Subconjunctivally applied naïve Tregs support corneal graft survival in baby rats

Antonia Hildebrand,[1,2] Christian Jarsch,[1] Yvonne Kern,[1] Daniel Böhringer,[1] Thomas Reinhard,[1] Johannes Schwartzkopff[1,3]

(The first two authors contributed equally to this study)

[1]University Eye Hospital, Killianstrasse 5, 79106 Freiburg, Germany; [2]Faculty of Biology, Albert-Ludwigs-University Freiburg, Germany; [3]Ophthalmology Clinic Knapp-Schwartzkopff, Lörrach, Germany

Purpose: Corneal transplantation is the most frequent and successful form of tissue transplantation in adults (<10% rejection). In young children, any corneal opacity should be corrected as early as possible to prevent lifelong visual impairment. However, the corneal graft rejection rate is dramatically increased in infants younger than 12 months of age (up to 85% rejection), and immunosuppressive therapy is particularly challenging in this age group. Regulatory T cells (Tregs) are a well-characterized T cell subpopulation with the potential to prevent autoimmune disorders or transplant rejection. Antigen-specific Tregs were shown to inhibit graft rejection in adult stem cell transplantation. Less is known about the role of naïve Tregs. The purpose of the present study was to elucidate the relevance of naïve Tregs in juvenile corneal transplantation in a baby rat keratoplasty model that reproduces the accelerated rejection in young patients.
Methods: Counts and inhibitory potential of Tregs were studied in spleens of 3- and 10-week-old rats. Unprimed Tregs (CD4+CD25+) were isolated from the spleens of 10-week-old Lewis rats and systemically or subconjunctivally administered in vivo in allogenic keratoplasty in 3- and 10-week-old Lewis recipient rats. In subconjunctival tissue, transcription was analyzed for induction of transforming-growth-factor beta (TGF-β).
Results: In 3-week-old rats, CD4 T cell counts, but not FoxP3 T cell counts were lower than in 10-week-old rats. The Tregs of both age groups had the potential to inhibit T cell activation in vitro. No significant delay in rejection was observed when Tregs were applied systemically before keratoplasty. However, subconjunctival application of Tregs abrogated rejection in 66.7% and 33.3% of the 3- and 10-week-old recipients, respectively. Analysis of the conjunctival tissue revealed a transplantation-induced increase in TGF-β transcription in the 3-week-old rats.
Conclusions: Our data suggest that local application of unprimed regulatory T cells may be a therapeutic strategy for preventing corneal graft rejection in young recipients.

Regulatory T cells (Tregs) have been described as suppressive T cells with roles in ocular immune privilege [1] and tolerance induction [2]. In 2003, Sakaguchi et al. described a CD4 positive T cell population that regulates T cell responses and prevents autoimmune disease in humans [3]. Since then, malfunctioning Tregs have been found to be responsible for many human immune disorders and diseases [4-6]. Tregs develop in the thymus and in the periphery in a distinct cytokine milieu. Thymus-derived Tregs are termed natural Tregs (nTregs), while induced Tregs (iTregs) from the periphery possess a certain plasticity and form under conditions such as elevated transforming growth factor beta (TGF-β) levels and self-antigen recognition [7]. TGF-β is a key cytokine in Treg development and can be used to generate Tregs in vitro [8]. Key surface markers of Tregs are CD4 and CD25 [9], while expression of the transcription factor FoxP3

correlates with Treg activation [10]. The ability of Tregs to modulate immune responses has been studied in adoptive transfer experiments and transplantation therapy, where Tregs suppressed immune pathologies such as graft versus host disease (GvHD) and allogenic transplant rejection [11-13].

Following transplantation, a graft may be attacked by the host's immune system because of genetically diverse human leukocyte antigen (HLA) loci [14]. In this case, patients receive long-term systemic immunosuppression therapy [15]. Since many side effects may ensue, early treatment of the mounting immune response would be of great benefit. Thus, the application of self-Tregs is a promising option in allogenic transplant recipients. Preclinical data on GvHD show that Tregs can help achieve tolerance in the recipient of an allogenic stem cell transplant [13,16].

Despite the genetic differences of donor and recipient, keratoplasty is one of the most successful organ transplantation procedures worldwide [17]. Adult patients with a low risk show more than 90% graft survival after 5 years with only little and short-term use of topical immunosuppressants. In

Correspondence to: Johannes Schwartzkopff, Ophthalmology Clinic Knapp-Schwartzkopff, Lörrach, Germany, Phone: +49 7621 422 350; FAX: +49 7621 422 3525; email: johannes.schwartzkopff@gmail.com

contrast, patients under the age of 12 months may suffer from rejection in more than 80% of the cases [18]. Therefore, it is of high interest to improve the treatment of young children, since the side effects of systemic immunosuppression are severe and difficult to control in these patients.

