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Donor-derived regulatory dendritic cell infusion and early immunosuppressive drug withdrawal in living-donor liver transplantation: a phase I/IIa trial

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Operational tolerance (OT) following complete immunosuppression withdrawal (ISW) is rare (~13%) in eligible adult liver transplant recipients when initiated 1-2-years post-transplant. Regulatory dendritic cells (DCregs) promote transplant tolerance in pre-clinical models and attenuate immune effector cells in humans. Here, we completed a first-in-human phase I/IIa trial (2-year recruitment; 5 ± 0.5 years follow-up) to evaluate the feasibility, safety and preliminary efficacy of pre-emptive donor-derived DCreg (ddDCreg) infusion 7-days pre-transplant in 15 prospective living-donor liver recipients. Two patients were excluded from analysis for reasons unrelated to the study. ISW began one year post-transplant in candidates with a quiescent/permissive protocol biopsy. ddDCreg infusions were safe, reproducible, and well-tolerated. One-year post-transplant, 8/13 patients were eligible for ISW, 4 achieved complete ISW, 3 remained off all immunosuppression for >1 year. These 3 remained drug-free for 3.0 ± 0.17-years, reflecting a 37.5% OT rate in ISW-eligible recipients. Given the exploratory nature of this trial, additional studies to evaluate efficacy are needed. ClinicalTrials.gov registration number NCT03164265

Development of safe and effective therapies that improve long-term allograft survival, while reducing the intensity and duration of conventional immunosuppression (IS) would significantly improve the health and financial burden of transplant recipients. More specifically, early IS drug withdrawal/minimization (ISW), or better, complete cessation within the first few years post-transplant, would spare patients the cumulative risk of cardiovascular, metabolic, infectious, neoplastic and/or renal side effects of long-term IS, particularly those

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associated with calcineurin inhibitors[1,2]. Testing of innovative strategies to reduce patient dependence on IS drugs early post-transplant is of special interest in liver transplantation, as the likelihood of achieving complete ISW without rejection or allograft loss is greater than in kidney transplantation[3–5]. Liver allografts are also better tolerated by the host's immune system, with the likelihood of safe ISW increasing over time, depending on number of years post-transplant and other factors[6].

Animal studies[7,8] and clinical observations[9] indicate that naturally-occurring, regulatory immune cells play an important role in maintaining allograft tolerance in steady-state. Moreover, extensive pre-clinical studies are persuasive regarding the ability of adoptively-transferred innate or adaptive regulatory cells to enhance allograft survival and promote donor-specific transplant tolerance[10–12]. These findings have provided a strong rationale for early-phase clinical testing of regulatory immune cells, particularly regulatory T-cells (Tregs) in liver and kidney transplantation, with results confirming safety[13–16]. However, unequivocal evidence of efficacy has yet to be documented. An alternative approach to Tregs is the evaluation of regulatory myeloid cells, including regulatory dendritic cells (DCreg) and macrophages[17,18].

The case for DCreg utilization is compelling since DCs regulate innate and adaptive immunity and inherently express major histocompatibility complex (MHC) class II[19–22]. When donor-derived (dd) DCregs are adoptively-transferred to allograft recipients before transplant they can induce antigen-specific tolerance and indefinite allograft survival[10,21,23]. Notably, the therapeutic effect of ddDCregs appears not to depend on their in vivo persistence, rather on the acquisition of donor-antigen and regulatory molecules by host antigen-presenting cells[24–26], a potential advantage over other cell therapy approaches. DCregs also regulate naïve and preformed memory T-cell (Tmem) responses[27–29] that cross-react with those to human leukocyte antigens (HLA)[30], representing a major barrier to long-term allograft survival[31,32]. Furthermore, using minimal IS in a robust, non-human primate renal transplant model, we have shown[33] that a single pre-transplant infusion of ddDCregs safely prolongs allograft survival, without host sensitization and with attenuation of donor-specific, alloreactive Tmem responses.

Encouraged by these pre-clinical findings, we conducted a first-in-human, phase I/IIa trial of pre-transplant ddDCreg infusion in adult living-donor liver transplantation (LDLT). ddDCreg infusion was performed combined with early ISW in eligible patients, commencing 12-months post-transplant. Early complete ISW, without intervention, lasting ≥1 year has been documented previously in only 13-16% of stable adult liver recipients eligible for ISW, when initiated 1+ or 3+ years post-transplant[34,35]. Our underlying hypothesis was that ddDCreg infusion would be safe, feasible and, by modulating the host immune response, would increase the likelihood of safe, complete ISW 24-36 months post-transplant. In earlier reports[36,37], we showed that ddDCreg infusion induces immunological changes that may be conducive to early, safe ISW and allograft tolerance. Here, we report the end-of-study (EOS) results of the trial. The findings demonstrate the feasibility and safety of "pre-emptive" ddDCreg infusion in adult LDLT. While limited by sample size and by differences between LDLT in the present trial and deceased-donor LT (DDLT) in historical early ISW studies, we show that a proportion of ISW-eligible, ddDCreg-infused patients can achieve operational tolerance (OT) early post-LT.

Results

Patient enrollment, demographics, and clinical characteristics

A graphical representation of the study schematic and anticipated drug weaning schedule is available in Fig. 1. In total, sixteen donor-recipient pairs were enrolled (Fig. 2a). To minimize immunologic confounders[38] and in line with our pre-specified protocol, 1 patient, prior to any intervention, was withdrawn from the study following a positive crossmatch after enrollment. Elutriated monocyte fractions were obtained, via leukapheresis, from the remaining 15 prospective donors. Good manufacturing practice (GMP)-grade ddDCregs were generated, as previously described[39], and infused into 15 recipients 7-days prior to transplantation. After transplantation, 2 additional participants were withdrawn from the study for health reasons deemed unrelated to the study, as indicated in Fig. 2a. Altogether, 13 ddDCreg-infused participants were included in the final analysis (Fig. 2a–c, Table 1).

The LDLT cohort's mean age and body mass index were 62 (interquartile range (IQR): 58–68) years and 30.0 (IQR: 25.8–33.9), respectively. The median Model for End-Stage Liver Disease score was 10 (IQR: 8–16). Most patients underwent transplantation for a primary diagnosis of alcohol-associated liver disease, followed by metabolic dysfunction-associated steatohepatitis and hepatocellular carcinoma (Table 1). Additional demographic, clinical, and donor information are in Table 1, S1–2. The median number of HLA-mismatches per patient

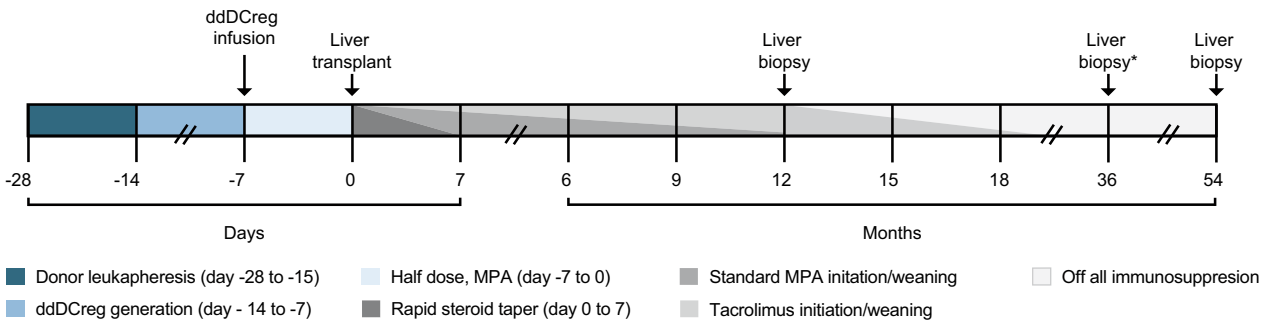

Fig. 1 | Graphical representation of the study schematic & anticipated drug weaning schedule. Following eligibility assessment and study enrollment, living donor liver transplant donors underwent 1 ($n = 12$ donors) or 2 ($n = 1$ donor) rounds of leukapheresis to acquire sufficient monocytes. Donor-derived regulatory dendritic cells (ddDCregs) were generated from the elutriated monocytes and infused into the transplant recipient 7-days prior to transplantation with daily mycophenolate (500-mg, twice daily) until transplant. Following transplantation, recipients received a rapid steroid taper over the first 7-days in addition to the standard-of-care (SOC) mycophenolic acid (MPA) and tacrolimus immunosuppression. A 12-month protocol biopsy was used to determine patient eligibility for complete MPA withdrawal and staged tacrolimus withdrawal. Tacrolimus withdrawal and weaning was expected to take less than 12 months and not to exceed 55 weeks from the eligibility evaluation from the 12-month protocol biopsy. A final end-of-study (EOS) liver biopsy was performed for all patients off IS and/or for patients who elected to undergo an additional EOS biopsy. Additional liver biopsies, as indicated by the *, were conducted for all patients all IS for a duration of one-year.

