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Reduced CD4^{+} CD25^{++} CD45RA^{-} Foxp3^{hi} activated regulatory T cells and its association with acute rejection in patients with kidney transplantation

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A R T I C L E I N F O

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A B S T R A C T

Background: It was found that regulatory T cells (Tregs) importantly affect the maintenance of the kidney graft. However, Tregs are a heterogeneous population with less to more suppressive activity. The aim of this study was to determine the effects of different subsets of Tregs, as well as their ratio to effector T cells (Teff), on kidney transplantation outcomes.
Methods: A total of 58 participants were enrolled in this study and divided into four groups: (i) first kidney transplant recipients (stable 1); (ii) second kidney transplant recipients (stable 2); (iii) transplant recipients with acute rejection (AR); and (iv) healthy control subjects. By using flow cytometer, the frequencies of CD4^{+} CD25^{++} CD45RA^{-} Foxp3^{hi} activated Tregs (aTregs), CD4^{+} CD25^{+} CD45RA^{+} Foxp3^{lo} resting Tregs (rTregs), CD4^{+} CD25^{+} CD45RA^{-} Foxp3^{lo} non-suppressive T cells, CD4^{+} CD25^{+} Foxp3^{-} cells Teff, and total Tregs were analyzed in all subjects.
Results: The frequency of aTregs (as well as the ratio of aTregs/Tregs) was significantly lower in the AR patients than the other three groups. In contrast to AR patients, stables 1 and 2 had a higher aTreg/Treg ratio than those in the control group. Although patients with AR had a significantly lower total Tregs than the other three groups, the balance of total Tregs and Teff was similar between patients with and without AR.
Conclusion: Patients with AR had poorer immunoregulatory properties than those with normal graft functioning, as well as those in the control group. These reduced immunoregulatory properties in patients with AR could lead to graft rejection.

1. Introduction

Transplantation is currently considered as the best treatment option for patients with end-stage organ failure (ESOF). However, the immune system of transplant recipients, including innate and adaptive immune cells, recognizes the damage-associated molecular patterns (DAMPs) or alloantigen of the allograft (e.g., MHC), which results in acute rejection (AR). AR has a negative impact on the transplantation outcome and could reduce the allograft survival rate. In this regard, immunosuppression drugs are used to control AR and increase the allograft acceptance rate.

Although AR is currently controlled well, chronic rejection still remains an elusive problem [1,2]. Some studies indicated that the immunoregulatory properties of transplant recipients are involved in operational tolerance and immunosuppression tolerance. It was also reported that patients with stable graft function had higher immunoregulatory cells (i.e., B and T regulatory cells) than the patients with either acute or chronic rejection [3–7]. A better understanding of the immunological properties of patients with normal graft function and those with rejection could lead to better control of patients and prevent AR.

CD4^{+} CD25^{+} Foxp3^{+} natural Tregs have a key role in the maintenance of homeostasis and suppression of self-reactive cells. High amounts of these cells are also associated with a better graft outcome

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[8–11]. Natural Tregs are considered as a heterogonous population based on the Foxp3 level and CD45RA expression. In this regard, they are divided into three different populations: (i) $CD4^+$ $CD25^{++}$ $CD45RA^-$ $Foxp3^{hi}$ activated Tregs (aTregs); (ii) $CD4^+$ $CD25^+$ $CD45RA^+$ $Foxp3^{lo}$ resting Tregs (rTregs); and (iii) $CD4^+$ $CD25^+$ $CD45RA^-$ $Foxp3^{lo}$ non-suppressive T cells [12]. Based on a study, aTregs are more suppressive than rTregs and express a high amount of inhibitory molecules, including cytotoxic T-lymphocyte associated protein 4 (CTLA4), lymphocyte activating 3 (LAG3), TNF receptor superfamily member 18 (TNFRSF18 [GITR]), CD39, and CD73 [1]. Another study indicated that rTregs could convert to aTregs and express a high level of Foxp3 upon activation [12]. The frequency of the $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs is broadly investigated in kidney transplant recipients [9,11]. However, there are poor data regarding the percentage of aTregs and rTregs in patients with AR and those with the first and/or second stable allograft. In the present study, we aimed to investigate the frequency of different populations of Tregs, as well as the frequency of total Tregs and $CD4^+$ $CD25^+$ $Foxp3^-$ T cells, as effector T cells (Teff).

