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BRIEF COMMUNICATION

AJT

Thymic function is a major determinant of onset of antibody-mediated rejection in heart transplantation

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Thymic function decreases progressively with age but may be boosted in certain circumstances. We questioned whether heart transplantation was such a situation and whether thymic function was related to the onset of rejection. Twenty-eight antithymocyte globulin–treated heart transplant recipients were included. Patients diagnosed for an antibody-mediated rejection on endomyocardial biopsy had a higher proportion of circulating recent thymic emigrant CD4+ T cells and T cell receptor excision circle levels than other transplanted subjects. Thymus volume and density, assessed by computed tomography in a subset of patients, was also higher in patients experiencing antibody-mediated rejection. We demonstrate that thymic function is a major determinant of onset of antibody-mediated rejection and question whether thymectomy could be a prophylactic strategy to prevent alloimmune humoral responses.

KEYWORDS
basic (laboratory) research/science, heart (allograft) function/dysfunction, heart transplantation/cardiology, immunobiology, immunosuppressant - polyclonal preparations: rabbit antithymocyte globulin, monitoring: immune, rejection: antibody-mediated (ABMR), thymus/thymic biology, translational research/science

1 | INTRODUCTION

Heart transplantation currently represents the best therapeutic option for patients with end-stage heart failure. Despite the advances in the field of transplantation and the development of potent immunosuppressive drugs, cardiac allograft rejection episodes are experienced by approximately 25% of patients at least once during the first year following heart transplantation.[1] Most heart-transplanted patients receive antithymocyte globulin (ATG) at the time of transplantation to induce a severe lymphodepletion expected to reduce the risk of acute rejection. In this context, a subsequent reconstitution of the immune system, based on thymopoiesis and homeostatic proliferation, is observed.[2]

The thymus is the primary site of T cell development, ensuring the formation of functional and self-tolerant T cells. The organ mainly involutes into fatty tissue after puberty as part of the aging process, which leads to changes in peripheral T cell subpopulation distribution; the naive T cell pool is reduced while memory T cell subset concomitantly expands.[3,4] Despite this involution, a residual thymic function persists in adulthood, although this vestigial thymic capacity varies between

Abbreviations: 0R, no evidence of rejection; AMR, antibody-mediated rejection; ATG, antithymocyte globulin; CM, central memory; CS, class-switched; CT, computed tomography; DSA, donor-specific antibody; EDTA, ethylenediamine tetraacetic acid; EM, effector memory; EMB, endomyocardial biopsy; HIV, human immunodeficiency virus; HTx, heart transplant; HU, Hounsfield unit; ISHLT, International Society for Heart & Lung Transplantation; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; rATG, rabbit antithymocyte globulin; RTE, recent thymic emigrant; TCMR, T cell mediated rejection; TEMRA, CD45RA+, terminally differentiated effector memory; Tfh, T follicular helper; TREC, T cell receptor excision circle; Tregs, regulatory T cells.

individuals. In addition, certain conditions may influence the extent to which thymic function is re-activated as revealed by the observations made after the introduction of antiretroviral therapy in human immunodeficiency virus (HIV)–infected lymphodepleted patients.[5-7]

The thymic function can be assessed in vivo in humans by measuring the thymic mass through computed tomography (CT) scan and also by measuring the frequency of recent thymic emigrant (RTE) T cells by flow cytometry performed on blood cells, as well as by quantifying by real-time polymerase chain reaction (PCR) the excised DNA molecules generated during thymocyte differentiation.[8] Indeed, during thymopoiesis, genetic rearrangements lead to the generation of fully functional TCRs. Byproducts of these processes, the T cell receptor excision circles (TRECs), are present in cells exported from the thymus but are progressively lost by their progeny because TRECs do not replicate during mitosis.

Given that heart transplant (HTx) patients are subjected to a thymus-targeted therapy combined with prolonged immunosuppressive treatments, we questioned in the present study whether heart transplantation was a clinical situation associated with a reactivation of the thymic function. Because the data obtained in several experimental models indicate that the thymus might be involved in transplantation tolerance,[9-12] we also analyzed whether thymic function was related to the onset of rejection in a cohort of HTx patients.

2 | MATERIALS AND METHODS

2.1 | Patients

Blood samples were obtained from 28 HTx consecutive patients of a single center (Bichat Hospital, Paris, France), collected at the time of routine endomyocardial biopsy (EMB) and classified in 3 subgroups according to the International Society for Heart & Lung Transplantation (ISHLT) 2004 and 2013 guidelines[13,14]: T cell–mediated rejection (TCMR) (grade 2R or higher), antibody-mediated rejection (AMR) (pathological AMR2 or higher), and patients with no evidence of rejection (0R) if they had not experienced acute rejection at the time of EMB and blood sampling. Blood was collected in ethylenediamine tetraacetic acid (EDTA) and used to retrieve plasma and to isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using standard Ficoll-Paque procedures, subsequently stored at −150°C until analysis. Patients received intravenous induction therapy consisting of 1.25 mg/kg rabbit antithymocyte globulin (rATG; thymoglobulin, Genzyme/Sanofi, Saint-Germain-en-Laye, France) on days 1 to 5 after transplantation. Maintenance immunosuppressive therapy included calcineurin inhibitor, mycophenolate mofetil, and prednisone. A group of healthy subjects (n = 11) without heart transplantation was also studied. Blood samples from 10 other HTx patients who did not experience AMR or TCMR higher than 1R were retrospectively retrieved to study the different subsets of circulating lymphocytes before and 3 months following heart transplantation with rATG induction. These 10 patients also received maintenance immunosuppression consisting of calcineurin inhibitor, mycophenolate mofetil, and prednisone after transplantation.

