SDC, Materials and Methods

Animals

Male Brown Norway and Lewis rats (6-8 weeks old weighing 200 to 250 g) were purchased from Charles Rivers Breeding Laboratories, Germany. Animals were kept in specific pathogen-free conditions and care was carried out in strict accordance with the Swiss Laws in Animal Protection. All experimental protocols were approved by the Veterinary Authorities of the Canton Bern.

Drug preparation

Tacrolimus (LC Laboratories, Woburn, MA, United States), TGMS (AK Scientific, Union City, CA, United States), EDTA Hybri-Max (Sigma, St. Louis, MO, United States) and sterile water (B.Braun, Melsungen, Germany) were used for TGMS-TAC preparation as described previously[1].

Limulus amebocyte lysate test (Pyrogent 03 Plus, Lonza Group, Basel, Switzerland) was used for pyrogen detection according to manufacturer's instructions and TGMS-TAC was considered pyrogen-free if 1:10 dilution of hydrogel in sterile water resulted negative to the test.

TAC solution for systemic injections was prepared by dissolving TAC in Ethanol (absolute, Merck, Darmstadt, Germany) with addition of 1:1 Kolliphor EL (Sigma). The solution was further diluted in sterile saline 1:10 for injection.

Hind limb transplantation and monitoring

Hind limb transplantations were performed as described previously[2] using a 2-surgeon method.

All the operations were performed under continuous inhalation anesthesia. Isoflurane 5% (AbbVie AG, North Chicago, IL, United States) with oxygen (0.8 L/min) was used for the induction of anesthesia. Maintenance anesthesia employed 1-1.5 % Isoflurane with 0.6 L/min oxygen. All the rats were maintained at a normal body temperature using thermal pads. Surgeon 1 prepared both hind limbs of a donor Brown-Norway rat for transplantation. The hind limbs were harvested while keeping the whole inguinal fat pad with its lymph nodes in the graft and taking care not to injure the epigastric vessels that ensure its vascularization. The femoral artery and vein were prepared for anastomosis on a length of approximately 1-1.5 cm from the emergence of the epigastric vessels up to the inguinal crease. Concomitantly, surgeon 2 prepared the first Lewis recipient by removing the hind limb including the inguinal fat pat and performing a midfemoral amputation. The recipient vessels were prepared for anastomosis from the inguinal crease up to the emergence of the epigastric vessels. The first allograft was then reattached by surgeon 2 using and intramedullary osteosynthesis with a 19 G needle with blunted ends, after which preliminary fixation of the anterior muscle compartments was performed with resorbable 5-0 sutures. Venous anastomosis was performed next performed using the cuff technique with a polyimide cuff with an inner diameter of 1.19 mm (Vention Medical Inc Denver, United States). The arterial anastomosis was performed next in an end-to-end microsurgical technique using 10-0 ETHILON Nylon Suture (Ethicon Inc.,

Somerville, NJ, United States). The transplantation was completed after neurorrhaphy of the femoral nerve, sciatic nerve and approximation of the posterior compartment thigh muscles. Skin closure was performed with resorbable 5-0 Coated VICRYL (polyglactin 910) Suture (Ethicon Inc.) in a continuous fashion. Surgeon 1 performed the same procedure of transplantation of the second hind limb allograft to a second Lewis recipient after finishing graft procurement and euthanasia of the BrownNorway rat. The successfully transplanted rats were randomly assigned to the following 2 groups:

Group 1 - Systemically treated with daily subcutaneous injection in the neck fold of 1 mg/kg TAC solution (n=6); Group 2 – Treated with 1 ml TGMS-TAC loaded with 7 mg TAC subcutaneously in the graft every 70 days starting the day after the operation (n=6). In this group, 4 depots of TGMS-TAC of 250 µL each were injected in the zones of biceps femoris, gastrocnemius, tibialis anterior, and vastus muscles, taking great care to distribute the amount of drug as evenly as possible intra and interindividually. Reinjection time point was decided upon a pilot study showing that transplanted animals (n=5) treated with a single intragraft injection of 1 ml TGMS-TAC loaded with 7 mg TAC (POD 1) rejected their graft on POD 83.4 ± 6.7. Reinjection time point was defined as 14 days before rejection and set to POD 70.

After transplantation, animals were inspected on a daily basis for weight loss and signs of pain or distress[3]. Graft survival was monitored until endpoint – POD 280 or macroscopic grade 3 rejection. Graft rejection was evaluated macroscopically as grade 0 = no rejection, 1 = erythema and edema, 2 = epidermolysis and exudation, and 3 = desquamation, necrosis, and mummification. At sacrifice skin and muscle from the graft were formalin fixed (24 h), paraffin-embedded, and sectioned (3 µm). Hematoxylin-eosin as well as periodic acid-Schiff stained sections were graded by a pathologist blinded to the treatment regimens. Skin was graded according to Banff classification[4], while muscle necrosis and lymphocyte infiltration were graded as grade 0 – none, 1 – minimal, 2 – moderate, and 3 – extensive.

Tacrolimus kinetics analyses

TAC in blood (systemic), in graft skin biopsies (local), and in contralateral hind limb skin biopsies was measured at selected time points.

Peripheral blood was collected from the sublingual vein in EDTA coated tubes (Sarstedt, Nümbrecht, Germany) and stored at -20° C until use. TAC concentrations in blood were assessed using the Kit MS1100 (ClinMass Complete Kit, advanced, for Immunosuppressants in Whole Blood, RECIPE Chemicals + Instruments GmbH, Munich, Germany) and quantified by LC-MS/MS.

