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The Presence of a Marked Imbalance Between Regulatory T Cells and Effector T Cells Reveals That Tolerance Mechanisms Could Be Compromised in Heart Transplant Children

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Background. Regulatory T cells (Treg) are crucial for the induction and maintenance of graft tolerance. In pediatric heart transplant procedures, the thymus is routinely excised, removing the primary source of T-cell replenishment. Consequently, thymectomy joined to the effects of immunosuppression on the T-cell compartment may have a detrimental impact on Treg values, compromising the intrinsic tolerance mechanisms and the protective role of Treg preventing graft rejection in heart transplant children. **Methods.** A prospective study including 7 heart transplant children was performed, and immune cell populations were evaluated periodically in fresh peripheral blood at different time points before and up to 3 y posttransplant. **Results.** Treg counts decreased significantly from the seventh-month posttransplant. Furthermore, there was a significant increase in effector memory and terminally differentiated effector memory T cells coinciding with the fall of Treg counts. The Treg/Teffector ratio, a valuable marker of the tolerance/rejection balance, reached values around 90% lower than pretransplant values. Additionally, a negative correlation between Treg count and T effector frequency was observed. Particularly, when Treg count decreases below 50 or 75 cells/µL in the patients, the increase in the frequency of T effector CD4+ and CD8+, respectively, experiences a tipping point, and the proportion of T-effector cells increases dramatically. **Conclusions.** These results reveal that interventions employed in pediatric heart transplantation (immunosuppression and thymectomy) could induce, as an inevitable consequence, a dysregulation in the immunologic status characterized by a marked imbalance between Treg and T effector, which could jeopardize the preservation of tolerance during the period with the higher incidence of acute rejection.

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INTRODUCTION

Heart transplantation (HTx) has become the most effective treatment to correct end-stage heart failure due to congenital heart disease (CHD) and cardiomyopathy in children.

Regarding the last update of the *International Society for Heart and Lung Transplantation Registry*, there are now around 120 centers performing approximately 700 pediatric heart transplants yearly worldwide.[1] However, posttransplant complications remain an unresolved problem that leads

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All the authors have participated sufficiently in this work to take public responsibility for the content. E.B.-Q., J.L.-A., M.P., M.M.-B., M.C., N.G., and R.C.-R. participated in the design of the study. M.C., N.G., and E.P. were involved in the clinical monitoring and the collection of patient samples. R.L.-E., together with E.B.-Q. and J.L.-A. carried out the sample processing and the validation of the results. E.B.-Q., J.L.-A., and R.C.-R. performed the data analysis and the interpretation of the results. E.B.-Q., M.P., M.M.-B., and R.C.-R. participated in the writing of the article. R.C.-R. is responsible for the overall study, including research design, data analysis, and writing of the article.

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to death in a high percentage of heart transplant pediatric patients, mainly in the early posttransplant period, and give median survival rates between 14 and 24 h depending on the age at transplant. In the recent era, 13% of these patients require treatment for immune rejection in the first year posttransplant, and the rejection episodes in the first 1–2 y posttransplant are clearly associated with worse survival and higher incidence of cardiac allograft vasculopathy at longer-term.[1] A better knowledge of the alterations in the immune homeostasis and immune mechanisms of rejection in this critical period in heart transplant children is crucial to adopt specific measures to improve these figures. However, considering the immature pediatric immune system's peculiarities and the particular interventions such as thymectomy performed in heart transplant children, the current knowledge from adults or other pediatric transplants cannot be extrapolated to these particular circumstances.

Regulatory T cells (Treg) are a subset of CD4+ T cells with suppressive capacity, which is crucial for the induction and maintenance of tolerance, and therefore with a central role in transplant acceptance.[2] Treg's ability to delay/prevent graft rejection has been shown in animal heart transplant models, preventing the activation and expansion of effector T cells responsible for cellular rejection, or inducing B cell death responsible for humoral rejection.[3] Indeed, the increased presence of Treg has been identified as being important in inducing operational tolerance and allograft survival in adult and pediatric transplant recipients.[4,5] On the contrary, a deficit in the Treg subset could severely compromise the main mechanisms of peripheral tolerance, thus increasing the risk of graft rejection. In fact, the Treg to effector T-cell (Teff) ratio has been described to be crucial for either tolerance preservation or the development of graft rejection responses.[6-8]

The use of immunosuppression in transplanted patients aims to dampen the immune response against the graft, but its pleiotropic effect could also affect cell subsets crucial for developing tolerance, such as Treg.[9,10] Treg are originated in the thymus, which in the case of children requiring HTx, is routinely discarded to gain field exposure in the transplant surgery. Preserved thymic function and increased Treg values could be critical factors for lower rejection rates and increased survival in transplanted children than adults.[11] However, in the case of children undergoing HTx, the detrimental effect of the immunosuppressive drugs[10] combined with the limited capacity to regenerate Treg cells in the absence of the thymus could seriously deplete the Treg pool compromising their role in tolerance.

In this study, our objective was to determine whether immunosuppression and thymectomy in heart transplant children might induce a dysregulation in the T-cell compartment, especially over Treg, which could be a determining factor in the risk of acute rejection in the first month's posttransplant. Identifying at which time points and for how long the mechanisms of immune tolerance are impaired might improve the immune monitoring of these patients and tailor the therapeutic interventions to minimize the incidence of acute rejection in heart transplant children.

MATERIALS AND METHODS

Patients

We performed a prospective study of 3 y follow-up in 7 heart transplant children (mean ± SEM age = 6.22 ± 1.77 y at transplant) (Table 1) enrolled at the Hospital "Gregorio Marañón" (HGUGM) who had undergone HTx between July 2015 and May 2017. Three patients (42.86%) underwent HTx for CHD and the remaining 4 (57.14%) for different cardiomyopathies (Table 1). All patients were thymectomized (>90% thymic tissue resection) at transplantation, excluding patients 1, 3, and 6 that were already thymectomized during prior surgeries for CHD. None of the patients received induction therapy. All patients received immunosuppression with tacrolimus (TAC), mycophenolate mofetil (MMF), and methylprednisolone (Pred). Steroids were withdrawn from the 7th to the 14th-month posttransplant, depending on the patient's clinical outcome. In patient 3, MMF was discontinued at 6 mo posttransplant because of neutropenia, maintaining dual therapy with TAC and Pred during the rest of the follow-up. In patient 5, Pred was withdrawn at 14 mo posttransplant and was reintroduced approximately 7 mo later because of pericarditis, maintaining triple therapy with TAC, MMF, and Pred until the end of the follow-up. Patient 7 also maintained triple therapy without withdrawal of Pred throughout the follow-up because of pericarditis (Table 1). Immunosuppression levels for each patient over time are shown in Figure 1. None of the patients developed confirmed rejection episodes during the follow-up. The study was conducted after the HGUGM ethics committee's approval and according to the principles expressed in the Declaration of Helsinki. Informed written consent from the legal guardians were obtained before the patient's enrolment.

Sample Processing and Immune Monitoring

Peripheral blood samples (<3 mL) were drawn and analyzed within 2 h after the extraction. Samples were obtained before transplantation and immunosuppressive treatment (baseline), and the immune follow-up was performed with an interval of 15 d for the first 2 mo posttransplant, 30 d from the second month and 6 mo from the first year, coinciding with their scheduled revision visits. From 2 y and until the end of the follow-up, the samples were obtained in the scheduled time within a range of ±60 d. Samples were grouped considering the baseline sample, 0, and the following months posttransplant: 1, 2, 3, 4–6, 7–9, 10–12, 18, 24, 30, and 36. Samples from patients underlying an ongoing infectious process were not included in the study since they can alter the immunologic evaluation.

