Regulatory, Effector, and Cytotoxic T Cell Profiles in Long-Term Kidney Transplant Patients

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ABSTRACT
Animal studies have suggested a potential role for regulatory T cells (Tregs) in allograft tolerance, but these FOXP3+ cells seem to be an inherent component of acute rejection (AR) in human recipients of renal transplants. The balance between regulatory cells and effector/cytotoxic cells may determine graft outcome; this balance has not been described for chronic allograft injury. We investigated the expression of key regulatory, effector, and cytotoxic transcripts (i.e., FOXP3, T-bet, and granzyme B, respectively) in the grafts and peripheral blood of long-term-surviving renal transplant patients. We found that, whereas neither intragraft nor peripheral blood FOXP3 or T-bet mRNA could distinguish between rejection and nonrejection status, granzyme B (GrzB) mRNA could: It was significantly increased in the graft and significantly decreased in the peripheral blood of patients with chronic antibody-mediated rejection (CAMR). Quantifying peripheral blood GrzB mRNA demonstrated potential to aid in the noninvasive diagnosis of CAMR. In summary, these data affirm GrzB as a marker not only for AR but also for CAMR. In addition, we identified several previously unreported clinical or demographic factors influencing regulatory/effector/cytotoxic profiles in the peripheral blood, highlighting the necessity to consider confounding variables when considering the use of potential biomarkers, such as FOXP3, for diagnosis or prognosis in kidney transplantation.


Achieving clinical transplant tolerance has been a major goal and the subject of intense research in transplantation for more than 50 yr.1,2 In rodents, allograft tolerance is frequently associated with a specialized population of suppressor T cells, the so-called regulatory T cells, or Tregs.3 CD4+CD25++ Tregs can be distinguished from other cell types by their constitutive expression of the forkhead-winged helix transcription factor FOXP3.4,5 The detection of FOXP3 in the allografts and immune compartments of tolerant animals has drawn attention to the potential role of these cells in allograft tolerance in rodent models.6,7 However, there is now compelling evidence from studies in humans that FOXP3+ cells are also an inherent component of acute allograft rejection (AR), with increased FOXP3 reported in both the urine8 and the graft.9–11 Thus, it has become apparent that the

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presence of FOXP3 or Tregs may not dictate graft outcome but rather their abundance relative to their effector/cytotoxic counterparts. Indeed, several groups have also clearly established the cytotoxic molecule granzyme B (GrzB) as a reliable intragraft, urine, and peripheral blood marker of acute kidney graft rejection.12–18

Thus, although the implication of regulatory and effector responses has been addressed for AR, so far there have been no reports on the subject relative to later times posttransplant in cases of stable graft function or late renal allograft injury, such as calcineurin inhibitor (CNI) toxicity or chronic antibody-mediated rejection (CAMR). Therefore, the potential involvement of FOXP3+ cells and any relationship to clinical and pathological correlates at later times posttransplant remain unknown. This prompted us to investigate the regulatory, effector, and cytotoxic profiles in the graft and peripheral blood of long-term-surviving renal transplant patients with stable graft function or different types of chronic graft injury. For this purpose, the Treg master transcription factor FOXP3, the Th1 cell transcription factor T-bet,19 and the cytotoxic molecule GrzB20 were analyzed in the graft and peripheral blood mononuclear cells of long-term-surviving kidney transplant recipients experiencing stable graft function or loss of graft function through injury or rejection.

Here, we show that, whereas FOXP3, T-bet, and GrzB can all distinguish acute rejection from nonrejection, only GrzB can distinguish chronic rejection from other forms of late graft injury, including idiopathic transplant glomerulopathy. In fact, GrzB was significantly increased in biopsies but significantly decreased in the peripheral blood of patients presenting CAMR. Moreover, quantifying peripheral blood GrzB mRNA shows potential as a decision-making tool for CAMR. Finally, we found that the expression of each of these three molecules can be influenced by different clinical covariates. These data highlight the importance of future studies of gene expression in transplantation to be performed on large cohorts such that potential confounding factors can be taken into account when evaluating the diagnostic or prognostic capacity of these genes or when establishing a mechanistic basis for graft outcome.