2. Materials and methods

2.1. Study subjects

A total number of 58 participants were enrolled in this study as follows: A number of 19 patients were first kidney transplant recipients with normal graft function (stable 1) (mean age ± SD = 41 ± 10, 12 males, 7 females); 12 patients were second kidney transplant recipients with normal graft function (stable 2) (mean age ± SD = 42 ± 10, 7 males, 5 females); seven patients had AR (mean age ± SD = 43 ± 3, 3 males, 4 females); and 20 subjects were in the healthy control group (mean age ± SD = 37.3 ± 9.7, 4 males, 16 females). All patients had a < 10% panel-reactive antibody (PRA) and received their graft from living donors. Stables 1 and 2 had a creatinine level of ≤1.4 mg/dL with no sign of rejection or any allograft dysfunction at the time of sampling. AR of the third group was approved by biopsy. AR occurred in stable 1 within one year after transplantation, and stable patients are transplanted for < 10 years.

2.2. Sampling

The blood collection of stables 1 and 2 was done in the clinic of a medical doctor, and of the third group was done at Shahid Beheshti Hospital, Babol, Iran. Blood was immediately collected from patients with AR before any anti-rejection therapy. Written informed consent was obtained from all 58 participants, and the study was approved by the Institutional Research Ethics Committee, Shahid Beheshti Hospital, Babol University of Medical Sciences. The clinical and demographic characteristics of patients are shown in Table 1.

Table 1
Demographic and clinical characteristics of study subjects.

Features	Stable 1 (19)	Stable 2 (12)	AR (7)
Age (year)*	41 ± 10	42 ± 10	37 ± 8
Gender (male/female)	12/7	7/5	3/4
Cr (mg/dl)*	1.1 ± 0.2	1 ± 0.2	3.94 ± 5
BUN (mg/dl)*	19 ± 5	15 ± 5	37 ± 19
WBC ($\times 10^3/\mu l$)**	7.6 (4.8–13.7)	6.7 (4–9)	–
FBS (mg/dl)**	96 (78–181)	92 (71–128)	188 ± 70
TG (mg/dl)**	121 (99–331)	130 (51–518)	–
Cholesterol (mg/dl)*	183 ± 35	167 ± 23	–

Note. Cr: Creatinine; BUN: Blood urea nitrogen; WBC: White blood cell; FBS: Fasting blood surge; TG: Triglycerides; AR: Acute rejection.
* Data are shown as mean ± SD.
** Data are shown as median, range.

2.3. Immunosuppressive drugs

Maintenance immunosuppressive regimens were standard triple therapy, which consisted of calcineurin inhibitor (CsA microemulsion or Tacrolimus) and combined with mycophenolate mofetil (MMF) and prednisone.

2.4. Peripheral blood mononuclear cell isolation

The peripheral blood (6 mL) was collected into an EDTA anticoagulant tube. Peripheral blood mononuclear cells (PBMCs) were obtained from all participants by using the Ficoll-Hypaque gradient (Biowest, Nuaille, France) centrifugation. In brief, the peripheral blood directly added to the Ficoll-Hypaque gradient and was centrifuged at 400 x*g* for 30 min. The middle phase (i.e., PBMCc) was collected and washed with phosphate-buffered saline (PBS) at 300 x g for 12 min. PBMCs contained monocytes that could interfere with results. Thus, PBMCs were incubated in a culture plate (SPL, Korea) for 30–45 min in order to decrease the number of monocytes.

2.5. Phenotypic analysis of T cells

PBMCs were collected from the culture plate and were stained (0.6×10^6) for cell surface markers, i.e., CD4, CD25, and CD45RA, which followed by intracellular staining of Foxp3. Before staining, fragment crystallizable (Fc) blocker (Biolegend, USA) was used for blocks of Fc receptors. In brief, BB515-conjugated anti-CD25 monoclonal antibodies (mABs) (Clone 2A3, BD Biosciences, USA), PerCPCy5.5-conjugated anti-CD45RA mABs (Clone HI100, Biolegend, USA), APC-conjugated anti-CD4 mABs (Clone RPA-T4, BD Biosciences, USA), respectively, were added to the cellular suspension and were incubated at 4 °C for 20 min. After that, the cellular suspension was fixed and permeabilized via Fixation and Permeabilization Buffer Set (eBiosciences, USA), followed by intracellular staining via PE-conjugated anti-Foxp3 mABs (Clone 236A/E7, BD Biosciences, USA). The cells were read by a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed by FlowJo (version 7.6.1; Tree Star Inc., Ashland, OR, USA) software.

