Title

Selective decrease of donor-reactive Tregs after liver transplantation limits Treg therapy for promoting allograft tolerance in humans.

Journal

Science Translational Medicine, 14(669)

Authors

Tang, Qizhi
Leung, Joey
Peng, Yani
et al.

Publication Date

2022-11-02

DOI

10.1126/scitranslmed.abo2628

Peer reviewed

Published in final edited form as:

Sci Transl Med. 2022 November 02; 14(669): eabo2628. doi:10.1126/scitranslmed.abo2628.

Selective decrease of donor-reactive T_{regs} after liver transplantation limits T_{reg} therapy for promoting allograft tolerance in humans

Qizhi Tang[1,2,3,*], **Joey Leung**[1], **Yani Peng**[1], **Alberto Sanchez-Fueyo**[4], **Juan-Jose Lozano**[5], **Alice Lam**[1], **Karim Lee**[1], **John R. Greenland**[6,7], **Marc Hellerstein**[8], **Mark Fitch**[8], **Kelvin W. Li**[8], **Jonathan H. Esensten**[9,3], **Amy L. Putnam**[2], **Angela Lares**[2], **Vinh Nguyen**[1], **Weihong Liu**[2], **Nancy D. Bridges**[10], **Jonah Odim**[10], **Anthony J. Demetris**[11], **Josh Levitsky**[12], **Timucin Taner**[13], **Sandy Feng**[1,*]

[1]Department of Surgery, University of California, San Francisco, San Francisco, CA 94143, USA.

[2]Diabetes Center, University of California, San Francisco, San Francisco, CA 94143, USA.

[3]Gladstone-UCSF Institute of Genomic Immunology, San Francisco, CA 94158, USA.

[4]Institute of Liver Studies, School of Immunology and Microbial Sciences, King's College London University, London WC2R 2LS, UK.

[5]Bioinformatic Platform, Biomedical Research Center in Hepatic and Digestive Diseases, Instituto de Salud Carlos III, 28029 Madrid, Spain.

[6]Department of Medicine, University of California, San Francisco, San Francisco, CA 94143, USA.

[7]Medical Service, San Francisco VA Health Care System, San Francisco, CA 94121, USA.

[8]Nutrition Sciences and Toxicology, University of California, Berkeley, Berkeley, CA 94720, USA.

[*]Corresponding author: sandy.feng@ucsf.edu (S.F.); qizhi.tang@ucsf.edu (Q.T.).

Author contributions: Q.T. and S.F. conceptualized the study. N.D.B. and J.O. provided trial oversight. S.F., T.T., J. Levitsky, and A.J.D. acquired clinical data. J.H.E., A.L.P., A. Lares, K.L., V.N., and W.L. acquired darT$_{reg}$ manufacturing data. J. Leung, A. Lam, and K.L. performed alloreactive frequency assays. J. Leung performed spectral flow. Y.P. performed TCR sequencing analysis. Y.P., A.S.-F., and J.R.G. performed the RNA-seq experiment. J. Leung and V.N. isolated peripheral blood T_{regs} after darT$_{reg}$ infusion for deuterium analyses. M.F. and M.H. quantified deuterium in T_{regs}. S.F., T.T., J. Levitsky, and A.J.D. analyzed clinical data. Q.T. and J.H.E. analyzed darT$_{reg}$ manufacturing data. J. Leung, Y.P., A.S.-F., J.-J.L., K.L., M.F., M.H., K.W.L., and Q.T. analyzed mechanistic data. S.F., T.T., and J. Levitsky interpreted clinical data. Q.T. and J.H.E. interpreted darT$_{reg}$ manufacturing data. J. Leung, Y.P., K.W.L., A.S.-F., M.H., and Q.T. interpreted mechanistic data. Q.T. and S.F. drafted the manuscript, and all authors participated in the review and finalization of the manuscript.

Competing interests: Q.T. is a co-founder, shareholder, and scientific advisor of Sonoma Biotherapeutics. Q.T. is a consultant of eGenesis and Qihan Bio. Q.T. is a coinventor of the following patents: "Expansion of alloantigen reactive regulatory T cells" (US9801911B2) and "Regulatory T cells suppress autoimmunity" (US7722862B2). A.L.P. and W.L. are coinventors on the patent "CD127 expression inversely correlates with FoxP3 and suppressive function of CD4$^+$ T_{regs}" (US9012134B2). S.F. is a consultant of Quell Therapeutics and eGenesis. S.F. serves as the chair of DSMB for Quell Therapeutics. A.S.-F. is a cofounder and shareholder of Quell Therapeutics. J. Levitsky is an advisor and receives research grant support from Eurofins and Novartis. The other authors declare that they have no competing interests.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. RNA-seq data are available in GEO (accession number: GSE208111). The cell line K562-hCD40L for generating sBCs was made available to Q.T. under a material transfer agreement. All requests for other raw and analyzed data and materials will be reviewed by the corresponding authors to verify whether the request is subject to any intellectual property or confidentiality obligations. Patient-related data not included in the paper were generated as part of clinical trials and may be subject to patient confidentiality.

[9]Department of Lab Medicine, University of California, San Francisco, San Francisco, CA 94143, USA.

[10]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20852, USA.

[11]Thomas E. Starzl Transplantation Institute and Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15213, USA.

[12]Department of Medicine, Northwestern University, Chicago, IL 60611, USA.

[13]Departments of Surgery and Immunology, Mayo Clinic, Rochester, MN 55905, USA.

Abstract

Promoting immune tolerance to transplanted organs can minimize the amount of immunosuppressive drugs that patients need to take, reducing lifetime risks of mortality and morbidity. Regulatory T cells (T_{regs}) are essential for immune tolerance, and preclinical studies have shown their therapeutic efficacy in inducing transplantation tolerance. Here, we report the results of a phase 1/2 trial (ARTEMIS, NCT02474199) of autologous donor alloantigen–reactive T_{reg} ($darT_{reg}$) therapy in individuals 2 to 6 years after receiving a living donor liver transplant. The primary efficacy endpoint was calcineurin inhibitor dose reduction by 75% with stable liver function tests for at least 12 weeks. Among 10 individuals who initiated immunosuppression withdrawal, 1 experienced rejection before planned $darT_{reg}$ infusion, 5 received $darT_{regs}$, and 4 were not infused because of failure to manufacture the minimal infusible dose of 100×10^6 cells. $darT_{reg}$ infusion was not associated with adverse events. Two $darT_{reg}$-infused participants reached the primary endpoint, but an insufficient number of recipients were treated for assessing the efficacy of $darT_{regs}$. Mechanistic studies revealed generalized T_{reg} activation, senescence, and selective reduction of donor reactivity after liver transplantation. Overall, the ARTEMIS trial features a design concept for evaluating the efficacy of T_{reg} therapy in transplantation. The mechanistic insight gained from the study may help guide the design of future trials.

INTRODUCTION

Liver transplantation is a life-saving treatment for patients with end-stage liver disease, but immunosuppression required to prevent allograft rejection imposes substantial lifetime risks of morbidity and mortality for recipients (1, 2). Minimization of immunosuppression exposure may therefore yield substantial long-term health benefits. Among the primary solid organs that are transplanted, the liver is known for promoting immune tolerance (3). Liver transplant tolerance can spontaneously develop and be unveiled through immunosuppression withdrawal (4, 5). However, the rate of spontaneous tolerance is low, especially early after transplantation. Therapies that can accelerate the development of tolerance, thus reducing the cumulative burden of generalized immunosuppression, may offer substantial benefits. Because of the liver's robust regenerative ability, a rejection that is rapidly diagnosed and successfully treated does not incur long-term sequelae. Consequently, liver transplantation presents a safe context to evaluate experimental tolerance-promoting therapies that require cessation of conventional immunosuppression for demonstration of efficacy.

Regulatory T cells (T_{regs}) are essential for immune tolerance to both self and environmental antigens, and their ability to suppress unwanted immune activation can be therapeutically harnessed to induce transplant tolerance (6, 7). In solid organ and hematopoietic stem cell transplantation, T_{regs} are necessary for establishing tolerance (8). T_{regs} have a distinct therapeutic profile compared to small-molecule and biological immunosuppressive drugs. T_{regs} migrate to sites of inflammation and have many distinct immunoregulatory mechanisms to both respond and adapt to a spectrum of inflammatory conditions (9). T_{regs} are long-lived, and their therapeutic effect may persist beyond their lifespan through a process termed infectious tolerance (10). The polyclonal pool of T_{regs} contains a small fraction of donor alloantigen–reactive T_{regs} ($darT_{regs}$). $darT_{regs}$ that are activated by donor antigens can exhibit suppressive functions in the graft and draining lymph nodes to inhibit the activation of graft-rejecting effector T cells. Thus, on a per-cell basis, $darT_{regs}$ are more potent than polyclonal T_{regs} ($PolyT_{regs}$) for graft protection (11, 12). We hence developed a good manufacturing practice (GMP)–compliant $darT_{reg}$ manufacturing process to enable $darT_{reg}$ therapy in humans (13). Here, we report the design and results of the phase 1/2 study, "$darT_{regs}$ to enable minimization of immunosuppression in liver transplantation" (ARTEMIS, NCT02474199). The objective of the study was to evaluate the safety and efficacy of $darT_{regs}$ for the promotion of liver transplantation tolerance.

RESULTS

Clinical trial design

ARTEMIS was a single-arm, open-label trial to determine the safety and efficacy of a single intravenous dose of $darT_{regs}$ to facilitate immunosuppression minimization after liver transplantation. The primary endpoint was the percentage of individuals who could stably reduce immunosuppression by 75% with discontinuation of a second drug, if applicable, and the secondary endpoint was the percentage of individuals who could be completely weaned off immunosuppression for 1 year while maintaining biochemical and histological stability. ARTEMIS aimed to enroll 9 to 11 participants to receive $darT_{regs}$. This sample size was calculated on the basis of previous data from AWISH and WISPR studies showing that 33.6% of adult liver transplant recipients could tolerate a 75% reduction of calcineurin inhibitors (14, 15). With nine participants, a success rate of 77.8% (seven of nine) would provide 89% power at an α of 0.05 to conclude that this differed from the baseline rate of 33.6%, using an exact one-sided binomial test. Similarly, with 11 participants, a success rate of 73.7% (8 of 11) would provide 85% power to conclude that this differed from the baseline rate.