We determined by flow cytometry (*Gallios Cytometer*, Beckman Coulter) the frequency and absolute counts of CD4+ T cells, CD8+ T cells, and Treg (CD3+CD4+CD25+CD127 low), including markers for the following subsets: naive (CD45RA+CD27+), central memory (CM; CD45RA−CD27+), effector memory (EM; CD45RA−CD27−) and terminally differentiated effector memory (TemRA) (CD45RA+CD27−). Values and functional markers (CD39, CTLA4, Ki67, and HLA-DR) of Foxp3+ Treg cells (CD3+CD4+CD25+Foxp3+) and frequency of Foxp3+ Treg "recent thymic emigrants" (RTE) (CD45RA+CD31+) were also analyzed in PBMCs by intracellular staining as previously described.[12,13] Gating strategies for all these immune populations and subpopulations are shown in Figure 2.

Statistical Methods

Linear mixed-effects regression models were adjusted for evaluating the evolution of each immunologic covariate over time. Since each patient's measurements are not independent, this type of model assumes that measurements within each

TABLE 1.

Clinical parameters of the patients included in the study

ID	Sex	Diagnosis	Age at Tx	Immunosuppression	Steroids withdrawal	Observations	Rejection episodes	Infections
Patient 1	M	CHD	14 mo	TAC, MMF, Pred	7 mo post-Tx		No	
Patient 2	M	DCM	3 y	TAC, MMF, Pred	9 mo post-Tx		No	
Patient 3	M	CHD	2 mo	TAC, Pred	No	MMF discontinued for neutropenia	No	
Patient 4	M	RCM	9 y	TAC, MMF, Pred	12 mo post-Tx		No	CMV (d + 245)
Patient 5	F	NCC	11 y	TAC, MMF, Pred	14 mo post-Tx and reintroduced 7 mo after	Pred reintroduced because of pericarditis	No	
Patient 6	M	CHD	8 y	TAC, MMF, Pred	9 mo post-Tx		No	CMV (d + 120)
Patient 7	F	DCM	11 y	TAC, MMF, Pred	No	Pred maintained because of pericarditis	No	CMV (d + 262)

CHD, congenital heart disease; CMV, cytomegalovirus infection; DCM, dilated cardiomyopathy; F, female; M, male; MMF, mofetil mycophenolate; NCC, noncompaction cardiomyopathy; Pred, methylprednisolone; RCM, restrictive cardiomyopathy; TAC, tacrolimus; Tx, transplant.

child are more similar than between different children (autocorrelation). The same model was used for the group analysis of the phenotype frequencies along with a pairwise comparison of adjusted predictions using the Bonferroni test. For the correlations, samples from all patients at all time points were considered. They were tested using the nonparametric Spearman correlation coefficient, and a linear mixed-effects regression model was applied for the cutoff point analysis.

RESULTS

Treg Counts Significantly Decrease From the Seventh-month Posttransplant

We assessed the dynamic changes in T-cell populations over the first 3 y posttransplant, taking the pretransplant sample (baseline, 0) as the reference value (0% change). In the first 6 mo, we did not find significant variations of Treg counts (cells/µL) compared to baseline levels. However, the Treg number significantly decreased from the seventh month (−37.20% from BL; $P = 0.012$), reaching their lowest values at the end of the follow-up (−68.95%; $P < 0.001$) (Figure 3A). These results were confirmed by measuring Foxp3+ Treg counts, which also decreased significantly (Figure 3B). The decrease in the frequency and number of Treg and Foxp3+ Treg cells is shown in a representative patient comparing pretransplant and 3 y posttransplant values (Figure 3C).

Total CD4+ T-cell counts did not vary significantly throughout the follow-up, except for a short-term/punctual increase at the third-month posttransplant (+38.26%; $P = 0.039$) (Figure 4A), and therefore the decrease seems to occur specifically in the Treg subpopulation and not in other conventional CD4+ T cells. Besides, there were no significant variations in total CD8+ T-cell counts during the follow-up (Figure 4B).

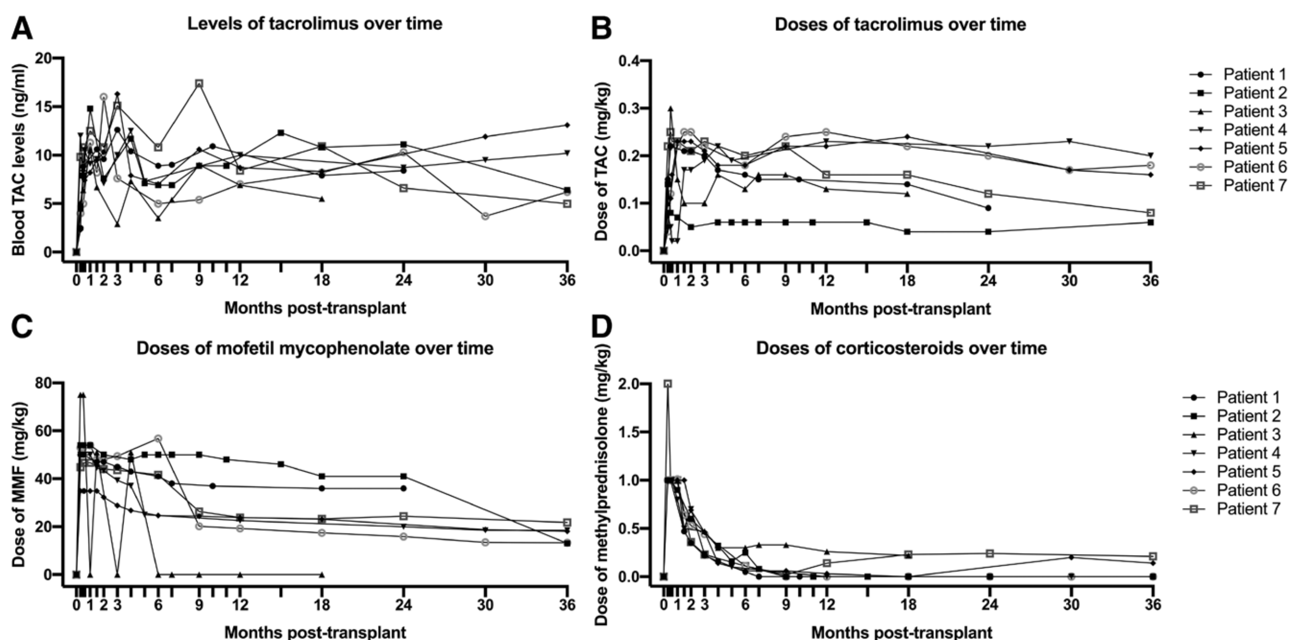

FIGURE 1. Immunosuppression levels of TAC, MMF, and methylprednisolone per patient over time. Drug levels for each patient at samples included in this study are shown. Blood TAC levels at ng/mL (A) and TAC doses at mg/kg (B) per patient over time. Doses of MMF at mg/kg (C) per patient over time. In patient 3, MMF was finally discontinued because of neutropenia. Doses of methylprednisolone (corticosteroids) at mg/kg (D) per patient over time. In patient 3, methylprednisolone was maintained. In patient 5 and patient 7, methylprednisolone was reintroduced or maintained, respectively, because of pericarditis. In the rest of the patients, corticosteroids were withdrawn from the 7th to the 14th-mo posttransplant. At time 0 (pretransplant), doses of all immunosuppressants were 0. MMF, mycophenolate mofetil; TAC, tacrolimus.