The study protocol (n°14-062) was approved by the local ethics committee of Paris 7 University (IRB 00006477) and patients gave written informed consent.

2.2 | Patient characteristics

The following clinical data were collected for each patient: recipient age and gender, primary heart disease, BMI, date of transplantation, donor age, presence of anti-HLA donor-specific antibodies (DSAs), and posttransplantation lymphocyte count. Positivity for DSA was detected by Luminex analysis (One Lambda, Canoga Park, CA) and defined as an antibody to donor HLA detected at mean fluorescence intensity (MFI) ≥1000.

2.3 | Assessment of RTE population

PBMCs were stained with the following fluorochrome-conjugated antibodies to quantify the RTE population: CD45-BV605, CD3-BV711, CD4-PerCP, and CD45RA-BV421 from Biolegend (San Diego, CA); CD8-PE-Cy7 and CD62L-FITC from BD Biosciences (San Jose, CA); and CD31-APC (clone 9G11) from R&D Systems (Minneapolis, MN). Analysis was performed using a LSRII flow cytometer and BD FACSDiva software 8.0 from BD Biosciences.

2.4 | TRECs analysis

Parallel quantification of the sjTRECs and the 13 DJβTRECs, together with CD3γ gene (used to normalize the data to the number of T cells) was performed using LightCycler technology (Roche Diagnostics). Intrathymic precursor T cell proliferation was evaluated through calculation of the sj/β-TREC ratio, as described.[8]

2.5 | Quantification of cytokines

The concentration levels of IL-7 and IL-15 in human plasma were assayed by Luminex according to the manufacturer's instructions (Luminex Screening Human Magnetic Assay, R&D Systems, Minneapolis, MN).

2.6 | Thoracic computed tomography

Thymic density and surface area were assessed by CT scan without contrast agent by an experienced cardiothoracic radiologist (P.O.).

2.7 | Statistical analysis

All analyses were performed using GraphPad Prism 6.0 for Mac OS X (GraphPad Software, San Diego, CA). Differences between groups were analyzed by Mann-Whitney *U* test and were considered statistically significant when the $P < .05$. Correlations were assessed by Spearman's rank correlation coefficient.

3 | RESULTS

3.1 | Study population and baseline characteristics

Characteristics of the study population are depicted in Table 1. Twenty-eight patients enrolled in the study between 2014 and 2016 were subdivided into 0R (n = 17), AMR (n = 6), and TCMR (n = 5) categories.

3.2 | Effects of rATG on lymphocyte subpopulations in the early posttransplantation period

We quantified the different subsets of lymphocytes to study depletion and recovery following rATG induction treatment in 10 HTx patients. Three months after transplantation, we observed a significant decrease in T cell to B cell ratio and CD4/CD8 ratio (Figure 1A). Over the same period, we noted a decrease in the percentage of CD4+ and CD8+ naive (CD45RA+ CD62L+), RTE (CD45RA+ CD62L+ CD31+), and central memory (CM) (CD45RA- CD62L+) T cells, with a concomitant elevation of CD45RA+ terminally differentiated effector memory (TEMRA: CD45RA+ CD62L-) T cells (Figure 1B), an unexpected result, since TEMRA cells are suspected to display lower homeostatic proliferative capacities.[15] Furthermore, these data indicate that lymphocyte subpopulations do not display the same sensitivity to rATG therapy.