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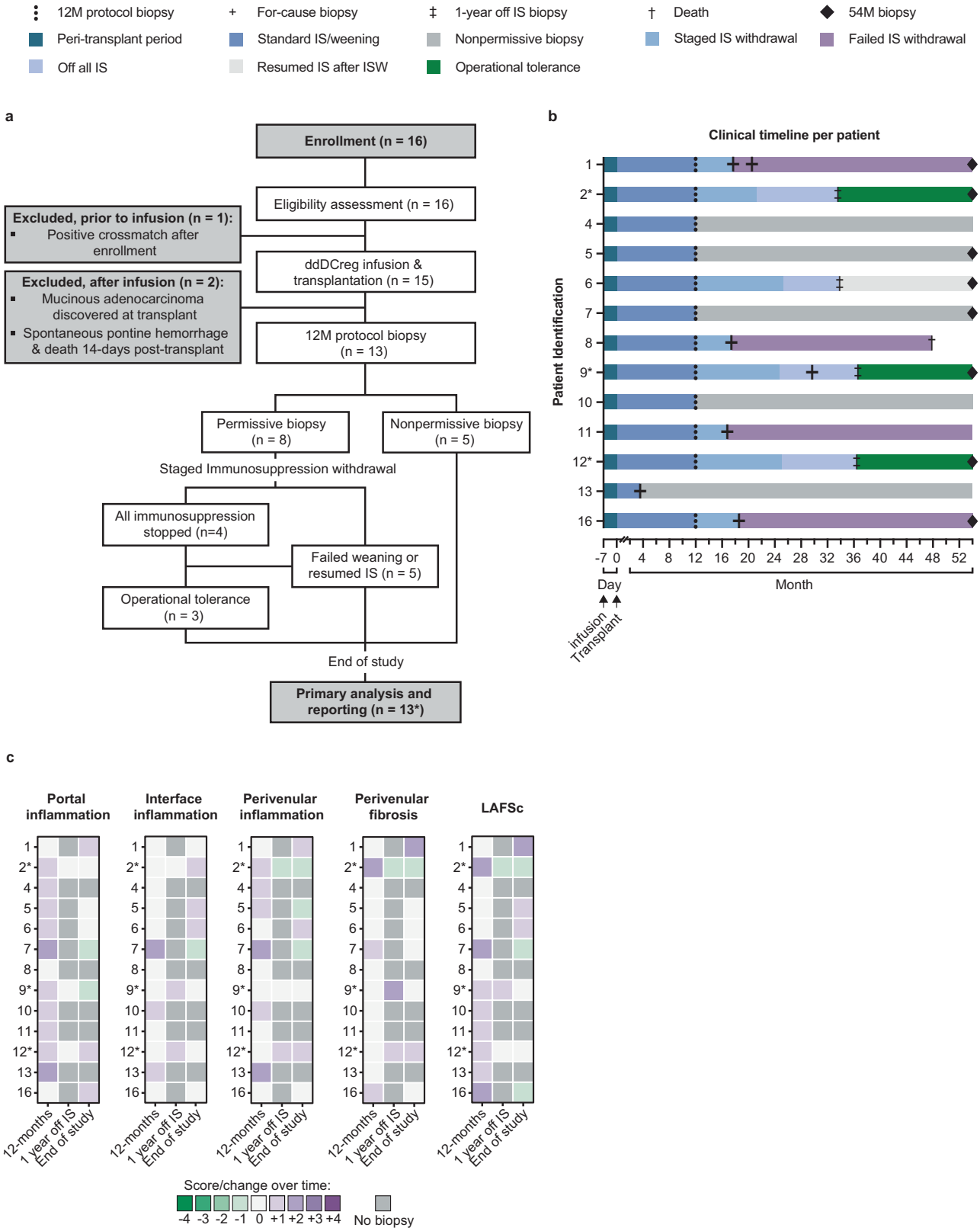

was 7, corresponding to a median total eplet-load of 50 (IQR: 21-60) (Table 1, S3–4). All tissue typing based-results are available in Tables S3, 4. Pre-transplant, pre-infusion, donor-specific antibody (DSA) was noted in 2 patients, although all 13 patients included in the final study were crossmatch-negative (Table 1, S5). Other patient-specific factors, such as recipient characteristics or operation-related details, appeared similar across the cohort (Table 1, S6).

Feasibility and safety

Donor leukapheresis was well-tolerated. Sufficient elutriated monocytes were obtained following one-round of leukapheresis from all but two donors, who underwent a second-round. No complete cell acquisition or manufacturing failures occurred; product release criteria are in Table 2. The mean dose and total number of ddDCregs infused were 5.2×10^6 DCregs per kg and 3.98×10^8 DCregs,

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Fig. 2 | Course and outcomes of the donor-derived regulatory dendritic cell (ddDCregs) infusion for living donor liver transplantation trial. a Modified Consolidated Standards of Reporting Trials (CONSORT) diagram of the Phase I/IIa, single-center, prospective, open-label, non-controlled, non-randomized, interventional, 16 patient cohort study in which low-risk, living donor liver transplant (LDLT) recipients received a single infusion of ddDCreg 1 week before transplantation. After enrollment and eligibility assessment, 1 patient was excluded secondary to a positive crossmatch. Following infusion and transplantation, two additional patients were excluded. In total, 13 patients underwent 12-month (M) protocol biopsies. Eight patients were eligible for staged immunosuppression (IS) withdrawal: of these, 4 were weaned off all IS, 3 of the 4 achieved operational tolerance and remained off all IS drugs until the end-of-study (EOS). The other patients with eligible, permissive protocol biopsies failed weaning ($n = 4$) or resumed IS following complete withdrawal ($n = 1$). *, one patient who failed weaning died 48.2 months post-transplant secondary to a geriatric fall and was included in the longitudinal analysis. **b** Modified swimmer plot demonstrating the clinical course and timeline for each individual patient included in the final study analysis. **c** Modified change heatmap depicting the initial evaluation and histologic changes, according to the Banff criteria, over time in the 1-year off IS and EOS biopsies compared to each patient's initial baseline eligibility biopsy (12 M protocol biopsy). Each row represents a single patient, and operationally tolerant patients are denoted by a *. To calculate change over time, absolute scores at baseline were subtracted from scores at follow-up for the following parameters: portal inflammation, interface inflammation, perivenular inflammation, perivenular fibrosis, and liver allograft fibrosis score (LAFSc). All score scales range from 0 to 3 except the LAFSc scale, which ranges from 0 to 9. Purple indicates an increase in score and green indicates a decrease in score; increasing intensity of either purple or green indicates a larger magnitude of change. Gray indicates no biopsy; white indicates no change.

Table 1 | Patient demographics and baseline clinical characteristics of the final ddDCreg cohort

Final study cohort ($n = 13$)											
Baseline demographic information:						Underlying disease and transplant-related characteristics:					
Recipient	Age	Sex	Race[a]	Weight (kg)	BMI	Primary diagnosis	MELD	DSA pre-DCreg infusion[b]	HLA MM	Total eplet load	Blood group (Donor-Recipient)
01	59	M	White	96	33.2	MASH	16	Negative	7	43	O + /O-
02	57	F	White	99	35.1	HCC	6	Negative	7	56	O + /O+
04	69	M	White	105	33.3	AALD	8	Negative	4	10	O + /O+
05	64	M	White	86	28.7	MASH	8	Negative	8	71	O-/O+
06	64	F	White	56	25.9	HCC	15	Negative	8	50	A + / A+
07	50	F	White	71	24.6	AALD	9	Negative	9	51	O-/ A+
08	68	F	White	72	27.1	MASH	9	Negative	6	38	O-/O-
09	62	M	White	128	38.2	AALD	7	Negative	0	0	O-/O+
10	62	F	-	64	25.6	HCV	10	Negative	2	14	O + / A+
11	66	F	White	68	24.2	AALD	17	Positive	5	21	O + /O-
12	69	F	White	73	27.5	MASH	17	Positive	8	66	O + /O-
13	67	F	White	88	34.4	HCC	10	Negative	9	65	O + /A+
16	48	M	White	97	32.4	AALD	20	Negative	11	60	O + /O+
Mean (±SD)	62 (7)	-	-	85 (20)	30.0 (4.6)	-	12 (5)	-	6 (3)	42 (24)	-
Median (IQR)	64 (58–68)	-	-	86 (70-98)	28.7 (25.8–33.9)	-	10 (8–16)	-	7 (4–9)	50 (21–60)	-

AALD alcohol-associated liver disease, *BMI* body mass index, *ddDCregs* donor-derived regulatory dendritic cells, *DSA* donor-specific antibody, *HCC* hepatocellular carcinoma, *HCV* hepatitis c virus, *HLA* human leukocyte antigen, *IQR* interquartile range, *kg* kilogram, *MELD* model for end-stage liver disease, *MASH* metabolic dysfunction-associated steatohepatitis, *MM* mismatch, *SD* standard deviation.

