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DISSERTATION

New drugs in prevention of experimental corneal graft rejection

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TABLE OF CONTENTS

I	Summary		2
	1	Abstract	2
	2	Abstrakt – Deutsch	3
	3	Abbreviations	4
	4	Introduction	5
		4.1 Immune privilege of cornea	5
		4.2 Corneal graft rejection	6
		4.3 Therapy	7
		4.4 Aim of the study	8
	5	Methodology	9
		5.1 Animals	9
		5.2 Surgical procedure	10
		5.3 Preparation of drugs	10
		5.4 Treatment protocol	11
		5.5 Graft assessment	12
		5.6 PCR	13
		5.7 Statistical analysis	13
	6	Results	13
		6.1 SEGRA	13
		6.2 Everolimus	14
		6.3 Spironolactone	15
	7	Discussion	16
	8	Bibliography	19
II	Affidavit, including declaration of own contributions		21
III	Print copies of the selected publications		23
IV	Curriculum vitae		41
V	Complete list of publications		43
VI	Acknowledgements		48

I Summary

1. Abstract

Background: Immune mediated graft rejection remains a major complication of penetrating keratoplasty (PKP), requiring more effective preventive measures. **Purpose:** We investigated the efficacy of selective glucocorticoid receptor agonist (SEGRA), everolimus and spironolactone on the prevention of allograft rejection in a MHC class I/II mismatch rat corneal transplant model. **Methods**: A total of 133 female Lewis rats underwent PKP. Syngeneic corneal grafting in 20 rats served as control for technical failure. All other 113 Lewis rats received allogeneic corneal grafts from Dark Agouti donors and were randomly assigned to receive the agent of interest, vehicle or remained untreated. ME-CD-SEGRA (0.25% SEGRA-containing microemulsion) and everolimus microemulsion (0.05 and 0.025%) were applied as topical treatment five times daily, while spironolactone suspension was given orally, 100 mg/kg/day. Therapy was applied daily for 35 days in SEGRA group and until the day of rejection in everolimus and spironolactone treated rats for graft survival analysis. Detection of immunological parameters was performed by testing mRNA expression of cytokines (IL-4, IL-10, IFN-γ, TNF-α) and T-cell markers (CD3, CD25) at predestinated time points. **Results:** Topical application of ME-CD-SEGRA significantly prolonged mean survival time (MST) of corneal grafts (42.2 ± 4.0 days) compared with untreated controls (11.7 ± 1.2 days, $p = 0.00003$) or animals that received the vehicle only (15.0 ± 1.5 days). ME-CD-SEGRA decreased intragraft mRNA expression of all tested cytokines and T-cell marker, in particularly significant for IL-4 compared to untreated controls ($p < 0.05$). Local administration of 0.05 or 0.025% everolimus was effective in prolonging the MST of corneal grafts (21 ± 6.57 days and 16.4 ± 2.3 days, respectively) compared to the vehicle group (13.3 ± 1.7 days, for both $p < 0.001$). At the same time, increased mRNA expression of IL-10 ($p = 0.015$) was detected in everolimus treated grafts. Spironolactone also significantly prolonged MST of corneal grafts (14.9 ± 2.0 days) compared with both vehicle- treated (12.3 ± 1.2 days, $p = 0.007$) and untreated controls (13.0 ± 1.0 days, $p = 0.01$). Spironolactone affected both systemic (down-regulation of CD25+ mRNA expression in spleen; $p < 0.01$) and local immune response (up-regulation of IL-10 mRNA expression in cornea; $p < 0.01$). **Conclusion**: All tested drugs significantly prolonged corneal graft survival following experimental keratoplasty. Overall, further clinical investigations of these agents may contribute to improve the outcome of keratoplasty.

2. Abstrakt (Deutsch)

Hintergrund: Immunmediierte Abstoßungsreaktionen sind Hauptursache des Transplantat-versagens nach perforierender Keratoplastik und stellen weiterhin eine Herausforderung dar. **Zielsetzung:** Wir untersuchten die Effekte eines selektiven Glucocorticoidrezeptoragonisten (SEGRA) sowie von Spironolakton und Everolimus hinsichtlich der Prävention einer Transplantatabstoßung in einem Ratten-Hornhauttransplantatmodell mit diskrepanter MHC Klasse I/II. **Methoden**: Es wurde an insgesamt 133 weiblichen Lewis-Ratten eine perforierende Keratoplastik durchgeführt. Syngene Hornhauttransplantationen an 20 Tieren dienten als Kontrolle für technische Probleme. 113 Lewis-Raten erhielten allogene Transplantate von Dark Agouti-Spendertieren. Die Empfänger wurden randomisiert den Therapiegruppen zugeteilt, erhielten das Wirkstoffvehikel oder blieben unbehandelt. ME-CD-SEGRA (0,25% SEGRA-haltige Mikroemulsion) oder Everolimus (als 0,05% bzw. 0,025% Mikroemulsion) wurden fünf Mal täglich topisch angewandt, während Spironolakton als Suspension 100 mg/kg pro Tag oral gegeben wurde. Die Therapie wurde bei der SEGRA Gruppe über 35 Tage postoperativ appliziert. Die Behandlung mit Everolimus oder Spironolakton wurde bis zum Zeitpunkt der Transplantatabstoßung durchgeführt. Zum Monitoring immunologischer Parameter wurde die Genexpression von Zytokinen (IL-4, IL-10, IFN-γ, TNF-α) und T-Zell Markern (CD3, CD25) im Transplantat zu vorbestimmten Zeitpunkten vorgenommen. **Ergebnisse**: Durch topische Anwendung von ME-CD-SEGRA konnte die mittlere Überlebenszeit der Hornhauttransplantate (42,2 ± 4,0 Tage) gegenüber der unbehandelten Kontrollgruppe (11,7 ± 1,2 Tage, $p = 0.00003$) oder Tieren, die nur die Wirkstoffvehikel erhielten (15,0 ± 1,5 Tage) signifikant verlängert werden. ME-CD-SEGRA verminderte die mRNA-Spiegel aller untersuchten Zytokine und T-Zell-Marker, insbesondere für IL-4 gegenüber unbehandelten Tieren ($p < 0.05$). Die lokale Anwendung von 0,05 oder 0,025 % Everolimus verlängerte die mittlere Überlebenszeit der Transplantate (21 ± 6.57 bzw. 16,4 ± 2,3 Tage) im Vergleich zur Wirkstoffvehikel-Gruppe (13,3 ± 1,7 Tage, für beide $p < 0.001$). Gleichzeitig wurde eine erhöhte IL-10 mRNA Expression ($p = 0,015$) im Transplantat nachweisbar. Spironolakton verlängerte ebenfalls signifikant die Überlebenszeit (14,9 ± 2,0 Tage) im Vergleich zur Wirkstoffvehikel Gruppe (12,3 ± 1,2 Tage, $p = 0,007$) und der unbehandelten Kontrollgruppe (13,0 ± 1,0 Tage, $p = 0,01$). Spironolakton beeinflusste sowohl die systemische (Senkung der CD25 mRNA Expression in der Milz; $p < 0,01$) als auch die lokale Immunregulation (Erhöhung der IL-10 mRNA Expression in der Hornhaut; $p < 0,01$). **Schlussfolgerung**: Alle getesteten Medikamente haben das Überleben des Hornhauttransplantats nach der Keratoplastik statistisch signifikant verlängert. Hiermit

verbunden besteht die Hoffnung, dass klinische Studien dieser Wirkstoffe den Erfolg nach Keratoplastik weiter vorantreiben könnten.