3.3 | HTx patients diagnosed for AMR have more CD4+ RTE T cells

A lower proportion of circulating CD4+ and CD8+ T cells with an RTE phenotype (CD45RA+ CD62L+ CD31+) was observed in HTx patients than in non-HTx subjects (Figure 2A and C). Among HTx patients, those diagnosed with TCMR and those without rejection had similar percentages of CD4+ or CD8+ RTE T cells. At variance, patients diagnosed for an AMR had a higher proportion of CD4+ RTE cells (mean: 14.2% of CD4+ T cells) than 0R ($P < .01$) and TCMR subjects did ($P < .05$; Figure 2A). In addition, there were more CD8+ RTE T cells in AMR patients in comparison with TCMR subjects (Figure 2C). As expected, the frequency of RTEs among CD4+ T cells was inversely related to age ($r = -0.5$, $P = .001$; Figure 2B). However, the differences observed in RTE percentages between groups could not be explained by the age of the patients, since the mean recipient age was similar in each group (Table 1). The proportion of effector memory (EM) cells among CD4+ and CD8+ T cells was lower in AMR patients than in 0R subjects (Figure 2D). A lower proportion of circulating T follicular helper (Tfh) cells among CD4+ T cells was observed in HTx patients than in non-HTx subjects (Figure S1B). Among Tfh cells, the percentage of Tfh1 cells was lower in HTx patients than in non-HTx controls, with a concomitant increase in the proportion of Tfh2 cells (Figure S1C-D). The proportion of Tfh1 cells tended to be lower in AMR than in 0R patients and was significantly lower than in TCMR subjects (Figure S1C). Conversely, AMR patients displayed significantly more Tfh2 cells than TCMR individuals did (Figure S1D). There was no statistically significant difference in the proportion of Tfh17 cells (Figure S1E), or in the percentage of regulatory T cells (Tregs) among CD4+ cells between the different subgroups of HTx patients (Figure S2). HTx patients displayed a significantly lower proportion of circulating B cells among CD45+ lymphoid cells than non-HTx controls (Figure S3B). There was however no difference in the repartition of B cell subpopulations (naive, transitional, memory, class-switched (CS) or not) among the different subgroups of HTx patients (Figure S3C-E). Altogether, these T and B cell phenotypic analyses indicate that AMR

TABLE 1 Characteristics of the heart transplant patients

	All (n = 28)	0R (n = 17)	AMR (n = 6)	TCMR (n = 5)
Mean recipient age, years (range)	45 (22-69)	46 (22-69)	42 (24-66)	47 (27-63)
Mean donor age, years (range)	42 (19-61)	43 (26-61)	40 (25-60)	39 (19-52)
Male recipients, n (%)	21 (75.0%)	13 (76.4%)	4 (66.7%)	4 (80.0%)
Mean recipient BMI, kg/m^2	25.1	24.8	24.7	26.4
Primary heart disease, n (%)				
Ischemic cardiomyopathy	12 (42.8%)	9 (52.9%)	1 (16.7%)	2 (40%)
Nonischemic cardiomyopathy	12 (42.8%)	5 (29.4%)	5 (83.3%)	2 (40%)
Valvular cardiomyopathy	2 (7.2%)	1 (5.9%)	0 (0%)	1 (20%)
Congenital heart disease	1 (3.6%)	1 (5.9%)	0 (0%)	0 (0%)
Miscellaneous	1 (3.6%)	1 (5.9%)	0 (0%)	0 (0%)
Mean time since transplant, months	21.1	23.3	18.7	16.4
Immunology				
Presence of DSA, n (%)	10 (35.7%)	2 (11.8%)	6 (100%)	2 (40%)
Immunodominant class I	3 (30%)	0 (0%)	2 (33.3%)	1 (50%)
Immunodominant class II	7 (70%)	2 (100%)	4 (66.7%)	1 (50%)

