

BRIEF DEFINITIVE REPORT

First-in-human therapy with Treg produced from thymic tissue (thyTreg) in a heart transplant infant

Esther Bernaldo-de-Quirós¹, Manuela Camino², Marta Martínez-Bonet¹, Juan Miguel Gil-Jaurena³, Nuria Gil², Diana Hernández-Flórez¹, Maria Eugenia Fernández-Santos⁴, Laura Butragueño⁵, I. Esmé Dijke^{6,7,8}, Megan K. Levings^{7,9}, Lori J. West^{7,8,10}, Marjorie Pion¹, and Rafael Correa-Rocha^{1,7}

Due to their suppressive capacity, regulatory T cells (Tregs) have attracted growing interest as an adoptive cellular therapy for the prevention of allograft rejection, but limited Treg recovery and lower quality of adult-derived Tregs could represent an obstacle to success. To address this challenge, we developed a new approach that provides large quantities of Tregs with high purity and excellent features, sourced from thymic tissue routinely removed during pediatric cardiac surgeries (thyTregs). We report on a 2-year follow-up of the first patient treated worldwide with thyTregs, included in a phase I/II clinical trial evaluating the administration of autologous thyTreg in infants undergoing heart transplantation. In addition to observing no adverse effects that could be attributed to thyTreg administration, we report that the Treg frequency in the periphery was preserved during the 2-year follow-up period. These initial results are consistent with the trial objective, which is to confirm safety of the autologous thyTreg administration and its capacity to restore the Treg pool.

Introduction

Despite the benefits of allotransplantation, the recipient's immune response can endanger graft viability and the patient's life. A promising alternative to the current immunosuppressive regime that could increase the success of allotransplantation is adoptive cellular therapy with regulatory T cells (Tregs). Studies in animal models demonstrated that Treg therapy confers indefinite graft survival without needing pharmacological immunosuppression (Tsang et al., 2009). Moreover, preserved Treg values are associated with operational tolerance in pediatric transplantation (Ohe et al., 2012; Schulz-Juergensen et al., 2013). Thus, several Treg-based clinical trials employing autologous Tregs purified from peripheral blood in solid organ allotransplantation are complete or ongoing (Oberholtzer et al., 2021; Orozco et al., 2022; Romano et al., 2019). Potential challenges limiting the efficacy of this approach include the low number of Tregs that can be isolated from peripheral blood and the lower quality (in terms of purity, survival, or phenotype stability) of adult-derived cells related to their higher state of cellular differentiation and ex vivo expansion (Arroyo Hornero et al., 2017; Hoffmann et al., 2006, 2009; Miyara et al., 2009). In addition,

the current approach requires the extraction of high volumes of blood to obtain sufficient Tregs, clearly a major limitation for pediatric patients.

To overcome these challenges, we pursued the employment of an alternative Treg source: discarded pediatric thymuses routinely removed in pediatric cardiac surgeries to gain adequate exposure to the retrosternal operative field (Bernaldo-de-Quirós et al., 2022a; Dijke et al., 2016). In vitro studies showed that these Tregs derived from the thymus have a stable phenotype and potent suppressive function, providing advantageous features that increase their therapeutic potential (Bernaldo-de-Quirós et al., 2022a; Dijke et al., 2016; MacDonald et al., 2019). To operationalize this approach, we developed a novel "good manufacturing practices" (GMP) protocol to produce large numbers of high-quality Tregs purified from thymic tissue (thyTregs). Next, we sought to assess the safety and efficacy of the autologous thyTreg therapy by initiating a clinical trial in infants undergoing heart transplantation. This is the first trial worldwide to administer a Treg therapy to transplanted children and the first to employ thyTregs in humans.

¹Laboratory of Immune-Regulation, Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, Spain; ²Department of Pediatric Cardiology, Hospital Gregorio Marañón, Madrid, Spain; ³Department of Cardiac Pediatric Surgery, Hospital Gregorio Marañón, Madrid, Spain; ⁴Cell Production Unit, Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; ⁵Pediatric Intensive Care Unit, Hospital Gregorio Marañón, Madrid, Spain; ⁶Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada; ⁷Canadian Donation and Transplantation Research Program Investigator, Edmonton, Canada; ⁸Alberta Transplant Institute, Edmonton, Canada; ⁹Department of Surgery and School of Biomedical Engineering, University of British Columbia, BC Children's Hospital, Vancouver, Canada; ¹⁰Department of Pediatrics, University of Alberta/Stollery Children's Hospital, Edmonton, Canada.

Correspondence to R. Correa-Rocha: rafael.correa@iisgm.com.

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Here, we report the results of the first patient treated with thyTregs after completing the final 2-year follow-up, providing interim data on the feasibility and safety of this new therapy and obtaining preliminary results about the achievement of the clinical endpoints of the trial. The ultimate success of this approach could open a new frontier in the management of immune tolerance.

Results and discussion

The thymus is routinely excised during pediatric heart transplantation and other cardiac surgeries, removing the primary source of T cell replenishment (including Treg cells). In transplantation, thymectomy and the effects of pharmacologic immunosuppression on the Treg compartment combine to produce a progressive decrease in Treg proportions from the seventh month after transplant which may compromise the protective role Tregs play in preventing heart transplant rejection in children (Bernaldo-de-Quiros et al., 2021; López-Abente et al., 2021). Strategies addressed to replenish the Treg pool in these patients could reduce the risk of rejection by restoring Treg-mediated suppressive mechanisms.

Numerous clinical trials using adoptive Treg cell therapy to prevent solid organ transplant rejection in adults are underway (reviewed in Oberholtzer et al. [2021]; Romano et al. [2019]). The standard autologous Treg therapy strategy used in the context of solid organ transplantation consists of Treg purification from blood, expansion ex vivo to obtain an adequate cell number, and return to the patient (Sánchez-Fueyo et al., 2020; Sawitzki et al., 2020; Todo et al., 2016). The results emerging from these trials are promising, but a potential limitation is the low/short therapeutic effect of the Treg infusion (Orozco et al., 2022). This could be due to the features of the administered Tregs since most matured or senescent Tregs, such as those from adults or obtained after extended ex vivo expansion cycles, would have reduced survival and phenotypic stability and may even lose their suppressive phenotype and acquire a proinflammatory phenotype (Hoffmann et al., 2006, 2009, 2011).

More recent studies show that the naïve Tregs, which are more abundant in cord blood Treg or Treg derived from thymic tissue, have a more stable demethylated FOXP3 phenotype (Arroyo Hornero et al., 2017) and are less likely to acquire a proinflammatory phenotype (Canavan et al., 2015) compared with CD45RA^{neg} Tregs. Treg cells coming from pediatric individuals would exhibit a more enduring naïve phenotype, but no trials of Treg therapy to prevent rejection have been performed in children due to the low number of Tregs that can be obtained from their limited blood volume (Bernaldo-de-Quirós et al., 2022b).