In mouse models, many millions of $PolyT_{regs}$ are required to induce transplant tolerance, which scales to several billions of T_{regs} in humans (16). The frequency of $darT_{regs}$ is estimated to be 10 to 20% within a $PolyT_{reg}$ population. Thus, for $darT_{regs}$, the effective dose for inducing graft tolerance in humans could be reduced by 80 to 90% to hundreds of millions (16). While preparing for the ARTEMIS trial, a pilot trial of T_{reg} therapy in liver transplantation was reported (17). Trial participants received a single infusion of autologous cell preparation enriched for $darT_{regs}$ 13 days after liver transplantation and 8 days after a single dose of cyclophosphamide. Immunosuppression was withdrawn gradually, and

7 of 10 treated patients met the criteria of the trial-defined end point of tolerance. A potential correlation of higher T_{reg} dose with successful immunosuppression withdrawal was observed: Tolerance was achieved in four of four participants who received greater than 300 × 10^6 $darT_{regs}$, two of three participants who received 100 × 10^6 to 300 × 10^6 $darT_{regs}$, and one of three participants who received fewer than 100 × 10^6 $darT_{regs}$. Thus, for ARTEMIS, we selected 300 × 10^6 to 500 × 10^6 $darT_{regs}$ as the target dose to permit complete immunosuppression withdrawal and 100 × 10^6 as a minimal infusible dose. Participants who received 100 × 10^6 to 300 × 10^6 were allowed to reduce immunosuppression by 75%.

We focused on participants 2 to 7 years after liver transplantation when the rate of spontaneous tolerance was below 15% (15, 18). Individuals in this early posttransplant period may derive greater benefit from minimizing immunosuppression to reduce risks of infections, malignancy, renal failure, and metabolic syndrome from cumulative drug exposure (2). Results from previous trials of immunosuppression withdrawal showed that 25% of the participants experienced graft rejection after a 50% reduction of immunosuppression (fig. S1) (14, 15), suggesting alloimmune activation at this step. Infusing $darT_{regs}$ just before the 50% dose reduction would minimize the negative impact of calcineurin inhibitor–based immunosuppression on the infused $darT_{regs}$ and might allow the cells to be activated by donor antigens to express suppressive function. With all the above considerations, we thus designed the ARTEMIS protocol as illustrated in Fig. 1A.

Clinical trial progression

The ARTEMIS trial enrolled participants in three centers, University of California, San Francisco (UCSF), Mayo Clinic Rochester, and Northwestern Medical Center. After reviewing the medical records of 322 living donor transplant recipients and identifying 25 eligible participants according to protocol-defined eligibility criteria (table S1), a total of 15 participants were enrolled between June 2016 and October 2018. Five were excluded before initiating immunosuppression withdrawal (Fig. 1B). The 10 participants who initiated immunosuppression withdrawal were considered as the intent to treat population (Table 1). One individual developed abnormal liver tests within 6 weeks after initiating drug withdrawal requiring an increase of immunosuppression, thus failing eligibility for $darT_{reg}$ infusion.

Nine participants reduced immunosuppression per protocol (tables S2 and S3), maintained stable liver tests, and were eligible for $darT_{reg}$ infusion. $darT_{reg}$ manufacturing was initiated for all nine participants, and five products met release criteria (table S4 and fig. S2), whereas the others were not released all because of insufficient dose. Five participants received $darT_{regs}$, two participants received the target dose (378 × 10^6 and 461 × 10^6 cells), and three participants received a lower dose (126 × 10^6, 136 × 10^6, and 190 × 10^6 cells; Fig. 1C). Four participants did not have infusible $darT_{reg}$ products. There were no adverse events related to $darT_{reg}$ infusion.

A total of five rejection episodes, all deemed serious by the trial protocol, occurred: one before and four after $darT_{reg}$ infusion (Fig. 1C and table S5). All participants were treated with increased immunosuppression, thus meeting the trial definition of rejection. All five

rejection episodes were deemed to be unrelated to darT$_{reg}$ infusion by the medical monitor, and all were resolved.

Among the 10 participants who initiated immunosuppression withdrawal, 2 (20%; 95% confidence interval, 2.52 to 55.61%) achieved the primary endpoint of reducing calcineurin inhibitor by 75% and discontinuation of a second drug for those who entered on a two-drug regimen. No participants attempted complete immunosuppression withdrawal. The trial was completed in December 2019 when all the enrolled individuals completed protocol-defined follow-ups.

darT$_{reg}$ manufacturing

A major challenge encountered during the ARTEMIS trial was darT$_{reg}$ manufacturing. Together, the UCSF GMP facility manufactured 22 darT$_{reg}$ products according to a process detailed in a master file submitted to the U.S. Food and Drug Administration (MF15431). We compared manufacturing data for individuals in the ARTEMIS trial to eight others for which freshly collected peripheral blood or leukapheresis products were also used as starting material, similar to ARTEMIS [excluding three that started with frozen cells and one that had conventional CD4$^+$ T cell (T$_{conv}$) outgrowth]. In general, lower cell numbers throughout the 16-day manufacturing process were observed for ARTEMIS participants than for non-ARTEMIS patients (Fig. 2A), leading to lower product yield (Fig. 2B). No correlation between T$_{reg}$ product yield and participant age was observed (fig. S3A). Viability of the T$_{regs}$ on day 16 of cell manufacturing was comparable between products that reached the infusible dose and those that did not (Fig. 2C).

Lower darT$_{reg}$ product yield correlated with a lower number of T$_{regs}$ purified from the starting material (Fig. 2D). For all participants enrolled in T$_{reg}$ therapy clinical trials supported by the UCSF T$_{reg}$ manufacturing program, absolute T$_{reg}$ counts in the peripheral blood were quantified using a T$_{reg}$ TruCount assay. Compared with participants in all other trials, ARTEMIS participants had significantly lower absolute T$_{reg}$ counts in the peripheral blood ($P = 0.0007$; Fig. 2E). In both ARTEMIS and non-ARTEMIS patients, we observed a trend of declining T$_{reg}$ counts with increased age (fig. S3B). The rates of T$_{reg}$ count decline were similar between ARTEMIS and non-ARTEMIS patients, losing about five T$_{regs}$ per microliter of blood per decade of life. ARTEMIS participants had lower T$_{reg}$ counts at all ages, suggesting an additional impact of being after liver transplantation (fig. S3B). Among ARTEMIS participants, those who did not receive darT$_{reg}$ infusion due to insufficient dose had a trend of lower T$_{reg}$ TruCounts. However, two participants with the highest T$_{reg}$ TruCount only had a partial dose produced, suggesting that other factors may have contributed.

A strong correlation was observed between darT$_{reg}$ product yield and T$_{reg}$ fold expansion during the 16-day culture period (Fig. 2F). To explore factors that might have affected T$_{reg}$ expansion to donor alloantigens, we assessed the correlation of T$_{reg}$ fold expansion, T$_{reg}$ donor age, counts of CD4$^+$ T cells, T$_{regs}$, monocytes, and granulocytes in the peripheral blood, the doses of tacrolimus and mycophenolate at trial enrollment, and numbers of human leukocyte antigen (HLA) class I and class II mismatches using multivariable correlation analysis (fig. S4). Negative correlations between T$_{reg}$ TruCount and age and between HLA

class II mismatches and granulocyte TruCount were observed. No correlation with other parameters was observed. Together, these analyses suggest the importance of T_{reg} counts and their responsiveness to alloantigen stimulation to T_{reg} product yield.

Pharmacokinetics of infused darT$_{regs}$

darT$_{regs}$ were manufactured in the presence of deuterated glucose, which resulted in the incorporation of deuterium into the genome of darT$_{reg}$ products. Deuterium enrichment in genomic DNA by gas chromatography–mass spectrometry can be used to track these autologous cells after infusion (19). Deuterium enrichment was detected in circulating T_{regs} in all infused participants. The signal peaked early after infusion and persisted for many months (Fig. 3A). The peak deuterium signals had a positive linear correlation with the number of darT$_{regs}$ infused (Fig. 3B), suggesting that the number of infused cells did not exceed an engraftment threshold. For participants 2 and 4, who received target doses of darT$_{regs}$ and were monitored for more than 300 days for deuterium, a pattern of two-phase decay was evident, as we have previously described in type 1 diabetes (19). A two-phase exponential decay model estimated that 80 to 90% of the infused darT$_{regs}$ were in the fast decay pool with a calculated half-life of about 50 days. The slow pool enrichment remained stable with very long half-lives estimated in both participants (Fig. 3C).

ARTEMIS T_{reg} characteristics

To investigate the cellular basis of low darT$_{reg}$ expansion, we performed RNA sequencing (RNA-seq) transcriptomic profiling of T_{regs} sorted from archived peripheral blood mononuclear cells (PBMCs) collected for T_{reg} manufacturing. The analysis included samples from eight ARTEMIS participants collected during stage 2 of immunosuppression withdrawal, six kidney transplant (KTx) patients 6 to 12 months after transplant on standard immunosuppression, two patients before KTx, and three patients with type 1 diabetes. These 19 samples were selected on the basis of T_{reg} manufacturing records to include 7 low, 6 medium, and 6 high expanders (defined by referencing the manufacturing records of comparable products; table S6). This sample set included those used in darT$_{reg}$ and PolyT$_{reg}$ manufacturing [high variability in T_{reg} expansion was also observed during PolyT$_{reg}$ manufacturing (20)]. Grouping these samples together may allow the identification of features shared by low expanders in both platforms. Gene set enrichment analysis of differentially expressed genes showing the highest correlation with T_{reg} fold expansion across all 19 samples revealed that low expansion was enriched in pathways of immune activation, cytokine signaling, and epigenetic regulation with an underrepresentation of pathways involved in translation regulation and ribosomal biogenesis (data file S1). Similar results were observed when the analysis was restricted to the ARTEMIS samples, which identified an increase in immune activation pathways and in negative regulation of T cell proliferation along with down-regulation of pathways involved in translation and ribosomal biogenesis (Fig. 4A and data file S2). These data suggest that low-expander T_{regs} have a distinct transcriptomic profile that includes both effector activation and senescence characteristics (21, 22).