2.6. Statistical analysis

Data were analyzed via SPSS 24 (IBM, Armonk, NY, USA) and GraphPad Prism software (Version 6, USA). First, the normality check of each parameter was assessed. The one-way analysis of variance (ANOVA) test was used for normally distributed data, and those with non-normal data were analyzed by the Kruskal-Wallis test. A *P*-value of < 0.05 was considered significant in all statistical tests. Data are presented as mean ± SD for normally distributed data and as median (interquartile range [IQR]) for non-normal data.

3. Results

3.1. Demographic and clinical characteristics of study subjects

The demographic and clinical features of kidney transplant recipients are shown in Table 1. The transplant recipient information is based on age- and sex-matched.The creatinine between stables 1 and 2 is the same.

3.2. Patients with acute rejection had lower level of circulating aTregs

To determine the frequency of $CD4^+$ $CD25^{++}$ $CD45RA^-$ $Foxp3^{hi}$ aTregs (II), $CD4^+$ $CD25^+$ $CD45RA^+$ $Foxp3^{lo}$ rTregs (I), and $CD4^+$ $CD25^+$ $CD45RA^-$ $Foxp3^{lo}$ non-suppressive T cells (III) (Fig. 1), PBMCs were isolated from patient and control groups. We showed that patients with AR had a significantly lower level of $CD4^+$ $CD25^{++}$ $CD45RA^-$

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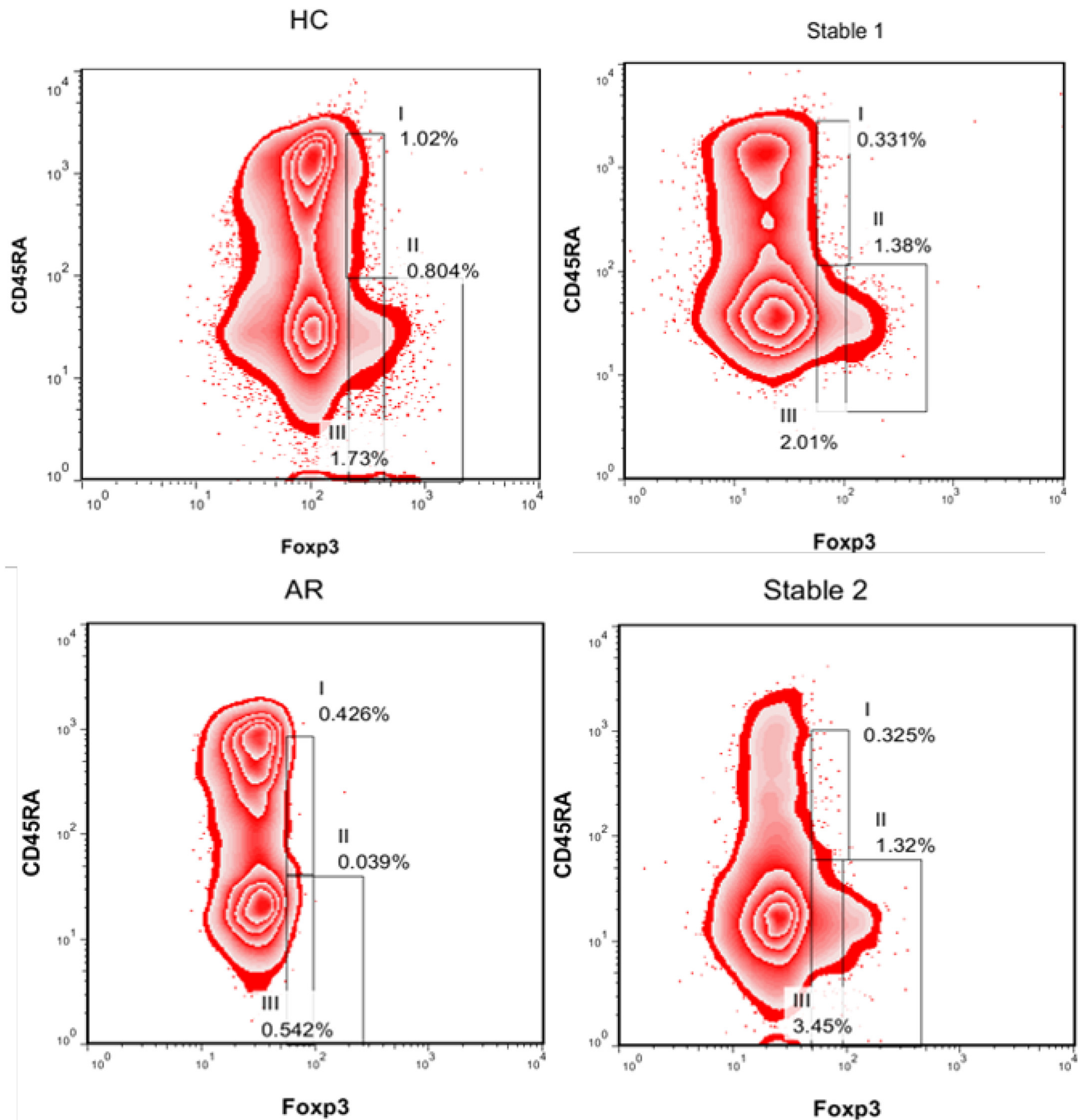

