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ORIGINAL ARTICLE

Efficacy of single-agent immunosuppressive regimens in a murine model of vascularized composite allotransplantation

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SUMMARY

We herein investigate the safety and efficacy of single-agent anti-rejection regimens in a mouse vascularized composite allotransplantation (VCA) model. Orthotopic hind-limb transplantations (Balb/c → C57BL/6) were performed using 6- to 8-week-old mice. A thirty-day regimen of either rapamycin, tacrolimus (both 1, 3, 5 mg/kg/day) or cyclosporine (25, 35, 50 mg/kg/day) was used. Primary endpoints were animal and graft survival, and secondary chimerism and regulatory T-cell levels. For rapamycin and tacrolimus given at 1, 3, and 5 mg/kg/day, median graft survival time (MST) was 23 days (18–28 days), 30 days (23–30 days), and 30 d (30–30 days) and 14 days (13–18 days), 30 days (16–30 days), and 30 days (30–30 days), respectively. For cyclosporine dosed at 25 and 35 mg/kg/day, MST was 15 days (12–18 days) and 21 days (14–27 days). Toxicity from CsA 50 mg/kg led to 100% mortality. Mixed chimerism levels were higher in rapamycin-treated animals than in tacrolimus-treated recipients ($P = 0.029$). Tacrolimus was superior in preventing leukocyte recruitment to the allograft. In murine VCA, no standardized immunosuppressive regimen exists, limiting comparability of outcomes and survival. Our data demonstrate that rapamycin and tacrolimus maintenance treatment at 5 mg/kg/day both yielded allograft survival (<grade 3 rejection) in all animals. Rapamycin displayed less toxicity and maintained mixed chimerism but was not as potent in controlling leukocyte recruitment compared with tacrolimus.

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Key words
chimerism, immunosuppression, mouse, solid organ transplantation, tissue transplantation, vascularized composite allotransplantation

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Introduction

The late 1990s featured the first attempts at composite tissue transplantation, such as hand and upper extremity transplantation, which, in contrast to solid organs, comprise different tissue types with varying levels of antigenicity including bone, muscle, vessel, nerve, and skin [1,2]. Thus far, vascularized composite allotransplantion

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(VCA) has clinically primarily been used in the context of hand, upper extremity, face, abdominal wall, uterus, and penis transplantation [3]. Over the past twenty years, VCA as a field has matured significantly into a treatment option for the reconstruction of severe tissue defects that cannot be managed conventionally [3,4]. As with solid organ transplantation, the success of VCA relies heavily on effective immunosuppressants that permit acceptance of heterogeneous tissue allografts once deemed too immunogenic to be maintained on conventional immunosuppression [5].

Historically, preclinical *in vitro* and *in vivo* models have driven innovation in transplantation science. Murine models dominate in current preclinical *in vivo* experimentation due to their cost-effectiveness and the relatively wide availability of transgenic and knockout strains that allow in-depth mechanistic analysis of various molecular pathways [6]. Despite widespread use of the murine model, there is no accepted immunosuppressive strategy with a well-defined dosing regimen, route of administration, or side effect profile. In the absence of consensus and appropriate references, immunosuppressive drugs are often dosed according to human clinical protocols. This fails to account for interspecies metabolic variation, leading to high rates of graft loss due to acute or chronic rejection and high morbidity and mortality in experimental animals from toxicity alone [7,8]. Further, studies are difficult to compare given variability in immunosuppressive regimens.

Our group has pioneered innovation in the creation and advancement of animal models across multiple organ systems [9] including cardiac [10], en-bloc chest wall, heart and thymus [11], pancreatic [12], abdominal wall [13], penile [14], hind-limb [15-17], hemi-face [18], and forelimb [19,20] transplantation. The goal of this study was to share our experience in the use of single-agent immunosuppression in murine VCA models through a review of the literature.

Methods and materials

Experimental animals

A 6- to 8-week-old male Balb/c (H-2K^{d}) served as donors and C57BL/6 (B6; H-2K^{b}) and B6.129(Cg)-Foxp3$^{tm3(DTR/GFP)Ayr}$/J (Foxp3^{DTR}) as recipients. All animals were purchased from Jackson laboratory (Bar Harbor, ME) and housed under standard conditions with unrestricted access to water and food. All experiments were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine (#MO19M240).

Orthotopic hind-limb transplantation

Balb/C hind limbs were transplanted into C57BL/6 and Foxp3^{DTR} recipients using a modified version of the technique previously described [16,17], outlined in brief below.

Donor procedure

The donor animal is sedated with 4% isoflurane inhalation (2% maintenance). After shaving and disinfection, a circumferential skin incision is made the groin. The femoral vessels are exposed and carefully isolated. The vessels are then ligated at the level of the inguinal ligament and transected, and the muscle groups of the hind limb and femur are transected at mid-thigh level. The graft is then flushed with 1 ml of heparinized saline, and polyimide cuffs are placed on the femoral vein and artery. Until transplantation, the graft is wrapped in gauze and stored at 4 °C.