[a]All patients identified as white, non-Hispanic or Latino; one declined to identify.

[b]All patients were crossmatch negative.

respectively. A target dose range of 2.5–10 × 10^6 ddDCregs per kg was achieved in 12 patients. A T-cell co-inhibitory (PD-L1) to co-stimulatory molecule (CD86) expression ratio of >1 was confirmed for all but one of the ddDCreg products. The mean ddDCreg anti- to pro-inflammatory cytokine (IL-10:IL-12) secretion ratio was 32.4. In patients who received an insufficient target dose (#13) or who received a product with a lower PD-L1:CD86 target ratio (#7), the IL-10:IL-12 ratio was 27 and 40, respectively, consistent with a tolerogenic DC phenotype. All infusions were monitored and well-tolerated, with no infusion reactions or adverse events (AEs) (Fig. 3). Pre- and post-infusion monitoring indicated mild presumptive hematopoietic cell sequestration and modest reductions in several laboratory tests (Fig. 3). As previously reported, intact infused ddDCreg were evident (mean 1.5% DC chimerism) in 8 of 9 recipients that could be analyzed 1-hour post-infusion (day −7). However, in these 8 patients, donor cells were no longer evident at later time-points (day −3 to the day of surgery); continued analysis performed 1-month post-transplant revealed intact donor cells (most likely graft-derived) in only 1 of the 9 patients.

The mean (± standard deviation) total hospital stay following transplantation was 14.0 ± 13 days (Table S6). One patient underwent reoperation following transplantation, whereas 5 patients were readmitted following discharge for non-ddDCreg-related issues (Table S6). At 12 months, no significant differences in biopsy-proven acute cellular rejection (BPAR), reoperation, readmission rates, vascular, biliary, or infectious complications were noted compared to a propensity-matched standard-of-care (SOC) LDLT reference cohort[37]. Infectious complications, until EOS, occurred in only 5 patients (Table S7). More importantly, only one per-protocol grade-4 AE occurred (Table S8): one patient (#8) died 48.2 months post-transplant secondary to a traumatic head injury.

DSA was routinely monitored throughout the study (Fig. 4, Table S5). Within the first-year post-transplant, 5 patients developed DSA. However, at 12-months, only 1 patient (#12) - who had pre-transplant DSA - exhibited persistent memory DSA. At 18-months, coinciding with ISW, 4 patients developed de-novo DSA. At EOS, 5 patients had circulating DSA. Of these 5 patients with EOS DSA, 2 were

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Table 2 | ddDCreg product generation and infusion-related information for the final cohort

Final study cohort cell product information ($n = 13$)[a]							
Recipient	**Rounds of donor leukapheresis**	**Elutriated donor monocytes (x10^9)**	**Generated ddDCregs (x10^8)**	**IL−10 to IL−12 ratio[b]**	**PD-L1 to CD86 ratio**	**Infused ddDCregs (x10^8)**	**Dose (x10^6 ddDCregs per kg)[c]**
01	1	1.9	6.03	7	5.2	5.43	5.3
02	2	1.6	3.46	10	4.5	3.11	2.9
04	1	2.0	6.25	26	17.8	5.63	4.3
05	1	1.7	3.74	22	3.5	3.54	4.1
06	1	2.0	4.98	48	4.5	4.73	8.3
07	1	3.0	4.99	40	0.9	4.56	8.7
08	1	1.6	3.20	45	2.5	2.98	4.0
09	2	2.5	5.65	52	3.9	5.48	4.2
10	1	1.5	5.68	79	4.4	5.47	8.3
11	1	2.4	2.45	15	2.0	2.21	3.2
12	1	1.3	3.79	34	8.7	3.48	4.6
13	1	0.7	1.41	27	6.9	1.37	1.6
16	1	1.6	3.77	17	7.0	3.68	4.1
Mean (±SD)	-	1.8 (±0.6)	4.26 (±1.47)	32.4 (±20.2)	5.5 (±4.2)	3.98 (±1.37)	5.2 (±2.1)
Median (IQR)	-	1.7 (1.6–2.0)	3.79 (3.46–5.65)	27 (17–45)	4.5 (3.5–6.9)	3.68 (3.11–5.43)	4.3 (4.1–6.1)

CD86 cluster of differentiation 86, *ddDCregs* donor-derived regulatory dendritic cells, *IL-10* interleukin-10, *IQR* interquartile range, *PD-L1* programmed death ligand 1, *SD* standard deviation.
[a]Release criteria: cell viability >70%, HLA-DR expression >80%, PD-L1 to CD86 mean fluorescence intensity ratio >1, mycoplasma negative, no organisms on gram stain, T- ($CD3^+$) and B-cell ($CD19^+$) contamination <1%, and endotoxin <5EU/kg of body weight per dose.
[b]IL-10 to IL-12 secretion ratio post-release following stimulation with J558-CD40L cell line.
[c]Target cell dose range: $2.5–10 \times 10^6$ ddDCregs per kg.

operationally tolerant (#2,12), 1 (#6) resumed SOC IS after 1-year off all IS, and 1 was ineligible for ISW (#13) (Figs. 2b, 4a, Table S5). The remaining patient, with EOS DSA, failed staged ISW (#1). Of note, only 3 patients (#1, 2, 12) at EOS had a strong immunodominant antibody, defined as a mean fluorescent intensity >5000 (Fig. 4a, Table S5). Moreover, C4d staining was positive in only 2/8 patients with available EOS biopsies (#1, 12) (Tables S9–13).

Given the variable pathogenicity of immunoglobulin G (IgG) subtypes and the greater occurrence of class II DSA in our cohort, IgG subtyping of DSA from 4 patients was performed (#2,6,8,12). Two patients displayed only elevated levels of IgG1 DSA (#2,6). The remainder exhibited the temporal transition of predominantly IgG1 to increasing IgG4 concentrations suggestive of chronic antigen exposure (#8,12) (Table S14).

Additional clinical monitoring, safety, and preliminary efficacy exploration

As previously reported[37], longitudinal monitoring of allograft function/outcomes including BPAR up to 12-months post-transplant revealed no significant differences between the ddDCreg-recipients and a propensity-matched SOC LDLT reference cohort. In fact, only one patient who received a sub-target dose of ddDCregs, experienced BPAR (#13) at 3.5 months post-transplant (#13). Following treatment (Table S13), she remained stable, without any additional issues, on her baseline IS until EOS.

At 12-months post-transplant, protocol biopsy analysis and eligibility assessment completed by the study pathologist, indicated that 8 ddDCreg recipients were eligible for ISW, according to published criteria[40] (Fig. 2a–b, Tables S11). The remaining five patients with either a nonpermissive biopsy or BPAR, all remained on their baseline IS and were stable throughout the remainder of the study (Fig. 2b, Tables S11, S13). For patients with a permissive protocol biopsy, complete mycophenolic acid (MPA) cessation occurred at 12.1 ± 1.4 months post-transplant (Fig. 4b–c). During staged tacrolimus ISW, a transient elevation in liver-related laboratory tests occurred at 18 months post-transplant (Figs. 4d–f, 5a–b). 4 of the 8 eligible patients (#1, 8, 11, 16) failed staged ISW (Fig. 2b, Table S13). Each patient who failed staged ISW, returned to their baseline IS, were monitored biochemically, and remained stable until EOS (Table S13).

The other 4 patients eligible for ISW achieved complete tacrolimus withdrawal 11.4 ± 2.2 months after their 12-month protocol biopsy (#2, 6, 9, 12) (Table S11, Figs. 2b, 4b, Supplementary Fig. 1). Three of these four patients had no overt evidence of T-cell mediated rejection one-year off all IS (operationally tolerant) and remained off all IS until the EOS (#2, 9, 12) (Table S11). The fourth patient (#6) - off all IS - experienced suspected immune re-activation and rejection two weeks after COVID-19 vaccination; they returned to their baseline IS without any further issues (Fig. 2b, Table S13). Compared to the historical benchmark of successful early ISW[34] without an additional intervention, the proportion of patients achieving OT, however, was not statistically significant ($P = 0.188$, 95% confidence interval: 0.09–0.76). All biopsy-related details (study and local pathologist evaluations), timing, and eligibility assessments for ISW/cessation/return to IS are available in Tables S9–13 with changes depicted overtime in Fig. 2c.