Previous studies have highlighted the relevance of Tregs in modulating cornea transplant rejection. Tregs are present in lymph nodes draining the orbit, and it has been shown in a mouse keratoplasty model that the ability of Tregs to prevent rejection correlates with FoxP3 expression [19]. Adoptive transfer of allogen-primed FoxP3+ lymph node Tregs prevented corneal graft rejection. In contrast systemic depletion of Tregs using an anti-CD25 antibody leads to accelerated rejection [20]. Currently, no data are available on adoptive transfer of Tregs from naïve rats (naïve Tregs) following keratoplasty and on possible age-dependent differences in Treg functions.

We established a rat model of corneal transplantation to differentially analyze rejection processes and immune pathology in baby and adult recipients [21-24]. In this baby rat keratoplasty model, 3-week-old Lewis recipients of a Fisher allograft show significantly earlier rejection compared to 10-week-old Lewis recipients, even in the absence of additional immunological challenges or risk factors.

We hypothesize that the accelerated graft rejection in the baby rat is due to an altered number or activation status of regulatory T cells. Thus, the aim of this study was to assess age-dependent characteristics of Tregs, such as numbers and inhibitory potential in vitro, and to investigate a potential therapeutic role of additional naïve Tregs in cornea transplant recipient rats of different age groups. Our data show that Treg counts were comparable in infant and adult animals and the inhibitory potential was similar. Systemic application of naïve

Tregs did not alter the time course of transplant rejection. However, subconjunctival (s.c.) application of naïve Tregs was associated with a significant increase in cornea graft survival. This effect was more prominent in baby transplant recipients suggesting a possible therapeutic use of Tregs in preventing cornea graft rejection in children.

METHODS

Animals and anesthesia: Inbred female Fisher (Rt1^{lv1}) and Lewis (Rt1^{l}) rats (Charles River, Sulzfeld, Germany) were used as the donors and recipients, respectively. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the IACUC of Freiburg University, Faculty of Medicine and the Regierungspraesidium Freiburg. The rats were anesthetized with a short inhalation of isofluran followed by an intraperitoneal injection of a mixture of ketamine (Essex, München, Germany), xylacin (Bayer, Leverkusen, Germany), and atropine (Braun, Melsungen, Germany). Animal euthanasia was done by CO_2 inhalation. All groups and treatments are shown in Table 1.

Corneal transplantation: Orthotopic penetrating kerato-plasties in all groups (Table 1) were performed as described [25]. Briefly, donor buttons from the central cornea were obtained using a 2.5 mm trephine and subsequently stored in conservation medium (Medium II; Biochrom, Berlin, Germany). The central cornea of a recipient was removed using a 2.0 mm trephine. The graft was fixed with eight inter-rupted sutures (11.0 Ethilon; Ethicon, Norderstedt, Germany). Finally, a blepharorraphy was applied for 3 days.

Clinical graft assessment and rejection kinetics: All grafts were examined daily and masked by two independent

TABLE 1. GROUPS AND TREATMENTS.

Group	Recipient age in weeks	Treatment	n	Survival until POD29, n(%)
allogenic	3	none	23	0 (0)
allogenic	3	CD4+CD25+ i.v.	4	0 (0)
allogenic	3	CD4+CD25+ s.c.	6	4 (66.7)
allogenic	3	CD4+ CD25- s.c.	4	1 (25)
allogenic	10	none	4	0 (0)
allogenic	10	CD4+CD25+ s.c.	6	2 (33.3)
allogenic	10	CD4+ CD25- s.c.	6	0 (0)
syngenic	3	none	8	8 (100)
syngenic	10	none	4	4 (100)

allogenic: Keratoplasty Fisher to Lewis, syngenic: Keratoplasty Lewis to Lewis, i.v.: intravenously, s.c.. subconjunctivally

investigators for signs of opacity according to an international score. Recipients with technical complications such as cataracts, infections, collapse of the anterior chamber, or massive hyphema were excluded. The time point of rejection was identified when an opacity score of 4 (complete opacity) was recorded.

T cell stimulation assay: Lymphocyte cell suspensions were made from Lewis spleens with Ficoll (Pancoll, PAN Biotech, Aidenbach, Germany) gradient centrifugation. Spleens were obtained from adult female Lewis rats and dissected in 100 µm cell strainers (BD, Heidelberg, Germany), rinsed with PBS (2.6 mM KCL, 1.47 mM KH_2PO_4, 138 mM NaCl, 8.0 mM $Na_2HPO_4*7H_20$, pH7.4; Life Technologies, Darmstadt, Germany), and spun down with 253 ×g for 7 min. Cells were resuspended in 10 ml PBS and layered onto 15 ml Pancoll solution and centrifuged for 25 min at 623 ×g. The resulting lymphocytes were washed in PBS and resuspended in Dulbecco's Minimum Essential Media (DMEM; BiochromAG, Berlin, Germany). Ninety-six-well flat-bottom plates (Greiner, Frickenhausen, Germany) were coated with each 2 µl CD3 (eBioG4.18, eBiosciences, San Diego, CA) and CD28 (clone JJ319). Lymphocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Eugene, CA), and a total of $1×10^5$ cells were added to each 96-well plate in 200 µl medium. For suppression assays, Tregs were added as indicated. Cell division was analyzed with flow cytometry on a fluorescence-activated cell sorting (FACS) Calibur (BD).