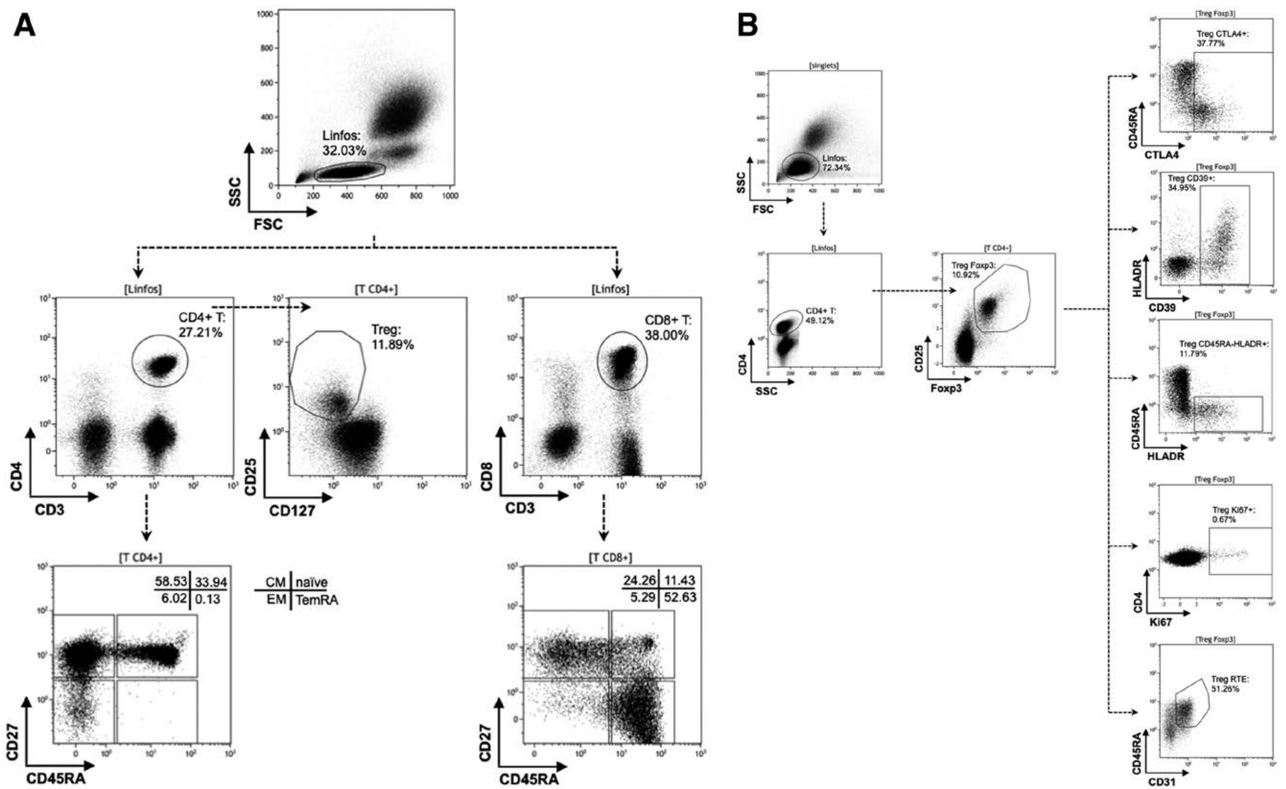

FIGURE 2. Flow cytometry gating strategy and representative dot plots for immune cell populations and subpopulations. Gating panels are shown for (A) lymphocytes, CD4+ T, Treg, and CD8+ T cells and their subsets: naive (CD45RA+CD27+), CM (CD45RA– CD27+), EM (CD45RA–CD27–), and TemRA (CD45RA+CD27–) analyzed in fresh whole blood samples. In peripheral blood mononuclear cells, gating panels are shown for (B) lymphocytes, CD4+ T and Foxp3+ Treg cells. Gated on Foxp3+ Treg, here we showed Treg CTLA4+, Treg CD39+, activated Treg (CD45RA-HLADR+), Treg Ki67+, and Treg RTE (CD45RA+CD31+). CM, central memory; EM, effector memory; FSC, forward scatter; Linfos, lymphocytes; RTE, recent thymic emigrants; SSC, side scatter; TemRA, terminally differentiated effector memory; Treg, regulatory T cells.

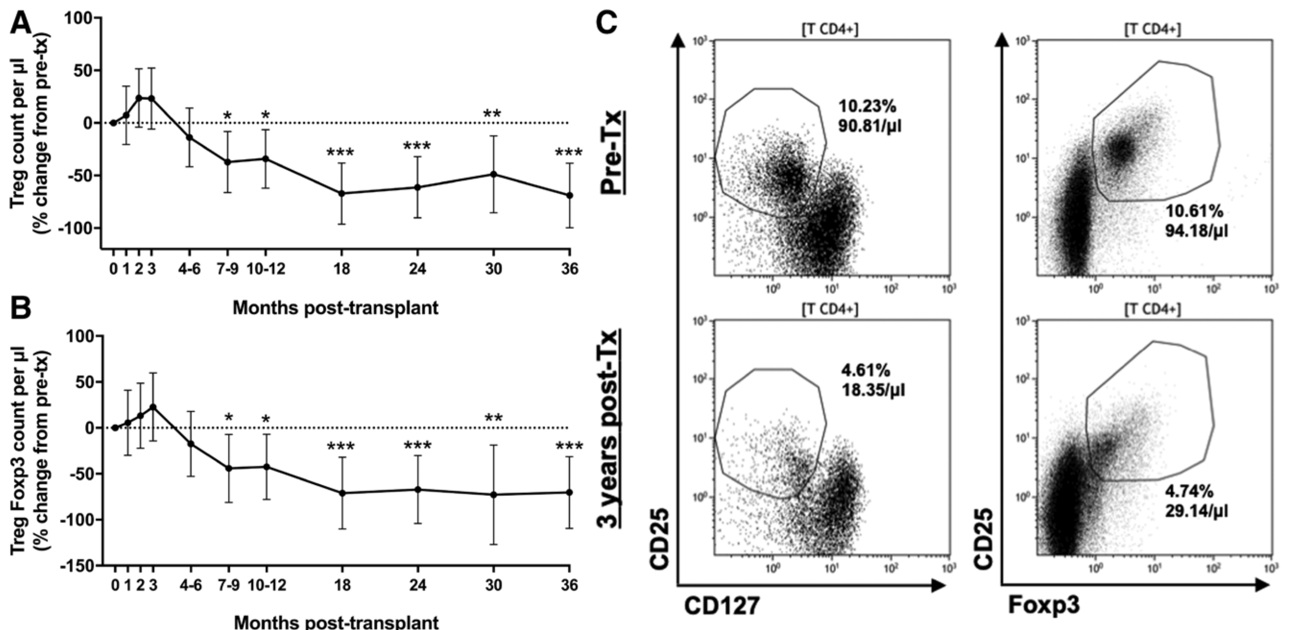

FIGURE 3. Percentage of change from the pretransplant sample (0) in Treg cell count (cells per µL of blood). Treg (CD4+CD25+CD127low) (A) and Foxp3+ Treg (CD4+CD25+Foxp3+) (B) change from pre-Tx. The dotted line represents the 0% change from pre-Tx. Samples were grouped considering the baseline sample, 0, and the following mo posttransplant: 1, 2, 3, 4–6, 7–9, 10–12, 18, 24, 30, and 36. The solid line represents the estimated mean with 95% CI at each point. If the CI does not include 0, a $P < 0.05$ will result. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Dot plots from a representative patient (C) showing the frequency and the cell number per µL of blood for Treg (left panels) and Foxp3+ Treg (right panels), comparing the pre-Tx with the 3 y post-Tx values in the same patient. Treg, regulatory T cells; Tx, transplantation.