Skin biopsies from the transplanted and contralateral limb were excised, weighed, snap frozen, and stored at - 20° C until use. The sample preparation was adapted using the MS1312 from Recipe as internal standard. TAC and internal standard were dissolved in 70 % (v/v) methanol solution. Standard spiking solution was prepared to build up a calibration curve between 0.3 and 65 ng/mL. The frozen tissues were gently thawed at room temperature. For blank matrix, samples skin samples without TAC treatment were used. A blank

matrix was prepared adding 1000 µL of precipitation solution to untreated tissue. A volume of 40 µL of internal standard solution and 960 µL of precipitation solution were added to the treated samples. All samples were then grinded with 5 stainless steel balls for 30 minutes at 25 Hz. The tubes were centrifuged 5 minutes at 4° C and 20'000 rcf. 500 µL of the tissue extract was filtered with a Mini-Uni Prep G2 vials (GE Healthcare, Chicago, USA). Chromatographic analysis was performed on an Acquity I-Class system (Waters, Milford, MA, USA) with ClinMass Complete Kits
(Immunosuppressants in whole blood, advanced – on-line analysis). The autosampler temperature was set at 10 °C and the autosampler needle was washed with a strong needle wash solution of isopropanol:methanol:acetontitrile:H2O (1:1:1:1, v/v). A solution of 20 % (v/v) methanol was used as weak needle wash. Analytes were ionized by electrospray ionization (ESI) in the positive mode and detected on a triple quadrupole mass spectrometer (Xevo TQ-S, Waters, Milford, MA, USA). The capillary and the cone voltage were set at 3 kV and 40 V, respectively. The source offset was set at 60 V, the desolvation temperature at 400° C, the desolvation gas at 1000 L/h, the cone gas at 150 L/h, the nebulizer at 7 bar and the source temperature at 150° C. The instrument was controlled via MassLynx (version 4.1, Waters). Data were acquired, integrated and processed with TargetLynx (MassLynx v4.1).

Analyses of immunosuppression-related toxicity

At sacrifice complete blood count was acquired (Sysmex KX-21N automatic hematology analyzer, Sysmex Corporation, Kobe, Hyōgo Prefecture, Japan). Kidney and liver function markers in plasma (creatinine, blood urea nitrogen and alanine aminotransferase, aspartate aminotransferase respectively) were submitted for analysis to the Center of Laboratory Medicine at the University Hospital of Bern. Kidney and liver samples were formalin fixed (24 h), paraffin-embedded, and sectioned (3 µm). Histopathological analysis (Hematoxylin and Eosin, Periodic acid– Schiff) were graded by a pathologist blinded to treatment regimens as described previously[5]. Results were compared to naïve age-matched Lewis rats.

Flow Cytometry analyses

For blood analyses, freshly drawn blood was collected in EDTA coated tubes at predefined time points for chimerism and Treg analyses. Erythrocytes were lysed with 10X RBC Lysis Buffer (Multispecies, Thermo Fisher Scientific, Waltham, MA, United States) and the cells were incubated for 15 min with Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific). After washing with 1xPBS 1%BSA, cells were incubated with the following antirat antibodies: Alexa Fluor 700 antirat CD45 antibody (BioLegend, San Diego, CA, United States), CD3-PerCP-Vio700, rat (Miltenyi Biotec, Bergisch Gladbach, Germany), APC/Cy7 antirat CD4 antibody (BioLegend), CD8b-PE-Vio770, rat (Miltenyi Biotec), and either FITC antirat CD25 antibody (BioLegend), or mouse antirat MHC Class I RT1Ac:FITC (Bio-Rad Laboratories, Hercules, CA, United States) for 15 min at 4°C. Cells were washed and permeabilized (eBioscience FoxP3 / Transcription Factor Staining Buffer Set, Thermo Fisher

Scientific) and incubated 30 min with anti-Helios-PE, human and mouse (Miltenyi Biotec) and FOXP3 monoclonal antibody (FJK-16s), eFluor 450 (Thermo Fisher Scientific), and acquired using a LSR II cytometer (BD Biosciences, San
Jose, United States) equipped with FACS Diva Software (BD Biosciences). Data were analyzed with Flow-Jo software (Tri-Star, Ashland, United States). Absolute cell number was estimated from the proportion of cells recorded by flow cytometry in the CD45+ gate multiplied by absolute mononuclear cell count measured using a Sysmex hematology analyzer in the same blood sample.
For skin analyses, at sacrifice graft and contralateral limb skin was minced (gentleMACS dissociator, Miltenyi Biotec, Bergisch Gladbach, Germany) and digested with Collagenase D (Roche, Basel, Switzerland) 1 mg/ml and DNAse I (Sigma) 200 µg for 1h at 37° C on agitation. Resulting suspension was filtered through 40 µm cell strainers (Falcon, Corning Inc., Corning, NY, United States) and overlaid on top of Ficoll-Paque PLUS Separation Media (GE Healthcare, Little Chalfont, United Kingdom). After centrifugation the ring of cells was collected, washed, and used immediately. Cell were stained, acquired and analyzed as described above.

DSA analyses in plasma

Plasma isolated at sacrifice was complement inactivated (46° C, 30 min) and incubated (1:10 in PBS) for 15 min with donor thymocytes ($1x10^6$ cells) pretreated with 3% BSA and purified mouse antirat CD32 (BD Biosciences) to block unspecific Fc receptors binding. After washing, cells were incubated for 15 min with Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific), washed and stained with CD3-PerCP-Vio700, rat (Miltenyi Biotec), goat $F(ab')_2$ anti rat IgG:FITC (Bio-Rad Laboratories) and R-Phycoerythrin AffiniPure $F(ab')_2$ fragment goat antirat IgM, µ chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) for 15 min at 4°C. After extensive washing, cells were acquired and analyzed as explained above. Minimum threshold of positivity for DSA in the plasma of transplant recipients was determined to be equal to the mean of naïve Lewis plasma plus 2 times its standard deviation.

