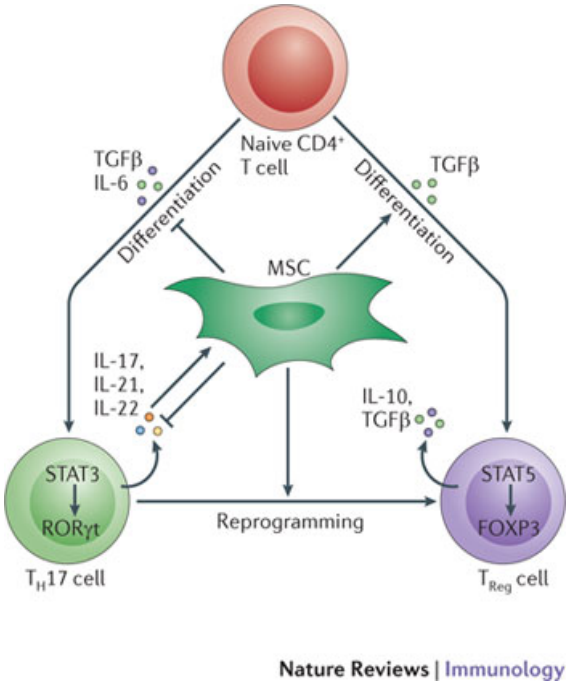
Posílení produkce Th2 cytokinů na úkor produkce Th1 cytokinů po podání MSC bylo sledováno i u mnoha *in vivo* modelů včetně exprimentální autoimunitní uveoretinitidy (experimental autoimmune uveoretinitis – EAU) (Zhang et al., 2011), alogenní transplantace rohovky (Jia et al., 2012), amyotrofické laterální sklerózy (Kwon et al., 2014) a mnoha dalších.

Treg lymfocyty a Th17 lymfocyty

Th17 lymfocyty chrání organizmus před extracelulárními patogeny a hrají klíčovou roli v některých zánětlivých onemocněních včetně roztroušené sklerózy a revmatoidní artritidy. Th17 lymfocyty mohou produkovat IL-17A, IL-17F, TNF-α, IL-1β, IL-23 i další cytokiny a jejich vývoj je indukován společným působením TGF-β a IL-6 (Wilson et al., 2007).

MSC brání prostřednictvím PGE-2 diferenciaci naivních $CD4^+$ T lymfocytů v Th17 lymfocyty a zároveň potlačují produkci IL-17, IL-22, IFN-γ a TNF-α u již diferencovaných Th17 buněk. V zánětlivém prostředí mohou MSC působit na Th17 lymfocyty a indukovat u nich fenotyp Treg lymfocytů (Obr. 3). Na molekulární úrovni vede působení MSC k epigenetickým změnám u Th17 lymfocytů projevujícím se zvýšenou expresí transkripčního faktoru Foxp3 (forkhead box p3) a navozením

schopnosti inhibovat proliferaci aktivovaných $CD4^+$ T lymfocytů (Ghannam et al., 2010).



Obr. 3: Vliv MSC na vývoj Treg a Th17 lymfocytů. MSC inhibují vznik Th17 lymfocytů a podporují přeprogramování diferencovaných Th17 lymfocytů na Treg lymfocyty. Naivní $CD4^+$ T lymfocyty mohou diferencovat pod vlivem TGF-β a IL-6 v prozánětlivé Th17 lymfocyty. Tento proces spočívá v aktivaci STAT3 (signal transducer and activator of transcription 3) a zvýšení exprese RORγt (retinoic acid receptor-related orphan receptor-γt). Naproti tomu, TGF-β podporuje tvorbu imunosupresivních $Foxp3^+$ (forkhead box P3) Treg lymfocytů přes aktivaci STAT5. MSC exprimují vysoká množství receptoru pro IL-17, který podporuje jejich proliferaci a přežívání, což může fungovat jako negativní zpětnovazebná smyčka, v níž MSC v reakci na IL-17 potlačují diferenciaci naivních $CD4^+$ T lymfocytů v další Th17 lymfocyty. MSC navíc přímo podporují vývoj Treg lymfocytů, inhibují produkci prozánětlivých cytokinů Th17 lymfocyty a také řídí přeprogramování Th17 lymfocytů na Treg lymfocyty. Převzato z Le Blanc et al., 2012.

Kultivace Th17 lymfocytů s MSC vede ke snížení exprese CD25 a produkce IL-17A. Tyto účinky byly zmírněny v případě, že bylo zabráněno mezibuněčnému kontaktu. K úplnému zablokování tohoto efektu pak vedlo použití selektivního inhibitoru COX-2. MSC rovněž inhibovaly funkce přirozeně se vyskytujících Th17 lymfocytů získaných z oblasti zánětu u modelu obstrukce močových cest (Duffy et al., 2011).

Treg lymfocyty představují specializovanou populaci T lymfocytů, jejímž úkolem je tlumit nežádoucí imunitní reakce a zajišťovat toleranci vůči vlastním antigenům. Fenotyp Treg lymfocytů je asociován s vysokou expresí transkripčního faktoru Foxp3, a naopak nízkou expresí CD127, což je spojeno s nástupem jejich regulační aktivity (Liu et al., 2006).

MSC svým působením navozují, regulují a udržují fenotyp a funkce Treg lymfocytů. MSC působí nárůst podílu $CD4^+CD25^{bright}Foxp3^+$ buněk majících zároveň sníženou expresi CD127. Treg lymfocyty kultivované v přítomnosti MSC po dobu 15 dnů si zachovávají své supresivní schopnosti, naopak nepřítomnost MSC v kultuře vede po 5 dnech ke ztrátě všech supresivních vlastností Treg lymfocytů (Di Iani et al., 2008).

Indukci Treg lymfocytů zajišťuje působení PGE-2 a TGF-β produkovaných MSC. Nezastupitelná role těchto dvou molekul byla potvrzena prostřednictvím inhibitoru a neutralizační protilátky, jejichž přidání vedlo k vymizení pozorovaného efektu (English et al., 2009; Melief et al., 2013).

B lymfocyty

Odpověď B lymfocytů je do značné míry závislá na kooperaci s Th lymfocyty. Vedle přímého působení MSC na B lymfocyty tedy může výsledný efekt souviset i s MSC zprostředkovanou inhibicí funkcí T lymfocytů.

MSC mají schopnost inhibovat proliferaci B lymfocytů a zdá se, že hlavním mechanizmem není indukce apoptózy, ale spíše zastavení buněčného cyklu B lymfocytů v G0/G1 fázi. MSC rovněž inhibují diferenciaci B lymfocytů v plazmatické buňky, což je spojeno s výrazným snížením produkce imunoglobulinu M (immunoglobulin – Ig), IgG a IgA. Ovlivněny jsou také chemotaktické vlastnosti B lymfocytů, které na svém povrchu exprimují méně chemokinových receptorů, což vede ke snížení chemotaxe. Naopak, exprese kostimulačních molekul a produkce cytokinů nebyla působením MSC ovlivněna (Corcione et al., 2006).

Ukázalo se, že pro inhibici proliferace a diferenciace B lymfocytů zprostředkovanou působením MSC je klíčová jednak přítomnost $CD4^+$ i $CD8^+$ T lymfocytů, jednak přímý kontakt mezi MSC a T lymfocyty, naopak kontakt MSC s B lymfocyty vyžadován není (Rosado et al., 2014).

Regulační B lymfocyty

Vliv MSC na vývoj Treg lymfocytů i dalších populací leukocytů se supresivním fenotypem je již poměrně dobře zdokumentován. Teprve nedávno však bylo imunomodulační působení MSC rozšířeno o populaci regulačních B lymfocytů (regulatory B cells – Breg), která se podobně jako Treg lymfocyty podílí na supresi

imunitní odpovědi, přičemž stěžejním mechanizmem jejich působení je produkce IL-10.

V případě klinické studie zaměřené na léčbu chronické GVHD vedla infuze MSC ke klinickému zlepšení, které bylo spojeno s nárůstem počtu $CD5^+$ Breg lymfocytů produkujících IL-10 a nižšími hladinami prozánětlivých cytokinů. MSC pravděpodobně podporují přežití a proliferaci $CD5^+$ Breg lymfocytů prostřednictvím aktivity IDO (Peng et al., 2014).

V jiné práci podání dvou dávek MSC potlačovalo průběh experimentální autoimunitní encefalomyelitidy. Léčebný efekt MSC souvisel zejména se snížením počtu Th17 buněk a posílením aktivity $CD1d^{high}CD5^+$ Breg lymfocytů. Zároveň došlo k poklesu počtu zánětlivých infiltrátů a k omezení demyelinizace míchy, což bylo spojeno s nižšími sérovými hladinami IL-6, TNF-α a IL-17 (Guo et al., 2013).

2.3.3.3 **Vliv prostředí na imunomodulační schopnosti MSC**

V souvislosti s výzkumem imunomodulačních schopností MSC existují četné studie poukazující na fakt, že MSC nedisponují svými supresivními schopnostmi konstitutivně, ale že je získávají až na základě působení zánětlivého prostředí. Zdá se, že MSC jsou vnímavé k signálům produkovaným zanícenou tkání a jsou schopny v reakci na tyto signály migrovat do daného místa a postupně rozvinout strategii zajišťující potlačení nežádoucí zánětlivé odpovědi, případně podpořit regeneraci poškozených tkání.

2.3.3.3.1 **Vliv prozánětlivých cytokinů na imunomodulační schopnosti MSC**

Podle některých prací je pro navození imunomodulační aktivity MSC klíčové působení prozánětlivých cytokinů – především IFN-γ, TNF-α a IL-1β (Krampera et al., 2006; Ren et al., 2008; Ren et al., 2009).

Prvním cytokinem studovaným v souvislosti s ovlivněním imunomodulačních schopností MSC byl IFN-γ, kdy Krampera et al. (2006) zkoumali supresivní aktivitu MSC podmíněnou přítomností IFN-γ produkovaného aktivovanými T lymfocyty a NK buňkami. Přidání neutralizační protilátky proti receptoru pro IFN-γ zcela zablokovalo supresivní účinky MSC na $CD4^+$ lymfocyty a částečně i na $CD8^+$ lymfocyty

(Krampera et al., 2006). Účinky IFN-γ byly potvrzeny *in vivo* na myším modelu GVHD, kde byly MSC stimulované IFN-γ efektivnější v potlačení reakce dárcovských T lymfocytů proti tkáním příjemce (Polchert et al., 2008).

Rovněž bylo zjištěno, že důležitou roli hraje v tomto ohledu i koncentrace IFN-γ, případně současná přítomnost dalších prozánětlivých cytokinů. IFN-γ samostatně nebo kombinované synergické působení IFN-γ s TNF-α nebo IL-1β navozuje imunosupresivní účinky MSC prostřednictvím zvýšené exprese COX-2 (English et al., 2007), IDO (Francois et al., 2011), iNOS (Romieu-Mourez et al., 2009) a superoxid dismutázy 3 (Kemp et al., 2010).

Důležitou roli hrají i hladiny IFN-γ, kterým jsou MSC vystaveny. Působení nízkých koncentrací IFN-γ zvyšuje u MSC expresi MHC gp II. To umožňuje, aby MSC v časné fázi imunitní reakce, kdy jsou hladiny IFN-γ v prostředí nízké, působily jako nekonvenční APC. Následně, v průběhu imunitní odpovědi, kdy koncentrace IFN-γ postupně stoupá, dochází ke snížení exprese MHC gp II, současné ztrátě schopnosti MSC prezentovat antigeny a k postupnému nástupu imunosupresivních schopností (Chan et al., 2006; Stagg et al., 2006).

MSC mohou navíc stejně jako DC zkříženě prezentovat exogenní antigeny a vyvolávat tak reakci $CD8^+$ T lymfocytů (Francois et al., 2009).

2.3.3.3.2 Vliv stimulace přes TLR na imunomodulační schopnosti MSC

Stimulace přes TLR může, stejně jako některé prozánětlivé cytokiny, významně ovlivnit imunomodulační vlastnosti a migraci MSC. MSC exprimují řadu povrchových i intracelulárně lokalizovaných TLR, které spouští signalizační kaskády regulující některé molekuly klíčové pro jejich imunomodulační aktivitu (Romiue-Mourez et al., 2009).

Studium vlivu stimulace MSC přes TLR však přineslo nesourodé výsledky svědčící na jednu stranu o podpoře imunosupresivních vlastností MSC, na straně druhé však o jejich prozánětlivém vlivu (Liotta et al., 2008). Tyto rozdílné závěry mohou být vysvětleny díky práci Waterman et al. (2010), která přinesla zajímavou teorii převádějící koncept polarizace makrofágů na prozánětlivých M1 a protizánětlivých M2 do oblasti MSC. Zjistili, že zatímco stimulace MSC přes TLR-4 podporuje převládnutí

prozánětlivého fenotypu spojené s produkcí TGF-β, IL-6 a IL-8, aktivace MSC přes TLR-3 indukuje protizánětlivý fenotyp vyznačující se zvýšenou expresí IL-4, IDO a COX-2 (Waterman et al., 2010). MSC by tak mohly být polarizovány v prozánětlivý či naopak protizánětlivý fenotyp na základě signalizace přes TLR, odrážející aktuální fyziologické podmínky, ve kterých se právě nachází v organizmu. Po migraci do zánětlivé tkáně mohou být stimulovány ligandy TLR-4 a přispívat k rychlé antimikrobiální odpovědi, avšak později při přetrvávajícím zánětu a poškození tkáně mohou být aktivovány přes TLR-3, utlumit nežádoucí zánět a podpořit regeneraci (Roumieu-Mourez et al., 2009).

Rovněž bylo zjištěno, že mnoho ligandů TLR podporuje migraci MSC. Ukázalo se však, že důležitý parametr je v tomto případě doba, po kterou jsou MSC ligandům vystaveny. Krátká stimulace přes TLR posilovala migrační schopnosti MSC, zatímco stimulace delší než 24 hodin tuto schopnost snižovala (Waterman et al., 2010).

Ligandy TLR i prozánětlivé cytokiny se mohou významně podílet na patogenezi některých onemocnění, u nichž je zvažována terapie pomocí MSC. Je tedy pravděpodobné, že terapeuticky podané MSC budou těmito molekulami ovlivněny. Z tohoto důvodu bude důležité načasovat podání MSC tak, aby prostředí v organizmu nemocného navodilo u MSC supresivní fenotyp.

2.3.4 **Terapeutické využití MSC**

Původním důvodem pro zvažované terapeutické využití MSC byl především jejich široký diferenciační potenciál a z toho vyplývající uplatnění v regenerativní medicíně. Později došlo k rozšíření jejich možného terapeutického potenciálu o oblasti využívající rozsáhlé imunomodulační schopnosti MSC. V současné době probíhá na základě poznatků získaných v preklinických testech více než 300 klinických studií sledujících efekt MSC na průběh a léčbu nejrůznějších onemocnění. V oblasti regenerativní medicíny se jedná především o studie využívající MSC pro léčbu poškození chrupavek a šlach, roztříštěných zlomenin kostí, osteogenesis imperfecta, osteoartritidy, těžkých poranění míchy, amyotrofické laterální sklerózy, infarktu, mrtvice, popálenin, jaterní cirhózy, retinitis pigmentosa, deficitu LSC, Duchenovy muskulární dystrofie, plicní fibrózy, rozštěpu rtu a patra a mnoha dalších. V oblasti

hematologie a transplantací kostní dřeně je to především léčba nedostatečné funkce štěpu po alogenní transplantaci HSC, akutní a chronické GVHD a těžké aplastické anémie. V případě autoimunitních onemocnění se již testuje terapie pomocí MSC u pacientů trpících ulcerózní kolitidou, diabetem mellitus, roztroušenou sklerózou, revmatoidní artritidou, Crohnovou chorobou a systémovou sklerodermií (čerpáno z www.clinicaltrials.gov).

Podstatou terapeutického účinku MSC v oblasti regenerativní medicíny není v řadě případů pouze diferenciace MSC v potřebný buněčný typ, ale také tvorba optimálního prostředí podporujícího proces hojení. MSC produkují celou řadu faktorů a molekul, které inhibují apoptózu, čímž minimalizují rozsah poškození, potlačují fibrózu a tvorbu jizev v postižené oblasti, modulují imunitní reakci a omezují tak nežádoucí zánětlivé poškození tkáně, stimulují angiogenezi a tím zajišťují tvorbu nového krevního zásobení a také podporují proliferaci tkáňově specifických progenitorových buněk (Caplan a Dennis, 2006).

V případě buněčné terapie pomocí MSC je velkou výhodou především jejich relativně snadná izolace a kultivace. Další výhodou je schopnost MSC selektivně migrovat do místa poškození. U myšího modelu alogenní transplantace srdce byly intravenózně podané MSC nalezeny v transplantátu a lymfoidních orgánech příjemce, kde se podílely na snížení reakce proti alogennímu štěpu (Ge et al., 2009).

Stále však existuje mnoho nevyjasněných otázek týkajících optimálního dávkování, načasování a způsobu podání testovaných MSC. Vzhledem k možné plasticitě imunomodulačního působení MSC bude klíčové, aby se jim po podání do organizmu pacienta dostalo optimální stimulace přes TLR a prozánětlivé cytokiny, která u nich povede k navození supresivního fenotypu.

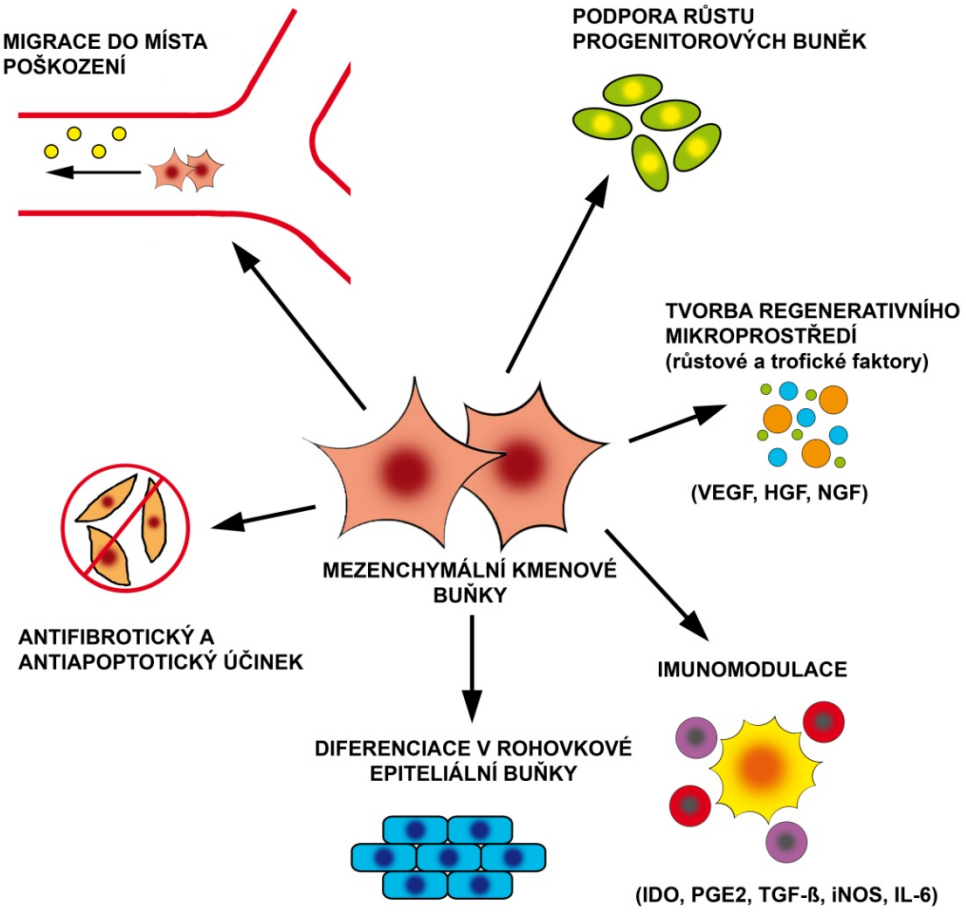
S terapií pomocí MSC jsou však spojená i jistá rizika, mezi něž patří především obavy z účinků MSC na růst nádorů a na protinádorovou imunitu, z tvorby nežádoucího buněčného typu *in vivo* a z nespecifické modulace imunitního systému, která může vést ke snížení obranyschopnosti vůči běžným patogenům.

2.3.4.1 Využití MSC v léčbě poškozeného povrchu oka

Poškození rohovkového epitelu může být spojeno s intenzivním zánětem, neprůhledností a neovaskularizací rohovky a s epiteliálními defekty, které mohou vyústit až v nevratnou ztrátu zraku. Již 24 hodin po poškození dochází k masivní infiltraci rohovky leukocyty. Během 48 hodin dojde k několikanásobnému nárůstu počtu makrofágů infiltrujících poškozenou tkáň, což je doprovázeno postupným zvyšováním počtu lymfocytů (O´Brien et al., 1998).

Konvenční léčba akutní fáze rohovkového zánětu v současnosti spočívá v potlačení zánětu a neovaskularizace prostřednictvím podávání protizánětlivých léků. V pozdější fázi pak může být provedena transplantace epiteliálních vrstev expandovaných z malého limbálního štěpu na různých podkladech. Omezená dostupnost LSC vhodných pro transplantaci a riziko rejekce při alogenní transplantaci vedlo k hledání jiných buněk vhodných pro tento účel. MSC byly vybrány pro svou schopnost diferenciace v epiteliální buňky a pro jejich imunomodulační vlastnosti nabízející možnost potlačit nežádoucí zánětlivou imunitní odpověď v poškozené rohovce. Modelové působení MSC při léčbě poškozené rohovky ukazuje Obr. 4.

Lan et al. (2012) sledovali účinek rohovkového poškození na mobilizaci endogenních MSC z kostní dřeně. Během 48 hodin došlo ke zdvojnásobení počtu cirkulujících MSC, které pravděpodobně souviselo se zvýšením hladiny chemokinů v rohovce i v krvi. Následné podání terapeutické dávky dárcovských MSC vedlo k rychlé regeneraci rohovky spojené s cílenou migrací podaných MSC do místa poškození (Lan et al., 2012). V jiné práci byly použity MSC předem diferencované v rohovkový epitel, které byly pomocí amniové membrány přeneseny na poškozenou rohovku, kde již 4 týdny po aplikaci snižovaly její neovaskularizaci a neprůhlednost (Jiang et al., 2010).



Obr. 4: Možné mechanizmy působení MSC při léčbě poškozeného rohovkového epitelu. Terapeutický účinek MSC souvisí s jejich schopností selektivní migrace do místa poškození, podporou růstu progenitorových buněk sídlících v rohovkovém epitelu, tvorbou regenerativního mikroprostředí prostřednictvím produkce růstových faktorů a imunomodulačních molekul, antifibrotickým a antiapoptotickým účinkem a možnou diferenciací v rohovkové epiteliální buňky.

V případě lidských MSC kultivovaných na amniové membráně, které byly použity v krysím modelu poškozené rohovky, terapeutický účinek spočíval spíše v potlačení zánětu a angiogeneze než v diferenciaci MSC v epiteliální buňky. V poškozených rohovkách léčených MSC došlo v porovnání s neléčenými kontrolami k významnému snížení počtu buněk exprimujících CD45 a IL-2 (Ma et al., 2006). U léčby pomocí MSC podaných injekčně pod spojivku došlo k významnému posílení obnovy rohovkového epitelu, snížení množství $CD68^{+}$ myeloidních buněk infiltrujících poškozenou rohovku a poklesu exprese TNF-α (Yao et al., 2012). V jiné práci byly MSC nanášeny na poškozenou rohovku pomocí válcovitého plastového aplikátoru, který zabránil mrkání a umožnil MSC přichytit se na poškozenou oblast. Bylo zjištěno, že jak MSC, tak částečně i opakované nanesení kondiciovaného média z kultur MSC

potlačovalo zánět a neovaskularizaci rohovky za současného snížení exprese IL-2 a IFN-γ, zvýšení exprese IL-10, TGF-β a IL-6 a omezení infiltrace rohovky $CD4^+$ buňkami (Oh et al., 2008).

Zajímavé výsledky přinesly i práce studující možnost využití imunomodulačního působení systémově podaných krysích MSC pro léčbu EAU. U zvířat léčených MSC došlo k zmírnění poškození sítnice, což bylo spojeno s poklesem infiltrace prozánětlivými buňkami a zvýšením počtu Treg lymfocytů. T lymfocyty získané ze zvířat léčených MSC produkovaly méně IFN-γ a IL-17 a více IL-10 (Zhang et al., 2011). Li et al. (2013) zjistili, že po podání MSC došlo k potlačení produkce Th1 a Th17 cytokinů, k současnému posílení produkce Th2 a Treg cytokinů a snížení množství IL-17 v komorové tekutině (Li et al., 2013a).

Další práce sledovala imunomodulační účinky systémově podaných MSC u modelu transplantace alogenní rohovky. Pooperační aplikace MSC prodloužila přežití transplantátu, došlo k inhibici proliferace zánětlivých T lymfocytů, ke snížení produkce Th1 cytokinů, posílení produkce Th2 cytokinů a zvýšení počtu Treg lymfocytů (Jia et al., 2012). Podobně tomu bylo i v další práci, kde intravenózně podané MSC zachycené v plicích působily přes produkci TSG-6 potlačení časného zánětu a snižovaly aktivaci APC v transplantované rohovce a spádových lymfatických uzlinách, což vedlo k prodlouženému přežití transplantátu (Oh et al., 2012).

2.4 **LSC**

Rohovkový epitel je obnovován pomocí malé populace LSC sídlících v bazální vrstvě limbu ve struktuře zvané Vogtovy palisády. Tato struktura poskytuje LSC unikátní mikroprostředí zajišťující jim ochranu před nežádoucími podněty, které by mohly zapříčinit jejich diferenciaci či apoptózu (Schermer et al., 1986; Gipson et al., 1989).

Pro LSC je typická malá velikost a pomalý buněčný cyklus, čehož se často využívá při jejich izolaci pomocí centrifugace na hustotním gradientu nebo na základě delšího zadržování značených DNA prekurzorů (Cotsarelis et al., 1989; Krulova et al., 2008). Doposud však nebyl nalezen jedinečný znak, který by byl exprimován pouze populací LSC. Pro jejich charakterizaci se nejčastěji využívá kombinace znaků asociovaných s kmenovými buňkami, jako jsou transkripční faktor p63 (Pellegrini et

al., 2001) a membránový transportér ABCG2 (ATP-binding cassette transporter group 2 protein) (Zhou et al., 2001; Budak et al., 2005). Současně musí být vyloučeny buňky nesoucí diferenciační znaky asociované s rohovkovým epitelem, mezi něž patří keratin 3 a 12 a konexin-43 (Chen et al., 2004).

Následkem onemocnění či poranění oka může dojít k deficitu LSC, který postupně vede k procesu konjunktivizace, při němž fenotypově odlišné buňky spojivkového epitelu penetrují rohovku a způsobují její neprůhlednost, chronické záněty a trvalé epiteliální defekty (Huang et al., 1991). V klinické praxi se při léčbě pacientů postižených deficitem LSC postupně upouští od transplantací rohovek nebo velkých limbálních štěpů a přistupuje se k transplantacím epiteliálních vrstev expandovaných z malého štěpu limbu na různých nosičích, jako je fibrinový gel (Marchini et al., 2012) nebo amniová membrána (Shahdadfar et al., 2012). V posledních letech byla ve snaze usnadnit přenos LSC při léčbě očních defektů vyvinuta řada nových biokompatibilních materiálů včetně kolagenových nosičů (Dravida et al., 2008), hydrogelů tvořených směsí chitosanu s kolagenem nebo elastinem (Grolik et al., 2012), nanovlákenných nosičů (Cejkova et al., 2014) nebo průhledné vrstvy vlasového keratinu (Feng et al., 2014). Omezená dostupnost LSC vhodných pro transplantaci a riziko rejekce při alotransplantaci vedlo výzkumné skupiny k hledání jiných buněk vhodných pro rekonstrukci nefunkčního nebo poškozeného rohovkového epitelu. Jednu z nejnadějnějších alternativ představují autologní orální slizniční epiteliální buňky (Sharma et al., 2012) nebo MSC kultivované na amniové membráně (Ma et al., 2006; Rohaina et al., 2014).