Recipient procedure

The recipient animal is identically sedated and prepared, and the same exposure is obtained. After dissection of the femoral vessels, the vessels are clamped and transected distally. The hind-limb graft is placed at its anatomical site using a 20-gauge intramedullary rod (BD Needles; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and absorbable 6-0 sutures (Polysorb®; Covidien, Dublin, Ireland) to reapproximate the musculature. The femoral vessels are reconnected via a non-suture cuff technique. After visual confirmation of blood flow, the skin is closed with non-absorbable nylon sutures (6-0 Ethilon®; Ethicon Inc., Somerville, NJ, USA). All animals received 0.1 mg/kg buprenorphine and 200 µl of enrofloxacin (Enroflox®; Norbrook Laboratories, Newry, UK) via subcutaneous (s.c.) injection. Animals are monitored on a heating pad until they were fully recovered before being returned to the animal housing facility. Using the previously described 4-grade rejection scale, we defined Grade 3 (skin epidermolysis) as the endpoint in this study (Fig. 1).

Immunosuppressive regimens and depletion of regulatory T cells

Starting at the day of hind-limb transplantation, all animals were treated with daily intraperitoneal (i.p.) injections of a single regimen of either cyclosporine (CsA; #C-6000; LC Laboratories, Woburn, MA, USA),

rapamycin (Rapa; #R-5000; LC Laboratories), or tacrolimus (Tac; Tecoland Corporation, Irvine, CA, USA) for 30 days. Dosages for CsA ranged from 25 to 35 and 50 mg/kg. For Rapa and Tac, dosages of 1, 3, and 5 mg/kg were tested. To deliver the immunosuppressants, an injection vehicle comprising tween 80 (2.3%; Sigma-Aldrich, Inc., Merck KGaA, Germany), polyethylene glycol average M_n 400 (26%; Sigma-Aldrich, Inc., Merck KGaA, St. Louis, MO, USA), and distilled water (71.7%) was used for a final volume of 200 μl per injection. In Foxp3^{DTR} recipients, depletion of regulatory T cells (Treg) was performed with an intraperitoneal injection of 1 μl of diptheria toxin (DT) on postoperative day (POD) 14 and 15.

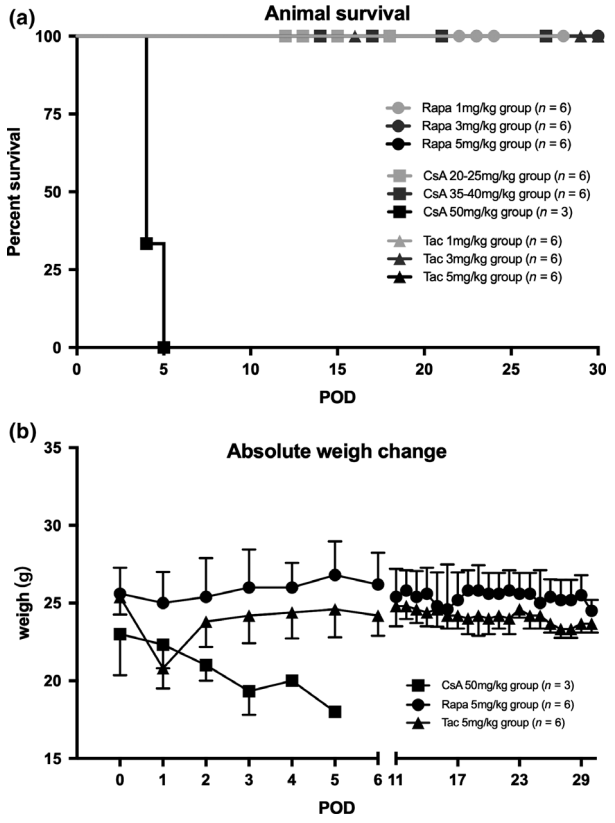

Figure 1 (a) Animal survival after orthotopic hind-limb allotransplantation and daily i.p. immunosuppressive therapy with rapamycin (1, 3, and 5 mg/kg/day), cyclosporine A (25, 35, and 50 mg/kg/day), and tacrolimus (1, 3, and 5 mg/kg/day; n = 5/group). All groups showed excellent animals' survival with the exception of cyclosporine 50 mg/kg/day. This dosage lead to animal death at day 4 (4–5). (b) Absolute weight change from pretransplant baseline weight. In the high-dose regimens, significant weight changes were observed in cyclosporine 50 mg/kg/day and tacrolimus 5 mg/kg/day treated animals. However, cyclosporine lead to a slow but progressive weight loss, which eventually contributed to animal death, tacrolimus showed a sharp decrease on POD 1 and quickly stabilized thereafter. CsA, cyclosporine A; g, gram; POD, postoperative day; Rapa, rapamycin; Tac, tacrolimus.

All animals were monitored daily for signs of rejection (Fig. S1), side effects of the tested immunosuppressants, and weight change. The grafts were also followed with photo-documentation weekly and upon reaching the endpoint using a Nikon COOLPIX P7100 camera (Nikon, Minato, Japan). The endpoint of this study was POD 30 or rejection grade 3 (epidermal sloughting) as VCA grafts are assessable by visual inspection. Lower rejection grades (erythema and/or edema) were defined as functioning grafts.