Our innovative approach employing discarded thymic tissue as a Treg source in combination with an optimized GMP manufacturing protocol overcomes these limitations, allowing the production of massive quantities of stable therapeutic Tregs with a naïve-like phenotype and optimal features. With our protocol, a single thymus can yield 300–3,000 million therapeutic thyTregs. By employing only a short 7-day culture to preactivate the thyTreg before infusion, we preserve their

phenotype, suppressive capacity, and stability, as described in this article. This enables Treg therapies to be used in the pediatric transplant context for the first time.

Phenotypic and functional properties of administered thyTregs

The first patient treated (thy1-01) was a 7-mo-old female (see patient description in Materials and methods section), whose excised thymic tissue weighed 8.9 g, yielding 7.6×10^9 thymocytes (854×10^6 thymocytes/g). Positive selection of CD25⁺ thymocytes, activation, and culture of resulting thyTregs was performed at the Cell Production Unit of Gregorio Marañón Health Research Institute (IISGM). We finally obtained 315×10^6 thyTreg cells for thy1-01, exceeding the dose required for the patient (Table 1). The viability of administered cells was 96.6% and the purity of thyTreg cells was 89.9% CD25⁺FOXP3⁺ cells (gating strategy is shown in Fig. S1).

In addition to FOXP3 expression, the Treg phenotype of thyTreg was further confirmed by comparing the expression of phenotypic markers associated with function and homing with peripheral-blood Tregs from the same individual. Infused thyTregs had high expression of cytotoxic T-lymphocyte-associated protein (CTLA-4), inducible T cell co-stimulator (ICOS), HELIOS, and CD27 (Fig. 1A). Furthermore, thyTregs expressed high levels of CCR4 and CD62L, indicating their putative ability to migrate to organs with large epithelial surfaces and to lymph nodes, respectively (Lamarche and Levings, 2018; Sather et al., 2007) (Fig. 1B). These results confirm that administered thyTregs have phenotypic features at least comparable with the Tregs that are present in the peripheral blood of the treated patients, and thus have the ability to exert the regulatory role of the endogenous Tregs.

Functional properties of the infused thyTregs were confirmed by (i) the release of high quantities of the suppressive cytokine IL-10 but negligible or undetectable values of IFN- γ , IL-4, or IL-17A (Fig. 1C) and (ii) a marked inhibition of the proliferation of CD4⁺ T and CD8⁺ T cells (Fig. 1, D and E), confirming their high suppressive capacity. Increased frequencies of effector CD8⁺ T cells have been related to a higher risk of rejection during the first year after transplant (Bernaldo-de-Quiros et al., 2021; Jacquemont et al., 2020), and the capacity of thyTreg to suppress the proliferation of CD8⁺ T cells could contribute to rejection prevention in treated patients.

The therapeutic use of adult peripheral Tregs, which can include induced and natural highly differentiated Tregs, may be limited by their phenotype instability of the cells, notably in a proinflammatory environment. These Tregs can lose their FOXP3 expression and suppressive phenotype and may switch to an inflammatory phenotype (Koenen et al., 2008; McClymont et al., 2011). We confirmed the stability of FOXP3 expression in the infused thyTreg cell product by analyzing the methylation profile of the Treg-specific demethylated region (TSDR) (FOXP3-ADS783) (Fig. S2A). An intermediate level of TSDR demethylation in thyTregs was observed in this female patient due to the methylation-mediated inactivation of one X-chromosome in females. The differential methylation pattern between thyTreg and conventional thymocytes (thyTconv) was observed not only

Table 1. Clinical characteristics of the pediatric heart transplant patients treated with thyTreg cells (thy1-01) or included in the control (non-treated) group

ID	Sex	Diagnosis	Age at Tx	Weight at Tx	Type of Tx	Age at thymectomy	thyTreg dose	thyTreg infusion
thy1-01	F	DCM	7 mo	6.1 kg	ABO incompatible	7 mo	122×10^6 ($20 \times 10^6/\text{kg}$)	Day +9 after Tx
Ctrl-1	M	CHD	14 mo	8.0 kg	ABO compatible	7 mo	-	-
Ctrl-2	M	CHD	2 mo	4.0 kg	ABO compatible	5 days	-	-
Ctrl-3	F	CHD	5 mo	5.0 kg	ABO incompatible	4 days	-	-
Ctrl-4	M	DCM	4 mo	6.5 kg	ABO compatible	4 mo	-	-

F, female; M, male; DCM, dilated cardiomyopathy; CHD, congenital heart disease; Tx, transplant.

in the *FOXP3* gene but in three other genes, including *CTLA-4*, *IKZF2*, and *ILR2A* (Fig. S2 A).

In addition, we exposed the patient's thyTregs to a cocktail of cytokines polarizing to Th1 (IL-2 and IL-12) or Th17 (IL-2, IL-1 β , IL-6, IL-23, and TNF- α) for 3–4 days and observed that thyTreg cells maintained their *FOXP3* expression intact (Fig. 1 F and Fig. S2). In addition, thyTregs produced negligible or undetectable quantities of IFN- γ and IL-17A compared with peripheral blood leukocytes cultured in the same polarizing conditions (Fig. S2, C and D), and the suppressive capacity (inhibition of CD4 $^+$ T and CD8 $^+$ T proliferation) of the thyTregs was also preserved in these proinflammatory conditions (Fig. S2, E and F).

ThyTreg administration, safety, and immunological and clinical follow-up

In addition to the first patient receiving the thyTreg therapy (thy1-01), four patients undergoing a heart transplant with the same immunosuppressive regimen but not receiving thyTreg therapy were also enrolled as a control cohort. Controls and the treated patient were clinically and immunologically followed up periodically for 2 years after transplant. The clinical trial design and a description of the immunosuppressive regime are included in the Materials and methods and in Fig. 2.

Patient thy1-01 was randomized and assigned to receive a dose of 20×10^6 cells per kg at day +9. No complications attributed to thyTreg administration were observed after thyTreg infusion and during the 2-year follow-up, suggesting that autologous thyTregs may be safe and well tolerated. Adverse events (AEs) in the treated patient were those commonly related to characteristics of the heart transplantation post-operative period and concomitant medications. Concretely, the AEs that required hospital admission according to the center's protocol were (i) swelling in sternotomy; (ii) gastroenteritis; (iii) weight loss and food rejection; (iv) otitis media and COVID. At the discretion of the clinicians responsible for patient follow-up, these events were not related to the therapy, fell within the usual events in these patients, and were resolved without complications. Since performing endomyocardial biopsies in such young children (<2 year) is contraindicated due to the risk of organ perforation, we have no histological data in these patients as an indicator parameter of rejection. Graft rejection episodes may be suspected by the appearance of clinical symptoms (fever, irritability, refusal of food, etc.), echocardiography alterations (myocardial thickening or hypertrophy,

atrioventricular valvular insufficiency, pericardial effusion, and diminished contractility), and/or heart rhythm alterations (flutter or ventricular tachycardia), which should improve after increasing immunosuppression. In patient thy1-01, all the mentioned parameters were normal and none of the clinical signs of suspected rejection appeared in this infant. It should be noted that none of the patients (treated or controls) received induction therapy and that the prednisolone dose was progressively reduced until it was completely withdrawn after the 10th–11th mo after transplant. Therefore, these preliminary results suggest that the combination of the thyTreg therapy with a reduction in the dose of immunosuppressants could be an approach to reduce the side effects of these drugs without increasing the risk of rejection.

