LABORATORY STUDY

Analysis of FOXP3 Gene and Protein Expressions in Renal Allograft Biopsies and Their Association with Graft Outcomes

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Abstract

Background: The transcription factor FOXP3 is increased in acute renal rejection, but its influence on graft outcomes is unclear. This study correlated FOXP3 with dendritic cells and graft outcomes. Methods: We assessed 96 kidney transplants undergoing allograft biopsy for cause. FOXP3 mRNA was analyzed by real-time polymerase chain reaction (PCR) and FOXP3 protein and DCsCD83+ by immunohistochemistry. Graft function and survival were assessed at 5 years post-transplantation, as well as by independent predictors of graft loss. Results: Intragraft FOXP3 gene and protein expression were significantly correlated (r = 0.541, p < 0.001). Both FOXP3 mRNA and protein were increased in patients with acute rejection (AR). High expression of FOXP3 mRNA or protein in biopsies did not correlate with clinical variables, but there was a trend to higher positive variation in the glomerular filtration rate (GFR) from biopsy to last follow-up. Patients with FOXP3-mRNA high had more DCsCD83+ in biopsy, but these cells did not associate with AR. Five-year graft survival was not influenced by either FOXP3 mRNA or protein expressions. Conclusions: FOXP3 mRNA and protein had a good correlation in archival renal graft tissue. Increased FOXP3 expression was found in AR and FOXP3 associated with high numbers of DCs. However, both FOXP3 mRNA and protein was not associated with better allograft outcomes.

Keywords: renal transplantation, graft rejection, regulatory T cells, FOXP3, dendritic cells

INTRODUCTION

The avoidance of allograft rejection and the development of long-term tolerance in transplantation have been a focus of research. Regulatory T cells (Tregs) and FOXP3 transcription factor promote a state of antigen-specific peripheral tolerance by suppressing activation and expansion of T effector cells under specific conditions, as demonstrated in experimental models.1-5

The transcription factor FOXP3 is the most specific marker of regulatory T cells, and is expressed in CD4+CD25+ suppressor cells but not in other lymphocytes, B cells, and NK cells, and is minimal in other T cells such as CD8+ lymphocytes.2,3,5,6 Clinical studies have identified an increased expression of the FOXP3 gene and/or its protein in renal graft biopsies with acute rejection and also in urine and blood compartments.7-12 However, the impact of Tregs FOXP3+ on graft outcomes seems conflicting in these reports. On the one hand, these cells could direct a FOXP3-induced immune response toward suppression of T effector cells, promoting renal graft acceptance with improved function, and both higher short-7 and long-term survival.10 On the other hand, FOXP3+ Tregs would not indicate alloimmunity per se but rather act as regulatory cells generated in the inflammatory response elicited by rejection, where Tregs would be involved in time-dependent stabilization of inflammation.12 Currently, the role of Tregs FOXP3+ during renal allograft rejection and their impact on graft outcomes remains unclear.

Antigenic stimulation is required for the suppressive action of Tregs, and antigen-presenting cells (APCs), such as dendritic cells (DCs), are involved in this process.13 Mature DCs induce the proliferation of Tregs

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under experimental conditions and have been used in vitro to expand antigen-specific Tregs that suppress graft-versus-host disease. Donor-specific antigen-reactive Tregs may develop in transplant recipients as their immune system is continuously exposed to graft alloantigens, thus sustaining FOXP3 generation, selection, and expansion to control graft rejection and induce and maintain tolerance.

Immunosuppressive drugs are believed to modulate the number and function of circulating Tregs and FOXP3 expression. Experimental and clinical evidence have shown favorable effects of mTOR inhibitors and negative effects of calcineurin inhibitors (CIs), but the concomitant use of other agents in clinical transplantation makes their individual effects difficult to understand.

The current study aimed to evaluate FOXP3+ gene and protein expression in indication biopsies of renal allografts and correlate both with antigen-presenting cells as well as graft outcomes. We hypothesized that dendritic cells would be present along with FOXP3 in interstitial infiltrates during acute rejection and in the late post-transplant period.

METHODS

Design
This is a cross-sectional study with collection of historical data.

Patients
Three hundred and thirty-eight renal transplants were performed at the Transplant Unit of Hospital de Clínicas de Porto Alegre (HCPA), southern Brazil, from August 2002 to December 2007. Of these, 106 underwent an indication graft biopsy; however, 10 patients were excluded because they did not have sufficient archival tissue, rendering 96 patients to be included in the study. Only the first biopsy of each patient was analyzed. The study protocol was approved by the Research Ethics Committee of HCPA, which is accredited by National Council of Research of the Brazilian Ministry of Health, and registered in the Office of Human Research Protections—OHRP—USDHHS, IRB 00000921. All patients signed the Informed Consent Form.