We additionally performed a 30-parameter spectral flow cytometry analysis focusing on markers of T cell activation and exhaustion using 23 PBMC samples that overlapped with

those in the RNA-seq analysis (table S6). After data acquisition of individual samples, events in the $CD4^+FOXP3^+HELIOS^+$ T_{reg} gate (fig. S5) for all samples were digitally pooled and displayed on Uniform Manifold Approximation and Projection (UMAP) to visualize marker expression as heatmaps (Fig. 4B). Most T_{regs} expressed CD45RO and FAS, indicative of previous antigen experiences. A subset of CD45RO T_{regs} expressed an HLA-DR^+ $CD25^{hi}$ and CTLA-4^+ phenotype. These markers were positively correlated with Ki67 expression, suggesting active cell cycling. A distinct $CXCR3^+$ T_{reg} population was observed, but it did not overlap with the HLA-DR^+ T_{regs}. Among HLA-DR^+–activated T_{regs}, a subgroup of $CD38^+$ expressed markers typically associated with T cell exhaustion, including programmed cell death protein 1 (PD1), killer cell lectin-like receptor G1 (KLRG1), and T cell immunoglobulin (Ig) mucin-3 (TIM3), whereas another subset highly expressed CD39 and T cell immunoreceptor with Ig and ITIM domains (TIGIT). Transcription factor inhibitor of DNA binding 2 (ID2) was high among all three subsets of activated T_{regs}. Interferon regulatory factor 4 (IRF4) expression was higher among the $CD38^+$ subset, and thymocyte selection–associated high mobility group box (TOX) was higher among the $CD39^+$ T_{regs}. T cell factor 1 $(TCF1)^{high}$ T_{regs} occupied an area of the UMAP that was nearly completely opposite of that of the HLA-DR+ Tregs.

T_{regs} in ARTEMIS participants showed an enrichment for the $CD38^+$ and $CD39^+$ T_{reg} subsets but relatively depleted of the $CXCR3^+$ T_{regs} (Fig. 4C). We then explored whether any of these T_{reg} phenotypes correlated with T_{reg} expansion during $darT_{reg}$ manufacturing. We found a significant inverse correlation between the percentages of HLA-DR^+ (P = 0.0226), $KLRG1^+$ (P = 0.0093), or $PD1^{hi}$ (P = 0.0251) T_{regs} and $darT_{reg}$ expansion and a significant (P = 0.0204) positive correlation between the amount of TCF1 expression and $darT_{reg}$ expansion (Fig. 4D and fig. S6). No correlation was found between the expression of CXCR3, ID2, IRF, or TOX and $darT_{reg}$ expression. These findings are consistent with RNA-seq results and suggest that the low-expander T_{regs} have features of activation and prior antigen experiences.

The liver is known for its ability to induce immune tolerance to antigens that it expresses (23, 24). Mechanisms of tolerance induction by the liver elucidated in mouse studies include trapping and deletion of activated effector T cells, induction of T cell anergy and exhaustion, and inducing T_{regs} (25–28). We therefore measured donor reactivity of T_{regs} in ARTEMIS participants using a modified mixed lymphocyte reaction assay, referred to as the alloantigen-reactive T cell frequency (ATF) assay, in which recipients' PBMCs were labeled with a cell tracker dye (CTD) and stimulated with CD40L-activated B cells from donors for 4 days (29). The proliferation of donor-reactive T_{regs} was readily measured and quantified using CTD dilution (fig. S7). We compared precursor frequencies of donor-reactive T_{regs} in ARTEMIS participants to a reference dataset of lung transplant recipients before and 1 year after transplant. The median (range) $darT_{reg}$ precursor frequency was 14.2% (4.7 to 38.5) before lung transplant, which remained similar 1 year after transplant. The median (range) precursor frequencies of $darT_{regs}$ in ARTEMIS participants were 7.9% (2.0 to 9.0), significantly lower than those in lung recipients, both before (P = 0.0059) and after transplant (P = 0.0123; Fig. 4E). To determine whether the low proliferation of ARTEMIS T_{regs} was due to deficiency in growth factors, we repeated the ATF assay in the presence of interleukin-2 (IL-2), IL-7, IL-15, IL-6, and tumor necrosis factor–α (TNF-α), all have

been shown previously to promote human T_{reg} expansion (30–35). None of these cytokines, either singly or in various combinations, changed the ARTEMIS participants' T_{reg} response to donor antigens (Fig. 4F and fig. S8). In contrast, T_{conv} and $CD8^+$ T cell responses to donor antigens were increased by IL-2, IL-7, and IL-15 (fig. S9).

To explore whether donor-reactive T_{regs} were deleted by the transplanted liver, we measured T_{reg} repertoire diversity in ARTEMIS participants using T cell receptor (TCR) sequencing. We reasoned that if as high as 10 to 20% of T_{regs} have direct alloreactivity to a mismatched donor, then their deletion would result in holes in the T_{reg} repertoire and reduced repertoire diversity. We sequenced TCRα and TCRβ chains of 60,000 T_{regs} purified from ARTEMIS ($n = 8$), post-KTx ($n = 6$), and control ($n = 8$) PBMCs. Repertoire diversity was measured using D50 indexes of the complementary-determining region 3 (CDR3) of TCRα and TCRβ chains. D50 is the percent of unique T cell clones that account for the cumulative 50% of total CDR3s counted in the sample (36). Thus, a diverse repertoire with equal representation of all clones has a D50 of 50. The median D50 of ARTEMIS T_{regs} was between 4.6 and 13.5 for TCRα and between 6.6 and 22.5 for TCRβ, not different from T_{regs} in post-KTx and control individuals (fig. S10A). Because CDR1 and CDR2 encoded by the V gene segments contribute to the recognition of allogeneic major histocompatibility complex (MHC) molecules (37), we compared TCRα and TCRβ V gene usage by T_{regs} from the three cohorts. No obvious changes in V gene usage were observed (fig. S10, B and C). Overall, these analyses show that T_{regs} in ARTEMIS participants remained similarly diverse when compared to normal controls and KTx recipients on immunosuppression.

T_{reg} donor reactivity after liver transplantation

Because ARTEMIS participants were enrolled 2 to 7 years after liver transplantation, no pretransplant baseline samples were available for direct comparison of T_{reg} donor reactivity before and after transplant. Therefore, we obtained, from the Immune Tolerance Network, peripheral blood samples collected in the AWISH study (NTC00135694) (15), a clinical trial of immunosuppression withdrawal trial in de novo liver transplant recipients. Longitudinal samples from 16 AWISH specimens collected before transplant as well as 6 months and 2 years after transplant were analyzed using the ATF assay. In pretransplant AWISH samples, between 27 and 63% of T_{regs} had diluted the CTD by the end of the 4-day assay (Fig. 5, A to C). After liver transplantation, proliferation induced by donor B cells was significantly reduced ($P = 0.0088$). In contrast, proliferation induced by a mixture of allogeneic B cells, selected to maximize the coverage of distinct HLA supertypes, did not change (Fig. 5, A and B). Further analyses of the dye dilution data show a reduced frequency of donor-reactive T_{reg} precursors after liver transplantation (Fig. 5D). Moreover, among the T_{regs} that entered the cell cycle, the percentage of T_{regs} that had divided five times or more, indicative of higher proliferative potential, was lower for posttransplant compared to pretransplant specimens. Conversely, the percentage of T_{regs} that had divided only once was higher (Fig. 5E). Together, these results show a substantial and selective reduction of T_{reg} donor reactivity as early as 6 months after liver transplantation, which persisted until 2 years after transplantation. Furthermore, the reduced reactivity can be attributed to either reduced frequency, decreased proliferative potential of darT_{regs}, or both.

To determine whether the reduced donor reactivity was limited to the T_{reg} compartment, we similarly analyzed the proliferative responses of $CD4^+$ T_{conv} and $CD8^+$ T cells. Donor-reactive $CD4^+$ T_{conv} showed a significant reduction of precursor frequency and proliferative potential by 2 years ($P = 0.0068$), but not at 6 months after transplant (fig. S11). Donor-reactive precursors of $CD8^+$ T cells were significantly reduced at both 6-month ($P = 0.0024$) and 2-year ($P = 0.0195$) time points (fig. S12, A to C); however, the proliferative potential of the remaining donor-reactive cells was mostly unchanged (fig. S12, D and E). For both $CD4^+$ T_{conv} cells and $CD8^+$ T cells, reactivity to a mixture of allogeneic B cells remained unchanged after transplantation, demonstrating that the observed changes were not due to generalized immunosuppression but selective to donor antigens. Overall, the kinetic analyses of T cell donor alloantigen reactivity showed a selective reduction of donor reactivity in all T cell compartments after liver transplantation, which may underlie the difficulties in manufacturing $darT_{regs}$ for the ARTEMIS trial.

DISCUSSION

ARTEMIS was designed to assess the safety and efficacy of $darT_{reg}$ therapy in liver transplant recipients. The trial design allowed for an assessment of both safety and efficacy with a modest number of participants. This efficient design is highly desirable for early-stage cell therapy trials with labor-intensive cell manufacturing processes. The efficacy endpoint, stable reduction, or complete cessation of immunosuppression after T_{reg} therapy is supported by the results of previous multicenter, prospective clinical trials of immunosuppression withdrawal in liver transplant recipients and supports an efficacy assessment of T_{reg} therapy.

The ARTEMIS trial achieved accrual of nine participants eligible for $darT_{reg}$ infusion; however, only five participants received $darT_{regs}$ and all infusions were well tolerated. Infused T_{regs} were detected in circulation in all participants, and evidence of a small long-lived subset of the infused T_{regs} was seen. An insufficient number of participants were treated for efficacy assessment. The failure to manufacture a full dose for most participants was associated with reduced T_{reg} counts and T_{reg} donor reactivity, possibly from years of exposure to the donor's liver under immunosuppression.

Mechanistic studies revealed dysfunction of $darT_{regs}$ after liver transplantation. Antigen recognition in the liver leads to deletion and hyporesponsiveness of antigen-specific $CD4^+$ T_{conv} cells and $CD8^+$ T cells in mouse models and clinical liver transplantation (25, 28, 38, 39), in contrast to no change after lung transplantation, and increased after kidney and small intestine transplantation (40–42). Although these previous studies in transplant recipients did not analyze T_{reg} dynamics, recognition of antigens in the liver leads to T_{reg} expansion in mouse models (26, 27, 43). We thus anticipated that $darT_{reg}$ frequency would increase after liver transplantation, especially when immunosuppression is gradually reduced to enable donor alloantigen recognition in a noninflamed environment that is conducive to T_{reg} induction. Contrary to this expectation, our mechanistic analyses together suggest a progressive process of T_{reg} activation, exhaustion, senescence, or deletion after liver transplantation.

