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PhD thesis:

Ex-vivo generation of regulatory T-cells expressing transcription factor foxp3 and their application in cornea transplantation

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Summary

This project investigated the therapeutic potential of allo-antigen specific regulatory T-cells in the context of penetrating keratoplasty. Two methods of ex-vivo generation of T regulatory cells have been explored in this study. Using recombinant retroviruses gene transfer of the transcription factor *foxp3* was achieved into allo-antigen specific primary rat T-cells. However, expansion of *foxp3* gene modified cells necessary for in-vitro and in-vivo studies, was found to be too cumbersome. The second approach exploited a non-depleting anti-CD4 antibody. Allo-primed mixed lymphocyte cultures were treated with low-dose anti-CD4 antibody resulting in an outgrowth of *foxp3* positive T-cells. CFSE labelled responder T-cells targeted with anti-CD4 treatment indeed confirmed preferential growth of regulatory T-lymphocytes. T$_{reg}$ generated by this procedure were studied by flowcytometry and found to express high levels of CD25, Ox-40 and ICOS, confirming their suppressor T-cell phenotype. To study T$_{reg}$ effects on the outcome of allo-graft survival, a full allogeneic cornea transplant model was established. Two different strain combinations were tested. First, a high responder LEW-DA model was set-up but was found to be too fragile and unreliable to serve as a preclinical model. A second novel strain combination was tested and discovered to be of low-responder characteristics and remarkably robust and highly suitable for in-vivo applications. The rejection process of the BN-PVG model was comprehensively studied using flowcytometric analysis of draining lymph nodes. Moreover, a gentle digestion protocol was established to isolate viable graft infiltration lymphocytes. Applying multi-parameter FACS six distinct cell populations were observed among which were CD8$^+$ cytotoxic T-cells, CD4$^+$ T-cells, CD3$^-$ CD8$^+$/- CD161$^{high}$ NK, CD3$^+$ CD8$^+$ CD161$^{null}$ NK-T-cells, CD161$^{null}$ large granular lymphocytes and MHC-2 positive cells. Additionally a serum analysis found evidence for IgM, IgG1 and IgG2a allo-antibodies in rejection animals. Finally ex-vivo generated T$_{reg}$ were tested towards their ability to prevent allo-graft rejection. CD3 sorted regulatory T-cell preparations with direct allo-antigen specificity did not prevent or delay rejection of allogeneic corneal grafts. The implications of this finding for the clinical application of adoptive T$_{reg}$ therapies have been discussed in detail.
Glossary

γ DA .................. irradiated dark agouti cells
γ PVG .............. irradiated PVG rat cells
ψ ..................... retrovirus packaging signal
amp .................. ampicillin resistance gene
APC ................... antigen presenting cell
BN ..................... Brown Norway rat
BSA .................. bovine serum albumin
CD ...................... cluster of differentiation
CFSE ............... Carboxyfluorescein succinimidyl ester
cfu .................. colony forming units
CMV ................ Cytomegaly virus
CTL ................... cytotoxic T-lymphocytes
CTLA ................... cytotoxic T-lymphocyte antigen
DA .................. dark agouti rat
DC ................ dendritic cell
DF .................... dilution factor
DMEM .............. Dulbecco’s Modified Eagle Medium
DTH ................ delayed-type hypersensitivity
EDTA ............... ethylene diamine tertaacetic acid
EGFP ............... enhanced green fluorescent protein
ELISA .............. enzyme linked immuno-sorbent assay
env .................. retroviral envelope protein
FACS ............... fluorescent activated cell sorter
FB .................. flat bottom cell culture plates
FCS ................ foetal calf serum
FMO ................ fluorescence minus one - gating control
foxp3 ............... forkhead box transcription factor 3
FSC ................. forward scatter
G-418 ............... geneticin derivate
gag .................. group associated antigen
GFP ................ green fluorescent protein
GIL .................. graft infiltrating lymphocytes
HEPES ......... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS .................. horse serum
IL2 ................... interleukin 2
IMS ................... industrial methylated spirit
IRES ................ internal ribosome entry site
KIR ................. killer inhibitory receptor
LEW ................ Lewis rat
LGL ................ large granular lymphocytes
LN .................. lymph node
LTR ................ long terminal repeat
MHC ................ Major histocompatibility complex
MLC ................ mixed lymphocyte culture
MLR ................ mixed lymphocyte reaction (same as MLC)
Mo-MLV .......... Moloney murine leukaemia virus
MSCV .......... murine stem cell virus
MW ................ molecular weight
MWCO .......... molecular weight cut-off
NEO$^R$ .......... phosphodiesterase gene, neomycin resistance
NFAT .......... nuclear factor of activated T-cells
NK ................ natural killer cell
NK-T .......... natural killer-like T-cell
PBMC .......... peripheral blood mononuclear cell
PBS .......... phosphate buffered saline
pol ........ reverse transcriptase polymerase
PVG .......... Piebald-Viral-Glaxo rat
rag .......... recombination activation gene
RB .......... round bottom cell culture plates
rcf .......... relative centrifugal force
RPMI .......... Roswell park memorial institute
RT .......... room temperature
RV .......... retro virus
SD .......... standard deviation
sem .......... standard error of mean
SSC .......... sideways scatter
SV40 .......... simian virus
T$_{\text{reg}}$ .......... regulatory T-cell
t$_{\text{rej-median}}$ .......... median time point of rejection
TCM .......... T-cell media
TCR .......... T-cell receptor
TLI .......... Total lymphoid irradiation
UF .......... ultra filtration
VC .......... retroviral concentrate generated with ultra filtration
VS .......... retroviral supernatant
VS Foxp3 .......... VS from the GP+E Foxp3-IRES-GFP packaging cell line
VS GFP .......... VS from the GP+E GFP packaging cell line
1 Introduction

1.1 Aim of the PhD project

The goal of this research project was to develop and implement a strategy to generate allo-antigen specific regulatory T-cells and their testing in a preclinical model of rat cornea transplantation to prevent allo-graft rejection. Rooted in translational and regenerative medicine this study was to provide knowledge and expertise in the development of novel immune-modulatory therapies for future clinical applications. Two methods for the ex-vivo generation of regulatory T-lymphocytes were to be investigated; a) retroviral gene-transfer of transcription factor \( \text{foxp3} \) or b) anti-CD4 treatment of allo-stimulated rat T-cells. In order to do so, a protocol for the culture and propagation of allo-antigen specific rat T-lymphocytes had to be developed and the optimal conditions for the afore mentioned strategies had to be determined. To foster the understanding of regulatory T-cells the in-vitro generated cells were to be characterised and subjected to an in-vitro functional assay. A necessary prerequisite for this task was the acquisition of scientific and technical skills to design and perform advanced multicolour flowcytometry and cell labelling techniques. Finally, a suitable cell purification approach for the in-vivo application of \( T_{\text{regs}} \) had to be adopted.

To test the possibility of ex-vivo generated T regulatory cells to prevent cornea rejection a fully allogeneic rat cornea transplant model had to be implemented. That included the choice of rat strains, animal welfare, pre- and post-operative care as well as assistance during the surgical procedure. Furthermore, the base characteristics of the transplant model were to be assessed followed by an in-depth investigation of the rejection process using serum analysis and FACS based immune-phenotyping techniques. Finally, the ex-vivo generated regulatory T-cells were adoptively transferred and the outcome of that therapeutic intervention had to be evaluated.
1.2 Research hypothesis

It is understood that allo-graft rejection following organ or tissue transplantation is a complex immunologic process which in many aspects resemble auto-immune diseases. Both processes frequently entail pathologic priming and expansion of antigen specific T-lymphocytes which in turn trigger a cascade of effector immune responses leading to tissue specific inflammation and organ failure. In recent years a T-cell population has been discovered which expresses the transcription factor foxp3 and carries all characteristics of a suppressor T-lymphocyte. These cells, termed ‘regulatory’ T-cells (T\textsubscript{regs}), have been identified to be the key players in sustaining peripheral tolerance and immune homeostasis. T\textsubscript{reg}, which display a TCR repertoire highly skewed towards self antigens, curtail auto-aggressive T-cells either by direct interaction, indirectly by modifying dendritic cell function and / or by creating an anti-inflammatory environment in inflamed tissues and lymphatic organs. These properties have made regulatory T-cells the ideal candidates for therapeutic strategies which aim at preventing allo-graft rejection in an antigen-specific manner.

It is hypothesised that by generating and adoptively transferring a foxp3 expressing T-cell population with direct allo-antigen specificity, it is possible to artificially create tolerance towards an allograft in the host. This is based on the following assumptions:

- Ex-vivo selected and expanded T-lymphocytes are functional and are not subject to excessive compartment contraction after re-infusion. Foxp3 expression is sufficiently stable and the T\textsubscript{reg} phenotype constitutes a terminal differentiation stage of a CD4+ T-lymphocyte.

- Further, T\textsubscript{regs} behave like memory T-cells in terms of longevity, hence providing life-time antigen specific immuno-suppression.

- The immuno-suppressive capacity of foxp3-T\textsubscript{regs} is decoupled from T-cell receptor specificity, thus making it possible to generate (read select) allo-antigen specific T\textsubscript{regs} or for that matter T\textsubscript{regs} with any desired TCR.

This hypothesis will be tested in a fully allogeneic rat cornea transplant model. That includes the following model specific assumptions:
• The direct pathway of allo-antigen recognition is sufficiently pronounced in cornea transplantation to provide a target for T_{reg} suppression.

• There exists a semi-direct pathway of allo-recognition, which would allow regulatory T-cells with direct allo-specificity to exert their suppressive properties on indirectly primed T-cells.

• Alternatively, by suppressing the direct initial stage of the cornea rejection the level of pro-inflammatory stimulus is reduced (i.e. the danger model hypothesis [1]). The reduction in danger signals would allow the eye to restore its immune-privileged status thus preventing chronic rejection by creating tolerance.

A graphical summary of the research hypothesis can be found in figure 1.2 on page 10.

1.3 Current understanding of T_{reg} biology

The term regulatory T-lymphocyte is broadly used to characterise cells with the ability to suppress other immune cells and numerous sub-populations have been assigned this attribute [2]. In order to avoid confusion, in the context of this work the term regulatory T-cells (T_{reg}) shall be exclusively used for CD4+ T-cells which express the transcription factor foxp3. Their role as key players for the maintenance of self-tolerance and the prevention of autoimmune diseases has been firmly established [3]. Under normal conditions individuals lacking regulatory T-lymphocytes rapidly succumb to auto-aggressive inflammatory processes [4].

The transcription factor foxp3  The forkhead box transcription factor P3 has been identified as the master regulatory gene controlling the development and function of regulatory T-cells [5, 6]. Loss-of-function mutations result in the scurvy phenotype in mice and immune disregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX also called XLAAD) in humans [7, 8]. In both species disease symptoms are very similar and can be summarized as multi-organ autoimmunity caused by excessive proliferation of T-lymphocytes, B- and NK-cells as well as a complete absence of T_{regs}. In mice foxp3 expression is restricted to the CD4+ T-cell subset. In rat and human however, significant mRNA levels of FOXP3 are also detected in CD8+ T lymphocytes with regulatory capacity. Gene expression profiling [9] and ChIP analysis [10, 11] have revealed that foxp3 controls more then
700 genes directly or indirectly through activation or repression of transcription. Many of its gene regulatory functions are exerted through interactions with NFAT and NFκB, for example the suppression of IL-2 expression [12, 13]. The human genetics of FOXP3 are more complex than in rodents. Three different isoforms have been discovered; a) the full length transcript and b) two shorter versions lacking either exon 2 or exon 7 [14]. The FOXP3Δ2 variant seems to confer regulatory capacity when over-expressed in naive T-lymphocytes whereas the role of FOXP3Δ7 is not fully understood [15, 14]. Moreover, transient FOXP3 expression is found in all activated human T-cells. Stable de-methylation of the FOXP3 promoter region together with histone acetylation is necessary for a stable transcription and essential for the T_reg phenotype [16, 17].

The ontology of regulatory T-cells Two distinct sub-sets of foxp3 expressing T-cells exist. First, the naturally occurring regulatory T-lymphocytes (nTregs), which arise from poorly defined precursors during thymic selection. A strong T-cell receptor-MHC-II interaction in combination with CD28 co-stimulation is required for the formation of Treg. Furthermore, IL-2 and IL-7 signalling are indispensable for complete Treg differentiation and survival. The number of regulatory T-lymphocytes maturing in the thymic environment is tightly controlled by yet undefined factors. Reconstitution of the thymus with additional Treg precursors does not lead to increased numbers of mature T regulatory cells in the periphery [18]. Expression of foxp3 is detectable at a late time point of the CD4+ single positive stage of thymic selection [19]. Although foxp3 is the lineage defining transcription factor it has been discovered that the expression of foxp3 merely consolidates the transcriptional program acquired earlier in the differentiation process of Treg [20]. Additionally it is believed that nTregs predominantly recognise self-peptides and prevent the activation of auto-aggressive T-cells which escape negative selection. However, the notion has been challenged recently and only further research will clarify the details of regulatory T-cell biology.

A second mechanism to differentiate regulatory T-cells exists in the periphery. Naive CD4+ helper cells activated in the presence of TGF-β and retinoic acid can convert into adaptive foxp3 expressing T-lymphocytes (iTregs) [21, 22, 23]. Certain gut associated CD103 expressing dendritic cells as well as splenic CD8+ CD205+ DCs are particularly adept at performing this conversion [24, 25]. Moreover, it was discovered recently, that adaptive Treg are in a reciprocal relationship with Interleukin-17
secreting pro-inflammatory CD4+ helper T-cells (Th-17 cells) and considerable plasticity exists between the two population of CD4+ T-lymphocytes [26]. With regard to their suppressive function nT\(_{\text{regs}}\) and iT\(_{\text{regs}}\) are largely indistinguishable although their gene expression profile differs significantly [20].

**The cell surface phenotype of T\(_{\text{regs}}\)** The cell surface markers expressed on regulatory T-cells have been the subject of intense investigation. It was discovered that T\(_{\text{regs}}\) display a similar phenotype to activated CD4+ helper T-cells, a fact that has significantly hampered research of this T-cell subset. Most notably T\(_{\text{regs}}\) express CD25, the high affinity receptor α-chain for interleukin-2 [27]. Additionally, more then 30 different cell surface molecules have been identified on suppressor T-cells. Some examples are CD62L, CD69, CD134 (Ox-40), ICOS, CD73, CTLA4, CD28, CD304 (neuropilin) and CCR6. A complete list was compiled by Sakaguchi et al. [28]. No unique cell surface protein distinguishes a T\(_{\text{reg}}\) from an activated T-lymphocytes although more recently it was found that lack of CD127 expression on human regulatory T-cells helps to discriminate them from activated effector cells [29].

**Immune-mechanisms of suppression:** Several immune-mechanisms have been proposed to explain the suppressor function of T\(_{\text{regs}}\) [26, 28]. Despite intensive research the matter has not been solved satisfactorily. Many of the suppressor mechanisms described hereafter are subject to debate and the lack of a conclusive theory for T\(_{\text{reg}}\) function constitutes the biggest gap in the understanding of this fundamental immune cell population. Lack of functional T\(_{\text{regs}}\) in healthy mice leads to activation and unchecked proliferation of NK cells, T and B cells, DCs, granulocytes, macrophages and monocytes, suggesting that the regulatory regime extends directly or indirectly to these cell types [4]. The following biologic properties of T\(_{\text{regs}}\) have been linked to their suppressor function:

- Secretion of immune-modulatory cytokines such as IL-10, TGF-β and IL-35. These secreted proteins have been found to modulate dendritic cell function and foster the induction of T\(_{\text{R}1}\) cells. However, IL-10 and TGF-β are redundant for the inhibition of naive conventional T-cells by T\(_{\text{regs}}\) [5].

- Evidence exists that T\(_{\text{regs}}\) directly influence the function of antigen presenting cells attenuating their ability to prime effector T-lymphocytes. This is achieved
through reverse CTLA4 and TIGIT signalling leading to a reduced expression of CD80/CD86 co-stimulatory receptors on dendritic cells and the secretion of IL-10 [30, 31]. Additionally LFA-1 and LAG-3 ligation have been implicated in this process [32, 33, 34].

- Direct killing of conventional activated T-cells by T\textsubscript{regs} has been reported in mice and human utilizing Granzyme B or A [35, 36]. Furthermore, human T\textsubscript{regs} are shown to express CD95L endowing them with a Fas mediated apoptosis induction property [37].

- Expression of CD39-CD73 was detected on regulatory T-lymphocytes. These two ectonucleotidases hydrolyse pro-inflammatory ATP thus directly impacting the environment for other immune cells [38].

- Galectin-1 has been proposed as a major component of the suppressor apparatus as blocking or disruption of the receptor significantly inhibits T\textsubscript{reg} functionality [39].

- The constitutive expression of high affinity Interleukin-2 receptor CD25 has lead researchers to speculate that T\textsubscript{regs} consume large amount of IL-2 depriving conventional T-cells of this crucial growth factor [40].

### 1.4 Strategies to generate regulatory T-cells

Routinely CD4+ CD25\textsuperscript{high} T\textsubscript{regs} are isolated from lymphatic tissues or PBMCs using fluorescence activated cell sorting (FACS) or magnetic assisted cell sorting (MACS). Expansion of these cells is achieved by polyclonal activation using anti-CD3/CD28 coated beads [41, 42], although artificial antigen presenting cells have also been used [43]. Furthermore, isolation and successful culturing of peptide- and allo-antigen specific regulatory T-cells has been reported [41, 44, 42]. Typically, expansion protocols require the addition of high concentrations of exogenous IL-2. However, Ouabed \textit{et al.} discovered a rare population of rat plasmacytoid dendritic cell which can expand T\textsubscript{regs} without additional cytokines [45]. Alternative methods have also been published. For example, in-vitro treatment of polyclonal T-cell cultures with super-agonistic anti-CD28 antibodies preferentially expands regulatory T-lymphocytes [46]. Additionally, it was discovered that non-depleting anti-CD4 antibodies, when added to activated T-cell cultures, favour the outgrowth of T-cells
with regulatory capacity [47]. This approach is investigated in more detail in this project.

