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Supplementary Information for:

Treg Inducing Microparticles Promote Donor-Specific Tolerance in Experimental Vascularized Composite Allotransplantation

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Supplementary Information Text

Materials and Methods

Animals

Six to eight-week-old male Lewis (LEW; $RT1^l$), Brown Norway (BN; $R71^h$), and Wistar Furth (WF; $RT1^u$) rats (Charles River Laboratories, Wilmington, MA) were used. Body weights at the time of transplants were ~300g. Animals were maintained under an Institutional Animal Care and Use Committee (IACUC) protocol in a specific pathogen-free environment at the University of Pittsburgh.

Hindlimb Transplantation

Using techniques developed in the University of Pittsburgh's Department of Plastic Surgery, hindlimbs from donor BN rats were transplanted to donor LEW recipients (1). Specifically, donor femoral vessels were anastomosed end-to-end to recipient femoral vessels. Femoral osteosynthesis was performed with an 18-gauge intramedullary rod.

TRI-MP Fabrication and Characterization

IL-2 and TGF-β1 microparticles (IL-2-MP and TGF-β1-MP, respectively) were prepared using a well-described double emulsion-evaporation technique (2). For IL-2-MP, 10μg of recombinant murine IL-2 (R&D Systems Minneapolis, MN) was mixed with 2 mg of BSA and 5 mM NaCl in 200 μl of de-ionized water. This solution was added to 4 ml of dichloromethane (DCM) containing 200 mg of poly lactic-co-glycolic acid (PLGA; RG502H, Boehringer Ingelheim Chemicals Inc., Petersburg, VA), and the mixture was agitated using a sonicator (Vibra-Cell, Newton, CT) at 25% amplitude for 10 sec, creating the first water-oil emulsion. This emulsion was then mixed with 60 ml of 2% polyvinyl-alcohol (PVA, MW ~25,000, 98% hydrolyzed; Polysciences) under homogenization (L4RT-A, Silverson, procured through Fisher Scientific) at 3000 rpm for 1 min, creating the second emulsion. The resulting double-emulsion was then added to 80 ml of 1% PVA, and DCM was allowed to evaporate with the solution placed on a magnetic stirrer at 600rpm for 3 hours. Subsequently, the microparticles were) washed 4 times in de-ionized water, and lyophilized (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 80 mTorr).

For TGFβ-MP, 2μg of recombinant human TGF-β1 (CHO cell-derived, PeproTech, Rocky Hill, NJ) was mixed with 10 mg D-mannitol, 1 mg of BSA, and 15 mM NaCl in 200 μl of de-ionized water. This solution was added to 4 ml of dichloromethane containing 200 mg of PLGA (RG502H), and the mixture agitated using a sonicator at 25% amplitude for 10 sec, creating the first emulsion. This emulsion was then mixed with 60 ml of 2% PVA (containing 125 mM NaCl) under homogenization at 3000 rpm for 1 min, creating the second emulsion. The resulting double emulsion was then added to 80 ml of 1% PVA (containing 125 mM NaCl), and placed on a magnetic stir plate (600rpm) for 3 hours to allow DCM to evaporate. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

Since Rapamycin is a lipophilic small molecule, Rapa-MP were prepared using a single emulsion-evaporation technique (2). Specifically, 2 mg of Rapamycin (LC Labs, Woburn, MA) dissolved in 100 μl DMSO was mixed with 4 ml of dichloromethane containing 200 mg of PLGA (RG502H). This solution was mixed with 60 ml of 2% PVA under homogenization at 3000 rpm for 1 min creating the microparticle emulsion. The resulting emulsion was then added to 80 ml of 1% PVA and placed on a magnetic stir plate (600rpm) for 3 hours to allow DCM to evaporate. Subsequently, the microparticles were centrifuged (200g, 5 min, 4 °C), washed 4 times in de-ionized water, and lyophilized.

For release assays: 10 mg of IL-2-MP or TGF-β1-MP were suspended in 1 ml of PBS with 1% BSA, and 10 mg of Rapa-MP was suspended in 1 ml of PBS (containing 0.2% Tween-80). Samples were then placed on an end-over-end roto-shaker at 37 °C. At daily time intervals, particle suspensions were centrifuged (250g, 5min), the supernatant removed, and the particles re-suspended in 1 ml of appropriate release buffer. IL-2 or TGF-β1 in the supernatant was measured using an ELISA (R&D systems, Minneapolis, MN), and Rapamycin was measured using spectrophotometry (absorbance at 278 nm). Surface characterization of MP was conducted using scanning electron microscopy (JEOL JSM-6510LV/LGS) and microparticle size distribution

determined by volume impedance measurements on a Beckman Coulter Counter (Multisizer-3, Beckman Coulter, Fullerton, CA).