Immunofluorescence analyses

Skin and muscle samples retrieved at sacrifice day were embedded in TissueTec - O.C.T. (Sakura Finetek, Alphen aan den Rijn, The Netherlands) on dry ice and sectioned (5 µm). Slides were stained with DAPI (4',6-diamidino-2-phenylindole, Boehringer Mannheim GmbH, Mannheim, Germany) and 1 of the following primary antibodies: goat antirat IgG-BIOT (Southern Biotech, Birmingham, AL, United States), goat antirat IgM-BIOT (Southern Biotech), mouse anti rat CD45RA (B Cells Only, Southern Biotech), polyclonal rabbit antihuman C3c complement (Multipurpose, Agilent, Santa Clara, California, United States), anticomplement C4c antibody (LifeSpan BioSciences, Seattle, WA, United States) or C5b-9, rat, mAb 2A1 (Hycult Biotech, Plymouth Meeting, PA, United States,). The following secondary antibodies were used: Streptavidin−Cy3 from *Streptomyces avidinii* (Sigma-

Aldrich), antirabbit IgG (whole molecule), F(ab′)2 fragment–Cy3 antibody produced in sheep (Sigma-Aldrich), donkey antisheep IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific) or goat antimouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 488 (Thermo Fisher Scientific). Slides were visualized with Leica DMI4000, LAS AF Software, Wetzlar, Germany. All images were captured with identical exposure times. Quantitative analysis of fluorescence intensity (integrated density) was performed by ImageJ software (https://imagej.nih.gov/ij/).

Statistical analyses

Statistical analyses were performed with Prism software (GraphPad Software Inc., La Jolla, CA, United States). Statistically significant data are presented as follows: *P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. Tests are specifically indicated under each figure.

References to SDC, Materials and Methods

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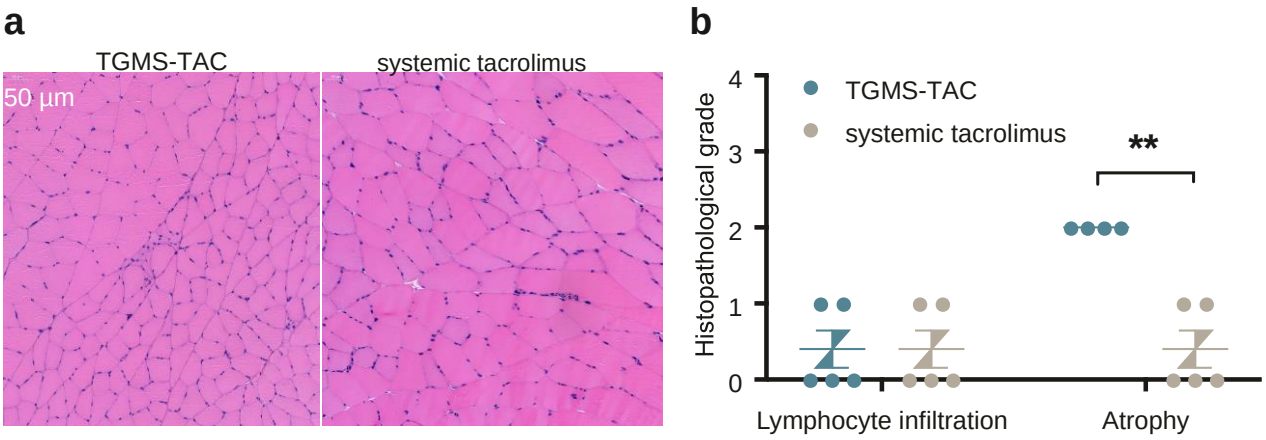

Figure S1: Atrophy and no rejection in graft muscle. (a) Representative histological hematoxylin and eosin stained sections of graft muscle collected at POD 280 from animals treated with TGMS-TAC or systemic tacrolimus and their corresponding (b) histopathological evaluation. Data are presented as individual values, mean ± s.d are indicated, **P<0.01 by Mann-Whitney test.