RESULTS

Intragraft Regulatory, Effector and Cytotoxic Profiles

Intragraft FOXP3 mRNA Cannot Distinguish Rejection from Nonrejection in Chronic Graft Injury.

We first sought to analyze the expression of FOXP3 in different types of chronic graft injury, including nonrejection (chronic CNI toxicity; n = 7), rejection (CAMR; n = 14),21 and the relatively rare diagnosis of idiopathic transplant glomerulopathy (TG; C4d and anti-HLA negative; n = 11). A comparison between biopsies with normal histology (n = 7) and with acute cellular rejection (n = 9) served as a positive control (Supplementary Figure 1). As expected, FOXP3 was significantly increased in acute cellular rejection (ACR) compared with biopsies with normal histology (P < 0.001; Supplementary Figure 1Ai). However, despite being detectable in all three types of chronic graft injury analyzed, FOXP3 transcripts here were detected in equivalent quantities (Figure 1A).

These data were confirmed at the protein level by quantifying the number of graft-infiltrating FOXP3+ cells by immunohistochemistry. Like FOXP3 mRNA, FOXP3+ cells significantly accumulated in ACR (P < 0.001; Supplementary Figure 1Bi) but were present at much lower levels in late injury with no significant differences (Figure 1B). Overall, the number of FOXP3+ cells per square millimeter within the graft correlated tightly with the quantity of FOXP3 mRNA (Spearman r = 0.71, P < 0.0001). FOXP3+ lymphoid cells were detected in the interstitium and in tubules in ACR (data not shown) but were present only in the interstitium in the case of chronic injury.

Figure 1. Quantification of intragraft FOXP3 mRNA and FOXP3+ cells in chronic injury of kidney allografts. (A) Quantitative PCR measurement of FOXP3 mRNA accumulation in kidney graft biopsies displaying signs of chronic injury (chronic calcineurin inhibitor toxicity, CNI tox, n = 7; idiopathic transplant glomerulopathy, TG, n = 11; or chronic antibody-mediated rejection, CAMR, n = 14). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the housekeeping gene HPRT with results expressed in arbitrary units (see Concise Methods for details). (B) Quantification of the number of FOXP3+ infiltrating cells per square millimeter in graft biopsies displaying chronic graft injury (CNI tox, n = 4, or CAMR, n = 9). (C) Staining of FOXP3 (rare blue cells indicated with arrows; ×40 magnification) or co-staining of FOXP3 (blue) and CD4 (brown) in chronic antibody-mediated rejection (×40 magnification + enlarged image).
and absent from tubules, glomeruli, and vascular lesions (Figure 1C, left-hand panel). In the CAMR samples, co-staining revealed that all FOXP3+ cells were CD4+ (Figure 1C, right-hand panel). Thus, FOXP3+CD4+ cells are present in the chronic graft injury but cannot distinguish between nonrejection and rejection.