Fig. 1. The analysis of different groups based on CD45RA and Foxp3.
Note. HC: Healthy control; AR: Acute rejection. The gating of aTregs, rTregs, and non-suppressive T cells are presented in each group.

$Foxp3^{hi}$ aTregs than stable 1 (P = .03), stable 2 (P = .005), and control (P = .039) group. The differences were not significant between stable 1, stable 2, and control group. However, stable 2 had a higher level of aTregs than stable 1 and control group (Fig. 2A). The control group had a significantly higher level of $CD4^+$ $CD25^+$ $CD45RA^+$ $Foxp3^{lo}$ rTregs than the AR patients (P < .001), stable 1 (P = .009), and stable 2 (P < .001) (Table 2). Moreover, stable 1 had higher level of rTregs than stable 2 (Figs. 1 and 2B). Stable 2 and control group had a significantly higher level of $CD4^+$ $CD25^+$ $CD45RA^-$ $Foxp3^{lo}$ non-suppressive T cells than stable 1 (P = .039 and 0.025, respectively) (Figs. 1 and 2C). The gating strategy and phenotypic analysis of T cells are shown in Fig. 3A and Table 2, respectively.

3.3. Patients with acute rejection had a lower level of circulating Tregs

Since the $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs have different populations on the basis of CD45RA expression and Foxp3 level, a different gating strategy was used to distinguish real Tregs. In this regard, we considered the rTregs (I) and aTregs (II) as total Tregs because the third population of $CD4^+$ $CD25^+$ $Foxp3^+$ conventional Tregs was shown as