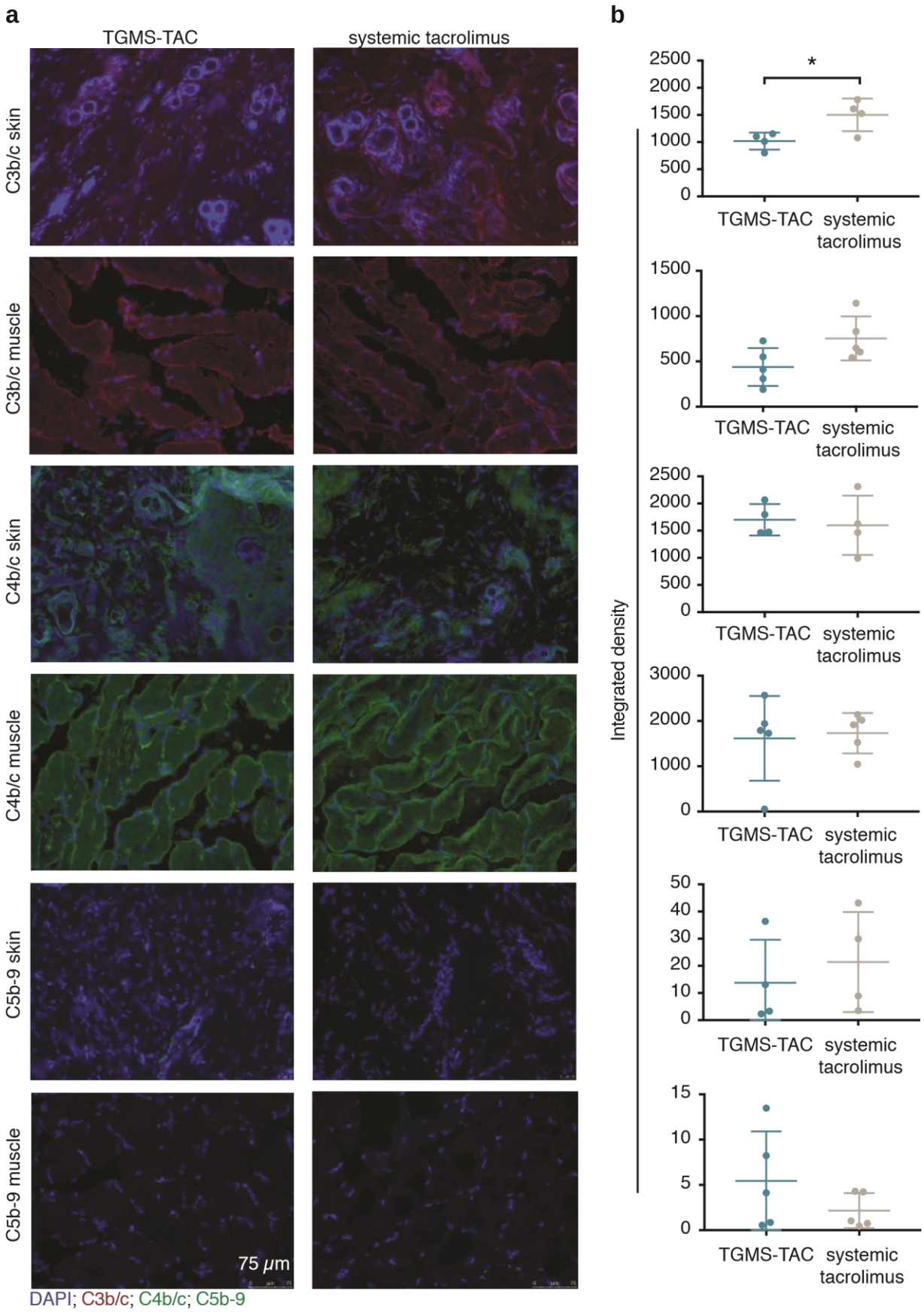

Figure S2: No complement deposition is detectable in long-term surviving grafts. C4b/c, C3b/c, C5b-9 deposition in graft skin and muscle retrieved at POD 280 from TGMS-TAC and systemic tacrolimus treated rats. (a) Representative results of immunostained cryo-sections with DAPI (nucleus), anti-IgG and anti-IgM antibodies and (b) their corresponding quantification. Data shown as individual values, mean ± s.d are depicted.
*<0.05 by Student's t-test.

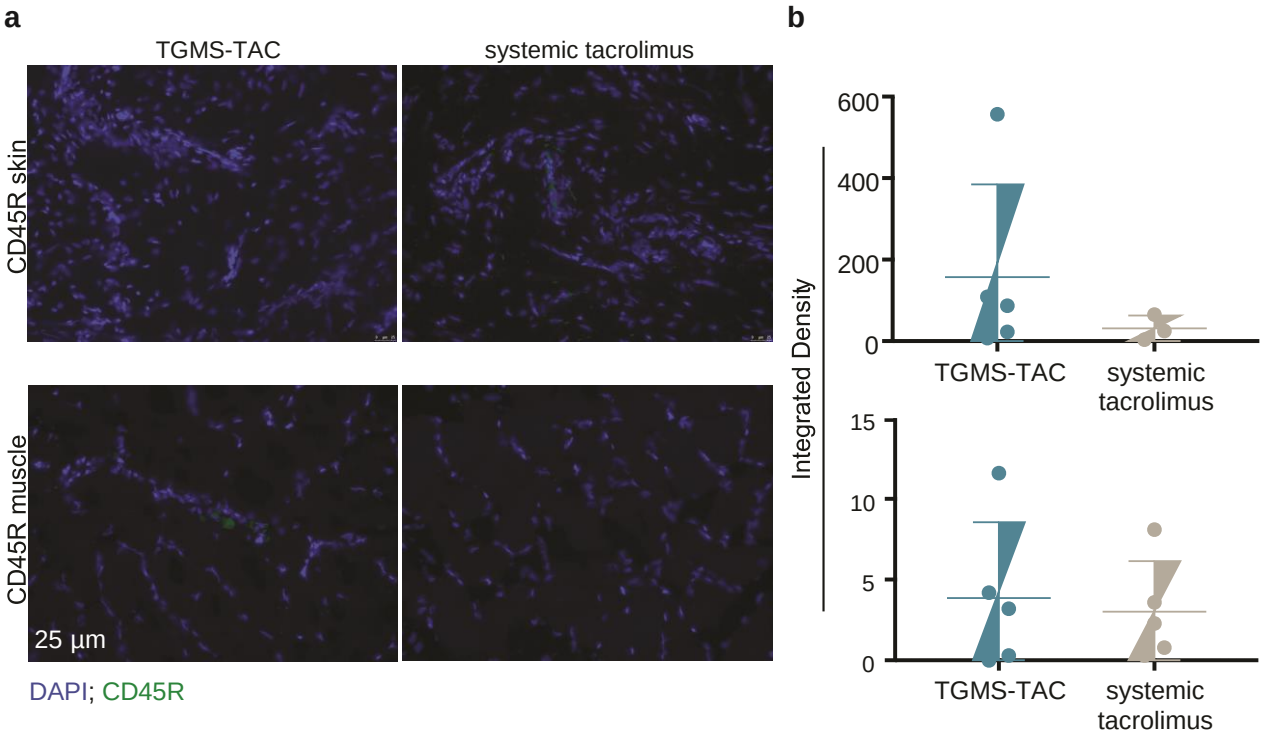

Figure S3: No B cell infiltration is detectable in long-term surviving grafts. CD45R+ cells infiltration in graft skin and muscle retrieved at POD 280 from TGMS-TAC and systemic tacrolimus treated rats. (a) Representative results of immunostained cryo-sections with DAPI (nucleus) and anti-CD45R+ antibody and (b) their corresponding quantification in graft skin and muscle. Data shown as individual values, mean ± s.d are depicted.
Statistical analysis - Student's t-test.