3. Abbreviations

ACAID	Anterior chamber-associated immune deviation
APCs	Antigen-presenting cells
α-MSH	Alpha-melanocyte-stimulating hormone
CD	Cluster differentiation
CRP	Complement regulatory proteins
CTL	Cytotoxic T-lymphocyte
DCs	Dendritic cells
DTH	Delayed type hypersensitivity
FasL	Fas ligand
GR	Glucocorticoid receptor
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
LCs	Langerhans cells
LFA	Lymphocyte function-associated antigen
MHC	Major histocompatibility complex
MCP	Monocyte chemotactic protein
MIF	Macrophage inhibitory factor
MIP	Macrophage inflammatory protein
MR	Mineralocorticoid receptor
mTOR	Mammalian target of rapamycin
NKT	Natural killer T-cells
PCR	Polymerase chain reaction
PD-L	Programmed death ligand
RANTES	Regulated on activation normal T-cell expressed and secreted
SEGRA	selective glucocorticoid receptor agonist
ME-CD-SEGRA	0.25% cyclodextrin-encapsulated SEGRA compound in a new microemulsion formulation
TCR	T cell receptors
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory cells
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

4. Introduction

Corneal transplantation is the oldest, most common and arguably the most successful type of transplantation performed throughout the world. In the USA alone, over 46,000 corneal transplants were performed in 2012 (according to American Eye Banking statistical report).
A graft placed into an avascular and non-inflamed bed is termed “normal-risk” and 2-years survival is about 90% (1). A graft placed into a presensitised host (with previous graft rejection) or a neovascularised, inflamed recipient bed is termed “high-risk” with approximate 50% surviving 2 years (1, 2).
Five-year survival times of renal, cardiac, liver and corneal transplants averages 75% (1). But keratoplasty patients, at least normal-risk ones, do not receive systemic immunosuppressive drugs and no HLA matching is performed. It can be assumed that normal-risk grafts at least enjoy some kind of immune privilege.

4.1 The **immune privilege** is primarily attributed to the avascularity of the cornea - absence of blood and lymphatic vessels, which prevents donor antigens from reaching the regional lymph nodes (afferent blockade). On the other hand, molecules expressed on the corneal endothelium (Fig.1) block the expression of effector T-cells and complement activation (efferent blockade). Anterior-chamber-associated immune deviation (ACAID) protects the graft through antigen-specific suppression of DTH (central protection) (3). Aqueous humour is in direct contact with the graft and it contains numerous anti-inflammatory and immunosuppressive molecules such as TGF-ß, α-MSH and VIP. This tolerogenic milieu and immature APCs provide the anergic status of Th1/2 cells and immune modulation/suppression by regulatory T-cells (Treg).

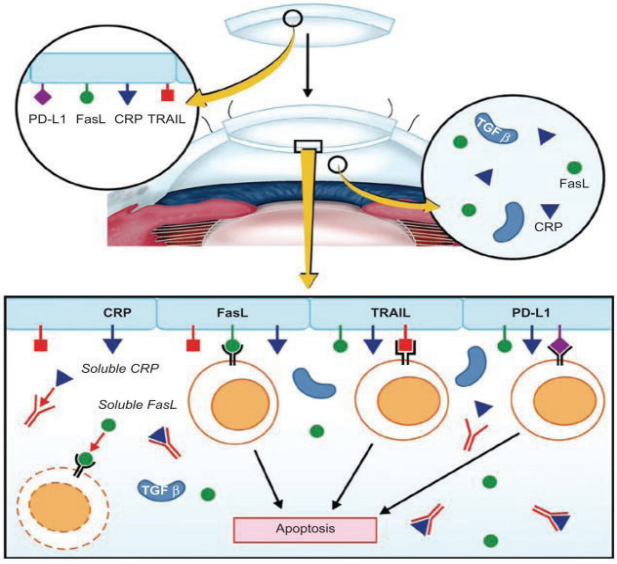

Fig. 1. Local factors contributing to immune privilege. *Niederkorn and Larkin. (3)*

VEGFR-2 blocks vessel formation in the cornea, **T regs** are induced by allografts and they inhibit induction and function of alloimmune T lymphocytes, **CRP** disable the component cascade and protect the corneal allograft from complement-mediated cytolyse, **FasL** induces apoptosis of activated T-cells and neutrophils, **TRAIL** induces apoptoses of activcated T-cells, **PD-L1** inhibits T-cell proliferation, induces their apoptosis, **MIF** inhibits NK cells and prevents cytolysis of MHC class I-negatice corneal endothelial cells.

4.2 Nevertheless, the immune privilege of the corneal allograft can fail, as significant numbers of corneal transplantation undergo **immunological rejection**. Data from the Australian Corneal Graft Registry shows that the 10-year-survival rate for corneal grafts averages 60% (4). Loosened transplant suture or herpetic infection recurrence have been shown to be events that threaten graft survival. Furthermore, vascularised recipient bed, rejected previous transplant and inflammation at the time of transplantation are high-risk-situations that worsen the prognosis of keratoplasty.

Numerous studies have tried to explain the conditions under which the corneal transplant is deprived of its immune privilege. A multi-step process is proposed. The first step is antigen presentation: migration and maturation of APC (which are immature in normal cornea) occurs via a direct or indirect pathway (Fig. 2). Once APCs entrap antigens, they migrate to the lymphoid organ and complete their maturation – and having matured, they can easily prime T lymphocytes and initiate an immune response (2).

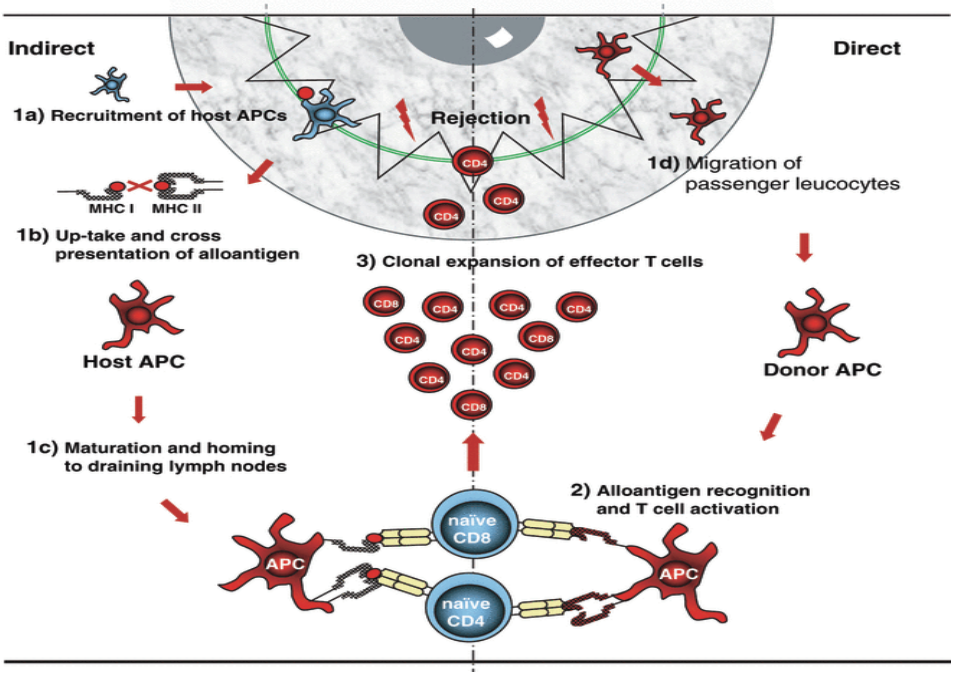

Fig. 2. Antigen presentation. *Pleyer and Schlickeiser (2)*

(1a) Recipient APCs migrate from the limbus centipetally into the corneal graft. There they either internalize and process alloantigens, or acqire shed donor MHC molecules (1b). Mature recipient APCs migrate to draining lymph nodes (1c) and present alloantigens indirectly or semidirectly, respectively. (1d) Donor APCs migrate out of the allograft and directly prime T-cells in the draining lymph nodes. (2) Alloantigen-specific T-cells recognize either presented corneal antigens or allogenic epitopes on the MHC molecules. (3) Activated T-cells proliferate and infiltrate the graft tissue.

Activation of T-cells by APCs is presented in Fig. 3.

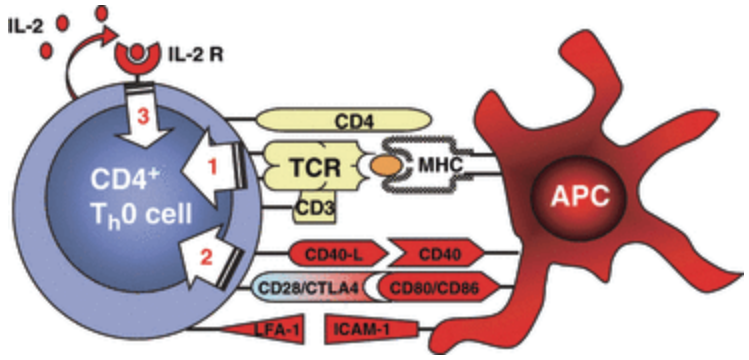

Fig. 3. Activation of T-cells by APCs. *Pleyer and Schlickeiser (2)*

The naïve T-cell can only be fully activated if it receives at least two different signals. Recognition of the peptide-MHC by the T-cell receptor delivers the antigen-specific signal 1. The interaction of co-stimulatory (CD80/CD86-CD28, CD40-CD40L) and accessory (ICAM-1-LFA-1) molecules (signal 2) leads to an amplification and completion of the signal triggering. The driving force for T-cell proliferation is the auto-/paracrine ligation of IL-2 and high-affinity (CD25+) receptor (IL-2 R). This is often referred to as signal 3.

