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Limbal stem cell transplantation and their utilization for ocular surface reconstruction

MUDr. Anna Lenčová

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Předseda oborové rady:	Prof. RNDr. Ivan Raška, DrSc.
Školící pracoviště:	Oddělení transplantační imunologie Ústav experimentální medicíny Akademie věd ČR Vídeňská 1083 142 20 Praha 4-Krč, Česká republika
Školitel:	Prof. MUDr. Martin Filipec, CSc.
Školitel-specialista:	Prof. RNDr. Vladimír Holáň, DrSc.

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Abstrakt

Cíle: Deficit limbálních kmenových buněk (LSC) patří mezi nejzávažnější onemocnění povrchu oka. Cílem dizertační práce bylo studium obnovy poškozeného povrchu oka. Proto byla zavedena limbální transplantace v experimentálním myším modelu. Byla provedena izolace LSC, přenos LSC a mesenchymálních kmenových buněk (MSC) izolovaných z kostní dřeně na nanovláknových nosičích na povrch poškozeného oka u myší a u králíků.

Materiály a metody: U myší BALB/c byla provedena syngenní, alogenní a xenogenní limbální transplantace. Po transplantaci byla sledována doba přežívání štěpů, imunitní reakce a účinky monoklonálních protilátek (mAb) (anti-CD4 a anti-CD8). Myší LSC byly rozděleny pomocí centrifugace na Percollovém gradientu a následně byla provedena analýza povrchových znaků LSC pomocí PCR a průtokové cytometrie. Na myším a králíčím modelu byly přenášeny LSC a MSC pomocí nanovláknových nosičů na poškozený povrch oka. Byl sledován *in vitro* růst buněk na nosičích, pooperační zánětlivá reakce a přežívání buněk na povrchu oka po přenosu nosičů.

Výsledky: K odhojení alogenních limbálních štěpů došlo promptně a v průběhu rejekce převažovala Th1 imunitní odpověď (IL-2, IFN-γ) mediovaná $CD4^+$ buňkami a NO produkovaným makrofágy. U xenotransplantátů převažovala Th1 i Th2 (produkce IL-2, IFN-γ, IL-4 a IL-10) imunitní odpověď. Pomocí anti-CD4 monoklonálních protilátek došlo k signifikatnímu prodloužení přežívání alogenních a xenogenních štěpů. Frakce získané po izolaci z vrchní (40% Percoll) a spodní (80% Percoll) vrstvy gradientu obsahovaly buňky s vysokou expresí znaků kmenových buněk a side-population fenotypu. Spodní frakce obsahovala buňky s expresí znaků $K12^-/p63^+$ vykazující vlastnosti blížší kmenovým buňkám na rozdíl od vrchní frakce. Společný přenos LSC a MSC výrazně inhiboval zánětlivou reakci v myším modelu. Přenos MSC na nanovláknech u králíků potlačil oxidativní stres rohovky způsobený poleptáním a podpořil proces hojení.

Závěr: U transplantace limbálních štěpů se neuplatňuje imunologická privilegovanost oka. Anti-CD4 monoklonální protilátky představují slibnou imunosupresi u alotransplantátů. Pomocí centrifugace na Percollovém gradientu lze získat dvě odlišné populace buněk vykazující vlastnosti kmenových buňek, ze kterých $K12^-/p63^+$ populace je bližší k LSC. Nanovláknové nosiče jsou vhodné pro léčbu poškozeného povrchu oka pomocí LSC a MSC v experimentálním modelu.

Klíčová slova: limbální kmenové buňky, deficit limbálních kmenových buněk, limbální transplantace, mesenchymální kmenové buňky, nanovláknové nosiče.

Abstract

Aims: Limbal stem cell (LSC) deficiency is one of the most challenging ocular surface diseases. The aim of this thesis was to study damaged ocular surface reconstruction. Therefore, a mouse model of limbal transplantation was established. Furthermore, LSC isolation, transfer of LSCs and bone marrow-derived mesenchymal stem cells (MSCs) on nanofiber scaffolds were studied.

Material and methods: Syngeneic, allogeneic and xenogeneic (rat) limbal grafts were transplanted orthotopically into BALB/c mice. Graft survival, immune response and the effect of monoclonal antibodies (mAb) (anti-CD4 and anti-CD8 cells) were analyzed. Mouse LSCs were separated by Percoll gradient; subsequently, they were analyzed for the presence of LSC and differentiation corneal epithelial cell markers and characteristics using real-time PCR and flow cytometry. Nanofiber scaffolds seeded with LSCs and MSCs were transferred onto the damaged ocular surface in mouse and rabbit models. Cell growth on scaffolds, post-operative inflammatory response and survival of transferred cell were analyzed.

Results: Limbal allografts were rejected promptly by the Th1-type of immune response (IL-2, IFN-γ) involving $CD4^+$ cells and nitric oxide (NO) produced by macrophages, contrary to the prevailing Th1 and Th2 immune responses (IL-4, IL-10) in xenografts. Anti-CD4 mAb significantly postponed the rejection in allografts and in xenografts. The lightest and densest fraction of the Percoll gradient were both enriched with cell populations with a high expression of stem cell (SC) markers and side-population phenotype. Contrary to the lightest (40%), the densest (80%) fraction contained $K12^-/p63^+$ cells with characteristics that were closer to SCs. In the mouse model, the nanofiber scaffolds with LSCs and MSCs suppressed the inflammatory reaction. In the rabbit model, the MSCs on nanofiber scaffolds reduced alkali-induced oxidative stress and significantly accelerated corneal healing.

Conclusions: Limbal grafts do not enjoy any privileged position of immunity in the eye. Anti-CD4 mAb treatment is a promising immunosuppressive approach after limbal allotransplantation. By centrifugation on Percoll gradient, two distinct populations of corneal epithelial cells with SC characteristics were separated, with the $K12^-/p63^+$ population being closer to LSCs. Nanofiber scaffolds can be useful for LSC and MSC transfer and future treatment of ocular surface injuries.

Key words: limbal stem cells, limbal stem cell deficiency, limbal transplantation, mesenchymal stem cells, nanofiber scaffolds.

1 GENERAL INTRODUCTION

The integrity of the corneal epithelium is essential for maintaining corneal transparency and ensuring visual function. The corneal epithelium is renewed by the cells, which migrate from the limbus. These cells originate from the LSCs, which are located in the basal layer of the limbus (Cotsarelis et al., 1989). The human limbus is described as a transition zone, 1.5-2.0 millimeter (mm) in width, between the transparent cornea and the opaque sclera, which is highly vascularized and innervated (Davanger and Evensen, 1971). The epithelium has 10 to 12 layers and overlies the stroma, which is radially arranged into the palisades of Vogt (Dua et al., 1994). The limbal area has several physiological attributes. The limbus has an important role in "barrier function", preventing encroachment of the conjunctival epithelium onto the cornea during homeostasis and the healing process (Thoft et al., 1979); (Tseng, 1989); (Huang and Tseng, 1991).