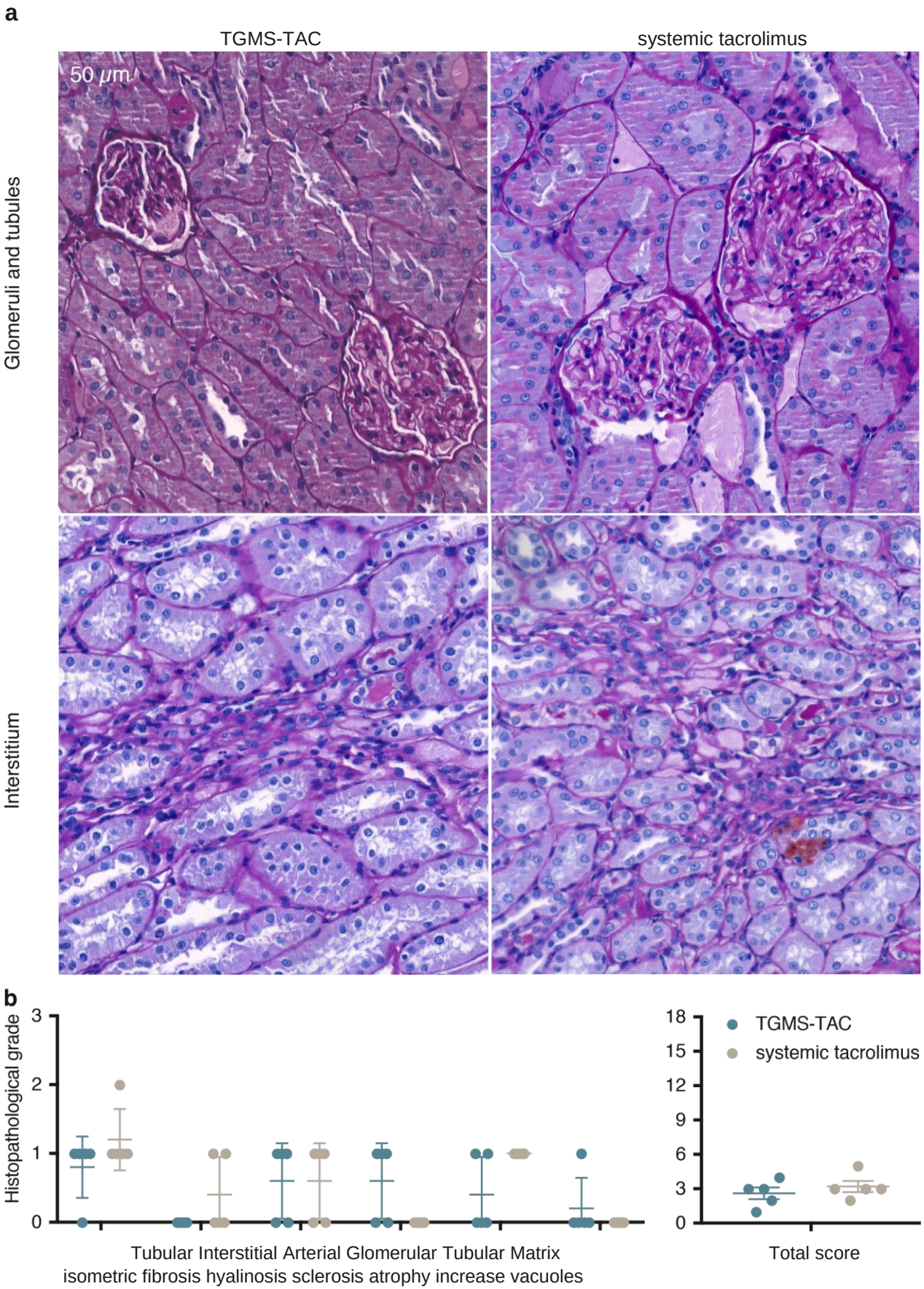

Figure S4: Minimal damage was observed in kidney of rats from both experimental groups. (a) Representative histological Periodic acid–Schiff (PAS) stained sections of kidney at POD 280 of animals treated with TGMS-TAC or systemic tacrolimus and their corresponding (b) histopathological evaluation according to the following semiquantitative pathologic scoring system for Calcineurin Inhibitor Nephrotoxicity: Tubular isometric vacuoles, Interstitial fibrosis, Glomerulosclerosis, Tubular atrophy and Mesangial matrix increase - None - Score 0, 1 to 25% - Score 1, 26 to 50% - Score 2, >50% - Score 3; Arteriolar medial hyalinosis - None - Score 0, <10% - Score 1, 11 to 30% - Score 2, >30% - Score 3. Statistical analyses of the differences between the 2 groups are shown. Data are presented as individual values, mean ± s.d. are indicated, **P<0.01, Mann-Whitney test.