The final step is rejection: activated T-cells proliferate and infiltrate the graft tissue. Adhesion molecules (ICAM-1, VCAM-1, LFA-1, FasL), cytokines (IL-1, IL-4, TNFα, IFNγ) and chemokines (RANTES, MCP, MIP) are involved in a complex network that mediates allograft response. It seems that it is DTH rather than CTLs that mediate corneal graft rejection (1).

4.3 Prevention of corneal graft rejection

Routine treatment after keratoplasty is presented in Table 1.

	PKP normal- risk	**PKP high- risk**	**DSAEK**	**DMEK**
Prednisolone topical	5x/day, from 4. week 3x/day, from 6. week 1x/day till 12 months, eventually long-term	5x/day, from 6. month 1x/day long-term	5x/day, from 4. week 3x/day, from 3 months 1x/day, from 6 months finish	5x/day, from 4. week 3x/day, from 3 months 1x/day, from 6 months finish
Prednisolone systemic	Eventually 3 days 1.5 mg KG	3-14 days 1.5 mg KG	Not indicated	Not indicated
CsA systemic	Not indicated	6-12 Months, by limbus KPL longer	Not indicated	Not indicated
MMF systemic (alternative to CsA)	Not indicated	1g/day over 14 days, den 2x1g/day 6-12 months	Not indicated	Not indicated

Table 1. Actual therapy to prevent immune rejection after PKP (penetrating keratoplasty), DSAEK (Descemet's Stripping Automated Endothelial Keratoplasty) and DMEK *(*Descemet Membrane Endothelial Keratoplasty). *Bertelmann and Pleyer (4)*

The therapy spectrum in high-risk keratoplasty is expanding. Apart from steroids, systemic application of Cyclosporin A (CsA) and Mycofenolat Mofetil (MMF) are common practise. Tacrolimus (FK 506) and Sirolimus (Rapamycin) are effective but could not achieve clinical importance due to high rates of side effects (5). Therefore, in general there is a tendency in ophthalmology to apply therapy locally to avoid the side effects associated with systemic use of drugs.

Topical corticosteroids represent the standard treatment for the prevention of corneal graft rejection, although they have side effects (glucocorticoid-induced glaucoma and cataract formation). In addition, they do not provide a guarantee against graft rejection. Alternative therapeutic options are therefore needed.

4.4 The aim of this study was to test the following drugs *in vivo* in rat keratoplasty model preferably topical. The drugs will be tested regarding local application on the cornea *in vivo* for the first time.

A) **Selective glucocorticoid receptor agonists (SEGRAs)** represent a new class of glucocorticoid receptor (GR) modulators that preferentially support transrepression-mediated anti-inflammatory activities (6). Since most of the steroid-associated side effects are thought to be due to transactivation-mediated activities of the GR, SEGRA compounds are expected to be safer than classical glucocorticoids. Corneal penetration of ME-CD-SEGRA was already tested *in vitro* with success (7). Anti-inflammatory properties of SEGRA were confirmed in various human ocular cells, including human corneal epithelial cells (6). SEGRA inhibited the release of multiple cytokines, including some key pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, MCP-1 and TNF-α. Recent studies suggest that SEGRA could avoid elevation of intraocular pressure which is one of the major side effects of steroid therapy (8).

B) **Everolimus** is a proliferation signal inhibitor derived from the naturally-occurring immunosuppressive macrolide, rapamycin (sirolimus). It belongs to mammalian target of rapamycin (mTOR) inhibitors and acts on a later stage of the T-cell cycle. The net effect is blockade of T-cell activation by preventing progression of the cell cycle from the G1 to the S phase. Everolimus is in use in solid organ transplantations and therapy for some tumours. It already showed immunosuppressive effects in experimental keratoplasty when applied systemically (9). Corneal penetration of everolimus microemulsion has been tested *in vitro* with success (10). Everolimus may therefore have the ability to prevent corneal graft rejection when applied topically. Furthermore, everolimus has been shown to prevent neovascularisation that can be an additional goal in keratoplasty.

C) **Spironolactone** is a well-known mineralocorticoid receptor (MR) antagonist. Interestingly, anti-inflammatory effects of this drug have been demonstrated in arthritis

patients (11) and these could be confirmed in this thesis regarding prevention of corneal graft rejection. Furthermore, antiangiogenic capacity of this drug was described in the rabbit cornea model. This might be of great interest as corneal neovascularisation is one of the most threatening conditions for corneal graft survival. Finally, dexamethasone - induced cataract formation occurs through activation of GR but not via the MR (12). Spironolactone has a much higher affinity for MR than it does for the GR. Probably, it might therefore indirect avoid cataractogenesis. This might be another important benefit of this drug.

Testing these drugs in experimental keratoplasty model could show us a better way to prevent corneal graft rejection in patients. SEGRA and everolimus have been tested *in vivo* locally for the first time using available methodology for drug preparation (7, 10). Furthermore, it was suggested to orally test spironolactone as previously reported to be efficient in the reduction of TNF levels in rats and in antiangiogenic activities in rabbit corneas. Nevertheless, this may be a challenge for the future to develop a novel methodology for local application of this drug. The rat model of corneal transplantation was used as a valuable standard model (14) to investigate underlying immune mechanisms and new approaches for prevention of corneal graft rejection.

Using the aforementioned drugs, we specifically intended to analyse:

1. Graft survival following therapy using statistical analysis such as Kaplan-Meier plots
2. Intragraft immunological parameters following therapy using PCR method
    - Th1-type cytokines (IFN-γ, TNF-α) screened as pro-inflammatory cytokines
    - Th2-type cytokines (IL-10, IL-4) analysed as mediators of graft tolerance
    - T-cell markers (CD3,CD25) will be required for T-cell activation.

5. Methodology

5.1 Rat model

Experiments were performed on inbred female rats (Charles River, Kisslegg, Germany) 200–250 g. Dark Agouti (DA; RT.1A av1) were donors and Lewis rats (RT.1A 1) were recipients. These strains are different with regard to the entire major histocompatibility complex (MHC) and they are not congenic. All animals were housed in wire-bottomed cages with controlled light/dark cycles, fed with a standard laboratory diet, and given free access to tap water. Only one eye of

each recipient underwent corneal transplantation, so no animal was blinded. All animals were handled in accordance with the USA National Institute of Health's ('Guidelines for the Care and Use of Laboratory Animals') and with German guidelines on the use of animals in research (Berliner Senatsverwaltung).

5.2 Surgical procedure

Orthotopic corneal transplantations were performed as previously described (14) under sterile conditions using an operating microscope. Prior to surgery all animals were anesthetised and the pupil was topically dilated to avoid complications. The 3.5 mm corneal button was trephined from each donor. Recipient corneas were trephined with 3.0 mm trephine. The donor graft was sutured into the recipient bed using a running suture.

After transplantation, antibiotic ointment was applied immediately to the eye.

Grafted animals were treated daily with the agent of interest or with vehicle, until the day of rejection in the survival analysis group, or until the predestinated time point in the PCR analysis group.

5.3 Preparation of drugs

A) SEGRA: The drug was kindly supplied by Schering (Berlin, Germany). As the water solubility of SEGRA is extremely low (2.6 mg/l at pH 7.4), the drug was completely solubilised in a vehicle consisting of a microemulsion system combined with hydroxypropyl-γ-cyclodextrin (HP-γ-CD) (ME-CD). The eye drop formulation contained 0.25% SEGRA compound, 15% HP-γ-CD and 84.75% of a transparent microemulsion (5% Castor oil, 10% Macrogol 300, 20% - Cremophor® RH40 65% aqua ad iniectabilia). This innovative drug formulation was previously developed and tested *in vitro* (7). The formulation was prepared under laminar airflow and filtered (sterile filter: cellulose acetate membrane 0.22 μm).

B) Everolimus: 0.05% and 0.025% emulsion was kindly supplied by Institute of Pharmacy, Free University Berlin, Germany. This everolimus-containing microemulsion was prepared according to the previous *ex vivo* study (10).

