SUPPLEMENTARY INFORMATION

Ex vivo expanded donor alloreactive regulatory T cells lose immunoregulatory, proliferation and anti-apoptotic markers after infusion into ATG-lymphodepleted, nonhuman primate heart allograft recipients

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SUPPLEMENTARY METHODS

Immunosuppressive regimens

To examine the influence of adoptively-transferred darTreg on heart allograft survival, monkeys CM115 and CM121 received rabbit ATG (Genzyme; Boston, MA) intravenously (i.v.) over 4 hours on days -3 and -1 before, and on days 6 and 13 after transplant at doses of 10, 5, 5 and 5 mg/kg, respectively. Methylprednisolone was given before each ATG infusion at doses of 5, 2.5, 2.5 and 2.5 mg/kg, respectively. Anti-IL-6R mAb (Actemra; Genetech, CA) was administered i.v. over 1 hour at 10 mg/kg on days -1, 6, 13 and 20, and then once every 4 weeks. Tacrolimus was given by intramuscular (i.m.) injection from day -3 to 14 (target trough levels: 10–15 ng/ml), followed by rapamycin (i.m.; LC Laboratories, Woburn, MA) from days 14 to 54 (target trough levels: 10–15 ng/ml), after which rapamycin was weaned slowly and discontinued completely on day 84. In darTreg tracking experiments (heart graft recipients CM102 and CM103), anti-IL-6R was discontinued at day 6 and no rapamycin was administered. In graft recipient CM220, that received delayed darTreg post-transplant, no IL-6R blockade was administered.

Monitoring of graft function

Heart allograft function was monitored by palpation under sedation (twice a week by two independent observers). A graft beating (palpation) score was used to assess graft function, with grade 3 representing strong/excellent, grade 2 moderate, grade 1 weak and grade 0 complete cessation of contraction. Graft injury was monitored by measuring relative creatinine phosphokinase (CPK) -MB relative index (RI) before transplantation and weekly post-transplant. In recipients CM115 and CM121, grafts were monitored until complete cessation of graft beating, at which timepoint the animals were euthanized. Recipients CM102, CM103 and CM220 were electively euthanized on days 18, 19 and 63, respectively.

Expansion of polyclonal and donor Ag alloreactive (dar) Treg

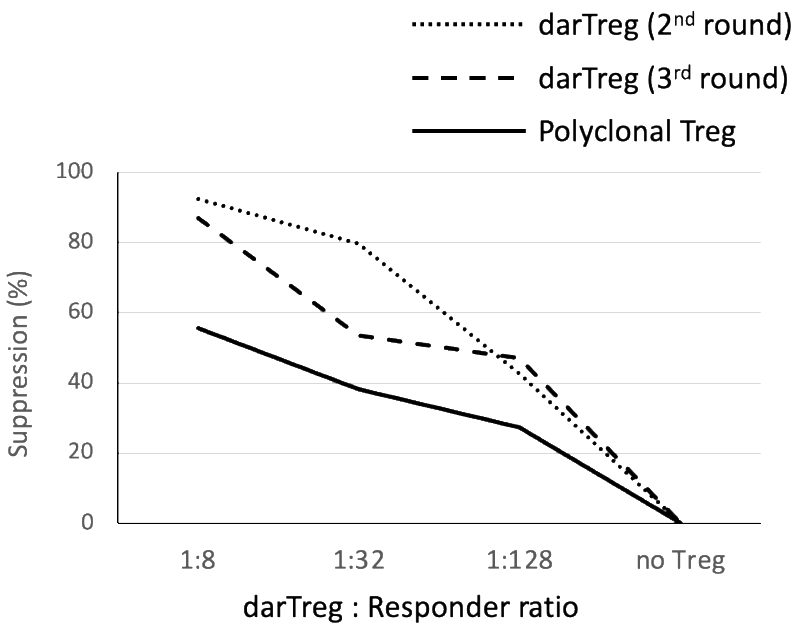
For the first round of polyclonal Treg expansion, freshly-isolated, flow-sorted Treg were cultured with irradiated (80 Gy) L-32 cells (artificial APCs) expressing CD32, CD80 and CD58 and loaded with anti-CD3 (BD Bioscience) for 7 days. For the first round of darTreg expansion, freshly-isolated Treg were cultured with CD154-activated B cells for 9 days. For the second and third rounds of expansion, round 1 polyclonal Treg or round 1 darTreg were cultured with L-32 cells at a T cell/APC ratio of 1 : 1 (7 – 8 days each round) in complete RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% v/v fetal bovine serum, 2 mM L-glutamine (Mediatech, Inc., Herndon, VA), 100 U/mL penicillin-streptomycin (BioWhittaker, Lonza, Allendale, NJ), 25 mM HEPES (Mediatech) and 55 mM b-2 mercaptoethanol (Invitrogen) in the presence of 300 U/mL recombinant human IL-2 (R&D Systems, Minneapolis, MN).

SUPPLEMENTARY FIGURES

Figure S1. Protocol for ex vivo expansion of autologous donor Ag alloreactive cynomolgous Treg (darTreg) and for comparison, polyclonal Treg.

Round	Day	Procedure
Round #1 (d 42 - 28 pre-tx)	0	• Flow sorting of recipient peripheral blood $CD4^{+}CD25^{hi}CD127^{lo}$ Treg • Coculture of Treg with donor CD40L-activated B cells (for darTreg) or L-32 cells (for polyclonal Treg)
	2, 4, 7	• Add IL-2 to the culture
	7, 9	• Harvest and cryopreservation of polyclonal Treg on day 7 and darTreg on day 9
Round #2 (14 days before Treg infusion)	0	• Harvest stored (Round #1) Treg • Coculture of Treg with L-32 cells
	0, 2, 4	• Add IL-2 to the culture
	7	• Harvest and split Treg
Round #3 (7 days before Treg infusion)	0	• Coculture of Treg with L-32 cells
	0, 2, 4	• Add IL-2 to the culture
	7	• Harvest and infuse Treg

Figure S2. Suppressive efficacy of autologous polyclonal Treg in comparison to darTreg.



Ex-vivo–expanded autologous polyclonal or darTreg were tested for their ability to suppress autologous T cell responses to allogeneic stimulators. darTreg from 2nd (dotted line) or 3rd (dashed line) rounds of expansion were used to suppress VPD450-labeled autologous T cell proliferation in response donor T cell-depleted PBMC (stimulators), in comparison to autologous polyclonal Treg (solid line). Percent divided cells was calculated using FlowJo software. Treg function is expressed as percent suppression of T cell proliferation calculated using the formula: (percent divided T cells without addition of Treg – percent divided T cells with Treg) / percent divided T cells without addition of Treg x 100%. darTreg exhibited superior suppressive function in

comparison to polyclonal Treg. Data are representative of Tregs from one of the heart allograft recipients (Figure 1).

Figure S3. Gating strategy for evaluation of darTreg phenotype by flow cytometry.