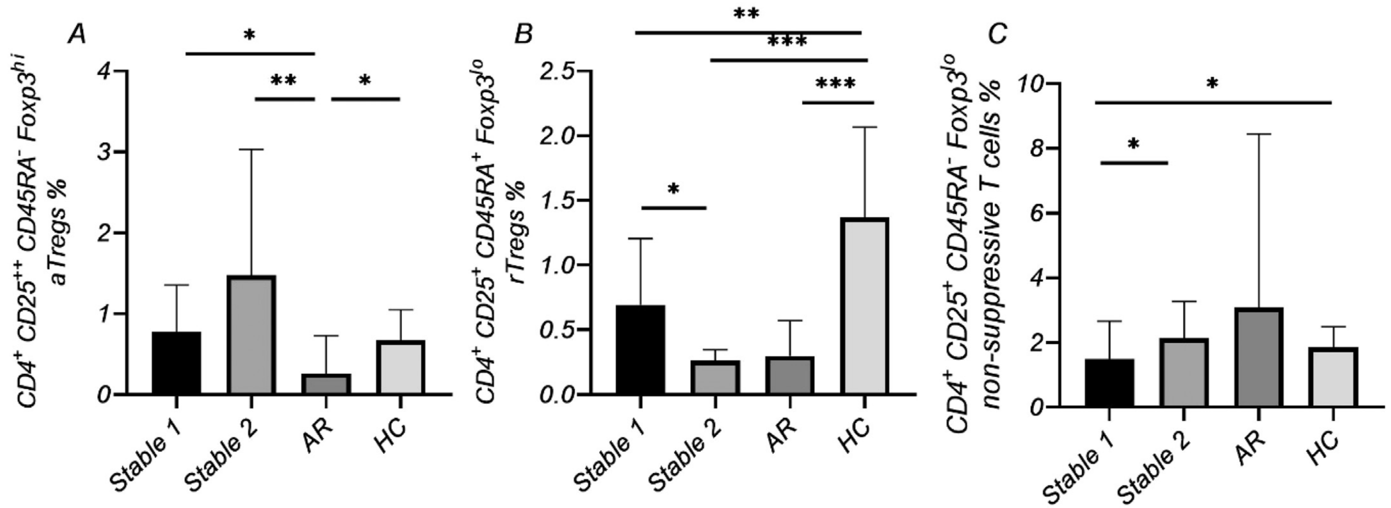

Fig. 2. The frequency of aTregs (A), rTregs (B), and non-suppressive T cells (C) are presented for all groups in different graphs.
Note. HC: healthy control; AR: Acute rejection.

Table 2
Frequency of different phenotype of T cell in the subjects.

Cell type*	Stable 1	Stable 2	AR	HC	P-value
$CD4^+$ lymphocyte**	27.6 ± 10.57	21.09 ± 6.97	21.11 ± 9.08	39.38 ± 10.87	< 0.001
$CD4^+$ $CD25^+$ $Foxp3^-$ Teff***	30 (25.95–39.55)	30 (11–37.9)	9.4 (8.71–12.7)	18.6 (15.45–21.87)	0.001
(aTregs + rTregs) Tregs***	1.3 (1–1.72)	1.6 (0.57–1.98)	0.26 (0.14–0.4)	2.2 (1.1–2.84)	0.011
Treg/Teff ratio***	0.045 (0.03–0.08)	0.043 (0.03–0.07)	0.032 (0.02–0.04)	0.103 (0.07–0.15)	0.006
$CD4^+$ $CD25^{++}$ $CD45RA^-$ $Foxp3^{hi}$ aTregs***	0.63 (0.46–0.88)	1.32 (0.39–1.71)	0.04 (0.02–0.16)	0.68 (0.42–0.92)	0.045
aTreg/Treg ratio**	0.5 ± 0.22	0.73 ± 0.21	0.28 ± 0.29	0.33 ± 0.1	< 0.001
$CD4^+$ $CD25^+$ $CD45RA^+$ $Foxp3^{lo}$ rTregs***	0.53 (0.38–0.77)	0.27 (0.19–0.32)	0.13 (0.13–0.37)	1.26 (0.88–1.86)	< 0.001
$CD4^+$ $CD25^+$ $CD45RA^-$ $Foxp3^{lo}$ non-suppressive T cells***	1.14 (0.91–1.57)	1.88 (1.18–2.94)	0.54 (0.42–1.78)	1.80 (1.21–2.19)	0.046

Note. HC: Healthy control; AR: Acute rejection.
* Data are shown from $CD4^+$ T cells.
** Data are shown as mean ± SD.
*** Data are shown as median (IQR).

non-Tregs (III), which produce a considerable amount of cytokines interleukin 2 (IL 2) and interferon $_\gamma$ (IFN-$_\gamma$) [12]. We showed that the frequency of Tregs were significantly lower in the AR group than in stable 1 ($P = .04$), stable 2 ($P = .044$), and control group ($P = .001$) (Figs. 1 and 4A). The differences were not significant in other groups, but control subjects had a higher frequency of Tregs than stables 1 and 2.

We also evaluated the aTreg/Treg ratio and found that AR had a lower ratio compared with stables 1 and 2 ($P = .055$ and < 0.001, respectively). However, the ratio was similar to the control group. Both stables 1 and 2 had a higher aTreg/Treg ratio compared with the control group ($P < .05$ and < 0.001, respectively) (Fig. 4C).