The primary endpoint of the trial is the repopulation of Treg cells in the patient, determined as the increase in Treg values in peripheral blood relative to pretransplant values or in comparison with a control cohort of non-treated patients (time frame: 24 mo). Transplanted patients in the control cohort (who did not receive thyTreg therapy) experienced a transient increase in the peripheral Treg frequency in the first weeks after transplant, which could be related to a response from the Treg to the inflammation associated with the transplant procedure. These control infants experienced a progressive decrease in the circulating Treg frequency 9 mo after transplant (Fig. 3 A), reaching levels 40% lower than pretransplant values at the end of the follow-up period, which agrees with a previous study (Bernaldo-de-Quiros et al., 2021). However, patient thy1-01 experienced an increase in Treg frequency after thyTreg infusion and maintained Treg values higher than pretransplant levels throughout the 2-year follow-up period. This contrasts with some trials administering Treg in transplanted adults, where increased circulating Tregs were noticeable from infusion but persisted for only 1 mo (Sánchez-Fueyo et al., 2020).

In addition to the percentage change in Treg frequency after transplant (Fig. 3 A), we also report values of the Treg frequency in total CD4 $^+$ T cells (Fig. S3 A). The reference value of Treg frequency in total CD4 $^+$ T cells in healthy infants younger than 1 year old is 8% (tolerance interval: 4–16%) (Schatorjé et al., 2012). Values of Treg percentage would be within the reference interval in both treated and control patients (Fig. S3 A), except that control patients seem to reach values <8% from 9 mo after transplant.

We also measured absolute Treg counts (cells per μl of blood), observing a higher increase in the absolute counts of peripheral

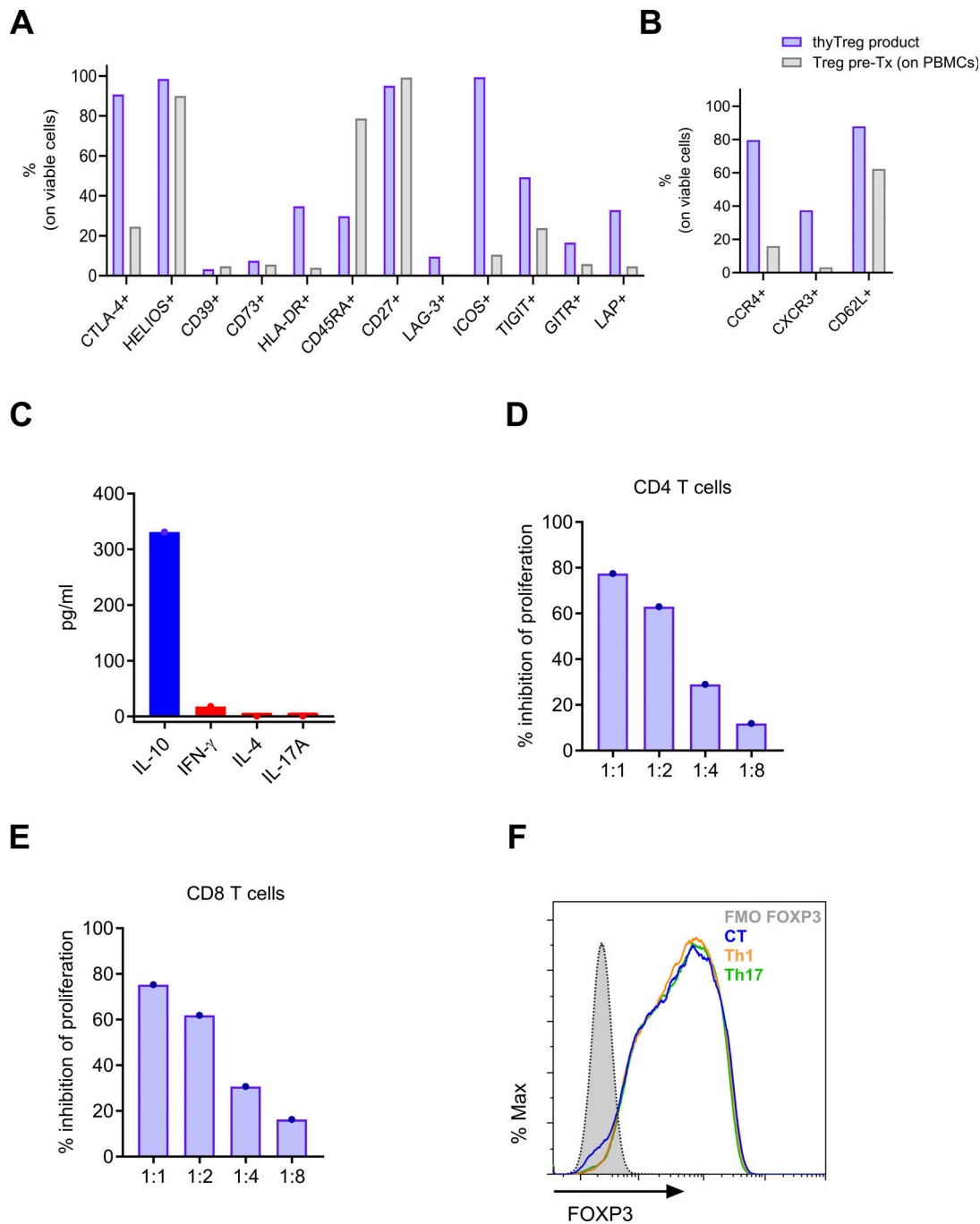


Figure 1. Characteristics of the infused thyTreg product. (A and B) Frequency of phenotypic/functionality (A) and homing (B) markers within viable cells of the thyTreg product infused to patient thy1-01 (purple) in comparison with the expression in peripheral Tregs analyzed on PBMCs (gray) from a blood sample collected immediately pretransplant (pre-Tx) from the same patient. **(C)** Quantification of cytokine release capacity of thyTreg in culture supernatant of infused thyTreg product. Anti-inflammatory cytokine IL-10 in blue; proinflammatory cytokines in red. **(D–F)** Suppressive capacity of infused thyTreg product, defined as % inhibition of CD4⁺ T cell (D) and CD8⁺ T cell (E) proliferation at the indicated ratios. Stability of FOXP3 expression in the thyTreg product under control (CT, blue) or Th1 (orange) and Th17 (green) proinflammatory conditions (F).

