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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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Abstract

BACKGROUND: Regulatory T cell (Treg) therapy is a promising approach to amelioration of allograft rejection and promotion of organ transplant tolerance. However, the fate of infused Treg, and how this relates to their therapeutic efficacy using different immunosuppressive regimens is poorly understood. Our aim was to analyze the tissue distribution, persistence, replicative activity and phenotypic stability of autologous, donor antigen allo-reactive Treg (darTreg) in ATG-lymphodepleted, heart-allografted cynomolgus monkeys.

METHODS: darTreg were expanded ex vivo from flow-sorted, circulating Treg using activated donor B cells and infused post-transplant into recipients of MHC-mismatched heart allografts. Fluorochrome-labeled darTreg were identified and characterized in peripheral blood, lymphoid and non-lymphoid tissues and the graft by flow cytometric analysis.

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Disclosure statement

The authors have no conflicts of interest to disclose.

RESULTS: darTreg selectively suppressed autologous T cell responses to donor antigens in vitro. However, following their adoptive transfer after transplantation, graft survival was not prolonged. Early (within 2 weeks post-transplant; under ATG, tacrolimus and anti-IL-6R) or delayed (6–8 weeks post-transplant; under rapamycin) darTreg infusion resulted in a rapid decline in transferred darTreg in peripheral blood. Following their early or delayed infusion, labeled cells were evident in lymphoid and non-lymphoid organs and the graft at low percentages (< 4% $CD4^+$ T cells). Notably, infused darTreg showed reduced expression of immunoregulatory molecules (Foxp3 and CTLA4), Helios, the proliferative marker Ki67 and anti-apoptotic Bcl2, compared with pre-infusion darTreg and endogenous $CD4^+CD25^{hi}$ Treg.

CONCLUSION: Lack of therapeutic efficacy of infused darTreg in lymphodepleted heart graft recipients appears to reflect loss of a regulatory signature and proliferative and survival capacity shortly after infusion.

INTRODUCTION

Cell therapy using regulatory T cells (Treg)[1, 2] has shown considerable promise for promotion of transplantation tolerance in animal models.[3–6] As a result, several centers have embarked upon early phase clinical trials designed primarily to assess the feasibility and safety of Treg therapy in organ transplantation.[7–11] Key questions yet to be resolved include the timing of cell infusion, optimal cell dosage, and selection of appropriate immunosuppressive and other drugs to preserve or enhance the cells' regulatory function.[5, 12–14] Moreover, the in vivo fate, tissue distribution, stability and longevity of adoptively-transferred Treg[15, 16] are largely unknown.

Nonhuman primates (NHP) are important pre-clinical models in organ transplant research.[17] We and others have observed[18, 19] that, when infused systemically into non-transplanted monkeys, fluorochrome-labeled, ex vivo-expanded autologous polyclonal Treg decline rapidly in peripheral blood during the first week post-infusion, though they persist at low levels in blood for at least 3 weeks.[19] In these studies, distribution of the infused Treg in native organs was not evaluated and could account for the diminished numbers of these cells in peripheral blood. Moreover, the dynamics of Treg migration, their preponderance relative to T effector cells, and their ability to suppress naïve versus memory T cell function[20] may variably impact their therapeutic efficacy after infusion.

We reported previously[21] that infusion of ex vivo-expanded autologous polyclonal Treg did not prolong survival of heart allografts in monkeys treated with anti-thymocyte globulin (ATG), an agent reported to expand Treg in vitro[22, 23] and promote Treg in vivo,[23–25] including in kidney-transplanted monkeys.[26] In the current study, we aimed to assess graft survival and to monitor infused donor antigen alloreactive (dar)Treg in peripheral blood, lymphoid and non-lymphoid tissues and the allograft of similarly-treated, NHP heart transplant recipients. To our knowledge, this is the first report on tracking and characterization of adoptively-transferred Treg in a preclinical NHP organ transplant model.

MATERIALS AND METHODS

Animals

Male cynomolgus monkeys (*Macaca fascicularis*) of Indonesian origin (3–5 kg; 5–7 years old) were obtained from specific pathogen-free colonies at Alpha Genesis, Inc, or the National Institute of Allergy and Infectious Diseases colony (both Yamassee, SC). Experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the NIH (publication number 86–23. Revised 1996). Environmental enrichment was provided.

Heart transplantation

Ten monkeys (including historical controls) received heart grafts from ABO-compatible, MHC-mismatched donors. MHC genotyping was performed as described.[27] Anesthesia, heterotopic intra-abdominal heart transplantation and graft monitoring were performed as detailed previously[21] and in the Supplementary Methods. In darTreg recipients CM115 and CM121, grafts were monitored until complete cessation of cardiac contraction, at which time the animals were euthanized. Graft survival was compared to historical controls with either no Treg infusion (CM117, CM116 and CM123) or with ex vivo expanded polyclonal Treg infusion (CM120 and CM118), reported previously.[21] darTreg recipients CM102, CM103 and CM220 were electively euthanized on days 18, 19 and 63 post-transplant, respectively.

Immunosuppression

Immunosuppressive regimens used are described in detail in the Supplementary Methods.

Expansion and function of donor Ag alloreactive (dar) Treg

$CD4^+$T cells were negatively enriched from fresh PBMC using NHP $CD4^+$T cell isolation kits (Miltenyi Biotech, Auburn, CA). $CD4^+CD25^+CD127^-$ Treg were then flow-sorted using a BD FacsAria (BD Biosciences, San Jose, CA). Isolated Treg were expanded using CD154-stimulated donor B cells for one week, followed by polyclonal stimulation using, artificial antigen-presenting cells (aAPCs; L-32 cells), kindly provided by Dr. Levings, University of British Columbia, Vancouver, Canada, as described[19, 21, 28] and in the Supplementary Methods (Figure S1). The ability of the expanded darTreg to suppress autologous alloreactive VPD450-labeled $CD3^+CD25^-$T cell (obtained from the same Treg donor) proliferative responses to prospective donor or third-party PBMC was determined in 5-day MLR.[29]

Tracking of infused darTreg

Expanded darTreg were labeled with either 4 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) or BD Horizon™ Violet Proliferation Dye 450 (VPD450, BD Biosciences) before infusion, as described.[19] At various times after infusion, mononuclear

cells were isolated from blood or tissues and the incidence of infused darTreg determined. Phenotypic analysis of the cells was performed in comparison with darTreg before infusion and endogenous (native) Treg. Tissue samples obtained from various organs were minced and digested with collagenase. The resulting cell suspensions were overlaid on Ficoll-Paque (GE Healthcare), centrifuged for 20 min at 1500 rpm and the buffy coat collected. .

Phenotypic analyses

Phenotypic analyses were performed using monoclonal (m) Abs directed against CD3 (clone SP34–2), CD4 (L200), CD8 (RPA-T8), CTLA4 (BNI3) and bcl-2 (Bcl-2/100) from BD Pharmingen (Franklin Lakes, NJ). Anti-CD25 mAb (BC96) was from eBioscience (San Diego, CA). Ki-67 (20Raj1) and Foxp3 (PCH101) mAbs were from Invitrogen (Carlsbad, CA), and CXCR3 (G025H7) and Helios (22F6) mAbs from Biolegend (San Diego, CA). Following live/dead staining with Zombie Aqua Fixable Viability Kit (BioLegend) at 4°C for 15 min, surface staining was performed with anti-CD3, CD4, CD8, CD25, CXCR3, and CTLA4. Afterwards, the cells were fixed and permeabilized for 45 min at 4°C using Fixation/Permeabilization buffer (eBioscienceTM; Invitrogen). Thereafter, intracellular staining was performed for Foxp3, Helios, Bcl2, Ki67 and CTLA4. Data were acquired on a LSR II or LSR Fortessa (BD Bioscience) flow cytometer and analyzed with FlowJo software (Tree Star).

RESULTS

Heart allograft survival

Two heart allograft recipients CM115 and CM121 (Figure 1A) received 4 weekly infusions of autologous, unlabeled ex vivo-expanded darTreg (Figure 1B) that exhibited superior ability to suppress autologous $CD3^+$T cell proliferation in response to donor than to 3rd party Ags at 1:8, 1:32 ($p<0.01$) and 1:128 ratios (Figure 1C). Moreover, their suppressive potency for MLR responses to donor was greater than that of ex vivo-expanded polyclonal Tregs[30] (Figure S2). Furthermore, darTreg expressed higher levels of CD25, Foxp3, CTLA4 and Helios, as well CXCR3 compared to autologous effector T cells (Figure 1D). Each recipient received 4 darTreg infusions on days 3, 10, 17 and 24 (Figure 1E).

Reductions in graft beating score were first observed on day 8 and 9, respectively. Graft contraction declined progressively thereafter until total cessation on days 84 and 85, respectively. Graft survival did not differ significantly from that of ATG-treated historical controls[21] that did not receive Treg infusion or received post-transplant infusions of polyclonal Treg (Figure 1F). These data indicate that infusion of darTreg into lymphodepleted recipient monkeys did not prolong heart graft survival, similarly to our previous observations using polyclonal Treg.