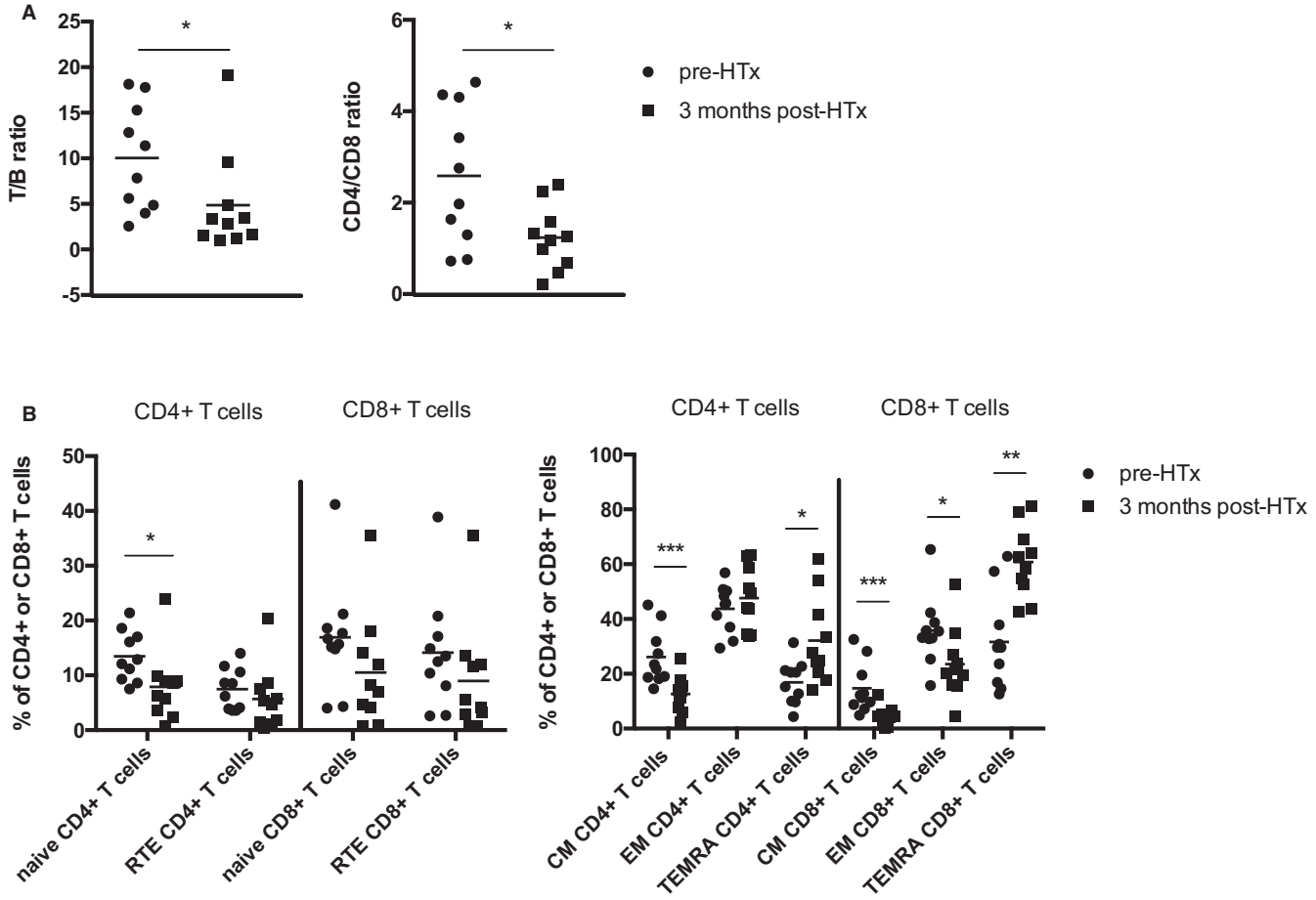

FIGURE 1 Effects of rATG on lymphocyte subpopulations in the early posttransplantation period. (A-B) The proportion of B and T lymphocytes and naive, RTE, CM, and TEMRA CD4+ and CD8+ T cells was assessed by flow cytometry in 10 patients, before and 3 months after heart transplantation. **P* < .05, ***P* < .01, ****P* < .001

patients tend to develop more Tfh2 cells while the TCMR patients tend to have more Tfh1 cells, and that the extent of B cell depletion does not account for the type of rejection that the patients will eventually develop.

3.4 | The larger CD4+ RTE T cell compartment in AMR patients is not due to a lower sensitivity to the rATG treatment

Distinct pretransplantation lymphocyte counts and/or initial lymphodepletion induced by the rATG treatment could account for the differences observed in patients who later developed an AMR. Although there was no difference in the pre-transplantation lymphocyte count among the different groups of HTx patients, the 24-hour posttransplantation lymphocyte count was significantly higher in patients who later developed an AMR (Figure 3A). The mean lymphocyte count measured on blood samples performed during the first 5 days after transplantation tended also to be higher in AMR patients (Figure 3A). We hypothesized that the lower lymphodepletion in patients that later developed AMR could be explained by a lower sensitivity to the rATG treatment due to distinct binding of rATG antibodies to their lymphocytes.

To address this question, we set up an assay wherein rATG binding on patients' lymphocytes was revealed with a fluorochrome-conjugated anti-rabbit antibody. First, we found that the fixation of rATG antibodies was more pronounced on T and B lymphocytes than on neutrophils, monocytes, and platelets (Figure 3B). The fixation of rATG, assessed by the MFI of the anti-rabbit antibody staining, on T cells, CD4+, RTE, and CD8+ T cells were similar in all groups of HTx patients (Figure 3C). We concluded that rATG bound equally well to T cells of AMR and other HTx patients and that the more prominent RTE population observed in AMR patients could not be explained by a lowered fixation of rATG on their circulating cells.

3.5 | Antibody-mediated rejection is associated with a more efficient thymopoiesis in HTx patients

Given that AMR patients appeared to be as sensitive as the other HTx patients to the rATG treatment, the higher percentage of CD4+ RTE T cells in their circulating blood could be due to a better thymopoiesis. To directly address this issue, we analyzed the distinct TREC molecules in peripheral blood cells as described.[16] We found that HTx patients had a lower sj/β-TREC ratio than non-HTx subjects (Figure 4A), indicating that transplantation itself and/or the