2.5 **Nanovlákenné nosiče**

V současné době je pro růst a přenos kmenových buněk k dispozici celá řada substrátů a nosičů včetně nanovláken, která v této souvislosti představují jednu z nejnadějnějších alternativ. Nejčastěji používaným způsobem přípravy nanovlákenných nosičů je elektrostatické zvlákňování neboli electrospinning, s jehož pomocí lze připravit vlákna o průměru v řádu desítek nanometrů až desítek mikrometrů s definovanou porozitou a hmotností na cm^2. Trojrozměrná struktura nanovlákenných nosičů připomíná strukturu extracelulární matrix a poskytuje kmenovým buňkám velkou plochu pro jejich přichycení a růst. Nanovlákenné nosiče mohou být připraveny z různých přírodních nebo syntetických polymerů, které jsou

vybírány především s ohledem na jejich co nejvyšší biokompatibilitu. Z přírodních polymerů se nejčastěji používá chitosan a kolagen, ze syntetických polymerů je to pak například polyamid (polyamide – PA) nebo polyvinylalkohol. Mezi nejdůležitější požadavky kladené na připravované nanovlákenné nosiče patří jejich stabilita ve vodném prostředí a optimální biokompatibilita. Zajímavou alternativu představuje příprava nanovláken, která mohou obsahovat léčiva nebo jiné bioaktivní látky (Wang et al., 2009). Možné využití nanovlákenných nosičů nesoucích MSC bylo již úspěšně testováno u řady zvířecích modelů patologických stavů včetně poškození povrchu oka nebo chrupavky (Li et al., 2005; Soleimani et al., 2010; Cejkova et al., 2013).

3 Cíle práce

- **Stanovit vliv cytokinového prostředí na imunomodulační vlastnosti MSC.**

  Imunomodulační účinky MSC jsou zprostředkovány celou řadu faktorů, jejichž produkce může být zásadně ovlivněna prostředím, ve kterém se MSC nachází. Proto bude sledován efekt působení protizánětlivých a prozánětlivých cytokinů na imunomodulační aktivitu MSC.

- **Určit vliv cytokinů produkovaných různě stimulovanými MSC na vývoj Treg a Th17 lymfocytů.**

  MSC produkují konstitutivně TGF-β, který řídí diferenciaci $CD4^+$ T lymfocytů v Treg lymfocyty. Zároveň mohou MSC za určitých okolností produkovat IL-6, přičemž kombinované působení TGF-β a IL-6 směřuje vývoj T lymfocytů k Th17 buňkám. Budou stanoveny faktory, které stimulují MSC k produkci IL-6 a tím k indukci diferenciace T lymfocytů v prozánětlivé Th17 buňky.

- **Zjistit, zda mohou intravenózně podané MSC ovlivněné různými cytokiny selektivně migrovat do oblasti poškozené rohovky.**

  Stejně jako mohou faktory z prostředí ovlivnit imunomodulační působení MSC, mohou mít také účinek na jejich migrační schopnosti. Bude sledován efekt stimulace MSC různými cytokiny na jejich schopnost selektivně migrovat do poškozené rohovky.

- **Stanovit, jaký účinek mají intravenózně podané MSC ovlivněné různými cytokiny na rozvoj časného zánětu v poškozeném rohovkovém epitelu.**

  Časná fáze zánětu v rohovkovém epitelu je spojena s infiltrací poškozené oblasti především myeloidními, později i lymfoidními buňkami. Bude určen efekt intravenózně podaných MSC na infiltraci rohovky různými populacemi leukocytů a na produkci IL-1, IL-6, IL-10 a NO v poškozené tkáni.

- **Testovat růstové vlastnosti MSC a LSC kultivovaných na nanovlákenných nosičích a možnost přenosu takto kultivovaných buněk na poškozenou rohovku.**

  Nanovlákenné nosiče mají komplexní trojrozměrnou strukturu, která poskytuje buňkám potřebnou oporu pro růst nebo diferenciaci. Nanovlákenné nosiče mohou být připraveny z různých materiálů, ale ne všechny materiály jsou vhodné pro růst daného typu buněk. Proto bude sledováno, zda jsou nanovlákna připravená z PA6/12 a nanovlákna nesoucí Cyklosporin A (Cyclosporine – Cs) vhodná pro růst a přenos MSC a LSC.

4 Seznam vlastních publikací

4.1 Seznam použitých publikací

Zajicova A, Pokorna K, Lencova A, Krulova M, **Svobodova E**, Kubinova S, Sykova E, Pradny M, Michalek J, Svobodova J, Munzarova M, Holan V. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant. 2010;19:1281-1290. IF = 3,571

Holan V, Chudickova M, Trosan P, **Svobodova E**, Krulova M, Kubinova S, Sykova E, Sirc J, Michalek J, Juklickova M, Munzarova M, Zajicova A. Cyclosporine A-loaded and stem cell-seeded electrospun nanofibers for cell-based therapy and local immunosuppression. J Control Release. 2011;156:406-412. IF = 7,261

Svobodova E, Krulova M, Zajicova A, Pokorna K, Prochazkova J, Trosan P, Holan V. The role of mouse mesenchymal stem cells in differentiation of naive T-cells into anti-inflammatory regulatory T-cell or proinflammatory helper T-cell 17 population. Stem Cells Dev. 2012;21:901-910. IF = 4,202

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Holan V, Zajicova A, **Javorkova E**, Trosan P, Chudickova M, Pavlikova M, Krulova M. Distinct cytokines balance the development of regulatory T cells and interleukin-10-producing regulatory B cells. Immunology. 2014;141:577-586. IF = 3,735

5 Výsledky

5.1 Role myších MSC v diferenciaci naivních T lymfocytů v protizánětlivé Treg nebo prozánětlivé Th17 lymfocyty

Svobodová E., Krulová M., Zajícová A., Pokorná K., Procházková J., Trošan P., Holáň V.

Stem Cells Dev. 2012;21:901-910.

MSC získané z kostní dřeně mohou modulovat imunitní odpověď a produkovat významná množství TGF-β a IL-6. Tyto dva cytokiny představují klíčové faktory, které recipročně regulují vývoj naivních T lymfocytů v Treg nebo Th17 lymfocyty. Tato studie dokazuje, že MSC a jimi produkované cytokiny účinně regulují expresi transkripčních faktorů Foxp3 a RORγt a kontrolují tak vývoj Treg a Th17 lymfocytů v populaci myších slezinných buněk stimulovaných aloantigeny nebo purifikovaných $CD4^+CD25^-$ T lymfocytů. Imunomodulační účinky MSC byly výraznější, pokud byly tyto buňky stimulovány tak, aby produkovaly TGF-β nebo IL-6 a TGF-β. Nestimulované MSC produkují TGF-β, ale neprodukují IL-6. Produkce TGF-β může být dále zesílena působením protizánětlivých cytokinů IL-10 a TGF-β na MSC. V přítomnosti prozánětlivých cytokinů naopak MSC produkují významná množství IL-6 a zároveň konstitutivně produkují TGF-β. MSC produkující TGF-β indukovaly přednostně expresi Foxp3 a aktivaci Treg lymfocytů, zatímco supernatanty získané kultivací MSC a obsahující TGF-β i IL-6 podporovaly expresi RORγt a vývoj Th17 lymfocytů. Při použití neutralizačních protilátek proti TGF-β a IL-6 byl účinek rozpustných faktorů v supernatantech z MSC zablokován. Výsledky ukázaly, že MSC mohou recipročně regulovat vývoj a diferenciaci naivních T lymfocytů v protizánětlivé $Foxp3^+$ Treg lymfocyty nebo prozánětlivé $ROR\gamma t^+$ Th17 buňky a tím mohou ovlivňovat autoimunitní a transplantační reakce.

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The Role of Mouse Mesenchymal Stem Cells in Differentiation of Naive T-Cells into Anti-Inflammatory Regulatory T-Cell or Proinflammatory Helper T-Cell 17 Population

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Bone marrow-derived mesenchymal stem cells (MSCs) modulate immune response and can produce significant levels of transforming growth factor-β (TGF-β) and interleukin-6 (IL-6). These 2 cytokines represent the key factors that reciprocally regulate the development and polarization of naive T-cells into regulatory T-cell (Treg) population or proinflammatory T helper 17 (Th17) cells. In the present study we demonstrate that MSCs and their products effectively regulate expression of transcription factors Foxp3 and RORγt and control the development of Tregs and Th17 cells in a population of alloantigen-activated mouse spleen cells or purified $CD4^+$ $CD25^-$ T-cells. The immunomodulatory effects of MSCs were more pronounced when these cells were stimulated to secrete TGF-β alone or TGF-β together with IL-6. Unstimulated MSCs produce TGF-β, but not IL-6, and the production of TGF-β can be further enhanced by the anti-inflammatory cytokines IL-10 or TGF-β. In the presence of proinflammatory cytokines, MSCs secrete significant levels of IL-6, in addition to a spontaneous production of TGF-β. MSCs producing TGF-β induced preferentially expression of Foxp3 and activation of Tregs, whereas MSC supernatants containing TGF-β together with IL-6 supported RORγt expression and development of Th17 cells. The effects of MSC supernatants were blocked by the inclusion of neutralization monoclonal antibody anti-TGF-β or anti-IL-6 into the culture system. The results showed that MSCs represent important players that reciprocally regulate the development and differentiation of uncommitted naive T-cells into anti-inflammatory $Foxp3^+$ Tregs or proinflammatory $ROR\gamma t^+$ Th17 cell population and thereby can modulate autoimmune, immunopathological, and transplantation reactions.

Introduction

The ability of mesenchymal stem cells (MSCs) to modulate immune response has been well documented in numerous models. MSCs inhibit mitogen- or antigen-induced cell proliferation, cytokine production, and generation of cytotoxic cells in vitro [1–4]. Various types of immune response, such as autoimmune, antitumor, or transplantation reactions, have been attenuated by the transfer of MSCs [5–8]. However, in some models, MSCs stimulate immune reactions as opposed to the immunosuppressive effects of MSCs usually observed [9–11]. So far, cell-to-cell contact, production of inhibitory molecules, or induction of regulatory T-cells (Tregs) has been implicated in the mechanism of immunomodulation mediated by MSCs [6,12–14]. However, the manifestation of individual mechanisms of MSC-mediated suppression differs among various models or between species.

MSCs are also potent producers of various cytokines. After mitogen stimulation or upon a contact with lymphocytes, they produce numerous cytokines; especially interleukin-6 (IL-6) and transforming growth factor-β (TGF-β) can be regularly found in MSC supernatants [15,16]. IL-6 and TGF-β have been shown to be the main factors reciprocally regulating the development of proinflammatory $ROR\gamma t^+$ T helper 17 (Th17) cells and anti-inflammatory $Foxp3^+$ Treg population [17,18]. In naive $CD4^+$ T-cells, TGF-β rapidly induces expression of both Foxp3 and RORγt regardless of the presence of IL-6 [19,20]. However, in the presence of IL-6 expression of Foxp3 is suppressed, expression of RORγt predominates, and the production of IL-17 is favored [19]. The crucial role in differentiation of T-cell subsets play concentrations of TGF-β. In low TGF-β concentrations IL-6 suppresses Foxp3 expression, and the development of Th17 cells prevails. On the other hand,

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higher concentrations of TGF-β favor the development of Foxp3$^+$ Tregs [21]. Although other cytokines can also modulate the development and the activity of Tregs and Th17 cells, TGF-β and IL-6 remain the most important cytokines that, in a concentration-dependent manner, can orchestrate the differentiation of Tregs and Th17 cells. As MSCs are a potent source of both IL-6 and TGF-β, they represent a candidate cell population that could significantly influence differentiation of Th17 cells and Tregs and in this way to modulate autoimmune, immunopathological, and protective immune reactions.

Here we show that MSCs produce variable levels of TGF-β and IL-6 depending on the presence of proinflammatory IL-1, IL-2, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), or anti-inflammatory (TGF-β, IL-10) cytokines and thus skew differentiation of naive CD4$^+$ T lymphocytes into Treg or Th17 direction. Although MSCs can also contribute to immunoregulation by other mechanisms involving production of inhibitory molecules [11,22,23] or a direct cell–cell contact [1,12], the cytokine-regulated production of TGF-β and IL-6 by MSCs offers explanation for the different levels and patterns of immunoregulatory properties of MSCs. The regulation of differentiation and development of T-cell subsets represents a novel property of mouse bone marrow (BM)-derived MSCs.

Materials and Methods

Mice

BALB/c and C57BL/6J (B6) mice of both sexes were obtained at the age of 8–12 weeks from the breeding unit of the Institute of Molecular Genetics, Prague, Czech Republic. The use of animals was approved by the local Animal Ethics Committee.

Isolation and culture of MSCs

MSCs were isolated from the BM of BALB/c mice. The BM from the femurs and tibias was flushed out, washed, and cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS; Sigma Co., St. Louis, MO), antibiotics (100 μg/mL of streptomycin and 100 U/mL of penicillin), and 10 mM HEPES buffer (hereafter referred to as complete DMEM). The cells were cultured at a concentration of 4×10^6 cells/mL in 6 mL of the culture medium in 25-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). After 24 h of incubation, the nonadherent cells were removed by washing and the remaining adherent cells were cultured with regular exchange of the culture medium and passaging of the cells to maintain an optimal cell concentration. After ~3 weeks of culture (2–3 passages), the cells were harvested by gentle scraping and immunodepleted of CD11b$^+$ and CD45$^+$ cells by magnetic activated cell sorting (MACS). In brief, cells were incubated for 15 min with CD11b MicroBeads and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD11b$^-$CD45$^-$ MSCs were isolated using the AutoMACS magnetic separation system (Miltenyi Biotec). The purity of sorted cells (>98% of cells were CD11b$^-$CD45$^-$) was confirmed by flow cytometry using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ).

Characterization of MSCs by flow cytometry

MSCs isolated using MACS were washed in PBS containing 0.5% bovine serum albumin and incubated for 30 min on ice with allophycocyanine (APC)-labeled monoclonal antibody (mAb) anti-CD44 (clone IM7; BD PharMingen, San Jose, CA), fluorescein isothiocyanate (FITC)-labeled anti-CD90.2 (clone 30-H12; BioLegend, San Diego, CA), phycoerythrin (PE)-labeled anti-CD105 (clone MJ7/18; eBioscience, San Diego, CA), APC-labeled anti-CD11b (clone M1/70; BioLegend), PE-labeled anti-CD31 (clone MEC 13.3; BD PharMingen), or FITC-labeled anti-CD45 (clone 30-F11; BioLegend). Dead cells were stained using Hoechst 33258 dye (Invitrogen, Carlsbad, CA) added to the samples 15 min before flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Differentiation of MSCs to adipocytes and osteoblasts

MSCs growing for 2–3 weeks and separated by magnetic cell sorting were cultured in a complete DMEM medium supplemented with specific adipogenic (containing 0.1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 mM indomethacine, and 0.5 μg/mL of insulin) or osteogenic (0.1 μM dexamethasone, 0.1 mM L-ascorbic acid, and 10 mM β-glycerolphosphate disodium salt pentahydrate) reagents [24]. Differentiation of cells was confirmed by staining with Oil Red O or Alizarin Red S.

Immunosuppressive properties of MSCs

Spleen cells (0.5×10^6/mL) from BALB/c mice were stimulated in a volume of 0.2 mL of RPMI 1640 medium (Sigma Co.) supplemented with 10% FCS, antibiotics, HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol (hereafter a complete RPMI 1640 medium) in 96-well tissue culture plates (Corning Co., Corning, NY) with 10 μg/mL of mAb anti-CD3 [25]. Purified MSCs were added to these cultures at the ratios of spleen cells to MSCs 5:1, 10:1, and 20:1. Cell proliferation was determined by adding ^{3}H-thymidine (0.5 μCi/well, Nuclear Research Institute, Rez, Czech Republic) for the last 6 h of the 72-h incubation period.

Preparation of MSC supernatants

MSCs purified by MACS were incubated at a concentration of 1×10^5 cells/mL in a volume of 1 mL of a complete RPMI 1640 medium in 24-well tissue culture plates (Nunc). The cells were cultured unstimulated or stimulated with 10 ng/mL of mouse recombinant IL-1α, IL-2, IL-6, IL-10, IFN-γ, or TNF-α (all cytokines were purchased from Immunotools, Friesoythe, Germany), 1 ng/mL of human TGF-β1 (PeproTech, Rocky Hill, NJ), or with 1 μg/mL of lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI). After 24 h of incubation period the adherent cells were washed several times with excess of medium to remove added cytokines. The cells were then incubated in fresh medium without cytokines. Since the presence of TGF-β in FCS could interfere with the TGF-β detection by ELISA, MSCs used for TGF-β production were first transferred into serum-free medium containing 200 μg/mL of bovine serum albumin

and then stimulated with cytokines. All supernatants were harvested after another 72-h period and the production of cytokines or NO was assessed.

Cytokine detection

The production of IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-γ, and TGF-β by MSCs was quantified by ELISA. For the detection of IL-4, IL-6, IL-10, and IFN-γ, the cytokine-specific capture and detection mAb purchased from PharMingen was used. IL-1β, IL-12, and TGF-β were measured using ELISA kits purchased from R&D Systems (Minneapolis, WN). To test the production of IL-17, spleen cells (0.8×10^6/mL) from BALB/c mice were stimulated for 96 h with irradiated (3,000 R) spleen cells (0.8×10^6/mL) from B6 mice in the presence of TGF-β, IL-6, or MSC supernatants. The concentrations of IL-17 in the supernatants were assessed using an ELISA kit (R&D Systems).

NO determination

Nitrite concentrations in MSC supernatants were measured using the Griess reaction [26]. In brief, 100 μL of the tested supernatant was incubated with 50 μL of 1% sulfanilamide (in 2.5% H_2SO_4) and 50 μL of 0.3% *N*-1-naphthylethylenediamine dihydrochloride (in 2.5% H_2SO_4). Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as a standard.

Regulation of IL-17 production and Foxp3 or RORγt expression

Spleen cells (0.8×10^6/mL) from BALB/c mice were stimulated in a volume of 1 mL of a complete RPMI 1640 medium (Sigma Co.) in 48-well tissue culture plates (Corning Co.) with irradiated (3,000 R) spleen cells (0.8×10^6/mL) from B6 mice. Recombinant human TGF-β (1 ng/mL; PeproTech), TGF-β (1 ng/mL) together with IL-6 (1 ng/mL; Immunotools), or the supernatants (30% of the volume) obtained after a 72-h incubation of MSCs were added to the cultures of stimulated spleen cells. The proportion of $CD4^+$ cells expressing Foxp3 or RORγt was determined after a 96-h incubation period by flow cytometry. The concentrations of IL-17 in the supernatants were determined by ELISA. In some experiments, purified $CD4^+CD25^-$ cells were used as responder cells. This subpopulation was isolated from spleen single-cell suspensions using a $CD4^+CD25^+$ Regulatory T-cell isolation kit (Miltenyi Biotec) and the AutoMACS magnetic separation system (Miltenyi Biotec), as we have described [27]. This procedure yielded >96% pure $CD4^+CD25^-$ cell population. Over 99% of these cells were $Foxp3^-$ and $ROR\gamma t^-$, and >86% of them expressed CD62L, a marker of naive T-cells (data not shown).

To determine the MSC-derived factors responsible for the regulation of T-cell differentiation, neutralization mAb anti-IL-6 (clone MP5-20F3; BioLegend) or anti-TGF-β1 (clone ab64715; Abcam, Cambridge, United Kingdom) at a concentration of 2 μg/mL was added to the cultures of spleen cells stimulated with alloantigens in the presence of MSC supernatants.

Detection of TGF-β and IL-6 gene expression by real-time polymerase chain reaction

MSCs were cultured untreated or were stimulated with IL-1α (10 ng/mL), TGF-β (1 ng/mL), or LPS (1 μg/mL). Total RNA was extracted from the cells using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. One μg of total 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 polymerase chain reaction (PCR) was performed on an iCycler (BioRad, Hercules, CA) as we have previously described [28,29]. The primers used for amplification were as follows:

GAPDH: 5′-AGAACATCATCCCTGCATCC-3′ (sense), 5′-ACATTGGGGGTAGGAACAC-3′ (antisense);

TGF-β: 5′-TGGAGCAACATGTGGAACTC-3′ (sense), 5′-CAGCAGCCGGTTACCAAG-3′ (antisense);

IL-6: 5′-GCTACCAAACTGGATATAATCAGGA-3′ (sense), 5′-CCAGGTAGCTATGGTACTCCAGAA-3′ (antisense).

The PCR parameters included denaturation at 95°C for 3 min, 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The CT values were within the range 25–28, 21–24, and 17–20 for *IL-6*, *TGF-β*, and *GAPDH*, respectively. Each single experiment was done in triplicate. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 s, and the data were analyzed on the iCycler Detection system.

Intracellular staining of Foxp3 and RORγt

The cultured spleen cells were harvested and washed with PBS containing 0.5% bovine serum albumin. Before intracellular staining, the cells were incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD4 (clone GK1.5; BioLegend) and Live/Dead Fixable Violet Dead Cell Stain Kit (Molecular Probes, Eugene, OR) for staining of dead cells. Cells were washed in PBS/0.5% bovine serum albumin, fixed, and permeabilized using eBioscience Foxp3 buffer staining set according to the manufacturer's instructions. For intracellular detection of Foxp3 and RORγt, the cells were stained for 30 min with PE-labeled mAb anti-mouse/rat Foxp3 (clone FJK-16s, eBioscience) or PE-conjugated mAb anti-mouse/human RORγt (clone AFKJS-9, eBioscience). Data were collected using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Detection of suppressor activity of Tregs

$CD4^+CD25^-$ spleen cells (0.6×10^6/mL) from BALB/c mice were incubated in 48-well tissue culture plates (Nunc) in a volume of 1 mL of complete RPMI 1640 medium unstimulated, with irradiated B6 spleen cells (0.8×10^6/mL) or with irradiated B6 cells in the presence of supernatants (30% of the volume) from MSCs that were either untreated or preincubated with IL-1α or TGF-β. After a 96-h incubation period, the cells were harvested and tested for their ability to inhibit mixed leukocyte reaction (MLR) BALB/c anti-B6 [27]. In this assay, BALB/c spleen cells (0.75×10^6/mL) were incubated with irradiated B6 cells (0.75×10^6/mL) in a volume of 200 μL of a complete RPMI 1640 medium, and the cells from the primary cultures were added to this MLR at a reactive cell to preincubated cell ratio of 4:1. Cell proliferation was determined after 96 h according to the ^{3}H-thymidine incorporation.

Statistical analysis

The results are expressed as the mean ± SD. Comparisons between 2 groups were analyzed by Student's *t*-test, and multiple comparisons were calculated by analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of MSCs

BM-derived adherent cells growing for 3 weeks in culture represent a heterogenous cell population with a variable cell size and morphology. After the MACS separation, the purified cells had a uniform spindle-shaped morphology. Over 98% of cells were negative for CD45, CD11b, and CD31 markers, but were positive for CD44, CD90, and CD105, the molecules attributed to murine MSCs (Fig. 1A). The MSCs were further characterized by their ability to undergo specific adipogenic and osteogenic differentiation (Fig. 1B) and by their capability to inhibit anti-CD3 induced T-cell proliferation (Fig. 1C), consistent with our previous results [27,28]. In addition, we confirmed a specific adipogenic and osteogenic differentiation of MSCs by expression of adipocyte- and osteoblaste-specific markers by real-time PCR (data not shown). Thus, these MACS-separated BM-derived adherent cells possess the phenotype, differentiation, and functional properties associated with MSCs.

Cytokine production by MSCs

Unstimulated MSCs did not produce detectable levels of IL-6 or any of the tested cytokines (IL-1β, IL-4, IL-10, IL-12, or IFN-γ). After stimulation with IL-1α or LPS, MSCs produced significant ($P < 0.001$) levels of IL-6, but not the other tested cytokines (Fig. 2A and unpublished observations). In contrast, MSCs spontaneously produced a high level of TGF-β and this production was decreased ($P < 0.05$) in the presence of IL-1α or LPS (Fig. 2A). Unstimulated MSCs also produce no detectable levels of NO. NO production remained very low even after stimulation with IFN-γ or LPS. However, significant NO production ($P < 0.001$) was detected if the MSCs were stimulated with LPS together with IFN-γ (Fig. 2B). Next we evaluated the effects of a panel of selected cytokines on IL-6 and TGF-β production by MSCs. We found that proinflammatory cytokines IL-1α, IL-2, IFN-γ, and TNF-α (and LPS) significantly stimulated IL-6 production, but anti-inflammatory cytokines IL-10 and TGF-β did not induce detectable levels of IL-6 (Fig. 2C). On the contrary, TGF-β, but not proinflammatory cytokines, enhanced ($P < 0.05$) production of TGF-β (Fig. 2C). Since the presence of TGF-β in the FCS could interfere with TGF-β detection by ELISA, the cultures for TGF-β production were performed in serum-free medium. To confirm the results from ELISA, MSCs were cultured in the serum-containing RPMI 1640 medium and the levels of *TGF-β* mRNA and *IL-6* mRNA were determined by real-time PCR. As shown in Fig. 2D, unstimulated MSCs

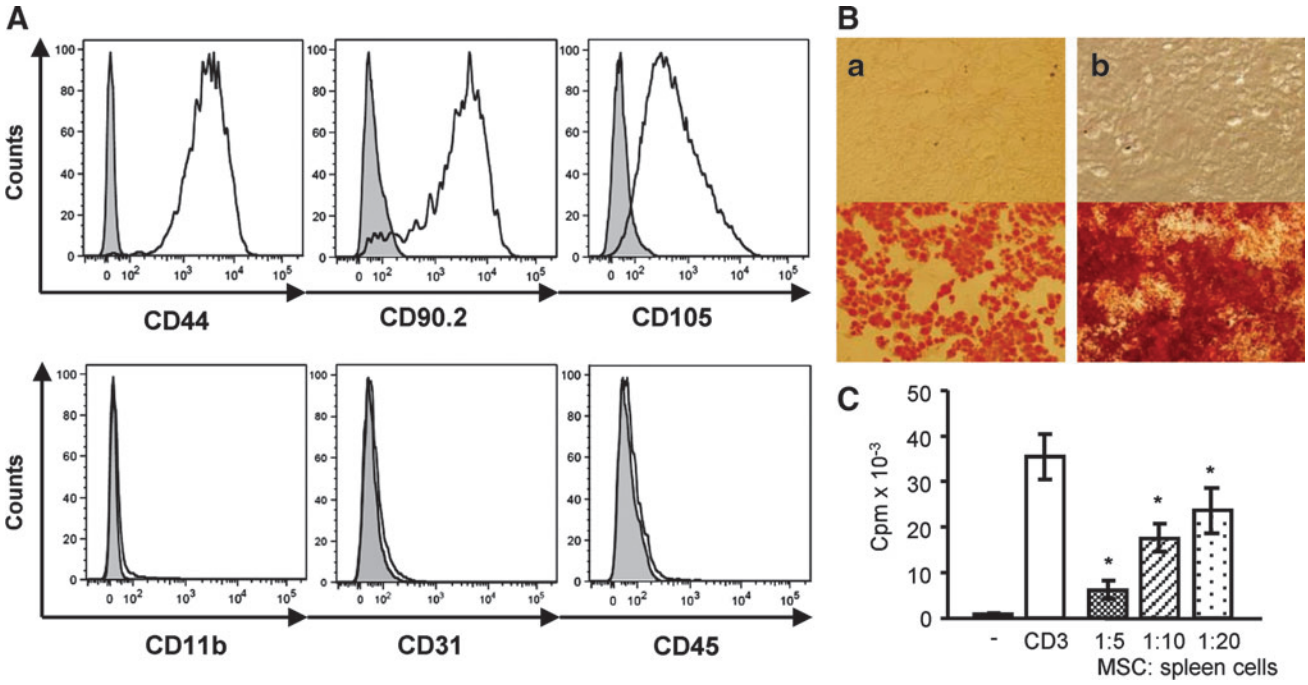

FIG. 1. Characterization of purified MSCs. **(A)** Expression of CD44, CD90.2, CD105, CD11b, CD31, and CD45 markers was assessed by flow cytometry. One of 5 similar experiments is shown. **(B)** The ability of MSCs to undergo **(a)** adipogenic and **(b)** osteogenic differentiation. The cultures without (*upper panel*) or with (*lower panel*) addition of differentiation agents were stained with Oil Red O for adipocytes or Alizarin Red S for osteoblasts. Original magnification: **(a)** 200×, **(b)** 40×. **(C)** Immunosuppressive properties of MSCs. Spleen cells were stimulated with mAb anti-CD3 in the absence or presence of MSCs (at the ratios 5:1, 10:1, and 20:1) and cell proliferation was determined. Each bar represents the mean ± SD from 4 individual experiments. Values with asterisk are significantly different (*$P < 0.05$) from the positive control. MSC, mesenchymal stem cell; mAb, monoclonal antibody.