Infusion of labeled darTreg early post-transplant

To track and characterize labeled darTreg infused early post-transplant, 2 monkeys (CM102 and CM103) were infused on days 3 and 10 (Figure 2A). The first darTreg infusion was labeled with CFSE and the second with VPD450 to distinguish the 2 populations in vivo. Of note, the first darTreg infusion was given 3 days before, while the second infusion 4 days

after the third (final) ATG dose (on day 6). The recipients were electively euthanized on days 18 and 19 post-transplant, respectively, at which times graft-beating scores as shown in Figure 2B.

Tracking of darTreg infused early post-transplant in peripheral blood

CFSE- and VPD450-labeled darTreg were monitored in peripheral blood following their infusion. The incidences of CFSE-labeled darTreg (% $CD4^+$ T cells) on days 5 and 6 after transplantation (i.e. 2 and 3 days after their infusion) were 1.4% and 0.4% in CM102, and 0.5% and 0.1% in CM103, respectively (Figure 3A). The incidences of VPD450-labeled darTreg on days 10, 12, 13 and 15 after transplantation (i.e. 30 min, 2, 3 and 5 days after infusion) were 21.8%, 26.7% 6.7% and 1.4%, respectively in CM102 and 12.1%, 21.9%, 8.4% and 1.4%, in CM103 respectively. Meanwhile, the percentages of CFSE-labeled darTreg in peripheral blood at the same time points were <0.1% in both recipients (Figure 3B). Although Treg transfer was not performed in the absence of ATG, these observations suggest that lymphodepletion may negatively impacts the incidences of infused darTreg, as evidenced by their more pronounced reduction after ATG infusion.

Detection of darTreg infused early post-transplant in host tissues and the allograft

Graft recipients CM102 and CM103 were electively euthanized on days 18 and 19 post-transplantation respectively, to examine CFSE- and VPD450-labeled darTreg in blood, lymphoid and non-lymphoid tissues and the allograft (Figure 4). In CM102, CFSE-labeled darTreg that were infused 3 days before ATG were not detected in peripheral blood or lymphoid or non-lymphoid tissues at the time of euthanasia. In CM103, CFSE-labeled darTreg were either minimally detected (0.01% of $CD4^+$ T cells), or could not be detected at the time of euthanasia. In both recipients however, VPD450-labeled darTreg, that were infused 4 days after ATG, were evident at higher levels in blood (0.8% and 1.8%), the heart graft (0.4% and 1.8%), kidney (0.06% and 2.4%) and liver (0.3% and 2%), but not in the thymus (Figure 4A). In both recipients, CFSE-labeled darTreg were not detected in secondary lymphoid tissues or bone marrow. On the other hand, VPD450-labeled darTreg were detected at very low levels in mesenteric (0.04% and 0.2%), axillary (0.05% and 0.4%), inguinal (0.02% and 1.8%) lymph nodes (LNs), spleen (0.2% and 0.8%) and in bone marrow (0.06% and 0.05%) (Figure 4B).

These observations show that ex vivo-expanded darTreg can be detected in secondary lymphoid tissues and native organs following their infusion into allograft recipient monkeys.

Characterization of darTreg infused early post-transplant in blood and lymphoid tissues

As CFSE-labeled darTreg were either minimally or not detectable, we could not evaluate their phenotype. Rather, we next examined the phenotype of VPD450-labeled darTreg at the time of euthanasia in blood, spleen and mesenteric, axillary and inguinal LNs. In both graft recipients (CM102 and CM103), VPD450-labeled darTreg were assessed for expression of Treg markers,- Foxp3, CTLA4, and Helios, the proliferation marker ki67, and the anti-apoptotic marker Bcl2, in comparison to endogenous (native) $CD4^+CD25^{hi}$ Treg (Figure 5). Expression of Foxp3 and CTLA4 by VPD450-labeled darTreg in all tissues examined was lower than by endogenous darTreg, while Helios expression was comparable.

Ki67 expression by VPD450-labeled darTreg was markedly reduced in comparison to that of endogenous darTreg. In CM102, Bcl2 expression by the infused darTreg was comparable to that of endogenous Treg, while in CM103, its expression by VPD450-labeled darTreg was lower than that by endogenous Treg. These observations suggest that ex vivo-expanded darTreg lose their regulatory signature, proliferative capacity and survival signals shortly after infusion into ATG-lymphodepleted allograft recipients.

Delayed infusion of autologous darTreg

Next, we evaluated whether "delayed" post-transplant infusion of ex vivo-expanded darTreg following ATG-mediated lymphodepletion would impact their survival and phenotype differently compared with early infusion. In one heart allograft recipient (CM220; Figure 6A), VPD450-labeled darTreg were infused intravenously on days 43, 50 and 57 post-transplant (Figure 6B). In this recipient, tacrolimus was discontinued and replaced by rapamycin on day 18, while no IL-6R blockade was administered.This recipient was electively euthanized on day 63. Graft beating score is shown in Figure 6B.

Detection of darTreg after delayed infusion in blood, host tissues and the allograft

The incidences of VPD450-labeled darTreg in peripheral blood on days 46, 50 and 57 post-transplant (i.e. 3, 7 and 14 days after their first infusion) were 9.2%, 3.4% and 3.5%, respectively (Figure 7A). At the time of euthanasia (Figure 7B), the darTreg were detected in blood (6.5%), the allograft (2.9%), native heart (1.6%) and graft-draining LN (1.5%). The VPD450-labeled darTreg were also detected in mesenteric (1%), right inguinal (2.5%), left inguinal (2.9%), right axillary (3.6%), left axillary (3.2%) LNs and spleen (2.7%). DarTreg were also found in low levels in kidney (0.7%), lung (0.7%), liver (2.4%), bone marrow (0.9%) and minimally in the thymus (0.09%).

Characterization of darTreg in blood after delayed infusion

On days 43, 46 and 50 post-transplant, the phenotype of infused darTreg in peripheral blood was compared to that of native (endogenous) $CD4^{+}CD25^{hi}$ Treg and VPD450-labeled darTreg immediately before infusion (Figure 8A). Thirty minutes after the first infusion (on post-transplant day 43), VPD450-labled darTreg in blood exhibited similar levels of Foxp3 and Helios to those of endogenous Treg. On post-transplant day 46 (3 days after the first infusion), Foxp3 and Helios expression was slightly reduced compared to native Treg. On post-transplant day 53, Foxp3 and Helios expression was similar. Of note, Foxp3 and Helios expression by VPD450-labeled darTreg on days 43, 46 and 53 after infusion was markedly lower compared to before infusion. CTLA4 expression by these cells was lower than that by native Treg and compared to before infusion. Ki67 and Bcl2 expression by VPD450-labeled darTreg was lower than that by native Treg. Notably, Ki67 expression by darTreg after infusion was markedly reduced compared to before infusion. These observations suggest that, in this setting, ex vivo-expanded darTreg in peripheral blood lose their regulatory phenotypic signature, proliferative and survival capacity over time after infusion.

Characterization of darTreg in lymphoid tissues

Similarly, VPD450-labeled darTreg and native (endogenous) $CD4^+CD25^{hi}$ Treg were evaluated in peripheral blood, spleen, mesenteric, axillary and inguinal LNs, and compared with the phenotype of VPD450-labeled darTreg immediately before infusion (Figure 8A,B). In peripheral blood and all lymphoid tissues, expression of Foxp3, Helios, CTLA4, Ki67 and Bcl2 by VPD450-labeled darTreg was markedly lower than that by endogenous Treg. Of note, at euthanasia (day 63), expression of each of these markers was markedly lower than that by VPD450-labeled darTreg before infusion. Collectively, these observations suggest that in this setting, infused darTreg lose their phenotypic signature, proliferative and survival capacity in native tissues, similarly to peripheral blood.

DISCUSSION

The kinetics of in vivo migration, tissue distribution, stability, replicative capacity and persistence/survival of ex vivo-expanded Tregs following their adoptive transfer in graft recipients are poorly understood. This is especially so in humans, in which early phase trials of polyclonal or darTreg in combination with various immunosuppressive regimens are currently underway in kidney and liver transplantation.[5] To date, ex vivo-expanded human autologous polyclonal Treg labeled with deuterium have been monitored in peripheral blood following their infusion into patients with autoimmune disease[31] or conventionally-immunosuppressed (tacrolimus, MMF, corticosteroid) kidney transplant recipients.[8] To our knowledge, no studies have been reported of monitoring adoptively-transferred darTreg in humans.

Our earlier work[21] showed that multiple infusions of polyclonal Treg into ATG-treated, profoundly lymphodepleted cynomolgus heart allograft recipients could exacerbate anti-donor immune responses. Others however,[32] using a different model, have reported prevention of MHC class I- and II- mismatched renal transplant rejection in rhesus monkeys given host T cells rendered anergic to donor Ag following post-transplant cyclophosphamide administration. In the present study, multiple infusions of autologous ex vivo-expanded darTreg were not associated with improved heart graft function or delayed graft rejection, similar to our previous observations with polyclonal Treg.[21]

The doses of darTregs that we infused ranged from 20.5 to 120×10^6/kg for each of 2–4 infusions per heart graft recipient. These doses are similar to or greater than the doses of autologous T cells rendered anergic to donor Ag and infused (total 102 +/− 67×10^6) 13 days post-transplant that prolonged renal allograft survival in 6 rhesus monkeys, inducing donor-specific tolerance in 50%.[32] They also resemble the single doses of similarly-generated Tregs ($14–36 \times 10^6$/kg) infused 12 days post-transplant into living donor kidney transplant patients (n=16) that, by contrast, exhibited high rates of rejection upon subsequent immunosuppressive drug withdrawal.[33] In other clinical studies of adoptive transfer of darTeg in living donor kidney transplantation, lower doses of darTreg cells have been targeted, i.e. 2×10^3-2×10^6/kg or $0.5–10\times10^6$/kg respectively in small numbers of patients at separate centers in the ONE Study[10] in which overall safety of cell therapy and similar 1-year graft survival compared to a reference standard of care group were reported recently. In human living donor liver transplantation, doses of $23.3–14.4 \times10^6$ T

cells rendered anergic to donor and administered 13 days post-transplant induced operational tolerance in 7/10 recipients.[7] A total of 300–500 × 10^6 darTreg have been targeted in a liver transplantation drug withdrawal study at UCSF (NCT02474199).