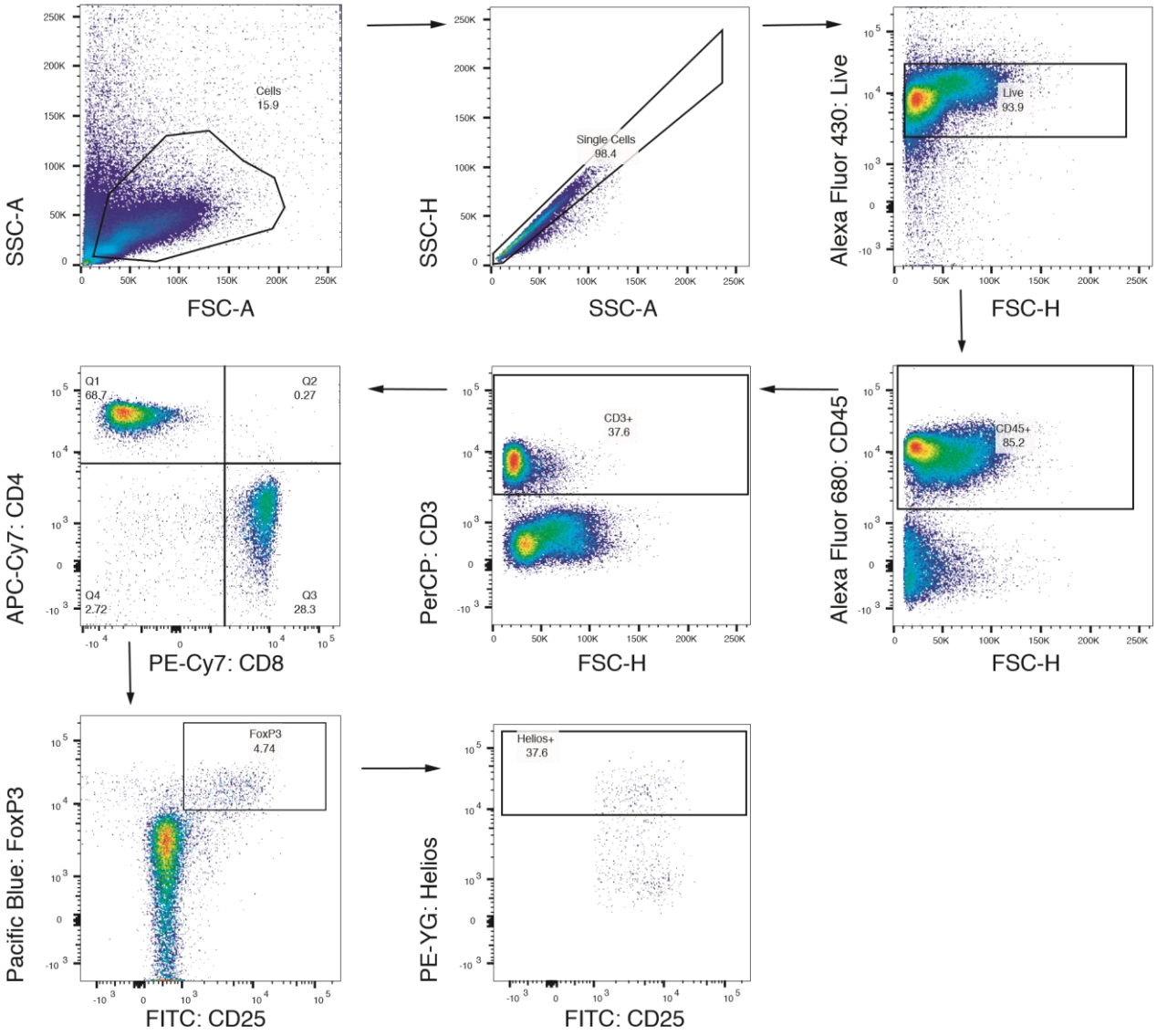

Figure S5: Gating strategy for enumeration of Treg cells in the peripheral blood. After identification of cells by their physical parameters (ie, forward and side scatter), single, viable (dim Viability Dye expression) CD45+ cells have been identified and gated. In the CD45+ gate, CD3+ cells have been selected and CD4+ population within them have been further investigated. Of those, FoxP3+, CD25high cells have been defined as Treg cells. Subsequently, Helios+ and Helios- Treg have been distinguished. All populations were expressed as frequency of CD45+ cells and the absolute cell number was determined using the complete blood cell count of the same blood sample. For skin analyses, due to inability to determine absolute white blood cell count, percent of parent population have been used to appreciate relative abundance of each population.