**Intragraft T-bet mRNA Cannot Distinguish Rejection from Nonrejection in Chronic Graft Injury.**

To gain insight into the level of potentially counteracting T effectors in the different forms of graft injury, mRNA for the Th1 transcription factor T-bet was measured. As previously reported, we confirmed T-bet as being significantly increased in ACR compared with grafts with normal histology ($P < 0.001$; Supplementary Figure 1Aii). However, there were no significant differences in chronic graft injury regardless of the rejection or nonrejection diagnosis (Figure 2A). T-bet+ cells significantly infiltrated the grafts with ACR compared with normal histology ($P < 0.05$; Supplementary Figure 1Bii), being detected occasionally in tubules and in the arterial intima (data not shown). Coexpression of T-bet and FOXP3 was not observed (data not shown). In late injury, T-bet+ cells were also detectable, with a significant increase in CAMR ($P < 0.01$; Figure 2B). In fact, CAMR cases often had quite prominent glomerular T-bet+ cells (Figure 2C, upper left-hand panel). In the CAMR samples, T-bet was expressed in >50% of CD4+ cells (Figure 2C, right-hand panel), in >50% of GrzB+ cells (Figure 2C, lower left-hand panel), and in rare B cells (<5%; data not shown). Thus, although there was a discordance between mRNA and protein in late injury, the number of T-bet+ cells infiltrating the grafts correlated significantly with T-bet mRNA levels (Figure 2C; Spearman $r = 0.61$, $P < 0.01$). These data indicate that the presence of T-bet mRNA cannot distinguish between nonrejection and rejection late after transplantation, but T-bet+ cells may do so, displaying a distinctive anatomical localization.

**Intragraft GrzB mRNA Can Distinguish Rejection from Nonrejection in Chronic Graft Injury.**

We next explored the contribution of cytotoxicity to the different forms of graft injury. First, we confirmed previous data showing a significant accumulation of GrzB transcripts in acute rejection ($P < 0.001$; Supplementary Figure 1Aiii). In addition, GrzB was significantly increased in CAMR versus CNI toxicity and TG ($P < 0.01$ and $P < 0.05$, respectively; Figure 3A). A similar trend of GrzB expression was observed after immunostaining for GrzB+ cells infiltrating the grafts with an increase in ACR (Supplementary Figure 1B) and a trend toward an increase in CAMR ($P = 0.07$; Figure 3B). In CAMR, GrzB expression was almost exclusively confined to CD8+ cells (>95%; Figure 3C, left-hand panel), whereas none coexpressed FOXP3 (Figure 3C, right-hand panel). For this molecule, no statistically significant correlation was detected between mRNA and protein (Spearman $r = 0.15$; NS). Thus, GrzB mRNA can distinguish late rejection from nonrejection.

**Peripheral Blood Regulatory, Effector, and Cytotoxic Profiles**

Peripheral Blood FOXP3 mRNA Correlates with Time Posttransplant But Not with Rejection or Nonrejection Status in Long-Term-Surviving Recipients.

To determine whether the regulatory, effector, and cytotoxic profiles were similar between the graft and the periph-
eral blood in long-term patients, we next analyzed PBMC from a largely independent group of patients with stable graft function with undetermined biopsy status \((n = 164),\) idiopathic transplant glomerulopathy \((n = 7),\) or CAMR \((n = 15)\) and healthy volunteers \((n = 15)\). As shown in Figure 4Ai, the blood FOXP3 mRNA followed the same pattern as that observed in the graft in that there were no significant differences between patients with or without rejection (Figure 4Ai). Of note, stable (STA) patients had significantly lower FOXP3 mRNA levels than healthy volunteers \((P < 0.01)\). Moreover, as determined in 6 patients with CAMR, FOXP3 levels in the PBMC were stable over time (Supplementary Figure 2). Finally, no differences were observed in peripheral blood FOXP3 mRNA between patients with stable graft function or acute cellular rejection early posttransplant (Supplementary Figure 1Ci).

Given the large number of STA patients included for analysis \((n = 164),\) we explored the potential confounding factors for FOXP3 mRNA expression in peripheral blood by multivariate linear regression analysis in these patients. Among the different covariates analyzed (Table 1 and Supplementary Table 3), the only significant association was between FOXP3 and time posttransplant, with a second-order polynomial relationship (increasing then decreasing) as illustrated in Figure 4Aii. Thus, FOXP3 mRNA levels in the peripheral blood of long-term-surviving renal transplant patients do not correlate with rejection or nonrejection status but vary with time posttransplant. This potential confounding factor merits further investigation in future studies of the relationship between FOXP3 and transplant rejection.