At EOS, the cohort's mean tacrolimus trough level was 2.8 ± 1.9 ng mL^{-1}, down from an initiation trough of 5.8 ± 3.3 ng mL^{-1} (Fig. 4b). However, the average tacrolimus dose, for those on IS at EOS, rose slightly above the cohort's baseline of 2.4 ± 1.6 to 2.7 ± 2.3 mg per day (Supplementary Fig 1). The urine protein excretion ratio (Supplementary Fig. 2a) also remained below 0.2 in 8/9 subjects at 36–48 months and random glucose levels at EOS decreased from baseline by a paired mean difference of 14.7 ± 45.2 mg dL^{-1} (Fig. 5c). Without accounting for EOS IS differences, mean estimated glomerular filtration rate (eGFR) increased from baseline to EOS, across the cohort, by a paired difference of 4.4 ± 27.8 mL per min per 1.73 m^2 (Fig. 5d). However, paired mean increases in systolic blood pressure (10.5 ± 16.3 mmHg) and triglycerides (98.9 ± 141.3 mg dL^{-1}) were noted at EOS (Fig. 5e, Supplementary Fig. 2b). Other factors, such as blood urea nitrogen (BUN) levels, creatinine, cholesterol, and diastolic blood pressure remained within expected ranges (Supplementary Fig. 2c–f). No new cases of hypertension, despite a rising cohort average, post-transplantation diabetes, hyperlipidemia, or hypercholesterolemia

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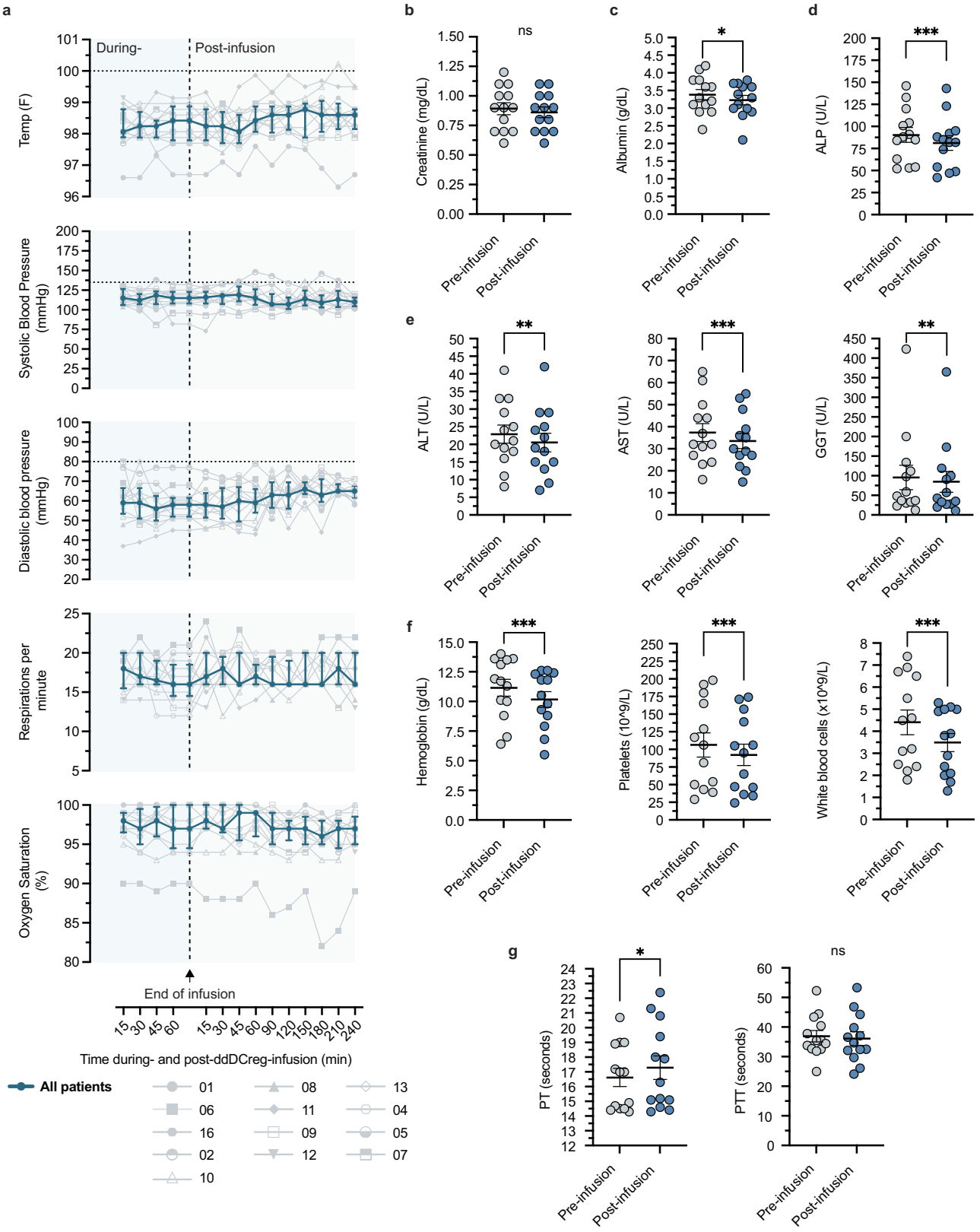

requiring medication or intervention were observed beyond the pre-existing instances identified at study inception. Mean hemoglobin, platelet, and white blood cell counts rose above baseline and remained within expected levels throughout the study (Supplementary Fig. 3a–c). Quality-of-life (QOL) evaluation demonstrated reduced metrics from 1-year post-transplant to EOS (Table S15).

Monitoring of inflammatory mediators, T-cell populations and anti-donor reactivity

Plasma mediators were measured sequentially using 32-plex meso-scale technology. Heatmaps depicting analyte clusters by timepoint (Fig. 6a) showed modulation of key pro-inflammatory cytokines (IL-12p70, IL-12/IL-23p40, TNF-b, GM-CSF, IL-17A, IFN-γ) and intercellular

Fig. 3 | Monitored clinical parameters during- and post-donor-derived regulatory dendritic cell (ddDCreg) infusion. a Spaghetti plots demonstrating temperature (top), blood pressure (middle-top, middle), respiratory rate (middle-bottom), and oxygen saturation (bottom) during and post-ddDCreg infusion (n = up to 13 per available samples per timepoint). Blue line indicates the cohort median with the interquartile range per timepoint. Arrow indicates the end of ddDCreg infusion and start of post-infusion monitoring. **b-g** Paired pre- and post-ddDCreg-infusion laboratory results for (**b**) creatinine, (**c**) albumin, (**d**) alkaline phosphatase (ALP), (**e**) alanine aminotransferase (ALT, left), aspartate aminotransaminases (AST, middle), and gamma-glutamyl transferases (GGT, right), (**f**) blood components such as hemoglobin (left), platelets (middle), and white blood cell count (right), as well as (**g**) prothrombin time (PT) and partial thromboplastin time (PTT). All pre- and post-ddDCreg infusion values (**b**–**g**) were compared utilizing a Wilcoxon paired-signed rank two tailed test (n = 13). Paired data are presented as mean values ± standard error of the mean. Statistically significant differences are indicated per plot *$P \le 0.05$ (albumin: $P = 0.0156$; PT: $P = 0.151$), **$P \le 0.01$ (ALT: $P = 0.0012$; GGT: $P = 0.0015$) and *** $P \le 0.001$ (ALP: $P = 0.0002$; AST: P = 0.0005; Hemoglobin: $P = 0.0002$; Platelets: $P = 0.0002$; WBCs: $P = 0.0002$).

adhesion molecules (VCAM-1; ICAM-1) from pre-transplantation, pre-infusion to EOS (Fig. 6a). Comprehensive longitudinal immunophenotyping of circulating $CD3^+$ T-cells (Fig. 6b, Supplementary Figs. 4–6) revealed decreasing percentages of $CD4^+$ compared with $CD8^+$ T-cells (Fig. 6b, Supplementary Fig. 4a–c). Within the $CD8^+$ T-cell compartment, a decrease occurred in PD-1^+ T-cells, but not in cells expressing EOMES or TIM-3, additional markers of exhaustion (Fig. 6b, Supplementary Fig. 5a–b). No substantial changes were observed in percentages or absolute numbers of Tregs ($CD3^+CD4^+CD25^{hi}CD127^-FOXP3^+$) (Fig. 6b, Supplementary Fig. 6a–b), or in the percentages of memory ($CD45RO^+$) and/or follicular T-cells ($CD4^+CD45RO^+CXCR5^+$) (Supplementary Fig. 6c–d). The gating strategy and representative plots are available in Supplementary Fig. 7a.

To investigate alloimmune reactivity, mixed leukocyte reactions were set up between recipient T-cells and donor or third-party stimulators at three timepoints: pre-infusion, 12-months, and 54-months post-transplant (EOS). Significantly lower proliferation of donor-specific T-cells, including IFN-γ-expressing $CD4^+$ and $CD8^+$ T-cells compared to third-party alloreactive T-cells was observed 12- and 54-months post-transplant, in contrast to similar degrees of alloreactivity before ddDCreg infusion (Fig. 6c, Supplementary Figs. 7b-c, 8a-c). This aligns with our previously published observations at 12 months post-LT[37], when ddDCreg-recipients, but not propensity-matched SOC LDLT patients, displayed lower proliferation of donor-specific, IFN-γ-expressing $CD4^+$ and $CD8^+$ T-cells compared with third-party alloreactive IFN-γ-expressing $CD4^+$ and $CD8^+$ T-cells.