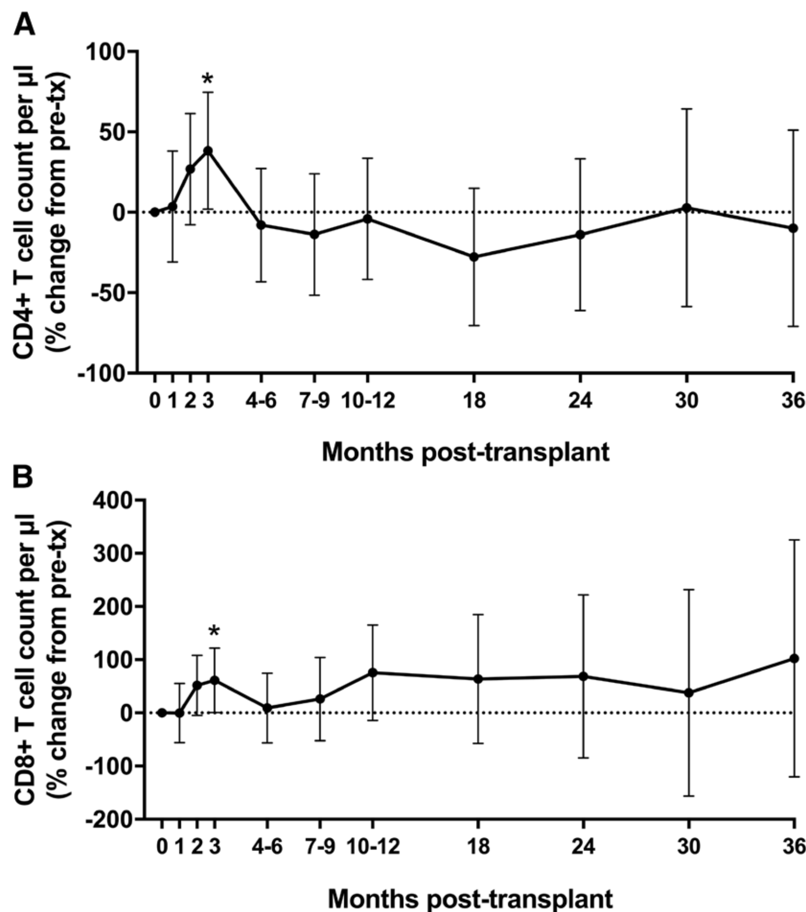

FIGURE 4. Percentage of change from the pretransplant sample (0) in CD4+ and CD8+ T cell count (cells per µL of blood). CD4+ T cell count (A) and CD8+ T cell count (B) change from pre-Tx. The dotted line represents the 0% change from pre-Tx. The solid line represents the estimated mean with 95% CI at each point. If the CI does not include 0, a $P < 0.05$ will result. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. CI, confidence interval; Tx, transplantation.

Markers Related to Treg Functionality and Proliferative Capacity Are Not Impaired After Transplant, but There Are Signs of Active Differentiation in the Treg Pool

In the light of the decreased Treg counts, we analyzed whether the expression of markers related to the functional and proliferative capacity of Treg could also be impaired. We found a significant increase in the frequency of CD39+ Treg (a marker of highly active and suppressive Treg with a regulatory effector/memory-like phenotype[14]) from the second-year posttransplant (+51.60%; $P = 0.009$), reaching their highest values at the end of the follow-up (+102.66%; $P < 0.001$) (Figure 5A). The frequency of CTLA4+ Treg, another critical marker of Treg functionality, kept comparable levels to baseline (Figure 5B). The frequency of activated Treg (CD45RA–HLADR+) was increased at the 18th month (+170.54%; $P = 0.044$), and an increasing (nonsignificant) tendency was observed along the rest of the follow-up (Figure 5C). No significant differences were found in the frequency of Ki67 (a marker present in all actively dividing cells) during the follow-up (Figure 5D), discarding a loss in the proliferation capacity of Treg as the cause of reduced Treg numbers. Therefore, although Treg counts were decreased in heart transplant children, the expression of markers related to the Treg functionality and proliferative capacity of remaining Treg does not appear to be impaired.

Nevertheless, the increase in the frequency of CD39+ Treg during the last 2 y posttransplant suggests a differentiation process in the Treg population to a more effector/memory-like phenotype. The increased differentiation in the Treg pool would also be confirmed by a decrease in the frequency of RTE Treg, identified as CD45RA+CD31+ Treg cells. In the absence of a functional thymus as a consequence of thymectomy, the CD31 marker was able to identify the thymic preformed Treg pool still presented in the transplanted patients after thymectomy.[15] The results showed that the frequency of Treg RTE significantly decreased from the 30th month (–50.07% from BL; $P = 0.018$), reaching values of –47.29% at the end of the follow-up ($P < 0.01$) (Figure 5E), indicating that the preformed RTE Treg pool was starting to deplete. Altogether, these results suggest that the Treg cell differentiation in conjunction with the lack of thymic replenishment causes the preformed Treg pool to begin to run low at the end of the follow-up.

Effector Subsets Within CD4+ and CD8+ T Cells Increase Dramatically After Transplant

It is known that the exposure to cumulative alloantigens in transplanted patients leads to a differentiation/activation increase within the peripheral T-cell pool.[16] Thus, although no significant variations were found in CD4+ and CD8+ T-cell counts during the follow-up, we were wondering whether