Clinical and Biopsy Data
Clinical data were collected retrospectively from renal transplant and pathology databases. Immunosuppression consisted of tacrolimus or cyclosporine, MMF or mycophenolate sodium, and prednisone. Six patients received rapamycin. Acute cellular rejection was treated with high dose IV steroids, thymoglobulin, or muromonab-CD3 (OKT3), and acute humoral rejection with plasmapheresis and intravenous immunoglobulin. Graft failure was defined as return to dialysis therapy or death.

The following variables were evaluated: age, gender, type of donor, pretransplant PRA against HLA class I and II, and HLA mismatches in loci A, B, and DR. Renal graft function was determined by the estimated glomerular filtration rate (eGFR) using the CKD-EPI equation at biopsy and at months 2, 6, 12, 24, 36, and last follow-up. The rate of improvement or decline in eGFR was estimated by ΔeGFR: last measured eGFR – eGFR at biopsy. ΔeGFR was adjusted for the total follow-up time of each patient. Delayed graft function, time between transplant and graft biopsy (days), and time between transplant and the last follow-up (months) were recorded. A random urine sample was collected to evaluate proteinuria as total urine protein-to-creatinine ratio. A renal pathologist who was blinded to clinical data evaluated histopathological findings of biopsies according to the Banff 2007 update.

RNA Extraction and cDNA Synthesis for Real-Time

RT-PCR of Paraffin-Embedded Tissue Sections
Four 5-μm sections of paraffin-embedded renal tissue were kept at −80°C and deparaffinized using xylene and 100% ethanol. Digestion of proteins and membranes was carried out for 3 h at 55°C in a lysis buffer containing EDTA (0.025 M), sodium sulfate (0.5%), Tris-HCl (0.01 mM, pH 8.0), NaCl (0.1 M), and proteinase k (3 μg/μL) (Invitrogen, Grand Island, NY, USA). Total RNA isolation was performed using the RNeasy Mini Kit purification protocol (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. Total RNA was quantified in the NanoDrop™ ND-1000 (Thermo Scientific, Waltham, MA, USA) and treated with DNase-I (Ambion, Grand Island, NY, USA) according to manufacturer’s instructions. Only RNA samples with an OD 260/280 ratio >1.8 were further processed. To test the efficacy of DNase treatment, a polymerase chain reaction (PCR) was performed with initiator oligonucleotides derived from a β-actin intronic gene (forward: GTGTGTGTGGGTAGGTAC and reverse: CATGTCACACTGGGGAAG). The reaction was performed in 40 cycles at 56°C. After treatment, total RNA was quantified again and cDNA synthesis was performed using 1 μg of total RNA, 0.25 μg of oligo dT18 and 3 μg of random oligo dN6 to a final volume of 10 μL, incubated at 65°C for 5 min and kept on ice. Ten microliters of a solution containing 1× buffer of enzyme SuperScript III, DTT 0.2M, dNTP20 mM, RNasin 40U (Promega, Madison, WI, USA) and SuperScript III 200U (Invitrogen, Grand Island, NY, USA) was prepared to a final volume of 20 μL. The solution was incubated at 25°C for 2 min and at 42°C for 2 h. To test for efficacy, a reverse transcription PCR (RT-PCR) was carried out using a housekeeping gene and cDNA as template. The reaction was performed with a paired temperature of initiators at 60°C (forward: 5’-GAAGGTGAAGTGGTGAAC-3’ and reverse: 5’-GGTGTCATTGATGGCAAC-3’) for 40 cycles. Gel electrophoresis on 8% acrylamide stained...
with AgNO₃ was performed. Only samples with amplification for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in RT-PCR were examined by real-time PCR.

**Quantitative Real-Time PCR for FOXP3**

The 5′ nuclease assay was performed using the ABI 7000 Sequence Detection System and TaqMan Universal PCR Master Mix containing AmpliTaq Gold® DNA polymerase, AmpErase UNG, passive reference (ROX), buffer, and dNTPs (Applied Biosystems, Foster City, CA, USA). The design and synthesis of the primer and fluorogenic probe for FOXP3 (ID: Hs 00203958_m1) mRNA were made by TaqMan Gene Expression Assays® (Applied Biosystems, Foster City, CA, USA) and had been tested and validated by the manufacturer. Due to its low intersample variability, 18S rRNA was used as an endogenous control (Eukaryotic 18S rRNA Endogenous Control, Applied Biosystems, Foster City, CA, USA). Gene expression assays consisted of 20× concentration (360 μM) of PCR primers and Taqman MGB® (Minor Groove Binding) probes. An intron-spanning primer was used to avoid genomic DNA amplification (Gene Expression Assays/Custom Primers and Probes; Applied Biosystems, Foster City, CA, USA). The TaqMan® probe was labeled with a reported dye (FAM, 6-carboxyfluorescein) on the 5′ end and a quencher dye (TAMRA, 6-carboxytetramethyl-rhodamine) on the 3′ end, except for the endogenous control 18S rRNA that was labeled with VIC reporter dye. Relative gene expression quantification was measured as fluorescence rise resulting from amplification and probe degradation. The average measurement from five samples of patients with histopathological diagnosis of “normal kidney” was used as the calibrator. The number of cycles at which fluorescence exceeds the detection threshold is called threshold cycle (Ct). A more specific template results in exceeding fluorescence earlier. The 2⁻ΔΔCt method was used in the assays, and steady-state mRNA levels were expressed as an n-fold difference relative to the calibrator.