Flow cytometry analysis of mixed chimerism and regulatory T cells

To distinguish donor from host mononuclear leukocytes, flow cytometry was performed on a BD™ LSR II cytometer (BD Biosciences, San Jose, CA, USA) with subsequent analysis with FLOWJO software (version 10.5.3). The percentage of donor cells circulating in host peripheral blood was calculated as described previously [21,22]. For mixed chimerism analysis, mouse peripheral blood was incubated after red blood cell (RBC) lysis with fluorescein isothiocyanate (FITC)-conjugated mAb directed against H-2d (SF1-1.1), PerCP-Cy5.5-conjugated anti-mouse CD3e (145-2C11), PE-conjugated B220 (RA3-6B2), and Alexa Flour 647-conjugated CD11b (M1/70) along with a non-Ag-specific FcγR-related binding blocker (2.4G2). Lymphocytes, granulocytes, and monocytes were initially identified via FSC/SSC ratio, then further selection via respective monoclonal antibody staining was conducted to identify B cells, T cells, and monocytes. Lineage-specific chimerism was defined as the percentage of H-2d$^+$ cells in their respective subset (Fig. 2a). Overall chimersim was defined as the percentage of all leukocytes that were found to be donor derived. For quantification of regulatory T cells (Fig. 2b), mouse peripheral blood was incubated after RBC lysis and permeabilization with APC-Cy7-conjugated CD3 (145-2C11), PE Cy7-conjugated CD4 (RM4.5), PerCP Cy5-conjugated Foxp3 (FJK-16s), fluorescein isothiocyanate (FITC)-conjugated mAb directed against H-2d (SF1-1.1), and a non-Ag-specific FcγR-related binding blocker (2.4G2). All mAbs were purchased from BD Pharmingen, BioLegend, and eBioscience. Gating strategies are shown in Fig. S2.

Histopathology

Upon reaching the study endpoint, skin samples of the transplanted hind limb were collected and animals were euthanized by CO_2 inhalation. Samples were fixed in

Transplant International 2020; 33: 948–957

10% neutral buffered formalin. Fixed tissues were further processed to paraffin in graded alcohols, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). All slides were reviewed by an expert veterinary pathologist (SEB) in a blinded fashion.

Statistical analysis

Results are expressed as median and range. Mann–Whitney *U* test or Kruskal–Wallis test was used for inter-group comparison. Kaplan–Meier survival curves and log-rank test were used to determine significance of differences in graft survival between groups. A two-sided *P*-value of < 0.05 was considered statistically significant. Prism Software package (GRAPHPAD Software 7.0; GraphPad, San Diego, CA, USA) was used for all statistical tests.

Results

Animal survival

No animal was lost in the Rapa- or Tac-treated groups irrespective of dosage. CsA, however, led to a substantial post-transplant mortality when administered at a dose of 50 mg/kg/day. All animals treated in this group died within 5 days after hind-limb transplantation, resulting in a statistically significant increased mortality ($P = 0.0046$) compared with all other treatment regimens (Fig. 1a, Table S1).

High doses of immunosuppressive agents resulted in differing weight changes from pretransplant baseline (Fig. 1b). In animals treated with Rapa 5 mg/kg/day, no weight changes were observed. In the Tac 5 mg/kg/day group, an initial drop of body weight was seen in the early postoperative phase with a median weight loss of 5 g (4–5 g) on POD 1, 2 g (1–2 g) on POD 2, and 1 g (0–2 g) on POD 3. This initial weight change did not result in an increase in mortality and subsequently stabilized. In contrast, animals treated with CsA 50 mg/kg/day presented with a slower but continuous weight drop. In this group, animals experienced weight changes starting from POD 2, when a median weight loss of 2 g (1–2 g) was observed. Weight loss increased over time with 3 g (3–5 g) on POD 3 and 6 g (6–6 g) on POD 4.

Graft survival

Untreated allografts showed a median graft survival of 8.5 (7–10) days (Fig. 2a). Treatment with Rapa 1 mg/