Study Design and Groups

Hindlimb recipients in all experimental and control groups received the same baseline IS protocol, consisting of FK506 (LC Laboratories, Woburn, MA) at a dose of 0.5 mg/kg, injected intraperitoneally (I.P.) daily from days 0-21. All rats also received two 0.5 ml doses of rabbit anti-rat lymphocyte serum (Accurate Chemical, Westbury NY) injected I.P. on POD -4 and 1. For rats also treated with TRI-MP, individual microparticle formulations, or combinations of two microparticle formulations, microparticles were injected subcutaneously in the lateral aspect of the transplanted limb (unless otherwise noted) at a concentration of 10mg/ml on POD 0 and 21. Animals treated with the complete TRI-MP system received 3mg of each microparticle formulation in 900μl sterile phosphate buffered saline (PBS). Some other animals received injections of the individual components of TRI-MP (3mg of each formulation in 300μl PBS), the pairwise iterations of TRI-MP (TGF-β1-MP + Rapa-MP, TGF-β1-MP + IL-2-MP, or IL-2-MP + Rapa-MP; 3 mg of each formulation in 600μl PBS), TRI-MP injected in the contralateral (non-transplanted) limb, or Blank-MP (empty microparticles).

Hindlimb Allograft Monitoring

Hindlimbs were monitored daily and scored for rejection (appearance grading) based on physical examination (1). Limbs were given a daily score using the following scale: Grade 0 (no rejection), Grade I (edema), Grade II (erythema and edema), Grade III (epidermolysis) and Grade IV (necrosis and "mummification"). Grafts were considered rejected when displaying signs of progressive clinical Grade III rejection.

Histology

Skin and muscle samples were obtained from the transplanted limbs of animals at their experimental endpoint (progressive grade III rejection or long-term survival >300days). Samples were fixed in 10% neutral buffed formalin, paraffin-embedded, sectioned at 5μm thickness, and stained with hematoxylin and eosin (H&E) for microscopic examination of tissue architecture and mononuclear cell infiltration.

Gene Expression

Gene expression profiles of inflammatory markers were evaluated in the skin and lymph nodes of long-term graft survivors, actively rejecting, and naïve rats. Total RNA was extracted from samples using TRI-reagent according to the manufacturer's instructions, and quantified using a NanoDrop 2000. For each reverse transcriptase assay, 4 μg RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit. Quantitative real-time PCR was then performed using VeriQuest Probe qPCR Mastermix, according to the manufacturer's instructions, with 5′ nuclease PrimeTime qPCR TaqMan primer-probe assays (Applied Biosystems) specific for IFNγ (Rn00594078_m1), TNFα (Rn99999017_m1), Perforin-1 (Rn00569095_m1), Serglycin (Rn00571605_m1), IL-17A (Rn01757168_m1), FOXP3 (Rn01525092_m1), TGFB1 (Rn00572010_m1), IL10 (Rn99999012_m1), EBI3 (Rn01527778_m1), and GAPDH (endogenous control, Rn99999916_s1). Target gene primers were FAM-MGB labeled, while the GAPDH endogenous control primer was labeled with VIC-MGB_PL. Duplex reactions (target gene + GAPDH) were analyzed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Expression of each target gene was calculated and normalized to GAPDH endogenous control and naïve tissue (from naïve animals or contralateral limbs) based on the Livak ($2^{-\Delta\Delta Ct}$) method.

Flow Cytometric Analysis

Draining and non-draining lymph nodes were harvested at the experimental endpoint (progressive grade III rejection or long-term survival >300days). Lymph nodes were then processed mechanically and strained through a 70μm filter to provide a single cell suspension. Cells were stained with fluorescently labeled antibodies for CD4 (OX-35), CD25 (OX-39), FoxP3 (FJK-16s), and IFN-γ (DB-1) (eBioscience, San Diego, CA). For intracellular cytokine staining, the cells were

placed in a 96-well plate overnight in cell culture media with Cell Stimulation Cocktail (plus protein transport inhibitor, eBioscience). Stained cells were then analyzed on a flow cytometer (LSR-Fortessa, BD Biosciences), and data were analyzed using FlowJo (Tree Star, Ashland, OR).