Isolation Treg: Regulatory T cells were obtained from the spleens of GFP transgenic [26] (kindly provided by Dr. Eiji Kobayashi, Jichi Medical University, Japan) and wild-type female Lewis rats. The spleens were dissected in a 100 µm cell strainer and washed with PBS for 5 min at 253 ×g. Cells were resuspended in PBS and layered on a Pancoll solution. After gradient centrifugation for 25 min and 623 ×g, splenic lymphocytes were retained from the interface. CD4 isolation was based on the magnetic-activated cell sorting (MACS) technique according to the manufacturer's instructions (Easy Sep PE Selection Kit; Stem Cell Technologies, Vancouver, Canada) and the anti-CD4-PE antibody (OX-38, eBioscience). The resulting CD4 positive lymphocytes were labeled with CD25 AF647 antibody (OX-39, AbD Serotec, Raleigh, NC) and underwent FACS sorting (MoFlow, Cytomation, Fort Collins, TX).

FACS staining: The spleen cells were dissected in 100 µm cell strainer and washed with PBS. $5×10^5$ cells were diluted in FACS buffer (PBS + 2% FCS (Biochrom) and 0.04% heparin (Braun, Melsungen, Germany)). Surface staining was conducted in FACS buffer and with 1 µg of CD4 PE

(clone OX-38) or CD25 AF647 (clone OX-39) per 10^6 cells. Red blood cells were lysed before accounting with cell lysis buffer (BD). Intracellular FoxP3 stain was performed with the FoxP3 staining kit and the anti-FoxP3 antibody (clone 150D/E4, eBiosciences). Cells were measured on a FACS Calibur.

RNA isolation: Conjunctiva was stored in RNAlater (peqlab, Erlangen, Germany) at −20 °C. Prior to isolation, the conjunctival tissue were dissected, and RNA was isolated via the High Pure RNA Tissue Kit (Roche, Basel, Switzerland). About 20 µl of RNA was reverse-transcribed in cDNA via Superscript RT and Oligo dT (Life Technologies) according to the manufacturer's instructions.

Quantitative PCR (qPCR): Semiquantitative PCR was performed with SYBR Green PCR Kit (Eurogentec, Cologne, Germany) following manufacturer's instructions and run on a Chromo 4 Bio-RAD PCR cycler. Analyses were performed with REST-384 version 2. Samples were run in triplets. Primers were designed via the online tool Primer 3 and produced by Metabion (Berlin, Germany). The following primers were used: β-actin primer, forward: 5′-CTA CAA TGA GCT GCG TGT GG-3′, reverse: 5′-CGG TGA GGA TCT TCA TGA GG-3′; TGF-β, forward: 5′-ATA CGC CTG AGT GGC TGT CT-3′, reverse: 5′-GAC TGA TCC CAT TGA TTT CCA-3′.

Statistical analysis: Corneal graft survival rates were compared using Kaplan–Meier survival curves and the log-rank test. The number of CD4+ and FoxP3+cells and inhibitory potential were analyzed using the two-tailed Student *t* test. Values of p<0.05 were considered statistically significant.

RESULTS

Age-dependent differences in Tregs: The relative number of Tregs was quantified in the spleens with FACS after staining for CD4 and FoxP3. The 3-week-old animals had 32.4±4.0% CD4+ T cells, while the 10-week-old animals had 40.0±2.2% CD4+ T cells (Figure 1A, n=11; p<0.0001). The percentage of FoxP3+ among the CD4+ cells in the 3-week-old rats was 6.0±1.5% and 7.1±1.2% in the 10-week-old rats (Figure 1B, n=11; p=0.85).