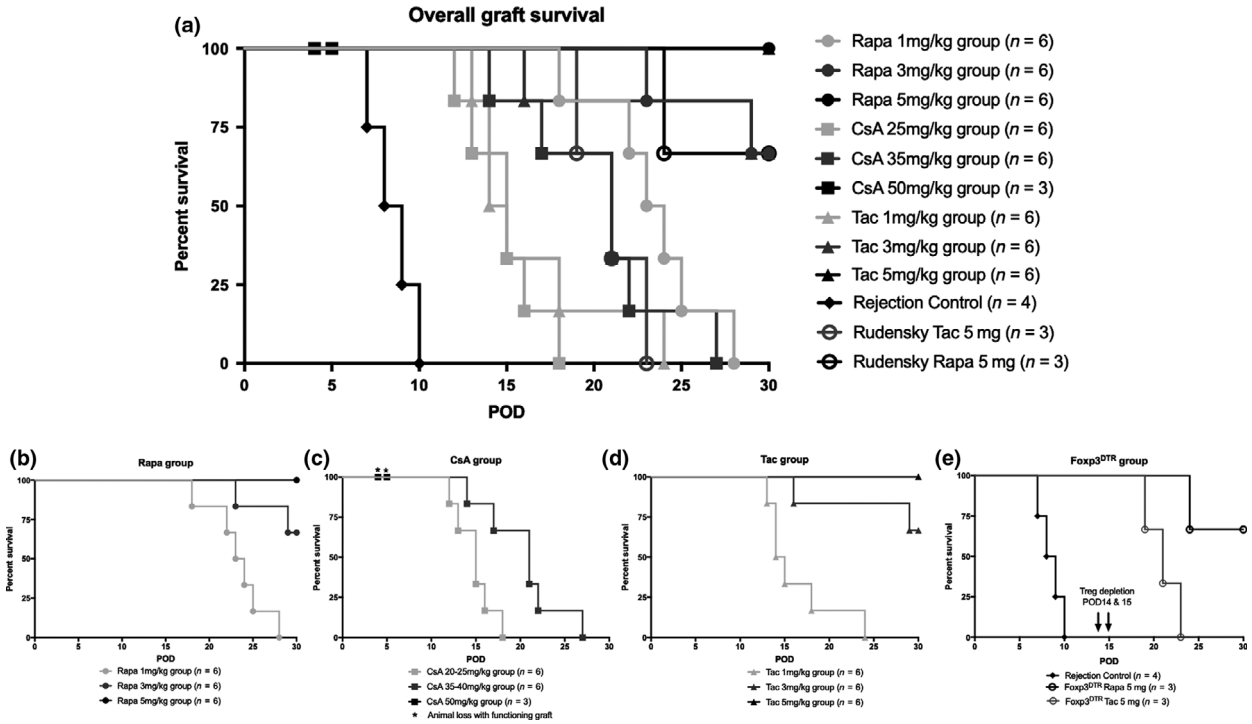

Figure 2 Overall, allograft survival (a), agent-specific allograft survival (b–d) and allograft survival after depletion of regulatory T cells on POD 14 (e). Rapamycin 5 mg/kg/day and tacrolimus 5 mg/kg/day were the only two immunosuppressive regimens that lead to 100% graft survival until POD 30. CsA, cyclosporine A; POD, postoperative day; Rapa, rapamycin; Tac, tacrolimus.

kg/day resulted in an extended median survival (MST) of 23 days (18–28 days), Rapa 3 mg/kg/day had a MST of 30 days (23–30 days), and, in the Rapa 5 mg/kg/day group, all grafts survived until the endpoint at POD 30 (Fig. 2a,b). Recipient treatment with CsA 25 mg/kg/day resulted in an MST of 15 days (12–18 days), and CsA 35 mg/kg/day yielded a MST of 21 days (14–27 days). CsA 50 mg/kg/day lead to early mortality without signs of rejection (Fig. 2a,c). Tac 1 mg/kg prevented allograft rejection for a median of 14 days (13–18 days), Tac 3 mg/kg/day for 30 days (16–30 days), and, in the Tac 5 mg/kg/day group, all grafts survived until POD 30 (Fig. 2a,d).

With 100% graft survival at POD 30, the Rapa and Tac 5 mg/kg/day groups displayed a statistically significant superior graft survival compared with CsA 25 and 35 mg/kg/day (both $P = 0.0005$) as well as Tac 1 mg/kg/day and Rapa 1 mg/kg/day (both $P = 0.0005$). No statistically significant difference in allograft survival was seen compared with Tac 3 mg/kg/day and Rapa 3 mg/kg/day (both $P = 0.14$).

Upon reaching the endpoint at POD 30, Tac 5 mg/kg/day treated animals displayed a macroscopically unaltered or minimally erythematous (grade 1) appearance. Histology showed no intragraft infiltrating cells and thus had a median rejection grade of 0 (0–0) (Fig. 3a,b). Rapa 5 mg/kg/day treated animals displayed macroscopic features of grade 1–2 rejection, and histology showed a median grade 2 (1–2) rejection at POD 30 (Fig. 3c,d).

Depletion of regulatory T cells on POD 14/15 (Fig. 2a,e) resulted in a significantly compromised graft survival in Tac 5 mg/kg/day treated animals with a MST of 21 (19–23; log-rank $P = 0.0018$) compared with nondepleted animals. The depletion of Tregs in this group leads to accelerated and aggravated rejection resulting in impaired graft survivial. Even though graft survival was worse in depleted Rapa 5 mg/kg/day treated animals [MST 30 (24–30); log-rank $P = 0.16$], this did not reach statistical significance. Statistical analysis of graft survival is presented in Table S1.

Hematopoietic chimerism

As hind-limb grafts carry vascularized bone marrow, peripheral mixed chimerism levels were measured to investigate the effect of conventional immunosuppression on their dynamics. In rejecting animals, overall chimerism levels dropped significantly with progressive allograft rejection ($P = 0.0002$). While median overall chimerism levels were 0.9% (0.8–1.2%) at POD 3, they significantly dropped to 0.4% (0.4–0.6%) and 0.1% (0.05–0.2%) on POD 5 and 7, respectively (Fig. 4).

Treatment with Rapa 1 and 5 mg/kg/day resulted in overall respective median mixed chimerism levels of 5.2% (4.3–7.2%) and 3.5% (2.0–10.0%) at POD 7 (Fig. 4). At POD 14, these levels dropped to 1.6% (0.8–8.6%) and 1.4% (0.3–10.4%) for Rapa 1 and 5 mg/kg/day, respectively. Thereafter, the mixed chimerism levels stayed relatively stable until POD 28 with 1.4% (1 mg/kg/day: 0.4–1.9%; 5 mg/kg/day: 0.6–7.9%) seen in both groups. Rapa 3 mg/kg/day displayed lower overall chimerism levels, which peaked on POD 14 with 1.8% (1.1–3.2%) (Fig. 4). Monocyte chimerism was most prominent in all three dosage regimens (Fig. S3c–e).