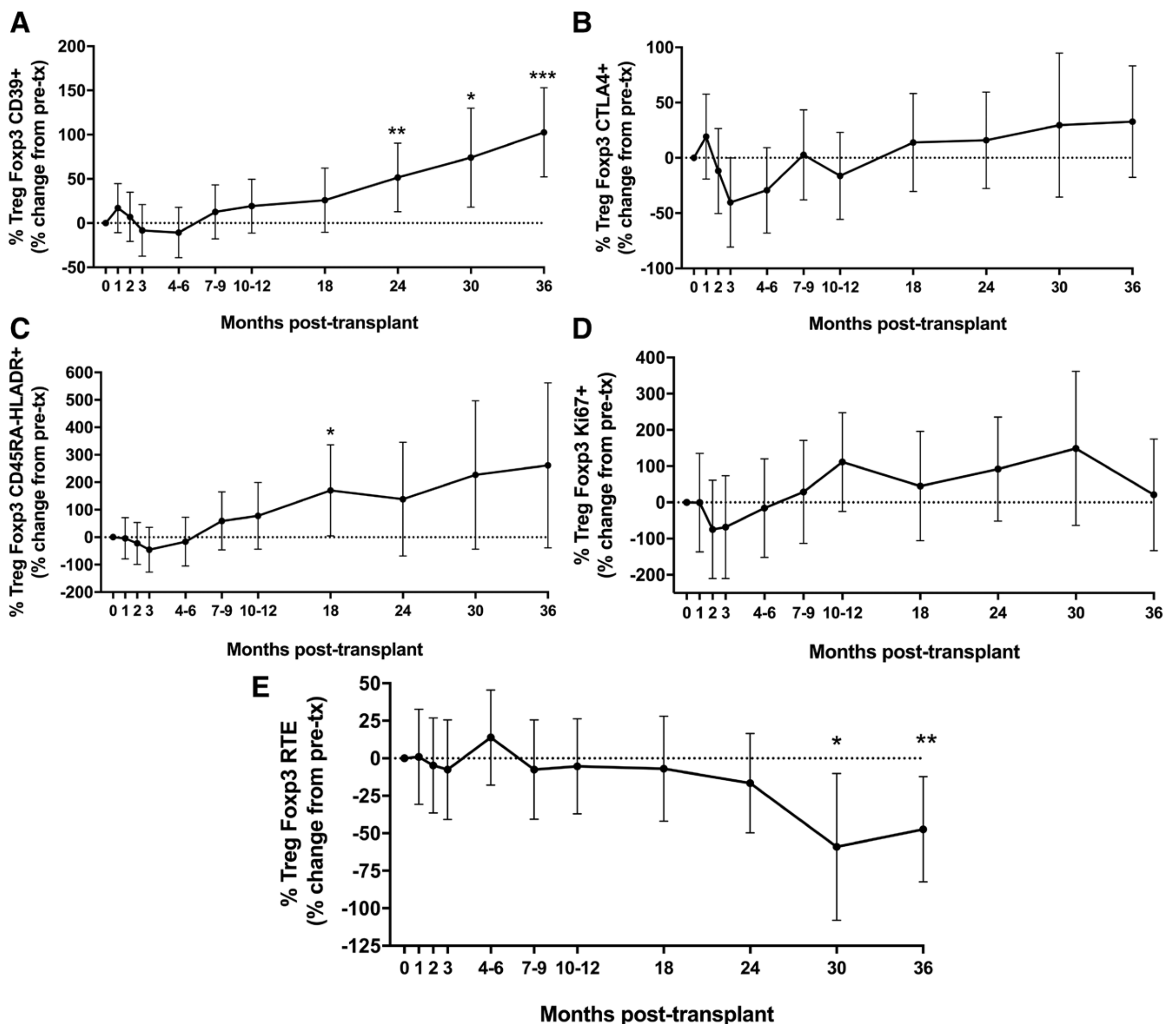

FIGURE 5. Percentage of change from the pretransplant sample (0) in the Foxp3+ Treg cell population. Frequency of Foxp3+ Treg CD39+ (A), Foxp3+ Treg CTLA4+ (B), Foxp3+ Treg CD45RA-HLADR+ (C), Foxp3+ Treg Ki67+ (D), and Foxp3+ Treg RTE (E) change from pre-Tx. The dotted line represents the 0% change from pre-Tx. The solid line represents the estimated mean with 95% CI at each point. If the CI does not include 0, a $P < 0.05$ will result. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. CI, confidence interval; RTE, recent thymic emigrants; Treg, regulatory T cells; Tx, transplantation.

their differentiation/activation status remained unchanged. Regarding CD4+ T-cell phenotypes, the frequency of EM cells significantly increased compared to baseline values from the fourth month (+393.02%; $P = 0.006$). EM CD4+ frequency reached the highest values (+793.45%; $P < 0.001$) at 2-y posttransplant to decrease afterward, coinciding with an increase of the most differentiated TemRA CD4+ subset at the end of the follow-up (+761.80%; $P = 0.004$) (Figure 6A). Frequencies of naive and CM CD4+ T cells did not change from baseline. We compared the behavior of these subsets employing the Bonferroni test. Significant differences in subset evolution were observed when analyzing EM versus naive, EM versus CM, and TemRA versus naive within CD4+ T cells (Table 2A). In the case of CD8+ T-cell phenotypes, the frequency of EM remains comparable to the baseline values ($P > 0.05$). However, the values of TemRA CD8+ T cells dramatically increased from the 10th month (+2953.86%; $P < 0.001$), reaching the highest values at 3-y posttransplant (+8350.75%; $P < 0.001$) (Figure 6B). The evolution of this TemRA CD8+ subset was significantly different from the other 3 phenotypes (naive, CM, and EM) (Table 2B). The increase in the frequency of the EM and TemRA subsets within CD4+ and CD8+ T is shown in a representative patient comparing the pretransplant with the 3 y posttransplant values (Figure 6C).

The Increase of Effector T Cells Is Concomitant With the Decrease in Treg Counts

Treg are characterized by their capacity to suppress the proliferation and function of effector T cells. Thus, we were interested in analyzing the Treg/Teffector (EM) ratio, which has been described as a valuable marker to monitor tolerance preservation.[17] We found a significant decrease from the fourth month for Treg/Teff-CD4+ (−27.88%; $P = 0.035$) and from the seventh month for Treg/Teff-CD8+ (−72.59%; $P < 0.001$), reaching minimum values at 3-y posttransplant

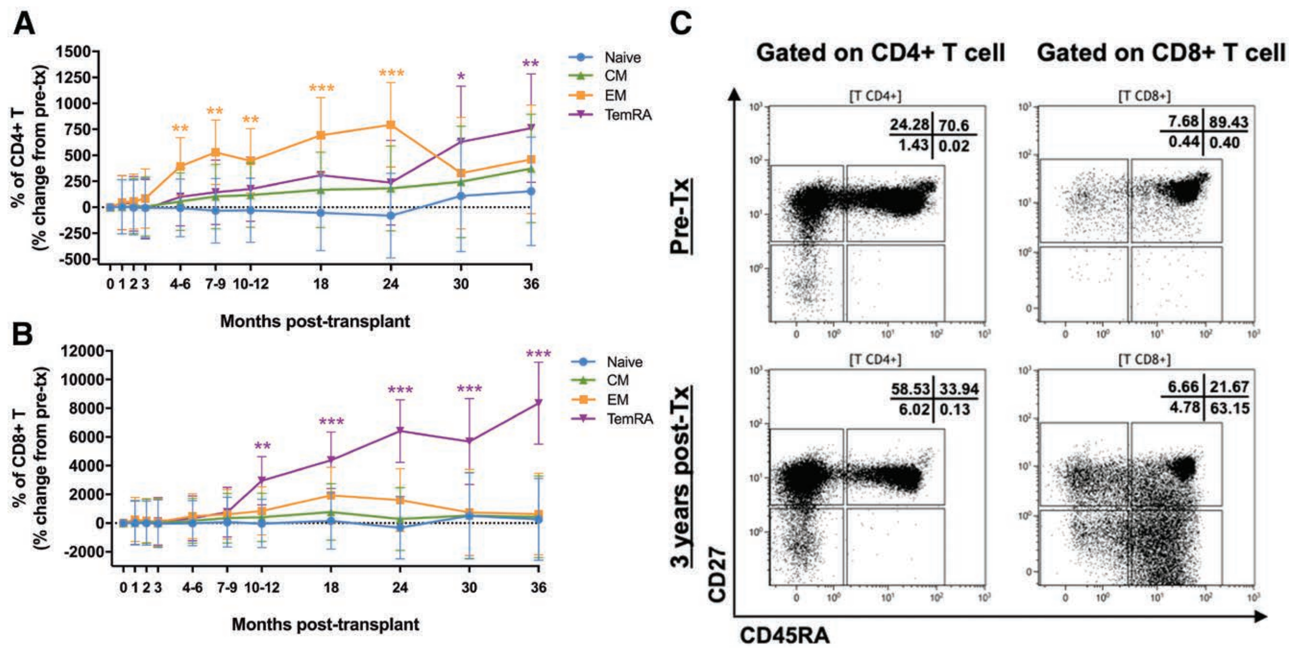

FIGURE 6. Percentage of change from the pretransplant sample (0) in the frequency of cell subsets within CD4+ and CD8+ T cells. Frequency of naive (in blue), CM (in green), EM (in orange), and TemRA (in purple) within CD4+ (A) and CD8+ (B) T cell populations change from pre-Tx. The dotted line represents the 0% change from pre-Tx. The solid line represents the estimated mean with 95% CI at each point. If the CI does not include 0, a $P < 0.05$ will result. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Dot plots from a representative patient (C) showing the frequency of naive (CD45RA+CD27+), CM (CD45RA−CD27+), EM (CD45RA−CD27−), and TemRA (CD45RA+CD27−) within CD4+ (left panels) and CD8+ (right panels) T cells, comparing the pre-Tx with the 3 y post-Tx values in the same patient. CI, confidence interval; CM, central memory; EM, effector memory; TemRA, terminally differentiated effector memory; Tx, transplantation.

(−82.95; $P < 0.001$ and −94.72%; $P < 0.001$, respectively) (Figure 7A and B). Additionally, we found a clear negative correlation between Treg counts and the Teff (EM) CD4+ and CD8+ cell frequency, which was consistent with the decreased Treg/Teff. The lower the Treg counts in peripheral blood, the greater the Teff CD4+ ($P < 0.001$) and CD8+ ($P < 0.001$) frequency (Figure 8A and B). Regarding TemRA cells, we also found a strong negative correlation between Treg counts and both CD4+ TemRA and CD8+ TemRA cells ($P < 0.001$).