Basal limbal cells have the characteristics of adult SCs. LSCs have a high proliferative potential *in vitro* compared to the epithelial cells in the peripheral and central cornea, a high capacity for error-free self-renewal, a long life span, slow cell cycle and the ability to retain labelled DNA precursors over a long period (Cotsarelis et al., 1989); (Pellegrini et al., 1999). LSCs undergo asymmetrical division and produce one SC to replenish the SC pool and one transient amplifying cell (TAC) (Daniels et al., 2001). TACs are fast-dividing progenitor cells and reside the basal epithelium of limbus and peripheral cornea. These cells give rise to post-mitotic and terminally differentiated cells in the suprabasal and superficial layers (Lehrer et al., 1998); (Schlötzer-Schrehardt and Kruse, 2005). The adult tissue-specific SCs express the side-population (SP) phenotype, which is characterized by the ability to efflux the DNA-binding vital dye Hoechst 33342 (Budak et al., 2005); (Umemoto et al., 2005). ATP-binding cassette sub-family G member 2 (ABCG2) is a transporter important for determining the limbal SP phenotype (Shaharuddin et al., 2013). The SP phenotype is a property of SCs (Shimano et al., 2003); (Zhou et al., 2001). Currently, a combination of positive markers specific for undifferentiated SCs (transcription factor p63, membrane protein ABCG2, vimentin and keratin K19), and an absence of markers typical for differentiated corneal epithelium (connexin 43, keratin K3 and K12) is used for LSC determination (Schlötzer-Schrehardt and Kruse, 2005). Moreover, SC morphology can be used in combination with putative SC markers (O'Sullivan and Clynes, 2007).

Loss of LSC source through primary disease or secondarily, due to ocular surface damage, can cause opacity, corneal cicatrization, impairment of transparency, and can lead to blindness (Dua et al., 2000). The correct assessment of LSC deficiency (LSCD) is crucial in clinical practice because these patients are not suitable candidates for conventional corneal transplantation. In these cases, lamellar or penetrating keratoplasty would provide only a short-term replacement of corneal epithelium in the recipient. It does not resolve the primary cause of the disease the loss of LSCs. The only way to cure LSCD is through transplantation of the whole limbus (Tan et al., 1996); (Holland, 1996); (Dua and Azuara-Blanco, 1999), or through transfer of *ex vivo* cultured LSCs (Pellegrini et al., 1997); (Rama et al., 2010). Treatment of total bilateral LSCD, in which a new source of LSCs must be provided via allografts from

living-related, cadavers or *ex vivo* cultured epithelial cells is more challenging (Dua, 2006). The disadvantage is immune non-histocompatibility with a high risk of rejection and the need for systemic immunosuppression (Holland, 1996); (Dua and Azuara-Blanco, 1999). The transfer of *ex vivo* cultured LSCs is currently a very favorable method for LSCD therapy. However, it is necessary to find an optimal scaffold for the SCs. Therefore, one of the objectives of our investigation was to test a new tool for LSC transfer. In bilateral LSCD, there is no autologous limbal tissue available for ocular surface reconstruction. Therefore, the search for alternative non-limbal autologous cells is a promising therapeutic approach for ocular surface reconstruction in regenerative medicine with the advantage of not needing systemic immunosuppression. MSCs represent one of the potential sources of autologous non-epithelial SCs with promising results for ocular surface reconstruction (Li and Zhao, 2014). However, there is a need for further studies to elucidate the mechanism of MSC effectiveness.

To date, there are limited numbers of experimental limbal transplantation studies. Further studies are necessary to improve understanding of the limbal graft and LSC survival, as well as their capacity to renew the ocular surface after transplantation. Further studies are also warranted to find effective immunosuppressive treatment. Animal models in this area can contribute substantially to improvement of LSCD treatment and, thus, improve the poor prognosis of this disease in clinical practice.

2 AIM

The aim of this thesis was to study the reconstruction of damaged ocular surface by transfer of LSCs in the mouse experimental model. Due to LSC deficiency, ocular surface reconstruction is one of the most challenging issues in current ophthalmology. The loss of LSC source through ocular surface damage leading to blindness can be resolved by LSC transplantation. The clinical course and cellular mechanisms of limbal graft rejection have to be recognized in order to develop a successful strategy to manage immune reaction in limbal allo- and xeno-transplantation. This thesis summarizes the results of limbal tissue transplantation, characterization of immune response and immunosuppressive therapy in the mouse model. In addition, the LSC isolation method, *ex vivo* culture of LSCs and MSCs on nanofiber scaffolds and their *in vivo* transfer were studied and applied for ocular reconstruction in the mouse and rabbit experimental model.

1. Firstly, we introduced an experimental model of orthotopic limbal transplantation into our laboratory to evaluate limbal allograft and xenograft survival. We decided to characterize the immune response to limbal graft, analyze donor-derived cell survival and assess the effect of systemic immunosuppression in the form of monoclonal antibodies.

2. Then we investigated an optimal LSC isolation method from limbal explant, which can be used for further SC-based experiments including LSC culture and transfer for ocular surface reconstruction in the experimental mouse model.

3. We intended to test a new nanofiber scaffold for SC transfer and analyze the effect of co-transfer of LSCs and MSCs onto the damaged ocular surface in the mouse model. We

investigated whether the selected nanofiber scaffolds are useful and suitable for SC culturing and transfer onto the ocular surface in the mouse model. We used our established limbal allotransplantation model to analyze the anti-inflammatory effect of the transferred cultured cells.

4. There is a need for further studies to elucidate the mechanism of MSC effect in chemical burns in the acute phase and to find a suitable scaffold for transfer before clinical application. The results from our previous experiments in the mouse model led us to investigate the effect of MSCs transferred on nanofiber scaffolds in further detail. We decided to study whether rabbit bone marrow-derived MSCs on nanofiber scaffolds effectively decrease alkali-induced oxidative stress in the rabbit cornea after chemical injury and whether this can contribute to the healing process.

3 MATERIAL AND METHODS

Detailed descriptions of the methods used are mentioned in the publications in the appendix of thesis.

3.1 Animals

Mice of both sexes of the inbred strains BALB/c and C57BL/6 (B6), rats of the inbred strain Lewis, and adult female New Zealand white rabbits were used for the experiments (Publication 1-4).

3.2 Surgical techniques, clinical evaluation and immunosuppression

Limbal transplantation in the murine model
In brief, donor limbal lenticule was circularly cut out from conjunctiva without scleral tissue and around the cornea. The corneal epithelium of the recipient ocular surface was debrided and the limbus was cut out. The donor limbal graft was placed orthotopically and was secured with 5 interrupted sutures. The eyelids were closed for 72 hours. In all experiments BALB/c mice were used as the recipients and BALB/c mice (syngeneic grafts), B6 mice (allografts) or Lewis rat (xenografts) as the graft donors (Publication 1).

Clinical evaluation of graft survival
Postoperatively, the ocular surface was observed daily using the operating microscope. A scoring scale ranging from 0 to 4 for corneal opacity was used to evaluate rejection (Maruyama et al., 2003). If the opacity score reached 2 (diffuse epithelial edema, opacity or both, obscuring iris vessels), the graft was considered as rejected (Publication 1).

Antibody treatment
Monoclonal antibodies (mAb) anti-CD4 (clone GK1.5) (Dialynas et al., 1983) and anti-CD8

(clone TIB 150) (Gottlieb et al., 1980) were injected intraperitoneally (Publication 1).

Transfer of nanofiber scaffolds
Nanofiber three dimensional (3D) scaffolds were prepared by electrospinning technology from polyamide 6/12. The scaffolds were transferred in two models. The first model was characterized by removal of corneal epithelium and limbus in BALB/c mice. The transferred LSCs from BALB/c mice were labelled with PKH26 dye and the fate of cell survival was analyzed on the cryosections postoperatively. In the second model, a strong immune response was induced by allogeneic limbal transplantation (CB7BL/6 donor, BALB/c recipient). The co-transfer of LSCs and MSCs was performed after this procedure. A control group with empty nanofibers and a group with no treatment were also studied. Real-time PCR was used to assess the postoperative inflammatory response in all groups (Publication 3).