While in previous tracking studies[19, 30] we monitored infused polyclonal Tregs in peripheral blood and secondary lymphoid tissue of immunosuppressed, non-transplanted cynomolgus monkeys, the present investigation, in which we have evaluated the therapeutic efficacy of infused darTreg and their fate in heart-allografted monkeys following lymphodepletion, provides new insights from this valuable, pre-clinical NHP model. Our dye labeling protocol does not impair NHP Treg viability or retention of Foxp3 expression following their in vivo transfer.[19] Our findings show, that while infused labeled darTreg can readily be detected by flow analysis in peripheral blood shortly after their systemic infusion, the ability to detect these cells diminishes progressively over the ensuing several days. The rapidity and extent of this decline appears to depend on the proximity of darTreg infusion to that of ATG, in that (although the delayed regimen was tested in only one animal and the results are therefore preliminary) delaying initiation of cell infusion from several days to one month after ATG results in a higher incidence of infused darTregs in the peripheral circulation, lymphoid tissues and the allograft. While this suggests that ATG may negatively impact Tregs, taking into account differences in the immunosuppressive regimens with which early and delayed darTreg infusions were combined, the data could also indicate that, while tacrolimus negatively impacts Treg, rapamycin is superior to tacrolimus in preserving NHP Tregs following their adoptive transfer, as suggested previously.[18] Under both experimental protocols however, we observed a rapid decline in transferred darTregs in the blood. This is consistent with our previous findings[19] and those of others[18] of an initial, short half-life of infused, dye-labeled polyclonal Tregs in the circulation of non-transplanted rhesus or cynomolgus monkeys, with very few cells persisting beyond 2 weeks. Importantly, this diminution in number could *not* be ascribed to proliferation of the darTreg (dye dilution), their selective accumulation in lymph nodes or bone marrow, or their phenotypic transformation. Cell death in the absence of appropriate expansion signals following their adoptive transfer, or immune-mediated elimination are possible underlying mechanisms. Notably, our findings are also in accord with similar pharmacokinetic profiles of adoptively-transferred human polyclonal Treg monitored using more human-applicable methods in hematopoietic stem cell transplant recipients[34] and kidney transplant patients,[8] and of chimeric Ag receptor (CAR) T cells in cancer patients.[35, 36]

Studies in mice[37] have shown that transferred darTregs, identified by congenic marker expression in cyclophosphamide-lymphodepleted islet allograft recipients, constitute nearly 50% of all Tregs early (4 and 6 days) post-transplant. While their migration pattern was in keeping with the trafficking of endogenous Treg from inflamed (graft) tissue to draining lymph nodes reported earlier,[38] the adoptively-transferred darTreg could barely be detected 14 days post-transplant, either in the graft or systemically,- in lymph nodes or spleen. This is in keeping with the low level of detection of transferred cynomolgus darTregs, either in the graft or lymphoid tissues, 1 or 4 weeks after their infusion early or late, respectively, after ATG administration in the present study. More recent monitoring of infused alloreactive (CAR) Tregs in mice using bioluminescence imaging has confirmed that they accumulate rapidly within skin grafts, with eventual migration to draining lymphoid tissue.[39] Notably,

expansion of adoptively-transferred darTregs has been reported in all tissues of mouse skin graft recipients when combined with IL-2 administration,[40] a possible means to mitigate their rapid loss/diminution.

While dye-labeling of infused darTreg may not be translatable to the clinical setting, it provided the important advantage (compared with deuterium labeling) of allowing us to directly examine the cells' phenotype in blood and tissues. In all host tissues examined, the transferred Treg exhibited lower levels of Foxp3 and the cell proliferation marker Ki67 than endogenous Treg, or the expanded darTreg before their infusion. Loss of Foxp3 by infused polyclonal cynomolgus or rhesus Tregs has been reported previously[18, 19] and taken to imply that the transferred cells lose their suppressive function. Unfortunately, as in these previous studies of polyclonal Treg, due to the low numbers of darTreg that persisted in the heart graft recipients, we were unable to isolate, re-purify and test their function directly. The current observations suggest that, as with polyclonal Tregs, the survival of transferred darTreg in lymphodepleted NHP recipients is short-lived and that, as reported in mice using different monitoring approaches, levels are maintained only at very low levels in lymphoid tissue and the allograft within the first few weeks post-infusion. Diminution of Foxp3 expression suggests loss of suppressive function over this period and that approaches are needed both to promote the longevity and to sustain the in vivo function of adoptively-transferred darTreg. Future application(s) of ex vivo-expanded Treg therapy may require approaches tailored to concomitant immunosuppressive regimens and the type of organ transplant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ag	antigen
ATG	anti-thymocyte globulin
CFSE	carboxyfluorescein succinimidyl ester
CTLA4	cytotoxic T lymphocyte antigen 4
Foxp3	forkhead box P3

IS	immunosuppression
MFI	mean fluorescence intensity
NHP	nonhuman primate
Teff	effector T cells
Tmem	memory T cells
Treg	regulatory T cells
VPD450	violet proliferation dye 450

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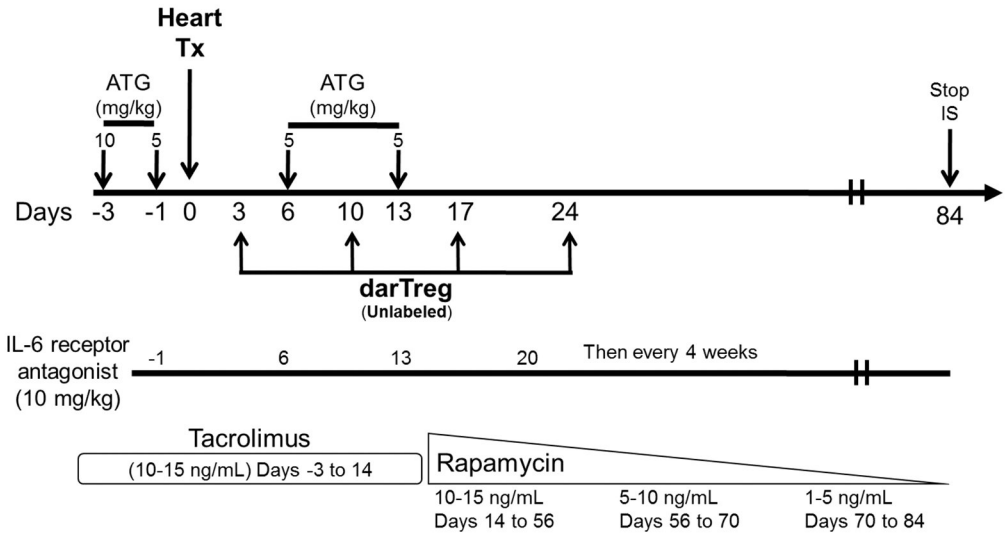

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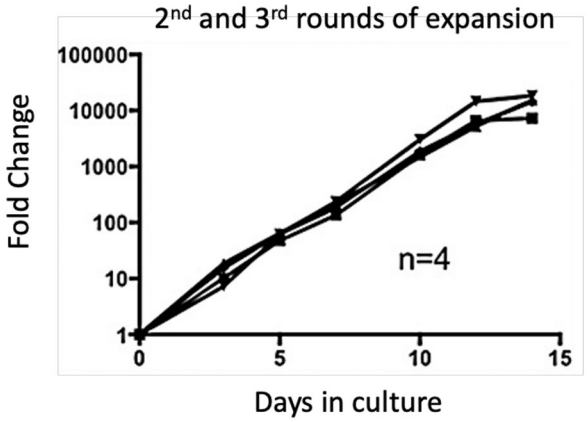