Treg in patient thy1-01 after thyTreg infusion compared with controls (Fig. 3 B). The Treg counts become comparable with controls from 9 mo after transplant to the end of follow-up despite total CD4⁺ T cells in this patient (Fig. 3 C) continuing to drop below control values. The immunosuppression and potentially also the thymectomy inevitably lead to a reduction in the CD4⁺ T counts that also affects Treg counts (Bernaldo-de-Quiros

et al., 2021). However, the thyTreg therapy may partially compensate for this effect and preserve the Treg proportion, which has been proved to be relevant for the risk of acute rejection and for maintaining an appropriate immune homeostasis.

We analyzed the evolution of the total Treg pool in the periphery by measuring the expression of markers associated with Treg function and homing (Fig. S3, B–D). Comparing pre- and

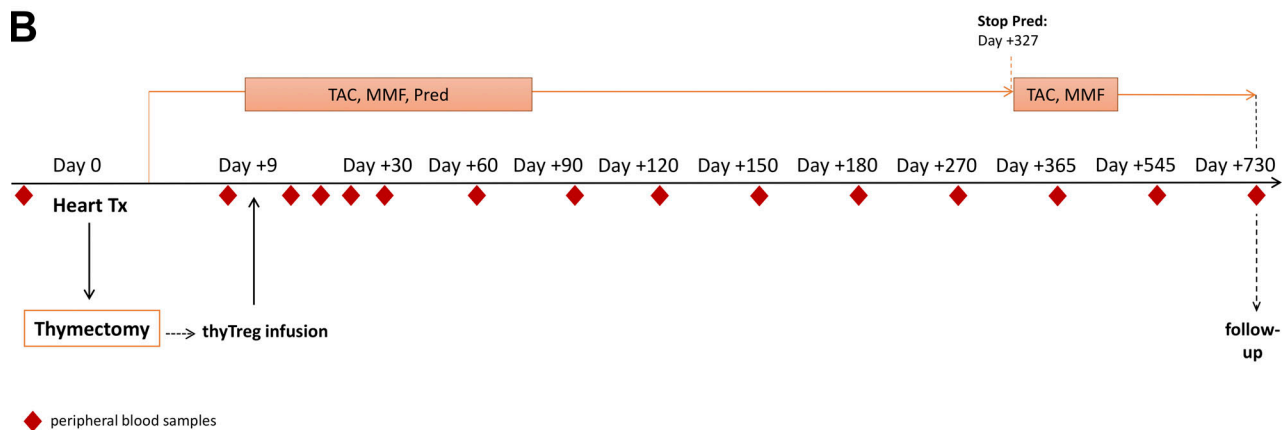
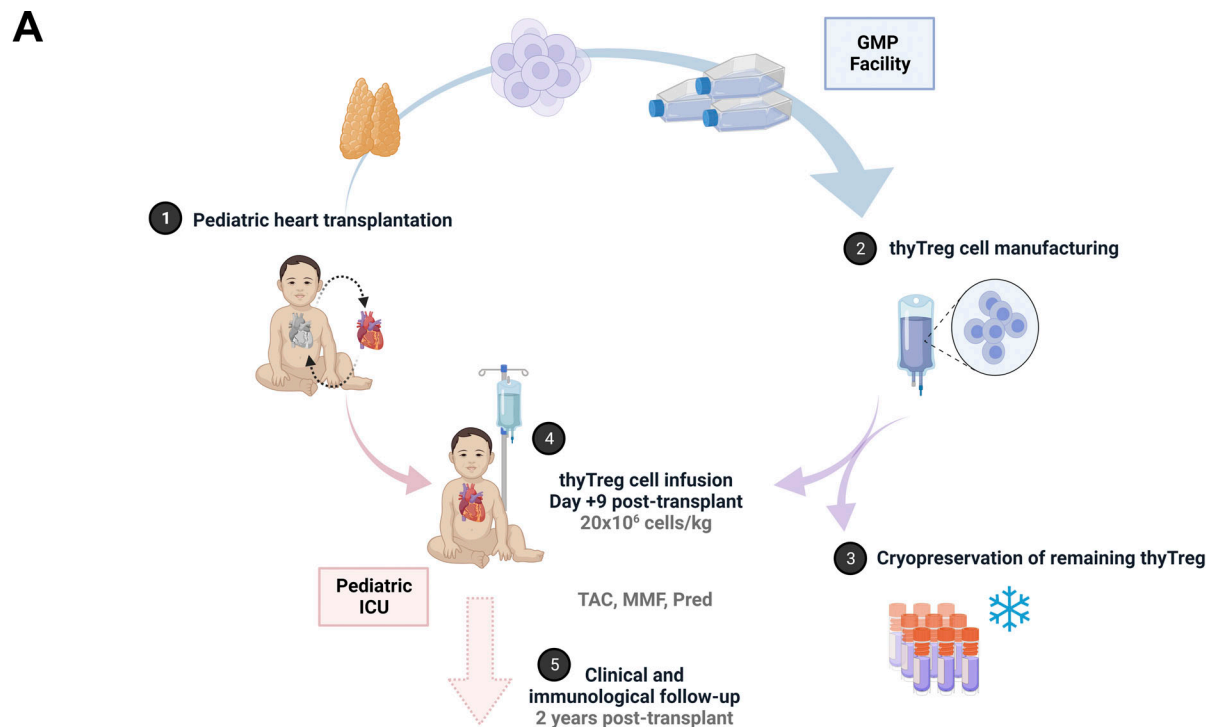


Figure 2. **Schematic representation of the clinical trial (NCT04924491).** (A and B) Graphic summary of the thyTreg infusion performed in patient thy1-01 (A) and peripheral blood sampling chronogram employed for both treated patient and control cohort (B).

post-infusion samples, we observed an increase in the proportion of FOXP3⁺ and HELIOS⁺ Tregs in periphery in the first month after infusion, but the expression of most of the markers in the Treg pool was not significantly modified by the infusion of thyTregs. We observed an increase in the proportion of HLA-DR⁺ Tregs in the first year after infusion, which could reflect an activation of the Treg pool. However, we also observed an increase in the proportion of ICOS⁺, TIGIT⁺, and GITR⁺ Tregs in the last year of follow-up. Higher expression of ICOS has been associated with increased Treg generation, proliferation, and survival abilities and higher suppressive capacities of Tregs, and is also associated with an increased expression of TIGIT

and GITR (Li and Xiong, 2020). Therefore, in addition to a preserved Treg frequency, the expression of markers related to Treg function is also preserved or even increased in the treated patient. Other mechanisms that could also contribute to the preservation of Treg frequency in the treated patient are the absence of the thymus in heart transplant patients, which could create “space” for the injected thyTregs to expand, or that the injected thyTregs could promote differentiation or recruitment of more endogenous Tregs by the mechanism described as infectious tolerance (Sullivan et al., 2020).