In order to truly generate regulatory T-cells ex-vivo two different avenues have been established. First, exploitation of the physiological function of TGF-β to convert naive CD4+ helper T-cells into foxp3 expressing lymphocytes with suppressive capacity [21, 22]. Retinoic acid the metabolite of vitamin A has been found to facilitate the conversion [48]. Furthermore, it has been established that addition of rapamycin to TGF-β treated T-cell cultures stabilises foxp3 transcription and blocks the outgrowth of potentially harmful Th-17 effector T-lymphocytes [49]. The second approach utilises forced expression of the transcription factor foxp3 by means of retroviral transduction. In the murine system ecotropic Moloney’s Murine Leukaemia Virus (MLV) and Murine Stem Cell Virus (MSCV) backbones prevail [5, 50, 51]. For ectopic expression of FOXP3 in human cells amphotropic recombinant lentivirus dominate [52, 53, 15]. Viral vectors generated for this approach are typically of similar design. A transcription cassette containing the therapeutic gene accompanied by a selection marker, which is frequently a green fluorescent protein (GFP). This strategy was also adopted for this project and a schematic of the recombinant retrovirus used is presented in figure 1.1. The gene-therapeutic strategy has two important advantages: a) foxp3 expression is decoupled from cell intrinsic gene regulation and thus more stable; b) the additionally conferred transduction marker facilitates the purification of ex-vivo generated T\textsubscript{regs} and allows in-vivo tracking of adoptively transferred cells.

![Schematic overview of the bi-cistronic retroviral expression vector (pL-Foxp3-IRES-GFP-SN) used in this project. Light blue genetic sequences are originating from the pLXSN plasmid (ClonTech\textsuperscript{®}). 5` LTR - long terminal repeat acting as integration site with intrinsic promoter activity for the transgene; \(\psi^+\) - retroviral genome packaging signal; MCS- multiple cloning site; Foxp3 - cDNA derived therapeutic gene; IRES - internal ribosome entry site; EGFP - optical reporter gene coding for enhanced green fluorescent protein; SV40 - simian virus 40 early promoter; NEO\textsuperscript{r} - neomycin resistance gene coding for phospho-diesterase; 3` LTR - the second integration site. A replication deficient retrovirus is produced by the GP\textsubscript{+}E86 packaging cell line [54], which has been stably transfected with the structural genes gag, pol and env, required for virus maturation, assembly and budding.](image-url)
1.5 Pathways of allo-recognition

**The direct pathway of allo-recognition:** This pathway has been associated with the acute rejection phase after allogeneic transplantation. All organs and tissues naturally harbour cells of haematopoetic origin for immuno-surveillance. In the context of transplantation such tissue borne immune cells may be activated as a result of the surgical trauma and re-perfusion injury and triggered to leave the graft. Donor derived antigen-presenting cells, also called passenger leukocytes, migrate into lymphatic tissues of the host where they initiate an allogeneic immune response by direct priming of host T-cells [55]. The polymorphic nature of the major histocompatibility proteins (MHC) is thought to be the key component of this mechanism. Due to allelic variations, donor MHC-complexes display diverse three dimensional folding patterns causing different amino acid residues to be exposed. Although thymic selection of T-cells ensures self-restriction of the T-cell receptor, host T-lymphocytes exhibit significant cross reactivity to non-self MHC molecules. The high precursor frequency of direct allo-responsive T-cells (5-10% of the T-cell pool) is believed to be the reason for the rapid and strong nature acute rejection. The involvement of the direct pathway following penetrating keratoplasty is controversial [56].

**The indirect pathway of allo-recognition:** As a result of normal cellular turnover or tissue damage following surgery and acute rejection, significant cell loss may occur in a graft. Apoptotic donor material is picked up and digested by host antigen presenting cells. Again due to allelic polymorphisms donor and host proteins may differ significantly in amino acid composition. Allo-peptides from major and minor histocompatibility determinants are presented to recipient T-lymphocytes on self-MHC-class-I/II and recognised as exogenous [55]. The result is the priming and clonal expansion of peptide specific T-cells, which subsequently orchestrate the rejection process. The indirect pathway can be considered a normal function of the immune system and it has been established that this pathway is critically correlated with chronic rejection [57, 58]. With regard to cornea transplantation it has been argued that the indirect pathway is the dominant mode of allo-recognition [59].

**The semi-direct pathway of allo-recognition:** This pathway was originally proposed by R. I. Lechler and collaborators [60]. Conceptual difficulties exist trying to explain the cross talk between direct and indirect pathway of allo-recognition. A particular problem poses the presence of CD8+ cytotoxic T-cells with direct allo-
specificity even at late stages of rejection when passenger leukocytes are thought to be eliminated. The mechanism proposes that host APCs can become bi-functional T-cell simulators for both the direct and the indirect responses. This is achieved by acquisition of intact donor MHC-molecules by recipient dendritic cells either by membrane transfer or exosomes for which ample in-vitro evidence has been accumulated [61, 62, 63]. Thus, indirectly primed CD4+ T-cells could provide 'help' to direct-specific CD8+ T-cells on the same APC, not violating the linkage paradigm that antigen-specific CD4+ and CD8+ T-cells are recruited through the same stimulator cell. The semi-direct pathway has so far not been conclusively demonstrated in-vivo and has to be considered hypothetical.

Allo-reactive NK - and NK-T-cells: In addition to the classical pathway of allo-recognition, the innate immune system is equally capable of non-self discrimination [64]. The function of NK- and NK-T-cells is tightly controlled by a set of activatory and inhibitory receptors [65]. For example, non-classical MHC-class-I molecules attenuate NK-cell activation [66]. Lack of self-MHC molecules on a somatic cell will instantly trigger NK cell mediated cytotoxicity. Both MHC-proteins as well as Killer Inhibitory Receptors (KIR) are subject to considerable genetic variation within one species [67]. Polymorphic NK-receptors expressed on donor cells are unable to ligate with inhibitory receptors on host NK-cells thus initiating immediate target cell lysis. The concept of natural killer-cell-mediated allo-recognition is summarised in the 'missing self hypothesis' [68]. Allo-NK responses following penetrating keratoplasty have been described in the literature [69, 70, 71]. A schematic overview of allo-recognition pathways and possible mechanisms of T_{regs} to influence those is presented in figure 1.2.
Pathways of allo-recognition and possible ways of regulatory T-cells with direct specificity to suppress effector mechanisms

Direct pathway

Indirect pathway

Figure 1.2: Graphical presentation of allo-recognition pathways in conjunction with the research hypothesis of this project. Left arm: Direct pathway of allo-recognition - Donor derived antigen presenting cells migrate out of the graft (passenger leukocytes) into secondary lymphoid tissues and stimulate CD4+ helper T-cells with direct allo-specificity which in turn provide pro-inflammatory cytokines to stimulate and expand CD8+ cytotoxic T-lymphocytes. Regulatory T-cells with direct allo-specificity suppress the activation of direct CD4+ helper T-cells either directly by immuno-suppressive cytokines, cell-cell interactions or by down-modulation of APC function. Middle arm: Putative semi-direct pathway of allo-recognition - Host antigen presenting cells pick up intact donor MHC molecules by membrane transfer, thus gaining the possibility to prime T-cells with direct and indirect allo-specificity. Through this double function of host APCs direct Trgs can block the priming of indirect allo-antigen specific T-lymphocytes. Right arm: Indirect pathway of allo-recognition - Apoptotic donor material is ingested by host APCs and allo-peptides are presented in the context of host MHC class 2 molecules to CD4+ helper T-cells which provide help to B-cells facilitating antibody maturation and isotype class switches. Trgs with direct specificity can only influence this pathway by indirect means such as bystander suppression or secreted cytokines such as TGF-β and IL-10. Not shown: Cross-presentation of allo-peptides to indirect CD8+ CTL. Due to disparate T-cell receptor specificity indirectly primed CD8+ T-lymphocytes can not engage directly with MHC-I molecules expressed on donor target cells. Their involvement in allo-graft destruction is not fully understood.
1.6 Penetrating keratoplasty

1.6.1 The eye - an immune privileged site

For a long time the eye has been known to be a special immunologic environment in which inflammatory processes are governed by a different set of rules to those observed in other tissues and organs. This phenomenon has gained the eye the status of an immune privileged site which attracted much attention from researchers and has been studied and reviewed intensively [72, 73, 74]. The eye in general and the ocular surface - the cornea - in particular are delicate structures and any damage can result in potentially life threatening loss of vision. The corneal endothelial single cell layer is essential in maintaining transparency of the cornea and has only marginal capacity to regenerate. In order to prevent tissue damage resulting from excessive inflammation following injury the eye is equipped with several intricate biological features to dampen inflammatory responses. The following immunologic mechanisms contribute to the immune-privileged status of the eye:

1. **Avascularity:** The healthy cornea lacks any blood or lymphatic vessels, a property with significant impact on graft infiltration by immune cells. Avascularity is actively maintained by secretion of soluble VEGF receptor-1 [75], and lymph-angiogenesis is inhibited by soluble VEGF-R2 [76].

2. **Soluble factors:** The aqueous humor is known to contain several immunosuppressive proteins such as VIP, α-MSH, TGF-β and CRP [73].

3. **CD95L expression:** Endothelial cells of the cornea have been found to express FAS-ligand, a membrane bound pro-apoptotic signalling molecule. Interaction of endothelial cells with cells of the immune system expressing FAS can induce apoptosis in the latter [77].

4. **Iris pigment epithelial cell:** It was discovered that IPE express co-stimulatory molecule CD86 (B-7.2) and membrane bound TGF-β [78]. During the process of extravasation from iris capillaries into the aqueous humor CD8+ T-lymphocytes engage in direct cell-cell contact with IPE. The interacting results in the formation of CD8+ regulatory T-cells expressing CTLA4 and foxp3 [79, 80]. These CD8+ suppressor T-cells have been shown to exert immunosuppressive functions on CD4+ T-lymphocytes thus attenuating T-cell mediated inflammation in the ocular environment.
5. **AICAID**: Anterior chamber associated immune deviation is a phenomenon which describes the induction of robust tolerance against allogeneic material when introduced into the anterior chamber under non-inflammatory conditions. The mechanism has been elucidated in mice to be a chain of events starting with the pick-up of antigens by F4/80+ antigen presenting cell in the eye followed by the migration of these APCs into the spleen where antigen is transferred to B-lymphocytes via the B-cell receptor [81, 82]. A complex interplay between B-lymphocytes, IL-10 secreting γ/δ-T-cells, NK-T-cells and CD8+ T-lymphocytes consequently leads to the formation of CD8+ foxp3+ allo-antigen specific T\textsubscript{regs} capable of curtailing CD4+ and CD8+ mediated DTH responses [83, 84, 85, 86].

### 1.6.2 Clinical challenges of cornea transplantations

Allogeneic corneal transplantation (also known as penetrating keratoplasty) is the most commonly performed transplantation procedure in humans. It is estimated that the annual number of procedure surpasses the 100,000 mark in western industrialised countries. Since its first successful implementation more then a 100 years ago by Eduard Zirm [87, 88] penetrating keratoplasty has helped to preserve vision for millions of patients. The procedure has steadily improved through novel surgical materials and techniques making it a routine procedure for the treatment of corneal dysfunction. However, the biggest obstacle has not been overcome. Despite the inherent immune privilege of the eye, corneal tissue is prone to non-self recognition by the host immune system and rejection of allogeneic grafts occurs at a high rate. A full fledged inflammation response is mounted involving all arms of the recipient immune system [89, 86]. Initiated by allo-independent danger signals resulting from the surgical trauma, activation of the adaptive immune system occurs and, in combination with innate immune responses graft destruction ensues. By studying animals models of penetrating keratoplasty it was learned that corneal tissue is endowed with multiple cell populations of haematopoetic origin [90, 91] which, in conjunction with host antigen presenting cells, initiate the rejection cascade. Furthermore, rejection is highly dependent on CD4+ helper T-cells which orchestrate Th1 as well as Th2 responses against the foreign tissue. Additionally, B-cells, NK-cells and neutrophils are heavily involved in the actual graft destruction process and neovascularisation is a risk factor strongly detrimental to graft survival [73]. Allograft survival frequencies after penetrating keratoplasty vary between 5-53% depending on mitigating factors.
Since there is no acute shortage of available corneas for transplantations patients are frequently re-grafted. However, the survival prognosis of secondary or tertiary corneal transplants decreases progressively [73]. Rejection episodes can occur even after years and allergic reaction or viral infections have been identified as possible trigger mechanisms [92, 93]. Notwithstanding the successful application of local immune-modulatory drugs such as glucocorticosteroids to delay rejection incidents, it is clear that true tolerance is not achieved even if the allo-graft is accepted for extended periods of time. Local manipulation of the rejection process has been successfully implemented in animals models utilising either direct topical application or gene therapeutic approaches. For example, overexpression of IL-10, NGF or blockade of T-cell co-stimulation by CTLA-4-Ig have all been shown to delay or prevent allo-rejection in preclinical models [94]. Systemic approaches aiming to exploit the immune mechanisms which maintain peripheral tolerance and the ocular immune privilege have received far less attention.
2 Materials and Methods

2.1 Mixed lymphocyte cultures

2.1.1 Isolation of primary lymphocytes

Male rats (LEW, DA, BN, PVG: age 8-14 weeks) were sacrificed in a CO$_2$ atmosphere. After skin disinfection with IMS the thorax was opened and blood withdrawn by heart puncture. Thymus and subcutaneous, cervical, brachial, inguinal, popliteal, mesenterial lymph nodes (LN) were resected with sterile surgical instruments and transferred into cold cell culture media. Lymph nodes and thymi were poured into a 100 µm cell strainer placed into a 6 cm Petri dish and covered with 5ml collagenase D digestion solution. Thymic tissue was additionally injected with collagenase D solution. Digestion was performed at 37°C for 30-45 minutes and stopped with an excess volume of ice-cold PBS+2mM EDTA. Primary lymphocytes were extracted by forcing the digested LN tissue or thymus through a 100 µm cell strainer with the piston of a sterile syringe. After isolation, cells were retrieved with a Pasteur pipette and rinsed through a 40 µm cell strainer and collected in a 50 ml screw cap tube. After centrifugation (400×rcf; 5 min.; 4°C) cell pellets were resuspended in ice-cold PBS+2mM EDTA and pelleted again, counted with a Neubauer chamber and stored on ice for further use.
Collagenase D digestion solution recipe:

+ RPMI + 25 mM HEPES
+ 1% fetal calf serum (57°C heat inactivated)
+ 2µg/ml Collagenase D (from powder)
+ 0.2µm sterile filtration
+ store at -20°C

Harvest of autologous serum: Blood was withdrawn by cardiac puncture using a 18G needle and transferred into a sterile 15ml screw cap tube. Coagulation was allowed to take place at room temperature. Clotted blood was then centrifuged (3000-4000×rcf; 10 min.; 4°C) and the resulting serum fraction was harvested with a sterile pipette and transferred into 1.5ml micro reaction tubes. After heat inactivation (30 min., 57°C) serum was stored at -20°C for later use.

Cell harvest from MLCs and density gradient purification: T-cells from MLCs were gently resuspended with a pasteur pipette and transferred into a suitable collection tube. Each well of the cell culture dish was rinsed with ice-cold PBS+2mM EDTA and the liquid collected as well. Cells were centrifuged at 400×rcf for 5 min. at 4°C, the supernatant discarded and the pellet was washed again with ice-cold PBS+2mM EDTA. After an additional centrifugation step the cell pellet was resuspended in cold PBS+2mM EDTA and 5ml cell suspension was transferred into a 15ml centrifugation tube. Using a 1000 µl pipette 2ml of ficoll gradient media (at room temperature) was carefully deposited underneath the cell suspension. Gradient centrifugation was performed at 350×rcf at RT for 20 minutes with deactivated centrifuge break. Cells floating at the inter-phase between ficoll and PBS were carefully removed with a 1000 µl pipette collected and washed twice with cold PBS+2mM EDTA.

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat ficoll: Pancoll ρ = 1,091g/l</td>
<td>P04-65500</td>
<td>Pan Biotech, Aidenbach, Germany</td>
</tr>
</tbody>
</table>
2.1.2 Isolation of thymic dendritic cells

Rat thymic dendritic cells were isolated using a discontinuous barrier floatation method exploiting the fact that DCs have a lower density than most other lymphocytes. Thymocytes isolated by collagenase digestion (see above) were resuspended in cold PBS+2mM EDTA (typically 20 ml per thymus) and 5ml cell suspension was transferred into a 15ml screw cap tube. Using a 1000 µl pipette 2ml of Nycodenz gradient media (at room temperature) was carefully deposited underneath the cell suspension. Gradient centrifugation was performed at 300×rcf at RT for 15 minutes with deactivated centrifuge break. Cells floating at the inter-phase between Nycodenz and PBS were carefully removed with a 1000 µl pipette collected and washed twice with cold PBS+2mM EDTA. When used for stimulator purposes, DCs were γ-irradiated with 12 Gray.

**Nycodenz density gradient media recipe:**

\[
\begin{align*}
+ & \text{ RPMI } + 25 \text{ mM HEPES (pre-warmed to 374°C)} \\
+ & 14.5\% \text{ (w/v) Nycodenz(from powder)} [\rho = 1.063g/l] \\
+ & 1\% \text{ fetal calf serum (57°C heat inactivated)} \\
+ & 0.45\mu m \text{ sterile filtration} \\
+ & \text{ store at 4°C}
\end{align*}
\]

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nycodenz® powder</td>
<td>1002424</td>
<td>Axis-Shield, Norway</td>
</tr>
</tbody>
</table>

2.1.3 Magnetic assisted cell sorting

Isolation of CD4+ T-cells was performed according to manufacturers instructions. Freshly isolated rat lymphocytes \((2 \times 10^8)\) were filtered through a 30µm and transferred into a 15 ml centrifuge tube. Then cells were washed once with 10 ml MACS buffer, centrifuged \((400\timesrcf, 5 \text{ min. } 4\text{°C})\) and resuspended in 1.8 ml MACS buffer. Subsequently, 200µl anti-CD4 microbeads were added and incubated for 10 minutes at 4°C. The labelling was stopped by adding an excess volume of MACS buffer.
followed by pelleting (400×rcf, 5 min. 4°C). The supernatant was discarded and the cell pellet was resuspended in 1ml MACS buffer. After placing the magnetic separation column into the magnet and priming with 3ml cold MACS buffer the cell suspension was added and allowed to pass through the column. Unlabelled cells were washed out of the column by rinsing three times with 3ml MACS buffer. Positively selected CD4+ cells were obtained by removing the separation column from the magnet, adding of 5ml MACS buffer and forceful flushing using the provided plunger. The separation was completed by a last centrifugation step (400×rcf, 5 min. 4°C). The resulting cells were resuspended in 2-3 ml TCM and counted. Typically, a cell number of $7 \times 10^7 - 1 \times 10^8$ could be expected from the above given starting material.