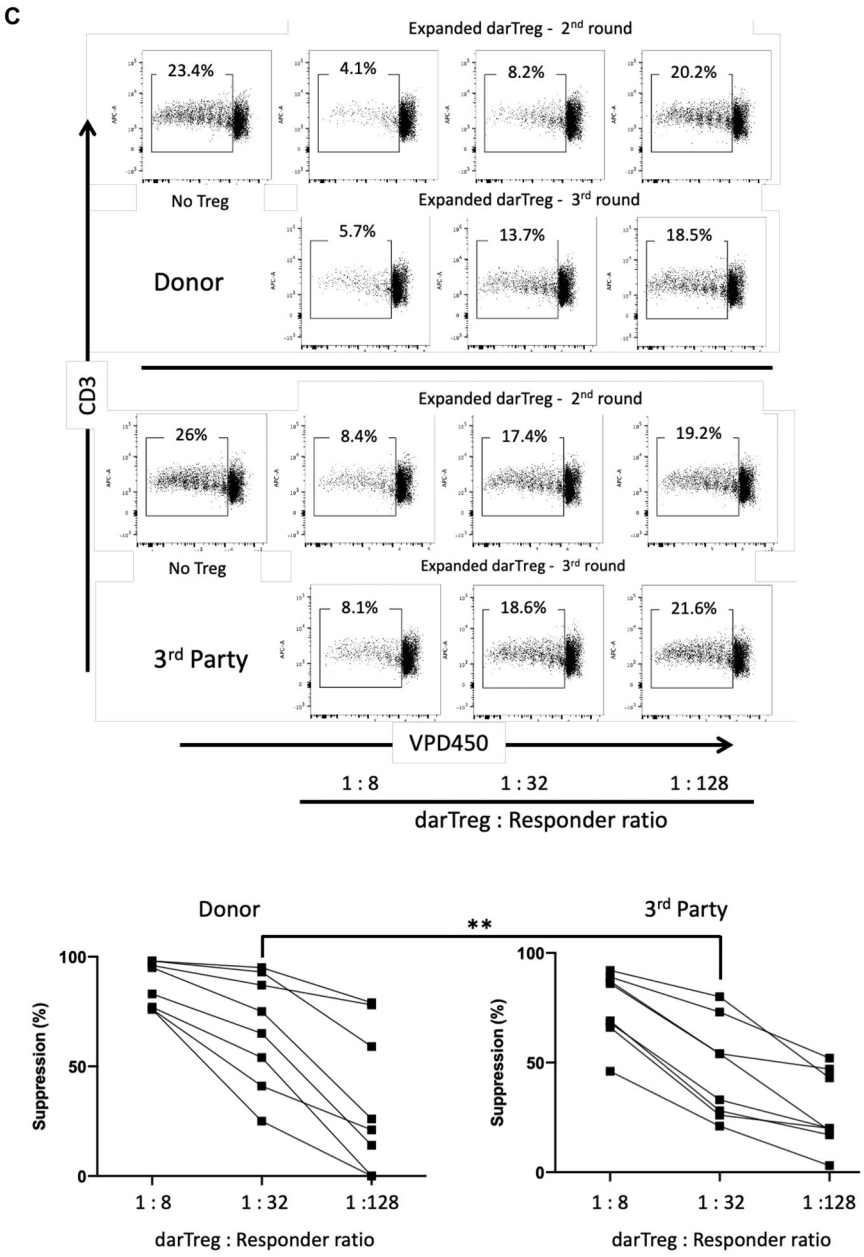

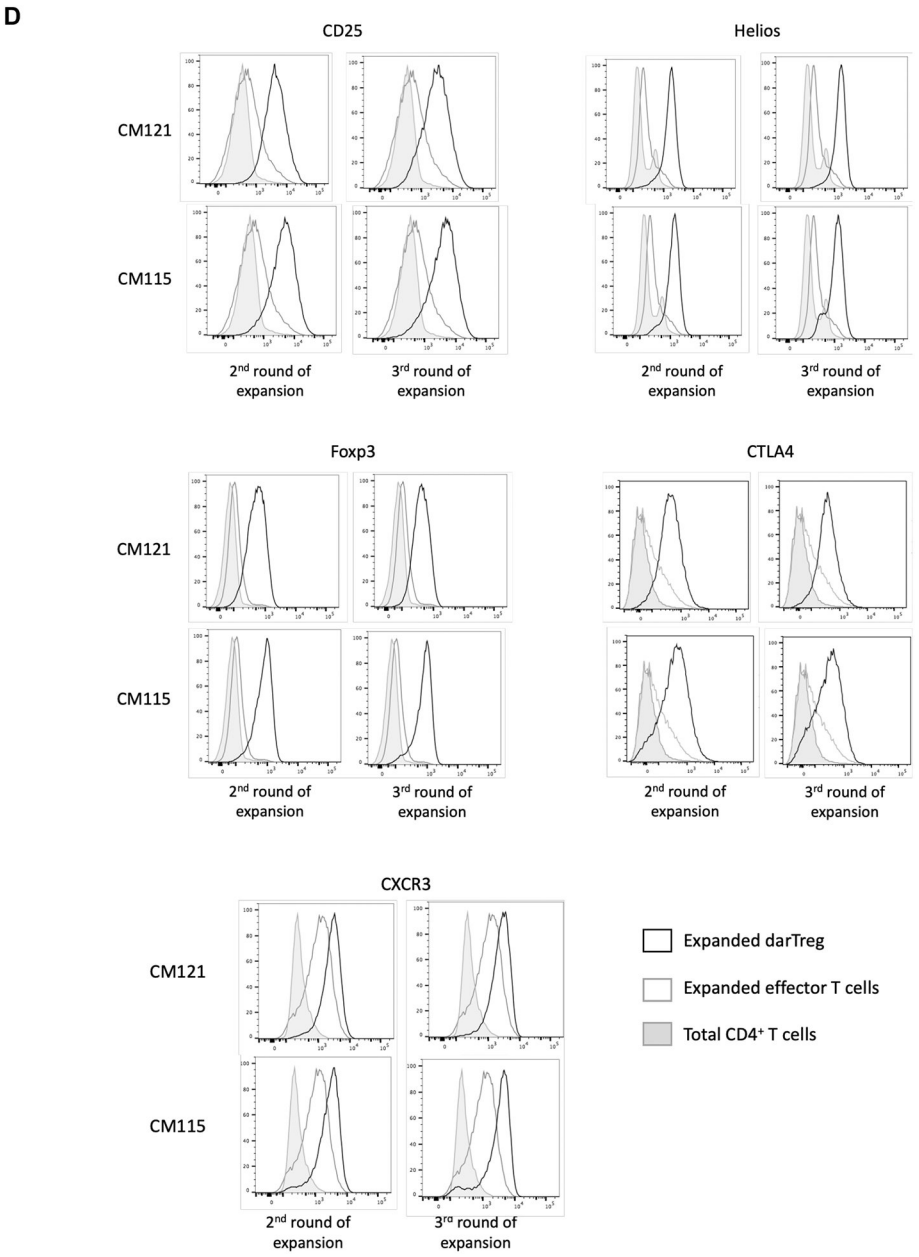

E

Timing, numbers and dosages of polyclonal Treg infusions

Animal ID	Time of infusion (POD)		Total polyclonal Treg infused (x10^6)	Polyclonal Treg (x10^6)/kg
CM120	Infusion #1	3	392	73
	Infusion #2	10	208	40
	Infusion #3	17	144	28
	Infusion #4	24	134	27
CM118	Infusion #1	3	596	132
	Infusion #2	10	588	134
	Infusion #3	17	154	35
	Infusion #4	24	422	98
	Infusion #5	31	110	26

Timing, numbers and dosages of darTreg infusions

Animal ID	Time of infusion (POD)		Total darTreg infused (x10^6)	darTreg (x10^6)/kg
CM115	Infusion #1	3	200	44
	Infusion #2	10	550	122
	Infusion #3	17	540	120
	Infusion #4	24	410	91
CM121	Infusion #1	3	280	47
	Infusion #2	10	540	91
	Infusion #3	17	580	93
	Infusion #4	24	370	63

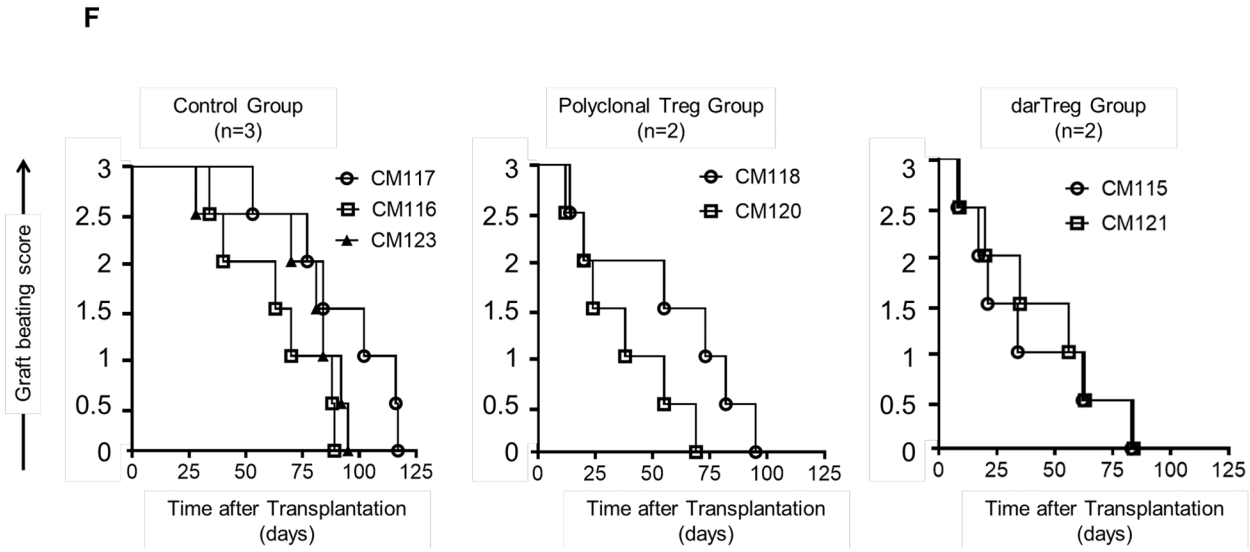

FIGURE 1.