C) Spironolactone: (Sigma Chemical, St. Louis, Mo., USA) was solubilised in 0.5% methylcellulose/0.5% Tween 80, as the water solubility of spironolactone is extremely

low. Vehicle (0.5% methylcellulose/0.05% Tween 80) preparation: methylcellulose powder was added to H2O and stirred until it completely dissolved (about 3-5 h); then Tween 80 was slowly added to the solution and well mixed. The suspension of compound and vehicle was sonicated for about 10 min, vortexed after 5 min and then again at the end of sonication. The compound was mixed well before administration to each animal, because of some precipitation during the preparation.

5.4 Treatment protocol

A total of 133 animals underwent orthotopic corneal transplantation. On 20 of them, syngeneic grafting was performed as control for technical failure. The remaining 113 Lewis rats underwent allogeneic corneal grafting from DA rats: 30 in SEGRA group, 37 in everolimus group and 46 in spironolactone group.

Corneal allograft (n = 113)	SEGRA group (n = 30)	Everolimus group (n = 37)	Spironolactone group (n = 46)
Graft survival analysis	SEGRA (n = 6) Vehicle (n = 6) Untreated (n = 6)	Everolimus 0.05 (n = 8) Everolimus 0.025 (n = 5) Vehicle (n = 8)	Spironolactone (n = 7) Vehicle (n = 9) Untreated (n = 16)
PCR analysis	SEGRA (n = 6) Untreated (n = 6)	Everolimus 0.05 (n = 8) Vehicle (n = 8)	Spironolactone (n = 8) Vehicle (n = 6)

Table 2. Summary of all experiments on corneal allografts

A) SEGRA: Allogeneic grafts in 18 LEW rats were randomly assigned to receive either: no therapy (n = 6), ME-CD (n = 6) as vehicle control group or ME-CD-SEGRA (n = 6). All treatments started at the day of surgery and were applied five times daily for 35 days. In order to investigate the effect of SEGRA treatment on intragraft cytokine expression, an additional 12 animals were used for PCR studies. They remained untreated (n = 6) or received the ME-CD-SEGRA formulation (n = 6). Day 7 was arbitrarily chosen for analysis since it could be presumed that at this time even untreated animals would not yet have been rejected their allograft. Further assumption is that at that time the drug would already have interfered with the immune response. Grafts were at that point explanted to analyse the intragraft mRNA expression pattern of the following cytokines: Il-4, IL-10, IFN-γ, TNF-α and CD3 as a T-cell marker.

B) Everolimus: Allogeneic grafts were carried out in 21 Lewis rats and randomly assigned to receive either: the drug vehicle (n = 8) or 0.05% everolimus in a microemulsion

formulation (n = 8) or 0.025% everolimus in a microemulsion formulation (n = 5). All treatments started at the day of surgery, and were applied 5 times daily until the day of rejection. Additional animals were used for PCR analysis in allogeneic transplants that received either the drug vehicle (n = 8) or 0.05% everolimus in a microemulsion formulation (n = 8). They were also analysed at day 7 following transplantation on IL-10, IFN-γ, CD3, CD25.

C) Spironolactone: For corneal survival analysis grafted animals were assigned to receive either spironolactone (n = 7), PBS (n = 9) or remained untreated (n = 16). Spironolactone suspension (100 mg/kg/day) and PBS (equal volume as spironolactone) were applied daily by gastric gavages starting on day 1 after transplantation, until the day of rejection. The chosen dosage of 100mg/kg/day has previously been reported to be efficient in the reduction of TNF levels in rats as well as in antiangiogenic activities in rabbit corneas. In additional recipients treated with spironolactone (n = 6) or PBS (n = 8), corneal neovascularisation was assessed daily under the microscope until day 12 following keratoplasty, and scored as previously described (14). These rats were sacrificed on day 12 for quantitative RT-PCR analysis. We tested the mRNA expression of cytokines (IL-4, IL-10, IFN-γ, TNF-α) and T-cell markers (CD3 and CD25) in the graft (locally) and in the spleen (systemically). Day 12 was selected based on our assumption that at day 12, the rejection was about to occur, resulting in a change of expression of cytokines.

5.5 Graft assessment

The transplanted corneas of the rats used for graft survival analysis were checked daily under the surgical microscope.

Corneal transparency as an indicator of corneal endothelial function and of graft endothelial injury was evaluated. Corneal opacity was graded as follows: 0, completely transparent cornea; 1, slight corneal opacity, but iris vessels clearly visible; 2, moderate corneal opacity, iris vessels still visible; 3, moderate corneal opacity, pupil only marginally visible; 4, complete corneal opacity, pupil not visible. Corneas exhibiting opacification of grade 3 or higher were considered rejected (14).

Corneal graft neovascularisation (NV) was observed in spironolactone group for PCR analysis. NV was scored as described in the literature (14): 0 - no vessels in graft or at graft margin, 1 - microscopically visible vessels at graft margin, 2 - vessels 25% in the graft, 3 vessels 50% in the graft and 4 - graft completely vascularised. Vascular caliber was scored as 0 - no vessels, 1 -

vessels only microscopically visible, 2 - vessels macroscopically visible and 3 – vessels clearly visible. Summarised results were obtained by averaging the scores at day 12.

5.6 Polymerase chain reaction

Detection of immunologic parameters in corneal allografts was performed by conventional reverse–transcription polymerase chain reaction (RT-PCR) and by quantitative real-time PCR (qPCR) (15). Corneal allografts were harvested at day 7 (in the SEGRA and everolimus groups) or day 12 (in the spironolactone group) and snap-frozen in liquid nitrogen. The isolation of RNA and subsequent analysis of mRNA-expression profiles by qPCR (ABI Prism 7700 Sequence Detection System) has been described elsewhere (15). Briefly, mRNA was prepared from each graft and reversely transcribed into cDNA. The cDNA was then analyzed for cytokine gene expression with qPCR. For the quantification of mRNAs of interest, the expression of a housekeeping gene (ß-actin) was set in relation to rat cytokine mRNAs (IL10 and IFNγ in all three groups and IL4 and TNFα in the SEGRA and spironolactone groups) and T-cell marker mRNAs (CD3 in all three groups and CD25 in the spironolactone and everolimus groups). All values are reported as means ± SEM.

5.7 Statistical Analysis

Graft survival was presented as mean survival time (MST) using Kaplan-Meier plots. MST was compared using Mantel-Haenszel survival analysis. For non-paired observations of qPCR data, unpaired Student's t-test was used if the values passed the normality test. If this was not the case, the non-parametric Mann-Whitney test was used. All values are reported as means ± SEM. A p value < 0.05 was considered statistically significant.

6. Results

In general, therapy was well tolerated in all animals. Rats receiving syngeneic graft remained clear beyond 60 days after transplantation.

6.1 SEGRA-treated corneal grafts showed a high survival rate (83%) during the 35-day treatment course. Untreated allografts were rejected within two weeks. SEGRA was highly effective in prolonging the MST of corneal grafts (42.2 ± 4.0 days) compared to untreated controls (11.7 ± 1.2 days, $p = 0.00003$) or animals that received the vehicle only (15 ± 1.5 days) (Fig. 4). Treatment of corneal allograft recipients with SEGRA decreased mRNA levels of all cytokines

and T-cell markers examined in the explants and was significantly decreased for IL-4 ($p < 0.05$) (Fig. 5). Although a clear down-regulation of all pro-inflammatory cytokines could be detected, the decrease did not reach statistical significance for all cytokines. Taken together, SEGRA significantly prolonged corneal graft survival following experimental keratoplasty.