Alkali injury in the rabbit model
In the rabbit alkali model, nanofiber scaffolds were prepared from polymer poly (L-lactid acid) by the needleless electrospinning procedure (Publication 3; Holáň et al., 2011). The alkali (0.15 N NaOH) was applied on the cornea of the right eye and then rinsed with tap water. The rabbits were divided into four groups. In the first group of rabbits the injured corneas remained untreated. In the second group, the MCSs were applied on the injured ocular surface after the injury and the eyelids were sutured for two days. In the third group, nanofiber scaffolds seeded with MSCs (in the fourth group nanofibers alone) were transferred onto the corneas immediately after the injury and the eyelids were sutured. The rabbit corneas were examined immunohistochemically, morphologically and for the central corneal thickness. The immunohistochemical, morphological and Real-time PCR analyses of the rabbit corneas were performed (Publication 4).

3.3 Cellular and molecular methods

Limbal stem cell isolation
Limbal tissue was obtained by scissor dissection. The tissue was centrifuged and followed by digestion with trypsin (Publication 2).

Mesenchymal stem cell isolation
Mouse MSCs were isolated from the bone marrow of BALB/c mice, rabbit MSCs from the bone marrow of adult New Zealand white rabbits (Publication 3 and 4).

Percoll gradient centrifugation
Limbal mouse cells were separated by Percoll density gradient centrifugation. Subsequently, several fractions were characterized for the presence of LSC markers and differentiation markers of corneal epithelial cells by Real-time PCR. The SP phenotype was determined by flow cytometry and the growth properties were analyzed *in vitro* (Publication 2).

Cell culture
Upon cell isolation, the mouse LSCs were cultured *in vitro*. For the growth on nanofibers, cells growing *in vitro* for 2-3 weeks were used (Publication 3). Mouse MSCs were isolated from the bone marrow of BALB/c mice. After 3 weeks of culturing, the cells were characterized phenotypically by flow cytometry and by their ability to differentiate into adipocytes (Publication 3). Rabbit MSCs were isolated from the bone marrow of adult New Zealand white rabbits and were cultured and characterized as described for mouse MSCs (Svobodová et al., 2012). After approximately 3 weeks of culture (2-3 passages), the cells were used for transplantation onto the ocular surface (Publication 4).

RNA isolation and reverse transcription from limbal grafts
Total RNA was extracted from the samples of limbal grafts using TRI Reagent (Molecular Research Center, Cincinnati, OH). Two g of total RNA were treated using deoxyribonuclease I (Sigma, St. Louis, MO) and used for subsequent reverse transcription (Publication 1).

Detection of limbal graft cell survival
Donor limbal cell survival was detected by Real-time PCR using primers specific for rat MHC class I molecule in xenogeneic model or for Sry (sex determining Y protein) in allogeneic model, where limbal grafts were grafted from C57BL/6 male donors on BALB/c female recipients (Publication 1).

Detection of cytokine and iNOS immune response
Levels of mRNA for various cytokines (IL-2, IFN-γ, IL-4, IL-10) and the expression of gene for inducible nitric oxide synthasis (iNOS) were detected by Real-time PCR (Publication 1).

3.4 Statistical analysis

The Mann-Whitney U test and the Students t-test were used for the statistical analysis (Publication 1).

4 RESULTS

4.1 Limbal tissue transplantation in a mouse model

Allografts, syngrafts and xenografts were grafted orthotopically in BALB/c mice. Graft rejection was assessed on the basis of clinical evaluation (corneal opacity score grade ≥ 2) and donor cell survival detection by RT-PCR postoperatively. The clinical observation of allografts and xenografts showed that limbal rejection was accompanied by limbal edema, limbal graft neovascularization, corneal neovascularization and opacity. The postoperative limbal edema was strongly developed in the xenogeneic model compared to the allogeneic and syngeneic model.

Postoperative corneal neovascularization was present in all quadrants in xenografts, less in allografts, unlike in the syngeneic model where only minimal peripheral neovascularization was present. While syngeneic limbal grafts survived permanently (> 28 days, n=10), the limbal allografts were rejected in 9.0 ± 1.8 days (n=14) and limbal xenografts in 6.5 ± 1.1 days (n=10).

The survival of donor limbal graft cells and the donor-derived cells on recipient cornea were detected by Real-time PCR using primers for MHC class I molecules in xenogeneic model and for Sry in allogeneic male-to-female transplantation. The clinical manifestation of graft rejection onset correlated with the kinetics of donor cell survival in the graft and on the recipient cornea in the allograft and xenograft model. Xenogeneic cells were detected on day 8 but not on day 12 after transplantation and allogeneic cells were detected up to day 14 after grafting in the limbal graft and on the corneal surface. When syngeneic grafts from male donors were grafted into female recipients, the male cells were still detected in the graft on day 28 after grafting.

The intragraft expression of cytokine response (IL-2, IL-4, IL-10, IFN-γ) and iNOS were detected by real-time PCR during the onset of graft rejection. Distinct patterns of intragraft gene expression of Th1 cytokines (IL-2 and INF-γ) and Th2 cytokines (IL-4 and IL-10) were detected during rejection of limbal allografts and xenografts. A significant expression of genes for Th1 cytokines IL-2 and IFN-γ was found in allografts, but the expression of genes for Th2 cytokines IL-4 and IL-10 did not exceed the levels in syngeneic grafts. Rejection of limbal xenografts was accompanied by the high expression of genes for both Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-10) cytokines. Significant iNOS gene expression was detected during rejection in both allografts and xenografts. The rejection reaction was prevented by systemic immunosuppression in the form of anti-CD4 mAb, and allograft survival was significantly prolonged (22.8 ± 4.2 days, n=8). The survival of limbal xenografts was postponed by anti-CD4 mAb (9.5 ± 1.8 days, n=6), but all grafts were rejected within 12 days. The administration of anti-CD8 mAb did not prolong the allo- and xenograft survival significantly (Publication 1).

4.2 Isolation and characterization of mouse limbal stem cells

We described that Percoll density gradient centrifugation is a convenient method of harvesting cells with SC characteristics from limbal tissue. These cells can subsequently be used for *in vitro* tissue culturing. In our experiments, the mouse limbal epithelial cells were separated and analyzed for expression of SC markers and characteristics (Publication 2).

In practice, a single cell heterogenous population was obtained after trypsin-dissociation of limbal tissue. Next, the cell suspension was separated by the Percoll gradient. The Percoll gradient centrifugation was used to separate the isolated cells into 5 individual fractions (40%, 50%, 60%, 70% and 80% Percoll gradient). Each fraction was characterized through Real-time PCR for both the presence of limbal SC markers (ABCG2, Lgr5, p63) and a differentiation marker of corneal epithelial cells (K12).

SCs express the SP phenotype based on the ability to efflux the DNA-binding dye Hoechst 33342. The SP phenotype is associated with ABCG2 expression. The SP phenotype was determined by flow cytometry. Cells retained in the lightest fraction (40% Percoll) and in the densest fraction (80% Percoll) of the gradient were both enriched for populations with a

high expression of the SC markers ABCG2 and Lgr5 and also expressed the SP phenotype. However, the lightest fraction (representing approximately 12% of total limbal cells) contained cells with the strongest spontaneous proliferative capacity and expressed the corneal epithelial differentiation marker K12. In contrast, the densest fraction (<7% of original cells) was K12 negative and contained small, non-spontaneously proliferating cells, which instead were positive for p63. Unexpectedly, cells from this fraction had the highest proliferative activity when cultured on a 3T3 feeder cell monolayer.