**Immunohistochemistry for FOXP3**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue with a double marker technique to identify FOXP3⁺ cells, with or without surface differentiation molecule CD8. Sections were heated in an oven at 60°C for 30 min, deparaffinized in xylene, rehydrated in absolute and 95% ethanol, and incubated for 5 min in 3% H₂O₂ in methanol to block endogenous peroxidase. Antigen retrieval was performed using Borg Decloaker™ pH 9.5 (Biocare Medical, Walnut Creek, CA, USA) in a pressure cooker and blocked with normal goat serum 1:50 and avidin D 1:10. Anti-CD8 (clone BC/1A5) monoclonal antibody (Biocare Medical, Walnut Creek, CA, USA) was diluted at 1:25 in Van Gogh Yellow diluent (Biocare Medical, Walnut Creek, CA, USA) and incubated overnight at 4°C. After biotin blockade, slides were incubated with Universal Link biotinylated goat purified anti-mouse IgG (Biocare Medical, Walnut Creek, CA, USA) for 20 min, followed by streptavidin–horseradish peroxidase (Biocare Medical, Walnut Creek, CA, USA) for 20 min, and developed with 3,3′-diaminobenzidine. All steps included washing with TBS/Tween 20. Slides were then incubated in sequence for blocking steps with 1% TBS/BSA for 20 min, 5% skim milk in TBS for 20 min and normal goat serum 1:50 and avidin D 1:10 for 20 min. A rabbit polyclonal anti-FOXP3 antibody (ab10563, Abcam, Cambridge, MA, USA) diluted at 1:800 in Van Gogh Yellow was incubated overnight at 4°C.

After biotin blockade, a biotinylated goat anti-rabbit IgG secondary antibody was used for 35 min followed by avidin-biotinylated alkaline phosphatase complex (ABC-AP, Vector Laboratories, Burlingame, CA, USA) for 60 min. All steps included washing with TBS/Tween 20 and only TBS before developing FOXP3 staining. Tissue sections were developed with Vector Blue™ alkaline phosphatase substrate (Vector) and mounted in Faramount (DAKO, Carpinteria, CA, USA).

According to previous data the vast majority of FOXP3⁺ cells express CD4 (around 96%) and few FOXP3⁺ cells express CD8. For technical reasons, we did not perform CD4 T cell staining, so we considered that total FOXP3 expression would be represented by single FOXP3⁺ cells plus double CD8⁺FOXP3⁺ cells.

**Immunohistochemistry for CD83⁺ Dendritic Cells**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue. Sections were heated in an oven for 30 min at 60°C, deparaffinized in xylene, rehydrated in absolute and 95% ethanol, incubated for 5 min in 4.5% H₂O₂ in methanol to block endogenous peroxidase. Antigens were retrieved with Antigen Decloaker™ pH 6.0 (Biocare Medical, Walnut Creek, CA, USA) in a pressure cooker, and blocked with normal goat serum 1:50 and avidin D 1:10. Anti-CD83 (ab49324, Abcam, Cambridge, MA, USA) monoclonal antibody (Biocare Medical, Walnut Creek, CA, USA) was diluted at 1:100 in Renaissance diluent (Biocare Medical, Walnut Creek, CA, USA), and incubated overnight at 4°C. After biotin blockade, slides were incubated with Universal Link biotinylated goat purified anti-mouse IgG (Biocare Medical, Walnut Creek, CA, USA) for 20 min, followed by streptavidin–horseradish peroxidase (Biocare Medical, Walnut Creek, CA, USA) for 20 min, and developed with 3,3′-diaminobenzidine. All steps included washing with phosphate-buffered saline. Slides were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany) and placed in permanent nonaqueous medium (Vector Laboratories, Burlingame, CA, USA).
Quantification of FOXP3 Protein Expression by Digital Image Analysis CD8^+ FOXP3^+ Cells and CD8^+ FOXP3^- Cells

FOXP3^+ cells were identified by one examiner (FVV) blinded to clinical data according to the following criteria: (a) blue nuclear staining in a single CD8^- FOXP3^+ cell; (b) double positive staining including blue nuclear staining for FOXP3 and surface brown staining for CD8, a CD8^- FOXP3^+ T lymphocyte.