There are likely multiple reasons for the dichotomous findings in humans and mouse models. We speculate that one contribution may be immunosuppression use in humans after liver transplantation (44). Calcineurin inhibitors are very effective at suppressing IL-2 expression, a cytokine essential for T_{reg} differentiation and survival. This may explain the low number of T_{regs} by TruCount in ARTEMIS participants. Moreover, calcineurin inhibitors and mycophenolate have been both implicated in inducing cellular senescence (45, 46), a phenotype of low-expander T_{regs} revealed in RNA-seq analyses of this study. The reduction of T_{reg} donor reactivity after liver transplant may explain why spontaneous transplant tolerance is rare, despite the deletion and inactivation of donor-reactive $CD4^+$ T_{conv} cells and $CD8^+$ T cells. Future studies are needed to define the cellular and molecular underpinnings of T_{reg} dysfunction after liver transplantation.

There has been intense interest in using low-dose IL-2 or CD25-dependent IL-2 muteins to selectively expand T_{regs} to treat autoimmune diseases (47, 48). In mouse models of transplantation, IL-2 therapy reverses the T_{reg} dysfunction induced by calcineurin inhibitors and promotes transplantation tolerance (49, 50). Recently, we have shown that low-dose IL-2 therapy increased not only the persistence of infused T_{regs} in the circulation but also the cytotoxic T cell signature in patients with type 1 diabetes (51). In ARTEMIS, we stimulated T_{regs} with donor antigens in vitro, in the presence of exogenous IL-2 and common γ chain cytokines, IL-7 and IL-15, to determine whether T_{reg} dysfunction could be reversed. Although the response of T_{regs} to the addition of these cytokines did not change, the proliferation of $CD8^+$ T cells markedly increased. These results echo the lack of donor-specific T_{reg} expansion following the in vivo administration of low-dose IL-2 to liver transplant recipients in a clinical trial (LITE, NCT02949492) that has a very similar design to ARTEMIS (52). Together, these results suggest that reduced IL-2 in transplant recipients maintained on calcineurin inhibitors may pose a substantial challenge to exogenous T_{reg} therapy and tolerance induction. IL-2 therapy may not have sufficient selectivity for T_{reg} enhancement in transplant recipients.

The ARTEMIS study has several limitations. The enrollment was restricted to living donor transplant recipients due to the need for donor B cells to manufacture $darT_{regs}$, thereby excluding deceased donor liver transplant recipients. Manufacturing $darT_{regs}$ for ARTEMIS was proven to be difficult. Mechanistic studies correlated an unexpected decrease in T_{reg} donor reactivity after liver transplantation with poor manufacturing outcomes, but the cellular and molecular underpinning of T_{reg} dysfunction after liver transplantation remains to be elucidated. It is presently unclear how the T_{regs} may be rescued if it is possible. Lastly, with only two participants receiving the target dose and a total of five infused participants, the study was not sufficiently powered to assess safety or efficacy of $darT_{regs}$. Moreover, four of the five participants treated with $darT_{regs}$ experienced an acute rejection episode during immunosuppression reduction, including a patient treated with the target dose of $darT_{reg}$. Although these rejections were assessed as unrelated to $darT_{reg}$ administration, these data are suggestive of limited efficacy of $darT_{reg}$ therapy in preventing graft rejection.

Future trial designs will need to address manufacturing challenges in $darT_{regs}$ after liver transplantation. With the advent of genome editing and synthetic biology, it is possible to engineer T_{regs} with desired alloantigen specificity without a need for donor tissue.

Additional features may be engineered to endow T_{reg} products with resistance to calcineurin inhibitors and independence from IL-2 (53). Alternative to genetic engineering of T_{reg} products, enrolling participants early after transplant before T_{reg} dysfunction is established may improve $darT_{reg}$ manufacturing. In addition, depleting donor alloantigen–reactive $CD4^+$ T_{conv} cells and $CD8^+$ T cells could be synergistic with T_{reg} infusion so that fewer infused T_{regs} can exert dominant immune regulation. In this regard, a pilot study of T_{regs} following cyclophosphamide-mediated depletion provides proof-of-concept data that demonstrate that intentional induction of liver transplantation tolerance is achievable (17).

MATERIALS AND METHODS

Study design

The ARTEMIS study aims to evaluate the safety and efficacy of autologous $darT_{regs}$ in liver transplantation. The trial enrolled male and female participants between 18 and 70 years of age 2 to 7 years after receiving a living donor liver transplant. The key eligibility criteria are summarized in table S1. Immunosuppression was reduced stepwise (tables S2 and S3), and $darT_{regs}$ were infused shortly before the 50% reduction of immunosuppression. The primary safety endpoint was the occurrence of Common Terminology Criteria for Adverse Events (CTCAE) grade 3 or higher adverse events including infusion reaction and cytokine release syndrome, grade 3 or higher infections as defined by the trial protocol, and any malignancy including posttransplant lymphoproliferative disease. The primary efficacy endpoint was the reduction of calcineurin inhibitor dosing by 75% and discontinuation of a second immunosuppressive drug, if applicable, with stable liver tests for at least 12 weeks. All infused participants (targeted and infusible doses) were permitted to reduce calcineurin inhibitor dose reduction by 75% and discontinue a second agent if applicable (primary endpoint). Only participants who received the targeted dose and successfully minimized immunosuppression as defined above would be offered the option of proceeding with complete immunosuppression withdrawal for assessing the secondary efficacy endpoint of achieving tolerance. More details on trial procedures can be found in Supplementary Materials and Methods.

$darT_{reg}$ manufacturing

Autologous $darT_{regs}$ were manufactured for individual trial participants at the UCSF GMP facility using a process previously published (13). Briefly, the process began with the production of stimulated B cells (sBCs) by two rounds of stimulation of donor PBMCs with irradiated K562 cells expressing CD40L. The resulting sBCs were irradiated and cryopreserved in CS10 medium. Recipient T_{regs} were purified from 1 U of whole blood or 5 liters of leukapheresis product using fluorescence-activated cell sorting (FACS) based on the phenotype of $CD4^+CD25^+CD127^{lo/-}$. The expansion cultures were initiated by mixing purified T_{regs} and thawed sBCs at a ratio of 1 T_{reg} to 4 sBCs in a T_{reg} expansion medium consisting of X-Vivo 15 base and 10% human AB serum supplemented with IL-2 (300 IU/ml). The cells were restimulated with anti-CD3– and anti-CD28–conjugated beads on day 11 and harvested on day 16. After debeading and quality control assessments, products that met the minimal infusible dose and the preset release criteria were formulated in infusion solution and released for infusion (table S4). For participants at Mayo Clinic and

Northwestern University, the products were shipped in containers validated to maintain temperatures between 2° and 10°C. Infusions were completed within 30 hours of final product formulation. Aliquots of archived darT$_{reg}$ products were submitted to EpigenDx for analysis of methylation of nine cytosines in the FOXP3 T$_{reg}$-specific demethylated region (assay ID: ADS783-FS2). Reagent information can be found in table S7.

Flow cytometry

Cryopreserved PBMCs were thawed, washed, and stained with fixable viability dye for 20 min before additional staining with cell surface fluorochrome-conjugated antibodies for another 30 min. The cells were then washed, fixed, and permeabilized in the Foxp3 transcription factor staining buffer set before staining with fluorochrome-conjugated antibodies for intracellular proteins for 60 min. All incubations were carried out in the dark at 4°C. Antibodies used for flow cytometry are summarized in table S8. The stained cells were washed before analysis on a Navios (Beckman Coulter) or an Aurora (Cytek) flow cytometer. Data analyses were performed using FlowJo (Tree Star Inc.) and Kaluza software (Beckman Coulter).

ATF assay

The assay was performed as previously described (29). Briefly, recipient PBMCs were labeled with a CellTrace Violet dye (Invitrogen) and mixed with appropriate donor sBCs at the ratio of 2 sBCs to 1 PBMC. To assess general alloreactivity, a mixture of six sBCs with distinct HLA types was used instead of donor sBC to stimulate the recipient PBMCs. The cells were cultured in RPMI 1640 medium supplemented with GlutaMAX, 10% human AB serum (Omega Scientific), 1% nonessential amino acids, 1% sodium pyruvate, and 1% penicillin/streptomycin (all reagents were from Gibco unless stated otherwise) for 84 to 96 hours before staining with antibodies to CD4, CD8, FOXP3, and HELIOS (table S8) as described above for flow cytometry. The samples were measured on a Navios (Beckman Coulter) flow cytometer, and CellTrace Violet dilution in T$_{regs}$ (CD4$^+$FOXP3$^+$HELIOS$^+$), T$_{conv}$ (CD4$^+$, not T$_{reg}$ gate), and CD8$^+$ T cells were analyzed using Kaluza software (Beckman Coulter).

Quantification of deuterium in circulating T$_{regs}$

PBMCs from patients who had received an infusion of darT$_{regs}$ were collected on days 1 and 6 and on months 1, 3, 6, 12, and 18. CD4$^+$CD25$^+$CD127$^{lo/-}$ T$_{regs}$ were sorted, and cell pellets were cryopreserved at −80°C. Samples were later used for batched analyses of deuterium content in the genomic DNA, as previously described (19).

TCR sequencing

Patient T$_{regs}$ were FACS-purified using CD4$^+$CD25$^+$CD127$^{lo/-}$ markers from PBMCs cryopreserved at the start of darT$_{reg}$ manufacture. At least 60,000 T$_{regs}$ were sorted for each sample and preserved in TRIzol reagent (Invitrogen). Total RNA was extracted from each sample using the Direct-zol RNA Microprep Kit (Zymo Research), and 60 ng of total RNA was submitted to iRepertoire Inc. for TCRα and TCRβ repertoire sequencing using the

Illumina MiSeq platform. Data analysis was performed using the online analytic tool iRweb (iRepertoire Inc.)

RNA sequencing

RNA was extracted from FACS-purified $CD4^+CD25^+CD127^{lo/-}$ T_{regs} as described above for TCR sequencing. RNA libraries were prepared and amplified using TruSeq Stranded mRNA sample preparation with the corresponding kit (Illumina Inc.). Paired-end 101–base pair sequencing was done, reaching about a total of 50 million reads by sample. RNA-seq data analyses are described in detail in Supplementary Materials and Methods.