4.3 Limbal and mesenchymal stem cell transfer on nanofiber scaffolds for treatment of ocular surface damage in a mouse model

The LSCs and MSCs were successfully cultured and transferred onto the mouse ocular surface. Nanofiber 3D scaffolds were prepared by electrospinning from polyamide 6/12 (PA6/12). The copolymer PA6/12 was selected on the basis of the nanofibers stability in aqueous solutions and its biocompatibility for LSC and MSC growth (Publication 3).

We found that the metabolic and proliferative activities of LSC and MSC on plastic culture plates or on PA6/12 nanofiber scaffolds were comparable. The growth of LSCs and MSCs seeded on a plastic surface and on PA6/12 nanofiber scaffolds was similar: gradually increasing during a 48-hour incubation. The morphology of growing LSCs was analyzed based on the cell shape and organization of actin cytoskeleton. A comparable morphology of LSCs growing on PA6/12 and on the poly-L-lysin coated glass surface was found.

The LSC transfer and co-transfer of LSC and MSC onto scaffolds PA6/12 were performed on the damaged ocular surface of BALB/c mice. The damaged ocular surface was induced by corneal epithelium and limbus removal in BALB/c mice. The LSC from BALB/c mice were labeled with PKH26 dye, cultured on nanofiber scaffolds and transferred onto damaged ocular surface. The PKH26-labeled LSCs migrated to the corneal surface from the nanofiber scaffold and were detected on cryosections on day 7 and 14 postoperatively.

The immunosuppressive properties of MSCs were demonstrated *in vitro* by their ability to inhibit T-cell proliferation and IFN-γ production. To demonstrate the suppression of a local inflammatory reaction by the co-transfer of LSCs and MSCs *in vivo*, two experimental models were used. In the first model, the damaged ocular surface was induced by corneal epithelium and limbus removal in BALB/c mice. The second model combined the mechanical trauma and allogeneic (C57BL/6) orthotopic limbal transplantation in BALB/c mice. Both models were used in order to study the immunosuppressive effect of LSC and MSC. The co-transfer of LSC and MSC on the scaffolds was performed immediately after the ocular surface damage. The control group was treated with empty nanofiber scaffolds. The group with non-damaged eye was also analyzed. The postoperative inflammatory response after scaffolds transfer was assessed by Real-time PCR. The mechanical trauma induced a moderate inflammatory associated with IFN-γ and iNOS production. This response was inhibited by the co-transport of LSCs and MSCs. In the model of limbal allotransplantation, the LSC and MSC co-transport on nanofibrous scaffolds significantly inhibited the local strong inflammatory reaction characterized by expression of IL-2, IFN-γ and iNOS genes determined by real-time PCR. The inflammatory reaction was slightly

suppressed in the control group with cell-free nanofiber scaffolds and not affected in the group with no treatment.

4.4 Mesenchymal stem cell transfer on nanofiber scaffolds for treatment of chemical corneal injury in a rabbit model

In our study, we demonstrated the suppression of oxidative alkali-induced injury by transfer of MSCs on nanofiber scaffolds in the experimental rabbit model (Publication 4). The model of alkali injury was induced by the alkali (0.15 N NaOH) applied on the cornea of the right eye and then rinsed with tap water. In the first group of rabbits the injured corneas remained untreated. In the second group, the MCSs were applied on the injured ocular surface after the injury and the eyelids were sutured for two days. In the third group, nanofiber scaffolds seeded with MSCs (in the fourth group nanofibers alone) were transferred onto the corneas immediately after the injury and the eyelids were sutured. The rabbit corneas were examined immunohistochemically, morphologically and for the central corneal thickness.

The injured untreated corneas showed decreased expression of the antioxidant aldehyde dehydrogenase 3A1 (ALDH3A1) in the corneal epithelium, particularly in superficial parts, where apoptic death (detected by active caspase 3) was high. ALDH3A1 is an enzyme protecting the cornea from oxidative stress caused by chemical injury. High expressions of matrix metalloproteinase 9 (MMP9) and markers of lipid peroxidation and oxidative stress (malondialdehyde (MDA) and nitrotyrosine (NT)) were found in this untreated group. In contrast, the injured rabbit corneas treated with MSCs on nanofiber scaffolds showed a high expression of ALDH3A1 in the epithelium, which was similar to the control untreated corneas. A low expression of MMP9 and active caspase 3, absent expression of MDA and NT were detected in this group, similarly as in the control corneas. Immunohistochemical staining also confirmed significantly lower expression of IL-8 and IL-1β in the group treated with MSC compared to the group without treatment. The corneas were harvested on day 10 after an alkali injury in order to detect the gene expression of pro-inflammatory cytokines. The gene expression of IL-1β, IL-2 and IFN-γ determined by real-time PCR was significantly reduced in the group treated with MSC compared to the group without treatment. The expression of genes for the pro-inflammatory cytokines corresponded with their immunohistochemical expression.

At the end of the experiment (on day 15), the injured untreated corneas were vascularized (with high vascular endothelial growth factor (VEGF) expression) and numerous inflammatory cells (macrophages/monocytes) were present in the corneal stroma. The stromal inflammatory infiltration was also significantly suppressed in corneas treated with MSCs on nanofiber scaffolds. Clinically, a significant suppression of corneal neovascularization was detected in corneas treated with MSCs on nanofiber scaffolds compared to the untreated corneas with extensive neovascularization on day 15 and corresponded with neovascularization quantification by VEGF gene expression with real-time PCR. 10 days after chemical injury, corneal thickening was noticed in both groups. The corneal thickening and opacity after alkali injury have regained their normal characteristics only in the group treated with MSCs on nanofiber scaffolds. The results from injured corneas treated with nanofiber scaffolds alone and with MSCs without scaffolds

showed similar results to the untreated injured corneas.

5 DISCUSSION

5.1 Limbal tissue transplantation in a mouse model

LSCD is a blinding eye condition and its treatment is very challenging. The only way to treat severe LSCD and restore the patient's vision is via limbal tissue or cultured LSC transplantation. Successful ocular surface transplantation restores ocular surface stability, reduces symptoms and improves the patient's vision. For bilateral ocular surface disease, the limbal allograft must be used and the immune-associated rejection remains the main risk factor responsible for the low success rate. Several studies have shown beneficial effects of limbal transplantation in patients with LSCD (Dua and Azuara-Blanco, 1999); (Daya et al., 2000); (Cauchi et al., 2008). However, the major problem with limbal allotransplantation is the high risk of immune rejection and the necessity for systemic immunosuppression (Tsubota et al., 1999); (Daya et al., 2000). Conversely, the corneal allografts often survive with only topical prophylactic immunosuppressive treatment. This stark contrast between incidence of rejection in corneal and limbal allografts may be, in part, due to a stronger vascular supply, and a higher density of antigen presenting cells, such as Langerhans cells, in the limbal region (Gillette et al., 1982); (Niederkorn, 1995). Additionally, the corneal epithelium is a highly immunogenic ocular tissue and the epithelial rejection takes the main place after limbal transplantation (Treseler et al., 1985); (Qi et al., 2013). Therefore, the clinical course and cellular mechanisms of limbal graft rejection have to be recognized in order to develop a successful strategy to manage immune reaction in limbal transplantation.

Our experimental limbal transplantation confirmed high incidence of rapid rejection similar to that observed in other experimental studies (Mills et al., 2002); (Maruyama et al., 2003). Allogeneic donor cells were not detectable in the recipient 2 weeks after transplantation indicating potent and rapid immune rejection (Mills et al., 2002). To elucidate the immunological mechanism responsible for low survival rate of limbal grafts, we studied the murine model of limbal allotransplantation and developed a novel method of xenotransplantation. The clinical features of limbal rejection in patients after allogeneic limbal tissue transplantation include limbal congestion, edema, vessel dilatation and significant corneal opacity (Shi et al., 2008); (Baradaran-Rafii et al., 2013). Similarly to these observations, the rejection of limbal allografts and xenografts in our experiments was characterized by graft and corneal neovascularization, limbal graft edema and significant corneal opacity with more profound reactions in xenogeneic models.