**Peripheral Blood T-bet Correlates with Time Posttransplant, Recipient Age, and Immunosuppressive Treatment but not with Rejection or Nonrejection Status in Long-Term-Surviving Recipients.**

As shown in Figure 4Bi, like graft T-bet mRNA, there were no significant differences in blood T-bet mRNA between long-term patients with or without rejection. Likewise, no differences were observed in peripheral blood T-bet mRNA between patients with stable graft function or acute cellular rejection early posttransplant (Supplementary Figure 1Cii). As illustrated in Figure 4Bii, multivariate linear regression analysis of the STA patients (Table 1 and Supplementary Table 3) revealed that T-bet mRNA levels decreased significantly with time posttransplant \((P < 0.0001),\) increased significantly with recipient age \((P < 0.01),\) and were significantly lower in patients treated with FK-506 compared with those receiving cyclosporine A \((C\alpha)\) \((P < 0.05).\) Thus, T-bet mRNA levels in the peripheral blood of long-term-surviving renal transplant patients do not correlate with rejection or nonrejection status but are influenced by certain confounding factors. Like FOXP3, T-bet mRNA levels were also stable over time (Supplementary Figure 2).

**Peripheral Blood GrzB Can Distinguish between Rejection and Nonrejection Status in Long-Term-Surviving Recipients Despite Also Being Influenced by Donor Age and Type of Immunosuppressive Treatment.**

In contrast to its significant increase in biopsies with CAMR, GrzB was significantly reduced in the PBMC of patients with CAMR compared with those with long-term stable graft function (Figure 4Ci). However, no differences were observed between patients with stable graft function or acute cellular rejection early posttransplant (Supplementary Figure 1Ciii). As illustrated in Figure 4Cii, multivariate generalized modeling of the STA patients (Table 1 and Supplementary Table 3) showed that the quantity of GrzB mRNA in the peripheral blood increased significantly with donor age \((P < 0.01)\) and was significantly lower in patients treated with FK-506 compared with those receiving CsA \((P < 0.01),\) whereas the presence or absence of steroids did not significantly influence GrzB mRNA levels in the peripheral blood.
levels. Given that the CAMR patients were for the majority treated with FK-506, an additional analysis was performed to compare CAMR with STA patients treated only with FK-506. This revealed that the decrease in GrzB in CAMR was not due to their treatment with FK-506, because the significant decrease in CAMR was still observed \((P < 0.001); \text{Figure 4Ci}\). Like the other two molecules, GrzB levels in PBMC were stable over time (Supplementary Figure 2).

Next, we set out to determine whether measuring GrzB in PBMC could potentially be applied to decision making in clinical practice. For this purpose, we performed a receiver operating characteristic (ROC) curve analysis for GrzB to discriminate between STA and CAMR (Figure 5). The analysis showed a good discrimination with an area under the curve of 0.73 (95% confidence interval of 0.60 to 0.84). An optimal cutoff was also calculated at 1.23, representing an equivalent specificity and sensitivity of 0.67.

Thus, peripheral blood GrzB-expressing cells decrease during CAMR. Moreover, GrzB mRNA levels tend to be higher in the blood of patients with grafts from older donors and are controlled to a greater extent by the CNI FK-506. Finally, measuring GrzB in the peripheral blood shows potential as a decision-making tool in clinical practice and may be combined with other molecules to help diagnose CAMR based on a blood sample.

**DISCUSSION**

Although the implication of regulation and effector/cytotoxic pathways has been investigated in the context of acute rejection, to date, there have been no reports relative to this subject in the context of chronic injury. For this reason, we investigated the expression of key regulatory, effector, and cytotoxic marker transcripts (FOXP3, T-bet, and GrzB) in the grafts and peripheral blood of long-term-surviving renal transplant patients, with special focus on nonrejection (stable graft function, CNI toxicity, and idiopathic TG) and rejection (CAMR) and with ACR as a reference. The association of these three molecules with various clinical, demographic, and pathological parameters was additionally analyzed. Overall, our results show that the mRNA of GrzB, but not that of FOXP3 or T-bet, can distinguish between chronic rejection (CAMR) and nonrejection (CNI toxicity and idiopathic TG) late after transplantation. IL-17a was also analyzed but was not expressed at detectable levels in any of the samples (data not shown). Moreover, each of these molecules is influenced by specific clinical or demographic parameters.