**MACS buffer recipe:**

+ PBS + 2mM EDTA (Lonza)
+ 0.5% (w/v) BSA
+ 0.2µm sterile filtration
+ store at 4°C

<table>
<thead>
<tr>
<th>material</th>
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<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat CD4 Microbeads</td>
<td>130-090-319</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>LS separation columns</td>
<td>130-042-401</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>MACS Multistand</td>
<td>130-042-303</td>
<td>Miltenyi Biotec, Germany; <em>kindly provided by Dr. Tyrone Bowes, REMEDI</em></td>
</tr>
<tr>
<td>MIDIMacs separation magnet</td>
<td>130-042-303</td>
<td>Miltenyi Biotec, Germany; <em>kindly provided by Dr. Tyrone Bowes, REMEDI</em></td>
</tr>
<tr>
<td>Pre-Separation Filters</td>
<td>130-041-407</td>
<td>Miltenyi Biotec, Germany</td>
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### 2.1.4 General tissue culture consumables

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<th>Manufacturer, Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>15ml screw cap centrifugation tube</td>
<td>62.554.502 PP</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>50ml screw cap centrifugation tube</td>
<td>62.547.254 PP</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>T-75 TC flasks</td>
<td>83.1813.002</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>T-175 TC flasks</td>
<td>83.1812.002</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>5 ml, 10 ml, 25ml sterile, single-use serological pipettes</td>
<td>–</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>10 µl, 20 µl, 200 µl, 1000 µl aerosol-resistant pipetting tips</td>
<td>–</td>
<td>StarLab, Germany</td>
</tr>
<tr>
<td>40µm cell strainer</td>
<td>734-0002</td>
<td>BD Falcon, UK</td>
</tr>
<tr>
<td>100µm cell strainer</td>
<td>734-0004</td>
<td>BD Falcon, UK</td>
</tr>
<tr>
<td>50ml syringes (Luer-lock)</td>
<td>613-3925</td>
<td>BD Falcon, UK</td>
</tr>
<tr>
<td>0.45µm syringe filters</td>
<td>83.1826</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>0.2µm syringe filters</td>
<td>83.1826.001</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>Filtropur V 25, 500ml filter units 0.2µm</td>
<td>83.1822.001</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>96 MicroWell Plates Nunclon flat bottom wells</td>
<td>734-2073</td>
<td>Nunc, Roskilde, Denmark</td>
</tr>
<tr>
<td>12-well plates</td>
<td>150628</td>
<td>Nunc, Roskilde, Denmark</td>
</tr>
<tr>
<td>6-well plates</td>
<td>83.1839</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>6 cm Petri dishes</td>
<td>83.1801</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>10 cm Petri dishes</td>
<td>83.1802</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
<tr>
<td>1,8 ml sterile cryo-tubes</td>
<td>368632</td>
<td>Nunc, Roskilde, Denmark</td>
</tr>
<tr>
<td>3.5ml pasteur pipette</td>
<td>86.1171</td>
<td>Sarstedt Ltd, Ireland</td>
</tr>
</tbody>
</table>
2.1.5 Media, buffers and additives

- **standard cell culture media:**
  + Dulbecco’s Modified Eagle Medium (DMEM) - high glucose
  + 10% fetal calf serum (57°C heat inactivated)
  + 100 U/ml Penicillin
  + 100 µg/ml Streptomycin

- **T-cell media (TCM):**
  + RPMI 1640 + 25 mM HEPES
  + 2 mM L-glutamine
  + 2 mM L-asparagine
  + 100 U/ml Penicillin
  + 100 µg/ml Streptomycin
  + 1 ml/100ml non-essential amino acids
  ± 2% autologous serum (57°C heat inactivated)
  ± 10% FCS (57°C heat inactivated)
  ± 50-500 U/ml human recombinant IL2

- **cryo-conservation solution (freezy):**
  + 90% FCS
  + 10% DMSO

**Reconstitution of recombinant human IL-2**

According to WHO standards specific activity of 13,000U/µg was assumed for recombinant IL2. Lyophilised peptide was dissolved in 0.1M acetic acid. A stock solution of 100,000 U/ml was prepared by diluting the dissolved peptide in PBS+1%BSA (w/v; carrier protein). After sterile filtration (0.2µm) aliquots were stored at -80°C.
### 2 Materials and Methods

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS w/o Ca(^+) Mg(^+) + 2mM EDTA</td>
<td>BE02-017F</td>
<td>Lonza Biologics, UK</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate-Buffered Saline (D-PBS) (1X) liquid</td>
<td>14190-250</td>
<td>Invitrogen / GIBCO</td>
</tr>
<tr>
<td>RPMI-1640 +25mM HEPES</td>
<td>BE12-115F</td>
<td>Lonza Biologics, UK</td>
</tr>
<tr>
<td>DMEM- high glucose</td>
<td>BE12-604F</td>
<td>Lonza Biologics, UK</td>
</tr>
<tr>
<td>100× non-essential amino acids</td>
<td>M7145</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>100× Pen/Strep</td>
<td>P0781</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>G7513</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>A7094</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Fetal calf serum (Lot# 56K3395, non-USA origin)</td>
<td>F7524-500ml</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>recombinant human IL-2</td>
<td>200-02</td>
<td>Peprotech EC LTD., UK</td>
</tr>
<tr>
<td>DMSO (molecular biology grade)</td>
<td>41639</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>1× Trypsin/EDTA</td>
<td>T3924</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>1M HEPES</td>
<td></td>
<td>Lonza Biologics, UK</td>
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</table>

#### 2.1.6 Rat strains

<table>
<thead>
<tr>
<th>rat strains</th>
<th>supplier</th>
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</thead>
<tbody>
<tr>
<td>Lewis (LEW), haplo type: RT-1(^1)</td>
<td>Charles River Laboratories, UK; Harlan Laboratories, UK</td>
</tr>
<tr>
<td>Dark Agouti (DA) haplo type: RT-1(^{av1})</td>
<td>B&amp;K Universal Ltd, UK; Harlan Laboratories, UK</td>
</tr>
<tr>
<td>Brown Norway (BN) haplo type: RT-1(^n)</td>
<td>Harlan Laboratories, UK</td>
</tr>
<tr>
<td>Piebald-Viral-Glaxo (PVG) haplo type: RT-1(^e)</td>
<td>Harlan Laboratories, UK</td>
</tr>
</tbody>
</table>
2.2 Production of recombinant retroviruses

Ecotropic recombinant retroviruses with a Moloney’s murine leukaemia virus backbone were used in this study [54]. The generation of retroviral gene therapy vectors was not part of this project, but a detailed description of the method has been published here [95, 96]. Retroviral packaging cell lines were maintained in DMEM-high glucose + 10% FCS + Pen/Strep T-75 tissue culture flasks. For retrovirus production confluent cell lines were harvested by trypsination and seeded 1:10 in T-175 TC flasks and grown to full confluency. One day before virus harvest cell lines were split 1:3 into T-175 bottles and the media was changed to 30 ml TCM to generate the bulk quantities of retrovirus containing media for T-lymphocyte transductions. Retrovirus concentration: Membrane ultra-filtration was used to increase retroviral titers and to improve the transduction of rat T-lymphocytes. Retroviral packaging cell lines were grown to full confluency in T-175 TC flask trypsinated and split 1:3 into fresh T-175 flasks filled with 30ml standard DMEM +10% FCS. After 24 hours media was harvested and subjected to sterile filtration using a 0.45µm syringe filter. Ultra-filtration columns were sterilised by exposing them for 5 minutes to UV light irradiation. Then, 20 ml of filtered retrovirus containing media were added to an ultra-filtration column which was spun at 3000×rcf for 75 minutes at 4°C. Retroviral concentrates were carefully retrieved using a 200µl pipett and transferred into sterile 2ml microreaction tubes. Between 350-400µl of viral concentrate could be harvested from a single centrifugation column and were used immediately for transduction experiments.

<table>
<thead>
<tr>
<th>cell lines</th>
<th>origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP+E86 GFP packaging cell line</td>
<td>Flügel A., MPI Munich</td>
</tr>
<tr>
<td>GP+E86 Foxp3-IRES-GFP packaging cell line</td>
<td>generated by T. Ritter at the Institute for Medical Immunology, Charité-Universitätsmedizin Berlin, Germany</td>
</tr>
<tr>
<td>GP+E86 solo - empty packaging cell line</td>
<td>Flügel A., MPI Munich, Germany</td>
</tr>
</tbody>
</table>
### 2.3 Transduction of rat T-lymphocytes

Standard mixed lymphocyte cultures were set-up according to the following protocol: Naive CD4+ T-cells were isolated using MACS technology and seeded at a density of $2 \times 10^5$ cell/well with $\gamma$-irradiated thymic dendritic cells at a ratio of 1:3 in 96-well flat bottom plates. Cell were cultured in a total volume of 120µl TCM+2% autologous serum per well. Only the 60 inner wells of each culture plate were used for cell cultures, while the outer rim of well was filled with sterile PBS to reduce evaporation. MLCs were cultured for 72 hours before transduction. On day 3 fresh retrovirus containing media was harvested and sterile filtered using a 0.45µm syringe filter and supplemented with 1-2% viral concentrate from ultra-filtration columns. Polybrene was added as a transduction enhancer [97] at a final concentration of 4µg/ml. Carefully 70µl of T-cell media was removed from each well of the MLC plates and 180µl retrovirus transduction media was added.

**Spinoculation:** Plates were then spun at $2000 \times rcf$ for 2 hours at 32°C [98]. **Incubation:** After centrifugation transduced MLCs were cultured for 24 hours at a reduced temperature of 32°C to reduce thermal retroviral decay [99]. **Antibiotic selection of transduced T-lymphocytes:** After a 24 hour virus incubation period 180µl of exhausted retrovirus media was removed from each well and replaced with 180µl fresh TCM +10% FCS + 50-500 U/ml rhIL-2 + 4mg/ml G-418. From thereon media changes were performed every second day using the same media composition and antibiotics (G-418) concentration.

**Measurement of the transduction frequency:** On day 5 (2 days post transduction) a random sample of three wells was analysed using flow-cytometry. GFP fluorescence was used as an indicator for successful retroviral transduction.
2.4 Anti-CD4 antibody treatment of allo-MLCs

Standard mixed lymphocyte cultures were set-up according to the methods described in section 2.1 using CD4+ MACS sorted BN cells. T-lymphocytes were co-cultured with γ-irradiated PVG thymic dendritic cells at a ratio of 3:1 in anti-CD4 treated cultures. Isotype treated control T-cells were cultured at a T-cell/DC ratio of 7:1. Cell were seeded in 12-well plates in a volume of 2 ml at a cell density of $2 \times 10^6$ cell/ml. The anti-CD4 antibody RIB5/2 and the isotype control antibody were added directly at the beginning the T-cell culture. Predilution of the antibodies were made with TCM+10% FCS as diluent. No media change was performed on RIB5/2 cultures for the entire culture period. Due to the high proliferation in control T-cell cultures a media change was performed on day three using TCM + 10% FCS +50 U/ml rh-IL2. For that 1 ml exhausted media was carefully removed with a 1000 µl pipett and replaced with 1 ml fresh media. For the anti-CD4 titration experiments MLCs were analysed on days 4 and 6. Cells designated for in-vivo application were harvested on day 7.

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse anti rat CD4 - RIB5/2 (1mg/ml; IgG2a)</td>
<td>n.a.</td>
<td>Acris Antibodies, kind gift of Dr. Birgit Sawitzki, Institute for Medical Immunology, Charité-Universitätsmedizin Berlin, Germany</td>
</tr>
<tr>
<td>functional grade mouse IgG2a isotype (1mg/ml; clone: eBM2a)</td>
<td>12-4724-85</td>
<td>eBioscience, UK</td>
</tr>
</tbody>
</table>

2.5 In-vitro suppression assays

To test the suppressive capacity of regulatory T-cells an in-vitro co-culture system was used. Naive BN responder T-cells were freshly isolated, CD4+ MACS purified and subsequently CFSE labelled. These cell were seeded (at $5 \times 10^4$ cells per well) together with allogeneic γ-irradiated PVG thymic DCs at a 5:1 ratio in 96-well round bottom plates. Graded numbers of either T$_\text{regs}$ or T$_\text{conv}$ harvested from anti-CD4 treated MLCs or isotype controls were added to these cultures starting at a
ratio of 1:1 down to 64:1 in triplicates. The total media volume per well was 250 µl TCM + 10% FCS + 2% autologous serum. Suppression assay cultures were performed with and without the addition of 100U/ml rh-IL2. CFSE dye dilution of proliferating responder T-cells was measured at day 3 and day 4 of culture.

2.6 Flowcytometry

2.6.1 General cell surface staining procedure

Live/Dead staining: For most FACS applications in this study, exclusion of dead cells from analysis was essential. A commercially available dye was used, which fluoresces when excited by a short wave length violet laser. The obvious benefit of this was, that light emissions from the live/dead dye are recorded separately from all other cell surface stains, thus leaving other important detection channels in the cytometer open without the need to compensate fluorescent spill over from dead cells. Additionally, the reagent used was very bright, stable and fixable with formaldehyde, which constitutes a big advantage over classical dye such as 7AAD or propidium iodide.

The staining was performed according to manufacturer’s instructions. Freshly isolated lymphocytes or harvested cells are washed twice with cold PBS. Up to $1 \times 10^7$ cells were resuspended in 1ml PBS and 1µl Violet Live/dead stain was added and mixed thoroughly. Incubation was performed for 30 minutes on ice in the dark. The reaction was stopped by adding 5ml PBS followed by pelleting. The staining was completed by washing the cells once with FACS buffer. To preserve the staining intensity samples are shielded from ambient light as much as possible.

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (405 nm excitation)</td>
<td>L34955</td>
<td>Molecular Probes Europe BV/Invitrogen, UK</td>
</tr>
</tbody>
</table>

Endogenous biotin blocking: Due to the lack of a wide range of directly labelled antibodies in the rat, biotinylated anti-rat antibodies had to be used for complex multicolour FACS stains. Antibody binding was then visualised by secondary stain-
ing with streptavidin conjugated fluorophores. These secondary dyes typically produce a high background signal which hampers identification of positively labelled cells. To quench at least some of the background signal, endogenous biotin was blocked using high concentrations of free streptavidin.

Deviating from manufacturer’s instructions, cell were pelleted and resuspended in 500µl PBS. One to three drops of concentrated streptavidin were added and the sample was mixed thoroughly. Cells were incubated for 15 minutes on ice, then washed once with PBS (400×rcf for 5 min. at 4°C) and again resuspended in 500µl PBS. Unsaturated streptavidin was subsequently blocked by adding 1-3 drops concentrated biotin solution. After an incubation of 15 minutes on ice, cells were washed with FACS buffer centrifuged and resuspended in a appropriate volume of FACS buffer for continued stainings.

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous Biotin-Blocking Kit</td>
<td>E-21390</td>
<td>Molecular Probes Europe BV/Invitrogen, UK</td>
</tr>
</tbody>
</table>

**Cell surface marker stainings**  Cell surface antigens were stained according to standard procedures: A small volume (<50µl) of FACS buffer was pipetted into 1.2ml PPN FACS tubes and the appropriate amount of primary antibody was added. Typically, $1 \times 10^6$ cells were then added to each tube giving a final reaction volume of 100µl and starting the labelling procedure. Samples were incubated on ice for 20 minutes in the dark. Cells were then washed twice with 700µl FACS buffer (400×rcf for 5 min. at 4°C) and the supernatants discarded. For secondary stains, 50µl of 1:1000 pre-diluted streptavidin conjugated fluorescent dyes were added, resulting in a final dilution of 1:2000. The secondary reaction was performed for 20 minutes on ice in the dark. The protocol was finalised by washing samples twice with FACS buffer (400×rcf for 5 min. at 4°C) and filtering each sample using a 40µm nylon mesh if necessary.
**FACS buffer:**

- D-PBS
- 2% fetal calf serum (57°C heat inactivated)
- 0.09% (w/v) sodium azide
- 0.2µm sterile filtration
- store at 4°C

<table>
<thead>
<tr>
<th>material</th>
<th>supplier code</th>
<th>manufacturer, supplier</th>
</tr>
</thead>
<tbody>
<tr>
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<td>61226</td>
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**Analysis of FACS data:** Flowcytometry experiments were designed and analysed using the principles established by Herzenberg *et al.* [100, 101]. Briefly, instead of simple isotype controls hierarchical 'fluorescence minus one' (FMO) negative controls were used. The gating strategy was: single cells (FSC-A vs. FSC-H) → live/dead discrimination (Violet-A vs. APC) → morphological lymphocyte gate (FSA-A vs. SSC).

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2 Materials and Methods

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**secondary antibodies**

| RAM     | X-56  | rIgG1 | PE     | 340270   | BD      |

**Isotype controls**

| mIgG2ακ | -     | -     | APC    | 17-4724  | eBioscience |
| mIgG2ακ | G155-178 | -   | FITC   | 555573   | BD      |
| mIgG3ακ | A112-3 | -     | PE     | 559926   | BD      |
| mIgG3ακ | B10   | -     | FITC   | 11-4742-73 | eBioscience |
| mIgG1   | MOPC-21 | -   | FITC   | 555909   | BD      |
| mIgG1   | F8-11-13 | -   | PE     | MCA1209PE | Serotec |
| mIgG1   | F8-11-13 | -   | Bio    | MCA1209B | Serotec |
| mIgG1   | F8-11-13 | -   | AF647  | MCA1209A647 | Serotec |
| mIgG2b  | -     | -     | PE     | 12-4732  | eBioscience |
| hamIgG2αλ | Ha4/8 | -     | PE     | 553965   | BD      |

**secondary reagents**

| Sav-PeCy7 | -     | -     | PeCy7  | 557598   | BD      |
| Sav-      | -     | -     | PerCpCy5.5 | 551419   | BD      |
| PerCpCy5.5 | -   | -     |        |          |         |
2 Materials and Methods

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<td>FACSCanto with FACS-DIVA V5</td>
<td>BD Biosciences, UK</td>
</tr>
</tbody>
</table>

*analysis software*

FlowJo V7.1.6 for MS Windows   Tree Star, Inc., USA

2.6.2 Intracellular staining procedure for foxp3 detection

The flowcytometric detection of transcription factor foxp3 was conducted using a commercial kit from eBioscience. Permeabilization of cells and staining of foxp3 protein was performed exactly according to manufacturer’s instructions. Briefly, lymphocytes were stained with a live/dead stain and appropriate cell surface markers as described in section 2.6.1 on page 25 (typically anti-CD4 plus additional antibody). The resulting cell pellet was resuspended in 700µl freshly prepared 1× Fixation/Permeabilization working solution and incubated for at least 2 hours at 4°C (maximum possible permeabilization 18 hours). Cells were centrifuged at 400×rcf for 5 minutes and the supernatant was aspirated. Samples were washed twice with freshly prepared 1× Permeabilization buffer using a volume of 1 ml and 400×rcf + 5 minutes centrifugation steps. Pelleted cells were then resuspended in 100µl 1× Permeabilization buffer and 0.125µg anti-foxp3 antibody was added. The labelling procedure was allowed to take place for 20 minutes on ice in the dark. The reaction was stopped by adding 700µl of 1× Permeabilization buffer followed by centrifugation and aspiration of the supernatant. A last washing step was performed with FACS buffer. Samples were filtered through a 40µm nylon mesh and analysed immediately. Fixation of samples was not necessary since the Fixation/Permeabilization working solution already contained para-formaldehyde.
2 Materials and Methods

2.6.3 Fluorescent cell labelling technique for proliferation measurement

**CFSE labelling technique:** The green fluorescent cell tracer dye CFSE was dissolved in DMSO and adjusted to a stock concentration of 5mM. Freshly isolated lymphocytes \((2 \times 10^7\) per 15ml tube) were washed once with staining buffer (see recipe below) and pelleted \((400 \times \text{rcf for 5 min. at } 4^\circ\text{C})\). The supernatant was discarded and the cell pellet was resuspended in 1ml pre-warmed \((37^\circ\text{C})\) staining buffer. CFSE was added at a final concentration of \(1\mu\text{M}\) and mixed thoroughly. Samples were incubated at \(37^\circ\text{C}\) (water bath) for 6 minutes in the dark. The reaction was stopped by adding an excess volume of cold DMEM containing 10% FCS. Samples were incubated for further 5 minutes on ice before centrifugation \((400 \times \text{rcf for 5 min. at } 4^\circ\text{C})\). Cells were washed twice with 5ml DMEM+10%FCS before finally being resuspended in 1 ml TCM and counted. Typically a cell loss of 50-60% was expected. Cell labelling intesity was checked under a fluorescent microscope.
**Materials and Methods**

**SNARF-1 labelling technique:** Fluorescent cell labelling with the red dye SNARF-1 was performed as described above with the exception that a final concentration of 3µM was used and the initial incubation at 37°C was performed for 10 minutes.