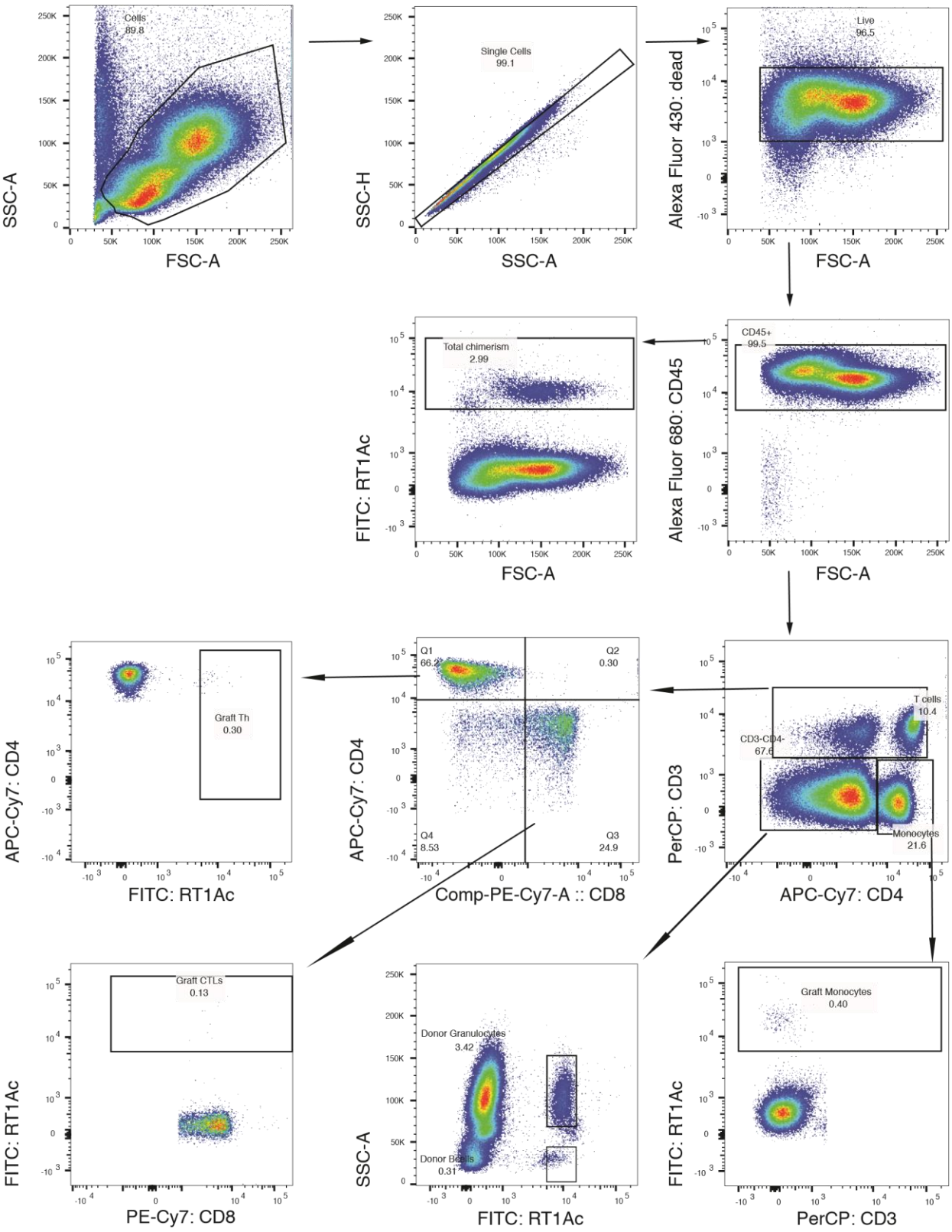

Figure S6: Gating strategy for the analysis of chimerism in the peripheral blood of hind limb recipients. After identification of cell by their physical parameters (ie, forward and side scatter), single, viable (dim Viability Dye expression) CD45+ cells have been identified and gated. In this gate the number of RT1Ac+ cells have been defined as "total chimerism" (ie, all donor-derived white blood cells). CD45+ cells have been further separated by their expression of CD3 and CD4. CD3+ cells have been defined as T cells and further separated to CD4+ T helper cells and CD8+ cytotoxic T cells. CD3-CD4+ cells have been defined as monocytes. From the CD3-, CD4- cells, SSChigh have been defined as granulocytes and SSClow cells as B cells. In each of those populations RT1Ac+ cells have been defined as their donor-derived counterparts. For blood analyses, complete blood cell count has been used to determine the absolute white blood cells count, corresponding to the parent CD45+ population. All populations were expressed as frequency of CD45+ cells and the absolute cell number was determined using the complete blood cell of the same blood sample. For skin analyses, due to inability to determine absolute white blood cell count, percent of parent population have been used to appreciate relative abundance of each population.