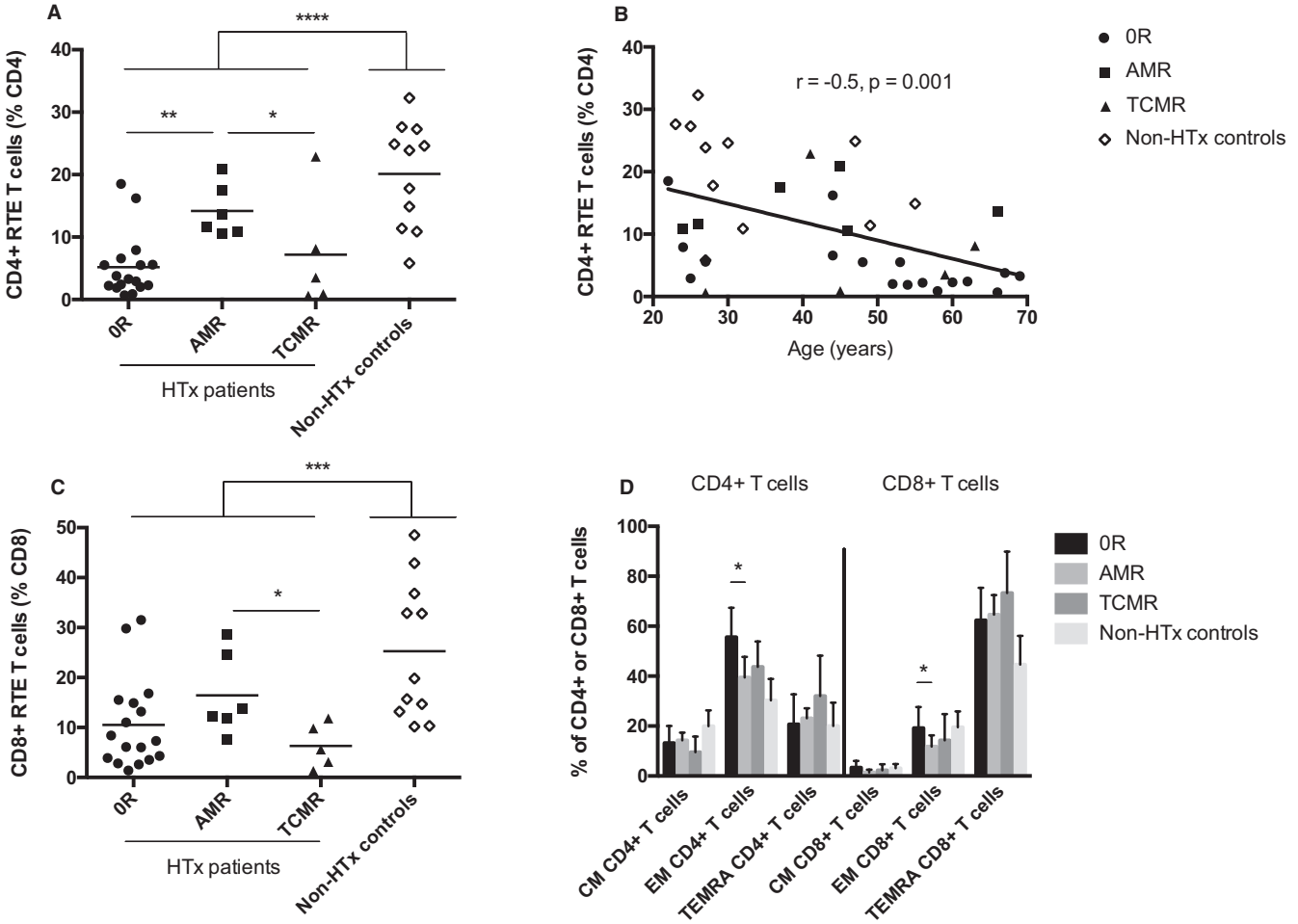

FIGURE 2 Analysis of T cells in HTx patients. (A-C) The proportion of circulating CD4+ and CD8+ T cells with an RTE phenotype (CD45RA+ CD62L+ CD31+) was determined by flow cytometry in 0R (n = 17), AMR (n = 6), and TCMR (n = 5) subjects, and in non-HTx controls (n = 11). (B) Correlation between age and proportion of RTEs among CD4 T cells. (D) The proportion of CM, EM, and TEMRA among CD4+ and CD8+ T cells was assessed by flow cytometry in 0R, AMR, and TCMR patients, and in non-HTx controls. *P < .05, **P < .01, ***P < .001, ****P < .0001

associated immunosuppressive regimen decreased thymic function. Remarkably, among HTx patients, those diagnosed for an AMR had a higher sj/β-TREC ratio than 0R and TCMR subjects (Figure 4A). The percentage of CD4+ RTE T cells was positively correlated with the sj/β-TREC ratio, confirming the predominant thymic origin of the CD45RA+ CD62L+ CD31+ CD4 T cells (Figure 4B). We next measured IL-15 and IL-7 plasma levels. IL-15 plasma level was significantly higher in HTx patients than in non-HTx subjects. There was however no difference in IL-15 plasma levels between the different subgroups of HTx patients, indicating that IL-15-induced peripheral homeostatic proliferation was not responsible for the observed expansion of CD4+ RTE T cells in the AMR group (Figure 4C). IL-7 levels of the patients and control subjects were all below the detection limit. Altogether, these results indicated that AMR patients had either an intrinsic better capacity to reactivate their thymic function or had a better preservation of their thymuses during the cardiothoracic surgery. We were able to get residual thymic tissue obtained from recipient patients during heart transplantation and could observe, embedded in the adipose tissue, lymphoid islets containing Hassall's corpuscles, confirming the persistence of thymic structures in transplanted patients from which they may be able to reactivate their thymic function (Figure S4).