**CFSE/SNARF-1 staining buffer:**

- D-PBS
- 0.1% fetal calf serum (57°C heat inactivated)
- 0.2µm sterile filtration
- store at 4°C

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<tr>
<td>SNARF-1 carboxylic acid, acetat, succinimidyl ester</td>
<td>S-22801</td>
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</table>

**2.6.4 Detection of allo-antibodies in rat serum**

Blood from surgical animals was collected according to the method described on page 15. However, after aliquoting serum samples were not subjected to heat inactivation. The serum was stored at -80°C.

Diluted serum (1:5 in FACS buffer) was incubated with $1 \times 10^6$ PVG splenocytes for 30 minutes on ice in a total volume of 100µl per test. Samples were washed twice with 700µl FACS buffer and pelleted (400×rcf for 5 min. at 4°C). In the fashion of a secondary FACS stain samples were then labelled with either anti-rat IgM [G53-238], IgG1 [MRG1-58], IgG2a [MRG2a-83] or IgA [MARA-1] (all from Antibodies-online, Germany). Positive control sera were generated by immunising naïve BN rats with $1 \times 10^6$ γ-irradiated (12 Gy) PVG thymic dendritic cells. Thymic DCs were obtained by density gradient centrifugation as described on page 16. Positive sera were harvested 14 days post immunisation.
2.6.5 Measurement of graft infiltrating lymphocytes and draining LN analysis

Cell isolation from corneal tissues: The central cornea containing the graft was marked with a 3 mm trephine and excised using vannas scissors. Dirt and iris pigments were removed by rinsing the tissue with sterile PBS. The excised graft was then transferred into a 2 ml screw cap tube containing 0.5 ml tissue digesting solution (see recipe below) and placed into a tube shaker/heater for 90 minutes (900 rpm @ 37°C). Collagenase digestion was stopped with cold PBS containing 2mM EDTA. All liquid and remaining tissue was poured into a 100µm cell strainer placed into a 6cm tissue culture dish. Undigested cornea tissue was then gently disintegrated with the help of a syringe plunger. After thoroughly rinsing the cell strainer and the collection dish with cold PBS/EDTA the cell suspension was collected in 15 ml Falcon tubes and stored on ice.

Cell isolation from draining lymph nodes: Ipsilateral submandibular as well as contralateral submandibular and brachial lymph nodes were carefully dissected from the animals and processed as described above for corneal tissue with the exception that the collagenase digestion time was reduced to 30 minutes.

Cornea Collagenase D digestion solution recipe:

+ RPMI + 25 mM HEPES
+ 5µg/ml Collagenase D (from powder)
+ 0.2µm sterile filtration
+ store at -20°C

2.6.6 Fluorescence activated cell sorting

T-lymphocytes were harvested according to the method described in section 2.1.1 on page 15. Cell were resuspended in 1ml FACS buffer without sodium azide. Regardless of cell number 0.4µg anti-CD3 antibody was added and the sample was incubated for 20 min. at 4°C in the dark. After the incubation, cells were washed once with FACS buffer and passed through a 30µm filter. After centrifugation (400×rcf for 5 min. at 4°C), the pellet was resuspended in sort buffer. The cell
density was adjusted to $5 \times 10^6$ cell/ml. The cell sorter was prepared for an aseptic sort according to the manufacturer’s instruction. The sorting procedure was performed using a 70$\mu$m nozzle, a 16-16-0 purity mask and commercial sheath buffer. Positively sorted cell were collected in sterile cold TCM.

**sort buffer recipe:**

- + PBS+ 2mM EDTA
- + 1% fetal calf serum (57°C heat inactivated)
- + 100 U/ml Penicillin
- + 100 $\mu$g/ml Streptomycin
- + 20mM HEPES
- + 0.2$\mu$m sterile filtration

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### 2.7 Rat cornea transplantation

#### 2.7.1 Surgical procedure

**General anaesthesia:** *Induction:* Animals were placed into an anaesthesia box connected to an Isoflurane vaporizer and pre-filled with a mixture of oxygen and Isoflurane. To reduce the discomfort to animals and to guarantee rapid induction the box was quickly flooded with Isoflurane (5% anaesthetic in 2 l / min medical oxygen) until rats were fully anaesthetized.
Surgical anaesthesia: Subsequently, animals were transferred onto a heated operating table and the snout was placed into a breathing tube connected to the anaesthetic machine. The volume percentage of Isoflurane was set between 1.5 - 2.5% depending on the size of the animal while the flow rate of oxygen was maintained at 2l/min. Deep anaesthesia was considered to be achieved when limb withdrawal and eye reflexes were abolished. Depth of anaesthesia was monitored by the breathing pattern of the animals.

Recovery from anaesthesia: The administration of Isoflurane was stopped and rats were allowed to breathe oxygen until they started to hyperventilate. Animals were then carefully placed into a clean cage lined with paper tissue towels. The recovery cage was placed onto a heating pad and covered with opaque operating drapes. Animals were allowed to remain in the recovery cage until fully awake, which was determined by observing normal inquisitive behaviour.

Pre-operative care: Before the surgical procedure, all animals were screened for ocular abnormalities as follows. Rats were anaesthetised and eyes were inspected under a surgical microscope. At 16× magnification the eyes were screened for large abnormalities such as corneal scratches and vascularisation, aspherical or asymmetric orbit, iris deformities and blood vessels from the iris extending into the anterior chamber. Furthermore, the central cornea was also inspected in detail at 40× magnification. A drop of saline solution (Alcon BSS) was applied, which caused an additional lensing effect, thus facilitating a closer inspection of the cornea for possible pigmentation and other abnormalities.

Pre-operative injections: Twenty-four hours before surgery animals were anaesthetised according to standard procedure and placed on a heating pad. The liquid to be administered (cell suspension or sterile PBS) were brought to 37°C to prevent discomfort in injected animals. The infusion of the total volume of 1ml was performed by tail vein injection using an 18G needle and a sterile single use syringe.

Note to the reader: All procedures described in the following paragraph were performed by a fully trained and licensed ophthalmic surgeon and not by the author himself. All procedures performed were conducted under animal licence no. B100/3852 and were approved by the Animals Ethics Committee of the National University of Ireland, Galway. In addition, animal care and management followed the Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science.
Surgery: Donor animals were humanly killed by CO₂ asphyxiation. Both eyes were excised and placed in sterile saline solution (Alcon BSS). Donor corneas were marked with a 3mm trephine and cut out using a small angled scissors. Before the corneal tissue was completely detached from the eyeball the first suture was introduced. Fully anaesthetised recipient animals were placed on a heated operating table which maintained body temperature at 37°C. Pupil dilating drops were reapplied if necessary and Tetracaine drops were administered until complete abolition of eye reflexes had occurred. Further eye movement was prevented by fixing the eye lobe with two 6-0 braided sutures. The graft bed was prepared by marking the central cornea with a 2.5mm trephine followed by excision of marked tissue. The donor cornea was placed on the recipient eye and sutured into place with 3 - 4 cardinal stitches. The wound was then completely closed by another 4-6 interrupted sutures. The suture knots were towed away from the donor tissue and knots were trimmed as short as possible. During the surgical procedure the eye was continuously moistened with sterile saline solution. No attempt was made to reconstruct the anterior chamber and wound closure was deemed successful when the eyeball started to re-inflate. The surgery was completed by another dose of Atropine drops and by covering the eye with antibiotic ointment containing chloramphenicol. The eyelids remained open. The approximate length of the procedure was 30 minutes. Animals that suffered iris damage during the surgical procedure were not allowed to recover from anaesthesia and were killed by being placed in a CO₂ atmosphere.
2.7.2 Post-op follow up on surgical animals

Post-surgery animals were inspected every second day. The status of the healing process was assessed and corneal opacification and progression of neovascularisation was recorded. (The protocol used for recording post-op parameters can be found in the appendix.) Animals were anaesthetised and inspected under an operating microscope. Corneal opacity of albino LEW rats was scored according to published criteria [102]:

- 0 - no opacity
- 1 - minimal opacity; iris capillaries visible
- 2 - moderate; large blood vessels of the iris visible
- 3 - strong opacity; only pupil margin visible
- 4 - complete opacity; anterior chamber not visible

Due to the fact that iris blood vessels are not visible in pigmented BN animals the scoring method was modified as follows:

- 0 - no opacity
- 1 - minimal; all iris details (crypts) visible
- 2 - moderate opacity; some iris details visible
- 3 - strong opacity; only pupil margin visible
- 4 - complete opacity; anterior chamber not visible

Post-surgical animals displaying any complications such as active bleeding into the anterior chamber, ocular infection, wound gapping (dehiscence), ruptured sutures or excessive epithelial shedding were excluded from the study and were euthanized.
### 2.7.3 Surgical materials

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**Ophthalmic surgery drugs**

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3 Results

3.1 Retroviral transduction of allo-antigen specific rat T-lymphocytes

The first approach to generate regulatory T-cells in this study was the forced expression of transcription factor foxp3. For this purpose recombinant retrovirus vectors were used to transduce allo-antigen stimulated rat T-lymphocytes. Two retroviral packaging cell lines (PCL) were provided. A) a GFP expressing control vector (GP+E GFP) and b) a retrovirus containing a bi-cistronic therapeutic gene construct (GP+E Foxp3-IRES-GFP). Both vectors additionally conferred an antibiotics resistance for selection purposes. Using intracellular antibody staining in conjunction with flow cytometry it was confirmed that the GP+E Foxp3-IRES-GFP PCL expressed the therapeutic gene foxp3 on a protein level (figure 3.1).

Figure 3.1: Flowcytometric confirmation of intracellular foxp3 expression in GP+E Foxp3-IRES-GFP retroviral packaging cell lines. GP+E GFP packaging cell lines served as negative control.
**Transduction of Mixed lymphocyte cultures**  MLCs were set up using CD4+ MACS sorted responder T-cells co-cultured with γ-irradiated thymic dendritic cells as antigen presenting cells. Most experiments were conducted using the LEW-DA strain combination. This set-up ensured the growth of allo-specific helper T-cells. Activated MLCs were transduced with freshly harvested retroviral supernatants on day 3 using an optimized protocol. Among other things optimization was achieved by reducing the culture temperature to 32°C for 24 hours after the retrovirus was added. This strategy typically increased the transduction frequency by 50-200% (n=3) as can be seen in **figure 3.2**. Successful gene transfer into rat T-lymphocytes was measured by flowcytometry detecting GFP expression. In preparative large scale transductions the percentage of transduced cells fluctuated between 13-25% for the GFP control vector and 5-14% for the foxp3 containing retrovirus (n>10). Applying a intracellular FACS staining procedure it was confirmed that primary T-cells exposed to the Foxp3-IRES-GFP vector expressed the protein foxp3 shortly after transduction (**figure 3.2 D**). To select GFP positive cells, antibiotic selection was commenced on day 4, 24 hours post transduction. Measuring the killing capacity of the antibiotic G-418 it was determined that un-transduced cells needed 5-6 days to completely succumb to the selective pressure (data not shown).

**Expansion of retro-virally transduced cells:**  Despite preparative scale transductions the number of GFP positive cells was too low for functional assays or in-vivo applications (typically <5 × 10⁶ from 5 × 10⁷ naive cells starting material). Hence the gene-modified T-lymphocytes needed to be expanded. This was attempted by restimulation experiments. MLCs were harvested on day 7 ficoll purified and re-cultured with γ-irradiated thymic donor DCs in the presence of G-418 and rhIL2. Despite numerous protocol modifications foxp3 transduced T-cells could not be expanded. Restimulation experiments failed repeatedly, either due to complete cell death or the outgrowth of GFP negative cells. An example of a failing culture is shown in **figure 3.3**. Contrary to ex-vivo generated Tregs, conventional GFP expressing T-cells could be propagated in-vitro. Cells would grow vigorously and form impressive proliferation clusters (see fig. 3.2C). Up to three cycles of re-stimulations were performed, achieving cell number in excess of 2 × 10⁸. However, even under the permanent influence of antibiotics a purity of greater then 85% could not be obtained. Also, GFP- T-cell cultures would fail occasionally for no apparent reason.
Figure 3.2: Representative results for the retroviral transduction experiments. Panel A+B: Optimization of retroviral gene transfer by reducing the incubation temperature to 32°C for 24 hours. Panel C: Fluorescence microscope image of conventional GFP transduced T-cells. Huge T-cell proliferation clusters can be observed at day 21 of culture after the third round of restimulation. Panel D: Confirmation of foxp3 expression in freshly transduced allo-primed MLCs. Lymphocytes were harvested 3 days after transduction with the Foxp3-IRES-GFP retrovirus or a GFP control vector and FACS sorted for GFP positive cells. Foxp3 transcription factor expression was detected by intracellular staining.
Figure 3.3: Representative FACS images of Foxp3-IRES-GFP transduced mixed lymphocyte cultures under antibiotics selection. Samples were taken on day 7 before the first restimulation, on day 17 after the second restimulation and day 23 after the third restimulation. FACS plots show the advancing decay of MLCs and the loss of GFP positive cells. Note the accumulation of non-viable GFP+ cells in the upper gate and the presence of GFP negative viable cells in the lower left gate.

Ultimately the approach of retroviral modification of primary rat T-lymphocytes was abandoned.

3.2 Anti-CD4 treatment of alloantigen-specific rat T-lymphocytes

To investigate the in-vitro effect of anti-CD4 treatment of allo-stimulated CD4+ T-cells an antibody titration experiment was devised. RIB5/2 was added to the T-cell cultures directly and intracellular foxp3 was measured on day 4 and 6. Addition of exogenous recombinant interleukin-2 was included as an additional parameter. The results in figure 3.4 demonstrate that the addition of anti-CD4 antibody significantly increases the percentage of foxp3 positive cells in allo-MLCs. On day 4 approximately 40% of all viable CD4+ cells expressed the transcription factor. That frequency was further increased by day 6. A dose dependent effect could not be observed and adding IL-2 did not significantly alter the outcome of the experiment. Microscopic evaluation of the T-cell cultures however revealed that high concentration of RIB5/2 significantly inhibited the proliferation of allogeneicly stimulated cells (data not shown). At 1µg/ml virtually no activated T-lymphoblast could be observed. Extended culture periods past day 6 or restimulation of MLCs at day 7
Figure 3.4: Summarised results for foxp3 detection in anti-CD4 treated of allo-antigen stimulated rat CD4+ T-cells. Bar diagram: Data resulting from RIB5/2 titration experiments. The effects of varying concentrations of anti-CD4 antibody and the addition of exogenous IL-2 were measured. Grey bars indicated mean values; black dots are individual measurements. FACS plots: Representative example for the data acquisition. Data shown generated from MLCs cultured for six day in the presence of rh-IL-2. n=3-4
Figure 3.5: Representative FACS images of CFSE labelled CD4+ BN T-lymphocytes cultured in the presence of allogeneic thymic dendritic cells. Cultures were treated with either 0.1\(\mu\)g/ml RIB5/2 antibody or 1\(\mu\)g/ml isotype control. Samples were analysed on day 6 of culture.

With thymic DCs did not further increase the frequency of regulatory T-cells. On the contrary, the percentage of foxp3 positive cells declined rapidly either due to complete decay of the cell culture at high RIB5/2 concentrations or by outgrowth of conventional T-cells at low concentrations of anti-CD4 antibody (data not shown). An interesting side observation is that anti-CD4 treatment of rat MLCs seemed to diminish the amount of CD4+ protein on T-lymphocytes (see FACS plots in fig. 3.4).