Interestingly, the negative correlation between Treg counts and Teff (EM) frequency showed 2 differentiated patterns, so that when Treg counts decrease below a number, the frequency of effector T cell increases sharply (Figure 9). Therefore, we tried to define which Treg value could be a cutoff point associated with an increased risk of effector T-cell spreading. We defined 25, 50, 75, and 100 Treg cells/µL as arbitrary cutoff points to perform the analysis. A cutoff point of Treg <50 cells/µL was related to the higher increase in CD4+ EM, since

TABLE 2.

Pairwise comparison between the subpopulations of CD4+ and CD8+ T cells

	Contrast	Delta method SE	Bonferroni z	Bonferroni P > |z|	Bonferroni 95% CI	
A. CD4+ T groups						
CM vs naive	109.852	60.390	1.82	0.413	−49.473	269.178
EM vs naive	**343.159**	**60.390**	**5.68**	**0.000**	**183.834**	**502.485**
TemRA vs naive	**214.613**	**60.390**	**3.55**	**0.002**	**55.288**	**373.939**
EM vs CM	**233.307**	**60.390**	**3.86**	**0.001**	**73.981**	**392.633**
TemRA vs CM	104.761	60.390	1.73	0.497	−54.564	264.087
TemRA vs EM	−128.546	60.390	-2.13	0.200	−287.872	30.780
B. CD8+ T groups						
CM vs naive	225.385	367.023	0.61	1.000	−742.916	1193.686
EM vs naive	613.289	367.023	1.67	0.568	−355.012	1581.590
TemRA vs naive	**2585.186**	**367.023**	**7.04**	**0.000**	**1616.884**	**3553.487**
EM vs CM	387.904	367.023	1.06	1.000	−580.397	1356.205
TemRA vs CM	**2359.801**	**367.023**	**6.43**	**0.000**	**1391.499**	**3328.102**
TemRA vs EM	**1971.897**	**367.023**	**5.37**	**0.000**	**1003.595**	**2940.198**

Pairwise comparison of adjusted predictions between naive, CM, EM, and TemRA of CD4+ (A) and CD8+ T (B) cells using the Bonferroni test. If the CI does not include 0, a $P < 0.05$ will result. Significant ($P < 0.05$) pairwise comparisons are highlighted in bold font.
CI, confidence interval; CM, central memory; EM, effector memory; TemRA, terminally differentiated effector memory.

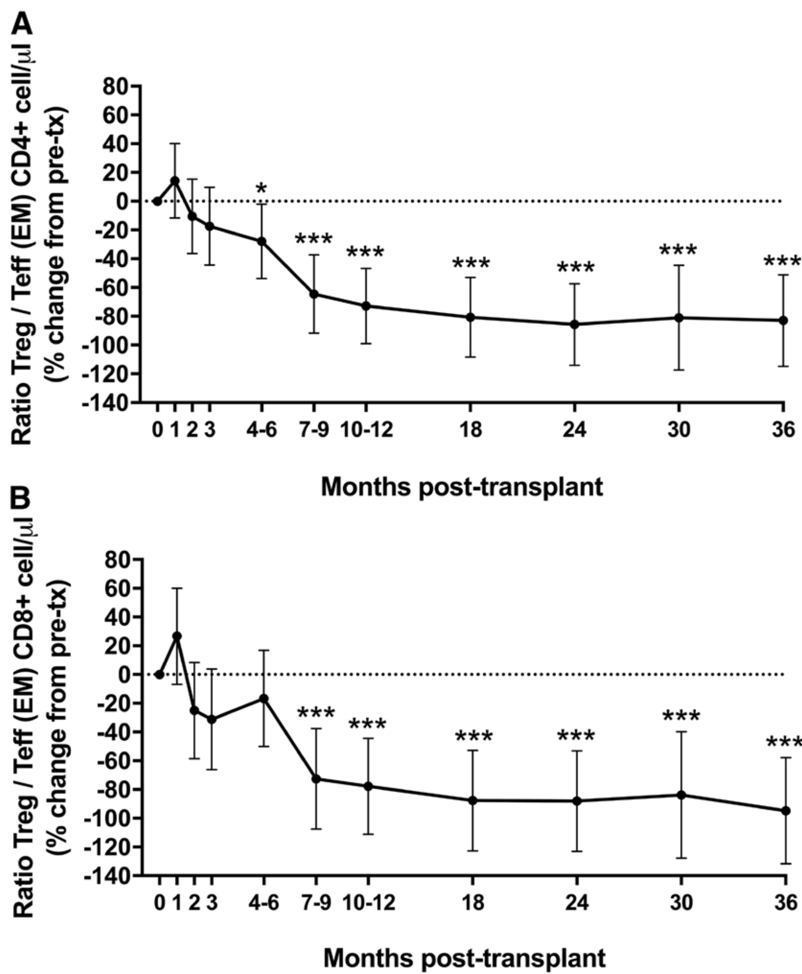

FIGURE 7. Percentage of change from the pretransplant sample (0) in the Treg to Teff (EM) CD4+ and CD8+ count (cells per μL of blood) ratio. Treg to Teff (EM) CD4+ count ratio (A) and Treg to Teff (EM) CD8+ count ratio (B) change from pre-Tx. The dotted line represents the 0% change from pre-Tx. The solid line represents the estimated mean with 95% CI at each point. If the CI does not include 0, a $P < 0.05$ will result. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. CI, confidence interval; EM, effector memory; Teff, effector T cells; Treg, regulatory T cells; Tx, transplantation.

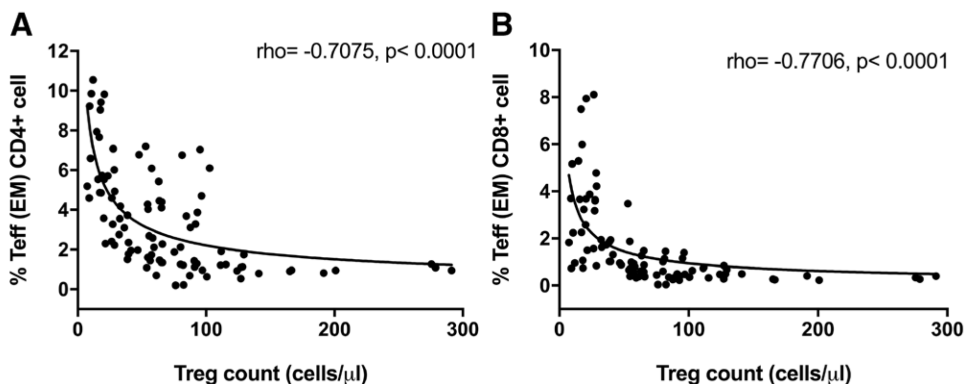

FIGURE 8. Correlation between Treg count (cells per μL of blood) and Teff (EM) CD4+ and CD8+ cell frequency. Samples from all patients at all time points were considered. Treg count and the frequency of Teff (EM) CD4+ (A) and Teff (EM) CD8+ (B) cells are shown. Correlations were tested using the Spearman correlation coefficient shown as rho value. $P < 0.05$ was considered significant. EM, effector memory; Teff, effector T cells; Treg, regulatory T cells.