There is much controversy about the fate of donor limbal graft and donor-derived cells on the ocular recipient's surface after limbal transplantation. Some studies showed that donor cells did not survive despite good clinical results (Williams et al., 1995); (Henderson et al., 2001)) while others confirmed long-term donor cell survival (Reinhard et al., 2004); (Djalilian et al., 2005); (Egarth et al., 2005). Several methods are described for the detection of donor

cell survival onto ocular surface after transplantation (Yin et al., 2013). In our allograft male-to-female model, the presence of corneal opacity (grade ≥ 2) coincided with the complete disappearance of allogeneic donor cells from both the limbus and corneal epithelium at 7-12 days after transplantation. To elucidate the limbal graft failure due to a technical issue, male donor limbal grafts were transplanted into female BALB/c recipients in a syngeneic model. Detection of donor male cells 4 weeks after surgery in the male-to-female model indicated that the prompt drop in allogeneic limbal cells is not due to surgical failure. No clinical signs of rejection of H-Y incompatible limbal grafts were observed in our study: similar to the results in H-Y incompatible corneal grafts in BALB/c mice (Hasková et al., 1997). In contrast, limbal grafts, incompatible in another relatively weak antigen enhanced green fluorescent protein, were rejected by the rat recipients (Keijser et al., 2006). These differences may reflect interspecies differences or suggest that BALB/c female mice are non-responder to the male-specific antigen. All limbal xenografts were rejected within 8 days of transplantation and no xenogeneic cells were detected by the real-time PCR beyond this period in the donors. These data suggest that limbal allografts and xenografts are promptly rejected and do not enjoy the immune privilege of the anterior part of the eye. The clarification of donor cell survival after the LSC transfer is essential for deciding immunosuppressive therapy duration in clinical practice.

Rejection of limbal allografts was associated with a strong Th1 cytokine response characterized by the expression of genes for IL-2 and IFN-γ in the rejected limbal grafts. Expression of the gene for IL-4 was not detected in rejected limbal allografts and also expression of the gene for IL-10, another Th2 cytokine, did not exceed the baseline levels in syngeneic limbal grafts. On the contrary, a strong expression of genes for Th2 cytokines IL-4 and IL-10, in addition to Th1 cytokines, was detected in rejected limbal xenografts. This pattern of cytokine expression during limbal graft rejection resembles cytokine profiles after corneal allo- and xenotransplantation, but the role of the Th2 cytokines in the rejection of xenografts is unclear (Pindjáková et al., 2005). Macrophages and their product nitric oxide have been shown to play a more important role in graft rejection. Mills et al. observed a significant infiltration of limbal allografts in rats by macrophages and we found a strong expression of the gene for iNOS in both rejected limbal allografts and xenografts (Mills et al., 2002). It has already been shown in various allo- and xenotransplantation models, including corneal transplantation, that the inhibition of iNOS activity may prolong graft survival (Krulová et al., 2002); (Strestíková et al., 2003)). The production of NO by graft infiltrating macrophages depends on the availability of IFN-γ, which was also detected during rejection of both limbal allo- and xenografts and is a key factor in delayed-type hypersensitivity (DTH) reaction. This suggests that strategies directed to inhibit IFN-γ (Th1 response), NO production and the DTH reaction should be beneficial in the promotion of limbal graft survival. Indeed, Maruyama et al. have shown that the Th2-biased immune system and the suppression of the DTH reaction may support the survival of limbal allografts in mice (Maruyama et al., 2003).

The success rate of limbal tissue allotransplantation differs among published studies from as low as 33.3% with inappropriate immunosuppression to as high as 77.2% with adequate immunosuppressive therapy (Miri et al., 2010); (Holland et al., 2012); (Tan et al., 2012). In clinical practice, various protocols of systemic treatment are being used after limbal allotrans-

plantation. There is evidence of the systemic use of steroids, cyclosporin (Tsubota et al., 1999), tacrolimus (Dua and Azuara-Blanco, 2000) and mycophenolate mofetil (Reinhard et al., 2004). It is widely recommended to use systemic immunosuppression for a period of at least 2 years postoperatively. The systemic therapy is associated with a serious side effects such as higher susceptibility to infectious and cancerous diseases. Therefore, a new approach would be welcomed for postponing the rejection in limbal allografting. In our study, we showed that targeting of $CD4^+$ cells by systemic application of mAb results in a suppression of the rejection reaction and in a significant prolongation of limbal allograft and xenograft survival. The effect of anti-CD4 therapy may be due to the elimination of $CD4^+$ T cells, which mediate the DTH reaction and are an important source of IFN-γ for iNOS expression (Krulová et al., 2002). In addition, the anti-CD4 antibody can inactivate $CD4^+$ macrophages, which play a role in both the afferent phase of transplantation reaction as antigen-presenting cells and in the effector phase as cytotoxic macrophages (Keijser et al., 2006); (Strestíková et al., 2003); (Slegers et al., 2004). It has been shown that a subpopulation of macrophages expresses CD4 molecules, and these $CD4^+$ macrophages have been shown to be involved in graft rejection (Wallgren et al., 1995). Nevertheless, anti-CD4 mono-therapy did not ensure a permanent limbal allograft survival. The rejections observed in the anti-CD4 treated recipients may be due to the activity of other $CD4^+$ cell-independent mechanisms (such as $CD8^+$ cell-activated macrophages, cytotoxic T cells, etc.). The results also showed that anti-CD8 treatment was not effective in the prevention of limbal allograft rejection, similar to the case of corneal transplantation (He et al., 1991); (Vítová et al., 2004). For the first time, our study demonstrates intragraft cytokine response in orthotopic limbal allo- and xenograft recipients and indicates the key role of Th1 response and $CD4^+$ cells in limbal graft rejection. Therefore, the strategies targeting $CD4^+$ cells as the main mediators of Th1 response and activators of macrophages for NO production were suggested to prevent limbal graft rejection. This suggestion was confirmed by the effectiveness of anti-CD4 treatment in the suppression of graft rejection in allogeneic limbal transplantation in the mouse model.

5.2 Isolation and characterization of mouse limbal stem cells

LSC-based therapy for LSCD has great potential. However, LSCs comprise just a minor fraction of the whole limbal tissue and there is a need to find an optimal isolation method. Indeed, the limbus has a very heterogeneous cell population with LSCs residing in the basal layer. At this epithelial level, there are several other cell types in the vicinity such as the immediate progeny, i.e. early TAC, melanocytes, Langerhans cells and corneal epithelial basal cells (Li et al., 2007). The transplantation of LSCs contributes to long-term homeostasis and the lack of SCs in the graft may be the cause of the failure (O'Callaghan and Daniels, 2011). Therefore, the ability to isolate a cell population with a high number of LSCs is essential for successful transplantation.