To confirm the hypothesis that anti-CD4+ treatment does result in the generation of allo-antigen specific T\(_{\text{regs}}\) CFSE labelled CD4+ T-cells were cultured in the presence or absence of RIB5/2. The results of these experiments are presented in figure 3.5. Two important observations could be made. Firstly, in control treated cultures it was observed that foxp3+ T-cells proliferate in parallel to conventional T-cells. Both populations underwent at least six cell divisions. Secondly, in anti-CD4 treated cultures the growth of conventional T-cells is markedly suppressed while T\(_{\text{reg}}\) still underwent cell cycling. However, it should be noted that also a substantial number of foxp3 positive cells did not proliferate in RIB5/2 cultures.
3.3 Cell surface marker analysis of rat regulatory T-cells

To better understand the nature of rat regulatory T-lymphocytes and to unravel the effect of anti-CD4 antibody treatment, a FACS analysis was conducted. Allo-antigen stimulated MLCs were cultured for seven days in the presence of RIB5/2 or mouse IgG2a isotype control. Subsequently, harvested cells were analysed for the expression of 12 cell surface markers. Furthermore, the data was contextualised with the cell surface marker profile of naïve, freshly isolated rat T-lymphocytes. The results are summarised in table 3.1 and a selection of cell surface marker stainings is shown in figure 3.6. The data indicates that RIB5/2 treatment significantly changed the expression of numerous markers or the frequency of cells expressing a certain cell surface antigen. In particular, anti-CD4 treatment significantly decreased the expression of co-stimulatory molecules CD28, ICOS and CTLA4 in both T\textsubscript{reg} and T\textsubscript{conv}, compared to isotype cultures. Additionally CD134 was attenuated in foxp3\textsuperscript{+} cells in RIB5/2 cultures. Most striking however, is the difference between T\textsubscript{regs} and T\textsubscript{conv} in anti-CD4 treated MLCs. For CD8, CD25, CD134, ICOS and CTLA4 foxp3\textsuperscript{-} cell showed a much lower staining intensity then foxp3 positive cells. In freshly isolated lymphocytes the frequency of T\textsubscript{regs} was approximately 3\% (n=3) and the cell surface phenotype was charachterised by high expression of CD25, CD134, ICOS and CD73. Compared to conventional naïve CD4\textsuperscript{+} T-cells, foxp3\textsuperscript{+} cells additionally showed increased levels of CD28, CD103, CD69 and CD62L. On the one hand regulatory T-cells in activated MLCs (isotype control) displayed a noticable increase in CD25, CD28, CD62L, CD134 and ICOS expression. On the other hand, the expression of CD44h, CD103 and CD69 was slightly reduced in activated T\textsubscript{regs} compared to naive foxp3\textsuperscript{+} lymphocytes.
Figure 3.6: Representative FACS histogram plots of selected cell surface markers analysed on T\textsubscript{reg} and T\textsubscript{conv} harvested from anti-CD\textsubscript{4} treated allogeneic MLCs and isotype control cultures. Results for extracellular CD25, CD134 and CD8 are shown as well as intracellular detection of CTLA4.
Table 3.1: Summarised results for the phenotypical characterisation of rat regulatory T-cells. Numbers given are an average ± SD of three independent measurements. All samples were multicolour stained with anti-CD4 and intracellular anti-foxp3 and one additional cell surface antigen. Tregs were identified by gating on foxp3 positive cells and compared to foxp3 negative CD4+ helper T-cells. Brown Norway RIB5/2 and isotype control treated lymphocytes were harvested after seven days of culture. Naive T-cells were freshly isolated from 8-12 week old animals.

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3.4 Establishment of a rat model for cornea transplantation

3.4.1 Selection of surgical animals

During the implementation of the cornea transplant procedure for the LEW-DA strain combination it was noted that some LEW rats designated for surgery showed ocular abnormalities. Concerned that these abnormalities constituted a confounding factor for transplant survival a screening program for all animals was initiated. In the course of the screening it was observed that almost all LEW animals showed some sort of imperfection of the eye. Example images are compiled in figure 3.7. Abnormal features with a high frequency of occurrence were an uneven corneal surface [fig. 3.7 D], blood vessels originating from the pupil margin extending into the anterior chamber [fig. 3.7 D], oval pupil and diminished pupil reflexes (miosis) in response to a strong light source [fig. 3.7 C]. Particularly troublesome was the observation of corneal impurities best described as pigments or deposits, which significantly reduced the transparency of the cornea [fig. 3.7 C]. For comparison other albino rat strains were scrutinized and similar ocular defects were observed in Sprague Dawley and Fisher F334 rats. In summary the eye of the LEW rats appeared immature and delicate. Furthermore, the surgeon noted that the corneal tissue of LEW animals was very floppy and lacked the rigidity seen in other pigmented rat strains and human tissue. In contrast to LEW rats all pigmented rat strains used (Brown Norway, Dark Agouti, PVG) showed none of the above mentioned abnormalities except for the deposits, which appeared at high frequencies. A representative example of such pigment/deposits can be found in fig. 3.7 B. Due to the high incidence of the ocular defects in LEW rats it was virtually impossible to select flawless animals for surgery. Consequently, only animals with excessive defects were excluded, in particular rats with uneven corneas and extra iris blood vessels. Only pigment free DA corneas were transplanted. For the BN-PVG strain combination only perfectly normal recipient and donor animals were selected for surgery.
Figure 3.7: Images of ocular abnormalities in otherwise healthy laboratory rat strains. Panel A: a healthy BN eye with pronounced iris details (crypts) and iris sphincter muscle visible; panel B: Image of a BN eye depicting the water drop lensing method to highlight corneal focal pigmentation (1); C: LEW eye with strong corneal deposits (2), oval pupil and decreased miosis; D: LEW eye with blood vessels protruding into the anterior chamber (3), an uneven cornea surface with depressions (5) and elevations (4). Magnification of all photographs 12.5-fold.
3.4.2 The Lewis Dark Agouti strain combination

The LEW DA full-MHC mismatch model is a popular transplant system with a rich body of research data available for comparison. Hence it was desirable to establish that strain combination for cornea transplantation. After achieving a 75% graft survival rate in the initiation phase using syngeneic donor corneas, grafting of fully allogeneic DA corneas was started. The condition of grafted corneas was inspected every second day and opacity, neovascularisation and other parameters were recorded and documented. Representative photographic images are compiled in figure 3.8. Several post-surgical complications were noted in transplanted animals. Most frequently anterior synechias were observed and often 50% or more of the pupil margin were adherent to the inside of the wound. These synechia rapidly accelerated the neovascularisation of the graft, which typically reached the transplanted cornea by day 8 or 9 in animals without anterior synechia. The main trigger for neovascularisation appeared to be the suture knots and removing those significantly slowed the development of new blood vessels. Furthermore, synechia coincided with intra-stromal iris bleeding and occasionally with haemorrhaging into the anterior chamber. After initial success in implementing the LEW-DA combination however, the survival rate of transplanted cornea started to decline. Post surgical animals often displayed no sign of wound healing with visible clefts between donor and recipient cornea. Failing grafts displayed high opacity scores (3-4) by day 6, when normal grafts typically showed signs of reduced post-operative opacity. Moreover, failing corneas were oedematous and frequently shedding of the epithelial cell layer was observed. The overall appearance of the eyes did not suggest bacterial infection and treating surgical eyes with artificial tears or antibiotic ointment did not ameliorate the situation. The rate of successfully transplanted corneas dropped far below the acceptable rate of 75% and, due to ethical and practical considerations, the LEW-DA model was discontinued.
Figure 3.8: Photographic images of the LEW-DA strain combination: A-H normal appearance of allogeneic grafts; J-M examples of failing corneal allo-grafts. A - Snapshot of the surgical procedure. B - early post-op animal (day 02) with minimal to moderate opacity (1-2). The arrow (1) indicates an anterior synechia and the distorted shape of the pupil indicates that the iris is not fully mobile and AC is shallow. Also visible is the normal wound oedema. C - Day 04 post op animal with minimal central opacity (OP=1). The arrow (2) indicates anterior synechia. Furthermore, beginning tissue hyperplasia around the suture knots can be detected. No visible neovascularisation. D - Example of a well healed corneal allo-graft (approx. day 10-12) with no opacity centrally and residual opacification around the wound. Sutures were removed on day 08. E - Slightly off centred corneal graft with beginning rejection (OP=1). The arrows indicate new blood vessels extending from the limbus towards the graft. F - Progressing rejection (op=2). Arrows highlight graft invading neovascularisation. Magnification of all images is 12.5×.
Figure 3.8: Cont’d: G - Advanced rejection (OP=3). Picture of the same animal as in F. Note the progression of neovascularisation. H - a fully rejected allo-graft (OP=4). The anterior chamber is no longer visible. The graft is elevated and aggressively neovascularised. J - post-mortem image of a failed corneal graft displaying epithelial shedding and early neovascularisation. K - failing cornea on day 06 (OP=4). The arrow indicates a large iris haematoma. L - lack of wound healing in a failing corneal allo-graft (OP=4). The left arrow shows a rift between donor and recipient tissue. The right arrow indicates coagulated blood, possibly originating from bleeding into the AC. Also visible is a halo of new blood vessels growing from the limbus towards the graft. M - a failed allo-graft (op=3) with shallow AC, irregular pupil and possible intra-stromal iris haemorrhage. Note the particular paleness of the eye. Magnification of all images is 12.5×.
### 3.4.3 The Brown Norway - PVG strain combination

In order to pursue the main goal of this study and due to the failure of the LEW-DA strain combination a new model for rat cornea transplantation had to be established. Based on the screening results of various rat strains (see chapter above) the following two rat strains were chosen. The Brown Norway rat (BN RT1n) served as graft recipient while the PVG rat (RT1c) was the designated cornea donor. Like the LEW-DA pairing, a combination BN-PVG constitutes a full allogeneic MHC mismatch model. After testing the suitability of BN animals for transplantation using syngeneic grafts (n=4), which were all accepted and healed completely, grafting of allogeneic corneas was initiated. The BN-PVG combination proved to be an immediate success. No major complications in post-op animals were observed and the overall drop-out rate was very low. The anterior chamber reformed quickly and eye lobes regained their spherical shape usually. Anterior synechias were still frequent, however the severity of which was much less pronounced than in the LEW-DA model. Typically only one spot of the iris was adherent to the graft and the mobility of the iris was not strongly affected. Occasionally synechia resolved spontaneously. The scoring method for opacity scores had to be slightly modified due to the pigmentation of the BN rat (see M&M page 35). However, detection of opacification was greatly facilitated by the dark background of a pigmented iris. Representative photographic images for the BN-PVG model including corneas with various degrees of cloudiness are summarised in figure 3.9.
Figure 3.9: Representative images of allo-grafted Brown Norway rats. A - example of a well healing corneal allo-graft (OP=0) before rejection, with residual opacity around the wound edge. B - a completely healed cornea (OP=0). Opaque patches around the wound and knots result from scar tissue. C - beginning of graft rejection (OP=1) Example of uniform haziness of the graft. Note the circumferential neovascularisation originating from the limbus. D - early rejection (OP=1 central) with strong focal opacity. The dashed arrow indicates the direction of rejection. Arrow 1 points to an anterior synechia. Arrow 2 highlights strong hyperplasia and neovascularisation around a suture knot triggering the rejection. E - example of post-op focal opacity at day 04 (OP=1-2). Note the still dilated pupil and wound oedema. F - another example of post-op focal opacity at day 04 (OP=1-2). Arrows indicate fibrin fibres in the anterior chamber a common feature of surgical trauma. Magnification of all images is 12.5×.
Figure 3.9: Cont’d:  

G - a fully rejected allo-graft (OP=3) with $360^\circ$ neovascularisation invading the cornea.  
H - a rejecting cornea (OP=2-3) with arrows indicating an endothelial rejection line seen occasionally.  
J - full rejection of a PVG cornea (OP=3-4). The arrow denotes an epithelial rejection line occasionally observable.  
K - appearance of a late rejecting cornea (OP=2-3) at day 26 with little neovascularisation involved.  
L - slit lamp image of K. Note the swollen cornea and the diffuse stromal opacity.  
M - slit lamp photograph of a fully healed cornea for comparison. Magnification of all images is 12.5×.
3.4.4 Rejection kinetics of LEW-DA and BN-PVG corneal allo-grafts

The most important parameter of every cornea transplant model is, of course, the time point of rejection, which is reached, according to conventional definition, when an allo-graft displays an opacity score of greater than three. Typically, survival rates are displayed using Kaplan-Meier survival plots, which can be found in figure 3.10 for both strain combinations used in this study. The rejection kinetics of LEW-DA and BN-PVG are strikingly different. While in LEW rats the onset of rejection is rapid and the time window for rejection narrow (min day 12; max day 16), BN animals are slow rejecters (min day 13, max day 27). Additionally, approximately 25% of all grafted BN rats spontaneously accept a PVG allograft. In contrast to LEW rats, which score maximum opacity quickly, BN rats exhibit delayed progress of rejection. Quite often many days passed between the observation of the first signs of an immune response and the arrival at the cut-off criteria (OP ≥ 3). An opacity score of four was rarely observed for BN recipients. Figure 3.4 also contains the survival curves for LEW rats originating from two different breeding colonies. Due to delivery shortages of one breeder, the supplier of LEW recipients had to be changed in the middle of an ongoing study. The baseline time point of rejection was re-established and it was noted that the new LEW rat strain rejected even more rapidly. The difference between both LEW -survival curves is statistically highly significant using both log-rank ($\chi^2 = 7.9; p = 0.005$) and Wilcoxon test ($\chi^2 = 8.2; p = 0.004$).
Figure 3.10: Summary of the non-parametric distribution analysis of allo-graft survival. The data displayed is right censored at day 42 - the end of the observation period. The rejection kinetics for LEW rats from two different sources is shown. CRL - breeding colony at Charles River Laboratories - UK, n=8; HARLAN - animals bred by Harlan UK LTD, n=8. The difference between LEW recipients is statistically significant at p=0.005 level using a log-rank test. Additionally, survival rates for the fully allogeneic BN-PVG (n=15) combination are given. Four allo-grafted BN recipients spontaneously accepted a PVG cornea (26.6%). Data from syngeneically grafted BN animals is given for comparison (n=6).
3.5 Comprehensive analysis of BN-PVG transplanted animals

3.5.1 Quantification of lymphocytes in draining lymph nodes

In order to better understand the novel strain combination of Brown Norway graft recipients and PVG cornea donors, a series of FACS analysis experiments was conducted on transplanted animals. The cellular composition of the draining ipsi-lateral submandibular lymph node (i-SM) was studied and compared to the contra-lateral LN (c-SM) or a distal brachial lymph node (d-Br), respectively. A photographic image of the draining submandibular lymph node in situ can be found in figure 3.15 on page 65. Using multi-colour flowcytometry the following lymphocyte populations have been analysed: CD4+ and CD8+ T-cells, B-cells, NK as well as NK-T-cells. The analysis was performed on actively rejecting graft recipients (allo-Tx-rej) and early allo- and iso-graft recipients (allo-Tx-d7; syn-Tx-d7). To determine the baseline values for the lymphocyte distribution, syngeneic long term survivors were included in the study (synTx-LT). Results and examples of FACS plots are displayed in figure 3.11. Actively allo-rejecting animals were dissected and it was observed that the ipsi-lateral submandibular lymph node was swollen and discoloured (see figure 3.15 A as an example). From the data presented in figure 3.11 one can deduce that the submandibular LNs in general have a strikingly different composition than distal brachial LNs. In cervical LN, B-cells were found to be the dominant population in all study groups accounting for roughly 50% of all isolated cells. Elevated cell numbers were found consistently in the draining LN (i-SM) of transplanted animals compared to the contra-lateral side due to swelling, although no discernable difference between the groups alloTx-d7, alloTx-rej and syn-Tx-d7 could be detected. From that it can be concluded that changes in lymphocyte composition are largely a result of the surgical trauma and the local inflammation that ensued afterwards, regardless of the MHC status of the graft. Concomittant to the analysis of the major lymphocyte compartments, the data for minor lymphocyte populations is shown in figure 3.11. A small reduction in frequency of CD4+ CD8+ double positive T-cells was observed in allo-recipients (allo-Tx-d07 and allo-Tx-rej) and syngeneically grafted animals on POD-07 compared to syngeneic long term survivors. Furthermore, a small increase in NK cell percentages was noticable in actively rejecting study animals (arrows in fig. 3.11).
Figure 3.11: Panel A: Representative FACS plot of CD4+ CD8+ staining used to count T-helper cells, cytotoxic T-cells and CD4+ CD8+ double positive T-lymphocytes. Events acquired: $2 \times 10^5$. Panel B: FACS plot example for B-cell detection. Panel C: Representative FACS plot for NK cell assessment. NK-T cell were confirmed by CD3 expression (not shown). Bar diagrams: Cumulative results for the quantification of major and minor lymphocyte populations in draining LN of cornea transplanted animals. An asterisk (*) indicates statistical significance at $p \leq 0.05$ determined by Mann-Whitney U-Test. Allo-Tx-d7 - animals allo-grafted and analysed at day 07 post op, n=6; allo-Tx-rej - animals displaying allo rejection of grafted corneas analysed after the onset of rejection, n=5; syn-Tx-d7 - syngeneicly grafted animals analysed at day 7 post-op, n=3; syn-Tx-LT - syn-grafted long term survivors analysed at the end of the observation period at day 42; n=3.
3.5.2 T-cell activation marker analysis

In order to identify T-cell activation characteristics for an allo-graft rejection T-lymphocyte sub-populations were screened (CD4+ SP and CD4+CD8+ DP) for the expression of CD25 and CD134 (Ox-40). The outcome was compiled in figure 3.12. Comparing iso-grafted long term survivors with early post-op groups (day 07) and rejecting animals revealed a modest increase in CD25 and CD25+CD134 DP expression in CD4+ single positive cells for the latter three groups. CD134 cell surface density was markedly elevated in draining LN (i-SM) of alloTx-d7 rats compared to actively rejecting animals. However, the same relative increase in CD134 expression is observed in iso-grafted control animals analysed on day 7 (synTx-d7) (fig. 3.12 upper bar diagrams). As a result of the swelling of draining LN absolute numbers of activated T-cells (CD25+, CD134+ and CD25/CD134 DP) were increased compared to quiescent LN of syngeneic long term survivors.

Using the same T-cell activation markers the small sub-population of CD4+CD8+ double positive T-cells was analysed. It was observed that allo-graft recipients (allo-Tx-d7 and allo-Tx-rej) harboured significantly more CD25 positive cells (figure 3.12 lower bar diagrams) than iso-grafted rats. Interestingly elevated CD25 expression was observed in all analysed lymph nodes of allo-graft recipients. Additionally, syngeneic long term survivors displayed the lowest frequency of CD25/CD134 double positive cells. In general CD4+CD8+ DP T-cells did not express CD134 without simultaneous expression of CD25, indicating a high activation status.