To address the question whether the Tregs of 3-week-old and 10-week-old rats act inhibitory, we studied the Tregs of both cohorts in a T cell stimulation assay in vitro. The splenic lymphocytes of the 10-week-old rats were stimulated with anti-CD3 and anti-CD28 antibodies and cocultured with or without the addition of Tregs from 3- or 10-week-old rats. The Tregs were sorted with flow cytometry using the surface markers CD4 and CD25 and were added in a 1:1 ratio to a
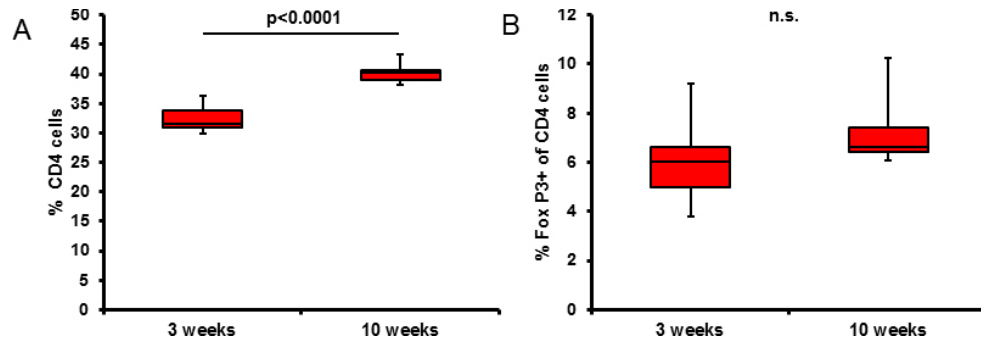
Figure 1. Tregs in 3- and 10-week-old rats. **A**: CD4 T cells in spleens of 3- and 10-week-old Lewis rats. Three-week-old rats have significantly fewer CD4 T cells, (n=11, p<0.0001). **B**: Amount of FoxP3+ CD4 T cells is similar in both age groups (n=11, p=0.85).

total of 10^5 cells per well. Cell proliferation was assessed as a loss of CFSE, and the ratio of divided to undivided cells was calculated (Figure 2). Adding Tregs clearly inhibited lymphocyte proliferation (p<0.01). Control T cell stimulation showed a ratio of 2.4 (n=4), while in the T cell stimulation with added Tregs, the ratio was reduced to 0.40±0.17 and 0.74±0.34, respectively. The Treg-mediated inhibition was not age-dependent (n=3 resp. n=9, p=0.141).

Intravenously applied Tregs do not reduce rejection: To test the inhibitory potential in vivo, the following experiment was conducted. The Tregs were sorted from the spleens of 10-week-old naïve rats using the surface markers CD4 and CD25. About 10^5 cells in 100 µl PBS were injected intravenously (i.v.) into the tail veins of the 3-week-old syngenic

recipients. One day later, keratoplasty was performed, and graft survival was assessed using the standard score. Figure 3 shows that the syngenic grafts were not rejected within the observation period (post-operative day [POD] 29 after keratoplasty), allogenic grafts were all rejected by day 11 (n=15), and allogenic grafts in animals treated with Tregs i.v. were rejected by day 12 (n=4).

Subconjunctivally applied Tregs reduce the rejection rate: About 10^5 Tregs in 30 µl PBS from 10-week-old naïve rats were applied to the conjunctiva at the end of surgery. CD4+CD25- cells were administered in controls. Recipients were clinically monitored until day 29 after keratoplasty. All baby rats with an allogenic transplant and without application of Tregs showed graft rejection "until day 11 after
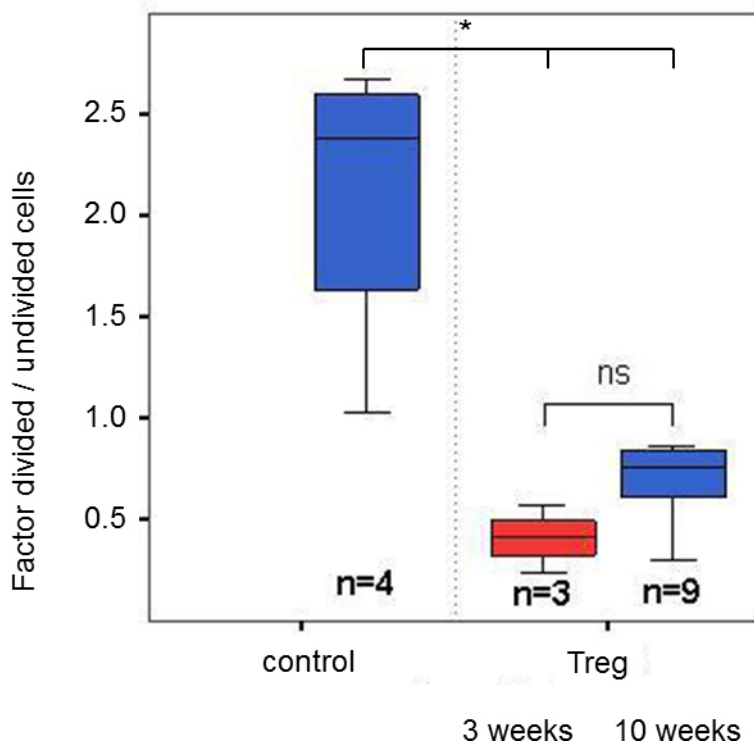