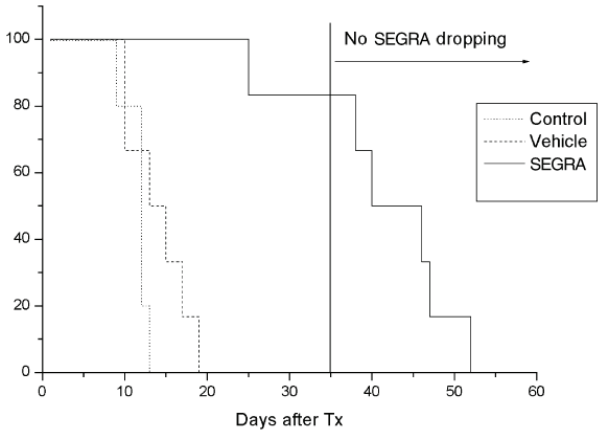

Fig. 4. Survival graph of corneal transplants folowing SEGRA therapy. SEGRA was effective in prolonging the graft MST significantly ($p = 0.0003$)

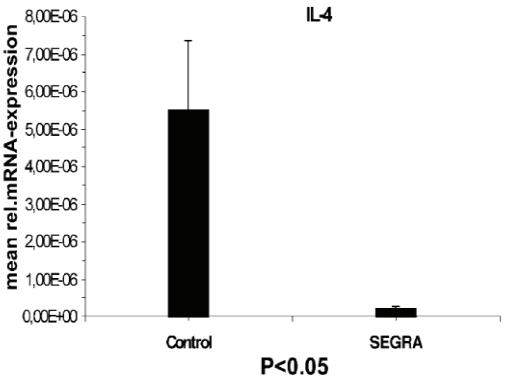

Fig. 5. mRNA expression of IL-4 was significantly decreased in SEGRA-treated grafts compared with untreated controls

6.2 Everolimus-treated corneas had significantly prolonged survival compared with the vehicle control group (Fig. 6). Specifically, the MST was 21 ± 6.57 days in the 0.05% everolimus group ($p < 0.001$) and 16.4 ± 2.3 days in the 0.025% everolimus group ($p < 0.001$). Allografts receiving the vehicle were rejected with a median survival time of 13.3 ± 1.7 days.

PCR studies on intragraft cytokine expression showed that the topical administration of everolimus increased the mRNA levels of CD25, IL-10 and IFN- γ , but this was significant only for IL-10 ($p = 0.015$) (Fig. 7).

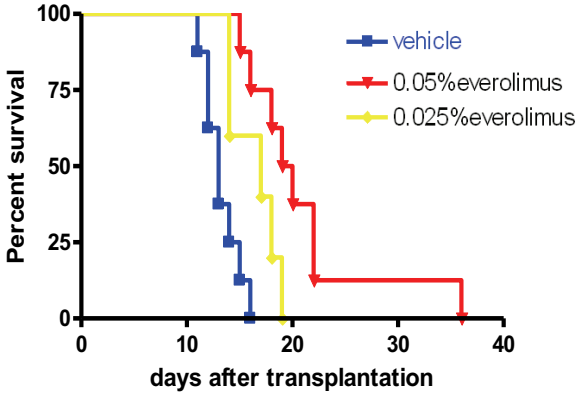

Fig. 6. Survival graph of corneal transplants after everolimus therapy. Compared with the vehicle control group, everolismus treated corneas had significaltly prolonged survival ($p < 0.001$)

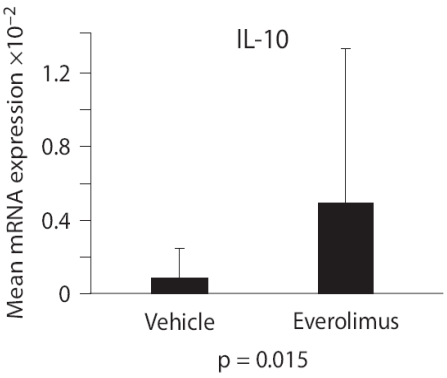

Fig. 7. mRNA expression of IL-10 was significantly increased in everolimus treated grafts compared with vechical-treated controls ($p = 0.015$).

6.3. Spironolactone-treated rats had a significantly prolonged MST of corneal graft (14.9 ± 2.0 days) compared with both PBS (12.3 ± 1.2 days, p = 0.007) and untreated controls (13.0 ± 1.0 days, p = 0.01) (Fig. 8). The PBS and control groups showed no statistically significant difference in MST (p = 0.13). The scores of extension of neovascularisation and vascular caliber tend to be decreased by spironolactone treatment compared with PBS treatment.

Treatment of corneal allograft recipients with spironolactone moderately suppressed the systemic immune response by down-regulation of CD25 mRNA expression (p < 0.01; Fig. 9) in the spleens of transplant recipients. It also affected local immune response by intra-graft up-regulation of IL-10 (p < 0.01; Fig. 10) in spironolactone-treated rats. However, a clear down-regulation of pro-inflammatory cytokines could be detected neither in corneal explants nor in spleens of transplant recipients.

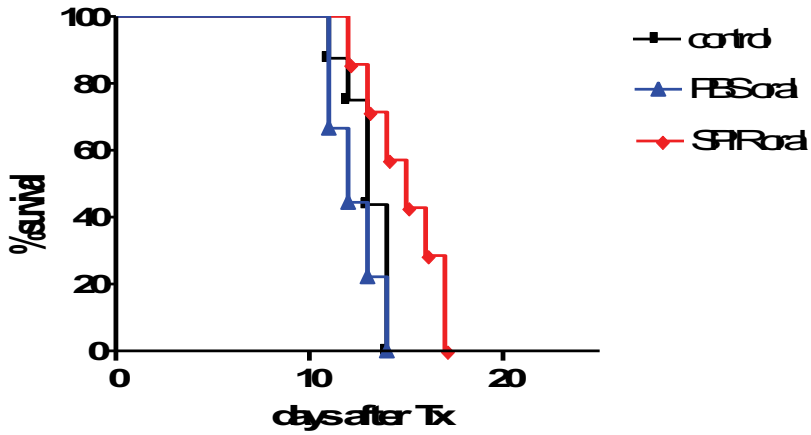

Fig. 8 Survival graph of corneal allogeneic transplants after Spironolactone (SPIR) therapy. Spironolactone-treated rats had a significantly prolonged MST of corneal grafts compared with both PBS (p = 0.007) and untreated controls (p = 0.01)

In summary, there is a clearly prolonged corneal graft survival using the aforementioned drugs following experimental keratoplasty.

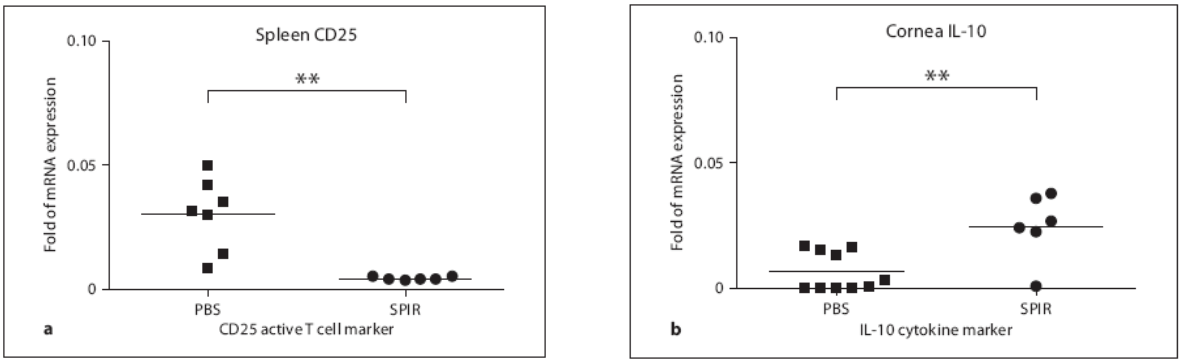

Fig. 9/10 Treatment of corneal allograft recipients with SPIR downregulated CD25 mRNA expression in the spleens of transplant recipients (**a**). It also up-regulated IL-10 mRNA expression in the grafts of SPIR-treated rats (**b**). The asterisks indicate a statistically significant difference (p < 0.01).

7. Discussion

The present results demonstrate that all three tested drugs prolonged corneal graft survival compared to controls. MST of corneal grafts was significant prolonged by application of all three drugs, particularly by SEGRA therapy. This finding is based on established investigation methods, using a rat model as well-characterized animal model of PKP (14). Rodent models of keratoplasty display many similarities to the human situation. They have permitted insights into the immunopathology of corneal allograft rejection and new treatment approaches. The underlying mechanisms of tested drugs were probably different according to the different expression of examined parameters of the immune process in the various groups.

Corneal allograft rejection is a T-cell-mediated, highly complex immune process. Notably, CD4+ is the master regulator of immune response. It has different pathways (Th1, Th2, Th17 or Treg) depending on the antigen. The T regulatory pathway, for example, has a calming role and it promotes graft survival. Th1-type cells predominantly produce IL-2 and IFN-γ, which mediate cellular immunity involving DTH (strongly suggested as mediator of corneal graft rejection). Th2-type cells produce various interleukins such as IL-4, IL-6, IL-10 and IL-13. They are proposed to mediate graft tolerance. Indeed, both Th1 and Th2 pathways are likely to contribute to corneal allograft rejection (2). They mutually regulate one another through their cytokine pattern.