CsA 25 and 35 mg/kg/day both showed macrochimerism (defined as multi-lineage chimerism levels> 1%) [23], which was stable until allograft rejection. For CsA 25 mg/kg/day, median overall chimerism levels were 1.3% (0.3–5.9), 1.3% (0.34–3.7%), and 1.9% (1.2–2.6%) on POD 7, 14, and 21, respectively. CsA 35 mg/kg/day showed chimerism levels of 1.0% (0.2–6.0%), 1.4% (0.4–2.2%), and 1.9% (0.2–2.9%) on POD 7, 14, and 21, respectively (Fig. 4). Similar to rapamycin, the monocyte lineage was predominant source of mixed chimerism (Fig. S3a,b).

Tac-treated animals showed the lowest overall chimerism levels. Animals treated with Tac 1 mg/kg/day showed median overall chimerism levels of 2.1% (0.3–4.9%), 0.6% (0.2–1.1%), and 1.6% (1.6–1.6%) at POD 7, 14, and 21, respectively (Fig. 4). Tac 3 mg/kg/day displayed macrochimerism levels at POD 7 of 1.1% (0.02–2.8%), but they subsequently dropped to 0.5% (0.1–1.4%), 0.3% (0.2–0.6%), and 0.2% (0.1–0.3%) on POD 14, 21, and 28. The Tac 5 mg/kg/day group was the only group that did not display overall macrochimerism at any time point. Mixed chimerism levels were 0.8% (0.1–1.6%), 0.7% (0.2–0.8%), 0.4% (0.2–0.7%), and 0.3% (0.1–0.6%) at POD 7, 14, 21, and 28 (Fig. 4). In contrast to the other groups, Tac 5 mg/kg/day treated animals initially showed a predominance of T-cell chimerism (Fig. S3f–h). When comparing overall chimerism levels in Rapa and Tac 5 mg/kg/day, the two groups with 100% graft survival until the endpoint at POD 30, Rapa displayed higher overall chimerism rate compared with Tac-treated animals (POD7, $P = 0.0079$; POD 14, $P = 0.4$; POD 21, $P = 0.3$; POD28, $P = 0.032$; Fig. 4). After Treg depletion in Foxp3^{DTR} recipients, a trend towards lower overall chimerism levels compared with nondepleted animals was observed for the Rapa and Tac 5 mg/kg/day

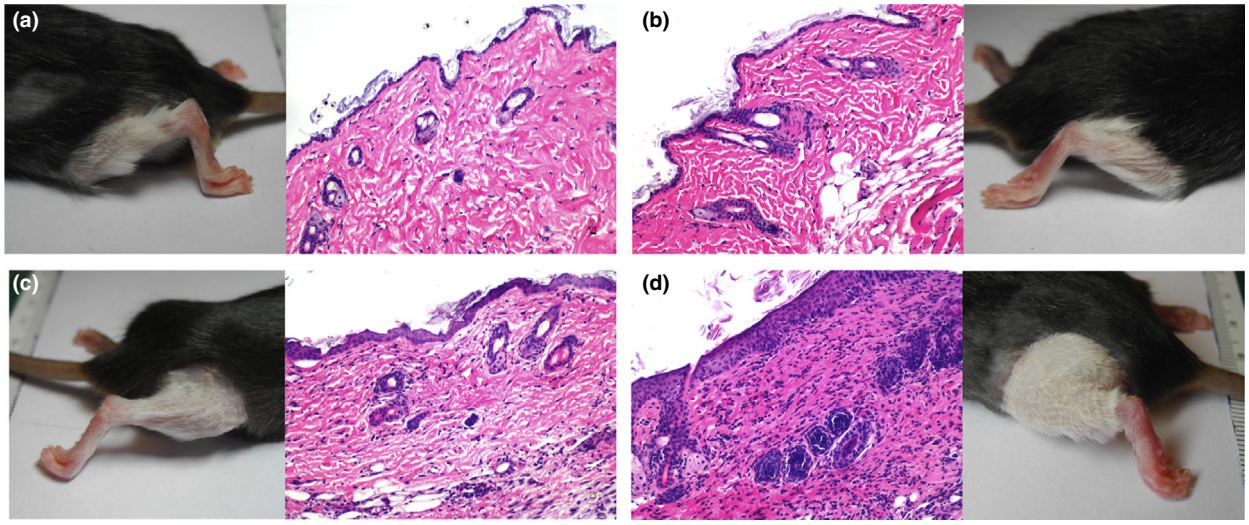

Figure 3 Marcoscopic and corresponding histopathologic assessment of graft skin at POD 30 using tacrolimus or rapamycin 5 mg/kg/day. Overall, both regimens allowed for allograft preservation (macroscopic < grade 3 rejection) in all animals at POD 30 (n = 6/group). In tac 5 mg/kg/day treated animals, macroscopic as well as histopathologic assessment revealed grade 0 rejection consistent with stable appearance of the tissue without leukocyte infiltration or other structural changes at POD 30 (a,b). Rapamycin-treated animals, in contrast, showed macroscopic signs of up to grade 2 rejection upon reaching the endpoint (c). Histology revealed a median grade 2 (b,c) rejection with a variable degree of leukocyte infiltration and epithelial hyperplasia. POD, postoperative day.