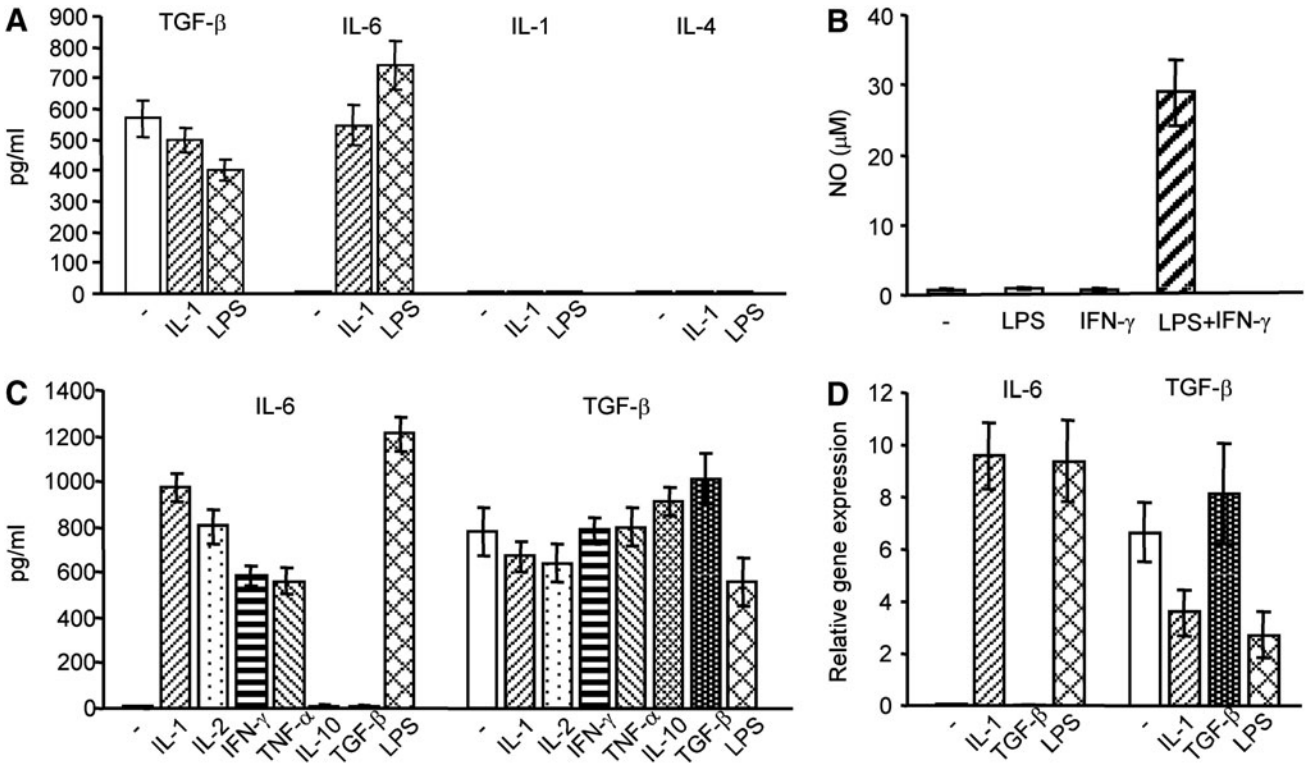

FIG. 2. Production of selected cytokines and NO by MSCs. MSCs were cultured unstimulated or in the presence of 10 ng/mL of IL-1α, IL-2, IL-10, IFN-γ, or TNF-α, 1 ng/mL of TGF-β, or 1 μg/mL of LPS. After a 24-h preincubation the cells were washed and cultured for another 72 h. **(A)** The presence of IL-6, TGF-β, IL-1β, and IL-4 in the supernatants was detected by ELISA. **(B)** The concentrations of NO were measured by spectrophotometry. **(C)** The effects of proinflammatory and anti-inflammatory cytokines on IL-6 and TGF-β production were assessed by ELISA. **(D)** Effects of IL-1α, TGF-β, and LPS on *TGF-β* or *IL-6* gene expression were detected by a real-time polymerase chain reaction. Each bar represents the mean ± SD from 4 to 6 independent experiments. TGF-β, transforming growth factor-β; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide.

expressed a significant level of *TGF-β* mRNA, but not *IL-6* mRNA. Expression of *TGF-β* mRNA was significantly ($P < 0.01$) decreased in the presence of IL-1α or LPS and slightly enhanced by exogenous TGF-β. Strong expression of *IL-6* gene was detected after stimulation of MSCs with IL-1α and LPS (Fig. 2D).

The effects of TGF-β and IL-6 on Foxp3 and RORγt expression and IL-17 production

Since MSCs can produce significant levels of IL-6 and TGF-β, we first tested the effects of recombinant IL-6 and TGF-β on IL-17 production by alloantigen-activated spleen cells. As shown in Fig. 3A, alloantigen-stimulated spleen cells produced a significant amount of IL-17 and this production was inhibited by TGF-β and considerably enhanced in the presence of TGF-β together with IL-6. These modulatory effects of IL-6 and TGF-β were confirmed when highly purified $CD4^+CD25^-$ cells were used as responder cells (Fig. 3B). To demonstrate that IL-6 and TGF-β also regulate expression of transcription factors Foxp3 and RORγt, which determine the development of Tregs and Th17 cells, respectively, we cultured spleen cells alone, with irradiated allogeneic cells or with allogeneic cells in the presence of TGF-β or TGF-β together with IL-6, and the percentage of $CD4^+$ $Foxp3^+$ and $CD4^+ROR\gamma t^+$ cells was determined by flow cytometry. As shown in Fig. 3C, the percentage of $CD4^+$ $Foxp3^+$ cells was significantly enhanced in the presence of TGF-β and this increase was reversed by IL-6. On the contrary, the percentage of $CD4^+ROR\gamma t^+$ cells was highest in the cultures containing TGF-β together with IL-6 (Fig. 3D).

Regulation of IL-17 production and Treg activity by MSC supernatants

Spleen cells were stimulated with irradiated allogeneic cells alone or in the presence of MSC supernatants. As demonstrated in Fig. 4A, supernatants obtained from the cultures of untreated MSCs significantly inhibited IL-17 production. This suppression was even more pronounced, when MSCs were pretreated with TGF-β or IL-10. On the contrary, the supernatants prepared from MSCs that were pretreated with IL-1α, IL-2, or LPS to produce IL-6 significantly enhanced the IL-17 production (Fig. 4A).

To demonstrate the effects of MSC supernatants on development of Treg activity, purified naive $CD4^+CD25^-$ spleen cells were activated with allogeneic cells in the presence of MSC supernatants. It was observed that supernatants from untreated MSCs or from MSCs preincubated with TGF-β activated Tregs with more pronounced suppressive activity than the supernatants from MSCs preincubated with IL-1 (Fig. 4B).

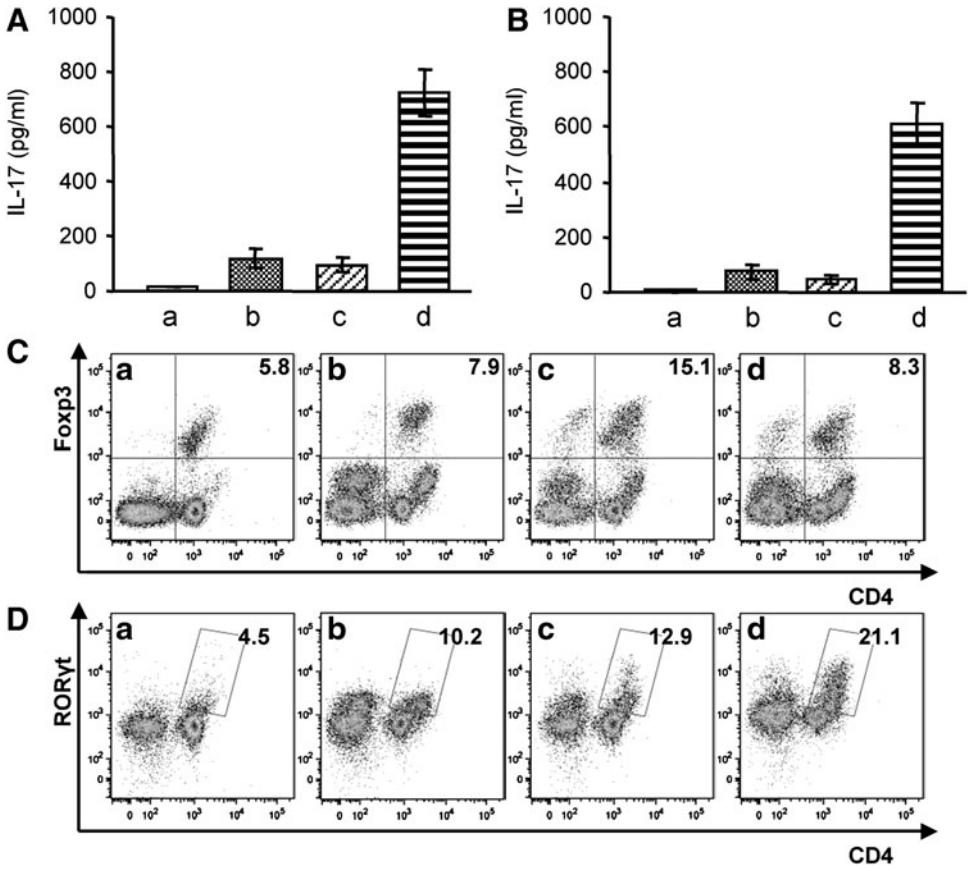

FIG. 3. The regulatory effects of TGF-β and IL-6 on IL-17 production and Foxp3 or RORγt expression in spleen cells stimulated with alloantigens. Spleen cells **(A)** or purified $CD4^+CD25^-$ cells **(B)** from BALB/c mice were cultured unstimulated **(a)**, stimulated with irradiated B6 spleen cells **(b)**, or were stimulated with B6 cells in the presence of 1 ng/mL of TGF-β **(c)** or TGF-β together with 1 ng/mL of IL-6 **(d)**. The concentrations of IL-17 **(A** and **B)** in the supernatants were measured after a 96-h incubation period by ELISA. Each bar represents the mean ± SD from 4 determinations. The percentage of $CD4^+Foxp3^+$ **(C)** or $CD4^+RORγt^+$ **(D)** spleen cells was determined by flow cytometry. One typical experiment of 5 determinations is shown.

Modulation of Foxp3 and RORγt expression by MSC supernatants

The supernatants containing TGF-β or TGF-β together with IL-6 were prepared by a 72-h incubation of untreated MSCs or MSCs that were preincubated for 24 h with IL-1α or TGF-β and then carefully washed to remove exogenous cytokines before preparation of MSC supernatants. The supernatants were added to the cultures of spleen cells stimulated with irradiated allogeneic cells, and the percentage of $CD4^+Foxp3^+$ and $CD4^+RORγt^+$ cells was determined. As demonstrated in Fig. 5A and B, the supernatants from untreated MSCs slightly increased the proportion of $CD4^+Foxp3^+$ cells. This increase was more apparent if the supernatants from MSCs pretreated with TGF-β (and containing endogenous TGF-β produced by MSCs) were used. On the contrary, supernatants from MSCs pretreated with IL-1α (which contain TGF-β and IL-6 produced by MSCs) rather decreased the percentage of $CD4^+Foxp3^+$ cells, but significantly enhanced the proportion of $CD4^+RORγt^+$ cells (Fig. 5C and D).

Effects of mAb anti-IL-6 and anti-TGF-β on the immunomodulatory properties of MSC supernatants

Supernatants from untreated MSCs (which contain TGF-β, but not IL-6) inhibited IL-17 production in cultures of alloantigen-stimulated spleen cells. Inclusion of neutralization mAb anti-TGF-β into these cultures abrogated the inhibitory effect of MSC supernatants (Fig. 6). On the contrary, supernatants from MSCs pretreated with IL-1α (which contain TGF-β and IL-6) significantly elevated IL-17 production. This enhancing effect of MSC supernatants was inhibited by mAb anti-IL-6 (Fig. 6). Since irrelevant, isotype-matched mAb added to these cultures did not abrogate immunomodulatory effects of MSC supernatants (data not shown), TGF-β and IL-6 were identified as the main factors produced by MSCs and responsible for regulation of IL-17 production.

Discussion

BM-derived adherent cells represent a morphologically and phenotypically heterogenous cell population. Especially in the mouse, a significant proportion of in vitro grown adherent BM cells express markers of hematopoetic precursors. To enrich MSCs in cultures of BM-derived cells, we performed depletion of $CD11b^+$ and $CD45^+$ cells by magnetic separation. The residual population of MSCs was characterized by a uniform spindle-shaped morphology, by the absence of $CD11b^+$ and $CD45^+$ cells and by expression of phenotypic markers associated with murine MSCs. These purified MSCs had the ability to specifically differentiate into adipocytes and osteoblasts and effectively inhibit T-cell proliferation, as it has been described for MSCs [6,29].

The purified MSCs spontaneously produced TGF-β, but without stimulation they produce no IL-6, IL-1, IL-4, IL-10, IFN-γ, or NO. After stimulation with proinflammatory cytokines (IL-1α, IL-2, IFN-γ, and TNF-α) or LPS, these MSCs secreted significant levels of IL-6, but not other tested cytokines (ie, IL-1β, IL-4, IL-10, IL-12, or IFN-γ). Anti-inflammatory cytokines TGF-β and IL-10 did not induce IL-6

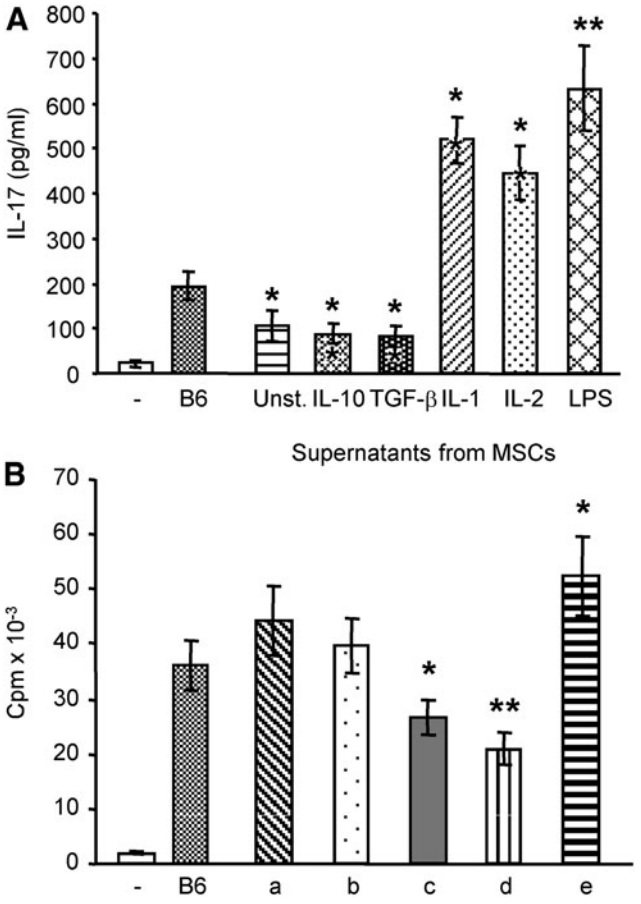

FIG. 4. Regulation of regulatory T-cell and T helper 17 cells development by supernatants from MSCs. **(A)** Spleen cells from BALB/c mice were cultured for 96 h unstimulated (−), stimulated with irradiated allogeneic cells (B6), or were stimulated with allogeneic cells in the presence of supernatants (30% of a total volume) from MSCs. For preparation of supernatants, MSCs were cultured untreated (unst.) or were preincubated with IL-1α, IL-2, IL-10, TGF-β, or LPS. Each bar represents the mean ± SD from 4 to 5 determinations. Values with *asterisks* are significantly different (*$P < 0.05$, **$P < 0.01$) from the control values in cultures without MSC supernatants. **(B)** The MLR BALB/c anti-B6 was set up, and purified $CD4^+CD25^-$ cells that were preincubated for 96 h with MSC supernatants were added into this culture. The reactive cells were cultured unstimulated **(−)** or were stimulated with irradiated allogeneic cells **(B6)**. The purified $CD4^+CD25^-$ cells were preincubated for 96 h unstimulated **(a)**, stimulated with irradiated B6 cells **(b)**, or were stimulated with B6 cells in the presence of supernatants from untreated MSCs **(c)** or from MSCs which were pretreated with TGF-β **(d)** or IL-1α **(e)**. Preincubated cells were added to MLR at a ratio of 4 reactive cells to 1 preincubated cell, and cell proliferation was determined. Values with *asterisks* are significantly different (*$P < 0.05$, **$P < 0.01$) from the control values (cultures without preincubated cells). Each bar represents the mean ± SD from 3 independent experiments. MLR, mixed leukocyte reaction.

production, but enhanced production of TGF-β. This cytokine production profile of purified mouse MSCs is similar to that described by Oh et al. [15] for human MSCs. Human MSCs produced spontaneously TGF-β, but not IL-6. IL-6 was induced by co-culture of MSCs with peripheral blood mononuclear cells, but other tested cytokines (IL-10, TNF-α, and IFN-γ) were not produced by human MSCs [15]. Similarly, increased expression of the gene for IL-6 and for some other growth factors was detected in human MSCs during culture with peripheral blood leukocytes [30]. Our results have shown that the production of TGF-β and IL-6 by purified MSCs is strictly regulated by cytokine environment.

It has been demonstrated in various models that TGF-β induces Foxp3 expression and plays a crucial role in the development of Tregs, whereas a combination of TGF-β and IL-6 leads to the activation of $ROR\gamma t^+$ Th17 cells and production of IL-17 [19–21]. To test the effects of MSCs and their supernatants on the differentiation of naive T-cells, we used a model of alloantigen-stimulated spleen cells. We confirmed that the production of IL-17 and expression of transcription factors Foxp3 and RORγt are reciprocally regulated by TGF-β and IL-6. Exogenous TGF-β added to the cultures of alloantigen-stimulated spleen cells induced Foxp3 expression, supported development of Tregs, and inhibited IL-17 production. On the contrary, the combination of TGF-β and IL-6 induced RORγt expression and enhanced production of IL-17. The addition of supernatants from untreated MSCs or from MSCs preincubated with TGF-β to the cultures of alloantigen-stimulated spleen cells induced Foxp3 expression and activation of Tregs, and resulted in an inhibition of IL-17 production. This inhibition of IL-17 production may be due to a negative effect of TGF-β on the development of Th17 cells and due to the effects of Tregs that are activated in the presence of TGF-β [21,27]. Kimura et al. [31] showed that IL-6 inhibits the activation of Foxp3 expression and supports expression of RORγt, which is a crucial factor responsible for the development of Th17 cells. An alternative explanation for the effects of TGF-β and other cytokines on the IL-17 expression in human naive T cells was presented by Manel et al. [32]. These authors showed that TGF-β upregulated RORγt expression, but at the same time inhibited its ability to induce IL-17 production. This inhibition was relieved by proinflammatory cytokines [32]. In accordance with the above observations, the supernatants from MSCs stimulated by proinflammatory cytokines induced lower levels of Foxp3 than supernatants from unstimulated MSCs, supported RORγt expression, and enhanced IL-17 production. Thus, MSCs and their supernatants can suppress or enhance Treg or Th17 cell development according to their activation status and their cytokine production. These observations are in agreement with the immunosuppressive properties of MSCs described in numerous models [1–4,6], but can also explain immunostimulatory effects of MSCs observed in other cases [9–11].

It has been shown that the immunomodulatory effects of MSCs are dose-dependent. At high ratios of MSCs to lymphocytes prevails suppression of lymphocyte proliferation, while lower concentrations of MSCs rather enhance cell proliferation [3,6]. Similarly, the switch between Foxp3 and RORγt expression is dependent on the concentrations of TGF-β and IL-6 [21]. Since the immunomodulatory effects of MSCs are mediated by factors present in MSC supernatants, the variable concentrations of TGF-β and IL-6 could explain these effects. We observed that TGF-β used at a wide range of concentrations (0.2–10 ng/mL) enhanced Foxp3 expression in alloantigen-activated T-cells, and that low concentrations of IL-6 (< 0.2 ng/mL) can redirect development of TGF-β-induced $Foxp3^+$ cells into IL-17-producing cells (data

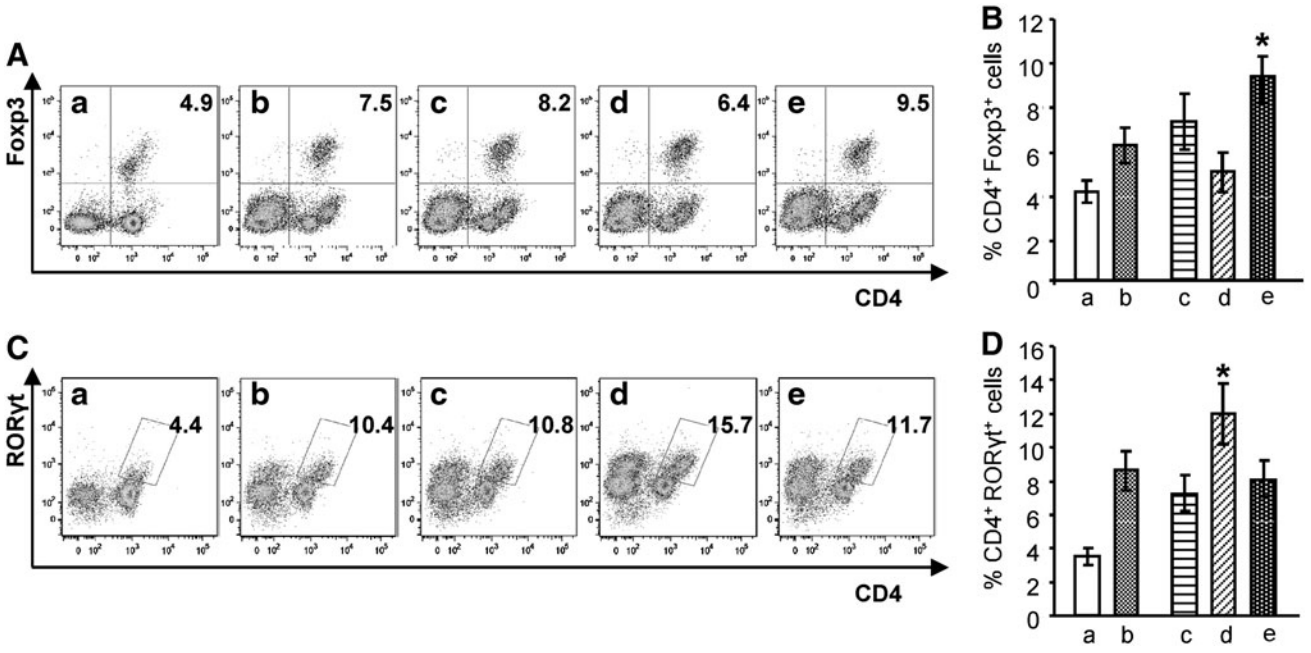

FIG. 5. Effects of MSC supernatants on Foxp3 and RORγt expression. The supernatants were prepared by a 72-h incubation of MSCs that were untreated or were preincubated with IL-1α or TGF-β, and were added to the cultures of BALB/c spleen cells stimulated with irradiated B6 cells. The percentage of $CD4^+Foxp3^+$ **(A, B)** or $CD4^+ROR\gamma t^+$ **(C, D)** cells was determined by flow cytometry after a 96-h incubation. **A** and **C** show representative flow cytometry dot plots of **(a)** unstimulated spleen cells, **(b)** spleen cells stimulated with alloantigens, **(c)** spleen cells stimulated with alloantigens in the presence of supernatant from untreated MSCs, **(d)** supernatants from MSCs pretreated with IL-1α, or **(e)** supernatants from MSCs pretreated with TGF-β. **B** and **D** show the mean ± SD of $CD4^+Foxp3^+$ or $CD4^+ROR\gamma t^+$ cells from 6 independent determinations. *Asterisks* indicate a significant difference ($^*P < 0.05$) from the control values (cultures without supernatants).

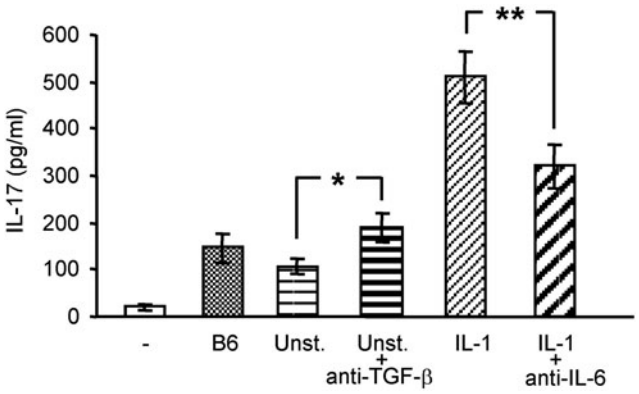

FIG. 6. Effect of neutralization mAb anti-TGF-β or anti-IL-6 on the ability of MSC supernatants to modulate IL-17 production. The supernatants containing TGF-β or TGF-β together with IL-6 were prepared by a 72-h incubation of untreated MSCs (unst.) or MSCs preincubated with IL-1α. These supernatants were added to the cultures of BALB/c spleen cells that were stimulated with irradiated B6 cells. Neutralization mAb anti-TGF-β or anti-IL-6 were added to these cultures at a concentration of 2 μg/mL. The levels of IL-17 in the culture supernatants were determined after a 96-h incubation by ELISA. Each bar represents the mean ± SD from 4 determinations. *Asterisks* indicate statistically significant difference ($^*P < 0.05$, $^{**}P < 0.01$).

not shown). However, the conditions in vivo, where cell–cell contact, membrane-bound cytokine molecules, and distinct local cytokine concentrations can be involved, may be quite different from those in vitro, and the immunoregulatory role of MSCs must be carefully controlled. Using neutralization mAb anti-TGF-β and anti-IL-6, we showed that TGF-β and IL-6 present in MSC supernatants are the main cytokines responsible for the reciprocal regulation of Treg or Th17 cell development by MSCs. Similarly, Liu et al. [16] showed that rat MSCs can inhibit or stimulate proliferation of myelin basic protein-specific T lymphocytes in a dose-dependent manner and that cytokines produced by MSCs are responsible for these immunomodulatory effects.