It is important to consider that the results shown in this first-in-human report are from a single patient and as such are

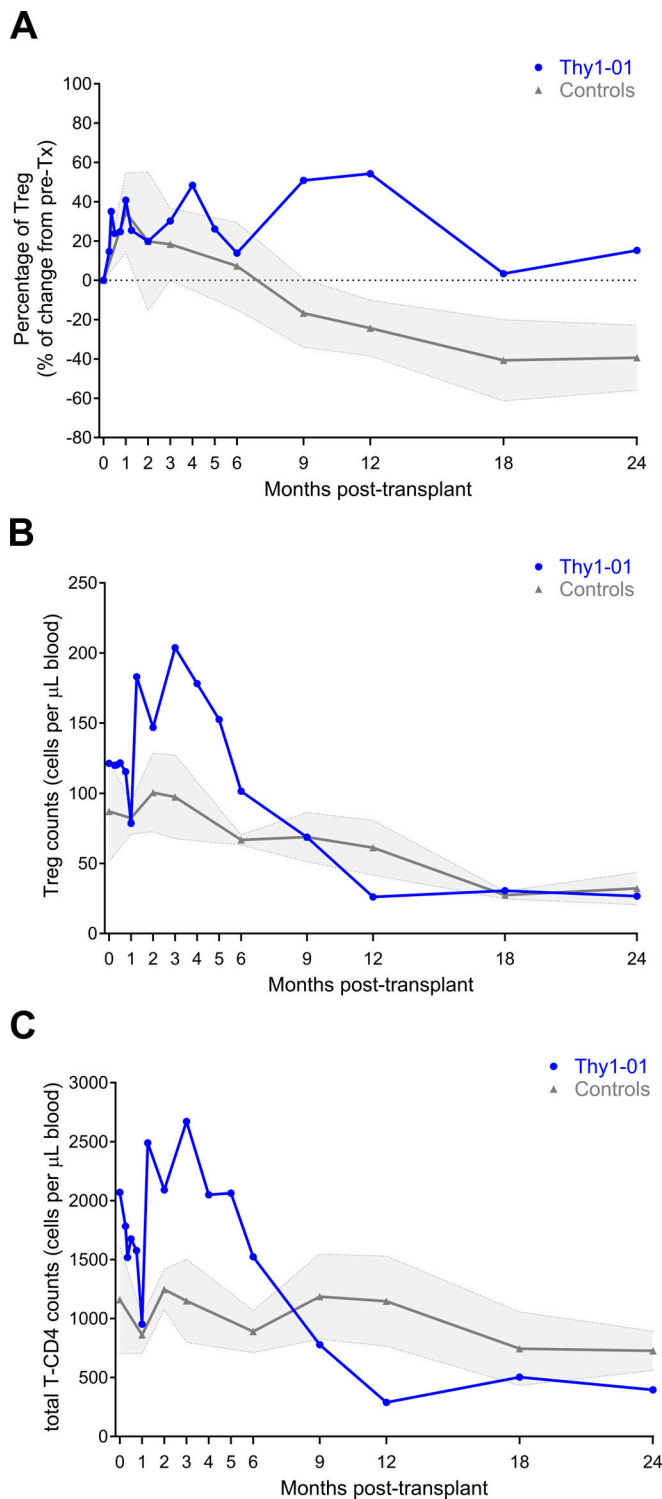


Figure 3. Peripheral Treg values in thyTreg-treated patients in comparison with age-matched control cohort. (A) Percentage of change from the pretransplant (pre-Tx) sample (0) in Treg frequency within CD4⁺ T cells of the patient treated and controls. The dotted line represents the 0% change from pretransplant values. **(B and C)** Treg (B) and total CD4⁺ T (C) absolute counts (cells per μL of blood) in thy1-01 and control cohort. Mean \pm 95% confidence interval in the control cohort ($n = 4$) is represented as a solid gray line. Whole peripheral blood samples from patient thy1-01 (blue) and controls (gray) at all time points were considered.

insufficient to draw definitive conclusions. Moreover, since thy1-01 is a pediatric patient recruited at 7 mo old, the amount of blood that can be safely collected in clinical follow-up is very limited. As a result, additional data such as the TCR repertoire of the Treg population, which would be useful to determine the dynamics of the Treg peripheral population after therapy, are not available. It will be necessary to complete the trial in more patients to confirm the efficacy of the therapy to reverse the drop in Treg, which could reduce the risk of graft rejection, but these initial results provide the first evidence for the feasibility and safety of this novel therapy.

This first-in-human employment of thyTreg paves the way for a novel generation of Treg therapies that could establish a new paradigm in preventing allograft rejection in heart transplant infants but also as a treatment for other immune-related disorders. Umbilical cord blood-derived Tregs have been employed as a partial match allogeneic therapy in adults to prevent graft-versus-host disease (Brunstein et al., 2016) with success, potentially attributable to a less differentiated or naïve phenotype conferring hypoimmunogenic features. Considering the potential for multiple therapeutic doses to be obtained from a single thymus (even without employing extensive expansion) and the potential hypoimmunogenic features related to the naïve-like phenotype of these thyTregs, this cell therapy could open the possibility of using off-the-shelf thyTregs to prevent rejection or to treat other inflammatory disorders or autoimmune diseases in both children and adults.

Materials and methods

Design and clinical endpoints

The patient described has been included in an exploratory and prospective phase I/II clinical trial to evaluate the safety and efficacy of transfusing autologous thyTregs to prevent rejection in children undergoing heart transplantation (EudraCT: 2018-003574-28; NCT04924491). The patient was treated at Hospital General Universitario Gregorio Marañón in Madrid (HGUGM), and together with the infants included in the control cohort, was transplanted and clinically followed at the Pediatric Intensive Care Unit (ICU) and Department of Pediatric Cardiology of HGUGM. The protocol was reviewed and authorized by local and federal authorities following current regulations and received the approval of the ethical committee of the HGUGM and the Spanish Agency for Medicines and Health Products (AEMPS) (detailed trial description and protocol are available at <https://clinicaltrials.gov/ct2/show/NCT04924491>).

The primary endpoint is to achieve a repopulation of Treg cells in the patient, determined as the increase of Treg values in peripheral blood with respect to pretransplant values or in comparison with a control cohort of non-treated patients, which are described to progressively decrease in heart transplant children (Bernaldo-de-Quiros et al., 2021).

In addition to safety (rate of toxicities), another surrogate endpoint is the incidence of suspected rejection episodes in the 2 years after transplant (the period with a high incidence of acute rejection).

Patient description

Here, we report results in the first case of a patient treated with thyTregs, who has completed the trial with a follow-up 2 years after transplant and after receiving the single thyTreg administration (Fig. 2). The first patient (thy1-01) receiving the thyTreg therapy was a female with dilated cardiomyopathy with a left bundle branch block. She was transplanted at 7 mo of age and weighed 6.10 kg, receiving an ABO-incompatible heart (donor: B; recipient: O) (West et al., 2001) with a donor:recipient weight ratio of 0.8:1. The parents of thy1-01 agreed to their child's participation in the study by signing the informed consent form.

The control group included four heart transplant recipients <2 years (mean age = 6.25 mo at transplant) (Table 1). All patients were thymectomized (estimated >90% thymic tissue resection) before transplantation.