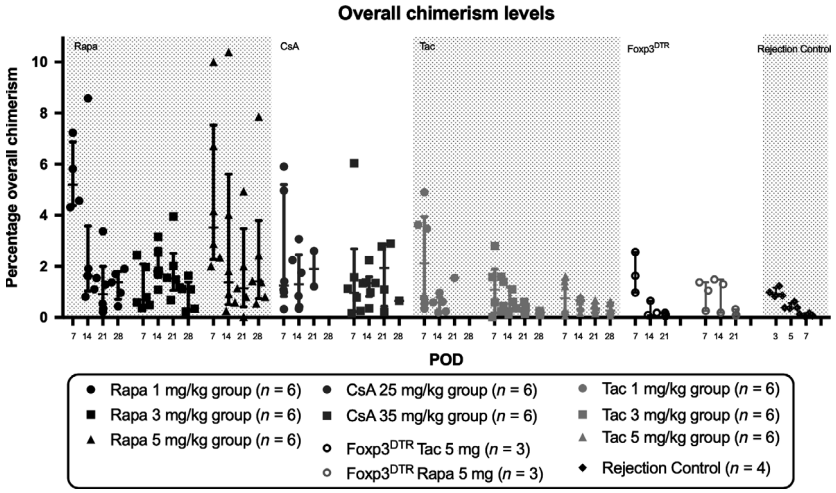

Figure 4 Overall chimerism levels on POD 7, 14, 21, and 28 for rapamycin, cyclosporin, tacrolimus, and Foxp3^{DTR} animals (n = 3–6/group and time point). POD, postoperative day.

groups with chimerism levels of 0.1% (0.08–0.3) compared with 1.1% (0.03–4.9; P = 0.4) and of 0.1% (0.06–0.2) compared with 0.4 (0.2–0.7; P = 0.071) on POD 21 (4). Corresponding to the decrease in overall chimerism levels, lineage-specific chimerism also decreased after depletion (Fig. S3i,j). An overview on overall and lineage-specific chimerism levels is shown in Tables S2 and S3.

Regulatory T cells

In rejecting animals, levels of Tregs significantly correlated with progression of rejection (POD 3 10.7% [9.7–11.7], POD 5 11.35% [9.9–13.2], POD 7 7.2% [6.6–9.9]; P = 0.044). In animals treated with a single immunosuppressive agent, median levels of Tregs ranged between 5.9% and 11.9% (3.05–14.2%) on POD 7 (P = 0.60). While Rapa 5 mg/kg/day treated animals displayed the lowest percentages, Tac 5 mg/kg/day

treated Foxp3^{DTR} animals demonstrated the highest (Fig. 5). By POD 14, all investigated groups expressed comparable median Treg levels (Rapa 5 mg/kg/day: 12.1% [7.2–23.4], Tac 5 mg/kg/day: 11.2 [6.7–16.7], Foxp3^{DTR} Rapa 5 mg/kg/day: 10.6% [7.3–11.1], Foxp3^{DTR} Tac 5 mg/kg/day: 11.9% [9.5–13]; $P = 0.63$). Treg depletion of Foxp3^{DTR} animals was confirmed by flowcytometry on POD16 (Foxp3^{DTR} Rapa 5 mg/kg/day: 0.2% [0.0–0.4], Foxp3^{DTR} Tac 5 mg/kg/day: 0.1% [0.2–0.3]). On POD21, the Treg percentages in Foxp3^{DTR} animals remained low (Foxp3^{DTR} Tac 5 mg/kg/day: 0.5% [0.2–0.6]; Foxp3^{DTR} Rapa 5 mg/kg/day: 1.5% [1.2–1.7]; $P = 0.0029$) (Fig. 5) while similarly high levels were seen in Rapa and Tac 5 mg/kg/day treated animals (Rapa 5 mg/kg/day: 11.3% [7.2–15.1], Tac 5 mg/kg/day: 11.2 [10.5–14.8]). On POD 28, percentages of Tregs were higher in Rapa 5 mg/kg/day treated animals at 14.6% compared with 10.3% (6.4–16.4%; $P = 0.09$) in Tac 5 mg/kg/day treated and 8.7 % (6.3–16.8%; $P = 0.17$) in wild-type C57BL/6 animals (Fig. 5).