Figure 2. Inhibitory potential of Tregs in a T cell stimulation assay. T cells were stimulated with CD3 and CD28 antibodies and Tregs of 3- and 10-week old rats were added in a co-culture experiment. Division of cells was traced with CFSE and ratio of divided to undivided cells was determined. Treg of both cohorts showed inhibitory potential (*p<0.01). Between the cohorts, no significant difference was observed (p=0.141).
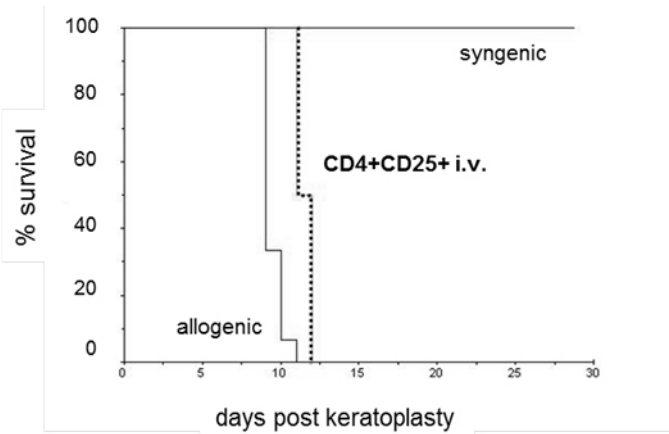
Figure 3. Graft survival after keratoplasty and i.v. Tregs. 3-week-old recipient rats received allogenic keratoplasty and obtained no additional cells (solid line, n=15) or Tregs intravenously (dotted line, n=4). Without cells, grafts were rejected at a median of POD 9, addition of Tregs into the tail vein resulted in a median rejection of day 11 after keratoplasty. Grafts of syngenic recipients (broken line) were not rejected until POD 29 (n=8).

keratoplasty (n=15), while the syngenic transplants were not rejected (n=8). In the group of six baby rats with s.c. application of Tregs, four did not reject the graft. In the control group, three out of four animals rejected the graft (Figure 4A). In the adult age group, two of the six 10-week-old recipients of an allogenic transplant and Tregs s.c. did not reject the grafts while all the controls (n=6) rejected the grafts until day 15 (Figure 4B). Adult recipients of an allogenic transplant without addition of cells reject the graft until day 15 (n=4), while syngenic recipients without addition of cells do not reject the graft (n=4). Results are summarized in Table 1.

Conjunctival TGF-β mRNA levels: In unrelated keratoplasties without application of Tregs, the conjunctival samples underwent mRNA analysis on days 3 and 14 and the day of
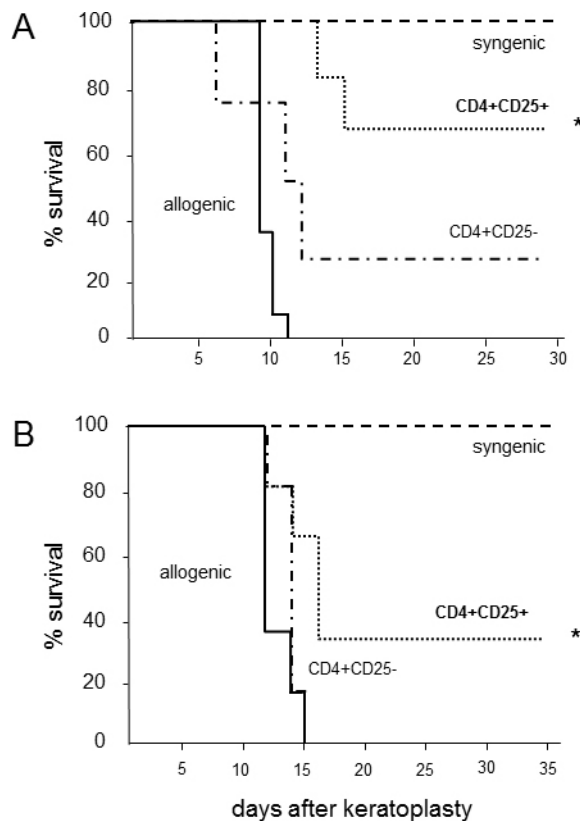

