Subconjunctivally applied naïve Tregs support corneal graft survival in baby rats

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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
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<sup>lv1</sup>) and Lewis (Rt1<sup>l</sup>) 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<sub>2</sub> inhalation. All groups and treatments are shown in Table 1.

Corneal transplantation: Orthotopic penetrating keratoplasties 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 interrupted sutures (11.0 Ethilon; Ethicon, Norderstedt, Germany). A blepharorraphy was applied for 3 days.

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

| 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 hyphaema 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 KH₂PO₄, 138 mM NaCl, 8.0 mM Na₂HPO₄·7H₂O, 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 1×10⁶ 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 disected in 100 µm cell strainer and washed with PBS. 5×10⁶ 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 10⁶ 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.

**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 TG-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
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..."
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 transplantation 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).
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, 10⁶ cells were administered to the tail vein of baby rat recipients 2 days 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 (allogeneic 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 10⁶ 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 10⁶ 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 overcome 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 Trl and Th3 cells [36].

Subconjunctivally applied Tregs improved corneal 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 corneal graft rejection with a particular benefit in young recipients.

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