Quantification of FOXP3 expression was performed by digital image analysis using the Image Pro Plus software, version 4.5 (Media Cybernetics, Bethesda, MD, USA). Slides were observed with a Zeiss microscope (model AXIOSKOP-40, Carl Zeiss, Oberkochen, Germany) and captured using a Cool Snap-Pro CS camera (Media Cybernetics, Bethesda, MD, USA). Non-consecutive random fields were selected using a Greek line method to avoid field overlapping (on average, 25 fields in each core). Positive event counting was performed under 400x magnification and a resolution of 2560 x 1920 pixels, with 1 mm corresponding to 5900 pixels and an image area of 0.1412 mm^2. The area (in pixels) of the field with no tissue or nonrenal tissue was subtracted from the total area, thereby correcting for positive events per area of renal tissue in that field. Single FOXP3^+ cells and double CD8^-FOXP3^+ cells were counted separately. The number of positive cells in the cortex was recorded, as well as cells located in the perivascular areas and aggregates at the corticomedullary junction, thus generating scores for analysis. Counting was performed on an ordinal scale, and the arithmetic mean of positive events (single FOXP3^+ cells plus double CD8^-FOXP3^+ cells/mm^2) was calculated for each core biopsy. The results were expressed as positive cells per mm^2.

Quantification of CD83^- Dendritic Cells

Sections stained for CD83^- dendritic cells were scored in coded slides by one examiner (FVV) who was blinded to clinical data. The number of positive cells in the cortex, in the perivascular areas, and at the corticomedullary junction was recorded at a 200x magnification. The area of the cortex was measured with a scaled magnifying glass (included glomeruli and vessels) and the data were expressed as the number of positive cells per mm^2.

Statistical Analysis

Descriptive statistics are presented as percentages for qualitative data and mean ± SD or median and interquartile ranges for quantitative data. The Shapiro–Wilk test was used to assess the normality of continuous variables. The chi-square test and ANOVA were used for group comparisons. The levels of FOXP3 mRNA and protein expression were analyzed by the Kruskal–Wallis test due to their asymmetric distribution. A logarithmic transformation was performed on FOX3 mRNA quantified to reduce asymmetric results. A post hoc analysis was carried out with Tukey honestly significant difference (HSD) correction for multiple comparisons. Correlations between FOXP3 gene and protein expression in biopsies and between FOXP3 and dendritic cells were assessed by the Spearman’s rank coefficient. The level of FOXP3 expression (high or low) was established by a receiver operating characteristics (ROC) curve. The cutoff value for the diagnosis of acute rejection was calculated as the largest area under the curve (AUC), and the highest sensitivity and specificity.

Graft function was measured as eGFR by the CKD-EPI equation at six time points, from biopsy to last follow-up (last serum creatinine, return to dialysis therapy, or death with a functioning kidney). The evolution of eGFR over time in the patients with acute rejection was analyzed using generalized estimating equation (GEE) and the Γ distribution with logarithmic function, comparing patients with high and low FOXP3 mRNA and protein expression. A ΔeGFR was calculated by this formula: final eGFR – eGFR at biopsy, adjusted by the time of follow-up in months.

The association between FOXP3 expression and graft survival (time between transplant and allograft failure or censoring for death with a functioning graft) was assessed by the Kaplan–Meier survival analysis, and differences in survival were measured by the log-rank test. Multivariate Cox regression analysis was used to identify variables predicting graft loss at the last follow-up, including variables with statistical significance in the univariate analysis. Data were processed and analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows version 18.0. The significance level was established at p < 0.05.

RESULTS

Tissue samples of five patients with normal graft histology served as calibrators to normalize FOXP3 mRNA expression and were not included in the analyses. The 91 remaining patients were included, of which 53 (58.2%) were male and 82 (86.3%) were Caucasian. Their mean age was 44.1 ± 12.8 years. Ninety-six percent were receiving CIs (cyclosporine or tacrolimus); 89% mycophenolate mofetil (MMF) or mycophenolate sodium; 6% rapamycin; and 2.2% azathioprine. Sixty (65.9%) patients underwent antibody induction therapy, of which 56% were treated with anti-CD25 (basiliximab), 2.2% with antithymocyte globulin (ATG), and 7.7% with OKT3 monoclonal antibody.

The patients were classified according to Banff 2007 Working Classification as no rejection (NR) (23 cases; 25.3%), acute rejection (AR) (39 cases;
42.9%), and interstitial fibrosis/tubular atrophy (IF/TA) (29 cases; 31.9%). No rejection included biopsies with acute tubular necrosis ($n = 12$), calcineurin inhibitor toxicity ($n = 5$), or borderline changes ($n = 4$). AR included acute cellular rejection ($n = 35$, two cases with mixed rejection) and pure antibody mediated rejection ($n = 3$). As anti-HLA donor antibodies were not measured at the time of the study, a presumptive diagnosis of antibody mediated rejection was defined by C4d-positivity and specific morphological features.