Figure 4. Graft survival after keratoplasty and s.c. Tregs. **A**: Graft survival after keratoplasty in 3-week-old recipients with subconjunctival Treg application. Application of Tregs led to 66.7% graft survival in 3-week-old recipients (n=6; *p<0.01), application of CD4+CD25- cells induced a graft survival of 25% and a median of POD11 (n=4), while without cells, grafts were rejected at POD9. Recipients of syngenic grafts did not reject until the end of the experiment on POD29 (n=8; *p<0.01). **B**: Graft survival after keratoplasty in 10-week-old recipients with subconjunctival application. In the control group (CD4+CD25-) the median of rejection was POD 12, in Treg group (CD4+CD25+) median was POD 16 and 37.5% of grafts survived POD35 (both n=6; *p<0.01) while in allogenic transplantations without addition of cells, rejection occurred on POD15 (n=4). In syngenic transplantations no rejection occurred until the end of the experiment on POD 35 (n=4).
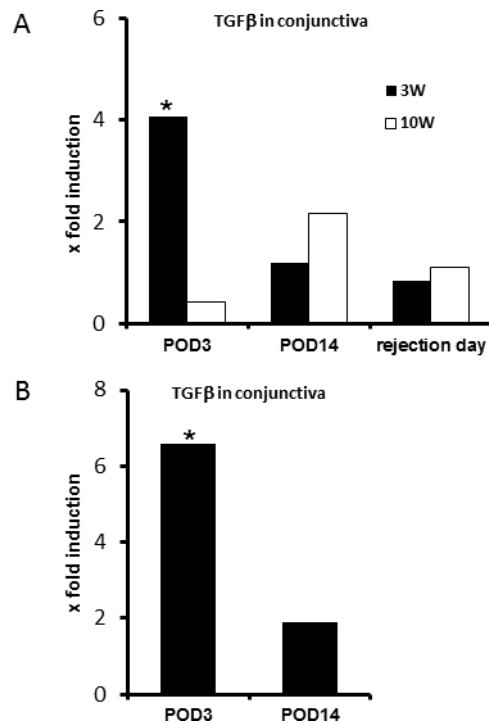
Figure 5. TGF-β in conjunctiva. **A**: Actin-corrected RT–PCR in conjunctival tissue of 3- and 10-week-old rats after keratoplasty, normalized to non-grafted eye. Transforming growth factor beta (TGF-β) transcription in the grafted eyes of 3-week-old rats is strongly induced on POD 3 (n=3, *p<0.01). **B**: Actin-corrected RT–PCR in conjunctival tissue after keratoplasty of the grafted eyes of 3-week-old rats, normalized to grafted eyes of the 10-week-old rats (n=3, *p<0.01).

rejection. The baby rats but not adult rats strongly induced conjunctival TGF-β mRNA on day 3 compared to the non-grafted eye (p<0.01, n=3; Figure 5A). Comparing TGF-β mRNA levels in the grafted eyes of the two cohorts, the baby rats revealed 6.6-fold higher levels than in the adult rats on day 3 after keratoplasty (p<0.01, n=3; Figure 5B).

DISCUSSION

In an analogy to humans, 3-week-old rats show accelerated transplant rejection compared to 10-week-old adult rats after keratoplasty [21]. Baby rats reject the cornea after a median of 9 days, while adult rats reject it after 15 days. Antigen-specific and FoxP3+ Tregs induce tolerance to an allogenic graft [20]. It has been shown in several settings of organ transplantation that recipients' Tregs can positively influence graft tolerance. However, corresponding data on cornea transplantation in young recipients are not available. We hypothesized that enhanced rejection in young recipients is associated with a lack or dysfunction of Tregs. To address possible general age-dependent differences, we characterized Treg counts and suppressive activity in 3- and 10-week-old Lewis recipient rats.

Baby rats exhibit fewer CD4 and similar FoxP3+ of CD4 cells compared to adult rats (Figure 1). The numbers are consistent with literature reporting a proportion of 5–10% regulatory T cells in mice, humans, and rats [27]. Fewer CD4

cells in baby rats can account for less pronounced adaptive immunity, but since CD4 cells consist of many different subpopulations with variable functions, a precise conclusion is difficult to draw.

Though numbers of Tregs were similar, the suppressive potential of Tregs may be age-dependent. To this end, we examined the ability of Tregs to suppress lymphocyte proliferation by adding Tregs of both age groups to activated lymphocytes and tracing cell divisions with CFSE. Both Treg cohorts showed the same ability to suppress lymphocyte proliferation (Figure 2). This characterization shows that Treg counts in recipient rats of the two age groups do not differ and the Treg inhibitory potential is equally strong in both groups.

CD4+CD25+FoxP3+ cells are essential for the balance between pro- and anti-inflammatory responses in the body. Two subsets of Tregs exist, the natural Tregs (nTregs), which are developed in the thymus, and induced Tregs (iTregs), which arise from peripheral conventional T cells [7]. iTregs have been shown to prevent human GvHD [28,29] when intravenously administered in patients. In many animal models of GvHD [30,31] and of solid organ transplantation, iTregs can induce tolerance [19]. The application route, specificity, and dose seem to be crucial in this issue. It has been established that FoxP3-expressing primed Tregs can abrogate rejection in a mouse model of corneal transplantation [19,32] and that Tregs are involved in allograft survival [32]. However, data

on the administration of a naïve Treg population to recipients of a corneal transplant have been missing.

We hypothesized that an additionally given naïve Tregs population can modulate rejection after keratoplasty. To address this issue, 10^5 cells were administered to the tail vein of baby rat recipients 2 day before keratoplasty. An insignificant delay in rejection was observed; the median rejection day was day 11 following keratoplasty in animals that received Tregs i.v. and day 9 in control animals. We cannot rule out that the median rejection day could increase in significance when a larger cohort is analyzed.