Cell Proliferation and Suppression Assays

Spleens from rats with long-term surviving hindlimb allografts and naïve rats were processed into single cell suspensions. Red blood cells were lysed and $CD4^+$ T cells were isolated using CD4 T cell enrichment columns according to the manufacturer's instructions (Miltenyi Biotech, Auburn CA). $CD4^+$ cells were then stained with anti-CD4 and anti-CD25. $CD4^+$ $CD25^-$ (Tconv) and $CD4^+$ $CD25^{hi}$ (Treg) populations were sorted using a fluorescence-activated cell sorter. To assess proliferative function, $CD4^+$ $CD25^-$ Tconv from long-term surviving or naïve rats were stained with VPD450 (BD Biosciences, San Jose, CA) and each was co-cultured/stimulated with irradiated splenocytes harvested from Brown Norway (BN) rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 dilution using flow cytometry (LSR-Fortessa) and FlowJo. The proliferative capacity of Tconv from long-term surviving rats was normalized to that of naïve rats.

The suppressive function $CD4^+$ $CD25^{hi}$ Tregs isolated from rats with long-surviving grafts and naïve rats was tested by assessing their ability to suppress Tconv proliferation in an MLR. $CD4^+$ $CD25^-$ Tconv from naïve rats were stained with VPD450 and co-cultured/stimulated with irradiated donor BN splenocytes and $CD4^+$ $CD25^{hi}$ Tregs were added at a ratio of 1:2 at the start of the culture. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using flow cytometry. Percent suppression was calculated as: {1 – [(% Proliferation of Naïve Tconv cultured with BN Splenocytes and Treg) / (% Proliferation of Naïve Tconv cultured with BN Splenocytes)]} x 100%.

MLR were also set up to test for antigen specificity of $CD4^+$ $CD25^{hi}$ Tregs isolated from long-term graft surviving rats. $CD4^+$ $CD25^-$ Tconv from naïve rats were stained with VPD450 and stimulated with either Brown Norway (BN) or Wistar Furth (WF) irradiated splenocytes in the presence of $CD4^+$ $CD25^{hi}$ Tregs isolated from long term surviving rats. At the end of the 7-day MLR period, proliferation was measured via VPD450 decay using flow cytometry. Percent suppression was calculated as: {1 – [(% Proliferation of Naïve Tconv cultured with BN or WF Splenocytes and Tregs) / (% Proliferation of Naïve Tconv cultured with BN or WF Splenocytes)]} x 100%.

Full Thickness, Nonvascularized Skin Graft Challenge

Donor specific tolerance was assessed *in vivo* in long-term surviving animals via secondary skin graft challenge. Skin allografts were harvested from donor strain (BN) or third-party strain rats (WF) and transplanted to the long-term survivors >300 days after VCA. Grafts were secured in place for 5 days and subsequently evaluated daily for signs of rejection. Rejection was defined as failure of the transplanted skin graft to "take" as evidenced by necrosis and wound contracture.

Statistics

Statistical analyses performed in GraphPad Prism, and all data are presented as mean ± standard deviation. Comparisons between two experimental groups were performed using a two-tailed Student's t-test, Welch's t-test, or Mann-Whitney U test, as appropriate. A one-sample t-test was used to compare fold changes in a single experimental group to a value of one, and differences in survival were identified using a log-rank test. For all tests, $p < 0.05$ was considered significant.