Discussion

Cellular immunotherapy is an innovative approach to reduce patient IS drug dependence and promote transplant tolerance. Here, we demonstrate that a single pre-emptive regulatory donor myeloid cell (ddDCreg) infusion in adult LDLT recipients is feasible and safe. Importantly, our approach differs from other regulatory immune cell therapy approaches for transplant tolerance in several ways: first, the regulatory cells are of myeloid not lymphoid origin; second, the infusions are healthy donor, not recipient-derived; third, ddDCregs inherently express donor MHC class II antigens; fourth, they function independently of in vivo persistence/replication[24–26]. Furthermore, no complete cell manufacturing failures occurred in our study in comparison to other regulatory cell therapy trials, where this issue has been encountered[41,42]. The clinical laboratory trends (e.g., eGFR, glucose, etc), absence of AEs, host-sensitization at 12 months post-transplant, and lack of new-onset long-term complications, also provide further safety assurance.

Mechanistic evaluation performed previously in the present patient cohort pre- and post-ddDCreg infusion[36], revealed acquisition by host antigen-presenting cells of donor exosomes co-expressing donor MHC-antigen and co-inhibitory PD-L1. In a relevant pre-clinical liver transplant model, this process, known as cross-decoration, results in down-modulation of the anti-donor T-cell response by host antigen-presenting cells[43]. In our current cohort, at the time of transplant, we observed an ensuing increase in Tregs relative to $CD8^+$ effector T-cells. At 12-months, down-modulation of effector T-cell responses compared to a propensity-matched SOC cohort, further suggested that ddDCreg infusion induces immunologic changes that may be conducive to early ISW[36,37]. Here, in follow-up, we demonstrate significantly weaker donor-specific versus third-party alloimmune responses at 12-months and at EOS in ddDCreg-infused patients, with reduced pro-inflammatory plasma mediators, such as GM-CSF and IL-12 as compared to the cohorts' pre-transplant, pre-infusion values.

Four of the eight stable liver recipients with permissive protocol biopsies at 12 months achieved complete ISW. Three of the eight (37.5%) achieved OT, i.e., one-year off all IS with no evidence of clinical dysfunction or overt T-cell mediated rejection. They also remained clinically stable, off all IS drugs for 3.0 ± 0.17 years (EOS). These exploratory findings compare with OT rates of 13%[34] and 16%[35], respectively, in eligible adult liver recipients participating in multicenter, non-interventional trials in which ISW commenced 1–2 years or ≥3 years post-transplant, respectively in patients with permissive biopsies. Moreover, only one patient exhibited de novo DSA at 12-months, while five patients, including two considered operationally tolerant, were positive at EOS. No evident correlation between ISW, DSA and allograft fibrosis was observed, although additional follow-up may be necessary to determine the inter-relationships between these factors and long-term allograft integrity.

While our clinical and mechanistic findings are encouraging, this study has limitations. The number of patients followed, albeit comparable to other early phase regulatory cell therapy trials, is small and the lack of a parallel ISW control group - due to the anticipated high levels of rejection without intervention - restricts generalization and definitive conclusions regarding true efficacy. The sample size justification does, however, reflect the goal of minimizing participant exposure while balancing the inflated error rate for certain AE outcomes and the lack of adjustment for multiples comparisons. This limitation was deemed acceptable given the patient population as well as the exploratory nature of the trial and therapy. Additionally, our historical comparison is based primarily on DDLT outcomes[34], rather than LDLT data, where our approach of utilizing ddDCregs would be different and may not be comparable, given the lower risk of rejection in LDLT when the living-donor is biologically related to the recipient (4/13 patients were first-degree relatives; although only 1 of these patients is off all IS), as compared to deceased donors[44]. The open-label, single-site design, without dedicated evaluation across patient subgroups, in a low-risk LDLT cohort introduces further possible confounders, including confirmation bias, that may complicate interpretation. Also, the QOL assessments did not demonstrate a positive effect; however, the coinciding emergence of COVID-19, along with patient-specific factors (e.g., family member deaths) and the lack of a pre-infusion evaluation, limits interpretation.

Nonetheless, our data show that a single, pre-emptive ddDCreg infusion in conjunction with early ISW in eligible adult LDLT patients is feasible, reproducible, and safe. While a proportion of ISW-eligible patients achieved OT, our study is exploratory, hypothesis-generating, and efficacy remains to be established. Forthcoming studies will evaluate efficacy in an appropriately designed/powered prospective trial in comparison to a randomized cohort, with a propensity score-adjusted comparison to historical data from early ISW trials without intervention. Moreover, an upcoming trial to be reported

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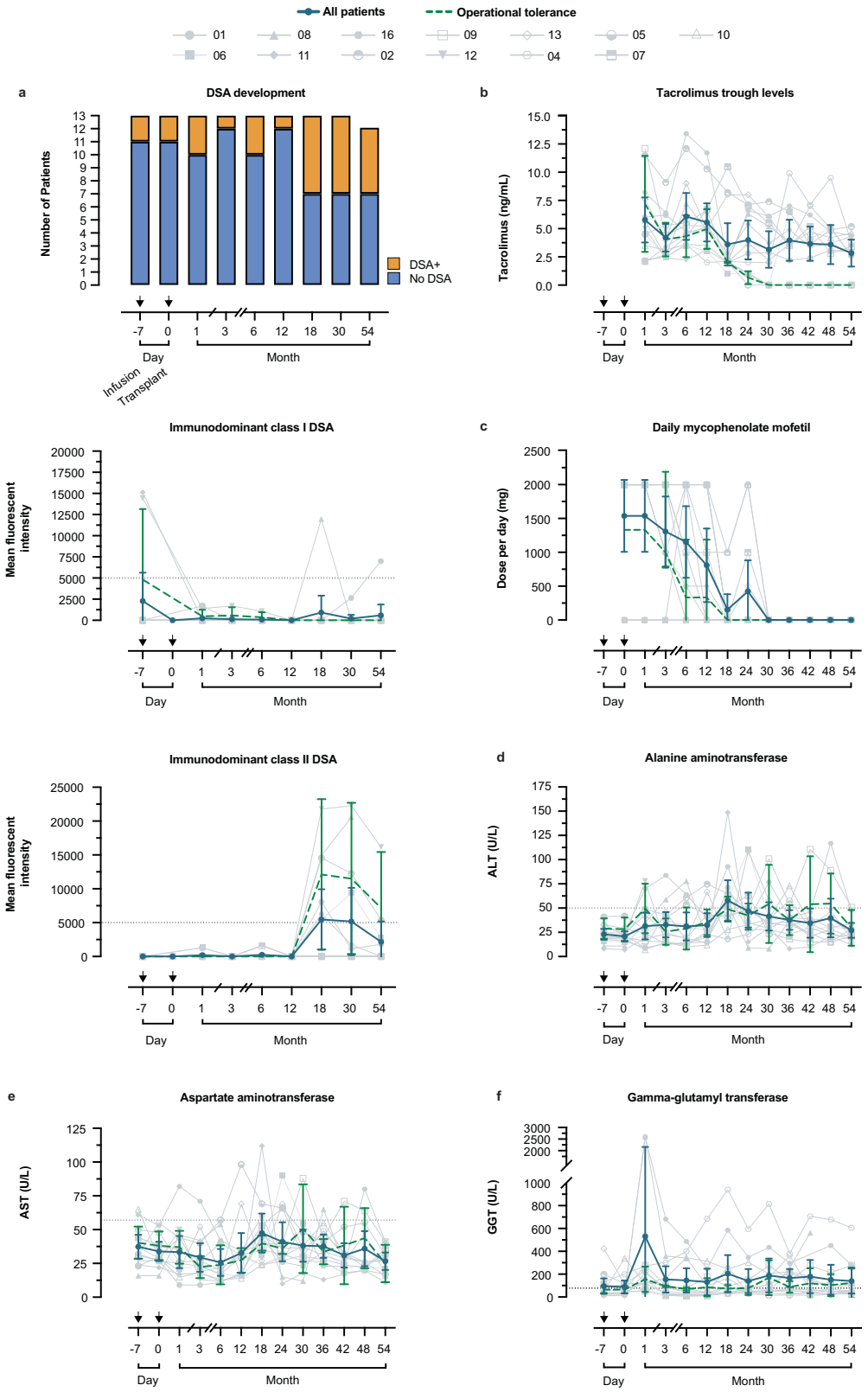

(NCT04208919; delayed ddDCreg infusion in LDLT 1-3 years post-transplant) and additional post-hoc analysis will help address the optimal timing and number of ddDCreg/infusions, as well as the most appropriate IS agent(s) for promotion of ddDCreg tolerogenicity/successful ISW. Lastly, modification of our current protocol for application in DDLT may be possible, given the recent, successful mobilization and apheresis-based collection of hematopoietic stem cells from deceased donors[45], although ddDCreg infusion would likely be post-transplant.