3.6 | Thymic surface area and density are higher in AMR patient

CT scan imaging was performed on 3 HTx patients to assess thymic surface area and density: P1 was a 66-year-old AMR patient, P2 was a 59-year-old TCMR patient, and P3 was a 66-year-old 0R patient. In P1 patient, the thymus had a surface area of 567 mm^2 with a density of -53 HU. Density was -85 and -99 HU in P2 and P3 patients, indicating a fatty replacement of thymic tissue, which did not allow for individualization of a residual thymus with measurable surface (Figure 5A-C). P1 had a higher proportion of circulating CD4+ RTE cells (12.3%), in sharp contrast with P2 (TCMR) and P3 (0R) patients, respectively, displaying 3.5% and 3.8% of circulating CD4+ RTE cells. As an additional control, we analyzed the CT scan of the thymus of a 66-year-old healthy subject (without heart transplantation) in whom we failed to individualize a residual thymus, with a measured density of -132 HU in the thymic locule (Figure 5D).

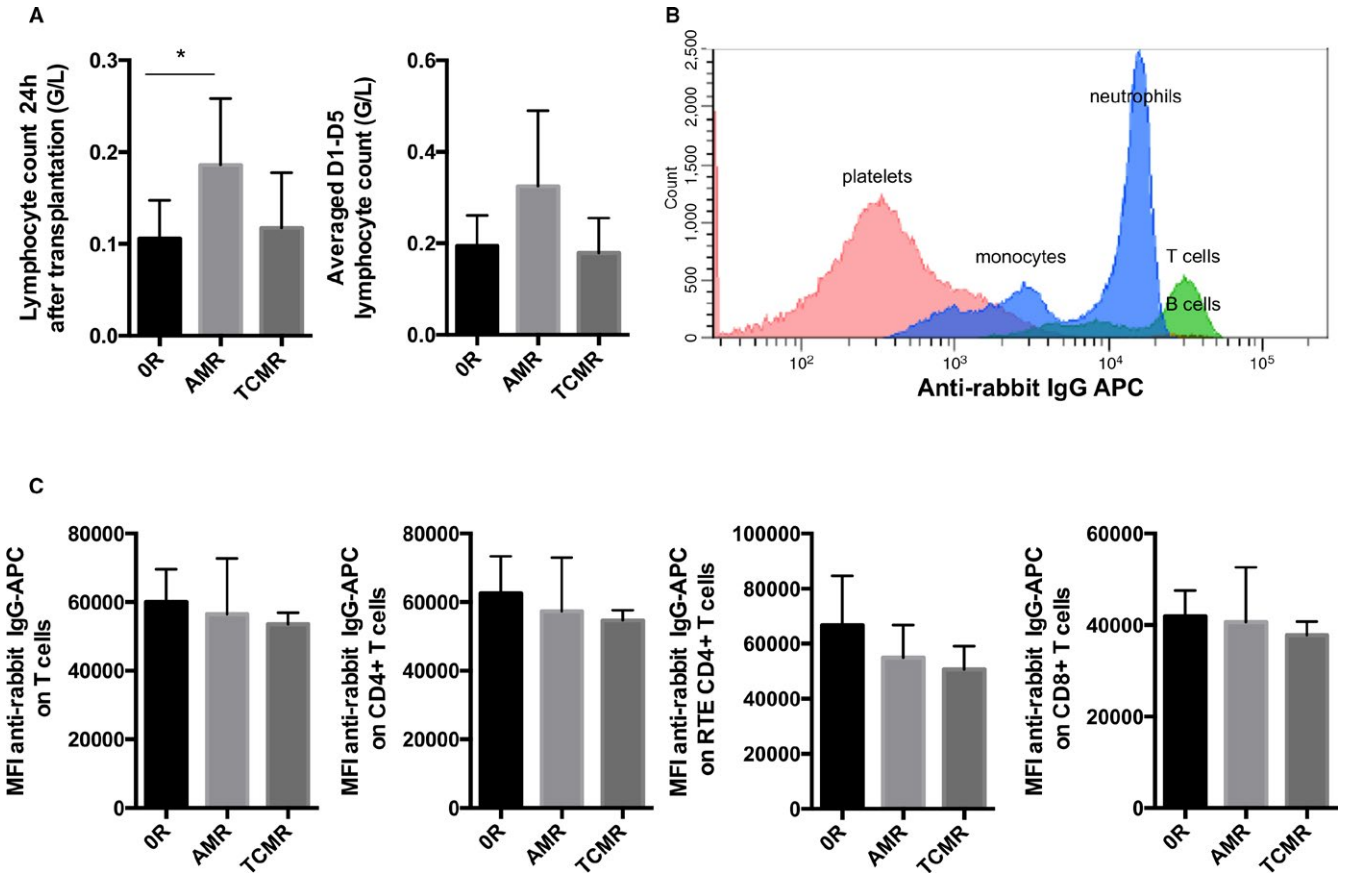

FIGURE 3 The larger naive RTE CD4+ T cell compartment in AMR patients is not explained by a difference of sensitivity to rATG induction. (A) Lymphocyte count 24 hours after transplantation and averaged lymphocyte count calculated from day 1 to day 5 (averaged D1-D5) after transplantation in HTx patients. (B) Fixation of rATG to circulating blood cells evaluated after incubation of rATG and subsequent detection by flow cytometry of its binding with anti-rabbit IgG coupled to APC. (C) Quantification by flow cytometry of IgG MFI (mean fluorescence intensity) on T cells, CD4+, RTE, and CD8+ T cells in the different subgroups of HTx patients. *$P < .05$ [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Our study is the first to demonstrate that thymic function is a key element in the onset of AMR in heart-transplanted patients. Indeed, patients diagnosed with AMR had higher proportion of RTEs and TREC levels than patients with cellular or no evidence of rejection.