Heart allograft survival in ATG-treated monkeys given autologous donor Ag alloreactive (darTreg) infusions or for comparison, polyclonal Treg infusions. A, Immunosuppressive regimen. Cynomolgus monkeys received rabbit ATG 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-6 receptor antagonist mAb 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 whole blood trough levels: 10–15 ng/ml), followed by rapamycin (i.m.) from days 14 to 56 (target trough levels: 10–15 ng/ml), after which rapamycin was weaned slowly and discontinued completely on day 84. B, ex vivo-expansion of darTreg (n= 4 separate ex vivo-expanded darTreg preparations); C, suppression of the proliferation of autologous T cells stimulated by donor or third-party stimulators by darTreg in MLR (top). darTreg were obtained from 2nd or 3rd rounds of expansion and are representative of 8 separate suppressive assays (bottom). Percent suppression of T cell proliferation is presented on the y-axis. x-axis shows darTreg : effector cell ratios. D, Phenotype of darTreg obtained from 2nd or 3rd rounds of ex vivo expansion. E, Timing, numbers and dosages of polyclonal (top) and darTreg (bottom). POD = post-operative day. F, Graft beating scores at various times post-transplant in monkeys infused with darTreg (n=2) and in non-infused (n=3) or polyclonal Treg-infused historical controls (n=2)

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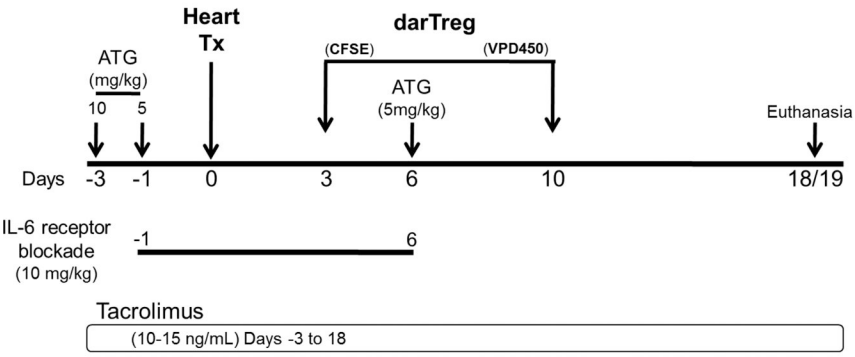

B

Animal ID	Time of infusion (POD)			Total darTreg infused (x10^6)	darTreg (x10^6)/kg	Label
CM102	Early post-transplant	Infusion #1	3	140	36.8	CFSE
		Infusion #2	10	280	77.8	VPD450
CM103		Infusion #1	3	90	20.5	CFSE
		Infusion #2	10	240	58.5	VPD450

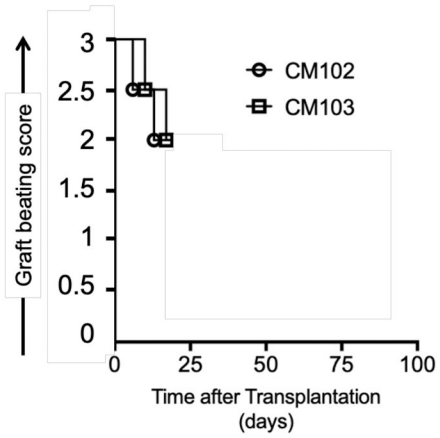

FIGURE 2.

Infusion of CFSE- and VPD450-labeled darTreg early post-transplant. A, The immunosuppressive drug regimen and darTreg infusion time points (days post-transplant) for 2 heart allograft recipient monkeys (CM102 and CM103) are shown. In each recipient, CFSE-labeled darTreg were infused on post-transplant day 3 (i.e. 3 days before ATG) and VPD450-labeled darTreg were infused on day 10 (4 days after ATG). Graft recipients were electively euthanized on day 18 (CM102) or 19 (CM103) for blood, allograft and host tissue sampling. B, Number of labeled darTeg infused at each time point into each graft recipient (top). Graft beating scores at various times post-transplant in CM102 and CM103 (bottom).

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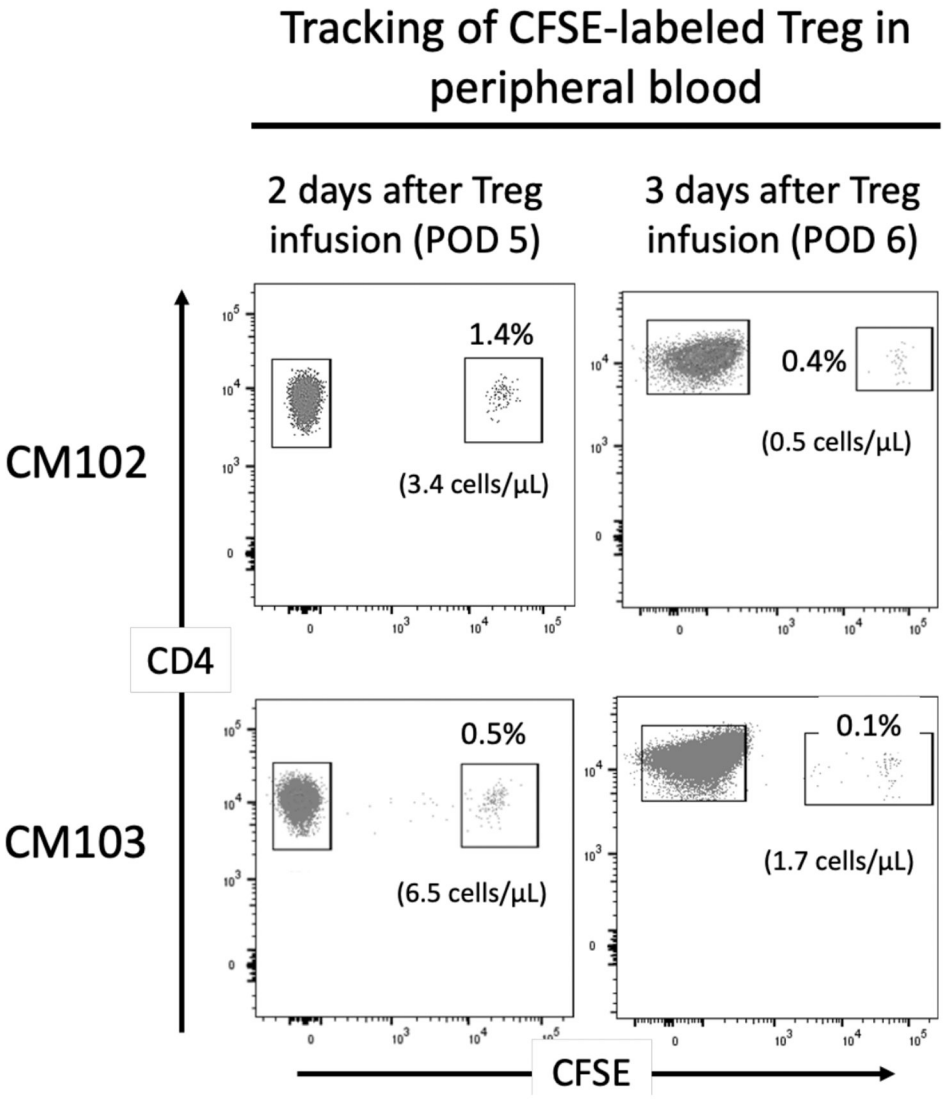