Statistical methods

Raw, individual-level data are presented in data file S3. For clinical data, categorical and continuous variables were compared using Fisher's exact and two-sample Student's *t* tests. Clinical statistical analyses were performed using SAS version 9.4 (SAS Institute). T_{reg} manufacturing and research laboratory data were analyzed using MS Excel and GraphPad Prism version 9.3.1. Statistical tests used for each dataset are provided in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank the following individuals for their contribution to the study: J. A. Bluestone for his advice during the design of this trial; T. Sledge for overall management and coordination of the clinical trial; J. Goldstein for regulatory support; D. Ikle, M. Sever, and K. Spain for statistical support; S. Blaschka, B. Block, A. Daud, J. G. Hanson, K. Harish, and D. Jakoubek for clinical trial administration, conduct, and coordination at UCSF, Mayo Clinic, and Northwestern University; L. Acevedo, F. Dekovic, G. Guinti, E. Lama, M. R. Lee, A. S. Leinbach, L. Masiello, B. R. Shy, A. Tam, and J. Xu for support of T_{reg} manufacturing and T_{reg} manufacturing database design and maintenance; J. Du for management of the UCSF Transplantation Research Lab; F. Vincenti for providing post-kidney transplant samples; and A. Shaked for providing the AWISH samples.

Funding:

The study is funded by a grant from NIAID 1U01AI104347 (to S.F. and Q.T.).

REFERENCES AND NOTES

1. Rodríguez-Perálvarez M, De la Mata M, Burroughs AK, Liver transplantation: Immunosuppression and oncology. Curr. Opin. Organ Transplant. 19, 253–260 (2014). [PubMed: 24685671]
2. Watt KD, Pedersen RA, Kremers WK, Heimbach JK, Charlton MR, Evolution of causes and risk factors for mortality post-liver transplant: Results of the NIDDK long-term follow-up study. Am. J. Transplant. 10, 1420–1427 (2010). [PubMed: 20486907]
3. Kubes P, Jenne C, Immune responses in the liver. Annu. Rev. Immunol. 36, 247–277 (2018). [PubMed: 29328785]
4. Feng S, Sanchez-Fueyo A, in Pediatric Hepatology and Liver Transplantation (Springer, 2019), pp. 625–652.
5. Thomson AW, Vionnet J, Sanchez-Fueyo A, Understanding, predicting and achieving liver transplant tolerance: From bench to bedside. Nat. Rev. Gastroenterol. Hepatol. 17, 719–739 (2020). [PubMed: 32759983]
6. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N, Regulatory T cells and human disease. Annu. Rev. Immunol. 38, 541–566 (2020). [PubMed: 32017635]

7. Tang Q, Bluestone JA, Regulatory T-cell therapy in transplantation: Moving to the clinic. Cold Spring Harb. Perspect. Med. 3, a015552 (2013). [PubMed: 24186492]
8. Romano M, Tung SL, Smyth LA, Lombardi G, Treg therapy in transplantation: A general overview. Transplant Int. 30, 745–753 (2017).
9. Tang Q, Bluestone JA, The Foxp3$^+$ regulatory T cell: A jack of all trades, master of regulation. Nat. Immunol. 9, 239–244 (2008). [PubMed: 18285775]
10. Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, Hori S, Waldmann H, Sustained suppression by Foxp3$^+$ regulatory T cells is vital for infectious transplantation tolerance. J. Exp. Med. 208, 2043–2053 (2011). [PubMed: 21875958]
11. Sanchez-Fueyo A, Sandner S, Habicht A, Mariat C, Kenny J, Degauque N, Zheng XX, Strom TB, Turka LA, Sayegh MH, Specificity of CD4$^+$CD25$^+$ regulatory T cell function in alloimmunity. J. Immunol. 176, 329–334 (2006). [PubMed: 16365425]
12. Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G, Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. Sci. Transl. Med. 3, 83ra42 (2011).
13. Putnam AL, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz MA, Trotta E, Szot GL, Liu W, Lares A, Lee K, Laing A, Lechler RI, Riley JL, Bluestone JA, Lombardi G, Tang Q, Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. Am. J. Transplant. 13, 3010–3020 (2013). [PubMed: 24102808]
14. Feng S, Ekong UD, Lobritto SJ, Demetris AJ, Roberts JP, Rosenthal P, Alonso EM, Philogene MC, Ikle D, Poole KM, Bridges ND, Turka LA, Tchao NK, Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants. JAMA 307, 283–293 (2012). [PubMed: 22253395]
15. Shaked A, DesMarais MR, Kopetskie H, Feng S, Punch JD, Levitsky J, Reyes J, Klintmalm GB, Demetris AJ, Burrell BE, Priore A, Bridges ND, Sayre PH, Outcomes of immunosuppression minimization and withdrawal early after liver transplantation. Am. J. Transplant. 19, 1397–1409 (2019). [PubMed: 30506630]
16. Tang Q, Lee K, Regulatory T-cell therapy for transplantation. Curr. Opin. Organ Transplant. 17, 349–354 (2012). [PubMed: 22790069]
17. Todo S, Yamashita K, Goto R, Zaitsu M, Nagatsu A, Oura T, Watanabe M, Aoyagi T, Suzuki T, Shimamura T, A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. Hepatology 64, 632–643 (2016). [PubMed: 26773713]
18. Benítez C, Londoño MC, Miquel R, Manzia TM, Abraldes JG, Lozano JJ, Martínez-Llordella M, López M, Angelico R, Bohne F, Sese P, Daoud F, Larcier P, Roelen DL, Claas F, Whitehouse G, Lerut J, Pirenne J, Rimola A, Tisone G, Sánchez-Fueyo A, Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. Hepatology 58, 1824–1835 (2013). [PubMed: 23532679]
19. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, Herold KC, Lares A, Lee MR, Li K, Liu W, Long SA, Masiello LM, Nguyen V, Putnam AL, Rieck M, Sayre PH, Tang Q, Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci. Transl. Med. 7, 315ra189 (2015).
20. Balcerek J, Shy BR, Putnam AL, Masiello LM, Lares A, Dekovic F, Acevedo L, Lee MR, Nguyen V, Liu W, polyclonal regulatory T cell manufacturing under cGMP: A decade of experience. Front. Immunol. 12, 744763 (2021). [PubMed: 34867967]
21. Lessard F, Igelmann S, Trahan C, Huot G, Saint-Germain E, Mignacca L, Del Toro N, Lopes-Paciencia S, Le Calvé B, Montero M, Senescence-associated ribosome biogenesis defects contributes to cell cycle arrest through the Rb pathway. Nat. Cell Biol. 20, 789–799 (2018). [PubMed: 29941930]
22. Zhang Y, Lu H, Signaling to p53: Ribosomal proteins find their way. Cancer Cell 16, 369–377 (2009). [PubMed: 19878869]
23. Thomson AW, Knolle PA, Antigen-presenting cell function in the tolerogenic liver environment. Nat. Rev. Immunol. 10, 753–766 (2010). [PubMed: 20972472]
24. Abrol N, Jadlowiec CC, Taner T, Revisiting the liver's role in transplant alloimmunity. World J. Gastroenterol. 25, 3123–3135 (2019). [PubMed: 31333306]

25. Mehal WZ, Azzaroli F, Crispe IN, Antigen presentation by liver cells controls intrahepatic T cell trapping, whereas bone marrow-derived cells preferentially promote intrahepatic T cell apoptosis. J. Immunol. 167, 667–673 (2001). [PubMed: 11441069]
26. Luth S, Huber S, Schramm C, Buch T, Zander S, Stadelmann C, Brück W, Wraith DC, Herkel J, Lohse AW, Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. J. Clin. Investig. 118, 3403–3410 (2008). [PubMed: 18802476]
27. Carambia A, Freund B, Schwinge D, Heine M, Laschtowitz A, Huber S, Wraith DC, Korn T, Schramm C, Lohse AW, TGF-β-dependent induction of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs by liver sinusoidal endothelial cells. J. Hepatol. 61, 594–599 (2014). [PubMed: 24798620]
28. Bénéchet AP, De Simone G, Di Lucia P, Cilenti F, Barbiera G, Le Bert N, Fumagalli V, Lusito E, Moalli F, Bianchessi V, Dynamics and genomic landscape of $CD8^+$ T cells undergoing hepatic priming. Nature 574, 200–205 (2019). [PubMed: 31582858]
29. Greenland JR, Wong CM, Ahuja R, Wang AS, Uchida C, Golden JA, Hays SR, Leard LE, Rajalingam R, Singer JP, Kukreja J, Wolters PJ, Caughey GH, Tang Q, Donor-reactive regulatory T cell frequency increases during acute cellular rejection of lung allografts. Transplantation 100, 2090–2098 (2016). [PubMed: 27077597]
30. Skartsis N, Peng Y, Ferreira LMR, Nguyen V, Ronin E, Muller YD, Vincenti F, Tang Q, IL-6 and TNFα drive extensive proliferation of human tregs without compromising their lineage stability or function. Front. Immunol. 12, 783282 (2021). [PubMed: 35003100]
31. Vignali D, Gürth C-M, Pellegrini S, Sordi V, Sizzano F, Piemonti L, Monti P, IL-7 mediated homeostatic expansion of human $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells after depletion with anti-CD25 monoclonal antibody. Transplantation 100, 1853–1861 (2016). [PubMed: 27306531]
32. Silva SL, Albuquerque AS, Serra-Caetano A, Foxall RB, Pires AR, Matoso P, Fernandes SM, Ferreira J, Cheynier R, Victorino RM, Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7. Oncotarget 7, 12163–12175 (2016). [PubMed: 26910841]
33. He X, Landman S, Bauland SC, van den Dolder J, Koenen HJ, Joosten I, ATNFR2-agonist facilitates high purity expansion of human low purity Treg cells. PLOS ONE 11, e0156311 (2016). [PubMed: 27224512]
34. Clark RA, Kupper TS, IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. Blood 109, 194–202 (2007). [PubMed: 16968902]
35. Veerapathran A, Pidala J, Beato F, Yu X-Z, Anasetti C, Ex vivo expansion of human Tregs specific for alloantigens presented directly or indirectly. Blood 118, 5671–5680 (2011). [PubMed: 21948174]
36. Hou D, Ying T, Wang L, Chen C, Lu S, Wang Q, Seeley E, Xu J, Xi X, Li T, Immune repertoire diversity correlated with mortality in avian influenza A (H7N9) virus infected patients. Sci. Rep. 6, 1–11 (2016). [PubMed: 28442746]
37. Colf LA, Bankovich AJ, Hanick NA, Bowerman NA, Jones LL, Kranz DM, Garcia KC, How a single T cell receptor recognizes both self and foreign MHC. Cell 129, 135–146 (2007). [PubMed: 17418792]
38. Taner T, Gustafson MP, Hansen MJ, Park WD, Bornschlegl S, Dietz AB, Stegall MD, Donor-specific hypo-responsiveness occurs in simultaneous liver-kidney transplant recipients after the first year. Kidney Int. 93, 1465–1474 (2018). [PubMed: 29656904]
39. Savage TM, Shonts BA, Lau S, Obradovic A, Robins H, Shaked A, Shen Y, Sykes M, Deletion of donor-reactive T cell clones after human liver transplant. Am. J. Transplant. 20, 538–545 (2020). [PubMed: 31509321]
40. Wang P, Leung J, Lam A, Lee S, Calabrese DR, Hays SR, Golden JA, Kukreja J, Singer JP, Wolters PJ, Tang Q, Greenland JR, Lung transplant recipients with idiopathic pulmonary fibrosis have impaired alloreactive immune responses. J. Heart Lung Transplant. 41, 641–653 (2022). [PubMed: 34924263]
41. Morris H, DeWolf S, Robins H, Sprangers B, LoCascio SA, Shonts BA, Kawai T, Wong W, Yang S, Zuber J, Tracking donor-reactive T cells: Evidence for clonal deletion in tolerant kidney transplant patients. Sci. Transl. Med. 7, 272ra210 (2015).