Here, we provide the first evidence that, in contrast to ACR, FOXP3 is not significantly increased in “alloimmune” chronic rejection (CAMR) compared with “nonalloimmune” chronic lesions such as CNI toxicity or idiopathic TG. This was true for both mRNA and protein. Relatively sparse infiltration of intragraft FOXP3+ cells coexpressing CD4 characterizes both acute and chronic forms of anti-
body-mediated rejection, in contrast to cell-mediated acute rejection.\(^9\) Of course, it is possible that some of the FOXP3 that we measured derived in part from non-Tregs (T cell receptor ligation in non-Tregs has been shown to transiently induce low levels of FOXP3\(^{23}\)). However, at least in the graft, the lack of FOXP3 and T-bet or FOXP3 and GrzB costaining together with the almost mutually exclusive distribution of FOXP3\(^+\) and T-bet\(^+\) cells within the graft biopsies suggests that the FOXP3\(^+\) cells that we observed within the grafts were more likely to be Tregs than activated T cells. In the peripheral blood, our finding of significantly reduced FOXP3 mRNA levels in transplant recipients in general versus healthy volunteers is supported by previous data showing that CNI in the maintenance regimen reduces peripheral blood Treg numbers in renal transplant recipients.\(^{24}\) We go further by showing that, unlike urine FOXP3 mRNA levels that are associated with renal function and predict outcome in the context of acute rejection,\(^8\) peripheral blood FOXP3 mRNA levels are not linked to renal function and cannot distinguish between patients who are rejecting their grafts chronically with deterioration of graft function and those that are enjoying good graft function. Thus, in contrast to urine, it seems unlikely that measuring FOXP3 in the blood will be useful for long-term monitoring of renal transplant recipients. The data that we present here also demonstrate a novel and atypical binomial time-dependent effect on peripheral blood FOXP3 mRNA levels, with FOXP3 initially increasing, reaching a maximum at around 12 yr posttransplant, and decreasing thereafter. This time-dependent effect could act as a confounding factor when samples associated with different clinical statuses are compared at different intervals after transplantation.

With regard to the effector transcription factor T-bet, our data confirm a previous report that this molecule is an intra-graft marker of acute rejection.\(^{22}\) We extend these findings by showing that, like FOXP3, T-bet mRNA is not a specific marker of chronic antibody-mediated rejection. Nevertheless, glomerular T-bet\(^+\) cells accumulated most prominently in CAMR, a novel finding that deserves further study. This discrepancy between mRNA and protein could be due to the smaller sample sizes used for immunohistochemistry or due to a small number of cells expressing a high number of transcripts in biopsies with CNI toxicity. Our finding that T-bet was lower in patients treated with FK-506 compared with those treated with CsA indicates that FK-506 may hold an advantage over CsA in terms of its ability to control the effector response. This could partly explain why FK-506 is more effective than CsA at inhibiting acute rejection in renal transplant patients.\(^{25}\) The gradual decline in T-bet with time posttransplant could possibly be due to chronic exposure to CNI. Peripheral blood T-bet mRNA levels additionally tended to be higher in older recipients, possibly the result of an age-related change in composition of T-bet-expressing peripheral blood cells. However, pe-
Peripheral blood T-bet could not distinguish rejection from stable function. Thus, like FOXP3, T-bet would probably be of no use for long-term monitoring of kidney transplant recipients.