Figure 3.12: Panel A: Representative FACS plot of activation marker expression on CD4+ single positive T-helper cell in draining LN. Events acquired: $2 \times 10^5$. Panel B: Example of CD25 and CD134 expression pattern on CD4+CD8+ double positive T-cells. Bar diagrams: Cumulative results for the quantification of T-cell activation status of helper T-lymphocytes. An asterisk (*) indicates statistical significance at $p \leq 0.05$ determined by Mann-Whitney U-Test.
3.5.3 Graft infiltrating lymphocytes

To advance the comprehensive analysis of the BN-PVG rejection process, lymphocyte populations which infiltrated the allograft (figure 3.13) were studied. For that purpose a gentle collagenase digestion procedure was designed and isolated viable cells were subjected to flowcytometric analysis. Six distinct cell types infiltrating a rejecting graft could be detected. It was found that CD4+ T-cells represented the largest single faction, constituting a quarter of all GIL. Approximately 50% of CD4+ cells were CD25 positive and a small subpopulation expressed MHC-class 2 molecules (fig. 3.13 F). CD4- MHC-2+ positive cells were observed, but could not be specified further. Additionally, using an anti-CD3/CD8/CD161 antibody combination, CD8+ cytotoxic T-cells as well as CD8+ CD161<sup>dull</sup> NK-T-cells and CD8<sup>+</sup>/CD161<sup>high</sup> CD3<sup>-</sup> NK cells were observed (fig. 3.13 E,G). The latter two cell-types constituted almost 50% of all infiltrating lymphocytes. Expression of CD25 by CD8+ cells was generally low (fig. 3.13 H). Whether CD8+ CTL or NK-T-cells differentially express CD25 could not be determined with the staining approach used. Moreover, a large granular cell type with CD161<sup>dull</sup> expression was detected. Those cells were not CD8 or MHC-2 positive (determined by back-gating strategy) and could already be detected at day 7 in allogeneic and syngeneic grafts (fig. 3.13 A,B).
Figure 3.13: FACS plots - Panel A + B: Allo-grafted cornea analysed at day 7 post-op. A) Forward-sideways scatter morphology of graft infiltrating cells. B) Representative image of CD161$^{\text{dull}}$ expression on LGL. No other cell type could be detected. Recorded events: $2 - 5 \times 10^3$. Panel C-H: FACS results of allo-rejecting corneas: Events acquired: $5 \times 10^3 - 3 \times 10^4$ per sample. C) FSC-SSC morphology of GIL. D) Detection of T-lymphocytes. E) Measurement of CD8 and CD161 NK markers on GIL. F) Sub-characterisation of CD4$^+$ T-cells and MHC-2 detection. G) Expression pattern of CD3 from populations gated in panel E. H) Measurement of CD25 T-cell activation markers on populations gated in panel D. Cut-off or positive CD25 expression was determined by performing isotype FMO samples on lymphocytes of draining LN from the same animal. For scaling reasons data is not shown in the histograms. Bar diagrams - summary of all lymphocyte specimen identified in rejected corneas (left diagram) and appropriate sub-characterisations (right diagram) n=5.
3.5.4 Serum allo-antibody measurement in grafted animals

To conclude the comprehensive analysis of the allo-graft rejection process in the BN-PVG keratoplasty model, the serum was screened for the presence of anti-donor immune-globulins. Serum harvested post-mortem from transplant recipients was incubated with donor splenocytes and and direct binding of allo-antibodies was visualised with fluorescently labelled anti-rat IgM, IgG1, IgG2a and IgA antibodies. Positively stained target cells were detected by flowcytometry. All surgical groups were included along with serum from animals with spontaneously accepted PVG-allografts. Positive control allo-anti-serum was generated by immunising BN rat with γ-irradiated PVG thymic dendritic cells.

As the results in figure 3.14 show, evidence was found for the presence of anti-PVG IgM, IgG1, and IgG2a immunoglobulins in allo-rejecting animals. At a serum dilution of 1:5 allo-transplanted animals analysed at POD-7 or graft acceptors did not exhibit anti-donor antibody responses. Similarly, anti-PVG antibodies could not be detected in iso-grafted cornea recipients.
Figure 3.14: Bar diagram - summary of allo-antibody screening in transplanted animals using a FACS based direct detection method. The presence of IgG1 infers a Th2 response, while the presence of IgG2a antibodies indicates a Th1-INF-γ induced class switch in B-cells. For IgM detection IgM+ B-cell were excluded from analysis by CD45RA staining (see fig. 3.11); IgA isotypes could not be detected. FACS plots - Display of the measurement method for direct binding of allo-antibodies to allogeneic target cells (PVG donor splenocytes); negative controls: serum from PBS injected animals harvested after day 14, positive controls: serum from animals injected with $1 \times 10^6$ γ-irradiated thymic dendritic cells collected after day 14.
3.6 In-vivo application of GFP transduced allo-specific T-cells

The original study design envisaged the adoptive transfer of GFP positive regulatory T-cells generated by retroviral transduction. Conventional allo-specific T-cells expressing GFP were designated as control cells. Despite the lack of foxp3 transduced T-cells, the negative control arm of the study was initiated. GFP positive conventional T-cells were harvested on day 2 after the second restimulation, ficoll purified and injected into LEW allo-graft recipients one day before surgery ($5 \times 10^6$ cells, n=4 total). One animal was sacrificed 24 hours after surgery and screened for GFP positive T-cells using flowcytometry. As expected, GFP transgenic T-lymphocytes were found in the lung, albeit at low numbers (109 GFP cells per $10^6$ recorded events). All remaining animals rapidly rejected their allo-grafts by day 6. Upon dissection, the ipsi-lateral lymph node was found to be swollen and discoloured (fig. 3.15 A). Flowcytometric analysis revealed the presence of GFP positive T-cells in the draining LN (fig. 3.15 C-D). The highest proportion of GFP cells was found in the ipsi-lateral LN ($1449/10^6$). The ipsi-lateral cervical and the contra-lateral LN harboured significantly less GFP transgenic cells ($449/10^6$ or $453/10^6$ respectively). Animals analysed at later time points (day 10 and day 13) showed markedly reduced numbers of GFP positive cells in the draining LN (<100/$10^6$, data not shown) to the point where the detection became very difficult. Analysis of spleens remained inconclusive, due to the high autofluorescence back ground. Unfortunately, the results could not be confirmed, due to the subsequent failure of the LEW-DA transplant model.
Figure 3.15: Homing behaviour of allo-antigen specific GFP transduced T-lymphocytes. Unpurified GFP positive T-cells \((5 \times 10^6)\) were adoptively transferred into corneal allo-graft recipients (LEW-DA combination) 24 hours before surgery. On the day of rejection (day 6) animals were dissected and draining LN screened for the presence of GFP expressing cells. Panel A: Photographic image of swollen ipsi-lateral submandibular lymph node of rapidly rejecting LEW recipients. Panel B: FACS analysis of ipsi-lateral sub-mandibular LN. Panel C: Ipsi-lateral cervical LN (below the submandibular LN). Panel D: Measurement of the contra-lateral LN (submandibular and cervical pooled).
3.7 In-vivo application of regulatory T-cells

![FACS plots demonstrating the high throughput flowcytometric cell sorting strategy to purify T-cells from allogeneic MLCs for in-vivo application. Panels A-C: Gating approach A: single cell gate to eliminate cell aggregates; B: morphological lymphocyte gate to exclude non-viable and excessively large cells; C: CD3 positive gate to sort T-cells. Arrows indicate gating hierarchy.](image)

To test the major hypothesis that allo-antigen specific T\textsubscript{reg}\textsuperscript{s} can prevent corneal rejection an in-vivo experiment was designed using the BN-PVG strain combination. Regulatory T-lymphocytes generated by anti-CD4 treatment of allo-MLC were adoptively transferred into corneal allo-graft recipients 24 hours before surgery. The time point of rejection was registered, Kaplan-Meier survival plots were constructed and the results are presented in figure 3.17. At first unpurified T\textsubscript{reg}\textsuperscript{s} were administered. These cell preparations potentially still contained viable PVG allo-stimulator cells, since only dead cell removal was performed using a ficoll gradient. Animals treated with unpurified T\textsubscript{reg}\textsuperscript{s} rapidly rejected their allo-grafts (n=3, median= day 09) and that approach was discontinued. A T-cell purification strategy was devised, which consisted of flowcytometric sorting of CD3+ cells. The approach is demonstrated in figure 3.16. Allo-graft recipients injected with purified cell preparations rejected significantly later (median= 22 days) then surgical animals treated with unpurified cells. However, compared to the PBS treated control group no prolongation of allo graft survival could be observed in T\textsubscript{reg} treated animals. Although both survival curves differ sightly and median survival times are unequal, the difference was not statistically significant (log-rank test: $\chi^2 = 1.04; p= 0.308$; Wilcoxon-test: $\chi^2 = 1.57; p = 0.21$; excluding long term survivors). When considering the fact that 26% of PBS injected allo-graft recipients spontaneously accepted the graft, the performance of the T\textsubscript{reg} FACS purified study group is in fact poorer.
As a matter of completeness the survival times of another study group are shown in figure 3.17. Conventional T-cells harvested from restimulated allogeneic MLCs were ficoll purified and injected. Similar to the unpurified T\(_{\text{reg}}\)-group, animals in that study section rejected rapidly (n=4, median= 9 days). However, the tissue culture approach used to generated those cell could not be adopted for anti-CD4 cultures, hence the data is not comparable. Due to the failure of anti-CD4 treated cell to prolong allo-graft survival a proper negative control group using conventional T-cells was omitted.

Figure 3.17: Kaplan-Meier plot of survival characteristics of allo-grafted animals receiving anti-CD4 generated regulatory T-cells and controls. Survival times of T\(_{\text{conv}}\) treated animals are not comparable to the T\(_{\text{reg}}\) groups, since they were generated with a different culturing protocol (data is shown for the sake of completeness). The difference in survival time between PBS injected control group and T\(_{\text{reg}}\) treated animals is not statistically significant (log-rank or Wilcoxon test). Legend: allo PBS - sham injected control group, n=15, \(t_{\text{rej-median}}=16\) excluding long term survivors; T\(_{\text{reg}}\) FACS purified - CD3 sorted RIB5/2 cultures, \(1.5 - 2.5 \times 10^6\) cells injected, n=9, \(t_{\text{rej-median}}=22\); T\(_{\text{reg}}\) unpurified - T-cell from RIB5/2 cultures obtained by ficoll gradient, n=3, \(t_{\text{rej-median}}=9\); T\(_{\text{conv}}\) unpurified - conventional allo-antigen specific CD4\(^+\) T-cells harvested after at day 02 after restimulation, ficoll purified and \(5 \times 10^6\) cell injected, n=4, \(t_{\text{rej-median}}=9\); syn BN - syngeneically grafted control animals, n=9.
4 Discussion

4.1 Retroviral transduction of primary allo-specific rat T-cells

The first challenge to this project was the establishment of a stable allogeneic mixed lymphocyte culture system. A recombinant retrovirus system was obtained and used subsequently to ectopically express the transcription factor \textit{foxp3}. Since Moloney based retroviruses require actively dividing cells to integrate into the host genome controlled T-cell proliferation conditions are absolutely crucial for successful transductions. A reproducible protocol was finally achieved by culturing CD4+ MACS sorted responder T-cells in the presence of thymic derived stimulator dendritic cells. Dendritic cells isolated from other lymphoid organs such as spleen and lymph nodes proved to be poor allo-stimulators (data not shown). A suitable transduction protocol was established by the author in a previous project [103] and some further optimisation were performed by lowering the viral incubation temperature to 32°C (see figure 3.2 on page 39). Despite low transduction frequencies retroviral gene transfer and short term expression of the therapeutic gene and long term expression a GFP marker in control T-cells could be achieved. However, selection by antibiotic G-418 and expansion of ex-vivo generated regulatory T-cells was not possible. The restimulation procedure appeared to be the vulnerable point of the protocol as this procedure would fail at a high rate. Various experimental modifications were tested of which only the addition of exogenous IL-2 was found to have an significant impact on cell survival after restimulations while for example murine T-cell growth factors - a crude supernatant from ConA stimulated Balb/c mice - had no significant effect contrary to literature reports [104, 105]. Alternatively, instead of using allogeneic stimulator cells for restimulation, polyclonal activation using anti-CD3/CD28 antibodies could have been utilised. The possibility was considered but rejected due to concerns that a single round of stimulation would not generate sufficiently pure populations of allo-antigen specific T-cells.
Only at a later stage of the project it was discovered that autologous serum was absolutely essential for productive MLC restimulations (data not shown). Due to the acute shortage of autologous serum its addition to allogeneic MLCs was restricted to the initial priming phase and could not be maintained for the entire T-cell culture period. By the time the discovery was made the retroviral gene transfer approach was already abandoned due to its ineffectiveness. For future projects it is thus recommended, although expensive, to commercially obtain rat serum and to pool and batch test self-made autologous serum.

Time considerations and security concerns lead to the choice of an existing ecotropic recombinant retrovirus system, despite the known disadvantages of low viral particle titers. Although an already optimised transduction protocol was used, not all possible improvements have been tried. For example a novel precipitation method was developed which allows the concentration of retroviruses up to a 1000-fold by means of polybrene flocculation [106]. This approach might have significantly increased the transduction frequencies consequently facilitating the selection and study of gene-modified cells. Additionally, the temperature at which retrovirus production was performed could have been lowered to 32°C as well, a methodical step which is reported to increase retroviral titers [107]. However, a shortage of incubation capacity at the laboratory precluded the implementation of this approach.

In addition to the frequent inability to expand retrovirally transduced T-cell cultures long term MLCs were occasionally overgrown by GFP negative T-cells despite continued antibiotic selection. That peculiar observation can be explained by the silencing of the retroviral promoter. The therapeutic gene expression cassette of the recombinant retroviruses used in this project was driven by the 5’ LTR endogenous promoter whereas the antibiotic resistance gene Neo(R) was under the control of a SV40 promoter. It is thus possible that foxp3 and GFP expression were abolished while the neomycin resistance conferring gene continued to be expressed by gene-modified T-lymphocytes. Anti-retroviral defence mechanisms are ubiquitous in mammalian cells [108, 109] and it is no surprise to observe that effect in primary rat T-cells. In the emerging field of inducible stem cell technology rapid silencing of retroviral promoter by dividing cell clones is even part of the essential experimental protocol to generate iPS [110, 111]. The effect could be prevented by using a T-cell specific promoter instead.

To mitigate both problems described above, different gene therapy vectors could
have been used in place of a Moloney leukaemia recombinant virus. In mice, the Murine stem cell virus back bone MIGR1 was utilised successfully for the forced expression of \( \text{foxp3} \) in primary T-cells in several studies [5, 112]. For human applications last generation lenti-viral vectors are commonly used to gene transfer \( \text{FOXP3} \) into CD4+ T-lymphocytes [52, 53, 15]. Both avenues, the cloning of a novel back bone or the establishment of a lenti-viral transduction system are time consuming and not entirely free of disadvantages. In particular the transient production of gene vectors using a lenti system is not ideally suited for preparative large scale transductions. Consequently, both approaches were not taken. In retrospect, it was prudent to try the established vector system, since it was used successfully in previous studies of GFP transduction into primary rat T-cells [113, 114, 115]. Hence the difficulties with \( \text{foxp3} \) gene transfer could not be anticipated. Nevertheless, due to its inefficiency the retroviral approach was discontinued.

### 4.2 Generation of T\(_{\text{regs}}\) by anti-CD4 treatment

The non-depleting mouse anti-rat CD4 antibody RIB5/2 has a long standing history of successful application in solid organ transplantation. Systemic administration of RIB5/2 completely abrogated allo-graft rejection in high responder liver, gut, kidney and heart transplant models [116, 117, 118, 119, 120]. Additionally it has been demonstrated that peri- and post surgical injections of anti-CD4 antibody prolonged corneal allo-graft survival [121, 122]. Most of these experiments were conducted before the discovery of \( \text{foxp3} \) expressing regulatory T-cells thus its precise mode of action remained elusive. It was speculated that RIB5/2 influenced the cell cycle, induced unresponsiveness and blocked post transcriptional cytokine synthesis [123]. The research was revigorated when Sawitzki et al. discovered that a similar anti-mouse antibody YTS-177 when added to allogeneic MLCs resulted in a high frequency of \( \text{foxp3} \) expressing T-lymphocytes [124]. As has been shown in the results section 3.2 on page 40 RIB5/2 displays the same properties. Its mode of action is however much more profane than initially hypothesised. A strong anti-proliferative effect and significant cell loss was observed in anti-CD4 treated cultures. Furthermore, Sawitzki et al. found that RIB5/2 treated cultures contained cells with a high expression of BAG-1, a protein with anti-apoptotic properties [125, 126]. Regulatory T-cells are known to be highly apoptosis resistance, thus it can be postulated that RIB5/2 induces programmed cell death in freshly primed and naive cells while
sparing apoptosis resistant cells such as T<sub>regs</sub>. The postulate is further corroborated by the observation that addition of IL-2, a cytokine well known to enhance T-cell survival [127] to anti-CD4 treated cultures abrogates the enrichment of foxp3+ T-cells [124]. Evidence for de-novo induction of foxp3 in anti-CD4 treated MLCs was not found (see figure 3.5). It can be concluded that RIB5/2 simply allows selective outgrowth of regulatory T-cells. Nevertheless, the anti-CD4 antibody RIB5/2 represents a highly valuable research tool to pre-select allo-antigen specific T<sub>regs</sub> in vitro.

### 4.3 In-vitro T<sub>reg</sub> suppression assays

Several attempts were made in this project to demonstrate the capacity of ex-vivo generated T<sub>regs</sub> to suppress allo-specific immune responses in-vitro. Experiments were performed according to published protocols. However, a consistent data set could not be produced. Primarily suppression assays failed due to the impurity of T<sub>reg</sub> preparations from RIB5/2 cultures. As has been demonstrated in figure 3.4 anti-CD4 treated MLCs still contained significant numbers of foxp3 negative lymphocytes. For these non-foxp3 cells the cellular composition of a suppression assay constitutes a restimulation experiment. Upon re-encounter with allogeneic stimulator cells such cells start to proliferate rapidly and vigorously. Naive CFSE labelled responder T-cells added as targets for suppression are at a significant disadvantage due to their inherent lag-phase, during which they were simply out-competed with regard to APC binding sites and nutrients. Suppression assay cultures were routinely overgrown by conventional T-cells. The effect was indeed so strong that CFSE-labelled target cells could hardly be detected by FACS on day 4 of culture (data not shown).

Typically T<sub>reg</sub> suppression assays are performed on freshly isolated CD25<sup>high</sup> purified cells not on pre-cultured lymphocytes. When cultured regulatory T-cells are used an sorting strategy that ensures a 90%+ purity is typically implemented. For example Oliveira et al. sorted CD62L+ CCR-7 positiv T<sub>regs</sub> from mouse anti-CD4 treated cultures and successfully performed suppression assays [47]. However, that approach could not be adopted in this study for two reasons. Firstly, no commercial anti-CCR7 antibody exist for flowcytometry in rats and chemokine receptor expression of rat T<sub>regs</sub> is unknown. Secondly, as demonstrated in table 3.1 conventional T-cells in rat anti-CD4 treated cultures still expressed CD62L at a high level.
Thus, a CD62L sort would not eliminate the contamination of $T_{\text{conv}}$ from regulatory T-cells preparations. Based on the cell surface phenotype of anti-CD4 generated Tregs presented table 3.1 a stain combination of CD8, CD25, CD134 is concivable to FACS sort regulatory T-cells from RIB5/2 cultures. Again this strategy could not be implemented due to practical reasons. The suppression assays as performed in this study already took 12 hours to set up. Time for an additional flowcytometric sorting step was not available. At least two people working simultaneously are required for this approach.