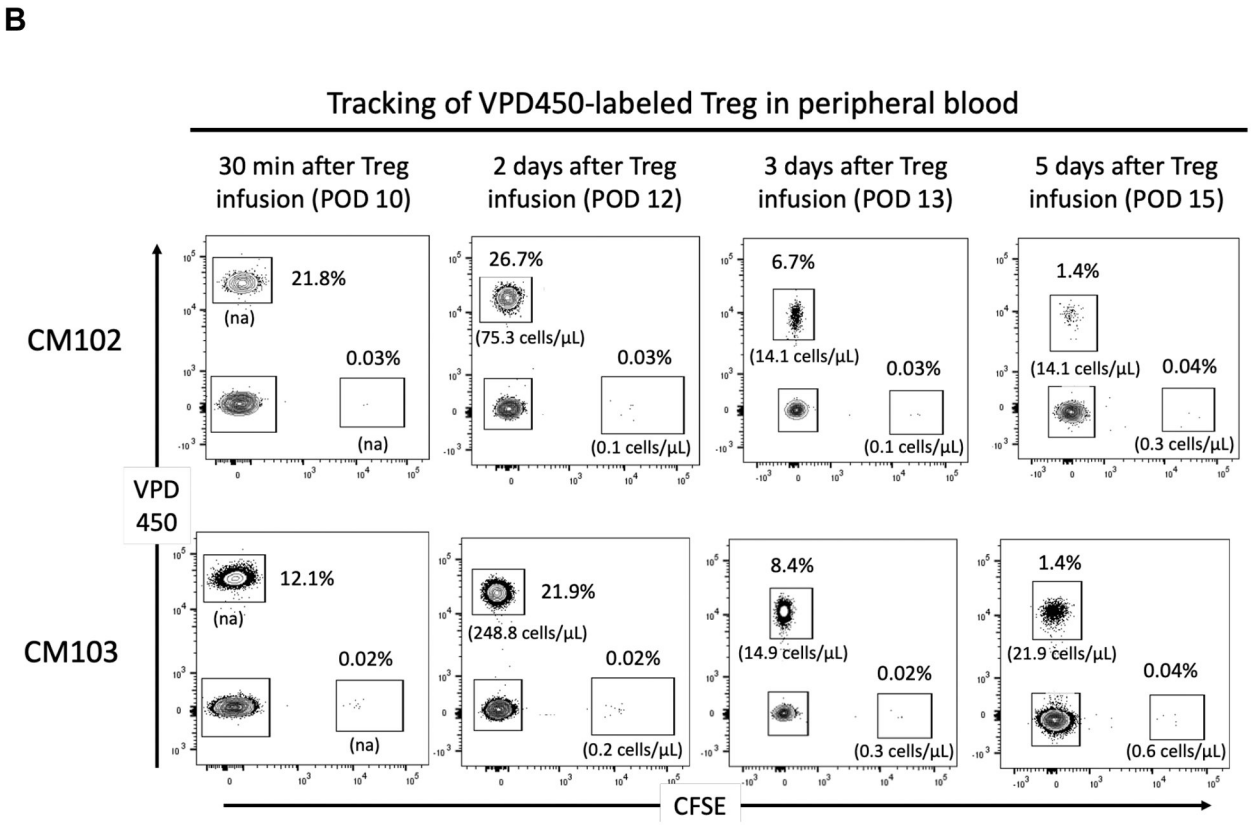

FIGURE 3.

In vivo tracking of CFSE- and VPD450-labeled darTreg infused early post-transplant in peripheral blood. A, Peripheral blood analysis of CFSE-labeled darTreg in heart allograft recipients (CM102 and CM103) on post-operative day (POD) 5 and 6 (i.e. 2 and 3 days respectively after their infusion). B, Peripheral blood analysis of VPD450- and CFSE-labeled darTreg in the same heart allograft recipients on POD 10, 12, 13, and 15 (i.e. 30 min, and 2, 3 and 5 days respectively after infusion of the VPD450-labeled darTreg,). Dot plots represent flow data after gating on total $CD4^+$ T cells. Absolute numbers of labeled darTreg (cells/µL) are also shown.

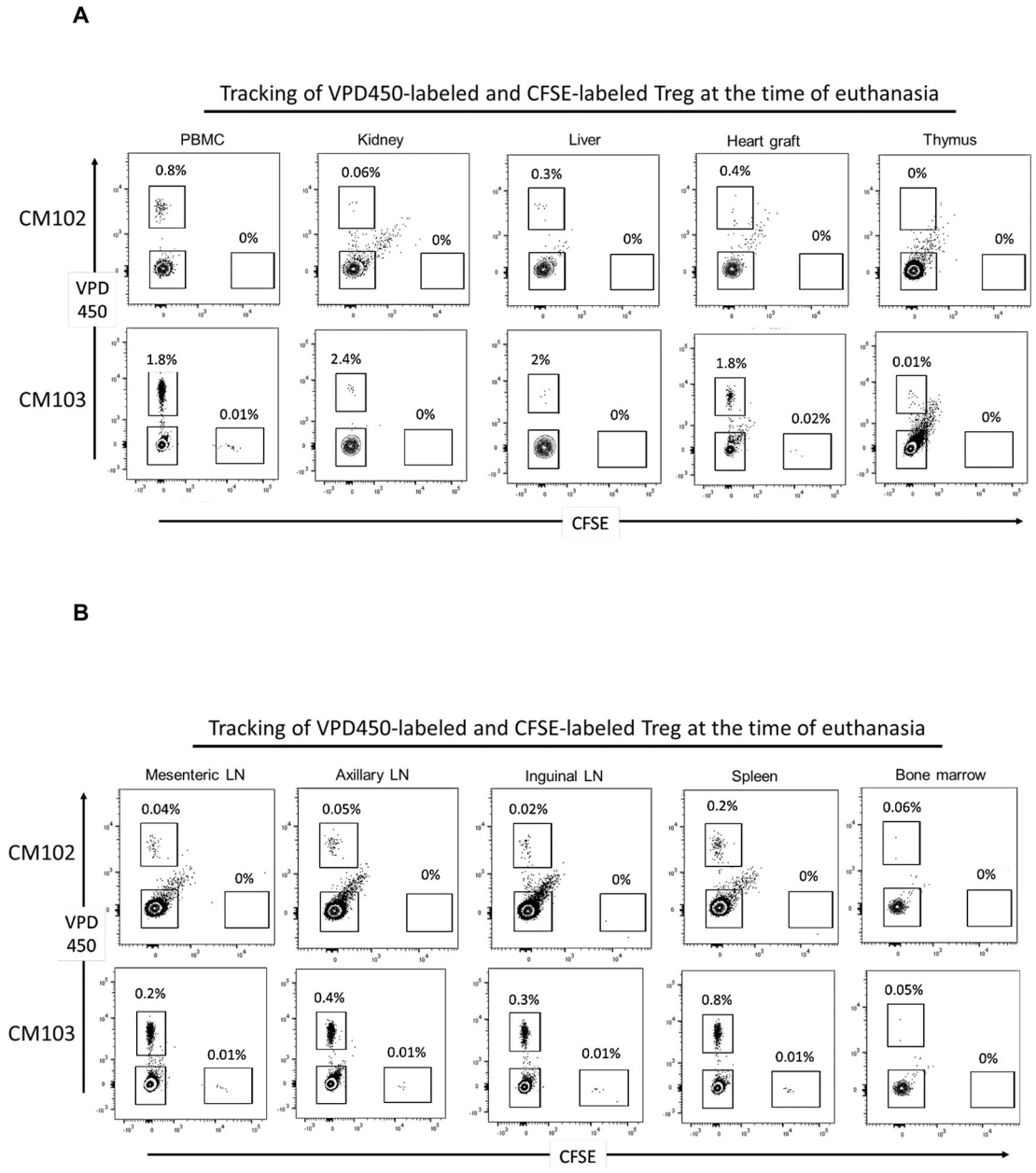

FIGURE 4.

Incidences of CFSE- and VPD450-labeled darTreg infused early post-transplant in peripheral blood, lymphoid tissues, non-lymphoid organs and the allograft at the time of euthanasia. A, Incidences of VPD450- and CFSE-labeled darTreg infused into monkeys CM102 and CM103 were evaluated 18/19 days post-transplant in (blood, kidney, liver and the heart allograft, thymus, and B, mesenteric, axillary and inguinal lymph nodes (LN), spleen and bone marrow. Dot plots represent flow data obtained after gating on total $CD4^+$ T cells. Absolute numbers of labeled darTreg (cells/μL) are also shown.

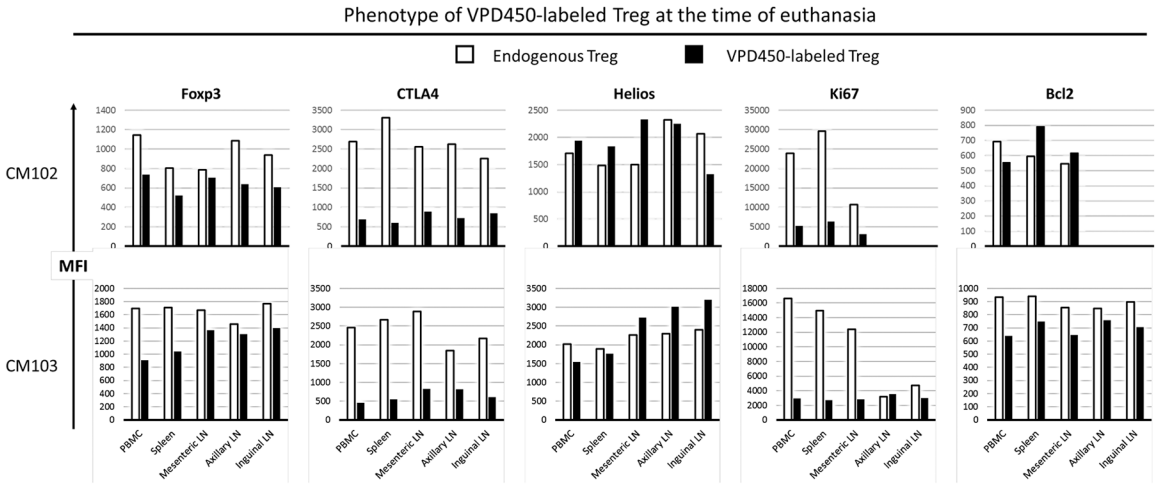

FIGURE 5.

Phenotype of VPD450-labeled darTreg infused early post-transplant in peripheral blood and lymphoid tissues at the time of euthanasia. In the 2 heart graft recipients (CM102 and CM103), the phenotype of VPD450-labeled darTreg was evaluated in blood, mesenteric, axillary and inguinal lymph nodes (LN) and spleen at the time of euthanasia 18/19 days post-transplant. MFI (mean fluorescence intensity) values of VPD450-labeled darTreg were determined simultaneously with MFI of native (endogenous) $CD4^{+}CD25^{hi}$ Treg.