None of the patients (controls and treated) received induction therapy. All patients received the same immunosuppressive regimen with tacrolimus (TAC), mycophenolate mofetil (MMF), and methylprednisolone (Pred). Pred was administered intraoperatively with two doses of 10 mg/kg, 1 mg/kg every 6 h on day 0, then 1 mg/kg every 12 h for the first month, and subsequently 1 mg/kg every 24 h with progressive reduction. Pred dose was reduced progressively until withdrawal within the first year after transplantation. Patient thy1-01 stopped the administration of Pred at day +327. MMF was administered at 600 mg/m², a pretransplant dose and continued after transplant every 12 h indefinitely. TAC was initially administered from day +3 after transplant at 0.1 mg/kg/day and target levels of 10 ng/ml in plasma from day +7 after transplant. Controls and the treated patient were clinically and immunologically followed up periodically for 2 yr after transplant (Fig. 2).

In addition to the first patient receiving the thyTreg therapy (thy1-01) reported here, five additional infants of a similar age and undergoing a heart transplant have been also included in the active arm of the study receiving the thyTreg infusion, but they have not still completed the follow-up.

ThyTreg therapy manufacturing and administration

The treated patient (thy1-01) received the same immunosuppressive regimen as the control group, and concomitantly, the infusion of autologous thyTreg purified from her thymus. Thymic tissue collected during the transplant procedure was processed in the GMP Cell Production Unit of IISGM, accredited by the AEMPS, to obtain the therapeutic dose. Thymic tissue was dissected mechanically to obtain a suspension of thymocytes and thyTreg cells were purified by immunomagnetic positive selection of CD25⁺ cells using the CliniMACS Plus (Miltenyi Biotec). ThyTreg cells were cultured in GMP conditions with 600 U/ml of GMP IL-2 and T-cell TransAct (both from Miltenyi Biotec). These 7 ± 2 days of culture have the goal of maintaining the cells viable while waiting for the patient to stabilize after the heart transplantation. Moreover, this short culture increases the purity of the cells, allows us to preactivate the cells before infusion, and to perform all the quality and safety controls in the therapeutic product prior to administration of the cells. When the patient thy1-01 was considered stabilized in the Pediatric

Intensive Care Unit (+9 days after transplant), the therapeutic product's quality release criteria and safety controls were performed. 122 × 10⁶ thyTreg cells (20 × 10⁶ cells per kg dose assigned) were diluted in lactated Ringer's solution (Braun) with 2% human albumin (Octalbin 200 mg/ml; Octapharma), dispensed in a transfer bag, and administered intravenously to the patient.

Phenotypic and functional analysis of infused thyTreg

The viability and phenotype of the thyTreg-infused product were evaluated by flow cytometry with a MACSQuant16 cytometer (Miltenyi Biotec), and data were analyzed using Kaluza software (Beckman Coulter). The gating strategy is depicted in Fig. S1. The infused product had a proportion of viable cells of 96.60% and a proportion of CD25⁺FOXP3⁺ cells (purity) of 89.90%. In vitro suppression assay was performed by coculturing Cell Trace Violet-labeled peripheral blood mononuclear cells (PBMC) obtained from buffy coats of healthy donors (provided by Madrid Transfusion Center) and thyTreg at different ratios in the presence of anti-CD3/anti-CD28 coated beads (Dynabeads; Gibco) in X-VIVO 15 media (Lonza) supplemented with 5% serum human AB (Sigma-Aldrich) and 600 U/ml of IL-2 (ImmunoTools). After 3 days, cells were analyzed by flow cytometry and the percentage suppression of proliferation was calculated according to the "Division index method" (McMurphy and Levings, 2012) within CD4⁺ and CD8⁺ T cells.

To evaluate thyTreg stability under proinflammatory conditions, thyTreg (and PBMC in parallel) were cultured and restimulated with TransAct alone or together with the following cytokines (ImmunoTools): IL-2 and IL-12 (polarizing condition to Th1); and IL-2, IL-1β, IL-6, IL-23, and TNF-α (polarizing condition to Th17). On day 3, cell culture supernatants were collected and the phenotype and function of thyTreg were analyzed. Quantification of secreted cytokines in cell supernatants was performed using ELLA Protein-Simple (Bio-techne) immunoassay technology. DNA methylation profile was analyzed by targeted Next-Gen bisulfite sequencing (NGS070V3 assay) performed by EpigenDx Inc.

Immunological evaluation of Treg values in peripheral blood

To evaluate the effect of thyTreg infusion in the peripheral Treg compartment, Treg frequency in blood was compared to pretransplant values and a control cohort of age-matched heart transplant recipients receiving the same immunosuppressive regimen but without thyTreg therapy. 100 μl of whole peripheral blood was periodically collected (Fig. 2 B) and stained with T cell surface markers (markers and gating strategy described in Bernaldo-de-Quirós et al., 2021 and Fig. S1). After surface staining, red blood cells were lysed using RBC Lysis/Fixation Solution (BioLegend). Samples were evaluated by flow cytometry with a MACSQuant16 cytometer and data were analyzed using Kaluza software.

Online supplemental material

We are including three supplemental figures that support and extend the findings and figures contained in the manuscript. Fig. S1 shows the flow cytometry gating strategy of thyTreg cells. Fig.

S2 shows additional characterization and stability of the infused thyTreg product. Lastly, Fig. S3 shows Treg frequencies and Treg pool characterization before and after infusion.

Data availability

The data underlying Table 1 and Figs. 1, 2, 3, S1, S2, and S3 are available in the published article and its online supplemental material. Raw data of this manuscript are not publicly available in any repository to preserve individuals' privacy under the European General Data Regulation. However, the data are available from the corresponding author upon reasonable request.

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procedures, clinical monitoring, and collecting patient samples. E. Bernaldo-de-Quirós and M. Martínez-Bonet have carried out the sample processing, immune analysis, and validation of the results. E. Bernaldo-de-Quirós, M. Martínez-Bonet, and R. Correa-Rocha have performed the data analysis and the interpretation of the results. E. Bernaldo-de-Quirós, M. Martínez-Bonet, M. Pion, I.E. Dijke, M.K. Levings, L.J. West, and R. Correa-Rocha have participated in the result discussion and writing of the manuscript. R. Correa-Rocha is responsible for the overall study, including research design, data analysis, and writing of the paper. R. Correa-Rocha is the principal investigator and promoter of the clinical trial.

Disclosures: E. Bernaldo-de-Quirós reported a patent to WO2019/166658A1 pending. M. Pion reported a patent to WO2019/166658A1 issued. R. Correa-Rocha reported personal fees from THYTECH outside the submitted work; in addition, R. Correa-Rocha had a patent to WO2019/166658A1 pending. No other disclosures were reported.