Methods

Study design

We conducted a single-center, prospective, open-label, non-controlled, non-randomized, interventional, institutional review

Fig. 4 | Donor-specific antibody (DSA) production, immunosuppressive drug levels, and hepatic-related labs from ddDCreg infusion to end of study. **a** Development of DSA across the study duration by patient count (top-left) and by immunodominant DSA subtype per patient (class I, left-middle; class II, left-bottom). Dashed line marks threshold for a strong DSA level, defined as a mean fluorescent intensity (MFI) of 5000. **b-c** Spaghetti plot depicting the immunosuppression taper by (**b**) tacrolimus trough levels and (**c**) dose per day of mycophenolate (Mofetil/CellCept) across all patients over time; patient 1012 received Mycophenolate Sodium/Mycophenolic Acid (Myfortic). **d-f** Spaghetti plots of (**d**) alanine aminotransferase (ALT), (**e**) aspartate aminotransaminases (AST), (**f**) gamma-glutamyl transferase (GGT) throughout the study (n = up to 13 per available samples per timepoint). Dashed line indicates the upper reference value for (**d**) ALT (72 U L^{-1}), (**e**) AST (57 U L^{-1}), and (**f**) GGT (78 U L^{-1}). All, blue line indicates the cohort mean with the 95% confidence interval per timepoint; dashed green line indicates the mean value with standard deviation for the subset of operational tolerance patients (n = 3). Arrows above each x-axis, as indicated in (**a**), mark the days of ddDCreg infusion and subsequent liver transplantation.

board–approved (University of Pittsburgh, STUDY19020267), cohort study of a single, pre-transplant infusion of ddDCregs (target dose: 2.5–10 × 10^6 ddDCregs per kg) with concurrent MPA (1/2 dose) 7-days before transplantation (Fig. 1). All patients provided written informed consent before participating in the study. An Investigational New Drug (IND) application was approved by the U.S. Food and Drug Administration (FDA) prior to the start of the study. GMP-grade ddDCregs were generated at the Immunologic Monitoring and Cellular Products Laboratory at the University of Pittsburgh Medical Center (UPMC)[39]. Infusions were conducted in low-risk LDLT recipients (ClinicalTrials.gov NCT03164265). All patients underwent clinical/mechanistic evaluation until EOS. All patients were on SOC IS (MPA and tacrolimus) for the first 12 months post-transplant, with weaning of MPA starting 6-months post-transplant (Fig. 1). A 12-month protocol biopsy and evaluation were conducted to determine tacrolimus weaning eligibility (Figs. 1, 2a–b). Participants who failed ISW, had a nonpermissive protocol biopsy, or a for-cause biopsy with T-cell mediated rejection, returned to SOC. The clinical course and histological progression over time for each patient are summarized and depicted in Fig. 2a–c. Additional study design information is available in the Study protocol (Supplementary Data 1) and in the Statistical Analysis plan (Supplementary Data 2).

Primary objectives, outcomes, and adverse event monitoring

The primary endpoints were feasibility, safety, and preliminary/exploratory efficacy of ddDCreg infusion to achieve complete ISW in LDLT recipients. AEs were established in the study protocol and monitored throughout. Preliminary/exploratory efficacy was based on the absence of rejection 1 year after complete ISW and compared to the historical benchmark of successful ISW (13%) commencing 1-2 years post-transplant in adult liver recipients in the absence of additional intervention[34]. Secondary endpoints were the following: influence of ddDCreg infusion and ISW on renal function, cardiovascular risk factors, QOL and DSA production; preliminary assessment of the impact of ddDCreg infusion and ISW on the alloimmune response. Additional objective information, AE reporting and safety monitoring details are available in the Supplementary information.

Reporting, patient population, and enrollment criteria

A modified CONSORT diagram[46] for a single arm trial was utilized to standardize reporting and improve study clarity. Donors aged 18-55 years, who met all standard institutional and United Network for Organ Sharing (UNOS) criteria and were negative for human immunodeficiency virus (HIV), human T-lymphotropic virus 1/2 (HTLV-1/2), hepatitis C and B, were eligible for liver donation. Only low-risk recipients between the ages of 18 to 75, irrespective of sex, race, or ethnic background, were eligible for LDLT and ddDCreg infusion. All females of childbearing potential required a negative urine or serum pregnancy test. Agreement for contraception was required for all female transplant recipients. All patients provided written informed consent. In total, 16 donor-recipient pairs were recruited between 2017-2020. However, only 15 patients underwent ddDCreg infusion and LDLT, due to a positive cross match identified in 1 patient before infusion. Thereafter, 2 additional patients were withdrawn from analysis due to medical conditions unrelated to ddDCreg infusion or LDLT. Clinical/immunologic monitoring was conducted by the living donor liver transplant team for all patients until the EOS, according to the Study Protocol. ISW, led by the LDLT team, was performed for all eligible patients included in the final analysis, according to the Study Protocol.

Flow cytometric/immunophenotypic analysis

Aliquots of whole blood from patients with available samples, at 7-days prior to and on the day of transplantation (prior to graft implantation) as well as at 1-, 3-, 6-, 12-, 18-, 30- and 54-months post-transplant were used for longitudinal immunophenotypic analysis by flow cytometry. All samples were stained with a mixture of fluorochrome-conjugated monoclonal antibodies (Table S16) in the dark for 30 min at room temperature. Cells were then incubated with 1x lysing buffer (BD) for an additional 10 min at room temperature. Tubes were washed twice with fluorescence activated cell sorting buffer. The cells were then fixed with a fixation/permeabilization buffer (eBioscience) for 40 min at 4°C. Intracellular staining was conducted by further permeabilization (eBioscience) and incubating the samples in the dark for 30 min at 4°C with a mixture of monoclonal antibodies (Table S16). Cells were then washed twice prior to data acquisition on a LSR Fortessa (BD). For supervised analyses, FlowJo was utilized. The gating strategy utilized for analysis with representative plots is listed in Supplementary Fig. 7a.

Complement-dependent cytotoxicity (CDC) crossmatching

Paired untreated and heat-inactivated frozen recipient serum aliquots were acquired per patient along with IgM heat-inactivated controls. After heat inactivation, sera were vortexed, centrifuged, and serially diluted in RPMI with 20% commercially prepared human AB serum to assess prozone/anti-complementary activity. Crossmatch trays (anti-human globulin (AHG) trays for T-cells; Amos-modified trays for B-cells) were arranged and built in HistoScope analysis software. Recipient sera and dilutions were dispensed into corresponding tray rows, followed by the addition of direct capture immunomagnetic cell isolates of donor T- or B-lymphocytes at 2.5–3.0 × 10^6 cells per mL (1 μL per well). Following incubation, trays were washed using PBS. For T-cell trays, AB complement diluted 1:2 with working AHG was added (5 μL per well) and incubated at room temperature for 60 min; for B-cell trays, undiluted DR complement was added (5 μL per well) and incubated 45–60 min. Fluoroquench was then added (5 μL per well) and cells were collected for fluorescence microscopy scoring. Controls were verified for expected performance, including negative AB and media wells, positive polyspecific and lineage-specific controls, and appropriate IgM heat-inactivation control results. Trays were then double scored in HistoScope and results were graded on a semi-quantitative cytotoxicity scale (negative to strong positive).