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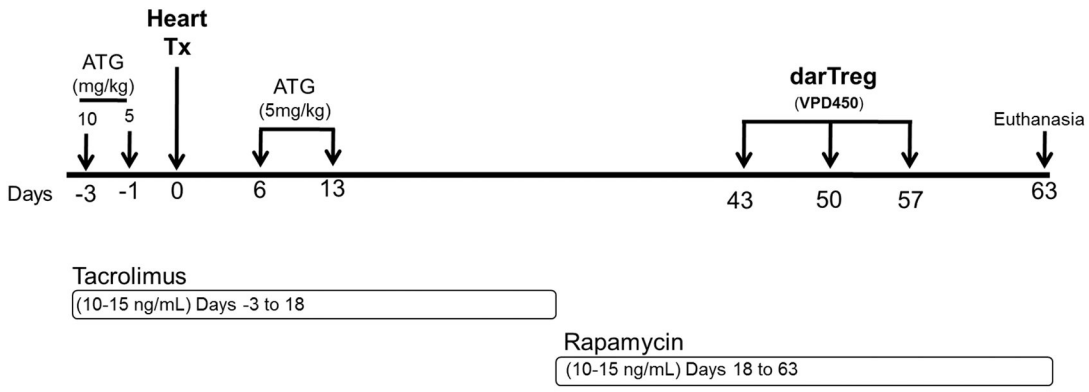


B

Animal ID	Time of infusion (POD)			Total darTreg infused (x10^6)	darTreg (x10^6)/kg	Label
CM220	Delayed post-transplant	Infusion #1	43	430	126.5	VPD450
		Infusion #2	50	628	174.4	
		Infusion #3	57	610	164.9	

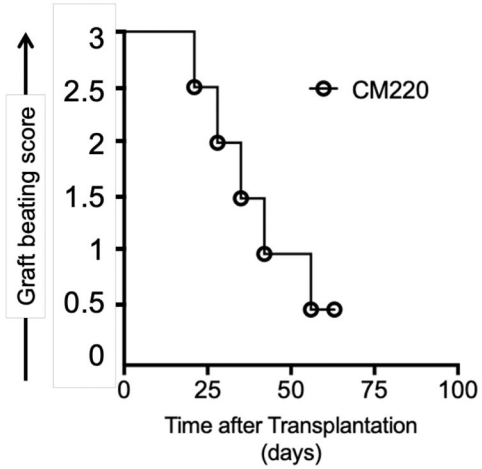
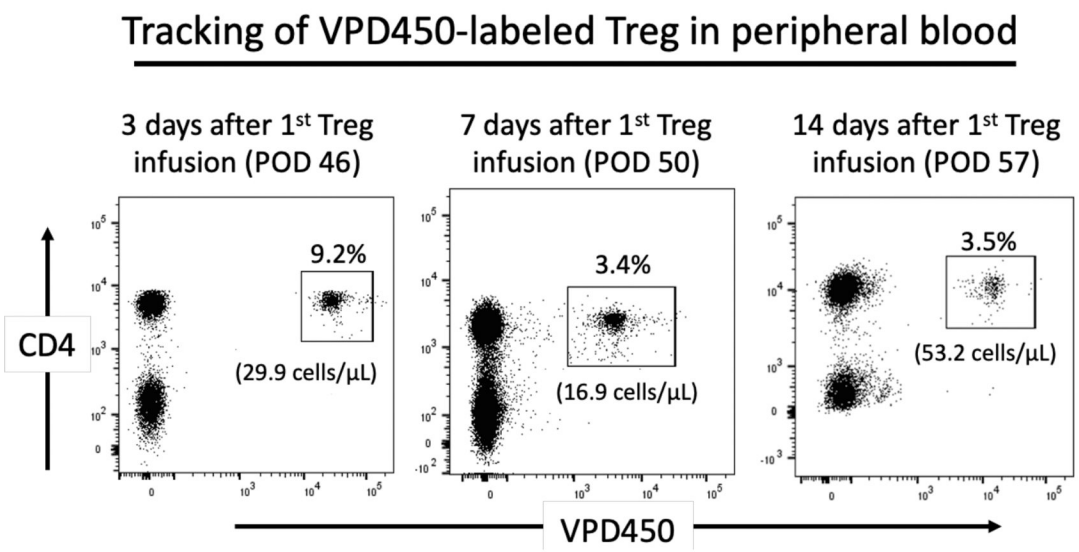


FIGURE 6.

Delayed infusion of darTreg post-transplant. A, Immunosuppressive drug regimen and darTreg infusion time points (days post-transplant) in one heart allograft recipient cynomolgus monkey (CM220). Infusions of VPD450-labeled darTreg were given on post-operative day (POD) 43, 50 and 57 (i.e. 30, 37 and 42 days after the final ATG infusion). B, Numbers of labeled darTeg infused at each timepoint (top). Graft beating scores at various times post-transplant in CM220 (bottom). The graft recipient was electively euthanized on POD 63 for blood, allograft and host tissue sampling.

A



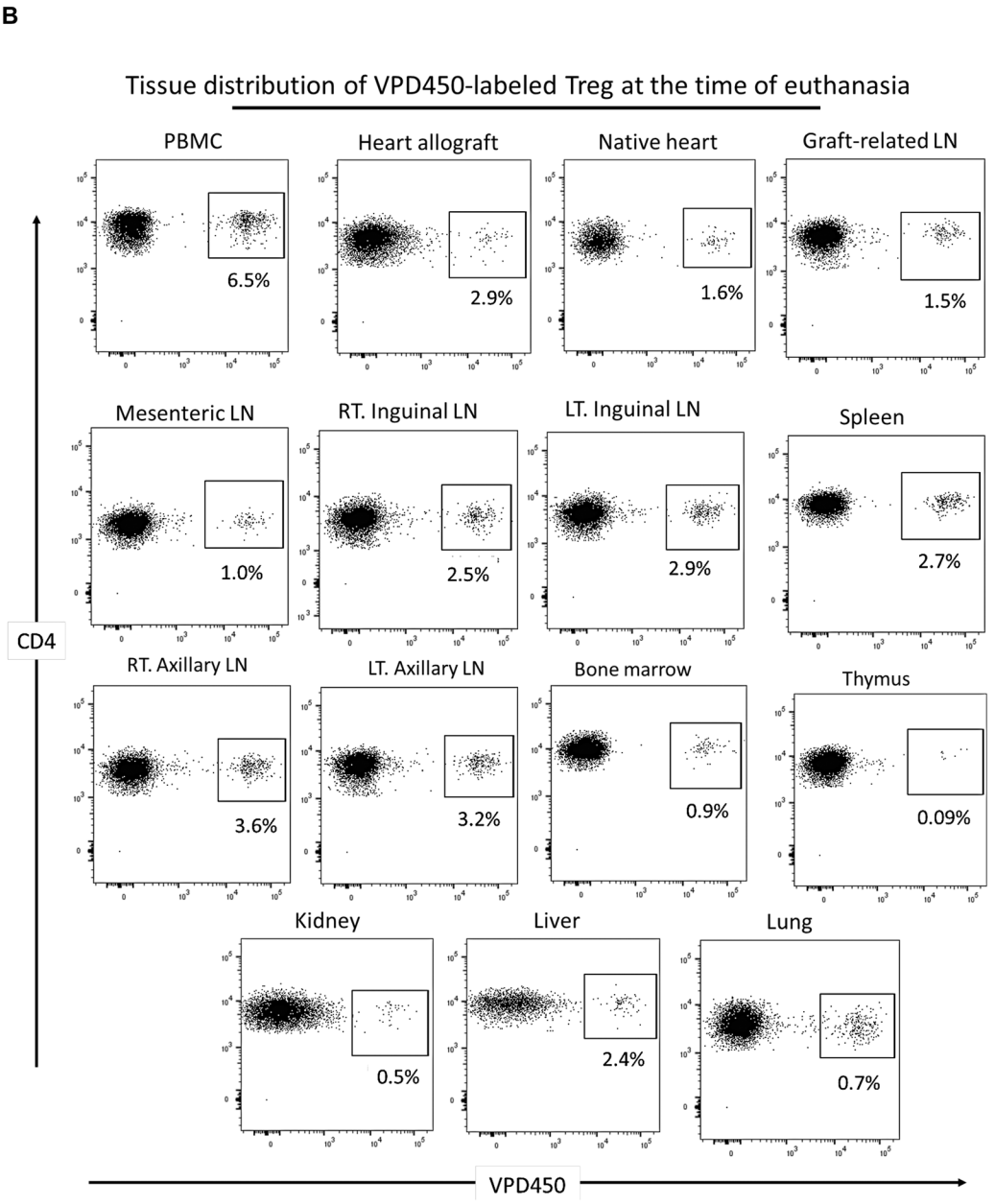
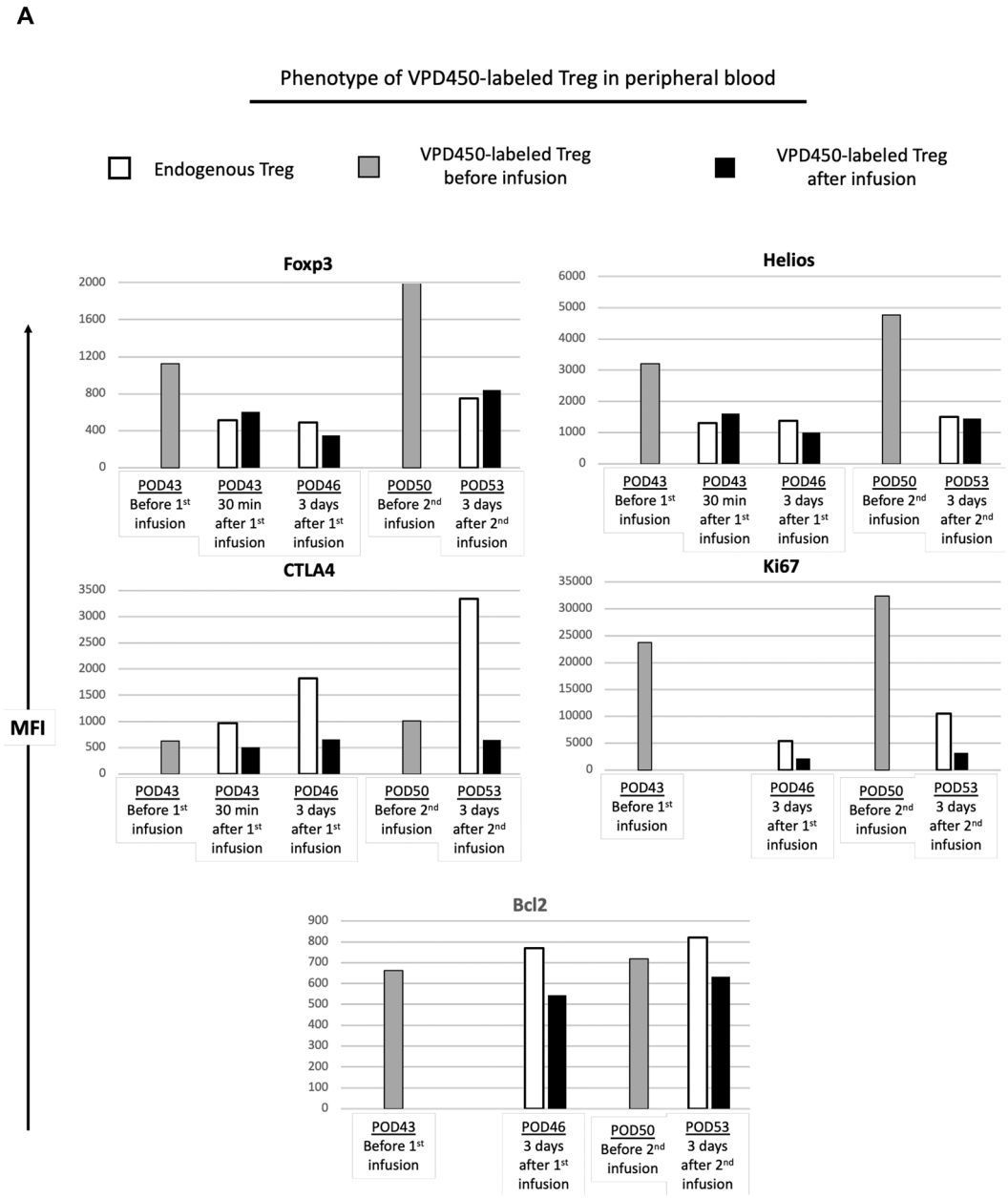