SEGRA-treated corneas tend to express lower mRNA of IFN-γ and TNF-α than controls. However, the difference in expression did not reach statistical significance. Since IFN-γ and TNF-α are mainly released by T-lymphocytes, the tendency towards reduced CD3 mRNA expression in SEGRA-treated recipients is consistent with this finding. Recent *in vitro* study demonstrated indeed inhibitory effects of SEGRA on multiple inflammatory cytokine release in primary human ocular cell cultures (6). In human corneal epithelial cells, SEGRA significantly inhibited IL-6 and TNF-α. Considering that only one time point in our study was arbitrarily chosen for PCR analysis, it might be speculated that the cytokine/T-cell marker expression may differ during the later follow-up.

Interestingly, in our study, SEGRA-treated corneas expressed lower levels of Th2 cytokines such as IL-4 ($p < 0.05$) than untreated ones. IL-4 mRNA expression was reduced in CTLA4Ig-treated corneas too (15). At this point, the role of Th2 cytokine seems to be less clear than that of Th1 (15). However, it is likely that both Th1 and Th2 cytokines play a critical role during corneal

allograft rejection. Thus therapies only modulating a Th1/Th2 shift may have a limited capacity on corneal allograft survival (15). Interestingly, SEGRA demonstrated immunomodulatory activity in rodent allergic dermatitis model, applied topically (6). Moreover, some studies suggest that allergic diseases increase the risk of corneal allograft rejection (3). Whether the success of SEGRA in the treatment of allergic dermatitis and keratoplasty shares some common mechanisms of action will require further research.

A growing body of evidence shows that mTOR inhibitors interfere with the immune response at a very early stage by affecting the dendritic cell (DC) function. In this connection, it has been shown that DC are able to secrete IL-10 instead of IL-12 and prevent acute allograft rejection. In our everolimus-treated group, we could register significantly increased mRNA levels of IL-10. Furthermore, the mRNA expression of the T-cell marker CD25 was 4.8-fold higher in everolimus-treated corneas compared to the controls in our study. Although this did not reach statistical significance, it might have contributed to the beneficial effect. Indeed, there is convincing evidence that Treg cells (CD4+CD25+) maintain immune tolerance and promote corneal allograft survival (3). Recently, it has been demonstrated that anti-CD25 antibodies abolish the immune privilege of corneal allografts. Thus, a tolerogenic role of CD25 may be relevant.
Moreover, lymphangiogenesis is strongly suggested to abolish the immune privilege of corneal allografts. Sirolimus has been shown to inhibit lymphangiogenesis in rat renal allografts by down-regulating VEGFR-3 protein expression. Anti-VEGFR3 antibody has been shown to promote corneal allograft survival by selective blockade of lymph vessel ingrowth (3). It would therefore be of great interest to investigate if everolimus inhibits lymphangiogenesis too, in particularly in the corneal allograft model.

Spironolactone-treatment has influenced both systemic and local immune response. Intragraft up-regulation of IL-10 in spironolactone-treaded rats could contribute to prolonged MST in this group. IL-10 inhibits cytokine synthesis by Th1 cells. Suppression of cytokine production may be associated with apoptotic potential of spironolactone. This might be an explanation for down-regulated active T-cells in the spleen observed in our study (down-regulated CD25). Temporal separation of cytokine suppression and apoptosis (late apoptotic action of spironolactone) may explain systemic but not yet local down-regulation of CD25 on day 12.
Spironolactone has been previously shown to inhibit corneal angiogenesis *in vivo*. Further investigation indicated a suppressed corneal neovascularisation in spironolactone-treated rats.

Spironolactone inhibits the proliferation of vascular endothelial cells but its effects on their migration and rearrangement in capillary-like structure needs further evaluation.
It is proven that dexamethasone-induced cataract occurs by activation of GR in lens epithelial cells, and not by activation of MR (12). Spironolactone has a very low affinity for the GR, it binds to MR and will therefore unlikely indicate cataractogenesis.

A concept of topical immunosuppression is promising, but is connected with limited effort. Influence on the afferent arm of immune response and avoiding of side-effects of systemic treatment are beneficial. However, it is confronted with limitation of topical influence on afferent arm of immune response. Investigations of local applications of some systemically successfully used drugs, such as Cyclosporin A and Mycophenolat Mophetil, remained on an experimental level (4). There are conflicting results on topical ophthalmic use of CsA. This may be probably related to poor drug penetration. On the other hand, topical Tacrolimus (FK 506) had success in one clinical study and was at least as effective in preventing graft rejection as topical steroids. However, further use was limited as a result of local discomfort. Topical Rapamycin did not succeed either. Interestingly, topical anti-TNF-α (infliximab) had success in experimental keratoplasty (1) but was not confirmed clinically. Recent findings regarding the clinical utility of topical VEGF inhibitors indicate that these agents are safe and effective treatments for corneal neovascularisation. Furthermore, recombinant human NGF for local application is also in process. However, these agents can be seen only as additional therapy in some cases of high-risk keratoplasty.

Despite all this efforts, it is foreseeable that corticosteroids are likely to remain a main option for local treatment of keratoplasty patients. SEGRA is likely to be an alternative agent showing similar anti-inflammatory activities with displaying less dominant side effects. Further research with the aim of evaluating the precise mechanisms of allograft rejection are of great importance. This may help to develop new solutions for immunological modulation and consequently improve success rate of corneal transplantation.

8. Bibliography

1) Chong EM, Dana MR. Graft failure IV. Immunologic mechanisms of corneal transplant rejection. Int Ophthalmol. 2008;28(3):209-22. Review.

2) Pleyer U, Schlickeiser S. The taming of the shrew? The immunology of corneal transplantation. Acta Ophthalmol. 2009;87(5):488-97. Review.

3) Niederkorn JY, Larkin DF. Immune privilege of corneal allografts. Ocul Immunol Inflamm. 2010;18(3):162-71.

4) Coster DJ, Williams KA. The impact of corneal allograft rejection on the long-term outcome of corneal transplantation. Am J Ophthalmol, 2005;140:1112-1122.

5) Bertelmann E, Torun N, Pleyer U. Present state of the art in topical and systemic immunosuppression after keratoplasty. Klin Monbl Augenheilkd. 2013;230:505-511.

6) Zhang JZ, Cavet ME, VanderMeid KR, et al. BOL-303242-X, a novel selective glucocorticoid receptor agonist, with full anti-inflammatory properties in human ocular cells. Mol Vis. 2009;15:2606-16.

7) Knapp-Ulrich S: Einfluss von Cyclodextrinen auf die okulare Verfügbarkeit von Antiglaukomatosa und Immunsuppressiva in vitro / in vivo. HU-Berlin (2004) PhD thesis: www.edoc.hu-berlin.de/dissertationen/knapp-ulrich-sigrid-ursula-2004-09-13/HTML

8) Stamer WD, Hoffman EA, Kurali E, et al. Unique response profile of trabecular meshwork cells to the novel selective glucocorticoid receptor agonist, GW870086X. Invest Ophthalmol Vis Sci. 2013;54(3):2100-7.

9) Reis A, Megahed M, Reinhard T, et al. RAD, a new immunosuppressive macrolide in murine corneal transplantation. Graefes Arch Clin Exp Ophthalmol. 2001;239(9):689-92.

10) Baspinar Y, Bertelmann E, Pleyer U, et al. Corneal permeation studies of everolimus microemulsion. J Ocul Pharmacol Ther. 2008;24(4):399-402.

11) Bendzen K, Hansen PR, Rieneck K. Spironolactone inhibits production of proinflamatory cytokines, including tumor necrosis factor-alpha and interferon-gamma, and has potential in the treatment of artritis. Clin Exp Immunol. 2003;134(1):151-8.

12) Klauber N, Browne F, Anand-Apte B et al. New activity of spironolactone: inhibition of angiogenesis in vitro and in vivo. Circulation 1996;94:2566-2571.

13) Gupta V, Awasthi N, Wagner BJ. Specific activation of the glucocorticoid receptor and modulation of signal transduction pathways in human lens epithelial cells. Invest Ophthalmol Vis Sci. 2007;48(4):1724-34.

14) Pleyer U: Immunobiology and prevention of allograft rejection after experimental keratoplasty. Buren, Aeolus Press, 1996, pp 22-23.

15) Gong N, Pleyer U, Yang J et al: Influence of local and systemic CTLA4Ig gene transfer on corneal allograft survival. J Gene Med 2006;8:459-467.