<50 Treg counts a significant negative association with EM CD4+ of −0.1071 ($P < 0.001$) is detected. In other words, the frequency of EM CD4+ increases by 0.1071% per unit of Treg diminished (Table 3A and Figure 9A). In the case of Teff (EM) CD8+, a Treg count <75 cells/μL showed the highest potency to determine a significant negative correlation with the frequency of EM CD8+ T (−0.0464; $P = 0.011$) (Table 3B and Figure 9B). Considering all the samples included in the study, the 75 and 50 Treg/μL cutoffs were reached around the 6th and 12th month, respectively (Figure 10). In summary, our results show that during the first 3 y after transplant, when Treg count decrease <50 or 75 cells/μL, the increase in the frequency

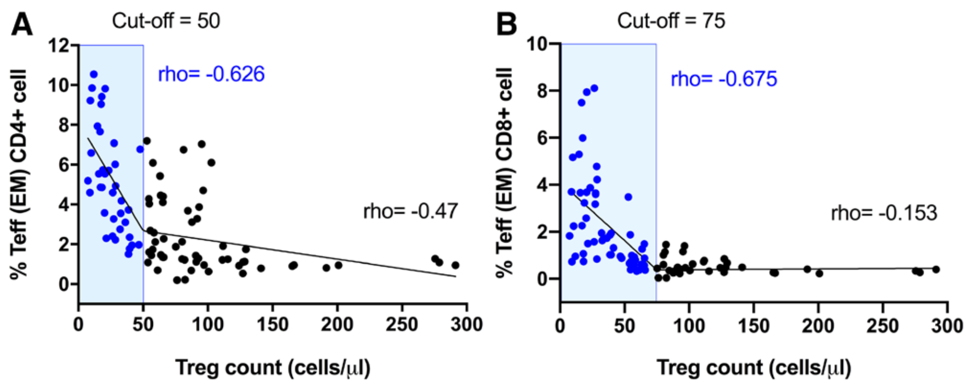

FIGURE 9. Correlations below and above a selected statistical cutoff point between Treg count (cells per μL of blood) and Teff (EM) CD4+ and CD8+ cell frequency. Samples from all patients at all time points were considered. Treg count <50 cells/μL (A) and Treg count <75 cells/μL (B) showed the highest potency to determine a significant negative correlation with the frequency of Teff (EM) CD4+ (A) and Teff (EM) CD8+ (B) cells, respectively. Correlations were tested using the Spearman correlation coefficient shown as rho value. Values below these selected statistical cutoff points are shown in blue. EM, effector memory; Teff, effector T cells; Treg, regulatory T cells.

of Teff (EM) CD4+ and CD8+ experiences a tipping point and the proportion of T-effector cells increases dramatically. This could indicate that Treg numbers below these thresholds are not sufficient to control the risk of Teff spreading.

DISCUSSION

In this prospective study, we describe the immune changes over the T-cell compartment observed right after transplant and during a follow-up of 3-y posttransplant in a cohort of heart transplant children. We have shown at which time points and for how long these patients experienced a relevant immune dysregulation, hallmarked by a decrease in Treg numbers and an increase in effector T cells, critical players for either tolerance preservation or development of graft rejection.

The observed Treg count changes cannot be attributed to the physiologic changes in T-cell populations by the age, because as described by Schatorjé et al,[18] in the period from 2 to 16 y, the Treg counts hardly vary. Treg cells can be generated extrathymically by differentiation in the periphery (induced Treg cells), but approximately 80% of the Treg repertoire originates in the thymus[19]; therefore, thymectomy should have a clear impact on the Treg counts. Besides, in the context of a pediatric heart transplant, thymectomy coexists with chronic immunosuppression over the patient's lifetime. Although CD4+ and CD8+ T cells are the primary target cells to control potentially graft rejection by immunosuppressants and these cells are also produced by the thymus, we have not found relevant changes in total CD4+ and CD8+ T cells counts during the follow-up. However, heart transplant children presented a marked decrease in Treg numbers from the seventh-month posttransplant, being the most beneficial population, the most affected in these transplanted patients.

The consequences of thymectomy over the T-cell compartment have been extensively studied in pediatric cardiac surgery.[19-21] Children undergoing neonatal thymectomy showed a significant reduction in the frequency and the total number of CD4+ and CD8+ T cells. More specifically, the naive T cell population shrinks, accompanied by the concomitant increase of the memory T cell subset due to homeostatic proliferation in an attempt to restore the T-cell pool.[20,21] Similarly, thymectomy has been related to decreased Treg counts that could lead to an increased homeostatic proliferation of Foxp3+ cells. However, in the case of Treg, this proliferation could carry a loss of its suppressive capacity compromising their role in the maintenance of immune tolerance.[19,22] Regarding thymectomy in transplanted patients, to our knowledge, there are only 2 cross-sectional studies in heart transplant children. In the first study, Ogle et al showed that individuals that undergo thymectomy plus transplantation (and immunosuppressive regime) in infancy have a dramatic decrease in the T-cell repertoire,[23] but the study does not analyze Treg cells. In another recent study, Mengrelis et al compared children undergoing HTx and children who simply underwent thymus excision without immunosuppression.[24] Interestingly, they showed that the relative proportion of Treg was lower in the transplantation group, which seems to indicate a more detrimental effect over the Treg population when thymectomy is accompanied by immunosuppression.

TABLE 3.
Correlations between Treg count (cells per μL of blood) below statistical cutoff points and Teff (EM) CD4+ and CD8+ cell frequency

	Coef.	Robust SE	z	P > |z|	95% CI	
A. EM CD4+ T						
Treg < 25 cells/μL	−0.0721	0.0827	−0.87	0.383	−0.2341	0.0899
Treg < 50 cells/μL	**−0.1071**	**0.0258**	**−4.16**	**0.000**	**−0.1576**	**−0.0567**
Treg < 75 cells/μL	−0.0523	0.0145	−3.62	0.000	−0.0807	−0.0240
Treg < 100 cells/μL	−0.0310	0.0141	−2.20	0.028	−0.0586	−0.0034
B. EM CD8+ T						
Treg < 25 cells/μL	0.0146	0.0550	0.26	0.792	-0.0933	0.1223
Treg < 50 cells/μL	−0.0546	0.0378	−1.44	0.149	−0.1287	0.0195
Treg < 75 cells/μL	**−0.0464**	**0.0183**	**−2.53**	**0.011**	**−0.0823**	**−0.0104**
Treg < 100 cells/μL	−0.0316	0.0111	−2.84	0.004	−0.0534	−0.0098

25, 50, 75, and 100 Treg counts (cells/μL) were determined as arbitrary cutoff points based on statistical values. Treg counts <25, <50, <75, and <100 cells/μL and the frequency of Teff (EM) CD4+ (A) and Teff (EM) CD8+ (B) cells are shown. If the CI does not include 0, a $P < 0.05$ will result. For Teff (EM) CD4+ and CD8+, the cut point of Treg with the highest potency to determine a significant negative correlation is highlighted in bold font.
CI, confidence interval; Coef, Spearman correlation coefficient; EM, effector memory; Teff, effector T cell; Treg, regulatory T cell.

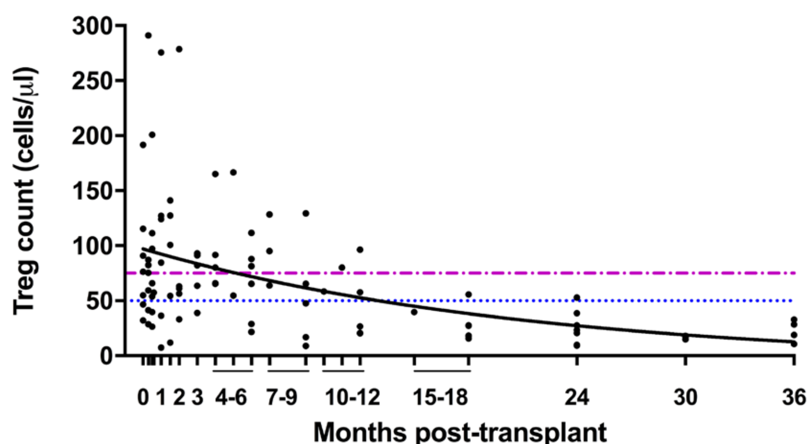

FIGURE 10. Evolution of Treg counts (cells per µL of blood) along the 3-y follow-up. Treg values from all the collected samples are shown. The black line represents the trend line. Dotted pink and blue lines represent the 75 and 50 Treg/µL cutoffs, respectively. Treg, regulatory T cells.