Suppression assays are a common tool to study regulatory T-cells properties. However, the artificiality of these in-vitro experiments is recognised and in-vivo experiments are usually required to unequivocally demonstrated suppressive capacity of $T_{\text{regs}}$. An in-vivo suppression assay design shall be presented thereafter which could potentially circumvent the problem of impure regulatory T-cell preparations. A small inoculum of viable allogeneic dendritic cells is injected subcutaneously into the the foot paw of naive animals. Simultaneously a high cell number ($1 \times 10^7$) of naive syngeneic CFSE labelled T-cells is injected intravenously which will serve as responder T-cells. Allo-antigen specific regulatory T-cells are injected on the same day or 24 hours before. Donor derived dendritic cells are expected to migrate into the draining lymph nodes which are well defined for the foot paw. After 4-6 days the popliteal and inguinal ipsi-lateral lymph nodes are excised and analysed by flowcytometry for signs of proliferation of CFSE labelled cells. Allo-antigen specific T-cell suppression will be demonstrated if CFSE dye dilution is lower in $T_{\text{reg}}$ treated animals compared to $T_{\text{conv}}$ injected subjects. This proposed assay is essentially a variation of experiments performed frequently with TCR-transgenic T-cells. That experimental design is often applied to study DTH responses and should in principal also work in a allogeneic setting. The explanatory power of such an in-vivo suppression experiment is much greater than an artificial in-vitro assay.

4.4 The rat cornea transplant model

4.4.1 Comparison between the LEW-DA and BN-PVG model

The establishment of a cornea transplant model was tested on two different strain combination. First the popular high responder LEW-DA combination was tried, due to the rich body of published data on this model. Rejection times between POD
12-16 recorded in this study is well within the range of published data. Interestingly LEW rats from two diverse breeding colonies rejected DA allo-grafts at different median time points. The variation was found to be statistically significant. Such disparate behaviour of laboratory animals is often explained by genetic drift resulting from generation of inbreeding of isolated colonies. Thus it is assumed that LEW rats from different manufacturers are no longer genetically and biologically identical. Instances of genetic drift are occasionally reported in the literature \[128, 129\]. As stated in the results section 3.4.2 on page 48 it was very difficult to properly established the LEW-DA combination, due to a high failure rate. Post-surgical animals would form anterior chambers poorly and develop excessive anterior and possibly posterior synechias. It is speculated that this caused blockage of aqueous humor circulation resulting in increased intra-ocular pressure. The clinical appearance of intra-stromal iris haemorrhage, sloughing of epithelium and wound dehiscence are consistent with a severe glaucoma. For ethical and practical reason the model was discontinued.

Alternatively, a novel strain combination was attempted. Due to delayed rejection kinetics and the occurrence of spontaneously accepted grafts the BN-PVG pairing can be classified as a low-responder model. The keratoplasty model proved to be extraordinarily robust with low post-operative failure rate. This property made it an ideal choice for preclinical studies and for training purposes. Furthermore, the dark pigmented background of the BN iris greatly facilitated the evaluation of transplanted corneas. Even minute opacifications could be detected. The clinical appearance of the rejection process in general does seem to resemble the human situation more closely as was demonstrated by the observation of epithelial and endothelial rejection lines. These typical features of the early rejection phase were never seen in LEW recipients.

Upon researching the subject a strong preponderance of high responder strain combinations was noted in the rat system in contrast to mice. Particularly popular are LEW-DA and LEW-BN allogeneic transplant models. The reasons for this are mostly practical. Since the rejection time point can be narrowly predicted experiments are much easier to plan. There is however no biological foundation for the use of high responder models. Rapid rejection is not typical for allogeneic keratoplasty in humans \[93\]. Moreover, the reasons for fast and uniform rejection with certain strain combinations is not understood. For the LEW rat it can be argued that the reported Th-1 bias of its immune reactions might accelerate graft rejection. Also,
the LEW eye seemed immature and an impaired immune privilege might increase rejection times. Finally, the Lewis rat is reported to be prone to auto-immune diseases which indicates that the immune system of said rat strain is easily disturbed [130, 131, 132].

Adding another low-responder strain combination to the arsenal of possible pre-clinical models for keratoplasty seems a desirable goal. Especially, since the over-reliance of certain strain combinations with their incompletely understood idiosyncrasies might lead to incorrect conclusions about the rejection process in humans and the value of some immune-modulatory strategies to prevent allo-graft destruction.

4.4.2 Exploration of the BN-PVG model

The BN-PVG fully MHC mismatched model constitutes a novel strain combination which has not been used previously. Hence no literature references for graft survival times and data on the immunologic reactions of the rejection process are available. In order to advance the understanding of the new model and the study of corneal allo-rejection in general, a series of post-mortem analysis was conducted on surgical animals.

Analysis of draining lymph nodes  Dissecting actively rejecting allo-graft recipients, it was observed that the ipsi-lateral submandibular lymph nodes were visibly swollen and discoloured. That observation was made in LEW recipients as well as BN animals after penetrating keratoplasty, indicating a pronounced immune reaction. The critical role of cervical LN in the allo-rejection mechanism has been demonstrated extensively. Surgical removal of cervical LN as conducted by Yamagami et al. [133] resulted in indefinite graft survival in allo-grafted mice. Furthermore, using a fluorescent tracer dye the same researchers could identify the afferent lymph flow into cervical LN. These results are further corroborated by the detection of passenger leukocytes in draining LN from GFP transgenic corneal allo-grafts [134] as well as tracking studies of artificial antigens applied topically to transplanted corneas [135]. As such, the identification of the graft draining lymph node seems unambiguous. Using multi-parameter FACS the composition of the draining LN was measured. Contrary to expectations no clear pattern of allo-antigen specific inflammation associated with the rejection incident could be detected. The data set was very heterogeneous and difficult to interpret. In relative terms a small proportional reduction of CD4+ cells can be seen in the ipsi- compared to the contra-lateral
Discussion

Side of allo-rejecters which is caused by a reciprocal expansion of B-lymphocytes. However, the difference was statistically not significant and was also observed in iso-grafted control animals at POD-7. Due to the swelling of the draining LN an absolute increase of all leukocyte populations was detectable. Again, these results could not be definitively attributed to the allo-rejection process, since the same observation was made in syngeneicly grafted study subjects on POD-7.

**T-cell activation markers:** To detect allo-primed T-cells the cell surface activation markers CD25 and CD134 were chosen because they are commonly used and because they performed excellently when studying in-vitro cultures of allo-activated T-cells (data not shown). A certain amount of T-cell activation background due to normal immunological processes in non-sterile animals was expected, hence the choice to focus on an inflamed lymph node rather then spleen and blood. Contrary to expectations no unambiguous pattern of allo-specific activation in draining LN was found. Due to enlargement of the draining LN increased absolute numbers of CD25+, CD134+ and CD25+CD134+ double positive cells were detected. Additionally at POD-7 CD4+ CD134+ cell counts were increased in allo- and iso-grafted recipients. From that it was concluded that signs of inflammation in the draining submandibular LN are largely indistinguishable from inflammation caused by the surgical trauma in syngeneic recipients and the resulting breach of the immunological barrier of the eye. The only evidence for an allo-specific immune response stems from the observation of increased frequencies of CD4+CD8+ double positive T-cells in allo-grafted animals. CD8alpha expression on CD4+ T-cells is not observed in mice. It is however a feature of rat and human CD4+ T-lymphocytes and is considered an activation marker [136, 137]. Furthermore, Kenny et al. determined that this peculiar subpopulation is dominated by Th-1 helper T-cells [138]. Given the clear difference seen between allo- and syn-grafted animals with regard to CD25 expression in CD4+CD8+DP cells, it is hypothesised that truly allo-antigen-specific T-cells may be found in that compartment. However, only a sorting and in-vitro analysis can confirm this assumption.

Two explanations can be brought forth to explain the data. Firstly, the number of allo-antigen-specific T-cells is dwarfed by bystander activation of helper T-cells due to the surgical trauma and the normal background of activated T-lymphocytes responding to environmental antigens. Secondly, although the ipsi-lateral LN exhibits
clear signs of inflammation, the allo-graft rejection process is not exclusively limited to that location and spill-over into the contra-lateral lymph node occurs. If this was true, then no significant differences between ipsi- and contra-lateral side would be observed. To exclude that possibility therefore, all cervical LN should be analysed separately. Alternatively, sterile animals could be used to eliminate any possible background. This strategy is however very cost intensive and sterile or gnoto-biotic animals were not available on site.

**Methodological critique:** From a design perspective the study conducted could be improved in several ways. For example, the analysis of T-cell activation status could include more markers, such as CD40, CD137, CD62L or CD71 \[139, 140\]. Also, based on the in-vitro data of activated MLCs in table 3.1, CD28 and ICOS may be good candidates. Due to the relative shortage of directly labelled anti-rat antibodies, it would be difficult to incorporate all of these surface stains into a multicolour FACS stain. Although modern flowcytometers can acquire up to 12 parameters simultaneously, the choice of fluorescently labelled anti-rat antibodies rarely permits the usage of more the 5 colours at a time. Alternatively, instead of focussing on cell surface markers, an intracellular cytokine staining could be adopted. For example detection of intracellular Interferon-γ (INF-γ) and Interleukin-4 would allow a direct assessment of Th1 or Th2 associated immune reactions. Alternatively, in-vitro restimulation of isolated CD4+ and CD8+ T-lymphocytes isolated from surgical animals in combination with an Elispot assay specific for secreted Inf-γ, IL-4 or IL-2 could be used \[141\]. This approach was adopted by Huq *et al.* and demonstrated the presence of indirectly primed CD4+ helper T-cells during corneal allo-graft rejection \[59\]. However, due to the high precursor frequency of direct allo-antigen specific T-cells, this experimental strategy is limited to the study of indirectly primed T-cells. A further optimisation could be achieved by simultaneously measuring phenotypes of circulating blood leukocytes. During the course of this study it was determined that only small amounts of blood were needed for a serum allo-antibody test. Thus, the majority of acquired blood samples could have been used for lymphocyte isolation and measurement instead.

More importantly, the study design could be improved by the better choice of negative control animals. Syngeneic control animal were analysed on day 42 after...
complete healing of the graft. This approach bears the disadvantage that relatively old animals were measured. The effects were quite prominently seen in the absolute numbers of lymphocytes in draining LN. The total number of leukocytes increased proportionally with the age of animals. Allo-grafted animals analysed in this study were much younger than the syngeneic long-term survivors and consequently harboured significantly less lymphocytes in all lymph nodes. This effect can be avoided by including age matched syngeneic controls. In practical terms this means for each allo-rejecting subject a time matched iso-grafted control animals should be studied. This would also allow to discern the allo-rejection process better than simply relying on POD-7 allo and iso-grafted transplant recipients.

4.4.3 The role of B-cells in corneal allo-graft rejection:

Most studies using penetrating keratoplasty rely entirely on corneal opacity scoring to determine the immune reaction against allogeneic grafted tissue. In this study a serum analysis was tested and found to be a good proxy-parameter for the allo-rejection process. Particularly intriguing is the observation of anti-donor IgG2a antibodies. A Th-1 immune response must have occurred, which provided interferon-γ for the antibody class switch from IgG1 to IgG2a [142]. Furthermore, the presence of IgG1 allo-antibodies is also indicative of robust indirect help by Th2 skewed CD4+ T-lymphocytes.

In contrast to other cell types B-cells and allo-antibodies do not garner much attention in the context of allo-graft rejection. Complement and antibodies can be detected in human aqueous humor [143], and allo-antiserum from rejecting animals has been found to confer complement dependent as well as independent cytolysis [144, 145]. Interestingly, antigen-presenting B-lymphocytes also appear to play a significant role in the maintenance of the immune-privileged status of the cornea by inducing CD8+ suppressor T-cells [81]. Preliminary experiments conducted in this project attempted to demonstrate direct binding of allo-antibodies to allogeneic PVG corneas. Using two-photon microscopy, corneal wholemounts were analysed after incubation with allo-serum from rejecting animals or positive control sera. Conclusive evidence for a direct binding of allo-ABs could not be detected. However, it is premature to exclude that possibility, since only naive healthy corneas have been tested instead of inflamed grafted tissues. The determination of the exact epitopes recognised by the allo-antibodies is a challenging task and was not attempted in this project. However, if this experiment were conducted in mice the proportion
of antibodies directed against MHC class I and II could have been examined. By using target cells from MHC class I or II knock-out animals respectively, the reduction in measured fluorescence intensity could be correlated with the amount of allo-antibodies directed against these molecules.

4.4.4 NK-cells and the corneal rejection process

Upon studying the immune response elicited by corneal allo-grafts, more and more evidence was accumulated that NK- as well as NK-T-cells play a non-redundant role during a rejection episode [71]. Intriguingly high numbers of cells expressing NK-markers were found in animals models of penetrating keratoplasty as well as in human samples of aqueous humor. In both instances allo-reactivity was demonstrated by in-vitro lysis of allogeneic donor cells [69, 146]. Furthermore, it was demonstrated that baby rats reject corneal allo-grafts predominantly through cytotoxic NK-cell mechanisms [70]. The novel strain combination established in this project might be especially suited to investigate the role NK- and NK-T-cells play in the allo-rejection process of corneal transplants. Not only is the full length genome sequence available for the Brown Norway rat [147], but NK- receptors are also particularly well studied for BN and PVG animals [148, 149, 64]. Since NK-T-cells with a regulatory phenotype are known and IL-10 secreting NK-T-cells have been implicated in the induction of ACAID, these cells may represent a worthwhile target for immune-modulatory intervention in the context of corneal transplants.

4.4.5 Graft infiltrating Lymphocytes

Collagenase digestion as a method to isolate lymphocytes has been used previously in mouse studies of inflamed corneas [150]. However, the technique has not been applied on allo-grafted tissue so far. FACS measurements of the corneal rejection process have so far only been performed on aqueous humor samples in rat and human [69, 146]. Using the rapid digestion protocol for corneas and the subsequent FACS analysis of isolated cells the presence of CD25 positive and negative CD4+ helper T-cells and CD8+ cytotoxic T-lymphocytes could be confirmed. Moreover, it was demonstrated that the often observed NK-marker CD161 [69, 70] is expressed on three distinct cell populations. In fact the majority of CD8+ expressing cells are either NK-T-cells or CD3- NK cells. Furthermore, a cell population with a CD161\textsuperscript{dull} phenotype was identified and was already present as early as day 07 in syngene-
ically and allogeneically grafted animals. It is assumed that these cells are activated monocytes as described by Scriba et al. [151]. Whether these cells are identical to ED2+ macrophages as reported by Larkin et al. remains to be determined [152]. Monocytes and macrophages are of particular interest and importance in the uptake of allogeneic material and the initiation of an allo-antigen-specific immune response. It has been shown that, when monocytes infiltration into the graft is blocked by a toxic drug allo-graft rejection can be completely prevented [153].

The method to isolate and analyse GIL could be further improved by adding FACS stainings for intracellular antigens, for example T-cell secreted cytokines such as INF-γ, IL-4 or TGF-β. Additionally, the presence of T\textsubscript{regs} could be determined by intra-nuclear foxp3 labelling, thereby enabling the investigation of tolerance induction in the ocular environment. However, the experimental approach is also limited by the requirement of inflamed tissues as source material, which provides sufficient cell numbers for analysis. In this study lymphocytes from healthy corneal specimen could not be isolated.

So far, immuno histochemistry has been the method of choice for studying graft infiltrating lymphocytes. The method is however, difficult to establish and laborious, effectively limiting the amount of processed samples. The isolation method developed in this project is much more rapid and has the added benefit of isolating viable cells from grafted corneas. The collagenase digestion protocol has the potential to revigorate the study of corneal infiltrates by analysing all lymphocyte populations separately. Due to the fact that graft infiltrating lymphocytes have not been subjected to functional assays or analysed in detail several important question remained unanswered so far.

1. **CD4+ helper T-cells:** The allo-specificity of these cells is uncertain. Performing in-vitro restimulation experiments on GIL isolated from rejecting corneas will help determine the precise specificity of these cells. This experimental approach will also allow for the determination whether CD4+ cells are actively engaged in killing allogeneic target cells. Furthermore, an in vitro culture of CD4+ cells isolated from grafts would allow for the possibility of a measurement of secreted cytokines and chemokines. Additionally the T-cell receptor repertoire could be studied and possible biases in TCR usage uncovered.

2. **CD8+ cytotoxic T-lymphocytes:** Analogous to CD4+ T-cells the allo-
specificity, cytokine profile and killing capacity of CD8+ T-cells remains poorly characterised.

3. **NK-T-cells**: The precise phenotype of NK-T-cells infiltrating the graft is unknown. By spectratyping the T-cell receptor of CD8+ CD161\textsuperscript{dull} cells it would be possible to determine whether Type I or Type II NK-T-cells infiltrate the graft. Messenger RNA analysis or in-vitro culture could also reveal the cytokine profile of these cells.

4. **Natural killer cells**: NK cell function is tightly regulated by the pattern of NK receptors both activating such as NK1.1, and inhibiting, such as KIR type cell surface proteins [67]. The exact involvement of these receptors in the allograft rejection process is mostly unclear. Although some functional assays on NK cells isolated from aqueous humor have been conducted [69, 146], demonstrating lytic activity against allogeneic target cells, the precise mechanism has not been elucidated. It is not known whether non-self recognition is triggered by allogeneic non-classical MHC class-I molecules or by polymorphisms in other inhibitory NK-receptors.

5. **Monocytes**: The cytokine and chemokine expression pattern of graft infiltrating monocytes has not been resolved on a population level. Additionally, it may be the case that infiltrating monocytes are actively involved in killing allogeneic target cells.

### 4.5 In-vivo application of regulatory T-cells

In order to test the main hypothesis of this project allo-antigen specific regulatory T-lymphocytes were generated in-vitro using anti-CD4 treatment of mixed lymphocyte reactions. Cultured T\textsubscript{regs} were then anti-CD3 FACS sorted to remove donor antigen presenting cells and adoptively transferred into recipient animals 24 hours before allogeneic corneal transplantation. T\textsubscript{regs} were tested in the BN-PVG strain combination and $2.5 \times 10^6$ cells per animals were injected. Graft survival was monitored as described above. No significant delay or prevention of graft rejection was observed in graft recipients treated with ex-vivo generated foxp3 expressing T-cells. In fact no spontaneous graft acceptance was observed in the treatment group and it has to be concluded that the T\textsubscript{reg} study group had poorer graft survival compared to the PBS injected control animals. Several explanations can be proposed to explain
this disappointing observation.