There was no significant difference between the groups regarding demographic variables or transplant characteristics, except for the time interval between transplant and graft biopsy that, as expected, was higher in the IF/TA group. The baseline eGFR was similar in the three groups.

There were no differences among the groups in the level of pre-transplant panel reactive antibodies, HLA mismatches, and graft function at the time of renal biopsy. Table 1 shows the main demographic and clinical characteristics along with transplant-related factors according to the histopathologic classification.

### Table 1. Demographic and clinical characteristics of renal transplant recipients.

|                | NR ($n = 23$)       | AR ($n = 39$)       | IF/TA ($n = 29$)     | $p$-Value |
|----------------|---------------------|---------------------|----------------------|-----------|
| Age (years)    | $46 \pm 15.0^a$     | $42.8 \pm 12.0$    | $43.9 \pm 11.8$      | 0.626     |
| Gender (male)  | 12 (50)$^b$         | 29 (71)            | 15 (50)              | 0.122     |
| Ethnicity/skin color (white) | 22 (91) | 34 (83) | 26 (87) | 0.595     |
| HLA mismatches (A, B, DR) | 18 (75) | 30 (73) | 23 (77) | 0.485     |
| Induction therapy (basiliximab/ATG/OKT3) | 4.26 $\pm$ 1.1 | 3.6 $\pm$ 1.0 | 4.0 $\pm$ 0.9 | 0.068     |
| Last pre-transplant PRA (%) | 14/0/2 | 25/1/3 | 12/1/2 | 0.808     |
| Time between Tx and biopsy (days) | $60 (11.5–345)^c$ | 29 (11–315) | $615 (270–1695)$ | <0.001 |
| Cr$_s$ (mg/dL) | 4.3 $\pm$ 2.8       | 4.8 $\pm$ 2.8      | 3.7 $\pm$ 1.4        | 0.185     |
| eGFR (mL/min/1.73 m$^2$) | 22.7 $\pm$ 14.9 | 19.8 $\pm$ 13.1 | 20.2 $\pm$ 9.6 | 0.649     |

| Notes: NR, no rejection; AR, acute rejection; ATG, anti-thymocyte globulin; IF/TA, interstitial fibrosis/tubular atrophy; PRA, panel reactive antibodies; Tx, transplant; Cr$_s$, serum creatinine; eGFR, estimated glomerular filtration rate (CKD-EPI). |
|---|---|---|---|---|
| $^a$Mean $\pm$ SD. |
| $^b$n (%). |
| $^c$Median and interquartile range. |

### Table 2. FOXP3 mRNA and FOXP3 and CD83 protein expression in renal allograft biopsies.

|                | NR ($n = 23$)       | AR ($n = 39$)       | IF/TA ($n = 29$)     | $p$-Value |
|----------------|---------------------|---------------------|----------------------|-----------|
| Log$_{10}$ FOXP3 RNAm | 1.80 $\pm$ 1.13$^a$ | 2.61 $\pm$ 1.47$^c$ | 1.70 $\pm$ 1.10$^d$  | 0.024     |
| FOXP3$^+$ (cells/mm$^2$) | 0 (0–1.35)$^b$  | 3.60 (0.57–11.35)$^{e,f}$ | 1.77 (0–2.86)       | <0.001    |
| CD83 (cells/mm$^2$) | 0.41 (0.22–0.92)   | 0.62 (0–3.55)       | 0.79 (0.35–2.28)$^f$ | 0.523     |

| Notes: NR, no rejection; AR, acute rejection; IF/TA, interstitial fibrosis/tubular atrophy. |
|---|---|---|---|---|
| $^a$x $\pm$ SD. |
| $^b$Median (IQR). |
| $^c$AR versus NR, $p = 0.039$. |
| $^d$AR versus IF/TA, $p = 0.035$. |
| $^e$AR versus NR, $p < 0.001$. |
| $^f$AR versus IF/TA, $p = 0.034$. |
| $^g$AR versus IF/TA, $p = 0.065$. |
Expression was analyzed at three time intervals after transplant: ≤6 months, 7–12 months, and >12 months when the number of patients was more equally distributed. Taking all biopsies together, FOXP3 mRNA did not decrease significantly from the first month to 1 year post-transplant. Stratifying for each Banff category, a slight increase of FOXP3 mRNA was found in patients without rejection and those with acute rejection from 1 to 12 months. The levels decreased thereafter. In the IF/TA group, there was a slight decrease up to 12 months remaining in the same levels thereafter. However, quantitative differences among these time intervals were not statistically significant, which is probably related to the small sample size in each time interval.

Type of induction therapy was not associated with a specific level of FOXP3 mRNA or protein at biopsy. A reliable analysis of the role of rapamycin in augmenting FOXP3 expression was precluded by the small number of patients in this therapy.