We first evaluated thymic activity by the quantification of peripheral CD45RA+ CD62L+ CD31+ CD4 T cells and showed that this population was more prominent in patients diagnosed with AMR. This phenotype characterizes the population of RTEs, which are naive peripheral T cells that have recently emigrated from the thymus.[17] TRECs correspond to small circular DNA produced during the recombination of the genomic segments encoding the TCR alpha and beta chains. Calculation of the sj/β-TREC ratio is recognized as the most reliable measure of thymic output, reflecting intrathymic proliferation of T cell precursors.[18] The strong positive correlation observed between the proportion of RTEs among CD4+ T cells and sj/β-TREC ratio confirmed that our initial quantification of RTEs by flow cytometry was an accurate reflect of thymic function. Two subsets of naive peripheral CD4 T cells can be distinguished by expression of CD31: the first one expressing CD31 is highly enriched in RTEs and the second subset comprises naive CD4+ T cells that have proliferated in the periphery and have downmodulated CD31 expression following TCR-induced activation.[17] Peripheral proliferation of CD31+ naive T cells can also be driven by cytokines, especially IL-7 and IL-15, without loss of CD31 expression.[19] The positive correlation between the percentage of RTEs and sj/β-TREC ratio, as well as the absence of difference in IL-15 plasma levels between the different subgroups of heart-transplanted patients, suggested that CD31+ T cells had indeed a thymic origin rather than resulting from cytokine-induced homeostatic proliferation. These results have indicated that a better thymic function predisposes HTx patients to AMR. In line with this, Bamoulid et al recently demonstrated that a high proportion of RTEs before transplantation could also predispose ATG-treated renal-transplanted patients to an acute cellular rejection, thereby suggesting that pretransplantation immune senescence could decrease the risk of acute rejection.[20] In a previous study, Morgun et al found higher TREC levels in HTx patients experiencing acute rejection.[21] Nonetheless, this work could not investigate AMR patients because it was conducted in 2004, before the standardization of histologic criteria of acute AMR.

These results were unexpected, since previously published data showed that thymic function was required for tolerance induction.[9-12] Although our results and those from this group appear conflicting, they