It has been reported that antigen-specificity and the application route of Tregs are crucial to tolerance development [33]. Thus, in a second attempt, Tregs were administered locally into the conjunctiva of the grafted eye. In this proximity to the antigen (allogenic corneal cells), functional induction of Tregs appears more probable. Rejection was delayed or abrogated in the baby and adult rats (Figure 4). The baby rats showed 66.7% graft survival, while the adult rats had only 33.3% surviving transplants. The more pronounced effect of Tregs in baby rats compared to the adult rats may be due to the use of equal cell numbers in both age groups, resulting in different dosages. The total number of effector T cells (Teffs) is higher in adult rats, so the influence of 10^5 Tregs may be smaller than in baby rats. We speculate that the residing Teff cell population in adult animals may have been too large to be efficiently regulated by 10^5 Tregs. In heart transplantation, it was shown that Treg to Teff ratios can influence graft rejection [34]. The poor effect of systemically administered Tregs may be a consequence of dilution as animals were not irradiated and resident Teffs could override the grafted Tregs. Cells may also be lost due to attached bovine serum albumin or preparatory antibodies, priming them for destruction.

Baby rats treated with CD4+CD25- cells also exhibited slightly delayed rejection. We used the surface markers CD4 and CD25 rather than intracellular FoxP3 to differentiate the Tregs from the non-Tregs. T cell plasticity may have allowed TGF-β-induced Treg transformation from CD4+FoxP3- cells [8,35]. Further CD4+ subsets known to be suppressive are Tr1 and Th3 cells [36].

Subconjunctivally applied Tregs improved cornea graft survival, and the beneficial effect was stronger in baby recipients. TGF-β is a crucial factor for converting T cells to Tregs in vitro [8]. Because the subconjunctival environment may influence administered cells, we quantified TGF-β mRNA levels by qPCR in conjunctival tissue. Baby rats showed stronger TGF-β transcription in response to cornea transplantation than adult rats (Figure 5). Although we did not

apply antigen-specific Tregs, the proximity to the graft and enhanced conjunctival levels of TGF-β may trigger suppressive Treg function. The fact that TGF-β is more expressed in the conjunctiva of young recipients and the proximity to the graft may explain the successful application of non-primed Tregs in this model. In baby rats, this effect may be enhanced, since TGF-β transcription increased rapidly after keratoplasty. At later time points, Tregs may have spread (and partly died), and cells could no longer be influenced by TGF-β in the conjunctiva. Our data strongly suggest that unprimed Tregs can be used in prophylactic therapy to prevent cornea graft rejection with a particular benefit in young recipients.

REFERENCES

1. Whittum JA, McCulley JP, Niederkorn JY, Streilein JW. Ocular disease induced in mice by anterior chamber inoculation of herpes simplex virus. Invest Ophthalmol Vis Sci 1984; 25:1065-73. [PMID: 6469490].

2. Thurau SR, Caspi RR, Chan CC, Weiner HL, Nussenblatt RB. Immunologic suppression of experimental autoimmune uveitis. Fortschritte Ophthalmol 1991; 88:404-7. [PMID: 1838532].

3. Hori S, Takahashi T, Sakaguchi S. Control of autoimmunity by naturally arising regulatory CD4+ T cells. Adv Immunol 2003; 81:331-71. [PMID: 14711059].

4. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155:1151-64. [PMID: 7636184].

5. Singh B, Read S, Asseman C, Malmström V, Mottet C, Stephens LA, Stepankova R, Tlaskalova H, Powrie F. Control of intestinal inflammation by regulatory T cells. Immunol Rev 2001; 182:190-200. [PMID: 11722634].

6. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev 2001; 182:18-32. [PMID: 11722621].

7. Lee HM, Bautista JL, Hsieh CS. Thymic and peripheral differentiation of regulatory T cells. Adv Immunol 2011; 112:25-71. [PMID: 22118406].

8. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 2004; 172:5149-53. [PMID: 15100250].

9. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998; 188:287-96. [PMID: 9670041].

10. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299:1057-61. [PMID: 12522256].

11. Hoffmann P, Ermann J, Edinger M. CD4+CD25+ regulatory T cells in hematopoietic stem cell transplantation. Curr Top Microbiol Immunol 2005; 293:265-85. [PMID: 15981484].

12. Wang XN, Haniffa MA, Holtick U, Collin MP, Jackson G, Hilkens CM, Holler E, Edinger M, Hoffmann P, Dickinson AM. T-cell suppression of CD8+ T-cell-mediated graft-versus-host reaction requires their presence during priming. Transplantation 2009; 88:188-97. [PMID: 19623013].

13. Safinia N, Leech J, Hernandez-Fuentes M, Lechler R, Lombardi G. Promoting transplantation tolerance; adoptive regulatory T cell therapy. Clin Exp Immunol 2013; 172:158-68. [PMID: 23574313].

14. Zinkernagel RM, Doherty PC. The discovery of MHC restriction. Immunol Today 1997; 18:14-7. [PMID: 9018968].

15. Lee RA, Gabardi S. Current trends in immunosuppressive therapies for renal transplant recipients. Am J Health Syst Pharm 2012; 69:1961-75. [PMID: 23135563].