Correlation between FOXP3 mRNA and Protein and between FOXP3 and Dendritic Cells in Renal Tissue

The Spearman rank coefficient showed a significant positive correlation between FOXP3 gene and protein expression in renal graft biopsies (r = 0.541, p < 0.001). Patients in the FOXP3high group had a higher number of dendritic cells in biopsy: 0.86 (0.25–4.0) versus 0.44 (0–1.12) cells/mm², p = 0.032. As shown before, CD83+ dendritic cells were present in higher numbers in biopsies with IF/TA when comparing to NR, but it did not differ from AR (Table 2). Taking only the biopsies with acute rejection, no correlation was found between FOXP3 mRNA and CD83+ cells (r = 0.166, p = 0.313) or FOXP3 protein and CD83 (r = 0.136, p = 0.408). Taking only the IF/TA for analyses, the biopsies uncovered a trend toward a positive correlation between FOXP3 and CD83+ protein expression (r = 0.350, p = 0.06), but not for FOXP3 mRNA and CD83+ protein expression (r = 0.166, p = 0.313).

Association between FOXP3 mRNA and Protein Expression and 5-Year Graft Function and Survival

The estimated GFR at 5 years post-transplantation was similar in patients with FOXP3high and FOXP3low groups. Only at 2 years, eGFR tended to be higher in...
the high FOXP3 compared to the low FOXP3 group: 40.6 (29.3–58.0) versus 33.5 (19.3–44.7) mL/min/1.73 m² (p = 0.06). However, at the last follow-up there was no difference between the two groups: 27.4 (11.1–51.6) versus 20.0 (10.8–34.1) mL/min/1.73 m² (p = 0.266). Analysis of Generalized Estimated Equation in patients with acute rejection demonstrated a trend toward better evolution of eGFR in group FOXP3 mRNAhigh as compared to FOXP3 mRNAlow (Wald Chi-Square: 2865; p = 0.090), which was independent of the eGFR time point measured (Moment-FOXP3 mRNA: Wald chi-square: 1.998; p = 0.736), as presented in Figure 2. No difference was found between FOXP3high and FOXP3low protein expression (Wald Chi-Square: 0.143, p = 0.706).

In a secondary analysis, we considered good graft function at 5 years post-transplantation to be an eGFR equal or higher than 30 mL/min/1.73 m². Patients in this range of eGFR had a higher FOXP3 mRNA [2.60 (1.56 to 3.50) vs. 1.93 (0.54 to 2.86) log10 RNAm, p = 0.023] and protein expression [3.6 (0 to 9.96) vs. 0.71 (0 to 3.16) cells/mm², p = 0.035] as compared to patients with eGFR lower than 30 mL/min/1.73 m². Moreover, patients with a positive ΔeGFR over time showed significantly higher FOXP3 protein expression in graft biopsy [1.958 (0.087 to 7.815) cells/mm²] as compared to those that had a negative change in eGFR [0.519 (0.0 to 2.512) cells/mm², p = 0.015]. Analyzing FOXP3high and FOXP3low groups, there was a trend toward a positive ΔeGFR in patients with high FOXP3 mRNA expression [5.2 (–0.6–30.9) and 1.07 (–6.7–15.6) mL/min/1.73 m², respectively, p = 0.08], suggesting an improvement in renal function up to 5 years of transplant. However, after adjusting the ΔeGFR by total time of observation for each patient, this difference disappeared.

The cumulative 5-year survival of renal grafts was not influenced by FOXP3 mRNA expression, which was 61% and 65.5% for patients in the FOXP3high and FOXP3low groups, respectively (p = 0.69, log-rank test) as shown in Figure 3. Multivariate Cox regression was performed to assess independent factors associated with graft survival (Table 3). Donor type, HLA mismatches, and FOXP3 expression in biopsy were not associated with graft loss up to 5 years. Higher eGFR at renal biopsy was associated with a protective effect against graft loss (HR = 0.94, 95% CI 0.90–0.98, p = 0.014), and there was a trend to higher risk of graft loss in patients with IF/TA as Banff histology (HR = 3.10, 95% CI 0.86–11.2, p = 0.082).
Table 3. Independent factors associated with graft loss in multivariate Cox regression model.