Regarding the cytotoxic molecule GrzB, we present novel data showing that the mRNA of this molecule is not only increased in acute rejection as reported previously but also in CAMR. This increase was absent in other forms of late graft injury that are not due to alloimmune mechanisms such as chronic CNI toxicity or idiopathic TG. It is currently unclear as to whether idiopathic TG is CAMR sampled at an inactive time or is due to other causes. According to our results, the two can be distinguished by GrzB mRNA (Mann–Whitney test; P < 0.05 for both the biopsy and the PBMC), suggesting CAMR may be the active phase of the same glomerular disease or that idiopathic TG is caused by a GrzB-independent mechanism. For this molecule, measuring the mRNA appeared more sensitive than measuring the protein. We additionally show for the first time that, in contrast to acute rejection where GrzB is increased in the PBMC, during CAMR GrzB is significantly decreased. Furthermore, ROC analysis revealed that measuring GrzB mRNA may have potential as a decision-making tool in clinical practice to diagnose CAMR in a minimally invasive manner. Given that GrzB transcripts were significantly reduced in peripheral blood but significantly accumulated in biopsies of patients with CAMR, it is tempting to speculate that cytotoxic cells migrate from the blood to the graft during this form of rejection. However, because our blood and biopsies were from largely independent patients, this “mirror image” hypothesis would need confirmation on simultaneous blood and biopsy samples.

We also show here that other factors also influence GrzB in the peripheral blood, notably donor age and type of CNI. The positive association with donor age may be related to the fact that older kidneys are thought to be more immunogenic. The positive association with donor age may be related to the fact that older kidneys are thought to be more immunogenic. It is currently unclear as to whether idiopathic TG is CAMR sampled at an inactive time or is due to other causes. According to our results, the two can be distinguished by GrzB mRNA (Mann–Whitney test; P < 0.05 for both the biopsy and the PBMC), suggesting CAMR may be the active phase of the same glomerular disease or that idiopathic TG is caused by a GrzB-independent mechanism. For this molecule, measuring the mRNA appeared more sensitive than measuring the protein. We additionally show for the first time that, in contrast to acute rejection where GrzB is increased in the PBMC, during CAMR GrzB is significantly decreased. Furthermore, ROC analysis revealed that measuring GrzB mRNA may have potential as a decision-making tool in clinical practice to diagnose CAMR in a minimally invasive manner. Given that GrzB transcripts were significantly reduced in peripheral blood but significantly accumulated in biopsies of patients with CAMR, it is tempting to speculate that cytotoxic cells migrate from the blood to the graft during this form of rejection. However, because our blood and biopsies were from largely independent patients, this “mirror image” hypothesis would need confirmation on simultaneous blood and biopsy samples.

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Thus, overall, we show distinct profiles of expression of regulatory, effector, and cytotoxic molecules in the grafts and blood of long-term-surviving renal transplant patients, each molecule being influenced by different clinical and demographic parameters. In fact, whereas neither intragraft nor peripheral blood FOXP3 or T-bet mRNA can distinguish rejection from nonrejection status in long-term-surviving renal transplant patients, GrzB can indeed do so, with a possible migration of GrzB-expressing cells from the blood to the graft during CAMR. These data affirm GrzB as an important marker not only for acute rejection but also for chronic antibody-mediated rejection. Moreover, our study has identified several previously unreported clinical and demographic factors influencing regulatory/effector/cytotoxic profiles in the peripheral blood. These data also underline how important it will be for future studies of gene expression in transplantation to be performed on large cohorts allowing for potential confounding factors to be taken into account when evaluating diagnostic, predictive, or mechanistic biomarkers. Analyzing other markers of CAMR, such as Tribbles-1 or TLR4 markers of acute rejection, such as the cytotoxic molecules Perforin and Fas-L, or other regulatory T cell-associated markers, such as GITR, could help to better understand the pathogenesis of CAMR and contribute to its diagnosis.

**CONCISE METHODS**

**Patients**

The study was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biologic Risks. The study was organized into two parts; the first based on graft biopsies and the second on peripheral blood.