Patients included in this study received TAC, MMF, and corticosteroids, and a possible explanation for the specific decrease in the Treg population could be a higher susceptibility of Treg to these immunosuppressants in comparison to conventional T cells. Tregs are strongly dependent on IL-2 because of their constitutively high expression of CD25 and amplified intracellular signal transduction downstream of the IL-2 receptor.[10] Although other cytokines can substitute the function of IL-2 on conventional T cells, IL-2 is indispensable for Treg development, homeostasis, and function,[25] and because Treg do not produce IL-2, they are dependent on IL-2 from other T cells and dendritic cells. TAC directly impairs Treg activation, proliferation, and survival, but it can also affect Treg indirectly by limiting IL-2 production by Teff,[10] and in the absence of an appropriate turnover by thymic production, the IL-2 deficit could promote the Treg-count decrease in the periphery. Regarding MMF, the effect on Treg is still controversial. In vitro experiments indicate that MMF does not alter Treg phenotype or even can promote Treg dominance over Th17 cells,[10] and kidney transplant recipients receiving MMF showed higher Treg levels than those treated with everolimus.[26] However, the MMF administration in animal models receiving Treg therapy reduced the efficacy of the Treg preventing rejection.[27] Finally, corticosteroids seem to benefit Treg prevalence and activity facilitating TGF-β signaling and Foxp3 expression.[10] Indeed, in our cohort, steroids were withdrawn from the 7th to the 14th-month posttransplant, which interestingly coincides with the period when the drop of Treg counts became remarkable. Certainly, the rapid steroid withdrawal could be one of the determinant factors that push this drop in Treg counts due to their benefit on the Treg proportion.[10] Besides, in the 3 patients in whom steroid therapy was maintained until the end of the follow-up, they received lower levels of corticosteroids from the seventh-month posttransplant, and the same marked decrease in Treg counts was observed.

There are very few studies reporting Treg values in pediatric patients transplanted with other organs when thymectomy is not performed to elucidate whether the immunosuppression alone also produces a similar Treg reduction. Stenard et al analyzed Treg values in 11 pediatric liver allograft recipients with a mean age of 53 mo receiving methylprednisolone, tacrolimus, and discontinued steroids.[28] Treg absolute counts were not analyzed in this study, but the authors report that the mean percentage of Treg was comparable between before transplantation and posttransplantation. These data could suggest again that thymectomy, along with chronic immunosuppression, would have more weight in the fall of Treg than immunosuppressants alone. Additionally, this study showed that Treg values were lower in pediatric liver allograft recipients during acute rejection in comparison with levels when the graft was stable, confirming their crucial role in preventing rejection.

Therefore, regarding which is the cause of the Treg fall, it is difficult to know whether the main causative factor for Treg impairment is thymectomy or immunosuppression. Probably there is a summative effect of both factors, as suggested by Mengrelis et al,[24] that produces the observed Treg impairment.

We have shown that the frequency of effector subsets within CD4+ and CD8+ T cells (EM and TemRA) starts to increase dramatically after transplant, coinciding with Treg's fall. T-cell effector subsets, particularly TemRA CD8+ T cells, are considered the main initiators of rejection, either cellular or humoral,[29] and responsible for transplant failure. TemRA CD8+ T cells are generated from the CM subset upon homeostatic proliferation,[30] and they are more resistant than other T-cell phenotypes to induced cell death and immunosuppression,[31] which could be the reason why TemRA CD8+ increases more drastically from the 10th-month posttransplant in our cohort of heart transplant children than other phenotypes. It has been reported an association between high TemRA CD8+ T cells and a higher risk of rejection during the first year posttransplant,[32,33] and also an association to a higher risk of long-term graft dysfunction in adult kidney-transplant patients.[34] However, despite the dramatic increase in the frequency of TemRA CD8+ in our patients, no rejection episodes appeared in the 3-y follow-up in this cohort. The incidence of immune rejection in heart transplant children in the first 5y posttransplant is lower than 15%,[1] and considering that only 7 patients were studied, the incidence of rejection in this small cohort of patients is not conclusive. Therefore, we cannot rule out that the increase in effector T cells, notably TemRA CD8+ cells, will not jeopardize graft survival in heart transplant children.

The hypothesis of this study is that the emergence and expansion of Teff, and particularly TemRA CD8+ cells, could be related to the observed Treg fall in the early period

posttransplant. Treg play a pivotal immunomodulatory role in suppressing through several mechanisms these exacerbated effector T-cell responses.[35] The balance between Treg and Teff has been postulated as a critical factor to prevent alloresponses and acute rejection,[7,36] either in numbers or in overall functional predominance.[35] In fact, low Treg numbers during the first month posttransplant have been associated with acute rejection in heart transplant adult patients.[37] In our study, we observed that patients presented a gradual and marked decrease in the Treg to Teff (EM) CD4+ and CD8+ T cell ratio from the fourth month, and low Treg counts were correlated to higher frequencies of EM and TemRA CD4+ and CD8+ T cells. Interestingly, this imbalance between Treg and Teff posttransplant coincides with the period of higher risk of acute rejection, and its effect persisted until the end of the follow-up, at 3 y posttransplant. Furthermore, previous studies in the group have shown that heart transplant children transplanted for >4 y still presented an immune dysregulation that even leads to the development of secondary chronic complications, such as atopic dermatitis.[38]

In the 7 heart transplant patients analyzed, we defined that a Treg count <50 cells/µL correlated with the highest frequencies of Teff (EM) CD4+, and Treg count <75 cells/µL with the highest frequencies of Teff (EM) CD8+. In our cohort, these values represent a potential risk cutoff point indicating that when Treg numbers decrease below 50 or 75 cells/µL, Treg cells could be insufficient to control the spreading of Teff cells leading to a more favorable environment for the development of graft rejection.

Summing up, although interventions derived from the HTx procedure (immunosuppression and thymectomy) are needed to carry out transplants, they could induce, as an inevitable consequence, a dysregulation in the immunologic status of transplanted children characterized by a marked decrease in Treg cells. We hypothesize that the diverse naive Treg reservoir already presented at birth, after normal fetal thymic development, and before thymectomy,[19] could protect against allogeneic T cell responses, at least in the short-term. In fact, our data indicate that the expression of markers related to Treg functionality and proliferative capacity were not impaired after transplant. However, an increased differentiation of the Treg pool, reflected by the decrease in the Treg RTE frequency, could lead to a depletion of existing Treg cells. The pressure exerted by immunosuppressants on the Treg population, having lost the ability to replenish the Treg pool by the absence of the thymus, overpasses the compensatory homeostatic proliferation leading to a progressive decrease in the Treg counts.

In the light of these results and the evidence supporting the crucial role of Treg to prevent rejection, we suggest that monitoring the balance between Treg and Teff could identify risk markers that may help to anticipate graft damage before a biopsy, leading to improved patient outcomes in these patients. New approaches addressed to prevent the Treg decrease or restore the pool of these cells could have an impact on the prevention of graft rejection, and therefore in reducing the mortality in heart transplant children. Interventions such as (1) the election of immunosuppressive drugs with lower impact on Treg; (2) supplementation with vitamin D, which has proven to increase Treg survival[39] and to reduce the risk of acute rejection[40]; (3) or even a cell therapy with Treg cells,[41,42] could counteract the Treg/Teff imbalance. In adults, adoptive transfer of Treg has provided promising results,[43] and we are currently recruiting patients in a clinical trial of heart transplant children treated with autologous thyTreg,[44] which could establish a new paradigm preventing rejection in the field of solid organ transplantation.

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