| Variable                        | Crude hazard ratio (95% CI) | p-Value | Adjusted hazard ratio (95% CI) | p-Value |
|---------------------------------|-----------------------------|---------|-------------------------------|---------|
| Acute cellular rejection        | 2.41 (0.66–8.78)            | 0.181   | 2.12 (0.58–7.73)              | 0.252   |
| IF/TA                           | 3.18 (0.88–11.4)            | 0.076   | 3.10 (0.86–11.2)              | 0.082   |
| eGFR at biopsy                  | 0.95 (0.91–0.98)            | 0.012   | 0.94 (0.90–0.98)              | 0.014   |
| Type of donor                   | 0.81 (0.32–2.05)            | 0.666   | NI                            |         |
| HLA I/II mismatches             | 0.99 (0.66–1.48)            | 0.984   | NI                            |         |
| FOXP3+ (cells/mm²)             | 0.93 (0.38–2.27)            | 0.889   | NI                            |         |
| Log₁₀FOX3 mRNA                 | 0.97 (0.68–1.26)            | 0.633   | NI                            |         |
| ≥2.5 FOXP3+ (cells/mm²)        | 0.75 (0.32–1.75)            | 0.509   | NI                            |         |
| ≥2.36 log₁₀FOX3               | 1.21 (0.54–2.72)            | 0.630   | NI                            |         |

Notes: CI, confidence interval; eGFR, estimated glomerular filtration rate; NI, not included in the multivariate regression model.

DISCUSSION

The transcription factor FOXP3 is the most specific marker of regulatory T cells (Tregs) and is expressed in CD4⁺CD25⁺ suppressor cells. In vitro and in vivo experimental models have demonstrated that production of Tregs in the periphery by FOXP3 transfection in naïve T cells can lead to tolerance induction and graft acceptance.²¹,²² Many clinical studies have identified an increased expression of the FOXP3 gene and/or its protein in kidney grafts with acute rejection using different techniques.⁷–¹²,²³,²⁴ However, the clinical significance of the presence and magnitude of FOXP3 expression remains not completely understood.

In this study, we investigated FOXP3 mRNA and protein expression in the paraffin tissue of kidney graft biopsies for cause. Both gene and protein expression were significantly higher in patients with acute rejection, confirming previous findings.⁷–¹²,²³,²⁴ FOXP3 gene amplification correlated positively and significantly with protein expression, showing that the detection of gene transcripts as much as protein expression is feasible in formalin-fixed, paraffin-embedded tissue.

Recent clinical studies have evidenced that different immunosuppressive agents can promote stimulation, inhibition, or even noninterference in the number and function of FOXP3⁺ Tregs.¹⁷,²⁵–²⁸ We could not find a difference in the intragraft expression of FOXP3 comparing basiliximab to ATG and OKT3 for induction therapy, as well as between calcineurin inhibitors and sirolimus-based regimens (data not shown). However, the limited number of patients on sirolimus precluded a robust analysis. Similarly, other clinical studies also did not show an association between type of immunosuppression and FOXP3⁺ Tregs in kidney graft recipients.¹²,²⁹

This study was not designed to determine the variability of intragraft FOXP3 expression over time, because each patient had only one for cause biopsy and not-sequential protocol biopsies. When considering biopsy analysis obtained at different time points after transplantation, it is suggested that FOXP3 expression is higher in the initial 6 months and remains relatively stable up to 1 year, then begins to decline thereafter. The early increment is probably related to a more intense graft inflammation, as previously shown by other authors.⁷,⁸,³⁰ The decrement observed after 12 months is most probably related to the occurrence of chronic rejection in which fewer CD4⁺CD25high Tregs and FOXP3 mRNA in peripheral blood have been described.⁹,²³,³¹ These data could suggest that immune regulatory mechanisms failed to protect the graft against T effector cell chronic injury. In contrast, other authors found higher levels of FOXP3⁺ Tregs late post-transplantation, postulating that more Tregs are needed over time to control persistent inflammation to avoid chronic graft injury and provide graft acceptance and better outcomes.³² Alternatively, this phenomenon could not indicate alloimmunity, but simply reflect the process of natural stabilization of inflamed sites operated by T regulatory cells, not associated with better graft survival, as suggested by Bunnag et al.¹² These authors reported increased FOXP3 expression in late kidney allograft biopsies as compared to early biopsies, which was correlated with inflammation, injury, and repair assessed by pathogenesis-based transcript microarrays.