As it was expected, the control group had a significantly higher frequency of $CD4^+$ lymphocytes than stable 1 ($P = .003$), stable 2 ($P < .001$), and AR group ($P = .003$). The differences were not significant between other groups, but stable 1 had higher $CD4^+$ lymphocytes than stable 2 and AR group (Fig. 4B).

3.4. Observation of greater effector T cells in stable patients

$CD4^+$ $CD25^+$ $Foxp3^-$ cells were considered as Teff. Teff were significantly higher in stables 1 and 2 than in those with AR ($P < .001$ and = 0.004, respectively). Moreover, Teff were significantly higher in stable 1 than in the control group ($P < .01$ [Fig. 5]). AR patients had lower Teff than control subjects, but the difference was not significant. Since the activation or inhibition of immune response depends on stimulatory and inhibitory signals, we evaluated the Treg/Teff ratio in the subjects. The ratio of Tregs/Teff was not significantly different between stables 1 and 2, as well as the AR group. However, all patients in stables 1 and 2, as well as the AR group, had a significantly lower Treg/Teff ratio than the control group (Fig. 5).

4. Discussion

The main cause of graft failure is immune responses against the allograft. Various immune cells, including dendritic, T, B, natural killer cells, and neutrophil, could be activated against the allograft. However, the balance between stimulatory and regulatory cells could change the fate of the allograft outcome. In other words, different immunological properties of transplant recipients contribute to tolerance or rejection of the allograft [1,13]. $CD4^+$ $CD25^+$ $Foxp3^+$ natural Tregs (as main cells) contribute to graft tolerance. The higher amount of Tregs is associated with stable graft function. Regarding CD4, CD25, and Foxp3, several studies reported that patients with stable graft function had a higher frequency of Tregs than patients with either chronic or acute rejection [8–11].

Fig. 3. Gating strategy and concomitant expression of CD25 and Foxp3.
Note. Lymphocytes were gated and separated based on CD4. CD4$^+$ lymphocytes were analyzed based on CD45RA and Foxp3 to achieve Tregs, and then Tregs were gated to separate the aTregs (I), rTregs (II), and non-suppressive T cells (III) (A). When CD4$^+$ lymphocytes were gated based on CD45RA and CD25, only the fractions of I, II, and III were Foxp3$^+$ (B). Moreover, aTregs, rTregs, and non-suppressive T cells, which were gated based on CD45RA and Foxp3, express a different amount of CD25 (C).