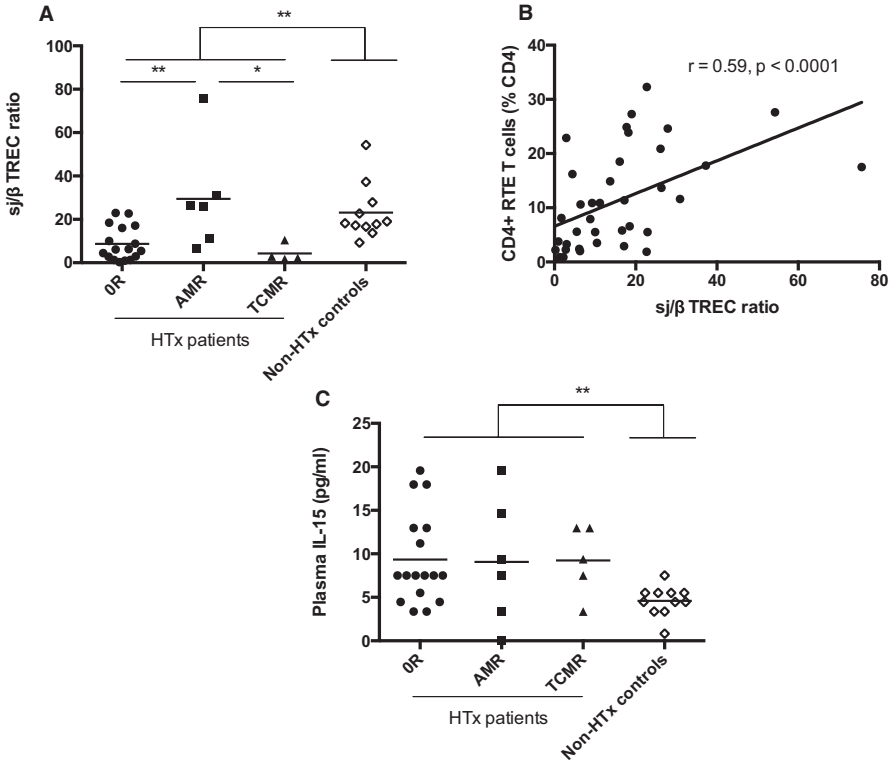

FIGURE 4 Antibody-mediated rejection is associated with a more efficient thymopoiesis in HTx patients. (A) Intrathymic precursor T cell proliferation was assessed by quantification of sj/β-TREC ratio by quantitative polymerase chain reaction, in HTx patients (n = 28) and non-HTx subjects (n = 11). (B) Correlation between CD4+ RTE T cell count and sj/β-TREC ratio. (C) Luminex quantification of IL-15 plasma levels in the different subgroups of HTx patients and non-HTx controls. *P < .05, **P < .01

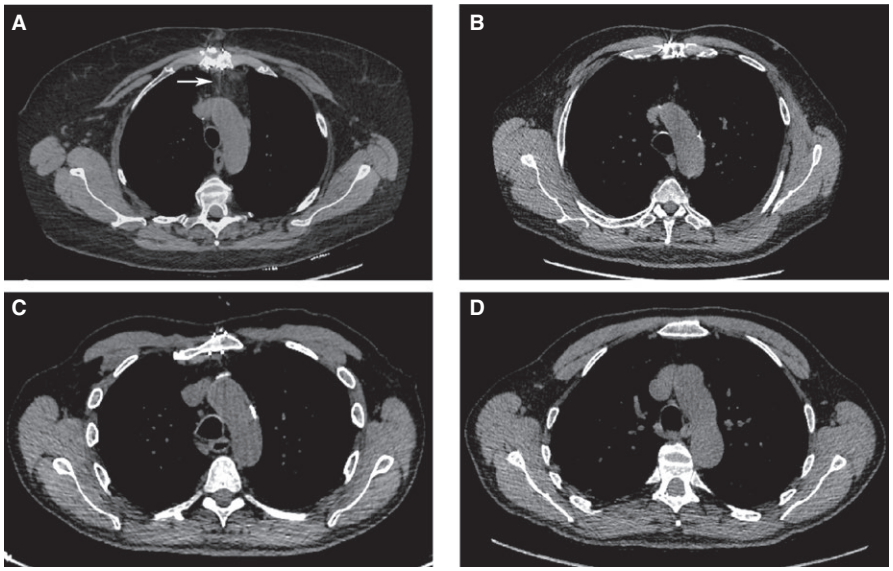

FIGURE 5 Thymic surface area and density are higher in AMR patient. Thymic surface area and density assessed by CT scan (axial plane) were strikingly higher in a 66-year-old AMR patient (A, white arrow), in contrast with a 59-year-old TCMR patient (B), a 66-year-old 0R patient (C), and a 66-year-old healthy subject (D) in whom residual thymic tissue was not detected

may instead indicate that the thymus could serve a dual role: on one side, it might promote T cell tolerance and prevent cellular-mediated rejection and on the other side, its overactivation, as observed in our AMR patients, may prompt humoral alloimmune responses through a mechanism that remains to be elucidated. Of note, it has been recently demonstrated that the perivascular space in the human thymus is a functional niche for viral-specific plasma cells.[22] We may speculate that it could host alloreactive plasma cells in transplanted patients, an issue that will be difficult to address, since the access to this tissue is rare, and even more in transplanted patients.

We found that thymic surface area and density were higher in AMR patient than in 0R or TCMR patients and that this parameter was correlated with the proportion of circulating RTEs. An extensive study assessed CT characteristics of the thymus in healthy adults and showed that the oldest patient with observable residual thymic tissue was 54 years old.[23] Of interest, we observed a large residual thymus in a 66-year-old HTx AMR patient. Thymic enlargement has already been observed in other pathological conditions, such as in HIV-infected patients after antiretroviral therapy introduction, and was an accurate reflect of thymic function.[7] To our knowledge, our study is the first to assess thymus by CT scan in transplanted patients and to correlate this finding with rejection onset.

The composition of the T cell repertoire in HTx patients mainly depends on the ATG-induced lymphodepletion and the subsequent lymphocyte replenishment achieved by thymopoiesis and peripheral

homeostatic proliferation. In this perspective, our study strongly suggests that thymopoiesis is a major determinant of AMR onset in HTx patients, even though the size of the studied cohort was limited and our results will need to be confirmed in a larger study and replicated in a validation cohort. AMR considerably reduces long-term function of the allografts and thymectomy might be a prophylactic strategy to prevent alloimmune humoral responses. Whether thymectomy should be proposed also as therapeutic strategy in established AMR will require additional studies to test if thymectomy can reduce DSA titers once they have appeared. Finally, the 3 most used thymic-function related markers, that is, assessment of blood TREC levels, quantification of RTE CD4+ T cells, and imaging evaluation of thymus, could represent an interesting tool set to identify patients at high risk of AMR.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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