16. Trzonkowski P, Szarynska M, Mysliwska J, Mysliwski A. Ex vivo expansion of CD4(+)CD25(+) T regulatory cells for immunosuppressive therapy. Cytometry A. 2009; 75:175-88. [PMID: 18855921].

17. Price FW, Whitson WE, Marks RG. Graft survival in four common groups of patients undergoing penetrating keratoplasty. Ophthalmology 1991; 98:322-8. [PMID: 2023753].

18. Althaus C, Sundmacher R. Keratoplasty in newborns with Peters' anomaly. Ger J Ophthalmol 1996; 5:31-5. [PMID: 8646177].

19. Chauhan SK, Saban DR, Lee HK, Dana R. Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation. J Immunol 2009; 182:148-53. [PMID: 19109145].

20. Hori J, Taniguchi H, Wang M, Oshima M, Azuma M. GITR ligand-mediated local expansion of regulatory T cells and immune privilege of corneal allografts. Invest Ophthalmol Vis Sci 2010; 51:6556-65. [PMID: 20702832].

21. Schwartzkopff J, Berger M, Birnbaum F, Böhringer D, Reinhard T. Accelerated corneal graft rejection in baby rats. Br J Ophthalmol 2010; 94:1062-6. [PMID: 19726423].

22. Mayer K, Reinhard T, Reis A, Niehues T, Claas FH, Sundmacher R. Differential contribution of natural killer cells to corneal graft rejection in 3-week-old versus mature rats. Transplantation 2003; 76:578-82. [PMID: 12923447].

23. Reis A, Megahed M, Reinhard T, Godehardt E, Braunstein C, Sundmacher R. Synergism of RAD and cyclosporin A in prevention of acute rat corneal allograft rejection. Cornea. Januar 2002; 21:81-4. .

24. Reis A, Reinhard T, Sundmacher R, Braunstein C, Godehardt E. Effect of mycophenolate mofetil, cyclosporin A, and both in combination in a murine corneal graft rejection model. Br J Ophthalmol. Juni 1998; 82:700-3. .

25. Schwartzkopff J, Schlereth SL, Berger M, Bredow L, Birnbaum F, Böhringer D, Reinhard T. NK cell depletion delays corneal allograft rejection in baby rats. Mol Vis 2010; 16:1928-35. [PMID: 21031017].

26. Inoue H, Ohsawa I, Murakami T, Kimura A, Hakamata Y, Sato Y, Kaneko T, Takahashi M, Okada T, Ozawa K, Francis J, Leone P, Kobayashi E. Development of new inbred transgenic strains of rats with LacZ or GFP. Biochem Biophys Res Commun 2005; 329:288-95. [PMID: 15721305].

27. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity 2009; 30:626-35. [PMID: 19464985].

28. Trzonkowski P, Bieniaszewska M, Juścińska J, Dobyszuk A, Krzystyniak A, Marek N, Myśliwska J, Hellmann A. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. Clin Immunol 2009; 133:22-6. [PMID: 19559653].

29. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, Defor T, Levine BL, June CH, Rubinstein P, McGlave PB, Blazar BR, Wagner JE. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood 2011; 117:1061-70. [PMID: 20952687].

30. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, Negrin RS, Fathman CG, Strober S. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. Blood 2005; 105:2220-6. [PMID: 15546950].

31. Tsang JY, Tanriver Y, Jiang S, Leung E, Ratnasothy K, Lombardi G, Lechler R. Indefinite mouse heart allograft survival in recipient treated with CD4(+)CD25(+) regulatory T cells with indirect allospecificity and short term immunosuppression. Transpl Immunol 2009; 21:203-9. [PMID: 19446634].

32. Guo X, Jie Y, Ren D, Zeng H, Zhang Y, He Y, Pan Z. In vitro-expanded CD4(+)CD25(high)Foxp3(+) regulatory T cells controls corneal allograft rejection. Hum Immunol 2012; 73:1061-7. [PMID: 22939904].

33. Semple K, Yu Y, Wang D, Anasetti C, Yu XZ. Efficient and selective prevention of GVHD by antigen-specific induced Tregs via linked-suppression in mice. Biol Blood Marrow Transplant 2011; 17:309-18. [PMID: 21224010].

34. Mirabet S, Gelpí C, Roldán C, Brossa V, Mendoza CA, Lopez L, Molto E, Alvaro Y, Martinez V, Padró JM, Roig E. Assessment of immunological markers as mediators of graft vasculopathy development in heart transplantation. Transplant Proc 2011; 43:2253-6. [PMID: 21839249].

35. Chen W, Wahl SM. TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. Cytokine Growth Factor Rev 2003; 14:85-9. [PMID: 12651220].

36. Okamura T, Fujio K, Sumitomo S, Yamamoto K. Roles of LAG3 and EGR2 in regulatory T cells. Ann Rheum Dis 2012; 71:Suppl 2i96-100. [PMID: 22460149].