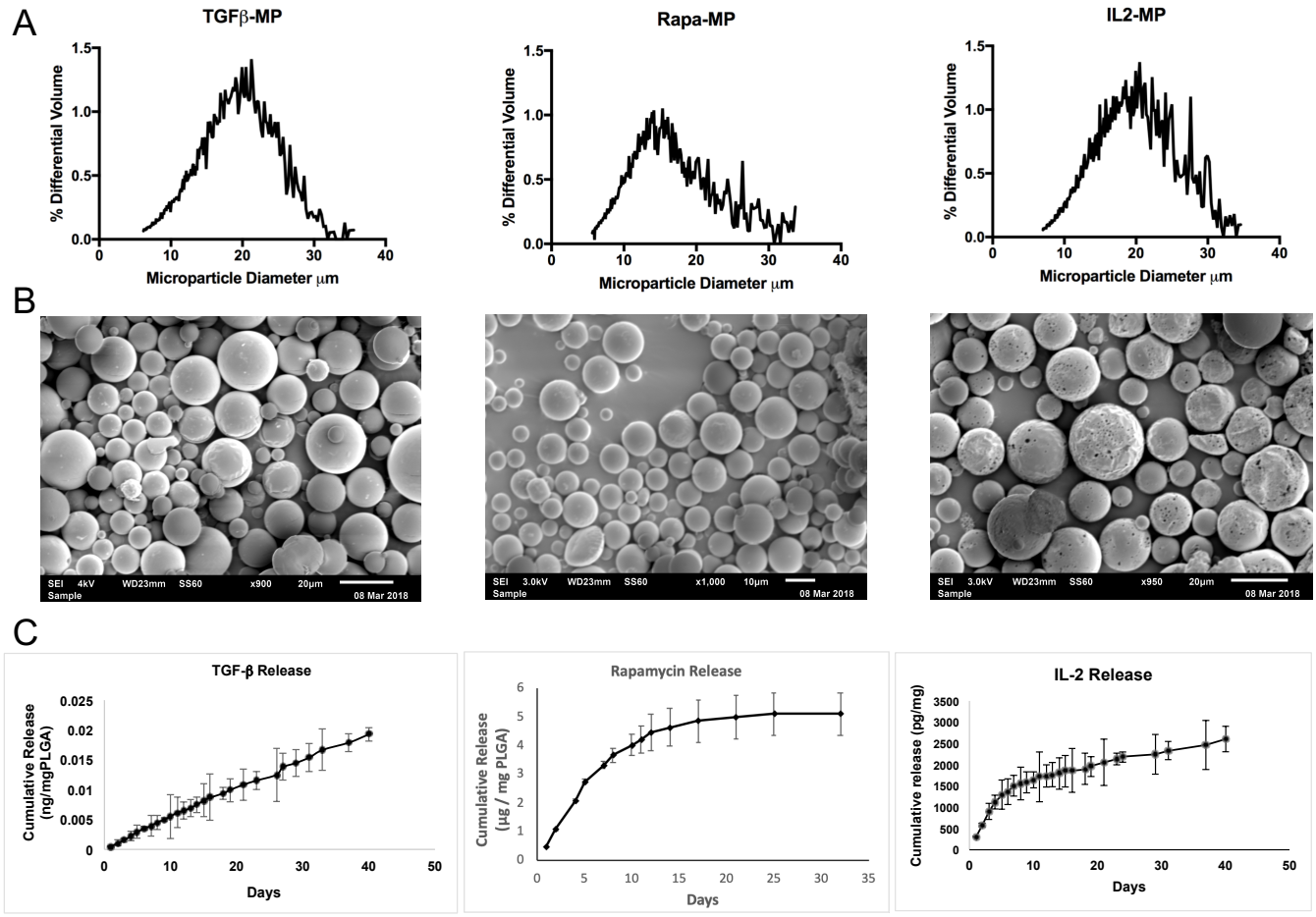

Fig. S1. Microparticle characterization. (A) Volume-weighted size distributions of TGF-β1-MP, Rapa-MP, and IL-2-MP engineered to be ~ 20 μm in diameter. (B) Representative SEM images of TGF-β1-MP, Rapa-MP, and IL-2-MP demonstrate a spherical outer surface. (C) Release kinetics of TGF-β1-MP, Rapa-MP, and IL-2-MP (N=3 for each formulation) demonstrate sustained release of each factor over 30-40 days.