Data on the influence of FOXP3 in graft outcomes are still controversial. In the present study, FOXP3³high expression correlated with greater eGFRs and a positive ΔeGFR between transplant and last evaluation, but no difference in absolute eGFR or a better graft survival rate was found at the last follow-up. In multivariate Cox regression, FOXP3 was not associated with a reduced risk of graft loss, as was a higher eGFR at the time of biopsy. Muthukumar et al.⁷ found significantly higher levels of FOXP3 mRNA in urine cells of renal transplant recipients with acute rejection that was inversely correlated to serum creatinine. These authors also reported a higher relative risk of graft loss at 6 months in patients at the lower quartile of log FOXP3 mRNA. In evaluating patients with subclinical acute rejection, Bestard et al.¹⁰ found that presence or absence of an infiltrate rich in FOXP3⁺ Tregs differentiated harmless infiltrates from injurious ones by predicting a better graft function at 2 and 3 years post-transplant. Corroborating this data, Zuber et al.³² extended these observations reporting that patients with inflamed fibrosis, but a high FOXP3/CD3 ratio within the graft, had a significantly better graft survival at 3 years after transplant. Louis et al.²³
described a reduced number of circulating FOXP3+ Tregs in patients with chronic rejection, but normal FOXP3 levels in stable grafts without evidence of rejection, or in those with tolerance. However, other studies did not demonstrate an association between FOXP3 and favorable graft function or survival outcomes even when restricting the analysis to patients with acute rejection. Unexpectedly, graft survival in patients with acute rejection who presented high expression of FOXP3 was even worse than in those with low FOXP3 expression, which could be related to severe interstitial inflammation. Recent studies could not demonstrate either a protective effect of FOXP3+ Tregs in graft function 1 year after transplant, or a reduction in the severity of allograft rejection. In a recent systematic review evaluating CD4+FOXP3+ Tregs and transplant outcomes in solid organ transplantation, Shan et al. included 22 case-control and cohort publications. In kidney transplantation, the serum creatinine level in FOXP3(high) group was significantly lower at 1 year as compared to FOXP3(low), while graft survival did not differ between the two groups. FOXP3+ Tregs in graft or urine associated better with transplant outcomes, than in peripheral blood. However, these conclusions may be biased due to the low quality and heterogeneity of the available studies. In summary, although FOXP3 molecule seems to have greater predictive value of graft outcome than Banff grading, neither up until now can distinguish clearly the type of intragraft operating cellular program, that is, tissue-destructive by effector T cells or tissue-protective by FOXP3+ Tregs. At the moment, the effect of FOXP3+ Tregs on graft outcomes at least in the long-term remains uncertain.

Few clinical studies evaluated if CD83 dendritic cells correlate with FOXP3+ cells morphologically. We hypothesized that antigen-presenting cells are present along with FOXP3 in interstitial infiltrates, as both molecules are involved in the process of generation and maintenance of immunological tolerance. Experimental studies in islet transplantation demonstrated that induced expansion of CD4+CD25+Foxp3+ Tregs by DCs in the presence of TGF-β1 can inhibit autoimmunity in non-lymphopenic mice. In another study it was shown that rapamycin-conditioned donor DCs effectively induced CD4+CD25+Foxp3+ Tregs (iTregs) in cell culture, which exerted donor-specific suppression in vitro and prolonged allogeneic islet graft survival in vivo. In this study, iTregs induced endogenous naïve T cells to convert into CD4+CD25+FOXP3+ T cells. Yamazaki et al. reported that allogeneic DCs in the presence of interleukin-2 in cell culture were much more effective than bulk spleen cells in expanding Tregs and in sustaining the expression of transcription factor FOXP3 in CD4+CD25+ T cells. These DC-expanded Tregs were potent suppressors of a mixed leukocyte reaction by CD25− CD4+ T cells, evidencing that Tregs can be expanded from a polyclonal repertoire by DCs.

There is a lack of studies in humans to explore if these experimental conditions could be translated to the clinical setting. In kidney transplantation, Haanstra et al. showed, in non-human primates with graft rejection, that CD83+ DCs predominated in nodular infiltrates along with a higher number of FOXP3+ cells, as compared to nonrejected grafts. In accordance with these results, we found a higher density of CD83+ DCs in biopsies with high expression of FOXP3. A recent study in human kidney grafts reported that an increased influx of myeloid dendritic cells during acute rejection was associated with interstitial fibrosis and tubular atrophy, predicting loss of graft function after the first year of transplant.

A higher number of CD83+ DCs was also found in IF/TA, a finding difficult to interpret as absolute levels of FOXP3 mRNA, in addition, FOXP3 protein in IF/TA was lower than in acutely rejecting grafts. We would expect such low levels in these biopsies and not the opposite. Perhaps one limitation of our study was our difficulty in accurately detecting DCs in kidney biopsies using the less sensitive peroxidase technique in paraffin-embedded tissue. Zuidwijk et al demonstrated high density of mature dendritic cells in clusters during acute rejection by immunohistochemistry in cryostat sections, perhaps because frozen tissue gives better antigen preservation. Nevertheless, presence of chronic damage could be better explained by other factors not related to failure of FOXP3+ cell function, but to chronic calcineurin inhibitor toxicity, donor-related factors, severity of rejection, low compliance, and polyoma virus infection, among other factors.

In conclusion, there was an increased FOXP3 expression in biopsies with acute rejection as expected. Graft biopsies presenting a high level of FOXP3 were found to be associated with a greater density of dendritic cells predominantly in IF/TA, a finding to be further explored. FOXP3 expression was not associated with better graft outcomes over a period of almost 5 years of follow-up, but greater amounts of both FOXP3 mRNA and protein were found in patients with higher eGFRs. To better explore the effects of FOXP3 expression on kidney graft function and survival, a larger and longer follow-up study is needed.

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