Tissue typing results

Next-generation sequencing (NGS) HLA genotyping for human HLA Class I (HLA-A, -B, -C) and Class II (HLA-DRB1/3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1) was performed using the Immucor MIA FORA NGS HLA Flex 11 Kit (Immucor, Peachtree Corners, GA) according to the manufacturer's instructions. Sequencing was carried out on Illumina MiSeq using a standard v2 flowcell and a 300-cycle MiSeq V2 reagent

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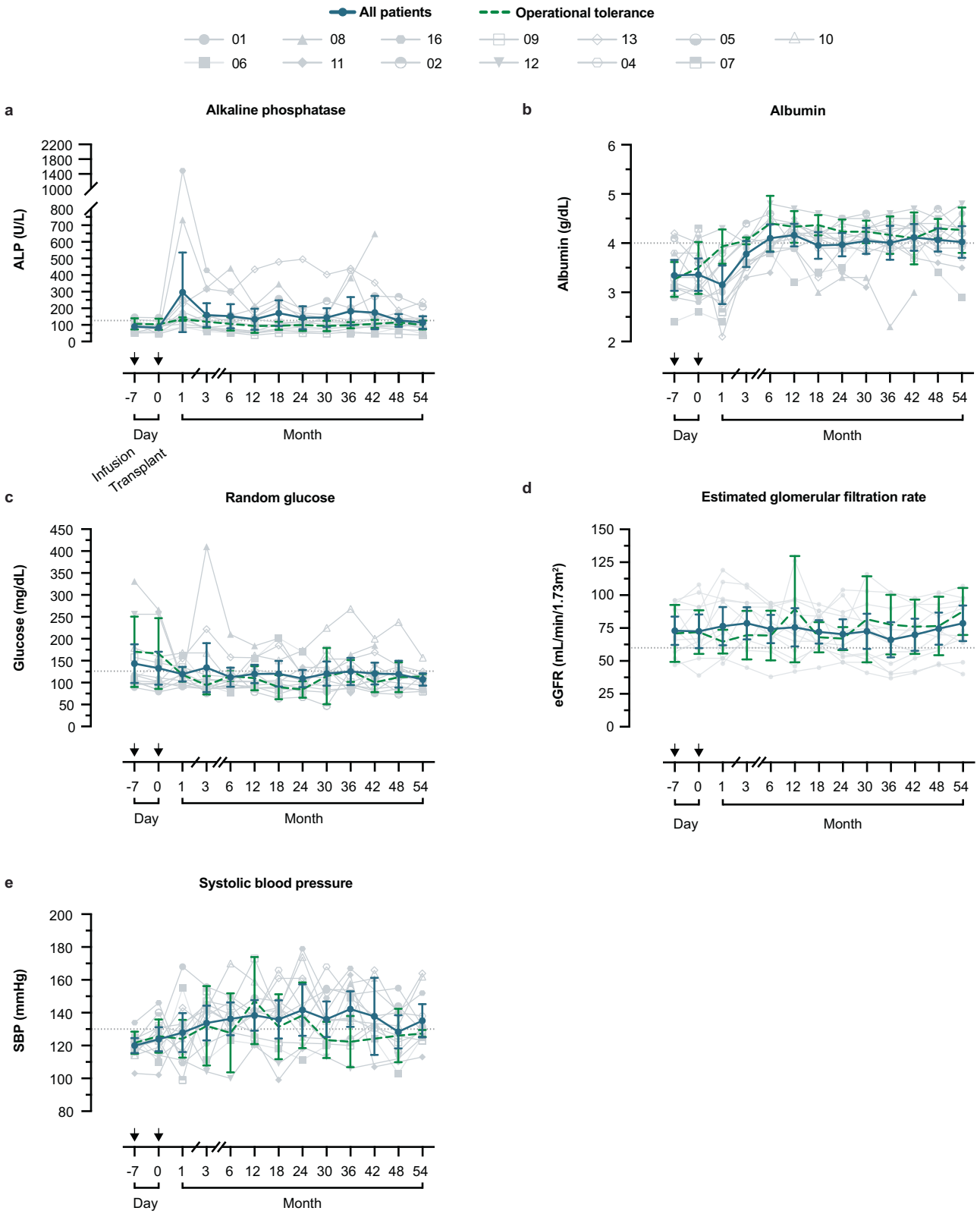

Fig. 5 | Longitudinal alkaline phosphatase, albumin, renal function, blood glucose and systolic blood pressure over the course of study. a-e Spaghetti plots of (**a**) alkaline phosphatase (ALP), (**b**) albumin, (**c**) random blood glucose, (**d**) estimated glomerular filtration rate (eGFR), and (**e**) Systolic blood pressure throughout the study (n = up to 13 per available samples per timepoint). Dashed line indicates the upper reference value for (**a**) ALP (126 U L^{-1}), the standard reference value for (**b**) albumin (4 g dL^{-1}), (**c**) a glucose value of 126 mg dL^{-1}, (**d**) an eGFR value of 60 mL/min/1.73 m^2, and (**e**) a systolic blood pressure value of 130 mmHg. All, blue line indicates the cohort mean with the 95% confidence interval per timepoint; dashed green line indicates the mean value with standard deviation for the subset of operational tolerance patients ($n = 3$). Arrows above each x-axis, as indicated in (**a**), mark the days of ddDCreg infusion and subsequent liver transplantation.

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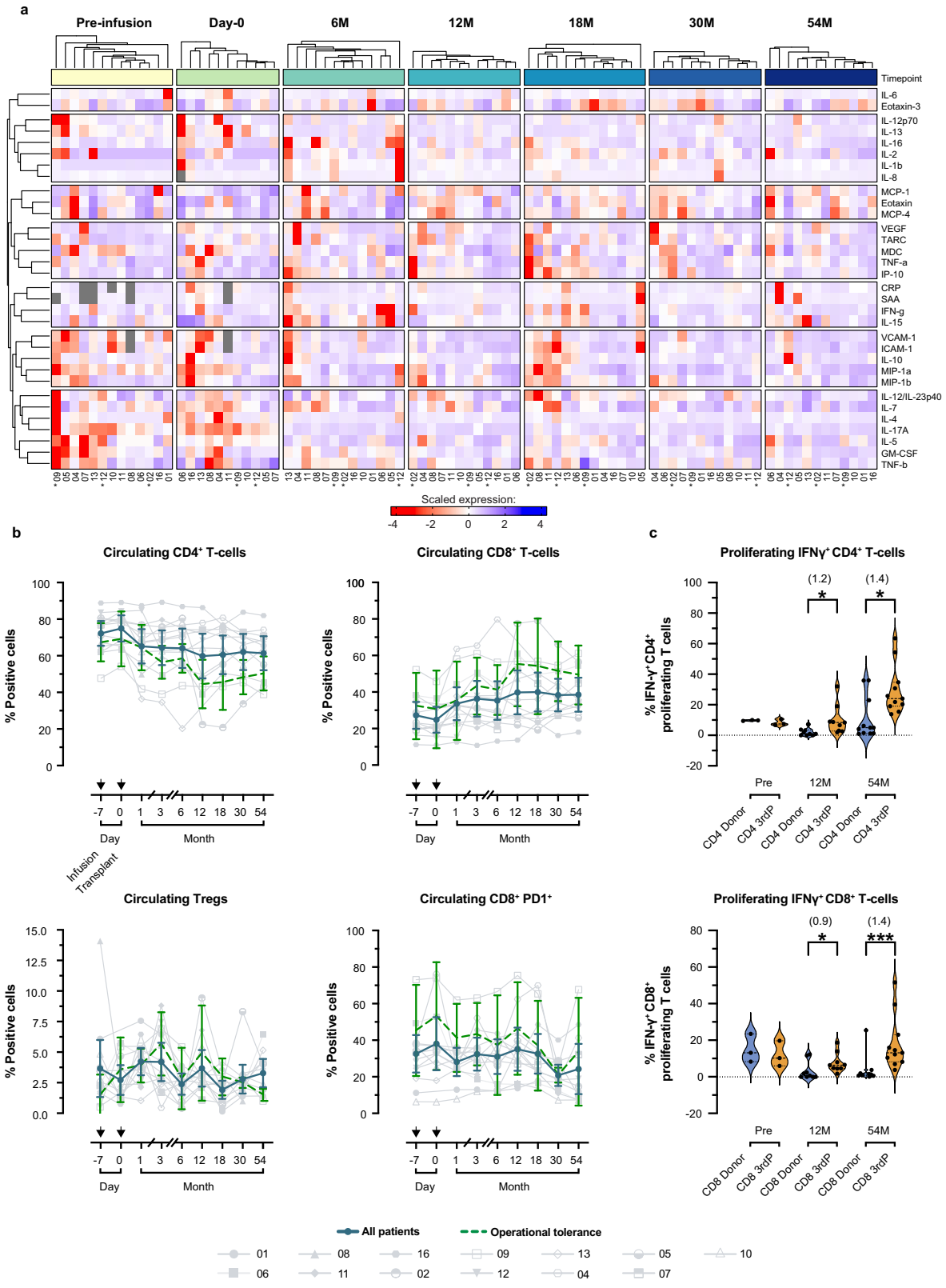

kit (Illumina, San Diego, CA). FASTQ sequencing data were analyzed by MIA FORA NGS HLA Flex software v4.5 with IMGT database 3360. When necessary, ambiguities were resolved by SSO and/or SSP HLA genotyping. Eplet mismatch load, across the cohort, was determined using the HLAMatchmaker version 4.0 for all HLA alleles (http://www.epitopes.net). The method was implemented via the R stats package v4.0.2.