42. Zuber J, Shonts B, Lau S-P, Obradovic A, Fu J, Yang S, Lambert M, Coley S, Weiner J, Thome J, Bidirectional intragraft alloreactivity drives the repopulation of human intestinal allografts and correlates with clinical outcome. Sci. Immunol. 1, eaah3732 (2016). [PubMed: 28239678]
43. Dangi A, Sumpter TL, Kimura S, Stolz DB, Murase N, Raimondi G, Vodovotz Y, Huang C, Thomson AW, Gandhi CR, Selective expansion of allogeneic regulatory T cells by hepatic stellate cells: Role of endotoxin and implications for allograft tolerance. J. Immunol. 188, 3667–3677 (2012). [PubMed: 22427640]
44. Azzi JR, Sayegh MH, Mallat SG, Calcineurin inhibitors: 40 years later, can't live without. J. Immunol. 191, 5785–5791 (2013). [PubMed: 24319282]
45. Luttropp K, Nordfors L, McGuinness D, Wennberg L, Curley H, Quasim T, Genberg H, Sandberg J, Sönnerborg I, Schalling M, Increased telomere attrition after renal transplantation—Impact of antimetabolite therapy. Transplant. Direct 2, e116 (2016). [PubMed: 27990481]
46. Welzl K, Kern G, Mayer G, Weinberger B, Säemann MD, Sturm G, Grubeck-Loebenstein B, Koppelstaetter C, Effect of different immunosuppressive drugs on immune cells from young and old healthy persons. Gerontology 60, 229–238 (2014). [PubMed: 24434865]
47. Klatzmann D, Abbas AK, The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. Nat. Rev. Immunol. 15, 283–294 (2015). [PubMed: 25882245]
48. Abbas AK, Trotta E, Simeonov DR, Marson A, Bluestone JA, Revisiting IL-2: Biology and therapeutic prospects. Sci. Immunol. 3, eaat1482 (2018). [PubMed: 29980618]
49. Whitehouse G, Gray E, Mastoridis S, Merritt E, Kodela E, Yang JH, Danger R, Mairal M, Christakoudi S, Lozano JJ, Macdougall IC, Tree TIM, Sanchez-Fueyo A, Martinez-Llordella M, IL-2 therapy restores regulatory T-cell dysfunction induced by calcineurin inhibitors. Proc. Natl. Acad. Sci. U.S.A. 114, 7083–7088 (2017). [PubMed: 28584086]
50. Kang H, Zhang D, Degauque N, Mariat C, Alexopoulos S, Zheng X, Effects of cyclosporine on transplant tolerance: The role of IL-2. Am. J. Transplant. 7, 1907–1916 (2007). [PubMed: 17617853]
51. Dong S, Hiam-Galvez KJ, Mowery CT, Herold KC, Gitelman SE, Esensten JH, Liu W, Lares AP, Leinbach AS, Lee M, Nguyen V, Tamaki SJ, Tamaki W, Tamaki CM, Mehdizadeh M, Putnam AL, Spitzer MH, Ye CJ, Tang Q, Bluestone JA, The effect of low-dose IL-2 and Treg adoptive cell therapy in patients with type 1 diabetes. JCI Insight 6, e147474 (2021). [PubMed: 34324441]
52. Lim TY, Perpinan E, Londono MC, Miquel R, Ruiz P, Kurt AS, Kodela E, Cross AR, Berlin C, Hester J, Issa F, Douiri A, Volmer FH, Taubert R, Williams E, Demetris AJ, Andrew L, Gilbert B, Lozano JJ, Martinez-Llordella M, Tree T, Sanchez-Fueyo A, Low dose interleukin-2 selectively expands circulating regulatory T cells but fails to promote liver allograft tolerance in humans. J. Hepatol. S0168–8278, 03065–3 (2022).
53. Ferreira LM, Muller YD, Bluestone JA, Tang Q, Next-generation regulatory T cell therapy. Nat. Rev. Drug Discov. 18, 1–21 (2019). [PubMed: 29880920]
54. Chandran S, Tang Q, Sarwal M, Laszik ZG, Putnam AL, Lee K, Leung J, Nguyen V, Sigdel T, Tavares EC, Yang JYC, Hellerstein M, Fitch M, Bluestone JA, Vincenti F, Polyclonal regulatory T cell therapy for control of inflammation in kidney transplants. Am. J. Transplant. 17, 2945–2954 (2017). [PubMed: 28675676]
55. Sawitzki B, Harden PN, Reinke P, Moreau A, Hutchinson JA, Game DS, Tang Q, Guinan EC, Battaglia M, Burlingham WJ, Roberts ISD, Streitz M, Josien R, Böger CA, Scottà C, Markmann JF, Hester JL, Juerchott K, Braudeau C, James B, Contreras-Ruiz L, van der Net JB, Bergler T, Caldara R, Petchey W, Edinger M, Dupas N, Kapinsky M, Mutzbauer I, Otto NM, Öllinger R, Hernandez-Fuentes MP, Issa F, Ahrens N, Meyenberg C, Karitzky S, Kunzendorf U, Knechtle SJ, Grinyó J, Morris PJ, Brent L, Bushell A, Turka LA, Bluestone JA, Lechler RI, Schlitt HJ, Cuturi MC, Schlickeiser S, Friend PJ, Miloud T, Scheffold A, Secchi A, Crisalli K, Kang S-M, Hilton R, Banas B, Blancho G, Volk H-D, Lombardi G, Wood KJ, Geissler EK, Regulatory cell therapy in kidney transplantation (The ONE Study): A harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials. Lancet 395, 1627–1639 (2020). [PubMed: 32446407]
56. Higdon LE, Lee K, Tang Q, Maltzman JS, Virtual global transplant laboratory standard operating procedures for blood collection, PBMC isolation, and storage. Transplant. Direct 2, e101 (2016). [PubMed: 27795993]

57. Dobin A, Gingeras TR, Mapping RNA-seq reads with STAR. Curr. Protoc. Bioinformatics 51, 11.14. 11–11.14. 19 (2015).

58. Li B, Dewey CN, RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, 1–16 (2011). [PubMed: 21199577]

59. Smyth GK, Ritchie M, Thorne N, Wettenhall J, LIMMA: Linear models for microarray data, in Bioinformatics and Computational Biology Solutions Using R and Bioconductor, Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S, Eds. (Springer, 2005).

60. Law CW, Chen Y, Shi W, Smyth GK, voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 15, R29 (2014). [PubMed: 24485249]

61. Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J, RankProd: A bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics 22, 2825–2827 (2006). [PubMed: 16982708]

62. Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA, DAVID: Database for annotation, visualization, and integrated discovery. Genome Biol. 4, 1–11 (2003).

63. Walter W, Sánchez-Cabo F, Ricote M, GOplot: An R package for visually combining expression data with functional analysis. Bioinformatics 31, 2912–2914 (2015). [PubMed: 25964631]

64. Yu G, Wang L-G, Han Y, He Q-Y, clusterProfiler: An R package for comparing biological themes among gene clusters. OMICS 16, 284–287 (2012). [PubMed: 22455463]

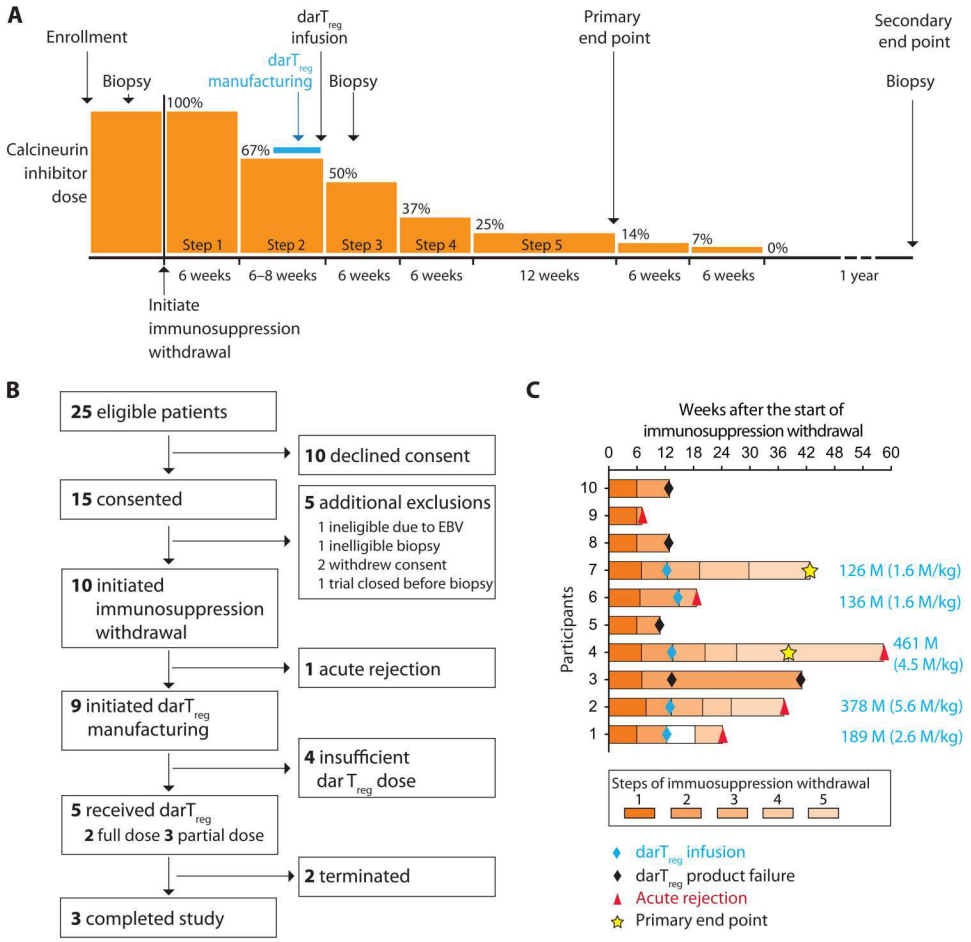

Fig. 1. ARTEMIS trial overview.