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Supplemental material

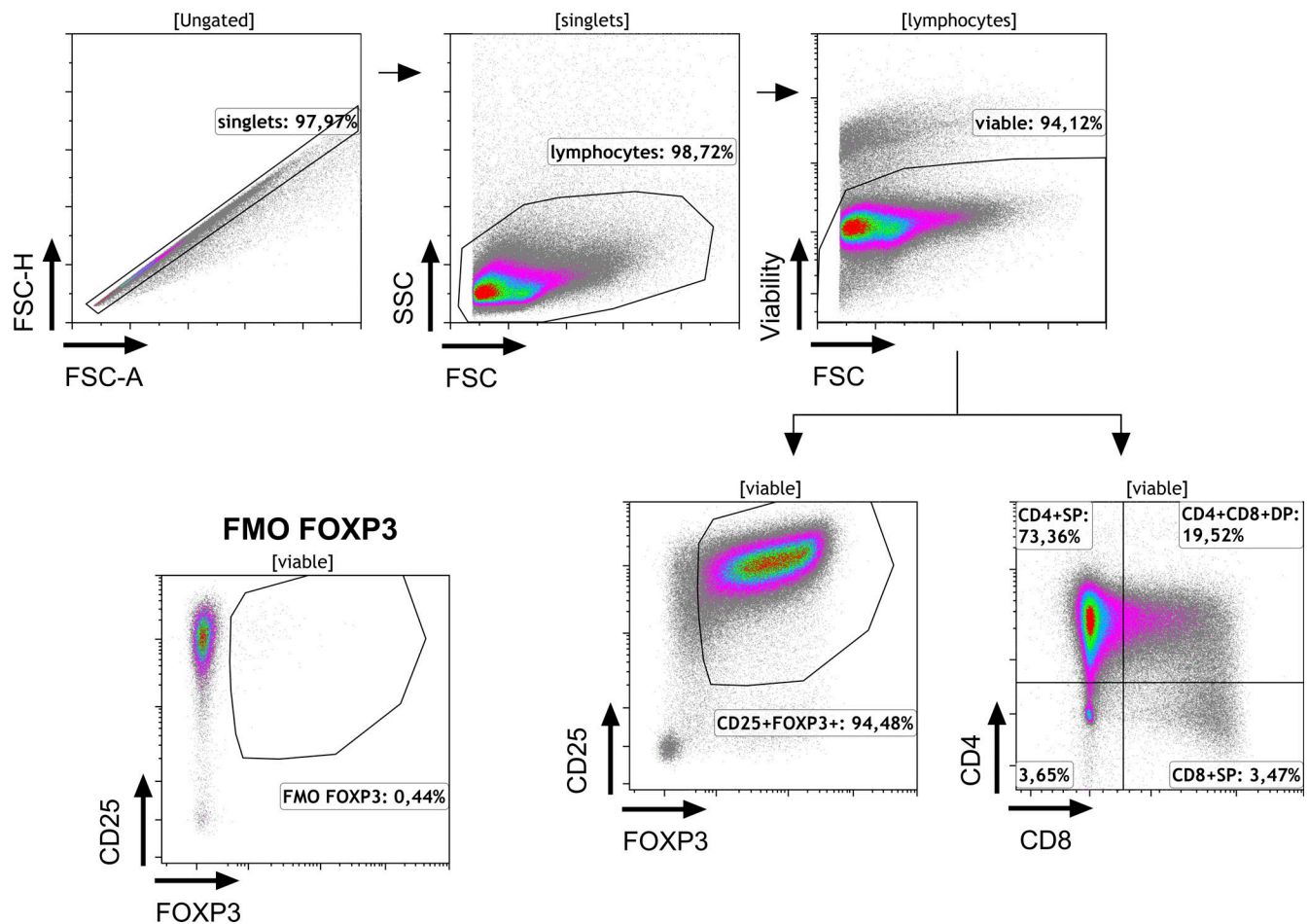


Figure S1. **Gating strategy of thyTreg cells.** Flow cytometry gating strategy and representative dot plots for immune cell populations and subpopulations. After gating on singlets, lymphocytes (thyTreg cells) were identified by size (forward scatter [FSC]) and complexity (side scatter [SSC]). Within this population, viable thyTreg were identified. Within viable thyTreg cells, we determined the purity in terms of CD25 and FOXP3 expression (CD25⁺FOXP3⁺). Fluorescence minus one (FMO) of FOXP3 is shown to determine background signal.