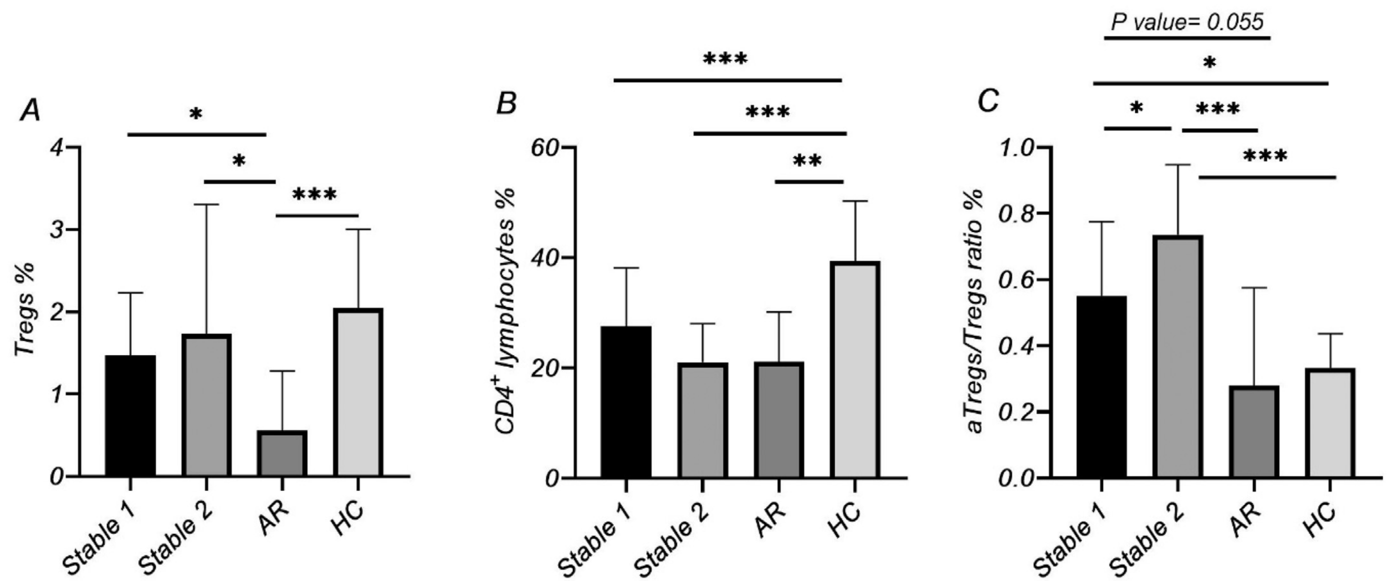

Fig. 4. The frequency of Tregs (A) and CD4$^+$ lymphocytes (B), as well as the ratio of aTregs/Tregs (C), are presented for all groups in different graphs.
Note. HC: Healthy control; AR: Acute rejection.

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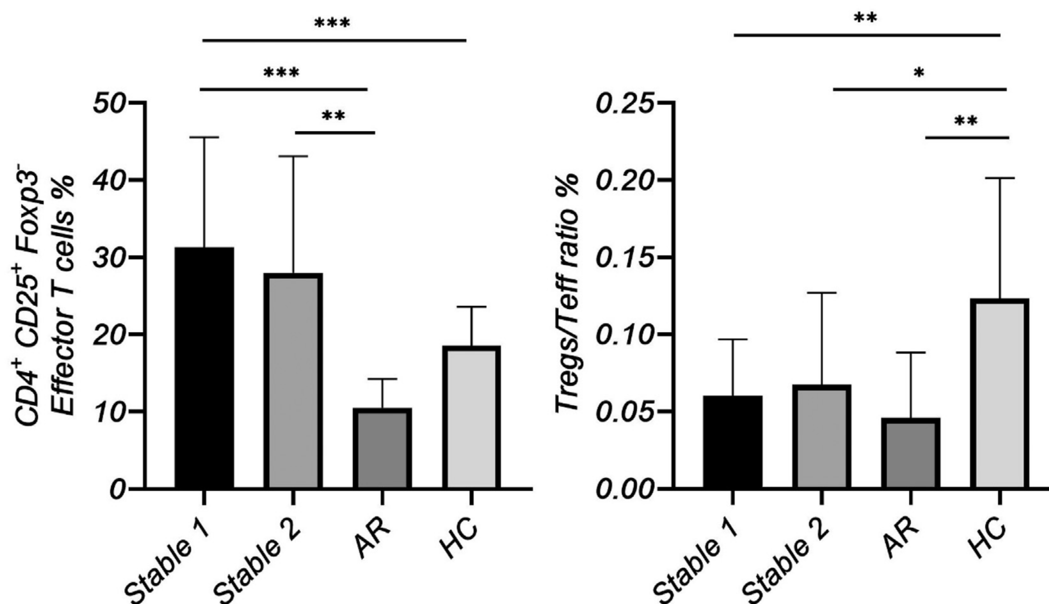

Fig. 5. The frequency of Teff and the ratio of Tregs/Teff are presented for all groups in different graphs.
Note. HC: Healthy control; AR: Acute rejection.

However, these Tregs may contain the third population (III) as non-suppressive T cells. In this regard, we considered the aTregs and rTregs as real total Tregs. Although CD25 was not used for gating Tregs, we showed that fractions I, II, and III differentially express CD25 (Fig. 3A and C). Moreover, when $CD4^+$ cells were evaluated based on CD45RA and CD25, only the fractions I, II, and III were $Foxp3^+$ cells (Fig. 3B). These results were consistent with Miyara and coworkers' study [12], and these three populations had a concomitant expression of CD25 and Foxp3. Both in vitro and in vivo analyses showed that rTregs could express a high level of Foxp3, down-regulate the expression of CD45RA, and become aTregs [12]. Here, we showed that patients with AR had remarkably a lower frequency of Tregs (rTregs + aTregs) in comparison to stables 1 and 2, as well as the control group. However, when Tregs and Teff of AR patients were analyzed together, a balance between Tregs and Teff was observed (discussed in the next fourth paragraph).