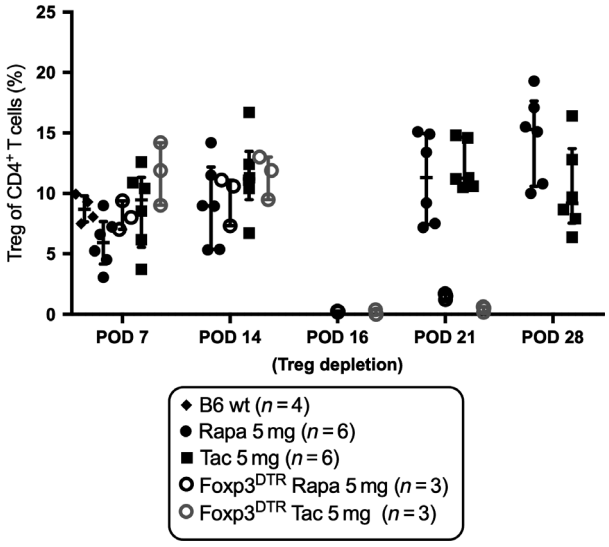

Figure 5 Percentage of CD25^{+}Foxp3^{+} cells (regulatory T cells; Treg) of all CD4^{+} cells at POD 7, 14, 21 and 28 for wild-type and Foxp3^{DTR} animals treated with Rapa and Tac 5 mg/kg/day and naïve B6 (B6 wt; n = 4–6/group). Treg depletion in Foxp3^{DTR} animals was confirmed on POD 16 (n = 3/group). POD, postoperative day, Rapa, rapamycin; Tac, tacrolimus; wt, wild-type.

Discussion

The murine hind-limb transplant model is one that is well-validated and has been published extensively by our group and others [9,17,24]. The extreme inconsistency within regimens, dosing, and survival highlights the fact that no standardized treatment has been reported to enable graft survival and subsequent mechanistic studies in VCA.

In our study, three different single reagents with three different dosages were tested. Dosages were based on the experience in various murine transplant models previously carried out by our group and on the current literature. In addition to animal and graft survival data, weekly mixed chimerism levels and percentages of peripheral blood regulatory T cells were collected. As early postoperative mortality was observed in higher dose regimens, animals were initially started on a reduced dose immunosuppressive regimen. For Tac, 0.5 mg/kg was given on POD 0 and 1, for CsA, 25 mg/kg was given on POD 0 and 1, and for Rapa, 1 mg/kg was given on POD 0. Thereafter, all animals received full doses of immunosuppression. With the exception of CsA 50 mg/kg, which resulted in toxicity and significant mortality, this initial low dosing prevented animal death for all other regimens. In the Tac-treated group, severe weight loss was observed in the initial postoperative period which may represent toxicity associated with this regimen. In contrast to CsA, however, this did not result in increased mortality. Though problematic in the clinical setting, we did not observe any differences in wound healing using Rapamycin, an observation similar to the of other immunosuppressive agents in this study.

The present study demonstrates MSTs of 14 days, 30 days (60%), and 30 days (100%) for Tac 1, 3, and 5 mg/kg/day and of 23 days, 30 days (80%), and 30 days (100%) for Rapa 1, 3, and 5 mg/kg/day, respectively. Kim et al performed an heterotopic cervical hind-limb transplant model using a fully mismatched strain combination (Balb/C [H-2^{d}] to C57BL/6 [H-2^{b}]). With daily subcutaneous injections of tacrolimus 3 mg/kg they reported, similarly to our results, a prolonged graft survival up to 30 days with 90% animal survival [25]. Xu *et al.* performed orthotopic fore-limb transplantations using the same strain combination and treated the animals with either IL-2/anti-IL-2 complexes (IL-2C), tacrolimus 1 mg/kg/day, and rapamycin 2 mg/kg/day or a combination of IL-2c and Tac or Rapa. Comparable to our experience, single-agent immunosuppression maintained the allograft rejection until POD 11–12 with i.p. Tac 1 mg/kg/day. In Rapa 2 mg/kg/day treated animals, their reported graft survival was 13–16 days fewer than to our results; this difference might be explained by the s.c. route of administration in the study. Combination of IL-2c and Rapa but not Tac led to a significantly prolonged allograft survival of 31–34 days or 46–51 days depending on time point (post- and pretransplant) of IL-2c injection. Interestingly, co-administration of IL-2c and Tac revoked the

effects of IL-2c administration. In those animals, a similar rejection pattern was seen as in Tac-only-treated animals [26]. Yan *et al*. performed orthotopic hind-limb transplants in different strain combinations (Balb/c [H-2^{Kd}] → Thy1-YFP [C57BL/6, H-2^{Kb}]; C57BL/6 [H-2^{Kb}] → S100-GFP [B6D2F1/J, H-$2K^{b/d}$]; S100-GFP [B6D2F1/J, H-$2K^{b/d}$] → C57BL/6 [H-2^{Kb}]) and saw that daily treatment with tacrolimus at 2 mg/kg/day, irrespective of combination, resulted in signs of rejection after 14 days and full necrosis after 28 days [27]. Lin *et al*. investigated osteomyocutaneous allografts (Balb/C [H-2^{d}] to C57BL/6 [H-2^{b}]) that were treated with rapamycin 3 mg/kg for the first 7 days after transplant and then every other day for three weeks. With this regimen, they reported an allograft survival of 45.8 ± 7.1 days, distinctively longer than the one seen in our study [28]. Zhu *et al*. treated heterotopic cervical hind-limb transplants i.p. with cyclosporine 3 mg/kg/day. This subtherapeutic dose did not prolong allograft survival, as median graft survival was with 7.2 days comparable to the survival of untreated animals in our group [29].