So far, various mechanisms have been proposed to explain the immunomodulatory effects of MSCs. Different cytokines and factors produced by MSCs were suggested as molecules responsible for MSC-mediated suppression [6,12,13,23]. Sato at al. [33] proposed that NO produced by MSCs plays a critical role in suppression of T-cell proliferation. A possibility that MSCs activate Tregs has also been suggested [14,22,34]. Our results have shown that TGF-β and IL-6 are the principal molecules responsible for the ability of MSCs to reciprocally activate or inhibit the development and functions of Tregs and Th17 cells.

The ability of MSCs to inhibit T-cell-mediated immune reactions has been used to prevent or treat autoimmune diseases [35,36], to suppress transplantation reactions [2,7,14,37], or to attenuate GVHD in humans and mice [38,39]. However, the results remain variable and the suppression was not achieved in all models [40,41]. The

suppression could be more pronounced if MSCs are modified with cytokines [36,40] or the treatment is combined with immunosuppressive drugs [37,41]. However, some discrepancies in efficiency of cytokine-treated MSCs still exist among individual models. While in our in vitro experiments IFN-γ-pretreated mouse MSCs produced TGF-β and IL-6, which stimulate IL-17 production, Polchert at al. [39] observed that IFN-γ-treated MSCs were more effective in the prevention of graft-versus-host reaction in vivo. These differences might reflect different models used and/or the involvement of distinct cell populations in proinflammatory reactions and in the graft-versus-host reaction. Altogether, the data indicate that the ongoing immune response and cytokine environment may significantly modify the effectiveness of MSC treatment.

It has recently been shown that human MSCs inhibit effector functions of Th17 cells and suppress IL-17 production [42]. Our results demonstrate that MSCs and their supernatants also inhibit the differentiation and development of Th17 cells from naive $CD4^+CD25^-$ precursors. Thus, MSCs inhibit proinflammatory Th17 cells on both the level of their activation and their effector function. These observations may explain the immunosuppressive effects of MSCs observed in models, such as transplantation or autoimmune reactions, where Th17 cells play an important role. However, the immunosuppressive function of MSCs can be reversed by IL-6 and, in some situations, MSCs can induce an extensive expansion of T-cell clones [30]. These interplays between MSCs, Tregs, and Th17 cells have to be taken into account when MSCs are used to treat autoimmune, immunopathological, or transplantation reactions.

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Author Disclosure Statement

No competing financial interests exist.

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5.2 Modulace časného zánětlivého prostředí v poškozeném oku pomocí systémově podaných MSC stimulovaných IFN-γ

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Tato studie sleduje účinek systémově podaných MSC na časnou fázi akutního zánětu v oku poškozeném hydroxidem sodným. Myši s poškozeným okem byly ponechány buď neléčené, nebo jim byly 24 hodin po poškození oka intravenózně podány MSC značené fluorescenční barvou, které byly buď neovlivněné, nebo byly stimulované pomocí IL-1, TGF-β nebo IFN-γ. Analýza buněčných suspenzí připravených z očí 72 hodin po poškození ukázala, že MSC specificky migrovaly do poškozeného oka, kde bylo nalezeno 30krát více fluorescenčně značených MSC než v oku kontrolním. Neovlivněné MSC i MSC stimulované IL-1, TGF-β nebo IFN-γ snižovaly počty leukocytů infiltrujících poškozené oko s výraznějším účinkem na myeloidní populace. Stanovení produkce cytokinů a NO v poškozeném oku potvrdilo, že nejúčinnějšího imunomodulačního efektu bylo dosaženo podáním MSC stimulovaných IFN-γ, které významně potlačovaly produkci prozánětlivých molekul IL-1, IL-6 a NO. Tato studie ukázala, že systémově podané MSC cíleně migrují do poškozeného oka a že MSC stimulované IFN-γ jsou nejúčinnější v potlačení akutní fáze rohovkového zánětu, ve snižování leukocytární infiltrace a v potlačení zánětlivého prostředí.

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ORIGINAL RESEARCH REPORT

AU1 ▶

Modulation of the Early Inflammatory Microenvironment in the Alkali-Burned Eye by Systemically Administered Interferon-γ-Treated Mesenchymal Stromal Cells

AU2 ▶

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The aim of this study was to investigate the effects of systemically administered bone-marrow-derived mesenchymal stromal cells (MSCs) on the early acute phase of inflammation in the alkali-burned eye. Mice with damaged eyes were either untreated or treated 24 h after the injury with an intravenous administration of fluorescent-dye-labeled MSCs that were unstimulated or pretreated with interleukin-1α (IL-1α), transforming growth factor-β (TGF-β), or interferon-γ (IFN-γ). Analysis of cell suspensions prepared from the eyes of treated mice on day 3 after the alkali burn revealed that MSCs specifically migrated to the damaged eye and that the number of labeled MSCs was more than 30-times higher in damaged eyes compared with control eyes. The study of the composition of the leukocyte populations within the damaged eyes showed that all types of tested MSCs slightly decreased the number of infiltrating lymphoid and myeloid cells, but only MSCs pretreated with IFN-γ significantly decreased the percentage of eye-infiltrating cells with a more profound effect on myeloid cells. Determining cytokine and NO production in the damaged eyes confirmed that the most effective immunomodulation was achieved with MSCs pretreated with IFN-γ, which significantly decreased the levels of the proinflammatory molecules IL-1α, IL-6, and NO. Taken together, the results show that systemically administered MSCs specifically migrate to the damaged eye and that IFN-γ-pretreated MSCs are superior in inhibiting the acute phase of inflammation, decreasing leukocyte infiltration, and attenuating the early inflammatory environment.

Introduction

A CHEMICAL BURN of the eye causes severe inflammation, corneal opacity, neovascularization, and epithelial defects, which can finally lead to a permanent loss of vision. A massive infiltration of inflammatory cells can be detected in the site of injury already 24 h after corneal damage. The number of infiltrating macrophages is amplified several fold within 36 h, and this increase is accompanied by a rising lymphocyte infiltration [1]. The conventional treatment protocols of the acute phase of corneal inflammation consist of inhibiting the inflammatory reaction and neovascularization by immunosuppressive drugs, but novel therapeutic strategies involving the transplantation of corneal epithelial sheets or limbal stem cells cultured on an appropriate carrier have been developed and successfully applied [2–5].

More recently, mesenchymal stromal cells (MSCs) have been suggested and tested as a promising therapeutic tool for the treatment of many disorders involving corneal defects. These cells have the potential to differentiate into various cell types [6], including epithelial cells [7–9], and thus they have been used for ocular surface reconstruction. Moreover, MSCs possess potent immunomodulatory properties and can influence various functions of immune cells, including dendritic cells, naive and effector T lymphocytes, and natural killer (NK) cells [10]. The immunomodulatory properties of MSCs have been documented in numerous in vitro and in vivo studies that demonstrate the ability of MSCs to prolong allograft survival [11,12], ameliorate experimental autoimmune disorders [13], or attenuate severe acute graft-versus-host disease [14].

The beneficial effects of MSCs consist not only in their ability to replace injured cells, but also in their modulation of the local proinflammatory microenvironment by the production of numerous immunomodulatory and trophic factors. It has been shown that the curative effect of MSCs on corneal injury can consist partly in the epithelial transdifferentiation of MSCs [7,15] and in the suppression of corneal inflammation [16–19].

This study was focused on monitoring and evaluating the effects of systemically administered MSCs on the early

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acute phase of inflammation in the alkali-burned eye using an experimental murine model. Since the immunomodulatory properties of MSCs can be modified by proinflammatory cytokines [20–22], we compared the effect of unstimulated MSCs and MSCs pretreated with interleukin (IL)-1α, interferon (IFN)-γ, or transforming growth factor (TGF)-β on the inflammatory environment in the eye.

Materials and Methods

Mice

Female BALB/c mice at the age of 8–12 weeks were obtained from the breeding unit of the Institute of Molecular Genetics (Prague, Czech Republic). The use of animals was approved by the local Animal Ethics Committee.

Isolation, culture, and purification of MSCs

Bone marrow for the cultivation of MSCs was isolated from the femurs and tibias of female BALB/c mice. The bone marrow was flushed out, a single-cell suspension was prepared using a tissue homogenizer, and the cells were seeded at a concentration of 2×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY), antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), and 10 mM HEPES buffer (hereafter referred to as complete DMEM) in 75-cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland). Nonadherent cells were washed out after 72 h of cultivation, and the remaining adherent cells were cultured for an additional 3 weeks (two passages) at 37°C in an atmosphere of 5% CO_2. Plastic-adherent cells were harvested by incubating the cells with 8 mL of 10 mM EDTA for 5 min 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 evaluated in terms of their purity and differentiation potential.

Phenotypic characterization of MSCs by flow cytometry

Unstimulated and cytokine-pretreated MSCs were washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and then incubated for 30 min on ice with the following anti-mouse monoclonal antibodies (mAbs): allophycocyanine (APC)–labeled anti-CD44 (clone IM7; BD PharMingen, San Jose, CA), phycoerythrin (PE)–labeled anti-CD105 (clone MJ7/18; eBioscience, San Diego, CA), APC-labeled anti-CD11b (clone M1/70; BioLegend, San Diego, CA), or fluorescein isothiocyanate (FITC)–labeled anti-CD45 (clone 30-F11; BioLegend). Dead cells were stained using 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).

Labeling of MSCs with PKH26 fluorescent dye

MSCs were labeled with a fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma) in order to monitor their fate after intravenous administration. MSCs were labeled according to the manufacturer's instructions with modifications introduced in the protocol to achieve optimal labeling for this type of cells. Particularly, a final concentration of 2 μM of PKH26 for 1×10^6 MSCs/mL and a 5-min incubation were determined to be the optimal conditions. The fluorescence intensity and homogeneity of the staining were tested by fluorescent microscopy (Inverted fluorescent microscope Olympus IX71, Center Valley, PA) and flow cytometry.

A model of corneal damage

Female BALB/c mice were deeply anesthetized by an intramuscular injection of a mixture of xylazine and ketamine (Rometar, Spofa, Prague, Czech Republic). The surface (corneal and limbal region) of the left eye was damaged by the application of 3-mm-diameter filter paper soaked with 8 μL of 1 N NaOH for 30 s. The eye was then thoroughly rinsed with 10 mL of PBS.

In vitro stimulation and the intravenous administration of MSCs

MSCs were labeled with PKH26 dye and incubated at a concentration of 0.5×10^6 cells/mL in a volume of 1.5 mL of complete DMEM in a 12-well tissue culture plate (Nunc, Roskilde, Denmark) for 24 h. MSCs were cultured either unstimulated or were pretreated with 10 ng/mL of mouse recombinant IL-1α (Immunotools, Friesoyte, Germany), 10 ng/mL of IFN-γ (Immunotools), or 2 ng/mL of human TGF-β (PeproTech, Rocky Hill, NJ). Stimulated MSCs were then harvested and centrifuged in an excess of serum-free DMEM in order to remove the rest of the serum and added cytokines. For intravenous application, 0.5×10^6 MSCs were resuspended in 200 μL of serum-free DMEM. The cell suspension was administered to mice through the tail vein using a 30G Omnican 100 syringe (B.Braun, Melsungen, Germany) 24 h after corneal damage.

Preparation of single-cell suspension from the eye and other organs

Single-cell suspensions from the whole eyeballs of both damaged and control eyes were prepared for flow cytometry analysis and cell culturing. The eyballs were cleaned of redundant tissue, cut into pieces, and centrifuged in 600 μL of HBSS to obtain a tissue extract for ELISA analysis. The pelleted tissue was then digested with 1 mg/mL of collagenase I (Sigma) in HBSS for 50 min at 37°C. To monitor the fate of PKH26-labeled MSCs after their intravenous administration, selected tissues and organs were obtained and digested to prepare single-cell suspensions for flow cytometry. Eyes, lung, and liver were digested in 1 mg/mL of collagenase I in HBSS for 50 min at 37°C. Lymph nodes (inguinal, brachial, cervical, and submandibular) and spleen were digested in 1 mg/mL of collagenase II (Sigma) in HBSS for 60 min at 37°C. A cell suspension from the bone marrow was prepared in the same way as in the protocol for the culture of MSCs.

Monitoring the migration of PKH26-labeled MSCs in the body

To determine the distribution of PKH26-labeled MSCs in the body 48 h after their intravenous administration, single-cell suspensions were prepared from several tissues and organs (eyes, lung, liver, spleen, lymph nodes, and bone marrow). For flow cytometry analysis, the concentration of the cells was adjusted to 1×10^6/mL in PBS containing 0.5% BSA. The number of PKH26-labeled cells was determined using an LSRII cytometer, and the data were analyzed by FlowJo software. To determine more precise location of $PKH26^+$ cells in damaged eye, single-cell suspensions were prepared from anterior segment, vitreous humor, and posterior segment of damaged eyes from mice treated with unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ. The cell suspensions were incubated with APC-labeled anti-CD44 mAb (BD PharMingen) and a total number of $PKH26^+CD44^+$ MSCs in particular segments was assessed by flow cytometry.

Immunofluorescent staining of frozen sections of damaged eyes

For frozen sectioning, damaged eyes of mice that were untreated or were treated with unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ were enucleated and immersion fixed in 4% paraformaldehyde for 1 h, followed by overnight cryoprotection in 15% sucrose. The eyes were embedded in optical cutting temperature medium and frozen sections at a thickness of 7 μm were prepared using a Leica CM 3050 cryostat (Leica, Wetzlar, Germany). The sections were refixed by 4% paraformaldehyde for 10 min, washed in PBS, blocked by 10% BSA in PBS containing 0.5% Triton X-100 for 30 min, and then incubated with APC-labeled anti-CD45 mAb (clone 30-F11; BioLegend) in blocking solution for 2 h at room temperature. After washing three times with PBS, slides were mounted with DAPI (Vectashield; Vector Labs, Burlingame, CA). $CD45^+$ leukocyte and $PKH26^+$ MSC infiltration were analyzed using fluorescent microscope Olympus Cell-R.

Detection of gene expression by real-time polymerase chain reaction

Total RNA was extracted from unstimulated and cytokine-pretreated MSCs using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. One microgram of total 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 polymerase chain reaction (PCR) was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) as we have previously described [23,24]. The sequences of primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-6, TGF-β, indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF), and cyclooxygenase 2 (COX2) used for amplification are presented in Table 1. The PCR parameters included denaturation at 95°C for 3 min, 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 s and were analyzed using StepOne Software version 2.2.2 (Applied Biosystems).

Samples for testing changes in the expression of genes for cytokines and iNOS in control and damaged eyes were prepared from fresh single-cell suspensions (IL-6) or obtained after culturing suspensions for 48 h (IL-1α, IL-10, or iNOS). In brief, cells (1×10^6/mL) from control and damaged eyes were cultured in a volume of 800 μL of RPMI 1640 medium (Sigma) containing 10% FCS (Gibco BRL), antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), and 10 mM HEPES buffer (hereafter referred to as complete RPMI) in 48-well tissue culture plates (Corning, Corning, NY) in the presence of 1.5 μg/mL of concanavalin A (ConA; Sigma) and 1.5 μg/mL of lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI) for a 48-h incubation period. The sequences of primers for GAPDH, IL-1α, IL-6, IL-10, and iNOS used for amplification are shown in Table 1.

Flow cytometry characterization of leukocyte populations infiltrating the damaged eyes

Single-cell suspensions prepared from control and damaged eyes were washed in PBS containing 0.5% BSA and incubated for 30 min on ice with the following anti-mouse mAb (all purchased from BioLegend): peridin-chlorophyll protein/Cyanine5.5 (PerCP/Cy5.5)–labeled anti-CD45 (clone 30-F11), APC-labeled anti-CD3 (clone 17A2), FITC-labeled

Table 1. Murine Primer Sequences Used for Real-Time Polymerase Chain Reaction

Gene	*Sense primer*	*Antisense primer*
GAPDH	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
TGF-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
IDO	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
iNOS	CTTTGCCACGGACGAGAC	TCATTGTACTCTGAGGGCTGAC
HGF	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
COX2	AGCCCACCCCAAACACAGT	AAATATGATCTGGATGTCAGCACATATT
IL-1α	TTGGTTAAATGACCTGCAACA	GAGCGCTCACGAACAGTTG
IL-10	ATTTGAATTCCCTGGGTGAGAAG	CACAGGGGAGAAATCGATGACA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TGF-β, transforming growth factor-β; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; HGF, hepatocyte growth factor; COX2, cyclooxygenase 2.

anti-CD4 (clone GK1.5), PE-labeled anti-CD8a (clone 53–6.7), PE-labeled anti-F4/80 (clone BM8), APC-labeled anti-CD80 (clone 16-10A1), PE-labeled anti-CD14 (clone Sa14-2), APC-labeled anti-CD11b (clone M1/70), FITC-labeled anti-Ly6G/Ly-6C (Gr-1) (clone RB6-8C5), and FITC-labeled anti-CD19 (clone 6D5). Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen) added to the samples 10 min before flow cytometry analysis. Data were collected using an LSRII cytometer and analyzed using FlowJo software. One hundred thousand events from each sample were measured. These events were gated for $CD45^+$ leukocytes after the exclusion of cell debris and dead cells and analyzed for particular markers.

Cytokine and NO measurement

Cytokines and NO were measured in tissue extracts and culture supernatants from control and damaged eyes. Tissue extracts were obtained during the preparation of single-cell suspensions from eyeballs (see above). Supernatants were obtained after culturing cell suspensions from control and damaged eyes. Cells (1×10^6/mL) were cultured in a volume of 800 μL of complete RPMI medium (Sigma) in the presence of 1.5 μg/mL of ConA and 1.5 μg/mL of LPS for a 48-h incubation period. The production of IL-1α, IL-2, IL-4, IL-6, IL-10, IL-17, and IFN-γ was quantified by ELISA. The production of IL-2, IFN-γ, and IL-6 was measured using cytokine-specific capture and detection of mAbs purchased from BD Pharmingen (San Diego, CA). IL-1α, IL-4, IL-6, IL-10, and IL-17 were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN). Only IL-1α, IL-6, and IL-10 were produced in significant concentrations and with enhanced production in the damaged eyes. The concentrations of NO in the supernatants were determined using the Griess reaction [25]. A mixture of 50 μL of 1% sulfanilamide and 50 μL of 0.3% N-1-naphthylethylendiamine dihydrochloride (both in 3% H_3PO_4) was incubated with 100 μL of the tested supernatant. Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as a standard.

Statistical analysis

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

Results

Characterization of MSCs

The purity and phenotypic markers of MACS-separated MSCs pretreated with cytokines were assessed with flow cytometry. The results showed that unstimulated and cytokine-treated MSCs were positive with a corresponding intensity for CD44 and CD105, which are the markers attributed to murine MSCs (Fig. 1A). On the other hand, <1% of the cells were $CD11b^+$ and <3% were $CD45^+$ (Fig. 1A); therefore, the population of bone marrow cells

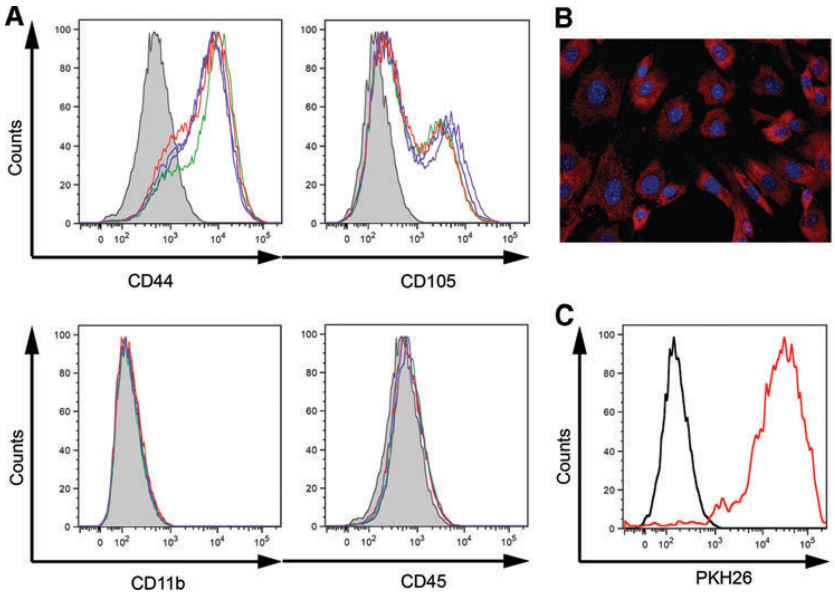

FIG. 1. Characterization of unstimulated mesenchymal stromal cells (MSCs) and MSCs pretreated with interleukin-1α (IL-1α), transforming growth factor-β (TGF-β), or interferon-γ (IFN-γ). **(A)** Flow cytometry analysis of CD44, CD105, CD11b, and CD45 markers expressed by unstimulated MSCs (*blue curve*) and MSCs pretreated with IL-1α (*green curve*), TGF-β (*red curve*), or IFN-γ (*violet curve*) in comparison with control unlabeled MSCs (*gray-tinted curve*). One of 3 similar experiments is shown. **(B)** Representative image of MSCs labeled with PKH26 fluorescent dye (red cell membranes) and maintained for 24 h in culture. The nuclei are blue (DAPI staining); original magnification is 400×. **(C)** Using flow cytometry, the fluorescence intensity of PKH26-labeled MSCs was analyzed after 24 h in culture (*red curve*) in comparison to control unlabeled MSCs (*black curve*). One of 3 similar experiments is shown. Color images available online at www.liebertpub.com/scd

was depleted of contaminating $CD11b^+$ and $CD45^+$ cells with a high efficiency. In addition, both unstimulated and cytokine-treated MSCs were able to undergo adipogenic and osteogenic differentiation (data not shown). Further, PKH26-labeled MSCs possessed a sufficient, detectable, and relatively homogenous fluorescent signal that could be detected using a fluorescent microscopy and flow cytometry even after cultivation (Fig. 1B, C).

Real-time PCR analysis of the expression of genes for immunomodulatory molecules was performed for further characterization of unstimulated MSCs and MSCs pretreated with IL-1α, TGF-β, or IFN-γ. As shown in Fig. 2, both unstimulated and pretreated MSCs expressed significant but variable levels of genes for TGF-β, HGF, and COX2. Moreover, MSCs pretreated with IL-1α and IFN-γ expressed a significant level of mRNA for iNOS. Only MSCs pretreated with IL-1α expressed the IL-6 gene and, on the other hand, only MSCs pretreated with IFN-γ expressed the IDO gene (Fig. 2).

Monitoring of the distribution of PKH26-labeled MSCs in the body

The distribution of $PKH26^+$ MSCs in the body 48 h after their systemic administration and possible differences in the migration of unstimulated and cytokine-pretreated MSCs were assessed using flow cytometry. Single-cell suspensions from the eye, lung, liver, and spleen; the inguinal, brachial, cervical, and submandibular lymph nodes; and the bone marrow were analyzed. A low number of $PKH26^+$ MSCs was detected in the lymph nodes, while higher numbers of $PKH26^+$ cells migrated to the spleen and bone marrow and the highest number of labeled MSCs was trapped in the lung and liver (data not shown). The analysis of suspensions prepared from damaged and control eyes showed that both unstimulated and cytokine-pretreated MSCs migrated preferentially into the damaged eye and that the number of $PKH26^+$ MSCs was more than 30-times higher in the damaged eye compared with the control eye (Fig. 3A). No significant differences were revealed between the migratory properties of unstimulated MSCs and MSCs pretreated with IL-1α, TGF-β, or IFN-γ (Fig. 3B). Flow cytometry analysis of single-cell suspensions from anterior segment, vitreous humor, and posterior segment of damaged eyes from mice treated with unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ revealed that ~20% of $PKH26^+$ $CD44^+$ cells detected in eye was present in anterior segment, 10% of labeled cells was detected in vitreous humor, and 70% of cells was present in posterior segment of the damaged eye (Fig. 3B).

Determination of leukocyte populations infiltrating damaged eyes after treatment with MSCs

Since both unstimulated and cytokine-pretreated MSCs preferentially migrated to the damaged eye, the potential effect of MSCs on the local environment was tested in the next experiments. Cell suspensions from control and damaged eyes from untreated mice or mice treated with systemically administered MSCs were labeled with selected combinations of mAbs and analyzed by flow cytometry. To test the lymphoid lineages, the percentage of $CD3^+CD4^+$

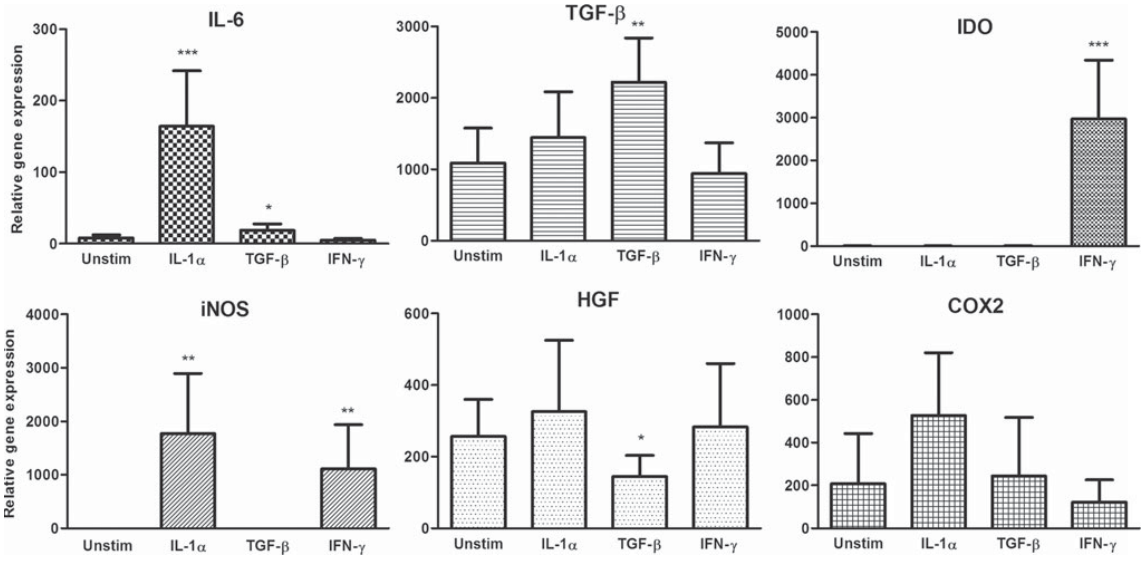

FIG. 2. Expression profile of genes for immunomodulatory molecules in unstimulated MSCs and MSCs pretreated with IL-1α, TGF-β, or IFN-γ. MSCs were cultured for 24 h unstimulated or were pretreated with 10 ng/mL of IL-1α or IFN-γ or 2 ng/mL of TGF-β. The expression of genes for IL-6, TGF-β, indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF), and cyclooxygenase 2 (COX2) was detected by real-time PCR. Values with asterisks are significantly different ($*P<0.05$, $**P<0.01$, $***P<0.001$) from the control value (unstimulated MSCs). Each bar represents the mean ± SD from six independent experiments.