DSA testing and IgG subtype analysis

Serum samples from transplant recipients were analyzed for circulating DSA pre-transplant and at 1, 3, 6, 12, 18, 30, and 54 months post-transplant by the histocompatibility laboratory at UPMC with the SOC Luminex single antigen bead (SAB) platform (One Lambda Thermo Fisher, Canoga, CA). The IgG subclass assay was performed utilizing

Fig. 6 | Plasma mediator analysis and longitudinal immune cell dynamics from donor-derived regulatory dendritic cell (ddDCreg) infusion to the end of study. **a** Heatmaps depicting 32-plex analytes at indicated timepoints. Each row depicts an analyte whereas each column depicts an individual patient (*, denotes operationally tolerant patients, $n = 3$). Heatmaps are hierarchically clustered (Euclidian distance with complete linkage) by row based on scaled gene expression and by patient at each timepoint. Gray marks plasma analytes without a value. **b** Longitudinal immune cell dynamics on selected cell types ($CD4^+$ T-cells, left-top; $CD8^+$ T-cells, right-top; Treg: $CD25^{hi}$ $CD127^-$ $FoxP3^+$ out of $CD4^+$ T-cells, left-bottom; $CD8^+$ $PD\text{-}1^+$, right-bottom) for all patients throughout the study. All, blue line indicates the cohort mean with the 95% confidence interval per timepoint; dashed green line indicates the mean value with standard deviation for the subset of operational tolerance patients ($n = 3$). Arrows above each *x*-axis, as indicated in the top left graph, mark the days of ddDCreg infusion and subsequent liver transplantation. **c** Incidences of IFN-γ^+ proliferating $CD4^+$ T-cells (upper-panel) and $CD8^+$ T-cells (lower-panel) cells in mixed-leukocyte reactions against donor or third-party (3rdP) cells at different timepoints: pre-infusion (Pre, $n = 3$), 12-months (12 M, $n = 9$), and 54-months (54 M, $n = 11$). In violin plots, each dot represents 1 patient; medians are indicated and interquartile ranges denoted by dashed lines. Statistical analyses among groups were performed using a two tailed Mann–Whitney *U* test given the small sample sizes and normality testing with the Kolmogorov-Smirnov test. Statistically significant differences are indicated per plot $^*P \le 0.05$ (IFN-γ^+ proliferating $CD4^+$ T-cells – 12 M: $P = 0.01$, 54 M: $P = 0.0128$; IFN-γ^+ proliferating $CD8^+$ T-cells – 12 M: $P = 0.03$), $^{**}P \le 0.01$, and $^{***}P \le 0.001$ (IFN-γ^+ proliferating $CD8^+$ T-cells – 12 M: $P = 0.001$). Effect estimates (Cohen D) are reported in parentheses for all statistically significant results.

the Luminex SAB platform with monoclonal antibodies specific for IgG subtypes 1-4, as described[47].

Mixed lymphocyte reaction (MLR) analysis

Aliquots of frozen peripheral blood mononuclear cells from liver recipients, donors, and third-party individuals were used for the MLRs. Responder cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). Donor or third-party stimulator cells were labeled with PKH-26, irradiated (20gy), and incubated at a 1:1 ratio for 5 days at 37 C in 5% CO2. On day five, cells were harvested and restimulated for an additional 6-hours with donor or third-party cells in the presence of Golgi Plug (Invitrogen). Live-dead discrimination was conducted with zombie-NIR (biolegend); cells were then surfaced stained with anti-CD3, -CD4, -CD8 (BD) and intracellularly for Interferon-gamma (BD) before acquisition on the spectral cytometer Aurora (cytek). Flow Jo software was used for all analyses. The gating strategy utilized for analysis is listed in Supplemental Fig. 7b and representative plots for each time point are displayed in Supplemental Fig. 7c.

Clinical and histologic allograft evaluation and monitoring

Permissive liver function tests (LFTs) were defined as alanine transaminase (ALT), aspartate transaminase (AST) and total bilirubin <2.5 times the upper limit of normal. All liver allograft biopsy evaluations were performed by the local and study transplant pathologists at the UPMC/University of Pittsburgh. The reads were utilized for the purposes of clinical management and ISW. All biopsies were evaluated based on the 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology for ISW eligibility and IS cessation criteria, which is also listed in Tables S17–18 [40]. For-cause biopsies (FCB) were implemented at the discretion of the clinical investigators. FCB occurred when ALT or AST levels were significantly elevated >2.5 times the upper limit of normal. All patients with evidence of rejection based on either a protocol biopsy or FCB, resumed SOC IS or returned to their baseline IS and were monitored biochemical for rejection resolution. All biopsy results, eligibility evaluation and assessment, and additional information regarding treatment of each episode of rejection are included in Tables S9–13.

Plasma mediator analyses

The multiplexed Meso Scale Diagnostics (MSD) platform (V-PLEX Human Biomarker 32-Plex Kit) was implemented, according to the manufacturer's instructions, for the evaluation of circulating cytokines, chemokines, angiogenesis and vascular injury factors. Available plasma aliquots, at 7 days prior to and on the day of transplantation, as well as 1-, 3-, 6-, 12-, 18-, 30-, and 54 months post-transplant were analyzed. Mediator analysis was completed utilizing two runs on the MSD platform. Expression values were scaled by individual analytes across all samples and runs.

Statistical analysis

Statistical analysis focused on patient safety outcomes and preliminary efficacy evaluation. Stopping rules and sample size justification were established before enrollment to provide sufficient power while maintaining a reasonable type-1 error. Stopping rules were designed to identify AE rates ≥ 10-30% above expected historical rates for each safety endpoint. The exact binomial distribution was used to calculate probabilities of correctly or falsely rejecting the null (i.e., detecting an increased AE rate). Statistical analyses were completed with SAS (v9.4). Cell product information, lab results, and demographic details/clinical history were summarized with descriptive statistics such as mean (standard deviation), median (IQR), and 95% confidence intervals. Where appropriate, paired differences of the mean were reported for changes in baseline and EOS lab values. Statistics used for pre- and post-ddDCreg infusion evaluation were calculated utilizing a Wilcoxon paired-signed rank test. Differences in MLRs were displayed utilizing violin plots with the median and IQR. Effect size analysis and a Mann-Whitney U test or Student's t test were utilized for comparisons across each timepoint in the MLRs in accordance with normality testing. Preliminary efficacy was established via proportion comparison to historical results (13.0%)[34]. Statistically significant differences, where appropriate, were indicated as $^*P \le 0.05$, $^{**}P \le 0.01$, and $^{***}P \le 0.001$. Hierarchical clustering was accomplished with Euclidean distance and complete linkage.

Data presentation and illustration

Figures were generated and prepared using Prism-graph Pad, R, and adobe Illustrator. Longitudinal immunophenotypic and other laboratory results were displayed utilizing spaghetti plots. The cohort's median and IQR or mean and `95% confidence interval were annotated in each spaghetti plot. Mean values, per timepoint, for operationally tolerant patients ($n = 3$) were also represented by a dashed green line in the aforementioned plots. The proportion of patients with and without DSA was represented by a stacked bar chart at each timepoint. A heatmap was generated for the plasma analytes utilizing the ComplexHeatmap package (v2.18.0) in R.

Data availability

Individual participant data (Source Data) that underlie the study results are provided with this report and will be publicly available at the time of publication, which includes the deidentified data necessary to reproduce the primary analyses and the values reported in the trial. The study protocol and statistical analysis plan (SAP) are provided as Supplementary Data 1 and 2, respectively. Donor and recipient informed consent forms are available via the ClinicalTrials.gov record (NCT03164265). Source data will be available indefinitely with this publication. To protect participant privacy, given the small cohort size and unique study design, patient directories/enrollment logs, direct identifiers, and any linkage keys will not be shared. Use of the publicly available Source data and results reported in this publication is permitted for appropriate scientific purposes consistent with the terms of

the publication and regulation. Source data are provided with this paper.

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Author contributions

A.H., A.W.T., F.G.L., A.Z., D.L., D.M.M. and M.A.S. conceptualized the study. C.M., L.M.T., Y.H., A.F.Z., E.M.A., D.L., D.M.M., and A.W.T. developed the methodology. All authors contributed to the writing, review, and editing of the manuscript. Clinical care and evaluation were completed by A.H., C.B.H., S.R.G., L.M.T., Y.H., B.D.E., and A.Z. Visualization of all the results and tables were generated by Y.H., C.M., and E.M.A. with final input from all authors. Formal analysis and experimental investigation were completed by C.M., Y.H., E.M.A., A.F.Z., D.L., A.Z., D.M.M. and A.W.T. All authors reviewed and agreed with the final submitted manuscript. All authors vouch for the accuracy and completeness of the data presented, as well as the fidelity of the study to the protocol.

Competing interests

A.H., F.G.L., D.M.M., and A.W.T. are co-inventors of University of Pittsburgh invention disclosures that concern protocols for clinical testing of regulatory DCs in human organ transplantation. A.F.Z., F.G.L., and A.W.T. are co-inventors of University of Pittsburgh invention disclosures and a provisional patent application for generation of human regulatory DCs for application in human organ transplantation. The remaining authors declare no competing interests.

Additional information

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