Our experiments have shown that cells sharing morphologic, phenotypical, and functional characteristics of LSCs can be found in the mouse limbus. LSCs are characterized by small size, a low replication rate, expression of certain markers such as a transporter ABCG2, p63, integrin 9, or K19, and expression of the SP phenotype, which have been described in human, rat and

rabbit models (Chen Z. et al., 2004); (Schlötzer-Schrehardt and Kruse, 2005); (Umemoto et al., 2005); (De Paiva et al., 2006); (Park et al., 2006)). We found two distinct, separable populations of corneal epithelial cells with SC characteristics (expression of ABCG2 and Lgr5, and SP phenotype) obtained from the mouse limbus by Percoll gradient centrifugation. The presence of SCs (less than 7% of original cells) was found in the densest fraction (80% Percoll gradient) containing small, non-spontaneously proliferating cells, positive for p63, and negative for K12. These cells occurred in a quiescent state and did not proliferate within the first 3 days in tissue culture, as has been described for SP cells in rabbits (Park et al., 2006). However, this cell population acquired a high proliferative activity when cultured on a 3T3 feeder cell monolayer. Thus, the quiescent cells from the densest fraction may require a specific niche to facilitate proliferation. The densest fraction also expressed the SP phenotype (>30% in comparison to 2%-5% in the whole limbus). All these characteristics mimicked the LSCs described in the human and rabbits more closely (Chen Z. et al., 2004); (Schlötzer-Schrehardt and Kruse, 2005); (De Paiva et al., 2006); (Park et al., 2006); (Umemoto et al., 2006).

A second cell fraction showing some characteristics of LSCs (positive expression of ABCG2 and Lgr5) was detected in the lightest cell population (40% Percoll gradient) and represented approximately 12% of the total limbal cell population and over 20% of the cells expressed in the SP phenotype. However, the light cell population was positive for the corneal differentiation marker K12. This population had the highest spontaneous proliferative capacity compared to unseparated limbal cells. Their proliferation response did not increase when they were cultured on a feeder cell monolayer.

The SP phenotype is a property of SCs (Shimano et al., 2003); (Zhou et al., 2001) and is associated with ABCG2 expression, however not all cells expressing ABCG2 exhibit the SP phenotype (Kim et al., 2002). The SP phenotype was reported in the conjunctival and limbal epithelium in humans and rabbits but not in the corneal epithelium (Watanabe et al., 2004); (Umemoto et al., 2005). Both populations contained cells expressing the SP phenotype based on the efflux of Hoechst 33342 dye. The number of SP cells in the unseparated mouse limbus was 3.8% of the total limbal cells, substantially higher than the number of slow cycling corneal epithelial cells found at the mouse limbus or the number of SP cells in human, rabbit, and rat limbal epithelia (De Paiva et al., 2005), (Umemoto et al., 2005); (Pajoohesh-Ganji et al., 2006); (Park et al., 2006). However, it corresponds to the number of SP cells found in the rat cornea (Umemoto et al., 2006). The studies of Umemoto and coworkers in humans (Watanabe et al., 2004), rabbits (Umemoto et al., 2006) and rats (Umemoto et al., 2005) showed that although the number of cells exhibiting the SP phenotype was less than 2% in the limbus, immunochemistry revealed that a larger proportion (approximately 10%) of limbal basal epithelial cells expressed ABCG2 transporter (Umemoto et al., 2005). Similarly, (Budak et al., 2005) suggested the existence of a significantly higher number of ABCG2$^+$ cells than SP cells. This discrepancy was explained by the differences in the transport activity of ABCG2. (Umemoto et al., 2005) also showed that in the rat, unlike the human and rabbit, the central cornea contains cells with the SP phenotype but that these cells expressed significantly lower levels of putative SC markers than the SP cells in the limbus (Umemoto et al., 2005). In addition, SP cells found in the rat cornea had a different profile on forward scatter analyses than SP cells in the limbus.

Our study showed that mouse limbal cells with the SP phenotype from the light cell fraction of the Percoll gradient (40%) had distinctive light-scattering properties from SP cells from the dense cell fraction (80%). It appears that the light cell fraction positive for K12 resembles the SP cells described by Umemoto et al. (Umemoto et al., 2005) in the rat central cornea rather than the basal LSCs. The interspecies differences exist in the distribution and properties of corneal epithelial cells with LSC characteristics and the mouse may represent a unique species that is different to human, rabbit, or rat.

5.3 Limbal and mesenchymal stem cell transfer on nanofiber scaffolds for treatment of ocular surface damage in a mouse model

Recent publications have shown promising results of SC-based treatment for ocular surface disorders. However, there is a need to find an optimal scaffold for the SC transfer. Nowadays, there is growing evidence of *in vitro* experiments using various electrospun nanofibers of various polymers to enable cell adhesion, proliferation and differentiation (Das et al., 2009); (Bhattarai et al., 2005); (Schindler et al., 2005). However, there is a limited evidence of *in vivo* use of nanofiber scaffolds in models of ocular surface disease. Therefore, we investigated whether these nanofiber polyamide scaffolds are useful and suitable for the SC culture and transfer onto the ocular surface in the mouse model. For the LSC isolation we used the Percoll method described in our previous experiments (Publication 2). To analyze the anti-inflammatory effect of the transferred cultured cells, our already established limbal allotransplantation model was used to induce a strong immune response (Publication 1).

The human AM is currently the most widely used carrier of LSCs (Tsai et al., 2000). However, due to human AM variability, the risk of infection transmission and crease formation it is not an ideal substrate and other scaffolds have been proposed for SC therapy (Levis and Daniels, 2009). Alternative scaffolds for cell culture and transfer have been used and include collagen scaffolds (Schwab et al., 2006); (Dravida et al., 2008), fibrin-based scaffolds (Rama et al., 2001); (Talbot et al., 2006), contact lens-based scaffolds (Di Girolamo et al., 2009) and synthetic polymers (Sharma et al., 2011). The synthetic (polyamide) scaffolds have advantage of biocompatibility, easy accessibility, no risk of infection transmission and good manipulation.

Previous experimental studies have shown that embryonic SCs can be grown and differentiated on nanofiber scaffolds (Nur-E-Kamal et al., 2006); (Smith et al., 2009). Our results with nanofiber scaffolds demonstrated that the scaffolds prepared by electrospinning technology from polyamide PA6/12 can be used for the culture and transfer of LSCs and MSCs. The 3D structure of nanofiber materials has a large surface area, which can mimic the extracellular matrix and is therefore supports cell growth and function. Up to day 14 after transfer, it was important for us to detect the labelled LSCs on the ocular surface. These results support the fact that nanofiber scaffolds PA6/12 are suitable for SC transfer and that 3 days of coverage is sufficient for LSC migration onto the damaged ocular surface. In our pilot experiments, we found that the nanofiber scaffolds from PA6/12 are stable with no cytotoxicity and surgical manipulation was simple. And the experimental study showed that these scaffolds are suitable for various cell types to be cultured (Dubský et al., 2012).

In our experiments, we found that the scaffolds without cells reduced the inflammatory reaction as well, but less so compared to the scaffolds with LSC and MSC. In view of these results, these scaffolds may serve as therapeutic bandage scaffolds for promoting epithelial healing. The advantage of this compared to human AM is easy accessibility, mechanical stability and no risk of infection transmission. However these scaffolds are not transparent and need to be removed. The 3D reconstruction of LSC niche in which the SCs are associated with adjacent epithelial and stromal cells has been demonstrated (Dziasko et al.,2014). Therefore the 3D structure of nanofiber scaffolds can mimic the extracellular structure of limbal niche. A transfer of scaffold with LSC in the limbal region may serve as a long-term source of LSCs and allow cell migration. However, further studies are needed to show that the scaffolds are able to allow the cells to maintain their SC properties and to serve as a niche.