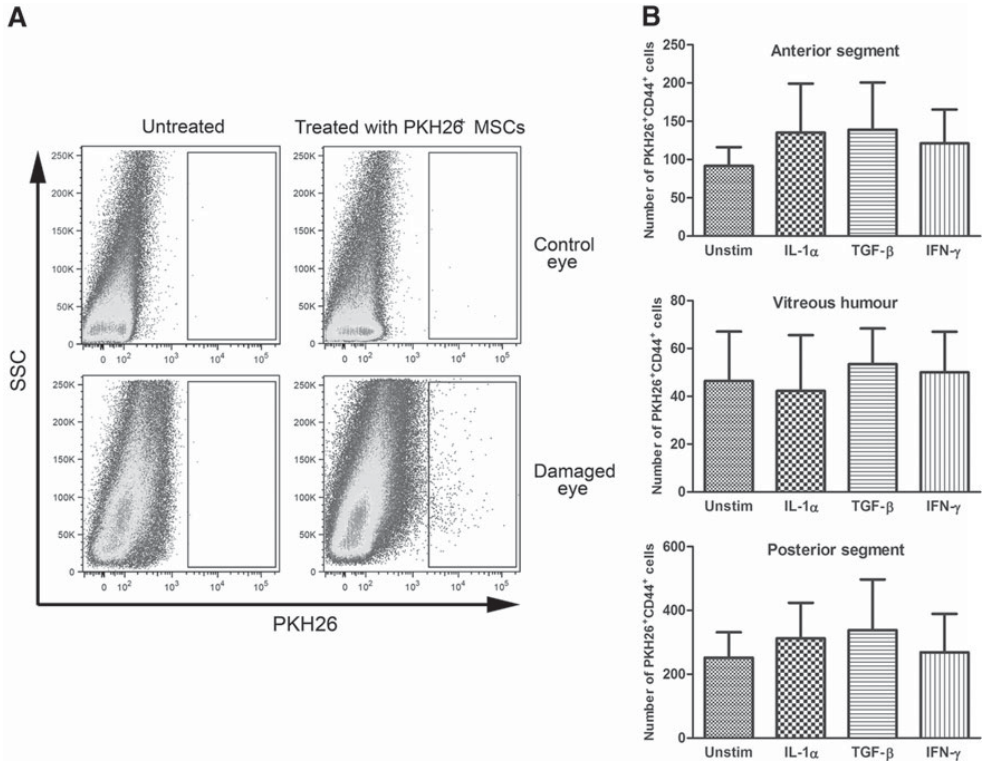

FIG. 3. Monitoring of the migration of PKH26-labeled MSCs into damaged and control eyes. **(A)** Representative dot plots showing flow cytometry analysis of single-cell suspensions prepared from control and damaged eyes from untreated mice and mice treated with unstimulated MSCs (MSCs pretreated with IL-1α, TGF-β, or IFN-γ showed a similar pattern of distribution.). A total of 200,000 events were analyzed after the exclusion of cell debris and dead cells, and these events were gated for $PKH26^+$. **(B)** Flow cytometry analysis of number of $PKH26^+CD44^+$ MSCs in anterior segment, vitreous humor, and posterior segment of damaged eyes from mice treated with unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ. Values with asterisks are significantly different ($*P<0.05$, $**P<0.01$, $***P<0.001$) from the control values (damaged eyes of mice treated with unstimulated MSCs). Each bar represents the mean ± SD from five independent experiments. ◀AU5

(T lymphocytes and NK-T cells), $CD3^+CD8^+$ (T lymphocytes and NK-T cells), and $CD19^+CD22^+$ (B lymphocytes) F4▶ cells was monitored. Figure 4 shows that the presence of all of the tested types of MSCs slightly decreased the percentage of damaged-eye-infiltrating lymphoid populations, but only MSCs pretreated with IFN-γ decreased the number of $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD19^+CD22^+$ cells significantly. The effect of MSCs on the myeloid lineage was tested by assessing the percentage of infiltrating $CD80^+$, $CD14^+$, $F4/80^+$, and $CD11b^+Gr\text{-}1^+$ (granulocytes and macrophages) cells. As demonstrated in Fig. 4, both unstimulated and cytokine-treated MSCs slightly decreased the percentages of $CD80^+$ and $CD11b^+Gr\text{-}1^+$ cells, but only MSCs pretreated with IFN-γ significantly decreased the percentages of all myeloid populations, while MSCs pretreated with TGF-β significantly decreased the percentage of $CD11b^+$ $Gr\text{-}1^+$ cells. In general, the effect of IFN-γ-pretreated MSCs was more profound on myeloid than lymphoid populations.

Microscopical analysis of frozen sections of damaged eyes confirmed the results from flow cytometry. As dem- F5▶ onstrated in Figure 5, a strong infiltration with $CD45^+$ leukocytes was observed in damaged eyes from untreated mice, and this infiltration was decreased in eyes from mice treated with MSCs.

Effect of MSCs on cytokine and NO production in control and damaged eyes

The flow cytometry data showed a significantly decreased infiltration of both lymphoid and myeloid cells in damaged eyes from mice injected with IFN-γ-pretreated MSCs. To extend this observation, we determined the production of the proinflammatory cytokines IL-1α and IL-6 and the secretion of NO in damaged eyes from mice treated with unstimulated or cytokine-pretreated MSCs. As demonstrated in Figure 6, systemic treatment with IFN-γ-primed MSCs ◀F6 significantly inhibited the local production of IL-1α, IL-6, and NO in the damaged eye. In contrast, the production of the anti-inflammatory cytokine IL-10 was not inhibited by MSCs (Fig. 6). These results were confirmed by real-time PCR analysis. The expression of genes for IL-1α and IL-6 in the damaged eyes of mice injected with IFN-γ-pretreated

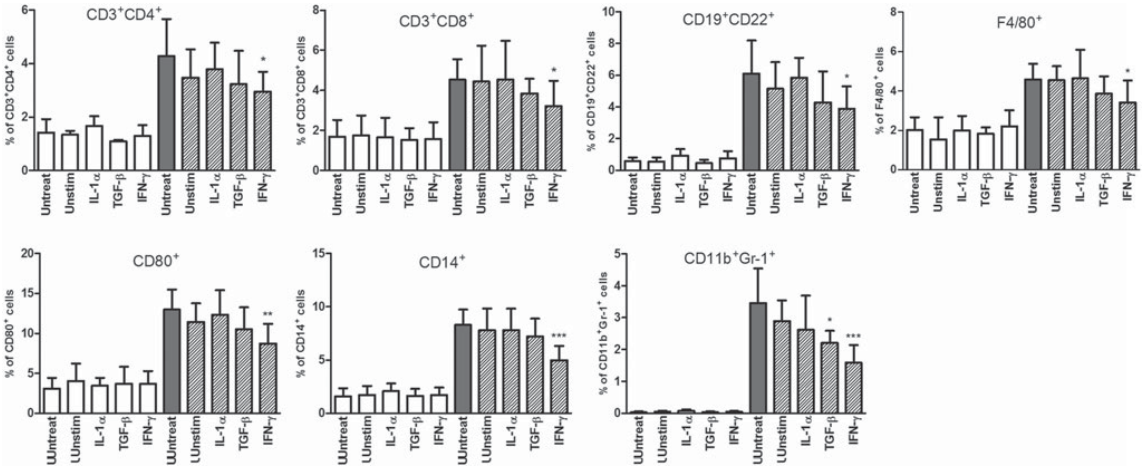

FIG. 4. Flow cytometry analysis of leukocyte populations infiltrating control (*white columns*) and damaged (*dashed and gray columns*) eyes of untreated mice and mice treated with the systemic administration of unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ (in the graphs labeled as Unstim, IL-1α, TGF-β, and IFN-γ). One hundred thousand events from each sample were measured. These events were gated for $CD45^+$ leukocytes after the exclusion of cell debris and dead cells and analyzed for the percentage of $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+CD22^+$, $CD80^+$, $CD14^+$, $F4/80^+$, or $CD11b^+Gr\text{-}1^+$ cells. Values with asterisks are significantly different ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) from the control values (damaged eyes of untreated mice). Each bar represents the mean ± SD from 10 independent experiments.

MSCs was significantly inhibited. Similarly, the expression of the gene for iNOS was significantly decreased in mice treated with IFN-γ-pretreated MSCs. In agreement with the results from ELISA, the expression of the IL-10 gene was not inhibited by MSCs (Fig. 7). F7 ▶

Discussion

The present study was designed to evaluate the effects of MSCs on immunological processes occurring in the eye in the early period after the ocular surface damage. We focused

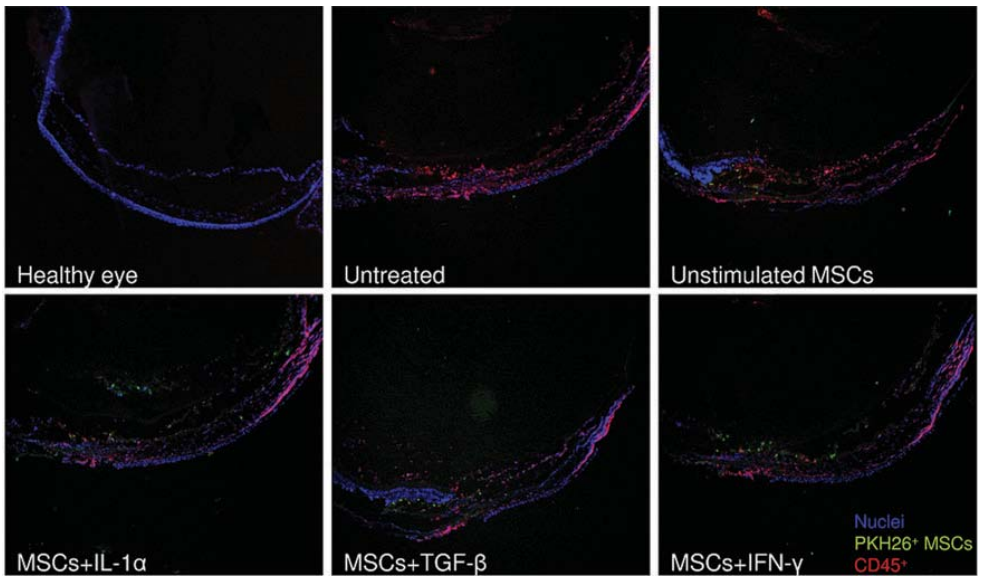

FIG. 5. Immunofluorescent staining of frozen sections of eyes from untreated mice and mice treated with unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ. Representative pictures show part of anterior segment (*right upper part* of image) and lateral segment of control healthy eye and damaged eye from untreated mouse and from mouse treated with unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ. The infiltration of the eye with MSCs labeled with PKH26 fluorescent dye (*green*) and $CD45^+$ leukocytes (*red*) is shown. The nuclei are *blue* (DAPI staining); original magnification is 40×. Color images available online at www.liebertpub.com/scd

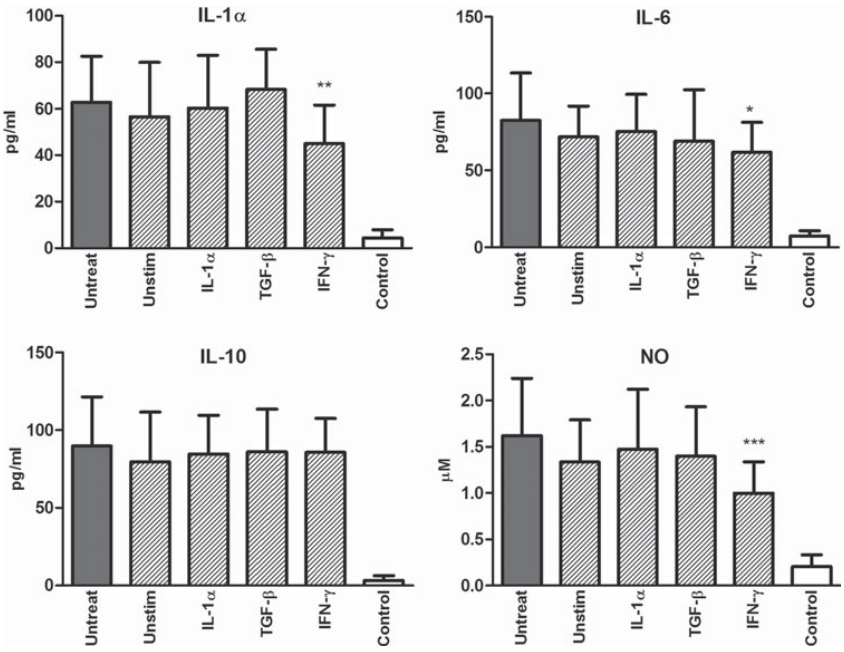

FIG. 6. The effect of MSCs on cytokine and NO production. Cytokine and NO production in control (*white column*) and damaged (*dashed and gray columns*) eyes of untreated mice and mice treated with the systemic administration of unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ (in the graphs labeled as Unstim, IL-1α, TGF-β, and IFN-γ). The level of IL-6 was measured by ELISA in tissue extracts obtained from freshly isolated eyes. The production of IL-1α and IL-10 was determined by ELISA in supernatants from cultures of control and damaged eyes after stimulation with concanavalin A (ConA; 1.5 μg/mL) and lipopolysaccharide (LPS; 1.5 μg/mL) for 48 h. The production of NO was measured in culture supernatants using the Griess reaction. Values with asterisks are significantly different ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) from the control values (damaged eyes of untreated mice). Each bar represents the mean ± SD from 12 independent experiments.

on the eye infiltration by different leukocyte populations and on the local cytokine microenvironment, but not on characterization of the eye surface regeneration, which occurs in the later phase after ocular surface damage. Majority of published studies investigated the effects of MSCs on the ocular surface regeneration, but not on immunological processes occurring in the eye within the first 3 days after ocular damage.

Since previous studies have demonstrated that cytokines can modulate the immunoregulatory properties of MSCs [20–22], we compared the therapeutic effects of unstimulated MSCs and MSCs pretreated with IL-1α, IFN-γ, or TGF-β. MSCs were administered intravenously 24 h after corneal damage, at the time when the number of eye-infiltrating myeloid and lymphoid cells markedly increases. The immunomodulatory effects of MSCs were evaluated 72 h after the injury.

Initially, we tested the expression of cell surface markers and the migratory properties of unstimulated and cytokine-pretreated MSCs. These cells were positive for CD44 and CD105 and no significant differences in the intensity of expression of the tested markers were detected. A similar conclusion was reached by Najar et al. [20], who tested a panel of endothelial, stromal, and adhesive markers in unstimulated MSCs and MSCs stimulated with a cocktail of proinflammatory cytokines; no differences were detected. In addition, we did not find any variation in the migration of unstimulated and cytokine-pretreated MSCs, and a comparable distribution of injected MSCs was detected within the tested organs and tissues. In another study, Hemeda et al. [26] assessed the effect of IFN-γ and TNF-α on MSCs, and TNF-α was recognized as the predominant regulator of MSC migration. On the other hand, pretreatment of MSCs with IFN-γ increased their migration to the inflammed intestine in an animal model of colitis [27].

Our results confirmed that systemically administered MSCs are able to specifically migrate to the damaged eye. We found a more than 30-times higher number of injected MSCs in the damaged eye compared with the control contralateral eye. These findings are in agreement with the observation of Lan et al. [28], who detected systemically administered MSCs on day 3 in the cauterized cornea but not in the contralateral cornea. Systemically administered MSCs were also detected in the cornea 14 days after their injection in a rabbit eye alkali-burn model [29] but not in the case of xenogeneic (human) MSCs administered systemically in order to heal a corneal injury in the rat [19].

Since both unstimulated and cytokine-pretreated MSCs preferentially migrated into the injured eye, we tested the effects of the engrafted MSCs on the infiltration of the damaged eye by populations of lymphoid and myeloid cells. All types of MSCs slightly decreased infiltration by the

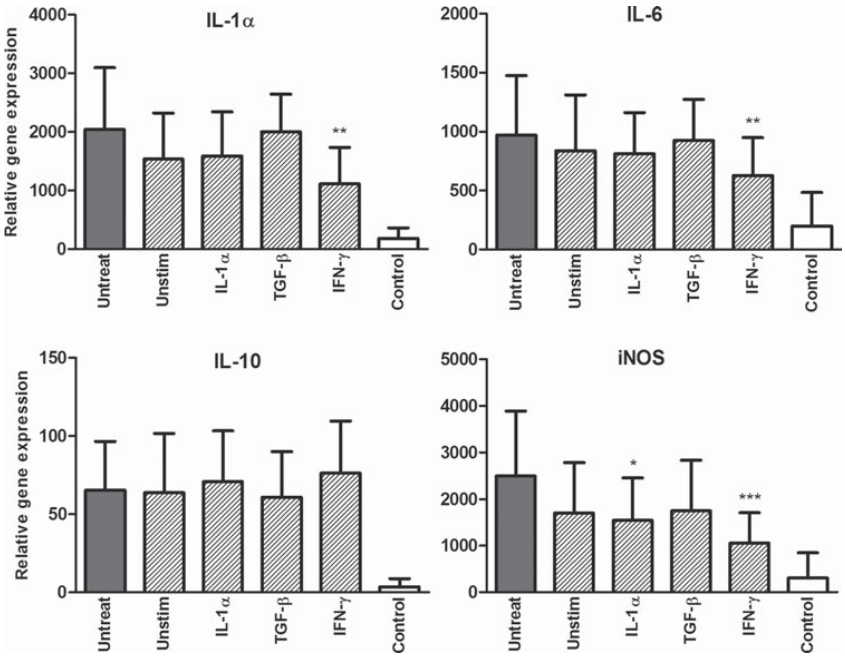

FIG. 7. The effect of MSCs on the expression of genes for cytokines and iNOS. The expression of genes for IL-1α, IL-6, IL-10, and iNOS in control (*white column*) and damaged (*dashed and gray columns*) eyes of untreated mice and mice treated with the systemic administration of unstimulated MSCs or MSCs pretreated with IL-1α, TGF-β, or IFN-γ (in the graphs labeled as Unstim, IL-1α, TGF-β, and IFN-γ) was measured by real-time PCR. The expression of the IL-6 gene was assessed in cells from freshly isolated eyes. The expression of genes for IL-1α, IL-10, and iNOS was determined in cells from cultures of control and damaged eyes after stimulation with ConA (1.5 μg/mL) and LPS (1.5 μg/mL) for 48 h. Values with asterisks are significantly different ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) from the control values (damaged eyes of untreated mice). Each bar represents the mean ± SD from eight independent experiments.

lymphoid populations, but only MSCs pretreated with IFN-γ decreased the number of $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD19^+CD22^+$ cells significantly. To date, a decreased expression of CD45 has been detected in damaged rat eyes transplanted with MSCs on an amniotic membrane [17], and a reduced infiltration of $CD4^+$ cells was observed in injured corneas of mice treated with MSCs [16]. Further, we found that MSCs pretreated with IFN-γ suppressed the infiltration of the damaged eye by $CD14^+$, $CD80^+$, and $CD11b^+Gr\text{-}1^+$ myeloid populations even more effectively than they inhibited infiltration by lymphoid cells. These results are in agreement with the findings of Yao et al. [18], who showed that the number of $CD68^+$ cells infiltrating the site of injury on day 7 was significantly lower in a group treated with MSCs [18]. In addition, the systemic injection of human MSCs significantly reduced the infiltration of neutrophils into the cornea on days 1 and 3 after injury in a rat model of a chemically burned eye [19]. These effects of MSCs on neutrophil infiltration are consistent with our observation that MSCs pretreated with IFN-γ strongly decreased the infiltration of the damaged eye by myeloid cell populations.

We next tested the effects of systemically administered MSCs on the early cytokine environment of the damaged eye. Although we tested a wide range of cytokines, that is, IL-1α, IL-2, IL-4, IL-6, IL-10, IL-17, and IFN-γ, only IL-1α, IL-6, and IL-10 were produced in this phase of inflammation in significant concentrations. We found that systemically administered MSCs stimulated with IFN-γ significantly decreased the production of IL-6 and IL-1α with the most profound inhibition of IL-1α production. This observation is in accordance with the findings of Roddy et al. [19], who showed in a rat model that the production of IL-1 on days 1 and 3 after injury was significantly decreased in corneas treated with human MSCs. On the other hand, Oh et al. [16] observed an increased production of IL-6 in rat corneas 3 weeks after injury and treatment with MSCs. This discrepancy could be explained by the different kinetics of IL-1 and IL-6 production. The peak of IL-1 production occurs at day 3 after injury, while the production of IL-6 culminates later [30].

Further, we detected a highly significant inhibition of iNOS expression and NO production in the damaged eye after treatment with MSCs prestimulated with IFN-γ. Since NO is a toxic and immunomodulatory molecule, its inhibition may represent another mechanism of the therapeutic action of MSCs.

In contrast to Roddy et al. [19], who found a significant increase in IL-10 production 3 weeks after the treatment of injured corneas with MSCs, we detected only a slight enhancement of IL-10 production in the damaged eyes of MSC-treated mice. This discrepancy can be due to the different time points used for IL-10 detection in our and Roddy's studies.

Since MSCs preincubated with various cytokines differ in their immunomodulatory effects, we tested the expression of genes for immunoregulatory molecules in unstimulated MSCs and in MSCs pretreated with IL-1α, IFN-γ, or TGF-β. Both unstimulated MSCs and cytokine-pretreated MSCs expressed significant levels of genes for TGF-β, HGF, and COX2. It has been already shown that these immunomodulatory molecules are constitutively expressed by MSCs [31]. On the other hand, we did not observe an increase in COX2 expression after stimulation with IFN-γ. English et al. [22] detected an increased expression of COX2 after the stimulation of murine MSCs with IFN-γ, but in their study a 20-times higher concentration of IFN-γ was used. We found that MSCs stimulated with IL-1α or IFN-γ expressed a significant level of the iNOS gene. Accordingly, the treatment of mouse MSCs with IFN-γ and any of three other proinflammatory cytokines (TNF-α, IL-1α, or IL-1β) induced the expression of several chemokines and iNOS [21]. In agreement with other studies [22,31,32], a significant increase in the expression of the IDO gene was observed in MSCs pretreated with IFN-γ.

Finally, MSCs stimulated with IFN-γ expressed significant levels of the genes for TGF-β, IDO, iNOS, HGF, and COX2; there were differences mainly in the expression of iNOS and IDO in comparison with the other types of MSC stimulation. Although iNOS and IDO are important immunomodulatory molecules [33–35], other immunoregulatory mechanisms are probably also involved in MSC-mediated immunosuppression. Nevertheless, our results clearly show that systemically administered MSCs rapidly migrate into the site of injury and attenuate the early phase of the inflammatory reaction. Infiltration by both myeloid and lymphoid cells and the local production of proinflammatory cytokines are decreased by MSCs, and MSCs pretreated with IFN-γ are superior in the inhibition of this early inflammatory microenvironment.

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Author Disclosure Statement

No competing financial interests exist.

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5.3 Léčba poškozeného povrchu oka pomocí LSC a MSC kultivovaných na nanovlákenných nosičích

Zajícová A., Pokorná K., Lenčová A., Krulová M., **Svobodová E**., Kubinová Š., Syková E., Přádný M., Michálek J., Svobodová J., Munzarová M., Holáň V.
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Terapie pomocí kmenových buněk představuje nadějný přístup jak léčit různá poškození, vrozená onemocnění nebo deficience kmenových buněk. Jedním z hlavních problémů spojených s léčbou pomocí kmenových buněk stále zůstává nedostupnost vhodného nosiče pro jejich růst a přenos. V této studii popisujeme růstové a metabolické vlastnosti MSC a LSC kultivovaných na trojrozměrném nanovlákenném nosiči připraveném z PA6/12. Nanovlákna byla připravena pomocí elektrostatického zvlákňování technologií Nanospider, která umožňuje vytvářet nanovlákenné nosiče mající definovaný průměr, pórovitost a základní hmotnost. Kopolymer PA6/12 byl vybrán na základě stability z něj připravených nanovláken ve vodném prostředí, biokompatibility a schopnosti fungovat jako nosič pro růst LSC, MSC a rohovkových endoteliálních a epiteliálních linií. Morfologie, růst a životnost buněk kultivovaných na nanovláknu byly srovnatelné s buňkami rostoucími na plastovém povrchu. Fluorescenčně značené $PKH26^{+}$ LSC kultivované na nanovlákenném nosiči byly přeneseny na poškozený oční povrch, kde bylo sledováno jejich přetrvání a přežití. Přenos LSC s MSC, které mají imunomodulační schopnosti, významně potlačil místní zánětlivou reakci a podpořil proces hojení. Výsledky ukázaly, že nanovlákna připravená z PA6/12 představují vhodný nosič pro růst LSC a MSC a pro jejich přenos za účelem léčby deficitu kmenových buněk a různých poškození povrchu oka.

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Treatment of Ocular Surface Injuries by Limbal and Mesenchymal Stem Cells Growing on Nanofiber Scaffolds

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Stem cell (SC) therapy represents a promising approach to treat a wide variety of injuries, inherited diseases, or acquired SC deficiencies. One of the major problems associated with SC therapy remains the absence of a suitable matrix for SC growth and transfer. We describe here the growth and metabolic characteristics of mouse limbal stem cells (LSCs) and mesenchymal stem cells (MSCs) growing on 3D nanofiber scaffolds fabricated from polyamide 6/12 (PA6/12). The nanofibers were prepared by the original needleless electrospun Nanospider technology, which enables to create nanofibers of defined diameter, porosity, and a basis weight. Copolymer PA6/12 was selected on the basis of the stability of its nanofibers in aqueous solutions, its biocompatibility, and its superior properties as a matrix for the growth of LSCs, MSCs, and corneal epithelial and endothelial cell lines. The morphology, growth properties, and viability of cells grown on PA6/12 nanofibers were comparable with those grown on plastic. LSCs labeled with the fluorescent dye PKH26 and grown on PA6/12 nanofibers were transferred onto the damaged ocular surface, where their seeding and survival were monitored. Cotransfer of LSCs with MSCs, which have immunosuppressive properties, significantly inhibited local inflammatory reactions and supported the healing process. The results thus show that nanofibers prepared from copolymer PA6/12 represent a convenient scaffold for growth of LSCs and MSCs and transfer to treat SC deficiencies and various ocular surface injuries.

Key words: Limbal stem cells (LSCs); Mesenchymal stem cells (MSCs); Nanofiber scaffolds; Ocular surface injuries; Inflammation; Tissue regeneration

INTRODUCTION

Stem cell (SC) therapy represents a promising approach to treating various inherited diseases or tissue injuries associated with SC deficiency. Adult (tissue-specific) SCs benefit from the ability to differentiate into the cell type for which they are committed and even from their ability to differentiate into other cell types (9,12). In addition, a population of SCs derived from bone marrow, called mesenchymal stem cells (MSCs), has immunosuppressive properties and thus can contribute to the healing process by inhibiting local inflammatory reactions (3,16,21).

One of the major problems associated with SC therapy remains the absence of a suitable carrier for the transfer of SCs to precise tissue locations. So far, various materials and scaffolds have been tested for the transportation of SCs. For example, macroporous hydrogels have been used to deliver MSCs for spinal cord injury repair (29) or self-assembling peptide nanofibers have been tested for myoblast transplantation in infarcted myocardium (7). To treat severe ocular surface damage and a deficiency in limbal SCs (LSCs), which are irreplaceable for corneal healing, various carriers for the culturing of LSCs and for their transplantation onto the recipient eye have been tested. They include fibrin glue (24), polymers or collagen sponges (26), and human amniotic membrane (30).

In the last years, promising scaffolds for the growth and transfer of various types of SCs have been offered

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by nanotechnology. Electrospinning processes can fabricate nanofibers with a diameter ranging from a few tens to hundreds of nanometers and with a defined porosity. The three-dimensional structure of nanofibrous materials has an extremely large surface area, and nanofibers can mimic the structure of extracellular matrix proteins, which provide support for cell growth and function. Nanofiber scaffolds can create specific niches where SCs can reside and maintain their unique properties. It has been shown that embryonic SCs or MSCs grow and differentiate on nanofibers comparably or even better than on plastic surfaces (10,20,27,33,34). We sought to determine whether adult tissue-specific SCs can also be grown on nanofiber scaffolds and whether these scaffolds can be used as carriers for cell transplantation in tissue regeneration.