1. **Insufficient number of regulatory T-cells injected:** Due to the difficulty in generating T\(_{\textrm{regs}}\) in-vitro no dose escalation study could be performed. Hence, it is possible that insufficient cell numbers were adoptively transferred into allo-graft recipients. The situation is further complicated by the fact that the injected cell preparations only contained approximately 50\% \(\text{foxp3}\) expressing cells. In rat model systems for EAE \(5 \times 10^6\) activated MBP specific T-lymphocytes are infused [154]. It is, however, speculative whether the conditions to break tolerance and induce an autoimmune disease and the induction of tolerance towards an allo-graft are comparable. Furthermore a T\(_{\textrm{reg}}\) marker was missing to establish the location and distribution of injected cells. Although some information about the homing behaviour was gathered from the injection of GFP transduced allo-specific T-cells, which were found to accumulate in the ipsi-lateral draining LN, the migration characteristics of regulatory T-cells might be different. Nonetheless, literature suggests, that T\(_{\textrm{regs}}\) do indeed preferentially home to draining LN and into inflamed tissues and even show shuttling between the two sites [155].

2. **Reduced importance of the direct pathway of allo-recognition:** Although the existence of passenger leukocytes in corneal allo-grafts has been established and these cells do migrated into the draining cervical lymph node of allo-graft hosts [134], several authors have claimed that indirect allo-recognition plays a dominant role in corneal graft rejection [56]. In-vitro studies on T-lymphocytes isolated from draining LN of allo-rejecters revealed the presence of indirectly primed CD4+ helper T-cells but no significant increase in directly responsive T-cells compared to the typical high back ground [59]. The current understanding of corneal allograft rejection maintains that the direct pathway is more relevant in high risk models of penetrating keratoplasty where allo-grafts are placed into pre-vascularised graft beds [59].

3. **Lack of a semi-direct pathway of allo-recognition:** Part of the hypothesis of this project was the assumption of a semi-direct pathway of allo-recognition. Its proposed mechanism would allow T\(_{\textrm{regs}}\) with direct specificity to suppress the indirect priming of allo-specific T-cells. To date, the semi-direct pathway has not been demonstrated conclusively in-vivo. Its presump-
tion rests entirely on in-vitro observations of membrane transfer carrying intact MHC class-II molecules and co-stimulatory receptors between immune cells [61, 62, 63]. The precise biological function of this process might, however, not be relevant to allo-recognition. For example it was determined that carry over of cognate MHC-II plus peptide from DCs to activated CD4+ cells facilitates the clonal expansion of recently primed T-cells independently of the priming APC. That mechanisms effectively liberates limited binding space on the APC to recruit and prime a new wave of naive T-cells [156]. Furthermore, the semi-direct pathway was conceived to explain the occurrence of CD8+ cytotoxic T-cells with direct specificity even at later stages of rejection where passenger leukocytes are thought to have disappeared [60]. It is, however, possible that activated donor endothelial cell take up the role of APCs and prime CTLs without the need of MHC-I transfer to host APCs and indirect help from CD4+ helper T-lymphocytes [157]. The presence of directly primed CD8+ cells was always assumed to be a necessary component of the allo-graft destruction process since indirectly primed CD8+ T-cells could not engage donor cells due to a disparate MHC-class-I receptor. Recent data suggest, however, that indirectly primed CD8+ T-cells can equally contribute to the rejection of a fully allogeneic graft. Valujskikh et al. found that H-2b restricted H-Y specific CD8+ T-cells reject male H-2k skin grafts in a cytokine dependent fashion without the need of MHC-I-TCR interactions [158]. In summary, the immunologic phenomena which led to the hypothesis may be explained by phenomena other than a semi-direct presentation pathway.

4. **Inactivity of T<sub>regs</sub>:** Regulatory T-cells are potent suppressors of naive T-cell responses. However, the suppressive activity is not absolute and can be modulated or switched off entirely. It was demonstrated that CD134 (Ox-40) signalling in T<sub>regs</sub> abolishes their suppressive capacity [159, 160], such that the rejection of allogeneic bone marrow and skin grafts could be triggered by in-vivo ligation of CD134 [161, 162]. Similarly to mice, rat T<sub>regs</sub> also express Ox-40 constitutively (see 3.3 on page 43) and it can be hypothesised that mechanisms of inactivation are identical. The FACS study of T<sub>reg</sub> phenotypes conducted in this study found that foxp3 expressing T-cells harvested from anti-CD4 treated cell cultures had a strongly reduced expression of CTLA-4 (see figure 3.6 on page 44) a fact indicative of intense CD134 signalling [163]. Although the subject was not investigated in this project, it was noted that,
in standard allogeneic MLCs a high number of foxp3 expressing T-cells was present (see figure 3.5 on page 42). The ratio of T\textsubscript{regs}/T\textsubscript{conv} was 1:5 at which allo-specific regulatory T-cells were not able to suppress the outgrowth of conventional CD4+ T-lymphocytes. It is thus possible that the same occurred in the in-vivo setting of allogeneic keratoplasty.

5. **Allo-specific responder T-cells escape T\textsubscript{reg} suppression:** Certain phenotypes of CD4+ T-lymphocytes are refractory to regulatory T-cell mediated suppression. In particular, memory T-cells are reported to be insensitive to T\textsubscript{reg} function [164]. The comprehensive analysis of draining lymph nodes of allo-rejecting animals revealed that the submandibular LN had a strikingly different composition then brachial control LN. In particular a high frequency of CD134+ CD4+ T-cells was present (see figure 3.12 on page 59). Ox-40 expression and ligation has been found to play a crucial role in memory T-cell formation [165]. Furthermore, the submandibular LN also drain the oral mucosa and as such are constantly exposed to environmental antigens. It may be reasonably concluded that the draining LN contain a high number of memory T-lymphocytes due to their exposed location. Since allo-antigen specific effector T-cells with direct specificity recruit randomly from the T-cell pool, a certain number of allo-reactive memory T-cells can be expected. With their lower activation requirements and rapid secretion of pro-inflammatory cytokines, they may have effectively circumvented the suppressive regime imposed by regulatory T-cells [166].

6. **Redundancy of allo-rejection mechanisms:** The investigation of transplant rejection biology has revealed a superfluency of allo-recognition and rejection mechanisms. Although it is generally believed that CD4+ helper T-cells play a dominant role in allo-graft rejection, alternative pathways exist. For example Niederkorn et al. and others found that CD4 knock-out mice still reject corneal allo-grafts albeit in a delayed manner [167, 168]. It is thus conceivable that adoptively transferred T\textsubscript{regs} functioned as expected and suppressed a naive T-cell response but the rejection of allo-grafted corneas was then executed by allo-reactive NK and NKT-cells in cooperation with host monocytes. The high number of polymorphisms found in NK-receptors of PVG rats compared to BN animals [148] is suggestive of a high susceptibility to allo NK-cell lysis of PVG donor cells and tissues. The observation of a strikingly high
number of NK and NK-T-cells in rejecting corneas supports this hypothesis (see figure 3.13 on page 61).

To summarise, given the multitude of possibilities it is difficult to determine which mechanism lead to the failure of the T\textsubscript{reg} adoptive transfer protocol. In principle all the above mentioned hypotheses could be tested. With the material constraints and limitations in manpower and time, however, it was not possible to test any of them in this project. It is clear, nonetheless, that more data and closer monitoring of the graft rejection process would be necessary to successfully implement a clinically relevant treatment strategy for the prevention of corneal all-graft rejection.

### 4.6 Regulatory T-cells in other transplant models

The unique property of regulatory T-cells to inhibit antigen specific T-cell responses has repeatedly lead to the proposal to utilise this outstanding feature to battle all-rejection processes [169]. At first a reductionist approach was chosen to demonstrate that T\textsubscript{regs} can indeed suppress allo-specific graft rejection in-vivo. Nude or rag-deficient mice were skin grafted and reconstituted with effector T-cells. Subsequently ex-vivo allo-primed nT\textsubscript{regs} were infused and it was observed that graft rejection was abolished [170, 171]. Further progress was made using TCR-transgenic models [172]. Chai \textit{et al.} generated HY-specific T\textsubscript{regs} using a retroviral vector for \textit{foxp3} transduction and demonstrated the ability of those cells to prevent the rejection of male skin grafts [50]. Advancing to fully immune-competent graft recipients it was noted that neither regulatory T-cells with direct nor indirect specificity were able to completely curb the rejection of skin and heart transplants. Joffre and colleagues showed that graft recipients needed to be pretreated with total lymphoid irradiation (TLI) and an allogeneic bone-marrow transplant for donor-specific T\textsubscript{regs} to confer long term tolerance [173]. Further research into the matter revealed, however, that TLI is not absolutely necessary to establish long term tolerance towards MHC disparate skin grafts [174]. Simultaneously, it was observed that T regulatory lymphocytes responding to the direct pathway of allo-recognition were in general insufficient to prevent graft destruction and chronic rejection was still observed in mouse and rat models of heart transplantation [173, 175]. Collectively, these observations are somewhat discouraging, since it highlights the complexity of tolerance induction and maintenance mechanisms and the role played by regulatory T-cells. Apparently ex-vivo generated T\textsubscript{regs} lack a crucial maturation or differentiation signal
to suppress host allo-immune responses for extended periods of time, since adoptive transfer of regulatory T-cells from permanent graft acceptors are fully capable of doing so [176]. All therapeutic interventions involving ex-vivo T<sub>regs</sub> either required adjunct immuno-suppression with tacrolimus [175] or rapamycin [177], ablation of CD8+ cytotoxic T-cells [177] or haematopoetic chimerism [173, 174]. For the latter requirement it can be hypothesised that donor dendritic cells resident in host lymphatic tissues are pivotal for the long term survival and function of allo-antigen specific regulatory T-cells [155, 178]. Donor allo-determinants expressed by the transplanted organ are seemingly insufficient to provide all necessary signals for the instruction of T regulatory lymphocytes to generate peripheral tolerance.

4.7 Critical analysis of the research hypothesis and conclusions

This project explored the possibility of direct allo-antigen specific regulatory T-cells to prevent corneal graft rejection in a fully allogeneic setting. The basic tenet of this hypothesis could not be confirmed though. Multiple factors could have lead to the failure of the T<sub>reg</sub> treatment protocol and the precise reason remains elusive. A certain risk was associated with the choice to focus on the direct pathway of allo-recognition as a leverage point to block allo-rejection since many researchers have argued for its reduced importance in the corneal transplant setting. Moreover, it was shown in various transplant models that direct responsive regulatory T-cells are insufficient to prevent allo-rejection when fully immune-competent hosts are used (see section above). The rational, however, to focus on the direct pathway was the feasibility of a clinically relevant ex-vivo T<sub>reg</sub> generation protocol instead of merely a proof of principle study. It was further hypothesised that a single dose of regulatory T-cells would tip the balance of the corneal immune response towards tolerance allowing the inherent immune privilege of the ocular surface to be restored. The occurrence of spontaneously accepted allo-grafts strongly favoured this assumption. Furthermore, retroviral gene transfer of transcription factor foxp3 was investigated in combination with long term T-cell culture to select and expand allo-antigen specific T-lymphocytes. The difficulties arising from this experimental approach were underestimated. Poorly understood culture conditions for rat allo primed T-lymphocytes were largely to blame for this. Most literature references describe the expansion of rat memory T-cells rather then the activation and expan-
sion of naive precursor T-lymphocytes. Apparently, the culture conditions differ to such an extent that an easy implementation of published protocols was not possible. Additionally, the retroviral vector system used in this project proved to be inadequate to confer long term expression of \textit{foxp3} in gene-modified T-cells. This finding was particularly surprising since identical gene therapy vectors performed well in mice. On the other hand, it could be demonstrated that anti-CD4 treatment of allogeneic mixed lymphocyte cultures is an attractive tool for ex-vivo generation of regulatory T-cells. In conjunction with a proper purification strategy, which could not be developed during this project due to time constraints, a promising source of donor-specific T_{regs} ready for preclinical testing could be established.

\textbf{Alternative approaches:} A different methodology could have been used to test the suitability of ex-vivo generated T regulatory cells. For example both pathways of allo-recognition could have been covered by using recipient\times donor F1 hybrid animals as stimulator cells in allo-MLCs and as cornea donors. This strategy would have allowed the generation of directly and indirectly primed allo-specific T-cells [173]. Additionally, memory T-lymphocytes with indirect allo-specificity could be generated by immunising naive animals with donor DCs and subsequent isolation of CD4+ cells from spleen and draining lymph node. Such cells could then be expanded in-vitro for extended periods of time using donor cell lysates added to syngeneic MLCs. These approaches were not pursued for two reasons. Firstly, since both methods can not be adapted for the human system they bear no significance for the development of a clinical grade adoptive T-cell therapy protocol. Secondly, constraints in the animal facility did not allow the breeding of hybrid animals and commercial acquisition was cost prohibitive. With regard to the ex-vivo generation of T_{regs}, a third method could have been used, i.e. the formation of regulatory T-cells using TGF-\beta and retinoic acid [24, 23, 22]. Adaptive iT_{regs} were investigated by a different researcher in parallel to this project (Kis \textit{et al. manuscript submitted}). Cells isolated from TGF-\beta+RA treated cultures were found to be suppressive in-vitro, however the frequency of \textit{foxp3} expressing cells was typically lower then after anti-CD4 treatment. The lack of a purification strategy and time considerations lead to a decision to forgo the preclinical testing of these cells.
Consequences for the clinical application of an adoptive T-cell therapy involving allo-specific T\textsubscript{regs}: Direct mixed lymphocyte reactions are technically possible in the human setting and represent an elegant way of generating on oligo-clonal population of allo-specific T-cells without the necessity of knowing the TCR specificity of each clone. The alternative of focusing on T-lymphocytes with indirect specificity is biologically and technically much more challenging. Although some immuno-dominant peptides have been isolated and characterised in mice and humans [179], clinical applicability is not clearly feasible at present. The low precursor frequency of peptide specific T-cells and the vast number of possible allo-peptides resulting from large numbers of haplotype combinations pose considerable obstacles. Some progress has been made however, and peptide libraries in conjunction with artificial APCs [180] or the utilisation of recombinant MHC molecules [181] (tetra- or pentamer technology) may constitute a clinically feasible approach to isolate indirect T-lymphocytes in the future.

The complexity of regulatory T-cell biology [28] in humans with its different sub-populations and the role of different splice variants of FOXP3 further hinder rapid progress from bench to bed site in this area. Furthermore, induced regulatory T-cells have been found to be much more unstable and to lose their suppressor function quickly in a strong inflammatory environment [182]. It was observed, for example, that iT\textsubscript{regs} can convert into IL-17 secreting effector T-cells [182] which would likely to be detrimental in the case of an ongoing allo-graft rejection episode. A method to deal with this phenomenon would be the use of retroviral vectors to force expression of FOXP3 independent of cell intrinsic gene regulation. However, as long as the safety concerns with regard to viral gene therapy vectors persist such a strategy is unlikely to advance to clinical studies.

Conclusions: Although some parts of this research project could not be completed successfully, the data acquired and the insight that was gained into the biology of regulatory T-cells represent a important mile stone on the way to developing a clinically viable adoptive T-cell therapy for the treatment of allo-graft rejection. The caveats and pitfalls of a simple regulatory T-cell injection approach have been exposed, highlighting the necessity to better understand the circumstances of ex-vivo generation. Furthermore, an in-depth understanding of the immuno-biology of corneal transplant rejection is absolutely pivotal to tailoring an immuno-modulatory
therapy. The establishment of a novel strain combination and protocol for rapid isolation of graft infiltrating lymphocytes will contribute to further research in this field. A strong case can be made for the importance of T regulatory cells in the maintenance of ocular immune privilege. It is thus likely that any future therapy will focus one way or another on this cell type, for the purpose of generating tolerance against transplanted allogeneic corneas. Whether a $T_{\text{reg}}$ transfusion approach will be taken and by which means allo-specific suppressor T-cells will be obtained is difficult to predict at the moment. More research is certainly needed to answer these questions. This research project has sought to contribute to the advancement towards a clinical therapy and to the emerging field of regenerative medicine.
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6 Publications by the author

A comprehensive flow-cytometric analysis of serum, draining lymph nodes and graft infiltrating lymphocytes during the rejection phase in a fully allogeneic rat cornea transplant model
Martin Maenz, Mourice Morcos, Thomas Ritter

Inhibition of alloantigen-specific immune response in a rat model by TGF-beta and retinoic acid-treated lymph node cells
Kornelia Kis, Martin Maenz, Thomas Ritter
*manuscript submitted*

Comparison of viral and nonviral vectors for gene transfer to human endothelial progenitor cells.

Gene-modified mesenchymal stem cells express functionally active nerve growth factor on an engineered poly lactic glycolic acid (PLGA) substrate.
6.1 Conference poster presentations

A comprehensive flow-cytometric analysis of serum, draining lymph nodes and graft infiltrating lymphocytes during the rejection phase in a fully allogeneic rat cornea transplant model.
Maenz M, Morcos M, Ritter T.
presented at the ARVO meeting 2010

Optimization of retrovirus-mediated gene transfer of Foxp3 in allo-antigen specific primary rat T-cells
Maenz M.; Kis K.; Ritter T.
presented at the annual meeting of the Irish society for gene and cell therapy (ISGCT)- Dublin 2008

Optimization of retrovirus-mediated gene transfer of Foxp3 in allo-antigen specific primary rat T-cells
Maenz M., Ritter T.
presented at the World Immune Regulation meeting (WIRM)- Davos 2007

Optimization of retrovirus mediated gene transfer in allo-antigen specific primary rat T-cells
Maenz M., Sawitzki B., Fluegel A., Volk HD., Ritter T.
presented at the meeting of the European society for gene therapy (ESGT) - Prague 2005
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Bibliography


Appendix
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age: | weight: | time in AF: |

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cornea donor:  □ BN (syn) AF code #:  □ PVG (allo) AF code #:  □ ............ (third party) AF code #: |

anaesthesia:  □ ISOFLURANE  
additional drugs:  □ Atropine 1%  □ Tropicamide 1%  □ Tetracaine 1%  □ Phenylepherine 2.5%  
antibiotic treatment:  □ Chloromycetin (chloramphenicol)  □ artificial tears  
suture:  □ discontinued-number of stitches:  □ continuous  

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If no; specify:  

**Day -01 donor inspection:** passed  □ yes  □ no  
If no; specify:  

**Un-blinding injections:**  

**Organ harvest:**  □ cornea  for  □ RNA  □ FACS sort  
□ LNs  for  □ RNA  □ FACS  
□ serum for allo-AB test  

**Other:**  

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Animal sacrificed - date:  

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If no – why:  

Pictures taken:  □ Yes  □ No  
Location:  

File closed:  □ Yes  □ No  

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Atropine drops: ☐ yes / ☐ no

Pictures taken: ☐ yes / ☐ no