We used the co-transfer of LSCs and MCSs on nanofiber scaffolds for ocular surface reconstruction in our experiments because these cells may act synergistically and may be both beneficial for ocular surface healing in clinical practice. The LSCs were used from the aspect that they serve as a source of SCs on damaged ocular surface and our experiments proved the migration of LSCs on ocular surface. MSCs were used as they have anti-inflammatory properties, which are proven in previous experimental studies (Ma et al., 2006); (Oh et al., 2008). Inflammation is one of the highest risk factors for the failure of SC based therapy for ocular surface reconstruction and the suppression of inflammation is crucial (Shortt et al., 2010). In our experiments, the co-transfer had immunosuppressive properties, significantly inhibited the local inflammatory reaction and supported healing of the damaged ocular surface.

Two main mechanisms are generally used for transferring cultured LSCs onto a damaged ocular surface. Primarily, the LSCs act as a source of SCs. Secondarily, the transferred LSCs may have a stimulatory effect on residual LSCs, even on an ocular surface with (a clinical diagnosis of) LSCD. This is supported by the fact that the long-term survival of transferred cells was not detected despite good clinical results (Rama et al., 2010). Additionally, the LSCs alone have immune-modulatory properties, which may be beneficial for suppressing inflammation and postponing the rejection (Holan et al., 2010). The co-transfer with MSCs may have another additional benefit, namely that MSCs have the potential to differentiate into different cell types, including the corneal epithelial cells (Pittenger et al., 1999); (Neuss et al., 2008); (Gu et al., 2009). However, there is controversy in current literature surrounding *in vivo* MSCs' ability to trans-differentiation into corneal epithelial cells (Reinshagen et al., 2011).

Used together, our results showed that the nanofiber scaffolds from PA6/12 polyamide are suitable for the growth and transfer of adult tissue specific SCs in the treatment of ocular surface disease in the mouse model. In the future, the nanofiber scaffolds may have a great potential to serve as a scaffold for a wider spectrum of adult SCs and could be used for treatment of various SC deficiencies in humans. However, further studies are needed to test SC survival and the preservation of SCs' properties after transfer onto the ocular surface.

5.4 Mesenchymal stem cell transfer on nanofiber scaffolds for the treatment of chemical corneal injury in a rabbit model

Chemical corneal injuries are one of the most common causes of LSCD. Alkali injuries cause extensive damage to the ocular surface, which can lead to loss of vision. After the injury, acute inflammation, corneal neovascularization, recurrent epithelial erosions and corneal ulcers are present. In the acute phase after injury, there is a need for a prompt anti-inflammatory therapy to reduce the risk of subsequent LSCD development. Appropriate anti-inflammatory treatment reduces the risk of LSCs exhaustion, which is believed to be the more common cause of LSCD than the primary LSC damage caused by the chemical injury. However, current therapy is not always efficient enough and it is necessary to look for alternative treatment options. The discovery of new effective treatment strategies may diminish the incidence of LSCD after chemical corneal injury, thus reducing the risk of vision loss and improving guarded prognosis. Currently, there is growing evidence that MSCs have great potential in regenerative medicine in terms of their anti-inflammatory and immune-modulatory properties (Uccelli et al., 2008); (Nauta and Fibbe, 2007); (Zhao et al., 2010). Based on our previous experience with SC transfer on nanofiber scaffolds (Publication 3), we studied the effect of MSCs after ocular surface injury in a rabbit model. The rabbit eye model is closer to the human eye and may have more similar applications in clinical practice.

Previous studies focused on healing after alkali injury in rats demonstrated the anti-inflammatory and anti-angiogenic effect of MSCs transferred onto human AM (Ma et al., 2006), applied topically (Oh et al., 2008) and injected subconjunctivally (Yao et al., 2012). Similar to these results, we found that MSCs transferred onto nanofiber scaffolds contributed to the healing process in the cornea after alkali injury. To elucidate the mechanisms of the healing process, we showed the positive effect of MSCs on nanofiber scaffolds on alkali-induce oxidative stress in the cornea for the first time. We found a higher antioxidant expression and lower expression of oxidative stress markers in the cornea after MSC therapy. On a cellular basis, the reduced stromal inflammatory infiltration by macrophages was found in the MSCs treated group during post-operative period. The expression of genes for pro-inflammatory cytokines (IL-1β, IL-6 and IFN-γ) was significantly reduced in the group treated with MSCs on nanofiber scaffolds compared to the group without treatment. The nanofiber scaffolds are suitable for SC growth and transfer and are easily transferred onto the ocular surface.

In view of the current knowledge, both mechanisms of MSCs (suppression of inflammation and differentiation into corneal-like epithelium) may participate in the healing process of ocular surface damage after alkali injury. The MSCs are able to differentiate *in vivo* into corneal epithelium-like cells after transfer onto damaged ocular surface (Gu et al., 2009). There are studies that demonstrate the therapeutic effects of MSCs for treating LSCD due to differentiation (Jiang et al., 2010); (Ye et al., 2006). However, the study of Reinshagen et al. did not prove the clinical improvement of LSCD in rabbits after the MSCs were injected under human AM secured on the corneal surface (Reinshagen et al., 2011). They described that the possible causes of therapy failure may be due to cell spread under conjunctiva or the washing out effect of tear film after transfer. Human AM has the disadvantage of inducing epithelial

repair accompanied by dense vascularization (Kim and Tseng, 1995); (Reinshagen et al., 2011).

For corneal transparency and to maintain good vision, it is essential to maintain the avascularity and uniform ultrastructure of collagen fibrils. Both attributes can be damaged after alkali injury. We found that corneal neovascularization was significantly suppressed after MSC transfer in alkali-injured corneas. This anti-angiogenic therapeutic effect may be given by a concurrent anti-inflammatory effect. But the exact mechanism is not known. Corneal swelling is a sign of corneal damage after alkali injury and can be measured by an ultrasound pachymeter (O'Donnell et al., 2006); (Cejka et al., 2010). We found that the MSCs transferred on nanofiber scaffolds normalized the corneal hydration after 10 days. Thus the corneal ultrasound pachymetry is a good tool for monitoring corneal restoration and thus following the healing process.

In conclusion, the use of autologous MSCs cells have the advantage of not inducing immune rejection. The MSCs are easy to isolate, therefore there is a high potential for clinical application. In addition, the rabbit eye model is closer to the human eye and, therefore, the results of our experiments may have more clinical applications.

6 CONCLUSIONS

The aim of this thesis was the reconstruction of the damaged ocular surface by LSC transfer in the experimental mouse and rabbit model. To achieve ocular surface recovery in LSCD, it is necessary to understand the cellular mechanism of LSC transplantation, immune response and graft survival. Based on the promising results of recent publications, there is a need to focus, not only on tissue transfer, but also on SC-based treatment for ocular surface disorders in more depth. The main conclusions of this study are as follows:

1. Allogeneic limbal grafts do not enjoy any immune privileged position of the eye and are rejected promptly by the Th1-type of immune response involving $CD4^+$ cells and NO produced by macrophages. Anti-CD4 treatment thus represents a promising immunosuppressive approach after limbal allotransplantation. Th1 and Th2 immune responses were detected in the xenogeneic model during the rejection. Depletion of $CD4^+$ cells significantly prolonged the limbal survival in the allograft and in the xenograft model. Limbal graft transplantation is a useful model for testing various immunosuppressive approaches. This surgical technique was successfully used for further experiments with nanofiber scaffolds and enabled a better understanding of the immune mechanism of SC transfer.

2. By centrifugation in Percoll gradient of epithelial LSCs, two distinct populations of corneal epithelial cells with LSC characteristics were separated in the 40% and 80% Percoll fraction of the gradient. The densest fraction (less than 7% of original cells) contained small, non-spontaneously proliferating cells, $K12^-/p63^+$, with a high *ex vivo* proliferative activity culturing on a 3T3 feeder cell monolayer. The $K12^-/p63^+$ population is closer to the primitive LSCs. Therefore this technique can be used for SC isolation from limbal

explant and subsequently for SC based therapy of ocular surface disorders. This method was used for our further experiments with LSC transfer and is still used as a standard method for LSC isolation in mouse model.