Using the original Nanospider electrospinning technology we prepared nanofiber scaffolds from a panel of natural and synthetic polymers and tested them for their biocompatibility and their ability to support the growth of various cell types (S. Kubinova et al., manuscript submitted for publication). On the basis of the stability of its nanofibrous architecture in aqueous solutions and its optimal biocomptibility, we selected copolymer polyamide 6/12 (PA6/12) for further studies. We characterized the growth properties of LSCs and MSCs on these nanofibers and used PA6/12 scaffolds for the transfer of LSCs and MSCs to treat ocular surface injuries in an experimental mouse model.

MATERIALS AND METHODS

Mice

Mice of the inbred strains BALB/c and C57BL/10Sn of both sexes at the age of 2–4 months were used in the experiments. The animals were obtained from the breeding unit of the Institute of Molecular Genetics, Prague. The use of animals was approved by the local Animal Ethics Committee of the Institute of Molecular Genetics. The animals were treated in accordance with the Principles of Laboratory Animal Care.

Materials and Nanofiber Preparation

The copolymer PA6/12 was purchased from Chemopharma (Wien, Austria). This material (10 wt%) was dissolved in 85 wt% formic acid (Penta Company, Fairfield, NJ) and heated at 50°C for 6 h. After reducing the temperature to room temperature, the material was used for electrospinning. A modified needleless Nanospider™ technology (U.S. patent No. WO205024101.2005), in which polymeric jets are spontaneously formed from liquid surfaces on a rotating spinning electrode, was used for the preparation of the nanofibers. This Nanospider technology flexibly enables the formation of fibers tens of nanometers to tens of micrometers in diameter. All nanofibrous samples used during this study were prepared at a basis weight of 3–5 g/m^2 and had nanofiber diameter ranging from 290 to 539 nm.

To test the stability of nanofibers in aqueuos solutions, the nanofibrous samples were cut into small pieces and soaked in deionized water in petri dishes. The water was exchanged every day. After a 7- or 14-day period of soaking the samples were dried at room temperature, and their nanofibrous architecture was analyzed using scanning electron microscopy (SEM).

LSCs, MSCs, Corneal Epithelial and Endothelial Cell Lines

LSCs were obtained by enzyme digestion from limbal tissues as we have recently described (13). In brief, limbal tissues from 10–12 BALB/c mice were cut with scissors and subjected to 10 short (10 min each) trypsinization cycles. The released cells were harvested after each cycle, centrifuged (8 min at $250 \times g$) and resuspended in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS, Sigma), antibiotics (100 U/ml of penicillin, 100 µg/ml of streptomycin), 10 mM HEPES buffer, and 2×10^{-5} M 2-mercaptoethanol. The cells were seeded into 12-well tissue culture plates (Nunc, Roskilde, Netherlands) and after 1 week expanded in 25-cm^2 tissue culture flasks (Corning, Schipol-Rijk, Netherlands). For the growth on nanofibers, cells growing in vitro for 2–3 weeks were used.

MSCs were isolated from femurs and tibias of BALB/c mice. 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/ml in complete RPMI-1640 medium in 25-cm^2 tissue culture flasks (Corning). On the following day 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. After 3 weeks of culturing, the cells were characterized phenotypically by flow cytometry (over 90% of them were MHC class II$^-$, CD86$^-$, and CD11b$^-$, but the majority was CD105$^+$) and for their ability to differentiate into adipocytes (data not shown).

Mouse corneal epithelial and endothelial cell lines, prepared by the immortalization of mouse corneal epithelial and endothelial cells (11), were also tested for their growth on nanofiber scaffolds.

Demonstration of the Immunosuppressive Properties of MSC In Vitro

Spleen cells (0.5×10^6/ml) from BALB/c mice were cultured in 200 µl of RPMI-1640 medium containing 10% FCS in 96-well tissue culture plates (Nunc), either

unstimulated or were stimulated with 1.0 μg/ml of concanavalin A (Con A, Sigma). MSC were added to these cultures at a ratio of MSCs to spleen cells of 1:2, 1:4, or 1:8. Cell proliferation was determined by incorporation of [^{3}H]thymidine (1 μCi/well, Nuclear Research Institute, Rez, Czech Republic) added to the cultures for the last 6 h of a 72-h incubation period. The cells were harvested using an Automash 2000 cell harvestor (Dynex, Chantilly, VA) and the radioactivity was determined. The presence of IFN-γ in the supernatants was assessed by an enzyme-linked immunosorbent assay (ELISA) using capture and detection anti-cytokine antibodies purchased from PharMingen (San Diego, CA) and following the instructions of the manufacturer.

Morphology of Cells Growing on Nanofibers and Plastic

LSCs or MSCs were cultured at various cell concentrations on nanofibers fixed in the inserts or on plastic surfaces. Nanofiber scaffolds were cut into squares (approximately 1.5 × 1.5 cm) and fixed into CellCrownTM^{24} inserts (Scaffdex, Tampere, Finland). The inserts with nanofibers were sterilized by UV light, soaked in sterile destilled water, washed in culture medium, and transferred into 24-well tissue culture plates (Corning). Fifty thousand cells in a volume of 700 μl of culture RPMI-1640 medium with 10% of FCS was transferred into each well. One or 2 days after seeding, the cells were fixed for 15 min in 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), and treated with Chemiblocker (1:20, Chemicon, Temecula, CA) and Triton X-100 (0.2%, Sigma). To label F-actin, the cells were incubated with Alexa fluor 568 Phalloidin (Molecular Probes, Invitrogen, Paisley, UK) diluted 1:300 in PBS containing 1% bovine serum albumin (Sigma) and 0.5% Triton X-100 (Sigma) overnight at room temperature. The nuclei were vizualized by using 4′,6-diamidino-2-phenylindole (DAPI) fluorescent dye (Invitrogen). Images were taken by a laser scanning confocal microscope (Zeiss, Jena, Germany).

Determination of Cell Proliferation

The proliferation of cells growing on plastic or nanofibers was determined according to [^{3}H]thymidine incorporation. The cells (50×10^3/well/700 μl of culture medium) were seeded into the wells of 24-well tissue culture plates (Corning) with or without inserts containing nanofibers. The plates were incubated for 24 or 48 h and cell proliferation was determined by adding [^{3}H]thymidine (3 μCi/well, Nuclear Research Institute) for the last 6 h of the incubation period. The radioactivity incorporated in cells growing on plastic or nanofibers was measured using a Tri-Carb 2900TR scintilation counter (Packard, Meridien, CT).

Determination of Metabolic Cell Activity

The metabolic activity of living cells was determined by the WST assay. The assay is based on the ability of living cells to cleave by mitochondrial dehydrogenases tetrazolium salts into water soluble formazan, which is then measured by spectrophotometry. Fifty thousand cells in 700 μl of RPMI-1640 culture medium were cultured in the wells of 24-well tissue culture plate (Corning) with or without inserts containing nanofibers for 24 h at 37°C in an atmosphere of 5% CO_2. WST-1 reagent (Roche, Mannheim, Germany) (10 μl/100 μl of the medium) was added to each well, and the plates were incubated for another 4 h to form formazan. Formazan-containing medium (100 μl) was transferred from each well into the wells of a 96-well tissue culture plate (Corning) and the absorbance was measured using a Sunrise Remote ELISA Reader (Grödig, Austria) at a wavelength of 450 nm.

A Model of the Damaged Ocular Surface and Cell Transfers

The recipient BALB/c mice were deeply anesthetized by an intramuscular injection of a mixture of xylazine and ketamine (Rometar, Spofa, Prague, Czech Republic). The surface (corneal region) of the right eye was damaged by epithelial debridement with a sharp needle (G23) and the limbus was cut out with Vannas scissors (Duckworth and Kent, Baldock, UK). To induce a stronger immune reaction in the anterior segment of the eye, an allogeneic limbus from C57BL/6 donors was grafted orthotopically to the recipients with a removed limbus according to the technique of Maruyama et al. (17). A 4-mm-diameter nanofiber circle (with or without SCs) was used to cover the limbal and corneal region and was sutured with four interrupted sutures using 11.0 Ethilon (Ethicon, Johnson & Johnson, Livingston, England) on the damaged ocular surface. The nanofibers with growing cells were transferred with the cell side facing down towards the ocular surface. For the cell transfer, equal numbers of LSCs and MSCs growing on the nanofiber scaffold were transferred, approximately 4×10^4 cells of each type. The eyelids were closed by tarsorhapy using one suture of Resolon 7.0 (Resorba, Nuremberg, Germany) for 72 h. An ophthalmic ointment compound containing bacitracin and neomycin (Ophthalmo-Framykoin, Zentiva, Prague, Czech Republic) was applied on the ocular surface for 3 days. The nanofiber scaffolds were removed from the ocular surface on day 3 after the operation.

To trace the fate and survival of LSCs after their

transfer onto the ocular surface, the cells were labeled with the fluorescent vital dye PKH26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma) according to the instructions of the manufacturer, cultured for 24 h on a nanofiber scaffold, and transferred on the damaged eye surface as described above. The recipients were killed 2, 7, or 14 days after cell transfer and the whole globes were dissected and placed for 1 h into 4% paraformaldehyde. Then the globes were transferred into a 15% sucrose solution in PBS for 24-h fixation; subsequently, cryosections at a thickness of 7 μm were prepared using a Leica CM 3050 S cryostat (Leica, Wetzlar, Germany). The nuclei were stained with DAPI. The presence of stained cells was analyzed using a fluorescent microscope.

Determination of Inflammatory Reaction by Real-Time PCR

The expression of genes for IL-2 and IFN-γ and for inducible nitric oxide synthase (iNOS) in cells from the ocular surface was detected by real-time PCR. The whole ocular surface (including the cornea and limbal region) was removed using Vannas scissors on day 7 after the operation and transferred into Eppendorf tubes containing 200 μl of TRI Reagent (Molecular Research Center, Cincinnati, OH). Total RNA was extracted using TRI Reagent according to the manufacturer's instructions. Total RNA (2 μg) was treated using deoxyribonuclease I (Sigma) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega, Madison, WI) in a total reaction volume of 25 μl using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed in an iCycler (BioRad, Hercules, CA) using the primers described in Table 1. iQ SYBR Green Supermix (BioRad) was used in all experiments. The PCR parameters included denaturation at 95°C for 3 min, then 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 on the iCycler Detection system, Version 3.1. Each single experiment was done in triplicate. The relative quantification model was applied to calculate the expression of the target gene in comparison to GAPDH used as an endogenous control.

Statistical Analysis

Analysis of data showed normal distribution and the results are expressed as mean ± SE. Comparisons between two groups were analyzed by Student *t*-test, and multiple comparisons were analyzed by ANOVA followed by Bonferroni post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Comparison of Metabolic Activity and Growth Properties of Cells Growing on PA6/12 Nanofibers or on Plastic Surfaces

LSCs, MSCs, and corneal epithelial or corneal endothelial cells were grown for 24 or 48 h on nanofibers (fixed in inserts) or on a plastic surface in 24-well tissue culture plates and their metabolic and proliferative activities were determined. As demonstrated in Figure 1, all four cell types had comparable metabolic activities and proliferative capacities irrespective of whether they grew on the plastic surface or on nanofibers. The growth of LSCs and MSCs on nanofibers was confirmed when the metabolic activity that corresponds to the number of living cells was determined at different time intervals. As shown in Figure 2, the metabolic activity of SCs gradually increased during the 48-h incubation period.

The Morphology of Cells Growing on Nanofibers

The shape of the cells and the organization of the actin cytoskeleton of LSCs growing on PA6/12 nanofibers were compared with those of cells growing on plastic surfaces. Figure 3 shows that the shape of the cells and the organization and thickness of the actin filaments formed in adherent cells were comparable between cells growing on nanofibers and plastic surfaces. Confocal and electron microscopy showed the penetration of LSCs into the nanofibrous structure and the growth of pseudopodia among the nanofibers (data not shown).

Transfer of LSCs and MSCs Using Nanofiber Scaffolds Onto the Damaged Ocular Surface

To prove that LSCs can be transferred using a nanofiber scaffold onto the ocular surface and that they can subsequently migrate from the scaffold onto the dam-

Table 1. Mouse Primer Sequences Used for Real-Time PCR

Gene	Sense Primer	Antisense Primer
GADPH	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
IL-2	GCTGTTGATGGACCTACAGGA	TTCAATTCTGTGGCCTGCTT
IFN-γ	ATCTGGAGGAACTGGCAAAA	TTCAAGACTTCAAAGAGTCTGAGG
iNOS	CTTTGCCACGGACGAGAC	TCATTGTACTCTGAGGGCTGAC

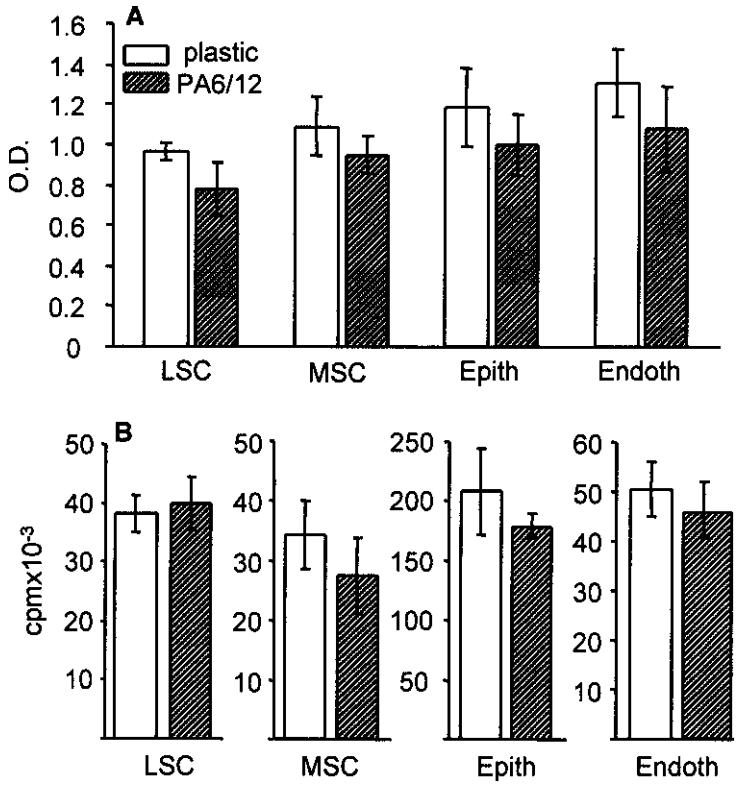

Figure 1. Metabolic and proliferative activities of LSCs, MSCs, and corneal epithelial and corneal endothelial cells growing on plastic or nanofibers. The same number of cells was seeded on plastic (24-well tissue culture plate) or PA6/12 nanofibers fixed in inserts. (A) The metabolic activity was determined by adding WST-1 reagent to the cultures for the last 4 h of a 24-h incubation period. (B) The proliferative activity was determined by adding [^{3}H]thymidine into the culture medium for the last 6 h of a 24-h incubation period. Each bar represents the mean ± SE from three to four determinations.

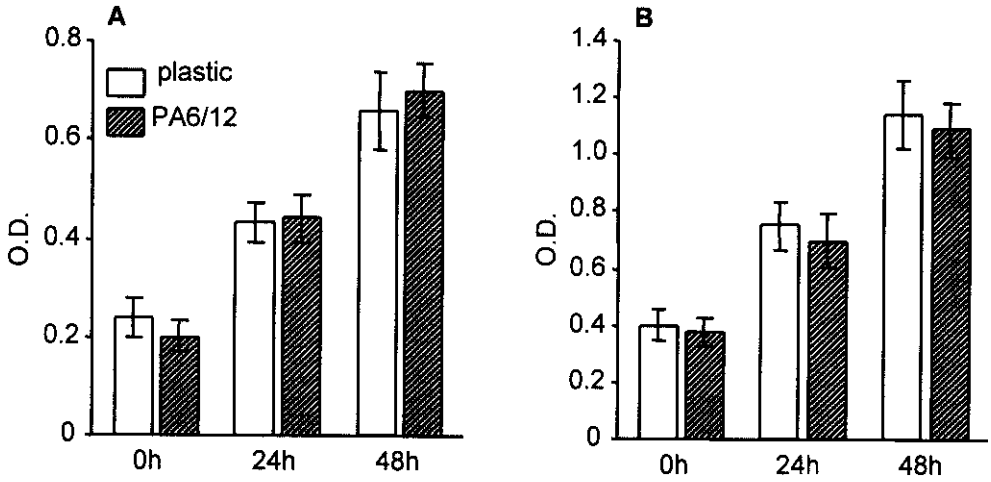

Figure 2. Comparison of the growth of LSCs and MSCs on plastic or nanofibers. Equal numbers of MSCs (A) or LSCs (B) were seeded into the wells of a 24-well tissue culture plate or onto PA6/12 nanofibers fixed in inserts and the metabolic activity of the living cells was determined at the beginning of culture (0 h) and after 24- or 48-h incubation. Each bar represents the mean ± SE from three determinations.

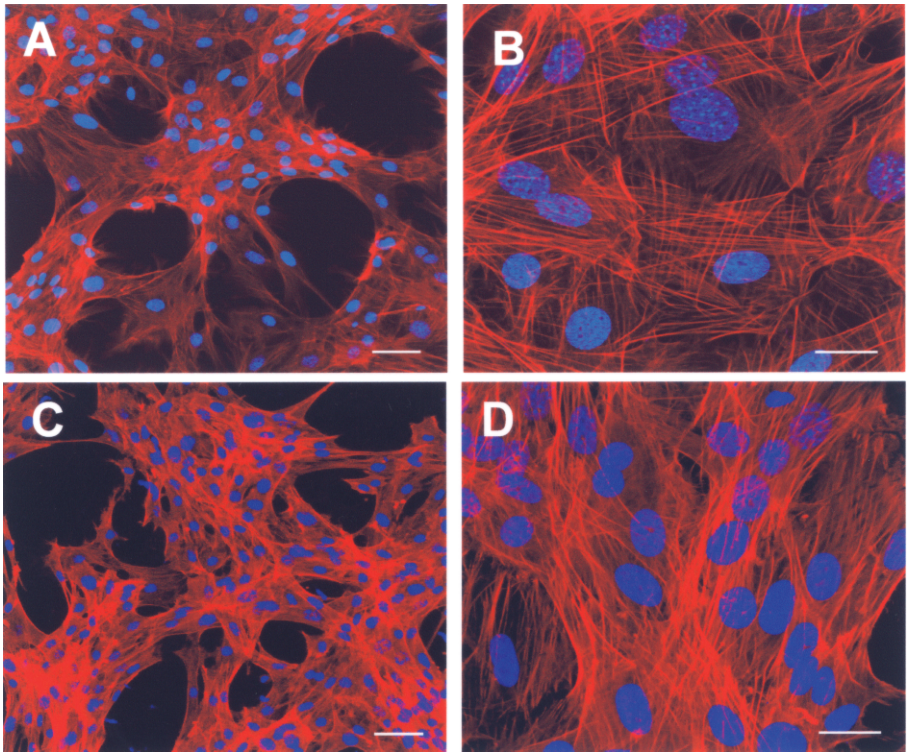

Figure 3. The morphology of LSCs growing on a glass surface or on PA6/12 nanofibers. The cells were cultured for 24 h on poly-L-lysine-coated glass inserts in 24-well tissue culture plates or on nanofibers fixed in inserts and were stained for F-actin with phalloidin (red filaments). The nuclei are blue (DAPI staining). (A, B) LSCs growing on the glass surface at two different magnifications. (C, D) LSCs growing on nanofiber scaffolds. Scale bars: (A, C) 50 μm; (B, D) 20 μm.

aged ocular surface, we labeled LSCs with the fluorescent dye PKH26, cultured them on PA6/12 nanofibers, and transferred them onto the damaged ocular surface. The globes were harvested at different time intervals after cell transfer and cryosections were prepared. As demonstrated in Figure 4, PKH26-labeled cells were clearly detected on the ocular surface on days 2, 7, and 14 after cell transfer.

Suppression of a Local Inflammatory Reaction by LSCs and MSCs

First, we demonstrated the immunosuppressive properties of MSC in vitro. Mouse spleen cells were stimulated with the T-cell mitogen Con A in the presence or absence of MSCs. As demonstrated in Figure 5, MSCs inhibited cell proliferation (Fig. 5A) and IFN-γ production (Fig. 5B) in a dose-dependent manner. No immunosuppression was observed if LSCs were used instead of MSCs, and the suppression by MSCs was preserved if MSCs were tested in a mixture with LSCs (data not shown).

To demonstrate the suppression of a local inflammatory reaction by the transfer of LSCs and MSCs in vivo, two models of ocular surface damage and treatment were used. In the first model, the ocular surface was mechanically damaged. The second model combined mechanical damage and the transplantation of allogeneic limbus. In the healthy, nondamaged eye, no detectable expression of IL-2, IFN-γ, or iNOS genes was found. The mechanical injury induced a moderate inflammatory reaction associated with the production of IFN-γ and iNOS. This response was inhibited after the transfer of nanofibers containing LSCs and MSCs (data not shown). The ocular surface damage associated with orthotopic limbal allotransplantation induced a strong inflammatory reaction characterized by the expression of the IL-2, IFN-γ, and iNOS genes (Fig. 6). This reaction was slightly inhibited by covering the eye surface with cell-free nanofibers and was significantly attenuated after the transfer of LSCs and MSCs growing on nanofiber scaffold (Fig. 6).

DISCUSSION

A growing body of recent studies has shown that electrospun nanofibers of various polymers allow the adhesion, proliferation, metabolic activity, morphology,

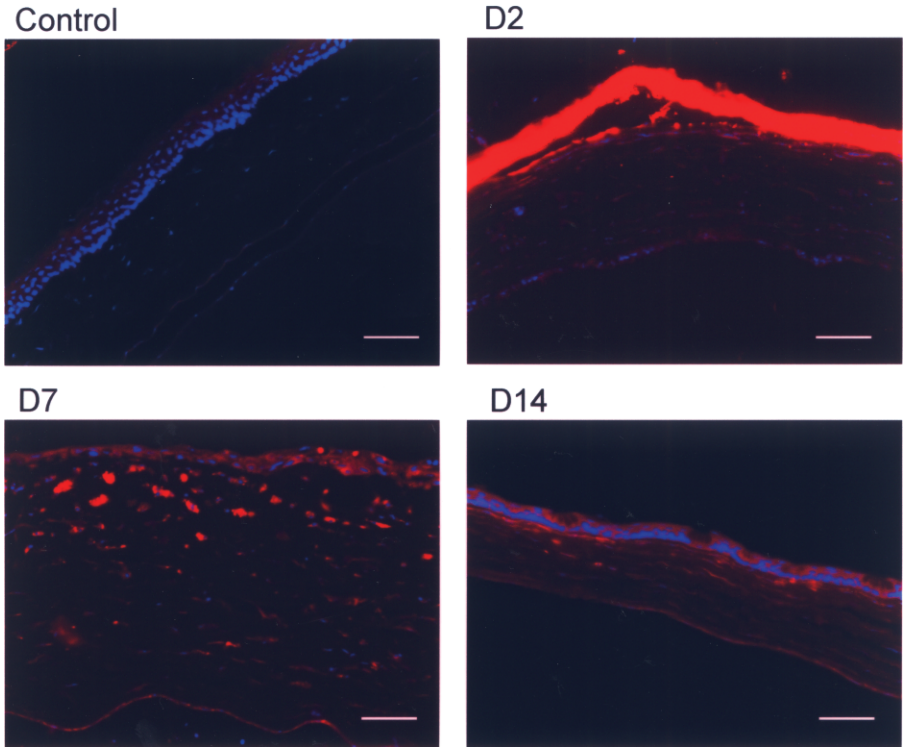

Figure 4. Detection of PKH26-labeled LSCs on the damaged ocular surface after their transfer on a PA6/12 nanofiber scaffold. LSCs were labeled with PKH26 and cultured for 24 h on nanofibers fixed in inserts. The nanofibers with cells were transferred and fixed (with the cell side facing down) for 3 days on the damaged ocular surface. The globes were removed 2, 7, or 14 days after the operation and 7-µm cryosections were prepared. The nuclei were vizualized with DAPI. The cryosections were prepared (A) from the control undamaged eye (without labeled cells), (B) from the eye 2 days after operation (the nanofiber scaffold with labeled LSCs is seen as a red lane, corneal epitheliun is removed), and from the eyes 7 (C) and 14 (D) days after the cell transfer (red stained cells are still present, the corneal epithelium is regenerated). Scale bars: 50 µm.

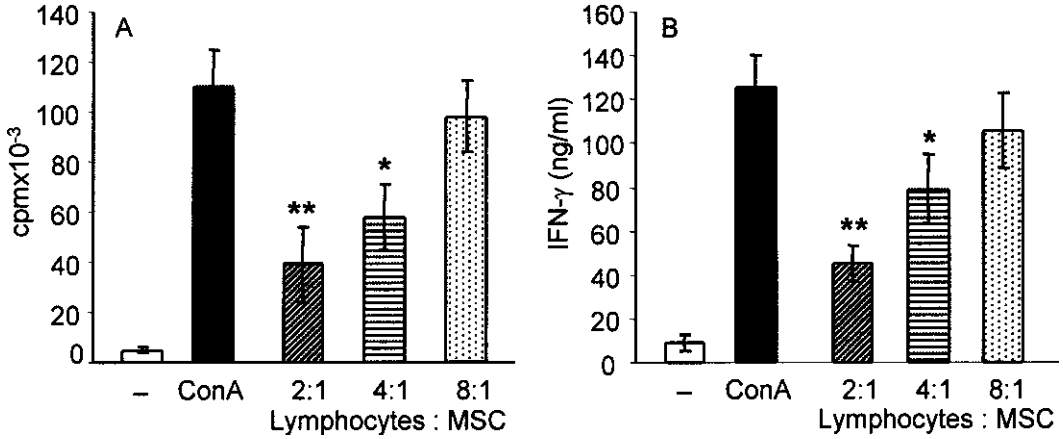

Figure 5. Immunosuppressive properties of MSCs in vitro. Spleen cells from BALB/c mice were cultured either unstimulated or stimulated with Con A (1.0 µg/ml) in the presence or absence of MSCs (at a ratio of 2:1, 4:1, or 8:1). Cell proliferation (A) was determined by [^{3}H]thymidine incorporation after a 72-h incubation period, the production of IFN-γ (B) was measured by ELISA in the supernatants after a 48-h incubation period. Each bar represents the mean ± SE from three experiments. *$p < 0.01$, **$p < 0.001$.