FIGURE 7.

Incidences of VPD450-labeled darTreg following their delayed infusion post-transplant in peripheral blood, lymphoid tissues and non-lymphoid organs at the time of euthanasia. A, Peripheral blood analysis of VPD450-labeled darTreg in CM220 on post-operative day (POD) 46, 50 and 57 (i.e. 3, 7 and 14 days respectively after their infusion). Absolute numbers of labeled darTreg (cells/μL) are also shown. B, Incidences of VPD450-labeled darTreg in blood, the heart allograft, native heart, lung, kidney, liver, graft-draining lymph node (LN), mesenteric LN, right (RT) and left (LT) inguinal LN, spleen, RT and LT axillary LN, bone marrow and thymus at the time of euthanasia, 63 days post-transplant. Dot plots represent flow data after gating on total $CD4^+$ T cells.



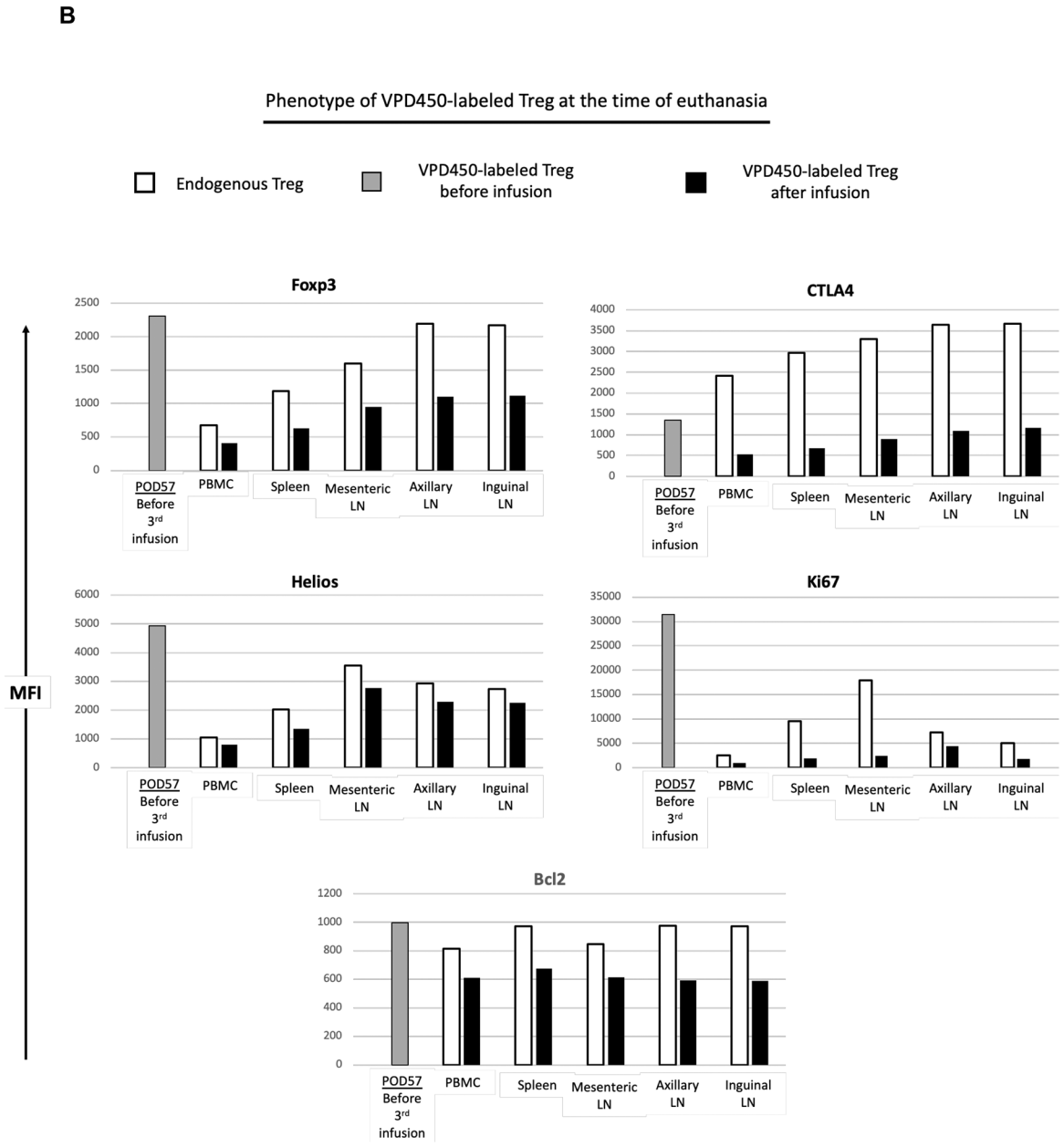


FIGURE 8.

Phenotype of VPD450-labeled darTreg following their delayed infusion post-transplant in peripheral blood and lymphoid tissues at the time of euthanasia. A, In monkey CM220, PBMC samples collected on POD 43, 46 and 53 were evaluated for VPD450-labeled darTreg phenotype in comparison to endogenous (native) $CD4^{+}CD25^{hi}$ Treg. For comparison, VPD450-labeled darTreg were analyzed on days 43 and 50 immediately before infusion. B, At the time of euthanasia, 63 days post-transplant, the phenotype of VPD450-labeled darTreg was evaluated in mesenteric, axillary and inguinal lymph nodes (LN), and spleen. MFI (mean fluorescence intensity) values of VPD450-labeled darTreg were determined simultaneously with MFI of native (endogenous) $CD4^{+}CD25^{hi}$ Treg. In A and B, white bars indicate native (endogenous) Treg; black bars indicate VPD450-labeled darTreg

in blood or lymphoid tissues after infusion. Gray bars indicate VPD450-labeled darTreg before infusion.