Mixed chimerism is a critical feature of murine tolerogenic VCA models and has been shown to correlate with allograft acceptance [23,30]. As the hind-limb transplant model includes vascularized bone marrow, peripheral mixed chimerism levels were measured. In our study, the progression of rejection correlated with the decline of mixed chimerism level ($P = 0.0002$) in untreated animals (Figs 2a and 4). Overall, mixed chimerism levels were higher in Rapa 5 mg/kg/day treated animals when compared to Tac 5 mg/kg/day treated ones ($P = 0.029$). Lower dosages of Rapa and Tac resulted in higher overall chimerism levels on POD7 (Rapa 1 mg/kg/day 5.8%, Rapa 3 mg/kg/day 1.2%, and Rapa 5 mg/kg/day 2.9%, $P = 0.0017$; Tac 1 mg/kg/day 2.1%, Tac 3 mg/kg/day 1.1%, and Tac 5 mg/kg/day 0.8%, $P = 0.32$), which might be explained by a dose-dependent myelosuppression. At later time points, however, low-dose Rapa was not sufficient to reliably suppress alloimmune activation, and the graft marrow was subsequently rejected resulting in a decline (POD7 vs. POD 28, $P = 0.083$) of donor chimera. In the Rapa 3 and 5 mg/kg/day groups, no statistically significant decline was observed (Rapa 3 mg/kg/day POD 14 $P = 0.09$, $P = 0.19$), suggesting that higher dosages of Rapa were more effective in preventing rejection. Similar results were seen in Tac-treated animals; however, overall mixed chimerism levels were low and thus the decline of donor-derived chimera was hard to quantify.

Tregs play a critical role in transplant immune regulation toward both self-antigens and allogeneic-antigens and have been demonstrated to prolong allograft survival in the setting of VCA [31-33]. The observation that Rapa 5 mg/kg/day treated animals displayed ($P = 0.029$) higher percentages of Tregs compared with Tac 5 mg/kg/day treated animals is in line with current literature demonstrating the expansile effect of Rapa on Treg levels [34-36]. This higher Treg percentage might also contribute to the higher mixed chimerism levels observed in this group. To further study the role of Tregs in this model, we used Foxp3^{DTR} mice and depleted Tregs on POD 14/15. Interestingly, the ablation of Tregs subsequently resulted in markedly reduced chimerism levels of 0.1% (0.1–0.2) at POD 21 for both, Rapa and Tac 5 mg/kg/day treated animals that translated in premature allograft rejection (Fig. 2e).

Limitations of this study include the low power associated with animal-based studies that could result in type II bias. We also did not see a statistically significant difference in graft survival between the 5 mg treatment groups and the 3 mg treatment groups. While those animals receiving 3 mg of either drug did not have 100% graft survival on histology and clinical examination, this cannot eliminate the possibility that the lower dose regimens are effective, and more power is needed to prove superiority. Even though rejection is a continuum, we decided to make a cutoff for graft survival at grade 3 (epidermolysis). We acknowledge that our cutoff level for graft function and loss is arguable; however, because of the often subtle changes in early rejection, this was the most practicable and reliable way to conduct this study and define a clear endpoint. The current study only investigates single-agent immunosuppressive regimens. Though induction followed by a combination immunosuppressive regimen is gold standard in the clinic, single-agent immunosuppression is standard practice in small animal models, especially when models require daily injections to reduce overall burden on experimental animals. Lastly, we did not follow the survival of the animals past POD 30, and thus, we cannot comment on long-term outcomes.

Conclusion

In our study of single-agent immunosuppressive regimens, we report 100% graft survival (< grade 3 rejection) at our 30-day endpoint using either tacrolimus at 5 mg/kg/day i.p. or rapamycin 5 mg/kg/day i.p. While acceptable allograft survival was seen with both drugs, treatment with tacrolimus resulted in lower histologic rejection scoring, whereas rapamycin leads to stable mixed chimerism levels. This study may be used to

inform and help standardize conventional immunosuppressive agent and regimen selection in future murine VCA transplant models.

Authorship

YG: performed operations and flowcytometry, performed analysis, performed literature review, and wrote and revised the manuscript; FM: conceptualized the study, performed operations, performed analysis, performed literature review, and wrote and revised the manuscript; JWE: conceptualized the study, performed literature review, and wrote and revised the manuscript SEB: performed histologic analysis; RK, SS, and GJF: wrote and revised the manuscript; BCO and GB: conceptualized, wrote and revised the manuscript.

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Conflict of interest

The authors have no conflicts of interest to disclose.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Macroscopic and corresponding histologic features of progressive acute rejection in orthotopic hind-limb transplantation in the mouse.

Fig S2. Gating strategy for analyzing (a) lineage-specific and overall mixed chimerism as well as (b) regulatory T cells after orthotopic murine hind-limb transplantation.

Fig S3. Lineage-specific chimerism on POD 7, 14, 21, and 28 for rapamycin, cyclosporin, tacrolimus and Foxp3^{DTR} animals (n = 3–6/group and time point).

Table S1. Survival analysis using the log rank test.

Table S2. Intergroup comparison of overall and lineage specific chimerism levels using Kruskal Wallis or Mann-Whitney test.

Table S3. Intergroup comparison of overall chimerism levels between Rapa 5 mg/kg/day and Tacrolimus 5 mg/kg/day in wild-type and Foxp3 ^{DTR} animals using the Mann Whitney test.

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