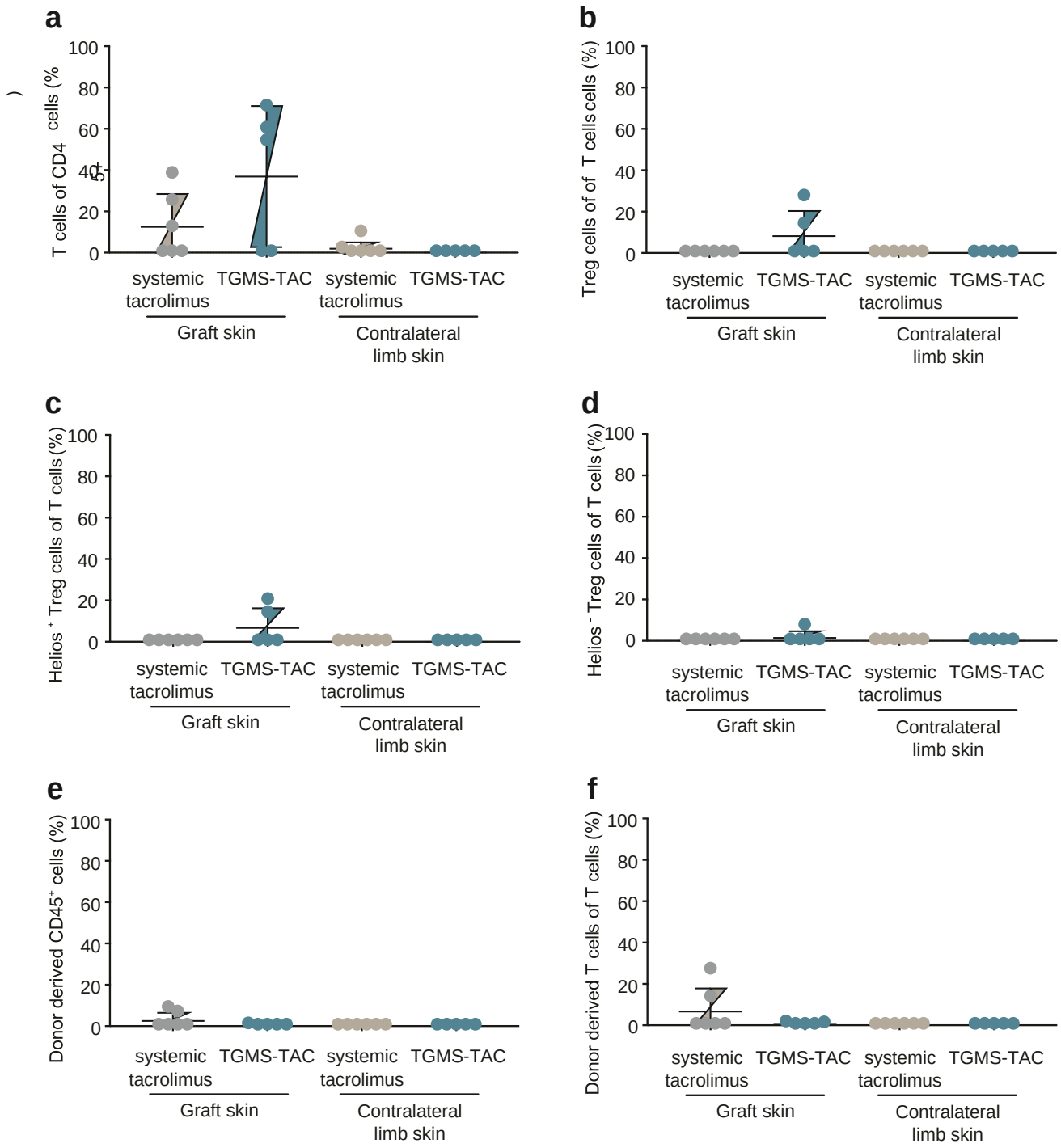

Figure S7: No significant changes in donor cell populations have been detected in graft skin. At sacrifice (POD 280) skin from both transplanted and contralateral native limb have been collected, minced and digested. Cells have been extracted and submitted to flow cytometric analyses, using the same antibodies and protocol from Figure 7 and 8. Total amount of T cells (a), Treg cells (b), Helios+ Treg cells (c) and Helios- Treg cells (d), as well as total donor-derived white blood cells (e) and T cells (f) have been analyzed according to the gating strategies described in supplementary figures 5 and 6. Populations have been presented as percent of their parent populations. Intragroup comparisons between graft and contralateral limb skin have been analyzed by paired Student's t-test. Intergroup comparisons between TGMS-TAC and systemic tacrolimus treated groups have been analyzed by unpaired Student's t-test.

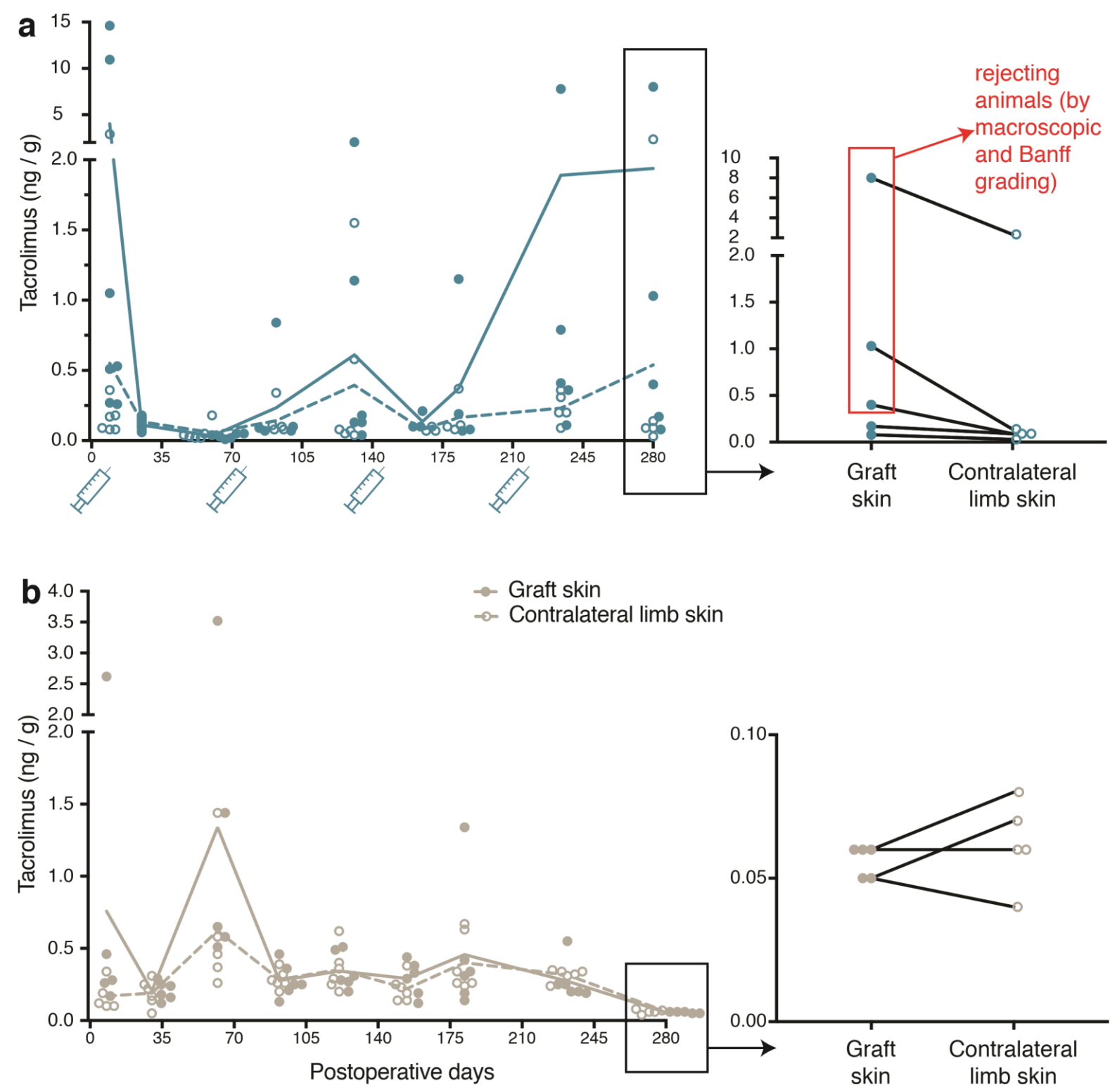

Figure S8: Trend towards higher tacrolimus levels in skin of transplanted limbs versus contralateral native limbs in rejecting TGMS-TAC treated animals. Tacrolimus measurements in graft and contralateral limb skin biopsies of (a) TGMS-TAC-treated and (b) systemic tacrolimus treated rats over time from Figure 4, with linear Y-axis. Zoomed in both panels are values from postoperative day 280 (endpoint). (a, zoomed-in panel) Tacrolimus levels in TGMS-TAC treated hind limbs are not significantly higher than in contralateral native limbs. However there appears to be a trend towards higher TAC skin levels in rejecting animals. (b, zoomed-in panel) No difference between TAC levels in transplanted and native limbs in systemically treated animals. TGMS-TAC reinjection time points are indicated. Data are shown as individual values and means. Individual values, when overlapping, have been manually shifted apart to left or right to increase visibility of each value.