II Affidavit

I, Ljiljana Otasevic-Wieschalla certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “New drugs in prevention of experimental corneal graft rejection”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date 20.08.2013 Signature

Declaration of any eventual publications

Ljiljana Otasevic-Wieschalla had the following share in the following publications:

Publication 1: Pleyer U, Yang J, Knapp S, Schäcke H, Schmees N, Orlic N, Otasevic L, De Ruijter M, Ritter T, Keipert S. Effects of a selective glucocorticoid receptor agonist on experimental keratoplasty. Graefes Arch Clin Exp Ophthalmol. 2005;243(5):450-5. Contribution in detail: Conduction and analysis of experiments, drug administration, participation in writing the manuscript, contribution for the revision of the manuscript.

Publication 2: Li XQ, Buch G, Otasevic L, Schlickeiser S, Bertelmann E, Pleyer U. Prolongation of corneal allograft survival by topical application of everolimus in experimental keratoplasty. Ophthalmic Res. 2008;40(6):309-14.
Contribution in detail: Conduction and analysis of key experiments, participation in writing the manuscript, contribution for the revision of the manuscript.

Publication 3: Otasevic L, Gong N, Ritter T, Mergler S, Pleyer U. Effects of spironolactone on corneal allograft survival in the rat. Ophthalmic Res. 2007;39(6):325-9. Contribution in detail: Active participation in planning the study and experiments, conduction and analysis of experiments, active participation in writing the manuscript, extensive contribution for the revision of the manuscript, presentation of the results at international congress (15th SOE Congress - European Society of Ophthalmology, 2005 in Berlin).

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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III Selected publications

1. Publication 1: Pleyer U, Yang J, Knapp S, Schäcke H, Schmees N, OrlicN, OtasevicL, De Ruijter M, Ritter T, Keipert S.Effects of a selective glucocorticoid receptor agonist on experimental keratoplasty.Graefes Arch ClinExpOphthalmol. 2005; 243(5):450-5. Impact factor 2.170

2. Publication 2: Li XQ, Buch G, Otasevic L, Schlickeiser S, Bertelmann E, Pleyer U.Prolongation of corneal allograft survival by topical application of everolimus in experimental keratoplasty.Ophthalmic Res. 2008; 40(6):309-14.
   Impact factor 1.561

3. Publication 3: Otasevic L, Gong N, Ritter T, Mergler S, Pleyer U.Effects of spironolactone on corneal allograft survival in the rat.Ophthalmic Res. 2007;39(6):325-9.
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V Complete list of publications

1. Otasevic M, Miljkovic-Selimovic B, Katic V, Tiodorovic B, Dinic M, Otasevic Lj. Helicobacter pylori i malignomi zeluca-prilog onkogenezi. Acta medica Medianae 1995; 1:59-65.

2. Otasevic M, Katic V, Miljkovic-Selimovic B, Dinic M, Otasevic Lj. Petrovic D. Helicobacter pylori infekcija i karcinom zeluca. Arch.Gastroenterohepatol 1996; 15(7): 41.

3. Otasevic M, Miljkovic-Selimovic B, Dinic M, Nagorni A, Katic V, Randjelovic G, Otasevic Lj. Zivic S, Tiodorovic B. Epidemiologija helicobacter pylori infekcije. Acta medica Medianae 1997; 6: 53-58.

4. Otasevic Lj, Zlatanovic G, Dinic M, Kostovska V, Veselinovic D. Ulcus Corneae-Microbiologycal Examination And Treatment. Abstrakt book,XI Congress of the European Society of Ophtalmology, Budapest 1997; 359.

5. Dinic M, Otasevic M, Tasic S, Otasevic Lj. Osetljivost pseudomonas aeurugonosa in vitro testiranjem prema aminoglikozidima i ureidopenicilinima. Zbornik rezimea, XXXI dani preventivne medicine, Niš 1997; 81.

6. Dinic M, Otasevic M, Miljkovic-Selimovic B, Otasevic Lj, Tasic S. Burkholderia cepacia-moguci izazivaci intrahospitainih inferencije. Zbornik rezimea, XXXI dani preventivne medicine, Niš 1997; 86.

7. Otasevic Lj. Zlatanovic G, Kostovska V. Kontuzione povrede oka lecene na Oftamoloskoj klinici u nisu u periodu od 1991-1996. Godine. Zbornik radova ,V kongres sudske medicine Jugoslavije sa medjunarodnim ucescem, H.Novi 1997; 347-351.

8. Zlatanovic G, Kostovska V, Otasevic Lj. Perforativne povrede oka lecene na ocnoj klinici u nisu od 1991-1996. GODINE. Zbornik radova,V kongres sudske medicine Jugoslavije sa medjunarodnim ucescem, H.Novi 1997; 405-409.

9. Kostovska V, Zlatanovic G, Otasevic Lj. Komparativna studija perforativnih I kontuzionih povreda oka. Medicina forensis 1997; 1:46-54.

10. Zlatanovic G, Stanojevic-Paovic A, Otasevic Lj. Moderna terapija retinalnog vaskulitisa. Medicinska istrazivanja 1998; 32(3): 31-35.

11. Dzunic B, Zlatanovic G, Otasevic Lj. Promene na ocima kod akutnog Trovanja metanolom. Acta medica Medianae 1998; 5: 23-28.

12. Dinic M, Otasevic M, Tasic G, Tasic S, Otasevic Lj. Streptococcus beta haemolyticus u raznovrsnom bolesnickom materijalu za period 1995-1998. Godine. Zbornik radova i sazetaka, Dani mikrobiologije Jugoslavije sa medjunarodnim ucescem, H.Novi 1998; 32-33.

13. Otasevic M, Dinic M, Miljkovic B, Djordjevic D, Tasic G, Otasevic Lj. Heliobacter Pylori And Duodenal Ulcus. Books of abstracts. I Congress of the Physicians of the Macedonian Preventive medicine, Ohrid 1998; 140.

14. Otasevic M, Stankovic-Djordjevic D, Miljkovic-Selimovic B, Dinic M, Otasevic Lj. Kliconoštvo kod kampilobakterioza. Zbornik rezimea, VIII kongres preventivne medicine Jugoslavije sa medjunarodnim ucescem, Beograd 1998; 86-87.

15. Tasic G, Stankovic D, Tasic S, Miladinovic N, Otasevic Lj, Konstantinovic Lj. Immunoglobulin-A-Specifice Serodiagnosis Of Chronic Chlamydial Infection. Books of abstracts, I Congress of the Physicians of the Macedonian Preventive medicine, Ohrid 1998; 160.

16. Zlatanovic G, Jaric-djordjevic V, Veselinovic D, Otasevic Lj. Level Of Immmunoglobulin A In Patients With Acute Anterior Uveitis And Seronegative Spondyloarthropatis (Ankylosing Spondylitis). Abstract book, Ochiul si patologia generala, Timosoara 1998; 12.

17. Kostovska V, Zlatanovic G, Otasevic Lj. Rezultati lecenja ambliopnih bolesnika. Acta medica Medianae 1998; 6: 69-79.

18. Otasevic Lj, Zlatanovic G, Kostovska V. Moguca uloga g-bakterija u etiopatogenezi APU i SpA. Zbornik rezimea, XIV kongres oftalmologa Jugoslavije, Cetinje 1998; 23.

19. Zlatanovic G, Jaric-Djordjevic V, Micic S, Kostovska V, Otasevic Lj. Najcesci uzroci uveitisa u decijem uzrastu. Zbornik rezimea, XIV kongres oftalmologa Jugoslavije, Cetinje 1998; 11.

20. Kostovska V, Smiljkovic V, Zlatanovic G, Veselinovic D, Otasevic Lj. Nasi rezultati lecenja ambliopije sa ekscentricnom fiksacijom. Zbornik rezimea, XIV kongres oftalmologa Jugoslavije, Cetinje 1998; 18-19.

21. Otasevic Lj, Zlatanovic G, Stanojevic-Paovic A, Nagorni A, Stamenkovic B. Pathohistological Finding Of The Intestinal Mucosa In Patients With Acute Anterior Uveitis (AAU) And Spondyloarthropathies (SpA). Uveitis in Third Millennium, Excepta Medica, International Congress Series 1204, 2000 Elsevier Science B.V., Proceedings of the Fifth International Symposium on Uveitis in Buenos Aires 2000; 251-254.

22. Zlatanovic G, Stanojevic-Paovic A, Otasevic Lj, Miljkovic-Selimovic B. Serological Finding In Patients With Acute Anterior Uveitis (AAU) And Spondyloarthropaties (SpA). Abstract book, Fifth International Symposium on Uveitis, Buenos Aires 2000; 60.