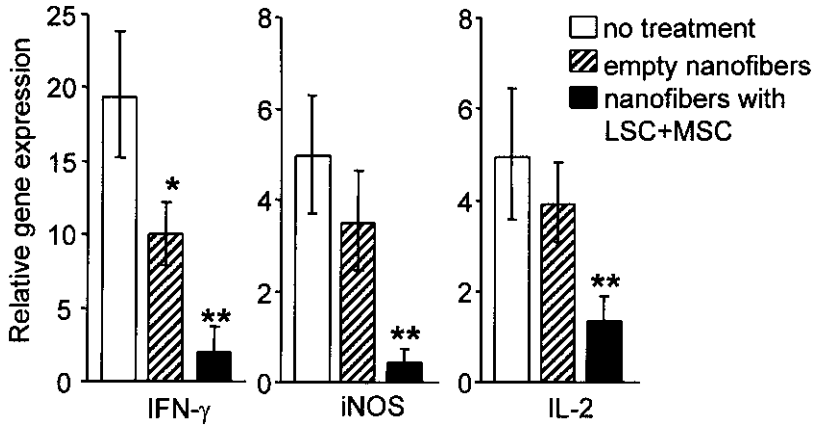

Figure 6. Suppression of the local inflammatory reaction after the transfer of LSCs and MSCs on a nanofiber scaffold. The inflammatory reaction was induced on ocular surface by epithelial debridement and orthotopic limbal allotransplantation. The ocular surface then remained untreated, covered with nanofibers without cells or covered with nanofibers with LSCs and MSCs. On day 7 after cell transfer the ocular surface was removed and the expression of genes for IL-2, IFN-γ, and iNOS was determined by real-time PCR. The comparative Ct method was used to determine the extent of the targeted gene expression normalized to an internal GAPDH control. Each bar represents the mean ± SE from three experiments (two mice in each experiment). $*p < 0.05$ in comparison to uncovered damaged eyes, $**p < 0.001$ in comparison to damaged eyes covered with cell-free (empty) nanofibers.

and organized assembly of different cell types in vitro (2,5,18,25,35). The constructs formed by nanofiber scaffolds and specialized cells have been suggested as perspective and promising tools for tissue engineering (14,15). However, the performance and behavior of nanofibrous materials in vivo are not well understood. Only scarce recent data are available to demonstrate the usefulness of nanofiber scaffolds in vivo.

We described here the preparation of electrospun nanofibers and their use as a scaffold to grow and transfer LSCs and MSCs to treat ocular surface injuries and SC deficiencies. The nanofibers were prepared by the original needleless Nanospider technology, which enables the creation of nanofibers from various polymers and of defined fiber diameter, porosity, and a basis weight. From a large panel of polymers tested in our pilot experiments, we selected copolymer PA6/12. This polymer turned out to be sufficiently biocompatible, forming nanofibers stable in aqueous solutions and suitable for the growth of limbal and mesenchymal SCs. In addition, differentiated corneal epithelial and endothelial cells were grown on PA6/12 nanofibers and their metabolic and proliferative activities were comparable with those of the same cells grown on plastic surfaces.

We have made a successful attempt to use the nanofiber scaffolds for the transfer of LSCs to treat ocular surface injuries or LSC deficiencies in an experimental mouse model. The limbus represents the region in the eye where SCs reside that are responsible for corneal renewal and repair (4,31). We have recently shown that the cells obtained by trypsinization of limbal tissue express the markers and characteristics of LSCs and that these cells can be propagated in vitro in tissue culture (13). Here we have shown that LSCs can be cultured on a polyamide nanofiber scaffold and that their proliferation, metabolic activity, and morphology when grown on nanofibers are comparable with those of cells grown on plastic surfaces. In addition, we have shown that LSCs growing on a nanofiber scaffold can be successfully transferred onto the damaged ocular surface. So far, human amniotic membranes have been used most frequently as a matrix for the growth and transfer of LSCs for therapeutic purposes (26,30). In spite of the use of human allogeneic amniotic material as a scaffold of LSCs for the reconstruction of the ocular surface, the beneficial effects of LSC transplantation have been reported (22). The use of biocompatible synthetic polymers for the preparation of nanofiber scaffolds for the growth and transfer of LSC would have apparent advantages.

We are aware that the limbal cell population that we transferred using the nanofiber scaffold was not a pure SC population. The population contained also differentiated cells originating from LSCs and other cell types of the LSC niche. Analysis of the gene expression of the transferred cells revealed that this cell population contained both differentiated epithelial cells (expressing CK12 and connexin 43) and cells expressing the putative LSC markers ABCG2, p63, and Lgr5 [(13), and unpublished data]. However, such a cultured population of

limbal epihelial cells is generally referred to as LSCs, and their therapeutic potential in the treatment of LSC deficiencies has been documented (22,32).

In addition to LSCs, we also grew on nanofiber scaffolds MSCs, which can differentiate to various cell types including corneal epithelial cells (8,19,23) and that have the ability to inhibit immunological reactions (1,6). We confirmed the immunosuppressive effects of MSCs in vitro by the inhibition of T-lymphocyte proliferation and by the suppression of cytokine production. It has been shown that the transfer of MSCs onto the damaged eye can support the healing process, but the effects of MSCs were attributed to the ability of MSCs to inhibit inflammatory reactions (15,21). In our experiments we cocultured LSCs and MSCs on nanofiber scaffolds and transferred them onto the damaged ocular surface where a local inflammatory reaction characterized by the expression of genes for IL-2, IFN-γ, and iNOS was induced by epithelial debridement and limbal allotransplantation. Analysis of the gene expression in tissue harvested from the damaged ocular surface 1 week after the injury showed that there was a significant suppression of the gene expression of the inflammatory proteins IL-2, IFN-γ, and iNOS, suggesting that the MSCs suppress the inflammatory reaction. This suggestion is supported by previous data showing that MSCs applied to the ocular surface can inhibit the local inflammatory reaction (15,21).

It has been demonstrated previously that embryonic SCs can be grown, propagated, and differentiated on nanofibers (20,28). Our results suggest that nanofiber scaffolds prepared from PA6/12 by electrospinning technology can serve as a convenient matrix for the growth of adult tissue-specific SCs and for their transfer to treat ocular surface injuries. Studies are in progress to test the survival and propagation of cells in terms of the persistance of SC markers and characteristics during their growth on nanofiber scaffolds and to monitor the presence of LSCs after their transfer onto the ocular surface in experimental LSC deficiency models.

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5.4 Využití nanovlákenných nosičů připravených technikou elektrostatického zvlákňování a nesoucích CsA a kmenové buňky pro buněčnou terapii a lokální imunosupresivní působení

Holáň V., Chudíčková M., Trošan P., **Svobodová E**., Krulová M., Kubinová Š., Syková E., Širc J., Michálek J., Juklíčková M., Munzarová M., Zajícová A.
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CsA je účinná imunosupresivní látka s nízkou rozpustností ve vodě. Proto byl CsA rozpuštěn v roztoku kyseliny polymléčné a z této směsi byly pomocí technologie elektrostatického zvlákňování připraveny nanovlákenné nosiče. Přidání CsA do roztoku kyseliny polymléčné ani proces elektrostatického zvlákňování neovlivnily strukturu nanovláken ani farmakologickou aktivitu CsA. Studium stability nanovlákenných nosičů s CsA při kultivaci ukázalo uvolňování CsA po dobu nejméně 96 hodin. Po umístění nanovláken s CsA na kožní alogenní transplantát došlo ke zpomalení uvolňování CsA, přičemž přibližně 35% léčiva bylo přítomno v nanovláknech 8 dnů po transplantaci. Přidání nanovláken s CsA do kultur slezinných buněk stimulovaných konkanavalinem A selektivně potlačovalo funkce T lymfocytů. Aktivita stimulovaných makrofágů ani jiných typů buněk nebyla potlačena v přítomnosti nanovláken obsahujících CsA. Překrytí kožních alotransplantátů nanovlákny s CsA významně potlačilo lokální produkci prozánětlivých cytokinů IL-2, IFN-γ a IL-17. Výsledky ukazují, že nanovlákna obsahující CsA mohou sloužit jako účinný nosič farmakologicky aktivních látek nebo buněk pro lokální potlačení zánětlivé odpovědi a buněčnou terapii.

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Cyclosporine A-loaded and stem cell-seeded electrospun nanofibers for cell-based therapy and local immunosuppression

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ABSTRACT

Cyclosporine A (CsA), a potent immunosuppressive drug with low water solubility, was dissolved in poly (L-lactic acid) (PLA) solution, and nanofibers were fabricated from this mixture by electrospinning technology. The addition of CsA into the PLA solution and the conditions of the electrospinning process did not influence the structure of the nanofibers nor affect the pharmacological activity of CsA. Study of the CsA release behavior in culture medium showed a release for at least 96 h. After the topical application of CsA-loaded nanofibers on skin allografts in vivo, the release was significantly slower and about 35% of the drug was still retained in the nanofibers on day 8. The addition of CsA-loaded nanofibers into cultures of mouse spleen cells stimulated with Concanavalin A selectively inhibited T cell functions; the activity of stimulated macrophages or the growth of non-T-cell populations was not suppressed in the presence of CsA-loaded nanofibers. The covering of skin allografts with CsA-loaded nanofibers significantly attenuated the local production of the proinflammatory cytokines IL-2, IFN-γ and IL-17. These results suggest that CsA-loaded electrospun nanofibers can serve as effective drug carriers for the local/topical suppression of an inflammatory reaction and simultaneously could be used as scaffolds for cell-based therapy.

1. Introduction

Nanofibers prepared by the electrospinning technology have turned out to be convenient and promising carriers for targeted drug delivery or as scaffolds for cell transfer in regenerative medicine. So far, a wide range of various molecules have been incorporated into the polymers used for nanofiber formation or bound on the surface of the nanofibers [1–4]. The release of therapeutic molecules from nanofibers depends on the type of polymer as well as the molecular weight and hydrophobicity/hydrophilicity of the drug. To modulate the kinetics of drug release from nanofibers, different forms of polymers or composite nanofiber structures, including nanofibers, nanoparticules or self-assembling proteins, have been tested [5–8]. These materials have proven to be suitable carriers for a number of molecules, including antibiotics, vitamins, growth factors and chemotherapeutics [9].

In addition, nanofibers have been successfully used as scaffolds for cell-based therapy in regenerative medicine. It has been shown that various cell types can be grown on nanofibers which represent an optimal matrix [10–13]. If allogeneic cells are used for cell-based therapy, local immunosuppression, in addition to a systemic suppression of immunity, would be useful for protecting the foreign cells from immunological rejection. For this purpose, the development of biocompatible nanofibers that could serve as a carrier of the cells and that could simultaneously release a selective immunosuppressive drug would be of the utmost priority.

To approach this task, we prepared and tested nanofibers that were fabricated by the original needleless electrospinning technology from a biocompatible polymer, poly(L-lactic acid) (PLA). Nanofibers prepared from PLA can be seeded with different cell types and can serve as carriers for cell-based therapy. We have recently shown that cell-seeded nanofibers can be succesfully used as a scaffold for stem cell transfer to treat ocular surface injuries and limbal stem cell deficiencies [13]. Here we demonstrate that PLA nanofibers can be simultaneously loaded with the immunosuppressive drug Cyclosporine A (CsA), a widely used selective inhibitor of calcineurin and T-cell functions. So far, CsA has been incorporated

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into various types of nanoparticules, nanostructures or micells to prolong its therapeutic efficacy after oral administration or ocular delivery [14–17]. We show here that CsA can be incorporated into PLA nanofibers during the non-jet electrospinning process. These nanofibers with encapsulated CsA can be used for the local suppression of harmful T-cell-mediated immune reactions and, after seeding with cells, could simultaneously serve as scaffolds for cell-based therapy.

2. Materials and methods

2.1. Mice

Mice of both sexes of the inbred strains BALB/c and C57BL/6Sn (B6) were used in the experiments at the age of 7–10 weeks. The animals were obtained from the breeding unit of the Institute of Molecular Genetics, Prague. The use of animals was approved by the local Animal Ethics Committee of the Institute of Molecular Genetics.

2.2. Materials and nanofiber preparation

The PLA polymer was purchased from Nature Works LLC (Minnetonka, MN). This material was dissolved in chloroform at 7 weight percent (henceforth abbreviated as wt%), and two other solvents, 1,2-dichlorethane (29 wt.%) and ethyl acetate (10 wt.%) (both purchased from PENTA, Prague, Czech Republic), were added to this solution. The mixture was stirred until a homogeneous polymer solution was obtained. CsA (TEVA Czech Industries, Opava, Czech Republic) was dissolved in the prepared polymer solution to selected concentrations (1, 2.5, 5 and 10 wt%) and the solution stirred until the drug was dissolved. The modified needleless Nanospider™ technology [18], in which polymeric jets are spontaneously formed from liquid surfaces on a rotating spinning electrode, was used for the preparation of the nanofibers. This Nanospider technology flexibly enables the formation of fibers tens of nanometers to tens of micrometers in diameter and the preparation of nanofiber matrices with masses per unit area ranging from 1 to 100 g per m^2. During this study, nanofiber materials with a mass per unit area of 10 g/m^2 and containing, if not indicated otherwise, 10 wt.% CsA were used. These nanofibers had a diameter ranging from 290 to 539 nm and contained 1 μg of CsA/mm^2. The morphology of CsA-free and CsA-loaded PLA nanofibers and their nanofibrous architecture were analyzed using scanning electron microscopy (SEM).

2.3. T-cell proliferation assay and assessment of immunosuppression in vitro

Single cell suspensions of spleen cells from BALB/c mice were prepared in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS, Sigma), antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin), 10 mM HEPES buffer and 5×10^{-5} M 2-mercapoethanol (hereafter referred to as complete RPMI 1640 medium). The cells (0.5×10^6/ml) were cultured in a volume of 200 μl of complete RPMI 1640 medium in 96-well tissue culture plates (Nunc, Roskilde, Netherlands) unstimulated or stimulated with 1.0 μg/ml of Concanavalin A (Con A, Sigma). To determine cell proliferation, ^{3}H-thymidine (1 μCi/well, Nuclear Research Institute, Rez, Czech Republic) was added to each well for the last 6 h of a 72-h incubation period. The cells were harvested using an Automash 2000 cell harvestor (Dynex, Chantilly, VA) and the radioactivity incorporated in the cells was measured using a Tri-Carb 2900TR scintilation counter (Packard, Meridien, CT).

To characterize the immunosuppressive potential of CsA-loaded nanofibers, nanofiber samples of variable size (ranging from 1 mm^2 to 25 mm^2) and with different concentrations of CsA (0, 1, 2.5, 5 or 10 wt.%) were added into wells with Con A-activated spleen cells. Free CsA at concentrations ranging from 1 pg/ml to 100 μg/ml was added to cultures of Con A-stimulated cells as a positive control.

2.4. Determination of the kinetics of CsA release from nanofibers in vitro

Samples of nanofibers (size 5×5 mm, mass per unit area 10 g/m^2, 10 wt.% CsA) were soaked in wells with 250 μl of RPMI 1640 medium with 10% FCS at laboratory temperature. The nanofibers were repeatedly transferred after 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h into new wells containing fresh medium. The supernatants from the individual wells after each transfer were harvested, stored at −20 °C and tested for the inhibition of Con A-induced T-cell proliferation and IL-2 production.

2.5. Proliferation of nonlymphoid cells

Limbal stem cells (LSCs) were obtained by enzyme digestion of limbal tissue from BALB/c mice as we have described [19]. Mouse mesenchymal stem cells (MSCs) were prepared from bone marrow isolated from the femurs and tibias of BALB/c mice as described [20]. The mouse embryonal fibroblast cell line 3T3 was obtained from the American Cell Culture Collection. LSCs, MSCs or 3T3 cells were cultured at a concentration of 25×10^3/ml in a volume of 200 μl of complete RPMI 1640 medium in 96-well tissue culture plates (Nunc) alone or in the presence of CsA-free or CsA-loaded nanofibers (sample size 3×3 mm, mass per unit area 10 g/m^2, 10 wt.% CsA). The proliferation of the cells was determined by ^{3}H-thymidine added to the cultures for the last 6 h of a 48-h incubation period.

To determine the growth of LSCs, MSCs and 3T3 cells on plastic and nanofibers, nanofiber scaffolds were cut into squares (approximately 1.5×1.5 cm) and fixed into CellCrown™^{24} inserts (Scaffdex, Tampere, Finland). The inserts with nanofibers were transferred into 24-well tissue culture plates (Corning Co., Corning, NY). One hundred thousand cells in a volume of 700 μl of complete RPMI 1640 medium were transferred into each well. The plates were incubated for 24 h, and cell viability and metabolic activity were determined by the WST assay [13]. The assay is based on the ability of living cells to cleave tetrazolium salts by mitochondrial dehydrogenases into water soluble formazan, which is then measured by spectrophotometry. In brief, WST-1 reagent (Roche, Mannheim, Germany) (10 μl/100 μl of the medium) was added to each well, and the plates were incubated for another 4 h to form formazan. The absorbance was measured using a Sunrise Remote ELISA Reader (Gröding, Austria) at 450 nm.

2.6. Production and detection of cytokines and nitric oxide (NO)

Spleen cells from BALB/c mice (0.5×10^6/ml) were stimulated with Con A (1.0 μg/ml) in a volume of 800 μl of culture medium in 48-well tissue culture plates (Corning) in the absence or presence of nanofiber samples (size 3×3 mm, mass per unit area 10 g/m^2, 10 wt.% CsA). The concentrations of IL-2, IFN-γ and IL-17 in the supernatants were assessed by an enzyme-linked immunosorbent assay (ELISA) using capture and detection anti-cytokine antibodies purchased from PharMingen (San Diego, CA) (IL-2 and IFN-γ detection) or R & D Systems (Minneapolis, MN) (IL-17 detection) and following the instructions of the manufacturers.

For the production of IL-1β, IL-6 and NO, mouse peritoneal macrophages (1×10^6 cells/ml) were stimulated with lipopolysaccharide (LPS, 1.0 μg/ml, Difco Laboratories, Detroit, MI) and IFN-γ (5 ng/ml) in the absence or presence of nanofibers. The concentrations of IL-1β and IL-6 in the supernatants were measured by ELISA using capture and detection antibodies purchased from R & D Systems (for detection of IL-1β) or from PharMingen (for IL-6 detection). The levels of NO in the supernatants were detected after 48 h using the Griess reaction [21].

2.7. Mouse models of skin grafting

Skin grafts from B6 donors were transplanted into BALB/c recipients according to the technique of Billingham et al. [22]. The grafts (size 8×8 mm) were covered with CsA-free or CsA-loaded nanofibers (size 10×10 mm, mass per unit area 10 g/m^2, 10 wt.% CsA). The grafts were removed on day 7 after transplantation and the grafted tissue was used for real-time PCR analysis to detect the expression of genes for proinflammatory molecules; alternatively, the graft explants were cultured in vitro for 48 h [23] and the concentration of IL-17 in the supernatants was assessed by ELISA.

To determine the kinetics of CsA release in vivo, the CsA-loaded nanofibers were removed 1, 2, 4 or 8 days after transplantation, and the amount of pharmacologically active CsA retained in the nanofibers was determined. The immunosuppressive potential (suppression of T-cell proliferation, inhibition of IL-2 production) of small samples from the collected nanofibers was compared with that of the original CsA-loaded nanofibers or with a pure CsA standard, and the percentage of CsA remaining in the nanofibers was calculated.

2.8. Characterization of the inflammatory reaction by real-time PCR

The expression of genes for the proinflammatory cytokines IL-2 and IFN-γ in cells from control or nanofiber-covered skin allografts was determined by real-time PCR. The details of RNA isolation, transcription and the PCR parameters have been described previously [13,20]. In brief, total RNA was extracted from the tissues using TRI reagent (Molecular Research Center, Cincinnati, OH). One μg of total RNA was used for subsequent reverse transcription. Quantitative real-time PCR was performed using an iCycler (BioRad, Hercules, CA). The following primers were used for amplification: GAPDH – 5′-AGAACATCATCCCTGCATCC (sense), 5′-ACATTGGGGGTAGGAACAC (antisense), IL-2 – 5′-GCTGTTGATGGACCTACAGGA (sense), 5′-TTCAATTCTGTGGCCTGCTT (antisense) and IFN-γ - 5′-ATCTGGAGGAACTGGCAAAA (sense), 5′-TTCAAGACTTCAAAGAGTCTGAGG (antisense). Each single experiment was done in triplicate. The relative quantification model was applied to calculate the expression of the target gene in comparison to GAPDH used as an endogenous control.

2.9. Statistical analysis

Each experiment was repeated at least three times, and the results are expressed as mean ± SD. Comparisons between two groups were analyzed by Student′s *t*-test, and multiple comparisons were analyzed by ANOVA. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Morphology of CsA-loaded nanofibers

Nanofibers from PLA polymer were prepared with various concentrations (weight percentage, wt.%) of CsA. At all tested concentrations (ranging from 1 to 10 wt.%), CsA did not influence the nanofiber formation and did not have any effect on nanofiber density, diameter, porosity or architecture (Fig. 1).

3.2. Immunosuppressive properties of CsA-loaded nanofibers

The addition of CsA-loaded PLA nanofibers, but not PLA nanofibers without CsA, into wells with Con A-stimulated spleen cells strongly inhibited T-cell proliferation (Fig. 2A). CsA-loaded nanofibers also inhibited in a dose-dependent manner the production of IL-2 (Fig. 2B), IL-17 (Fig. 2C) and IFN-γ (Fig. 2D) by Con A-stimulated spleen cells. The inhibition depended on the concentration of CsA in the PLA (Figs. 2A–D) and on the mass per unit area of the nanofiber material (data not shown). In contrast, the production of IL-1β (Fig. 2E) or NO (Fig. 2F) by LPS/IFN-γ stimulated macrophages was not inhibited in the presence of CsA-loaded nanofibers.

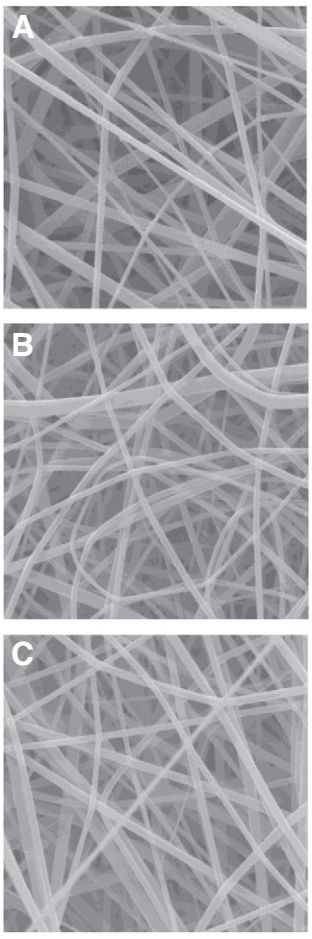

Fig. 1. SEM characterization of CsA-loaded PLA nanofibers. A - 0% CsA, B - 1 wt.% CsA, C - 10 wt.% CsA. Magnification: 5000×.

3.3. The kinetics of drug release in vitro and in vivo

To study the kinetics of CsA release from CsA-loaded nanofibers, samples of nanofiber material (size 5×5 mm, mass per unit area 10 g/m^2, 10 wt.% CsA) were soaked in 250 μl of complete RPMI 1640 medium, and at the indicated time intervals the nanofibers were transferred into the same volume of fresh medium. The presence of pharmacologically active CsA released into the culture media was assessed at individual time intervals by its ability to inhibit T-cell proliferation and IL-2 production. As demonstrated in Fig. 3A and B, a significant amount of CsA was detected in the samples harvested at intervals within the first 12 h of incubation, but the release of CsA continued subsequently for at least 96 h.

To evaluate the release behavior of CsA in vivo, CsA-loaded nanofibers covering skin allografts were collected 1, 2, 4 and 8 days after grafting, and the presence of CsA in the nanofibers was tested for its ability to inhibit Con A-induced T-cell proliferation and IL-2 production. The results showed that nanofibers collected at all tested days still retained a sufficient amount of CsA to effectively inhibit T-cell proliferation (Fig. 4A) and IL-2 production (Fig. 4B). A quantitative determination of CsA content in nanofibers collected at the individual time points showed, in comparison with the amount of CsA in the original nanofibers at time 0, that about 50% of the CsA was retained in the nanofibers on day 2 and about 35% of the original CsA content remained in the nanofibers on day 8 after grafting.

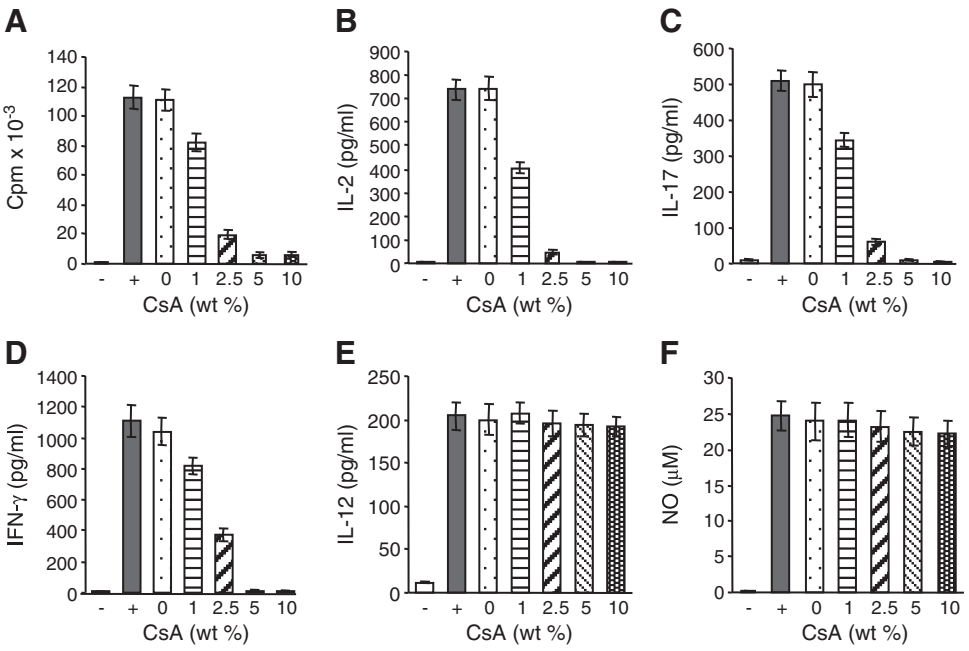

Fig. 2. Selective inhibition of T-cell proliferation and cytokine production by CsA-loaded PLA nanofibers. Mouse spleen cells were cultured unstimulated (−), stimulated with Con A (+) or stimulated with Con A in the presence of nanofiber material (size 2×2 mm, mass per unit area 10 g/m^2) with the indicated content (wt.%) of CsA. Cell proliferation (A) and the production of IL-2 (B), IL-17 (C) and IFN-γ (D) were determined. The production of IL-1β (E) and NO (F) by macrophages stimulated with LPS/IFN-γ in the presence of nanofibers with the indicated content of CsA was also assessed.

3.4. The growth of cells on CsA-loaded PLA nanofibers

Three types of cells, LSCs, MSCs and 3T3 fibroblasts, were cultured for 72 h in plastic tissue culture plates in the presence of CsA-loaded PLA nanofibers and cell proliferation was determined according to ^{3}H-thymidine incorporation. It was observed that the cells grew in the presence of CsA-loaded nanofibers comparably as in the presence

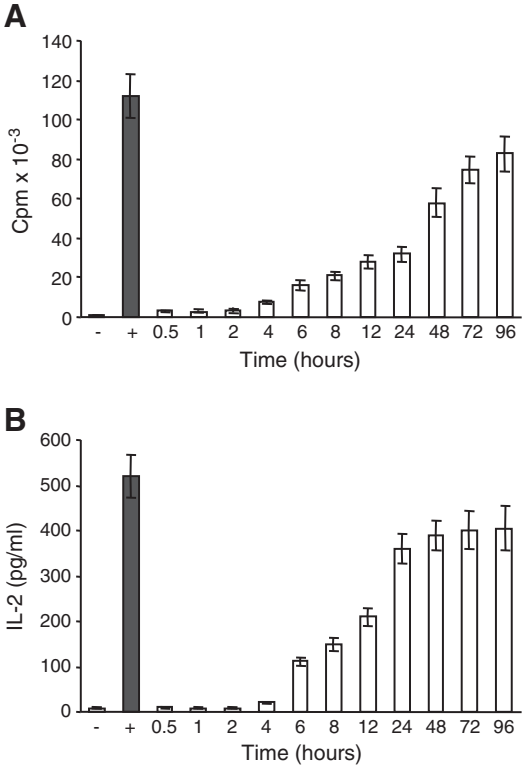

Fig. 3. The kinetics of CsA release from nanofibers in vitro. CsA-loaded nanofibers were soaked in culture medium and transferred at the indicated time intervals into fresh medium. Samples of medium after each transfer were harvested and tested for their ability to inhibit Con A-induced T-cell proliferation (A) and IL-2 production (B).