3. The nanofiber scaffolds from a polyamide 6/12 can be useful for growth and transfer of LSCs and bone marrow-derived MSCs, and can be utilized for future treatment of ocular surface injuries and LSCD. The co-transfer of LSCs and MSCs suppressed the inflammatory reaction and therefore improved corneal healing in the mouse model.

4. Bone marrow-derived MSCs on nanofiber scaffolds reduced the alkali-induced oxidative stress in the cornea after alkali injury and significantly accelerated corneal healing in the experimental rabbit model. The transferred MCSs protected against the peroxynitrite production, suppressed the cell apoptosis, matrix metalloproteinase levels and pro-inflammatory cytokine production. Reduced inflammation resulted in decreased corneal neovascularization after alkali injury.

The understanding of cellular and molecular mechanism of limbal tissue transplantation and finding of new treatment strategies may improve long-term limbal graft survival, clinical outcome and thus may be beneficial for patients with LSCD diagnosis. Additional to that, the better understanding of mechanism of SC-based therapy may give us more knowledge for future treatment options for patients with this severe diagnosis, especially in cases of bilateral LSCD. Therefore, this field of experimental ophthalmology has a great potential for patients with LSCD, who have a very poor prognosis. Further studies are necessary to determine the long-term effect of LSC transfer, the fate of donor cells on ocular surface in term of SCs properties and the long-term cell survival after the transfer of *ex vivo* cultured LSCs and MSCs on the ocular surface.

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8 LIST OF PUBLICATIONS

8.1 Publications related to the thesis

1. Lenčová A, Pokorná K, Zajícová A, Krulová M, Filipec M, Holáň V. Graft survival and cytokine production profile after limbal transplantation in the experimental mouse model. Transpl Immunol. 2011 Apr 15;24(3):189-94. (IF 1.912)

2. Krulova M, Pokorna K, Lencova A, Fric J, Zajicova A, Filipec M, Forrester JV, Holan V. A rapid separation of two distinct populations of mouse corneal epithelial cells with limbal stem cell characteristics by centrifugation on Percoll gradient. Invest Ophthalmol Vis Sci. 2008 Sep;49(9):3903-8. (IF 3.528)

3. Zajicova A, Pokorna K, Lencova A, M 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(10):1281-90. (IF 6.204)

4. Cejkova J, Trosan P, Cejka C, Lencova A, Zajicova A, Javorkova E, Kubinova S, Sykova E, Holan V. Suppression of alkali-induced oxidative injury in the cornea by mesenchymal stem cells growing on nanofiber scaffolds and transferred onto the damaged corneal surface. Exp Eye Res. 2013 Nov;116:312-23. (IF 3.017)

8.2 Published abstracts

1. Holan V, Zajicova A, Lencova A, Pokorna K, Svobodova E, Krulova M. (2009), Treatment of ocular surface injuries by the transfer of limbal and mesenchymal stem cells growing on nanofibrous scaffolds. Acta Ophthalmologica, 87: 0. doi: 10.1111/j.1755-3768.2009.205.x (IF 2.441)

2. Holan V, Pokorna K, Krulova M, Lencova A, Zajicova A, Jirsova K, Filipec M, Forrester J. (2010), The presence of cells with stem cell markers and characteristics in the central cornea of the mouse. Acta Ophthalmologica, 88: 0. doi: 10.1111/j.1755-3768.2010.345.x (IF 2.809)

3. Holan V, Krulova M, Pokorna K, Lencova A, Zajicova A, Filipec M. (2008), Two distinct populations of corneal epithelial cells with limbal stem cell characteristics in the mouse. Acta Ophthalmologica, 86: 0. doi: 10.1111/j.1755-3768.2008.418.x (IF 2.138)

4. Lencova A, , Pokorna K, Zajicova A, Filipec M, Holan V. (2008), Experimental models of orthotopic limbal transplantation in the mouse. Evaluation of allo- and xenograft survival and characterization of cytokine response. Acta Ophthalmologica, 86: 0. doi: 10.1111/j.1755-3768.2008.543.x (IF 2.138)

5. Lencova A, Filipec M, Holan V. (2011), Graft survival and cytokine production profile after limbal transplantation in the experimental mouse model. Acta Ophthalmologica, 89: 0. doi: 10.1111/j.1755-3768.2011.3144.x (IF 2.629)

6. Holan V, Zajicova A, Chudickova M, Lencova A, Trosan P, Svobodova E, Krulova M. (2011), Cyclosporine A-loaded and limbal stem cell-seeded nanofibers for the local suppression of inflammatory and transplantation reactions. Acta Ophthalmologica, 89: 0. doi: 10.1111/j.1755-3768.2011.452.x (IF 2.629)

8.3 Other selected presentations

Oral presentations:

1. Transfer of mesenchymal stem cells on nanofiber scaffolds for treatment of a chemical injury of the cornea (first author), 18th Nottingham Eye Symposium and Research Meeting 2014, Nottingham, UK

2. Treatment of corneal chemical injury by using mesenchymal stem cells (first author), National Congress of Czech Society of Ophthalmology (COS) 2013, Brno * Graft survival and cytokine production profile after limbal transplantation in the experimental mouse model (first author), EVER (Association for European Vision and Eye Research), 2011, Crete, Greece

3. Treatment of damaged corneal surface by nanofiber scaffolds (first author), COS 2010, Teplice

4. Limbal stem cell transplantation in the mouse (first author), 10th International Ocular Inflammation Society Congress (IOIS) 2009, Prague

5. Limbal stem cells in the eye of the mouse (co-author), IOIS 2009, Prague

6. Th1 and Th2 cytokine production after limbal transplantation in experimental model (first author), Cytokine Symposium 2009, Prague

Poster presentations:

1. Transfer of limbal and mesenchymal stem cells on nanofiber scaffolds for treatment of ocular surface damage (first author), ARVO 2010 (Association for Research in Vision and Ophthalmology), Ft Lauderdale, USA

   * Award: G. M. JAGGER TRAVEL GRANT ARVO 2010, Ft Lauderdale, Florida, USA

2. Analysis of limbal and corneal cells markers (first author), COS 2009, Prague

3. Experimental model of allogeneic limbal transplantation (first author), COS 2009, Prague

4. Anti-CD4 is effective immunosuppression after experimental allogeneic limbal transplantation (first author), ARVO 2009, Ft Lauderdale, USA

5. Experimental model of orthotopic limbal transplantation in the mouse model. Evaluation of allo- and xenograft survival and characterization of cytokine response (first author), EVER 2008, Portoroz, Slovenia

   * 2 awards: POSTER PRIZE Section Cornea/Ocular Surface, EVER 2008, Portoroz,

6. Slovenia; TRAVEL GRANT EVER 2008, Portoroz, Slovenia

7. Isolation and characterization of mouse limbal stem cells (co-author), Congress of Czech and Slovak Allergologists and Clinical Immunologists 2008, Prague

8.4 Publications not related to the thesis

1. Vesela M, Barakova D, Lencova A. Analysis of reasons of intraocular lenses explantation. Cesk Slov Oftalmol. 2014 Winter;69(4):170-3. Czech.

2. Lencova A. Case report: Keratitis after LASIK, Online Case Library - Ophthalmology, Case Presentations from Fellows of the Salzburg Medical Seminars International 2011