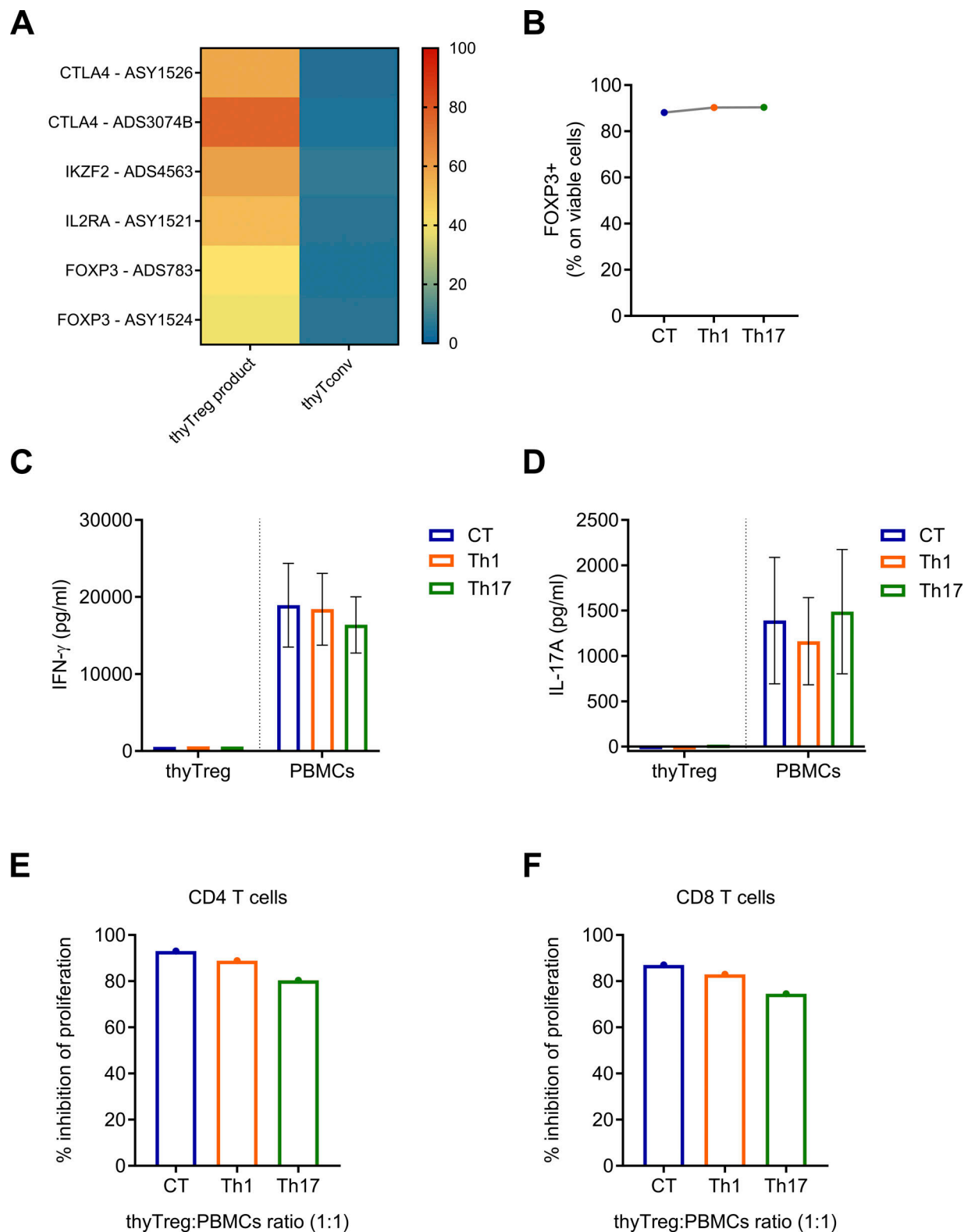


Figure S2. **Additional characterization and phenotypic and functional stability of the infused thyTreg product.** (A) Demethylation level of six genome regions located in four genes (calculated as the mean of demethylation of the CpGs contained in the region) within thyTreg cell product and thyTconv of patient thy1-01 (female). FOXP3-ADS783 corresponds to the TSDR region of FOXP3. An aliquot of the thyTreg cell product (thy1-01) was restimulated under control (CT, blue) or under Th1 (orange) and Th17 (green) proinflammatory conditions and evaluated after 3 days of culture. (B) The frequency of FOXP3 in viable cells is representative of the phenotypic stability. (C and D) Summary of quantitation of secreted IFN- γ (C) and IL-17A (D) by thyTreg or restimulated PBMC ($n = 3$) under proinflammatory conditions. (E and F) Summary of the suppressive capacity as inhibition of CD4⁺ (E) and CD8⁺ (F) T cell proliferation of thyTreg at ratio 1:1 in control or Th1 and Th17 switching conditions.

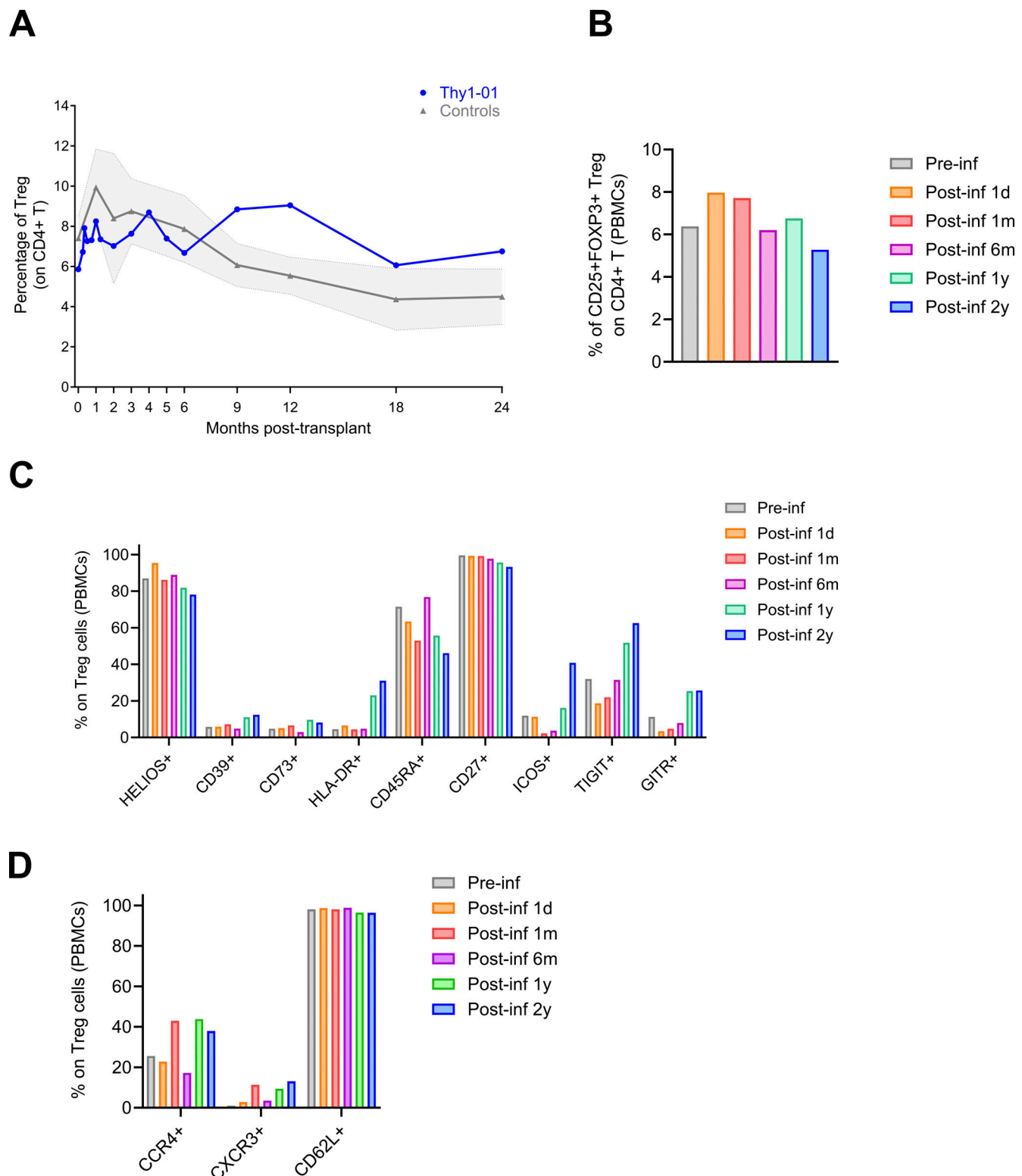


Figure S3. **Treg frequencies and characterization of the Treg pool before and after infusion.** (A) Treg frequencies within CD4⁺ T cells of the patient treated (thy1-01) and controls. Mean \pm 95% confidence interval in the control cohort ($n = 4$) is represented as a solid gray line. Whole peripheral blood samples from patient thy1-01 (blue) and controls (gray) at all time points were considered. Peripheral blood Treg cell compartment was analyzed in patient thy1-01 by flow cytometry at preinfusion (Pre-inf; 8 days after transplant), 1 day after infusion (Post-inf 1 day), 1 mo, 6 mo, 1 year, and 2 years after infusion in patient thy1-01. (B) Frequency of CD25⁺FOXP3⁺ Treg on CD4⁺ T cells analyzed on PBMCs from blood samples. (C and D) Frequency of phenotypic/functionality (C) and homing (D) markers within viable CD25⁺FOXP3⁺ Treg cells analyzed on PBMCs from blood samples at those time points during the follow-up.