23. Djordjevic-Jocic J, Zlatanovic G, Babic G, Jovanovic P, Zivkovic S, Vujanovic M, Otasevic Lj. Keratoconjunctivitis vernalis-klinicka studija. Abstract book, VI kongres oftalmologa Srbije, Beograd 2001; 33.

24. Jovanovic P, Zlatanovic G, Veselinovic D, Stankovic-Babic G, Djordjevic-Jocic J, Otasevic Lj. Akutni napad glaukoma nakon usmrkavanja kokaina-prikaz slucaja. Abstract book, VI kongres oftalmologa Srbije, Beograd 2001; 13.

25. Otasevic Lj, Zlatanovic G, Veselinovic D, Visnjic Z, Jovanovic P, Jocic-Djordjevic J, Miljkovic-Selimovic B, Dinic M. Possible Role Of Helicobacter Pylori In Etiopathogenesis Of Acute Anterior Uveitis. Abstract book, XIII Cogress of the European Society of Ophthalmology, Istanbul 2001; 302.

26. Visnjic Z, Visnjic M, Veselinovic D, Otasevic Lj, Kovacevic P. Merker Cell Carcinoma Of The Upper Lid - Case Report. Abstract book, XIII Congress of the European Society of Ophthalmology, Istanbul 2001; 133.

27. Djordjevic-Jocic J, Zlatanovic G, Babic G, Jovanovic P, Kostovska V, Otasevic Lj. Frequency Of Scleritis And Episcleritis Among Patients Suffering From Rheumatoid Arthritis And Other Systemic Immune-Mediated Diseases. Abstract book, XIII Congress of hte European Society of Ophthalmology, Istanbul 2001; 309.

28. Zlatanovic G, Djordjevic-Jocic J, Babic G, Jovanovic P, Kostovska V, Otasevic Lj. Comparing The Levels Of Circulating Immune Complexes Among Patients With Scleritis. Abstract book, XIII Congress of hte European Society of Ophthalmology, Istanbul 2001; 319.

29. Jovanovic P, Zlatanovic G, Veselinovic D, Kostovska V, Stankovic-Babic G, Otasevic Lj. Clinical Neuroophthalmology Features Of Plumb (Pb) Caused Acute Intoxication. Abstract book, XIII Congress of the European Society of Ophthalmology, Istanbul 2001; 323.

30. Selimovic-Miljkovic B, Otasevic Lj, Otasevic M, Dinoc M. Neke Gram negativne bakterije I akutni prednji uveitis i spondiloartropatije. Zbornik radova Dani preventivne medicine, XXXV sastanak, Nis, 2001;138-9.

31. Dinic M, Otasevic M, Miljkovic-Selimovic B, Stojanovic P, Djordjevic D, Tasic G, Otasevic Lj. Zastupljenost MRSA u raznovrsnom bolesnickom materijalu. Zbornik radova Dani preventivne medicine, XXXV sastanak, Nis, 2001;139-40.

32. Dinic M, Otasevic M, Miljkovic B, Tasic G, Stankovic D, Otasevic Lj, Stojanovic P. Uzrocnici hospitalnih infekcija izolovani iz aspirata bolesnika hospitalizovanih na Decijoj klinici KC Nis. Zbornik radova Dani preventivne medicine, XXXVI sastanak, Nis, 2002;77-8.

33. Otasevic Lj, Zlatanovic G, Miljkovic-Selimovic B, Milic S, Stamenkovic B, Jocic-Djordjevic J. HLA status bolesnika sa akutnim prednjim uveitisom i spondiloartropatijama. Balneoklimatologija, maj 2002; vol.26., supl.2., 79-83.

34. Djordjevic-Jocic J, Zlatanovic G, Otasevic Lj. Skleritis udruzen sa reumatoidnim artritisom i ostalim sistemskim bolestima vezivnog tkiva. Balneoklimatologija, maj 2002; vol.26., supl.2., 201-206.

35. Stamenkovic B, Stankovic A, Nedovic J, Stojanovic S, Dimic A, Deljanin-Ilic M, Djordjevic D, Otasevic Lj, Lovic M. Procena funkcionalnog kapaciteta kardiovaskularnog sistema u bolesnika sa reumatoidnim artritisom. Balneoklimatologija, maj 2002; vol.26., supl.2., 195-199.

36. Miljkovic-Selimovic B, Otasevic M, Stankovic A, Spasic M, Randjelovic G, Tasic-Miladinovic N, Otasevic Lj, Kocic B. Campylobacter And Other Gram-Negative Bacteria In Reactive Arthritis And Guillain-Barr Syndrome. Book of abstracts, Congress of the physicians of the Macedonian preventive medicine with international participation, Ohrid 2002; 66.

37. Otasevic Lj, Tomasevic B. Razvoj bilateralne katarakte usled hiperbaricne oksigenacije (HBO). Abstract book, XV Kongres oftalmologa Jugoslavije, Zlatibor 2002; 49.

38. Otasevic M, Stankovic-Djordjevic D, Miljkovic-Selimovic B, Otasevic Lj, Dinic M, Tasic G. Campylobacter lari i campylobacter upsaliensis. Novi entiteti u etiologiji kampilobakterioza. Acta medica Medianae 2002; 2: 39-49.

39. Dinic M, Otasevic M, Tasic G, Djordjevic D, Miljkovic-Selimovic B, Otasevic Lj. Karakteristike *vacA* genotipova Helicobactera pylori i korelacija sa ishodom infekcije. Acta Facultatis Medicae Naissansis 2003; 20(1):6-10.

40. Otasevic M, Dinic M, Miljkovic-Selimovic B, Otasevic Lj, Stojanovic P. Mikrobioloska dijagnoza *Helicobacter pylori* inifekcije. Acta Facultatis Medicae Naissansis 2003;20(1):22-8.

41. Orlic N, Otasevic Lj, Hartmann C, Metzner S, Meisel H, Pleyer U. Detection of Anti-viral Antibodies in the Aqueous Humor of Patients with Acute Exacerbation of Acute Posterior Multifocal Placoid Pigment Epitheliopathy (APMPPE). 7th Congress of the International Ocular Inflammation Society. Padova, Italy - May 25th to 30th, 2003.

42. Otasevic Lj, Zlatanovic G, Miljkovic-Selimovic B, Dinic M, Stankovic A, Stamenkovic B, Nagorni A. Helicobacter pylori – possible trigger of Acute Anterior Uveitis and Spondyloarthropaty. Acta Facultatis Medicae Naissansis 2003;20(1):83-88.

43. Otasevic M, Nagorni A, Stankovic-Djordjevic D, Dinic M, Otasevic Lj. Helicobacter pylori and gastric cancer. Archive of Oncology 2003 ;11(4) :233-7.

44. Otasevic L, Walduck A, Meyer TF, Aebischer T, Hartmann C, Orlic N, Pleyer U. *Helicobacter pylori* Infection in Anterior Uveitis. Infection 2005; 3:82-85.

45. Pleyer U, Yang J, Knapp S, Schäcke H, Schmees N, Orlic N, Otasevic L, De Ruijter M, Ritter T, Keipert S. Effect of a selective glucocorticoid receptor agonist on experimental keratoplasty. Graefe's Arch Clin Exp Ophthalmol 2005; 243:450-455.

46. Otasevic L, Zlatanovic G, Stanojevic-Paovic A, Miljkovic-Selimovic B, Dinic M, Djordjevic-Jocic J, Stankovic A. Helicobacter pylori: an underestimated factor in acute anterior uveitis and spondyloarthropathies? Ophthalmologica. 2007; 221(1):6-13.

47. Dinić M, Tasić G, Stanković-Dordević D, Otasević L, Tasić M, Karanikolic A. Serum anti-Helicobacter pylori IgA and IgG antibodies in asymptomatic children in Serbia. Scand J Infect Dis. 2007; 39(4):303-7.

48. Otasevic L, Gong N, Ritter T, Mergler S, Pleyer U. Effects of spironolactone on cornealallograft survival in the rat. Ophthalmic Res. 2007; 39(6):325-9. Epub 2007 Oct 23.

49. Li XQ, Buch G, Otasevic L, Schlickeiser S, Bertelmann E, Pleyer U. Prolongation of corneal allograft survival by topical application of everolimus in experimental keratoplasty. Ophthalmic Res. 2008 Oct; 40(6):309-14.

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