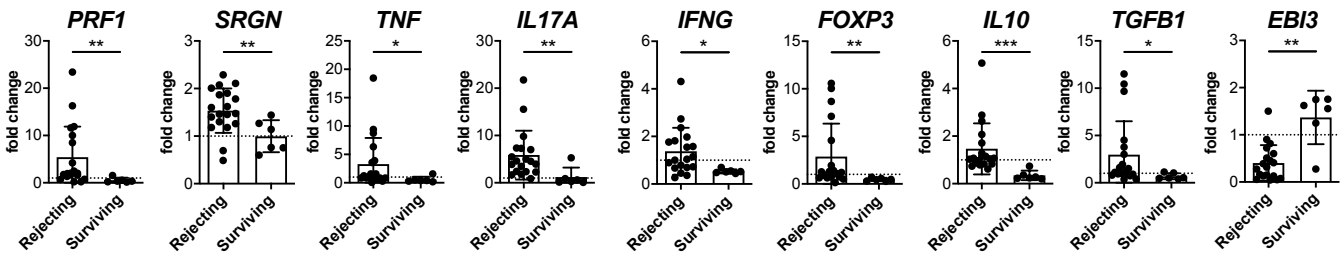

Fig. S2. Relative mRNA expression in hindlimb draining inguinal lymph nodes from rejecting allografts (No MP, Blank MP, soluble TRI, contralateral TRI MP, or combinations of 2 MPs; N=19, POD 33-45) vs. long-term surviving allografts (TRI-MP; N=6, POD >300). Expression levels are presented as fold changes ($2^{-\Delta\Delta Ct}$) relative to non-allograft draining inguinal lymph nodes from naïve rats or contralateral limbs (N=15-19). Bars represent mean ± SD, and dots represent values from individual rats. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$, and were determined by either two-tailed unpaired t-test or Mann-Whitney U test, as appropriate.

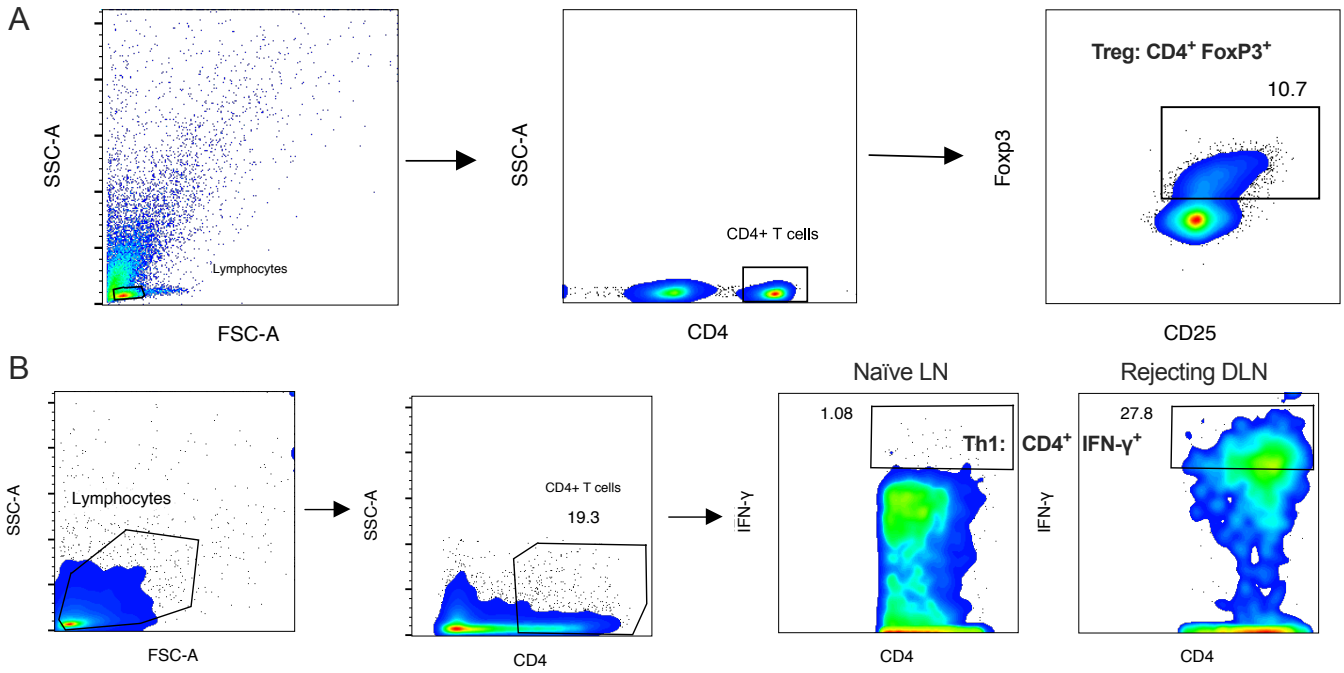

Fig. S3. Flow cytometry gating strategy for phenotypic analysis of $CD4^+$ T cells in allograft draining inguinal lymph nodes (as quantified in Figure 4). (A) $CD4^+$ $FoxP3^+$ Tregs and (B) $CD4^+$ IFN-γ^+ Th1 cells were identified by expression of CD4 and FoxP3 or IFN-γ.

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