The CD45 molecule (a transmembrane glycoprotein) is a phosphatase that regulates the kinase belonging to the Src-family kinases. CD45 has different isoforms, such as CD45RA, CD45RB, and CD45RC. According to T cell differentiation and function, CD45 isoforms are expressed on their surface and led to efficient T cell activation upon T cell receptor engagement [1]. Miyara et al. (2009) indicated that $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs contain a population that secretes inflammatory cytokines, such as IL-2, 17, and IFN-$_\gamma$. Thus, they categorized $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs into three populations, i.e., $CD4^+$ $CD25^{++}$ $CD45RA^-$ $Foxp3^{hi}$ aTregs, $CD4^+$ $CD25^+$ $CD45RA^+$ $Foxp3^{lo}$ rTregs, and $CD4^+$ $CD25^+$ $CD45RA^-$ $Foxp3^{lo}$ non-suppressive T cells [12]. Braza et al. (2015) demonstrated that patients with chronic rejection had a lower frequency of aTregs than the operational tolerance group. They also showed that patients with operational tolerance had aTregs with a higher level of CD39 and GITR [11].

In consistence with the previous study, we showed that AR patients had a lower frequency of aTregs than stables 1 and 2, as well as the control group. Although Braza's study had different groups compared with our study, patients with normal graft function and rejection exist in both the studies. However, some studies used a different marker to discriminate aTregs from others [14,15]. Schaier et al. (2012) used human leukocyte antigen (HLA) DR to determine those Tregs with high suppressive activity. They showed that DR^{hi} $CD45RA^-$ Tregs were lower in patients with acute rejection than patients without rejection [15]. Generally, these data highlighted the immunoregulatory properties of patients with stable graft function.

To better understand the immunoregulatory properties of study subjects, we evaluated the aTreg/Treg ratio and found a lower aTreg/Treg ratio in the AR patients than the patients in stables 1 and 2. This result (as well as aTregs results in stables 1 and 2, besides AR patients) suggests that patients with AR remarkably loss their immunoregulatory properties. These results also highlighted the role of immune characteristics of transplant recipients on the allograft outcome. The results between stables 1 and 2 were interesting. Although there was no significant difference between stables 1 and 2 regarding the aTregs and aTreg/Treg and Treg/Teff ratios, the frequency of these cells was higher in stable 2 than the frequency of them in stable 1. In general, patients in stable 2 had stronger immunoregulatory properties than in stable 1.

Regarding Teff, results are controversy [9,16]. We showed that patients in stables 1 and 2 had a significantly greater level of Teff than the AR patients. Moreover, stable 1 also had a higher level of Teff than the control group. Teff and total Tregs were lower in the AR patients than stables 1 and 2. We further evaluated the Treg/Teff ratio to get reliable data regarding the balance between Tregs and Teff. At first glance, it seems that patients with normal graft function should have lower Teff than patients with rejection. However, the Treg/Teff ratio was not significantly different between stables 1 and 2, besides AR patients. Although Teff were higher in stables 1 and 2 than the AR group, the Treg/Teff ratio was not significantly different between them, implying that there is a balance of Tregs and Teff in the transplant recipients.

In general, it implies that a lower frequency of total Tregs in the AR patients cannot participate in or facilitate allograft rejection. This is because AR patients also had a low amount of Teff, and the ratio of Tregs/Teff was not significantly different between transplanted subjects. However, both the aTregs and ratio of aTregs/Tregs were lower in the AR patients than the stable subjects.

We acknowledge that our study had limitations, including small sample size and lack of functional analysis of aTregs and rTregs. Future studies with a larger sample size are needed. Moreover, the assessment of HLA-DR and glycoprotein A repetitions predominant (GARP) on the aTregs will reveal the association between HLA-DR and GARP activated Tregs with $CD4^+$ $CD25^{++}$ $CD45RA^-$ $Foxp3^{hi}$ aTregs.

In conclusion, a balance was observed between Tregs and Teff in the

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transplanted subjects. However, we showed that stable patients had considerable immunoregulatory properties in which the aTregs and aTreg/Treg ratio were greater than patients with AR. The results of this study are consistent with other studies, which showed that stable patients generally have great immunoregulatory properties. In this regard, regulatory T cell therapy could be improved by using those Tregs with great suppressive activity. Switching immunological properties of transplant recipients to the regulatory ones may result in the reduced use of immunosuppressive drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

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