A graphical summary of the ARTEMIS trial protocol (**A**) and consort diagram (**B**) are shown. (**C**) Clinical progression of individual participants until they were off protocol due to rejection, manufacturing failure, or withdrawn from study. Doses of darT$_{regs}$ infused are listed in blue (M, million).

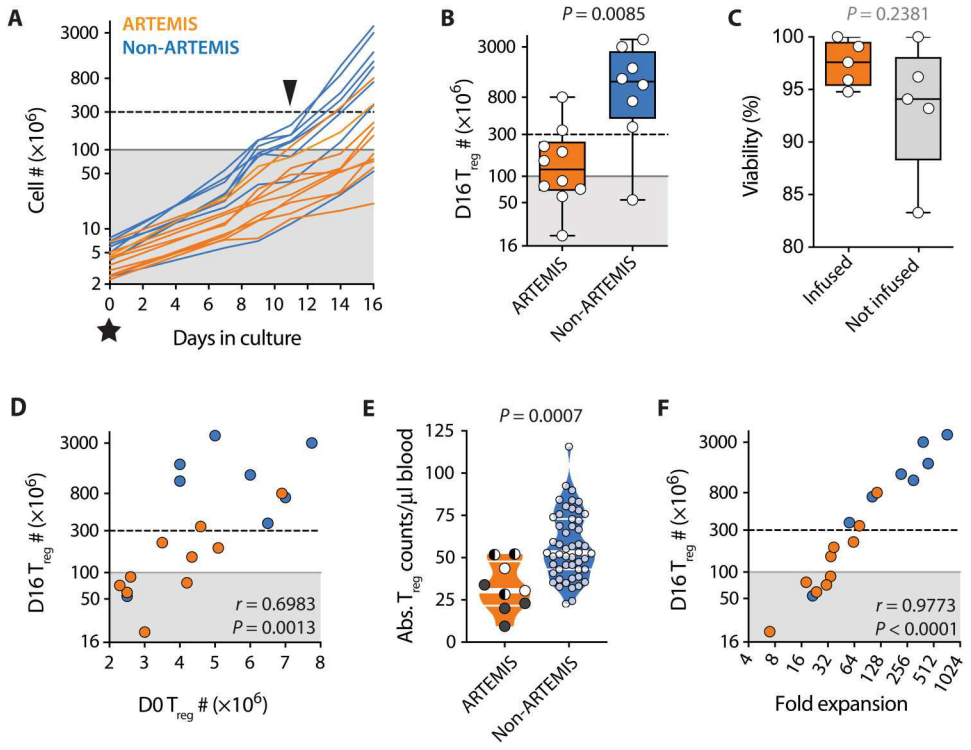

Fig. 2. ARTEMIS darT$_{reg}$ manufacturing outcome and correlative data.

(**A**) T$_{reg}$ expansion during the 16-day darT$_{reg}$ manufacturing for ARTEMIS (n = 10) and non-ARTEMIS individuals (n = 8). The star at day 0 indicates sBC stimulation, and the arrowhead at day 11 indicates aCD3/28 bead stimulation. (**B**) Day 16 darT$_{reg}$ (D16T$_{reg}$) product yield is shown for ARTEMIS (n = 10) versus non-ARTEMIS individuals (n = 8). Mann-Whitney test was used to assess the statistical significance of the difference between the two groups. (**C**) Day 16 viability was measured in infused (n = 5) and not infused (n = 5) darT$_{reg}$ products in the ARTEMIS trial. Mann-Whitney test was used to assess the statistical significance of the difference between the two groups. (**D**) Correlation between number of T$_{regs}$ purified on day 0 of culture initiation and day 16 darT$_{reg}$ product yield. Spearman correlation was used to assess the significance of the correlation. (**E**) Absolute (Abs.) T$_{reg}$ numbers per microliter of blood were measured using the T$_{reg}$ TruCount assay, and results from ARTEMIS (n = 9) and participants in other trials [type 1 diabetes, n = 25; pre-islet transplant, n = 4; pre-kidney transplant (KTx), n = 3; post-KTx, n = 9; pre-liver transplant, n = 3; pemphigus, n = 5; total, n = 51] were compared. The statistical significance of the difference between the two groups was assessed using an unpaired t test. For ARTEMIS, black symbols indicate products that failed to meet the minimal infusible dose. White symbols are products that achieved the full target dose. Half white symbols are products that achieved a partial dose. (**F**) Correlation between T$_{reg}$ fold expansion during the 16 days of culture and day 16 darT$_{reg}$ product yield. Spearman correlation was used to assess the significance of the correlation. In (A), (B), (D), and (F), the shaded area is below the 100 × 10^6 minimal infusible dose, and the black dashed line indicates the target dose threshold of 300 × 10^6. For all panels, ARTEMIS participants are represented in orange and non-ARTEMIS data points are shown in blue. (A), (B), (D), and (F) include

all darT$_{reg}$ GMP products manufactured at the UCSF facility except four (three due to the use of cryopreserved instead of fresh PBMCs as starting materials and one due to outgrowth of contaminating CD4$^+$ T$_{conv}$ cells). Data for ARTEMIS include 10 data points from nine participants because manufacturing was attempted twice for participant 3. (C) and (E) included all available data. Data in (B) and (C) are presented as box- and-whisker plots showing all data points. The spread of the whiskers represents the range, the height of the box represents the inner quartile range, and the line in the box represents the median of the dataset. Data in (E) are presented as truncated violin plots showing all data points. The median is indicated by the thick white line, and the inner quartile range is indicated by the thin white lines.

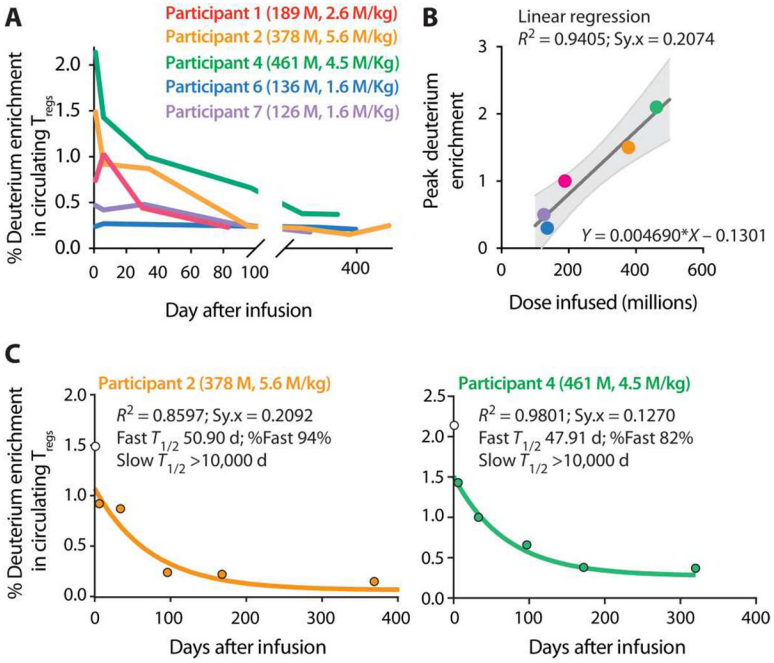

Fig. 3. Pharmacokinetics of T_{reg} products after infusion.

(**A**) Percent deuterium enrichment in the genomic DNA of peripheral blood T_{regs} collected after T_{reg} infusion. The numbers of infused darT_{regs} are indicated in the legend. (**B**) The relationship between the number of cells infused and the peak of deuterium enrichment was assessed using simple linear regression. (**C**) The biphasic exponential decay model was used to calculate the clearance rates ($T_{1/2}$) and relative size of the two distinct kinetic pools (fast and slow) of darT_{regs} for participants 2 and 4. Day 1 data points were excluded from curve fitting due to variable mixing and trafficking of the infused darT_{regs} at this early time point. All available data are included in (A) and (B). Decay curve analyses in (C) exclude day 1 data points (white circles) based on the reasoning that the infused T_{regs} may not have sufficient time to reach equilibrium.