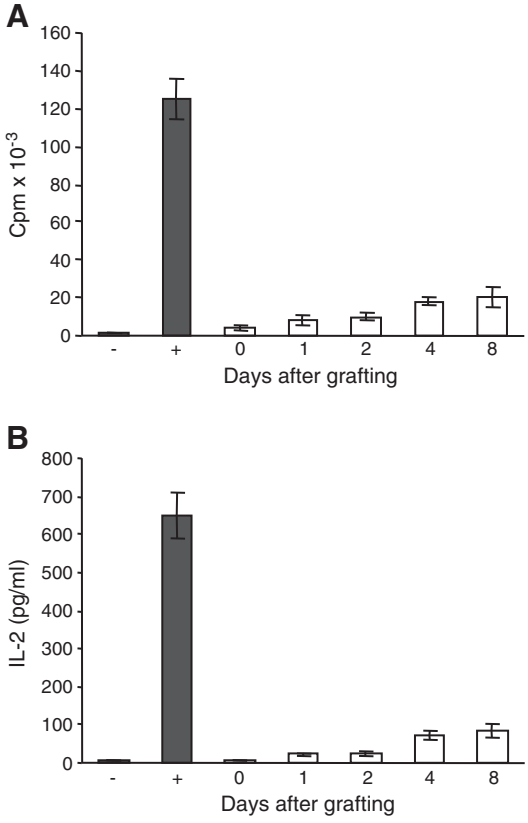

Fig. 4. The kinetics of CsA release in vivo. Nanofibers (10 wt.% CsA) covering a skin allograft were harvested on the day of grafting (day 0) and 1, 2, 4 and 8 days after transplantation. Small pieces (2×2 mm) of these nanofibers were added to cultures of Con A-stimulated spleen cells, and cell proliferation (A) and IL-2 production (B) were determined.

of nanofibers prepared from PLA without CsA or in wells without nanofiber samples (data not shown). In addition, the cells were seeded on plastic 24-well tissue culture plates, on PLA nanofibers without CsA or on CsA-loaded nanofibers, and cell viability and metabolic activity were determined after a 24-hour incubation period by the WST assay. Fig. 5 shows that all cell types grew on CsA-containing nanofibers comparably as on nanofibers without CsA or on tissue culture plastic.

3.5. Suppression of a local inflammatory reaction in vivo

The rejection of a skin allograft induces a local inflammatory reaction that can be detected by the expression of genes for the proinflammatory cytokines IL-2, IL-17 and IFN-γ (Fig. 6). Covering the sides of skin allografts with CsA-loaded nanofibers significantly inhibited the expression of the genes for IL-2 (Fig. 6A) and IFN-γ (Fig. 6B) and attenuated the production of IL-17 in cultured explants of skin allografts that had been covered by CsA-loaded nanofibers (Fig. 6C).

4. Discussion

The transplantation of organs or the therapeutic transfer of allogeneic cells requires extensive systemic immunosuppression to inhibit the deleterious immune response to antigens on the grafted cells. Similarly, immunosuppression is also the first choice of treatment to manage harmful inflammation. The systemic administration of immunosuppressive drugs is regularly associated with side effects that prevent the use of effective doses of the drugs. To avoid this problem, the local delivery of the drugs offers a promising solution. Searching for an optimal carrier for targeted drug delivery, nanotechnologies provide a perspective tool. So far, various nanoparticules, nanofibers, nanocomposites and other nanomaterials have been tested [4–8,14–17]. Here, we show that CsA, a potent inhibitor of T-cell functions, can be efficiently encapsulated into PLA nanofibers and used for the local suppression of T-cell reactivity and the inhibition of inflammatory reactions.

CsA represents one of the most widely used immunosuppressive drugs, but its use is limited by its side effects, such as nephrotoxicity,

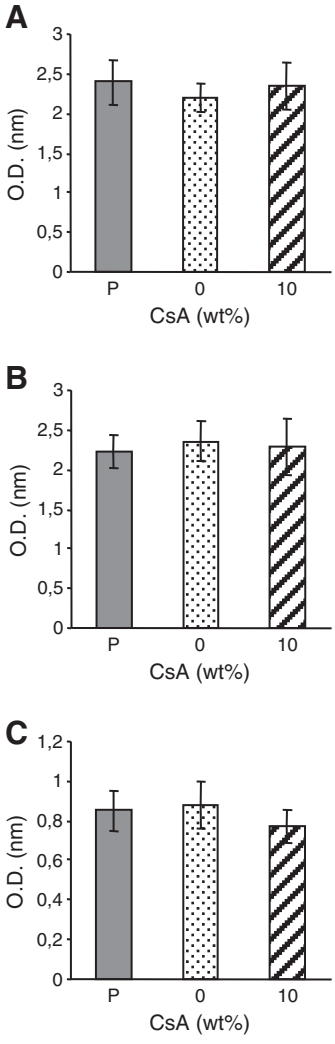

Fig. 5. The growth of LSCs, MSCs and 3T3 fibroblasts on CsA-loaded nanofibers. Mouse LSCs (A), MSCs (B) or 3T3 fibroblasts (C) were grown on plastic (tissue culture plates, P) or on PLA nanofibers with the indicated content (wt.%) of CsA, and cell proliferation was determined after a 24-h incubation period.

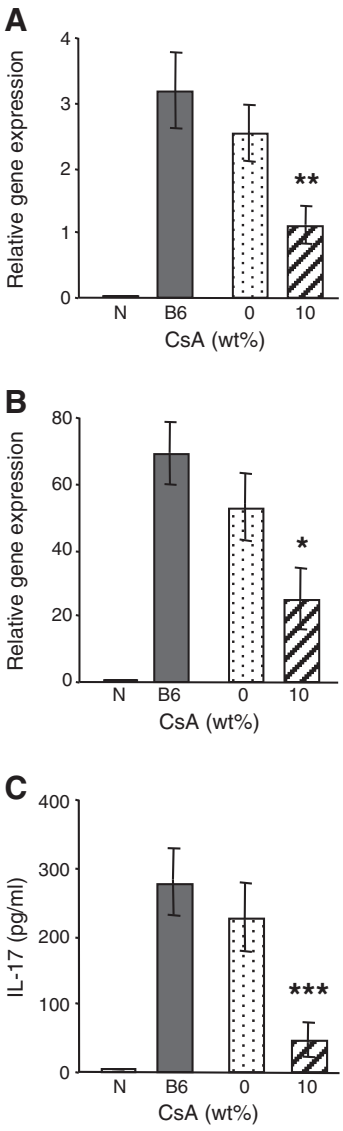

Fig. 6. Suppression of local inflammatory reactions by CsA-loaded nanofibers. The expression of genes for IL-2 (A) or IFN-γ (B) was detected in normal B6 skin (N), in untreated skin allografts (B6) or in allografts that were covered with nanofibers without CsA (0) or with 10 wt% CsA (10). The comparative Ct method was used to determine the extent of target gene expression normalized to an internal GAPDH control. In addition, explants of normal skin or of skin allografts were removed on day 7 after transplantation and were cultured for 48 h. The presence of IL-17 in the supernatants was determined by ELISA (C). Each bar represents the mean ± SD from 5 to 6 determinations. Values with asterisks are significantly different ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) from the positive control (allografts without nanofibers).

gingivitis and hirsutism, regularly observed at higher drug doses. At lower concentrations, CsA selectively inhibits T-cell functions without any apparent effect on other cell populations. Therefore, we dissolved CsA into a PLA solution and prepared nanofiber scaffolds with different concentrations (1–10 wt.%) of CsA. We calculated that 1 mm^2 of CsA-loaded nanofiber material with 10 wt.% CsA contains 1 μg of CsA. Although CsA has extremely low aqueous solubility (6.6 μg/ml) [24], it has been shown that concentrations of the drug as low as 50–200 ng/g of tissue are sufficient to suppress T-cell reactivity and inflammation [25]. The addition of CsA-loaded nanofibers into cultures of spleen cells stimulated with Con A inhibited T-cell proliferation and suppressed the production of T-cell cytokines. The production of IL-1β, IL-6 or NO by activated macrophages and the growth of LSCs, MSCs or 3T3 fibroblasts were not inhibited in the presence of CsA-loaded nanofibers. These results indicate that the incorporation of CsA into the PLA solution and the electrospinning of this mixture did not impair the immunosuppressive properties of CsA. Studies of the kinetics of CsA release in aqueous solutions revealed that the majority of CsA was released from the nanofibers into the culture medium within the first few hours, but a significant amount of CsA was retained in the nanofibers and gradually released for more than 96 h. The sustained release of CsA for a few days is different from the pattern of release of water-soluble small molecular weight substances which are rapidly released from nanofibers within a few minutes or hours [6,26]. The delayed release of CsA from the nanofibers may be associated with the very low solubility of CsA in aqueous solutions. Although attempts have been made to control drug release behavior [8], the release of a significant amount of the drug initially, coupled with a sustained release for a prolonged period of time, may be of great interest, especially in situations in which a harmful inflammatory reaction must be rapidly suppressed and then kept at an acceptable level. Moreover, the drug release profile in vivo may be quite different from that observed in aqueous solutions in vitro. Indeed, we observed that CsA-loaded nanofibers retain a substantial amount of the drug for more than 8 days when they are placed on skin allografts in vivo.

Although the potential use of drug-loaded nanofibers has been extensively discussed, there are only a few studies showing the effectiveness of drug-loaded nanofibers in biological systems in vitro [7,27,28] and in vivo [29,30]. Our data presented here show for the first time that CsA-loaded nanofibers fabricated from PLA polymer effectively and selectively inhibit the proliferation of activated T cells and suppress the production of T-cell cytokines in vitro. Moreover, using an experimental model of skin transplantation in mice, we showed that covering skin allograft with CsA-loaded nanofibers significantly inhibited the local production of the proinflammatory cytokines IL-2, IL-17 and IFN-γ.

In addition to their ability to serve as drug carriers, CsA-loaded nanofibers could be used as scaffolds for the growth and transfer of non-T-cell populations. We showed that LSCs, MSCs and 3T3 fibroblasts grow on CsA-loaded nanofibers comparably as on nanofibers prepared from a CsA-free PLA. Since nanofibers prepared from PLA polymer have comparable mechanical properties as nanofibers prepared from polyamid, which we recently described and used for LSC and MSC transfer to treat ocular surface injuries [13], CsA-loaded and simultaneously cell-seeded nanofibers appear to be potential scaffolds for cell transfer in regenerative medicine.

In conclusion, we showed that the immunosuppressive drug CsA can be incorporated directly into electrospun nanofibers without any loss of its pharmacological activity. Such CsA-loaded nanofibers can be used as drug carriers for the local suppression of inflammatory reactions and as cell scaffolds for tissue repair and regeneration. These observations suggest that CsA-loaded PLA nanofibers may be extremely useful for simultaneous cell-based therapy and the local suppression of T-cell-mediated immune reactions.

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6 Diskuze

Vývoj Treg a Th17 lymfocytů je řízen prostřednictvím působení TGF-β a IL-6, které mohou být produkovány i MSC. Zjistili jsme, že MSC spontánně produkují TGF-β, avšak bez stimulace neprodukují detekovatelné hladiny žádného z dalších testovaných cytokinů včetně IL-1, IL-4, IL-6, IL-10 a IFN-γ. Produkce IL-6, ale ne žádného dalšího z testovaných cytokinů, bylo docíleno stimulací MSC prozánětlivými cytokiny (IL-1, IL-2, IFN-γ a TNF-α nebo LPS). Ovlivnění MSC protizánětlivými cytokiny TGF-β a IL-10 neindukovalo produkci IL-6, ale naopak posilovalo produkci TGF-β. Tento cytokinový profil MSC odpovídá závěrům Oh et al. (2009), kteří zjistili, že lidské MSC spontánně produkují TGF-β, ale ne IL-6, jehož produkce však může být navozena následnou kultivací MSC v přítomnosti mononukleárních buněk periferní krve. Naše výsledky tedy ukázaly, že produkce TGF-β a IL-6 je přísně regulovaná cytokinovým prostředím, ve kterém se MSC nachází.

TGF-β indukuje expresi Foxp3 a hraje klíčovou roli ve vývoji Treg lymfocytů, zatímco kombinované působení TGF-β a IL-6 vede k aktivaci RORγt^{+} Th17 lymfocytů a k produkci IL-17 (Zhou et al., 2008). K testování účinku MSC a jejich supernatantů jsme použili model slezinných buněk stimulovaných aloantigeny. Potvrdili jsme, že produkce IL-17 a exprese Foxp3 a RORγt je regulovaná prostřednictvím TGF-β a IL-6. Exogenní TGF-β přidaný do kultur slezinných buněk stimulovaných aloantigeny indukoval expresi Foxp3, podporoval vývoj Treg lymfocytů a inhiboval produkci IL-17. Naproti tomu kombinované působení TGF -β a IL-6 indukovalo expresi RORγt a produkci IL-17. Přidání supernatantů z kultur neovlivněných MSC nebo MSC předem stimulovaných TGF-β ke slezinným buňkám stimulovaným aloantigeny indukovalo expresi Foxp3, aktivaci Treg lymfocytů a inhibici produkce IL-17. Inhibice produkce IL-17 je daná negativním účinkem TGF-β na vývoj Th17 lymfocytů a efektem Treg lymfocytů, které jsou aktivovány v přítomnosti TGF-β (Zhou et al., 2008). Naproti tomu IL-6 inhibuje expresi Foxp3 a podporuje expresi RORγt, který působí jako klíčový faktor odpovědný za vývoj Th17 lymfocytů (Kimura et al., 2007). Prokázali jsme, že supernatanty získané kultivací MSC stimulovaných prozánětlivými cytokiny indukují nižší expresi Foxp3 než supernatanty z nestimulovaných MSC, zároveň podporují expresi RORγt a posilují produkci IL-17. MSC a jejich supernatanty

tedy mohou prostřednictvím produkovaných cytokinů posilovat nebo naopak tlumit vývoj Th17 a Treg lymfocytů. Tyto závěry jsou v souladu s imunosupresivními schopnostmi MSC popsanými dalšími autory (Di Nicola et al., 2002; Le Blanc et al., 2003), ale také vysvětlují stimulační efekt MSC pozorovaný v jiných případech (Fang et al., 2006; Bocelli-Tyndall et al., 2009).

Použití neutralizačních protilátek proti TGF-β a IL-6 prokázalo, že IL-6 a TGF-β jsou cytokiny přítomné v supernatantech produkovaných MSC zodpovědné za regulaci vývoje Treg a Th17 lymfocytů. Podobně také Liu et al. (2009) prokázali, že krysí MSC mohou inhibovat nebo naopak stimulovat proliferaci T lymfocytů specifických proti myelinovému basickému proteinu a že právě cytokiny produkované MSC jsou odpovědné za tento imunomodulační účinek.

Existuje mnoho molekul a faktorů odpovědných za imunomodulační účinky MSC. Prokázali jsme, že TGF-β a IL-6 jsou klíčové molekuly produkované MSC a odpovědné za inhibici nebo naopak aktivaci vývoje a funkcí Treg a Th17 lymfocytů.

Ghannam et al. (2010) zjistili, že MSC inhibují efektorové funkce Th17 lymfocytů a potlačují produkci IL-17. Naše výsledky ukázaly, že MSC a jejich supernatanty inhibují vývoj Th17 lymfocytů z naivních $CD4^{+}CD25^{-}$ buněk, což znamená, že MSC inhibují Th17 lymfocyty na úrovni jejich aktivace i efektorových funkcí. Těmito závěry mohou být vysvětleny imunosupresivní účinky MSC pozorované v případě transplantací nebo autoimunitních onemocnění, kde Th17 lymfocyty hrají důležitou roli. Imunosupresivní účinky MSC však mohou být v některých případech narušeny působením IL-6, v důsledku čehož může dojít naopak k indukci proliferace T lymfocytů (Crop et al., 2010). Je tedy důležité, aby při zvažování léčby imunopatologických onemocnění pomocí MSC byla brána v úvahu spojitost mezi MSC a vývojem Treg, ale také Th17 lymfocytů. V současné době již existují práce, které přenesly poznatky o vlivu MSC na vývoj Treg lymfocytů *in vivo*. Z nejnovějších je to například indukce Treg lymfocytů u myšího modelu astmatu (Ogulur et al., 2014), EAU (Li et al., 2013a) a experimentální kolitidy (Li et al., 2013b).

V další části projektu jsme se zaměřili na efekt MSC na imunitní reakce probíhající v oku během prvních 72 hodin po poškození rohovkového epitelu pomocí 1 N hydroxidu sodného. Důraz byl v tomto případě kladen především na imunologické aspekty týkající se časné fáze rohovkového zánětu, nikoliv na sledování regenerace poškozeného rohovkového epitelu, která nastává v pozdější fázi a na jejíž aspekty se

zaměřila již řada publikovaných prací. MSC byly intravenózně podány 24 hodin po poškození, v čase, kdy významně stoupá počet leukocytů infiltrujících poškozenou rohovku. Imunomodulační efekt MSC byl testován 72 hodin po poškození.

Jelikož řada studií včetně naší prokázala, že cytokiny mohou významně ovlivňovat imunoregulační vlastnosti MSC (English et al., 2007; Ren et al., 2008; Svobodova et al., 2012), porovnávali jsme terapeutický efekt neovlivněných MSC a MSC stimulovaných IL-1, IFN-γ nebo TGF-β. Na počátku jsme potvrdili, že stimulace MSC pomocí IL-1, IFN-γ nebo TGF-β neovlivňuje expresi povrchových znaků ani migrační schopnosti takto ovlivněných buněk. Ke stejným závěrům dospěli i jiní autoři (Hemeda et al., 2010; Najar et al., 2012).

Naše výsledky rovněž potvrdily, že intravenózně podané MSC jsou schopny specificky migrovat do poškozeného oka, kde jsme detekovali 30krát více fluorescenčně označených MSC než v odpovídajícím kontrolním oku. Podobných výsledků bylo dosaženo i v jiných pracích, kde autoři nalezli systémově podané MSC v rohovce po 3 dnech od poškození kauterizací (Lan et al., 2012), ale ne v případě xenogenních lidských MSC podaných intravenózně za účelem hojení rohovkových defektů u krys (Roddy et al., 2011).

Následně jsme testovali účinek MSC na infiltraci poškozeného oka populacemi myeloidních a lymfoidních buněk. Všechny typy MSC snižovaly infiltraci oka lymfoidními populacemi, ale pouze MSC stimulované IFN-γ statisticky významně snižovaly počty $CD3^+CD4^+$, $CD3^+CD8^+$ a $CD19^+CD22^+$ buněk. K podobným výsledkům dospěli i jiní autoři, kteří pozorovali snížení exprese CD45 a infiltrace $CD4^+$ buňkami v rohovce léčené MSC (Ma et al., 2006; Oh et al., 2008). Zároveň jsme zaznamenali výrazné potlačení infiltrace poškozeného oka $CD14^+$, $CD80^+$ a $CD11b^+Gr\text{-}1^+$ myeloidními populacemi po podání MSC stimulovaných IFN-γ. Odpovídajících výsledků dosáhly i práce, které ukázaly významné snížení počtu $CD68^+$ a neutrofilů infiltrujících poškozenou rohovku u zvířat léčených pomocí MSC (Roddy et al., 2011; Yao et al., 2012).

Abychom stanovili účinek systémově podaných MSC na cytokinové prostředí utvářející se v časné fázi po poškození rohovky, testovali jsme produkci řady cytokinů včetně IL-1, IL-2, IL-4, IL-6, IL-10, IL-17 a IFN-γ, ale pouze IL-1, IL-6 a IL-10 byly produkovány v této fázi zánětu ve zvýšených koncentracích. Systémově podané MSC stimulované IFN-γ významně snižovaly produkci IL-1 a IL-6. K podobným závěrům dospěli i Roddy et al. (2011), kteří zaznamenali významné snížení produkce IL-1

v rohovkách ze zvířat léčených MSC po 1 a 3 dnech po poškození. Naproti tomu Oh et al. (2008) detekovali po 3 týdnech zvýšenou produkci IL-6 v poškozených rohovkách po léčbě MSC. Neshodu s našimi výsledky by mohla vysvětlovat rozdílná kinetika produkce IL-1 a IL-6. Vrchol produkce IL-1 nastává v oku 3 dny po jeho poškození, zatímco produkce IL-6 vrcholí později (Sotozono et al., 1997). Dále jsme zjistili významnou inhibici produkce NO a exprese iNOS v poškozeném oku po léčbě MSC stimulovanými IFN-γ. Protože je NO toxická a imunomodulační molekula, jeho inhibice může představovat další mechanizmus terapeutického působení MSC. Na rozdíl od práce Roddy et al. (2011), kteří detekovali významné zvýšení produkce IL-10 3 týdny po léčbě poškozené rohovky pomocí MSC, my jsme v naší práci zaznamenali pouze nevýznamné zvýšení produkce IL-10 v poškozeném oku příjemců léčených MSC. Tyto rozdíly mohou být opět dány odlišnými časy, ve kterých byly hladiny IL-10 měřeny.

Abychom určili, jaké molekuly a faktory mohou být zodpovědné za výše popsané efekty MSC stimulovaných IFN-γ, testovali jsme expresi genů pro IL-6, TGF-β, IDO, iNOS, HGF a COX-2 u neovlivněných MSC a MSC stimulovaných IL-1, IFN-γ a TGF-β. U všech typů MSC jsme naměřili konstitutivní expresi TGF-β, HGF a COX-2. Na rozdíl od práce English et al. (2007) jsme však nepozorovali zvýšení exprese COX-2 po stimulaci MSC pomocí IFN-γ, což mohlo být způsobeno tím, že v této studii byla pro stimulaci použita 20krát vyšší koncentrace IFN-γ. Dále jsme zjistili, že MSC stimulované IL-1 a IFN-γ exprimovaly vysoké hladiny genu pro iNOS. Podobně i v jiné práci ovlivnění MSC IFN-γ a dalšími prozánětlivými cytokiny indukovalo u MSC expresi různých chemokinů a iNOS (Ren et al., 2008). V neposlední řadě jsme zaznamenali významný nárůst exprese genu pro IDO u MSC stimulovaných IFN-γ, což je v souladu se závěry jiných prací (English et al., 2007; Ryan et al., 2007).

MSC stimulované IFN-γ exprimovaly geny pro TGF-β, IDO, iNOS, HGF a COX-2. V porovnání s neovlivněnými MSC a MSC stimulovanými IL-1 a TGF-β byly nalezeny rozdíly především v expresi iNOS a IDO. Ačkoliv iNOS a IDO patří k důležitým imunomodulačním molekulám, v účinku MSC byly pravděpodobně zapojeny ještě další faktory a molekuly. Naše výsledky však ukázaly, že systémově podané MSC selektivně migrují od poškozeného oka a potlačují časnou fázi rohovkového zánětu. Infiltrace rohovky populacemi myeloidních a lymfoidních buněk

a produkce prozánětlivých cytokinů byla snížena po podání MSC, přičemž nejvýznamnějšího účinku bylo dosaženo při použití MSC stimulovaných IFN-γ.

Předložené výsledky poukazují zejména na důležitost cytokinového prostředí, jehož působení mohou být MSC vystaveny, a které může výrazně ovlivnit jejich imunomodulační vlastnosti. V *in vitro* systému jsme prokázali významné účinky IL-1 a TGF-β na produkci TGF-β a IL-6 u MSC (Svobodova et al., 2012). V modelu poškozeného povrchu oka jsme rovněž použili MSC stimulované IL-1 a TGF-β, avšak jejich podání nezpůsobilo změny v proporci Treg a Th17 lymfocytů v poškozené rohovce (nepublikovaná data). Naproti tomu použití MSC stimulovaných IFN-γ významně ovlivnilo časnou fázi rohovkového zánětu. Tyto práce jsou příkladem toho, že účinky MSC pozorované v *in vitro* systému nemusí vždy nutně odpovídat účinkům sledovaným *in vivo*. *In vivo* jsou MSC vystaveny komplexnímu působení mnoha vlivů prostředí, a může být tedy velmi těžké předem stanovit, jaký efekt budou MSC u konkrétního onemocnění mít. Avšak mnohé studie prováděné na zvířecích modelech různých patologických stavů a v posledních letech i probíhající klinické testy využívající MSC k léčbě řady závažných a mnohdy i neléčitelných onemocnění přináší stále nové poznatky posouvající výzkum MSC dál. Příznivé výsledky přináší i výzkum nanovlákenných nosičů, které mohou nést na svém povrchu MSC nebo obsahovat CsA či jiné farmakologicky aktivní látky. Podle našich výsledků jsou taková nanovlákna účinná v tlumení imunitní reakce proti alogennímu limbálnímu nebo kožnímu štěpu (Zajicova et al., 2010; Holan et al., 2011). Tyto výsledky jsou v souladu i se závěry jiných autorů (Li et al., 2005; Soleimani et al., 2010; Cejkova et al., 2013).

7 Závěr

- **Vliv cytokinového prostředí na imunomodulační vlastnosti MSC.**

  Působení prozánětlivých cytokinů (IL-1, IL-2, IFN-γ a TNF-α) indukuje u MSC produkci IL-6. Stimulace MSC protizánětlivými cytokiny (TGF-β a IL-10) u nich posiluje produkci TGF-β. MSC mohou modulovat prostřednictvím produkce TGF-β a IL-6 vývoj T lymfocytů v Treg nebo Th17 lymfocyty.
  MSC konstitutivně produkují TGF-β, HGF a COX-2. Stimulace MSC prostřednictvím IFN-γ navíc indukuje expresi a aktivitu iNOS a IDO, což jsou významné imunomodulační molekuly, které mohou významně ovlivnit průběh imunitních reakcí.

- **Vliv cytokinů produkovaných MSC na vývoj Treg a Th17 lymfocytů.**

  Supernatanty získané kultivací MSC ovlivněných TGF-β indukují vývoj Treg lymfocytů. Takto vzniklé Treg lymfocyty přidané do směsné lymfocytární kultury byly schopny částečně potlačit reakci proti alogenním lymfocytům. Naproti tomu v přítomnosti supernatantů z MSC ovlivněných IL-1 dochází ke vzniku Th17 lymfocytů produkujících IL-17.

- **Migrace intravenózně podaných MSC ovlivněných cytokiny do poškozené rohovky.**

  Systémově podané MSC jsou schopny specificky migrovat do poškozeného oka, ve kterém lze detekovat 30krát více fluorescenčně označených MSC než v nepoškozeném kontrolním oku.

- **Účinek MSC ovlivněných cytokiny na rozvoj časného zánětu v poškozeném rohovkovém epitelu.**

MSC stimulované IFN-γ potlačují infiltraci poškozeného oka lymfoidními, ale především myeloidnímu buňkami. Současně potlačují produkci IL-1, IL-6 a NO v místě poškození.

- **Růstové vlastnosti MSC a LSC kultivovaných na nanovlákenných nosičích a přenos takto kultivovaných buněk na poškozenou rohovku.**

  MSC i LSC kultivované na nanovlákenných nosičích připravených z polymeru PA6/12 a na nanovláknech nesoucích CsA vykazují standardní růstové vlastnosti. Fluorescenčně značené MSC jsou detekovatelné v poškozené rohovce i 14 dnů po jejich přenosu pomocí nanovlákenného nosiče.

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