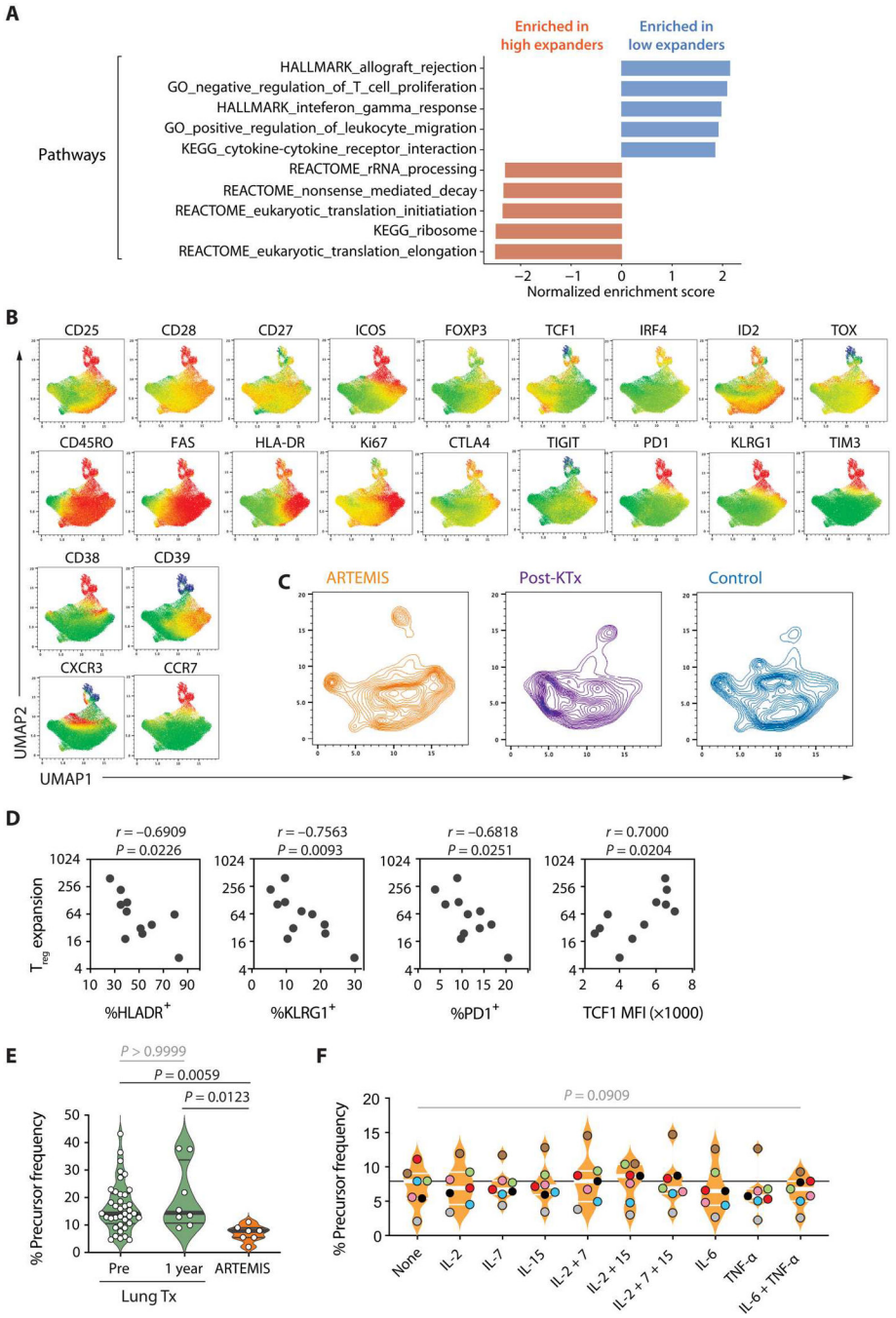

Fig. 4. Deep profiling of T_{regs} in ARTEMIS participants.

(**A**) Whole-genome transcriptomic analysis of ARTEMIS T_{regs} was performed using RNA-seq. Bars correspond to the top pathways over- and underrepresented in the list of genes differentially expressed between T_{regs} classified as low ($n = 5$) and high/mid expanders ($n = 3$) during T_{reg} manufacturing, as assessed by gene set enrichment analysis to have adjusted $P \leq 2.12 \times 10^{-5}$ with the highest (blue) or lowest (red) normalized enrichment score. (**B** and **C**) Spectral flow analysis of T_{regs} for ARTEMIS participants ($n = 8$), post-KTx individuals ($n = 7$), and nonimmunosuppressed control individuals (CTL; $n = 8$). (B) Data

from $CD4^+FOXP3^+HELIOS^+$ cells (n = 64,286 cells) from all individuals were pooled to create a UMAP to visualize marker expression as heatmaps. (C) Data from ARTEMIS (n = 23,704), post-KTx (n = 15,149), and CTL (n = 25,062) participants were separated and projected on the same UMAP in (B) for comparisons. (**D**) Spearman correlations of T_{reg}-expressed markers and $darT_{reg}$ expansion during manufacturing were analyzed. (**E**) Donor-reactive T_{reg} frequencies in ARTEMIS (n = 7) participants were compared with historical data obtained from lung transplant (Lung Tx) recipients before (n = 38) and 1 year after transplantation (n = 8). Kruskal-Wallis test with Dunn's multiple comparisons was used to determine the statistical significance of the differences. (**F**) The impact of added cytokines on ARTEMIS (n = 7) donor-reactive T_{reg} precursor frequencies was measured. Friedman's test with Dunn's multiple comparisons was used to determine the statistical significance of the differences. Two ARTEMIS samples were excluded from analysis in (E) and (F) due to low cell recovery from cryopreservation and insufficient events collected during flow cytometry data acquisition. Violin plots are used to summarize the data in (E) and (F). The center lines of the box-and-whisker plots are medians, the ranges of the whiskers are from the maximum to the minimum, and individual data points are shown, color-matched by donor, in (F). P values are listed above the graphs. Samples used are detailed in table S6. ARTEMIS participant 1 was excluded from the analyses due to the lack of banked samples, and participant 9 was excluded due to ineligibility for $darT_{reg}$ manufacturing.

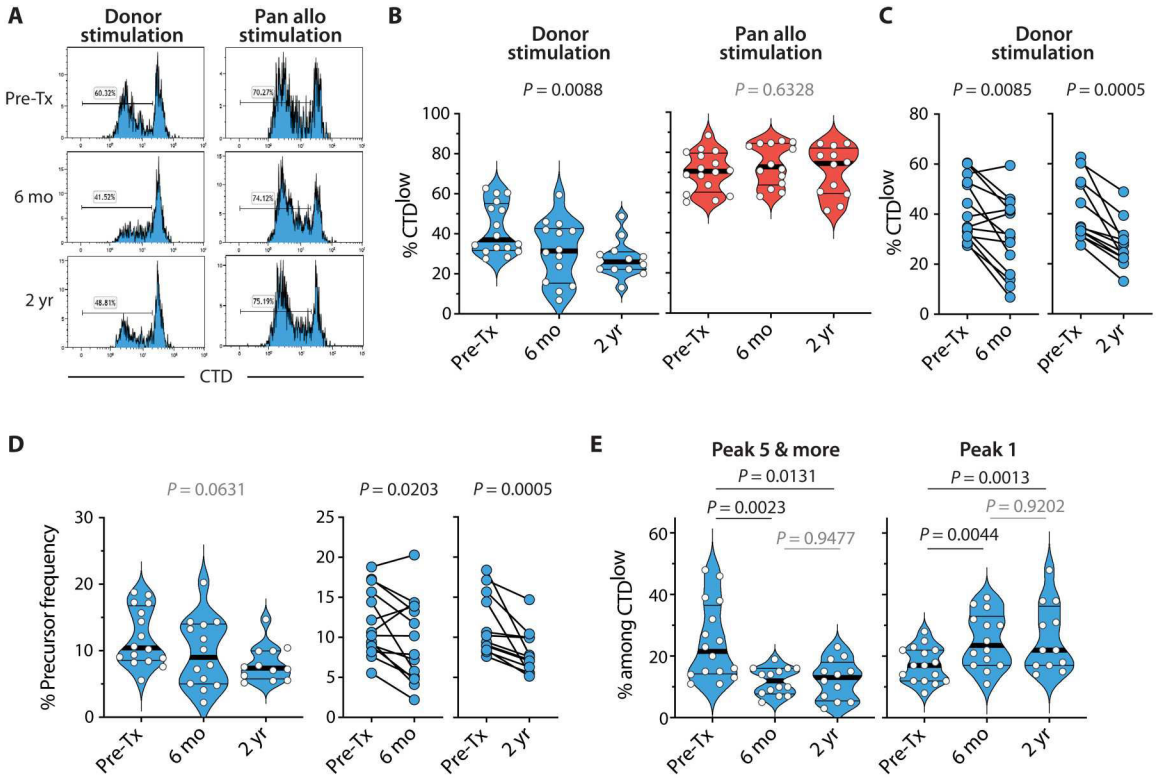

Fig. 5. T_{reg} donor reactivity before and after liver transplantation.

Liver transplant recipients' PBMCs banked from the AWISH study ($n = 16$) were labeled with CTD before stimulation with activated B cells from the liver donors or a panel of allogeneic (pan allo) B cells. CTD dilution was measured on day 4 of culture by flow cytometry. (**A**) Representative flow cytometric histograms of CTD dilution and analysis gates for CTD^{low} cells for $CD4^+$ $FOXP3^+HELIOS^+$ T_{regs}. (**B**) Violin plot summaries of percentages of T_{regs} in the CTD^{low} gate stimulated by donor or pan allo B cells. Individual data points are represented by white circles, the medians are shown as thick black lines, and the inner quartiles are marked with thin black lines. Mixed-effects analysis was used to assess the significance of the changes over time. (**C**) Paired analyses are shown comparing the percentages of CTD^{low} T_{regs} before transplant (Pre-Tx; $n = 16$) and at 6 months ($n = 14$) or 2 years ($n = 12$) after transplantation. Wilcoxon test was used to assess the statistical significance of the differences observed. (**D**) Frequencies of $darT_{reg}$ precursors were calculated and summarized. Mixed-effects analysis and Wilcoxon test were used to assess the statistical significance of the differences observed. (**E**) Summary of percentages of T_{regs} in the CTD^{low} gate that had five or more rounds of cell division (left) and those that had divided only once (right). Mixed-effects analysis and Tukey multiple comparison posttest were used to assess the significance of the changes over time. *P* values are listed above the graphs. In all panels, the 6-month time point excludes two samples and the 2-year time point excludes four other samples due to insufficient cells recovered after thawing. Details of samples used in the analyses can be found in data file S3.

Table 1.

Demographics for the participants who initiated immunosuppression withdrawal.

Donor	
Age (years)	33 (21–54) *
Male sex	5 (50%) †
White race	8 (80%) †
Hispanic ethnicity	2 (20%) †
Recipient	
Age at enrollment (years)	60 (24–70) *
Age at transplant (years)	55 (20–67) *
Time from transplant to screening biopsy (years)	3 (2–6) *
Male sex	6 (60%) †
White race	7 (70%) †
Hispanic ethnicity	2 (20%) †
Diagnosis	
Hepatitis C	4 participants
Cryptogenic/nonalcoholic steatohepatitis	3 participants
Metabolic/genetic	2 participants
Alcohol	1 participant
Time from transplant to screening biopsy (years)	3 (2–6) *
Alanine aminotransferase (U/liter)	18 (13–38) *
γ-Glutamyltransferase (U/liter)	16 (10–100) *
Alkaline phosphatase (U/liter)	74(61–87) *
Immunosuppression	
Tacrolimus monotherapy	6 participants
Tacrolimus + mycophenolate mofetil	3 participants
Cyclosporine + mycophenolate mofetil	1 participant
Donor-recipient HLA mismatches	
HLA class I (A, B, C; total of 6 alleles)	3 (0–5) *
HLA class II (DRB1 and DQB1; total of 4 alleles)	2 (1–3) *

* Median (minimum to maximum).

† Number of participants (% of all participants).