**Part 1: Comparison of Intragraft Regulatory, Effector, and Cytotoxic Profiles in Nonrejection versus Rejection.**

Forty-eight biopsies from 48 patients were included for analysis and were selected according to their diagnosis based on the Banff classification criteria. Three groups of biopsies with late graft injury were included: lesions of calcineurin inhibitor toxicity (CNI tox; n = 7, all negative for C4d and DSA), idiopathic transplant glomerulopathy (TG; n = 11, all negative for C4d and DSA antibodies), and chronic antibody-mediated rejection (CAMR; n = 14, all had transplant glomerulopathy associated with C4d staining and circulating DSA). The demographic and clinical data for all of these individuals are provided in Table 2. Controls included a group of protocol biopsies with normal histology and stable graft function taken at 1 yr posttransplant (STA; n = 7, all negative for C4d and DSA) and a group of biopsies for cause with acute cellular rejection (ACR; n = 9, all negative for C4d and DSA). Clinical data for these control groups are provided in Supplementary Table 1.

**Part 2: Comparison of Peripheral Blood Regulatory, Effector and Cytotoxic Profiles in Nonrejection versus Rejection.**

One-hundred ninety-nine blood samples from 15 healthy volunteers (HV) and 184 kidney transplant patients were included for the analysis of PBMC. The first group of transplant patients had, at the time of sampling, stable graft function at least 5 yr posttransplant with undetermined biopsy status (STA; n = 164). Other groups included patients with deteriorating graft function with biopsy-proven idiopathic TG all negative for C4d and DSA (TG; n = 7) or deteriorating graft function with biopsy-proven transplant glomerulopathy together with C4d and circulating anti-HLA (CAMR; n = 15, all had transplant glomerulopathy associated with C4d staining and circulating DSA). The demographic and clinical data for all of these individuals are provided in Table 3. Controls included a group of patients with stable graft function (STA; n = 10) and a group of patients with acute cellular rejection (ACR; n = 9, all negative for C4d and DSA) early posttransplant. Clinical data for these two groups are provided in Supplementary Table 2.
Biopsies (16 to 18 gauges) were homogenized with a PT 3100 Polytron Rotor-Stator (Kinematica, AG, Luzern, Switzerland). RNA was extracted using the QIAGen RNA microextraction kit (QIAGEN, Courtaboeuf, France), and quality and quantity were determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA). PBMC were separated by density centrifugation using Lymphosep, lymphocyte separation media (Bio West, Nuaille, France), and RNA was extracted using the TRIzol method (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. For STA and ACR patients only, blood was collected in PAXgene tubes (Becton Dickinson, Le Pont Claix, France) and processed according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using an RT-
Quantification of Infiltrating Cells

Immunohistochemistry for FOXP3 was performed on formalin-fixed, paraffin-embedded tissue as described, using a rabbit polyclonal anti-FOXP3 antibody (ab10563, Abcam, Cambridge, MA), a mouse monoclonal antibody to T-bet (4B10, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-GrzB (MAB3070, Chemicon, Temecula, CA). Sections were scored for positive cells in coded slides by one observer (RBC), according to previously published methods, and the results were expressed as the number of positive cells in the cortex per square millimeter. Double immunohistochemistry stains were performed to determine the phenotype of the cells expressing FOXP3, T-bet, and GrzB, using FOXP3 versus CD4 or versus T-bet, T-bet versus FOXP3, versus GrzB, versus CD4 (1F6, Biocare Medical, Concord, CA), or versus CD20 (L26, Dako, Carpinteria, CA), and GrzB versus CD8 (SP16, Biocare Medical) versus FOXP3 or versus T-bet. Each of these double stains was done on 3 samples of CAMR.

Statistical Analysis

Nonparametric methods were used for all of the comparisons between the groups with small sample sizes (CNI tox, TG, and CAMR). In particular, the nonparametric Mann–Whitney test was used to compare differences in FOXP3, T-bet, and GrzB expression between two groups. The nonparametric Kruskal–Wallis test followed by Dunn’s multiple comparison was used to compare differences in expression between three groups. A chi-square test was used to analyze differences in immunosuppression in the PBMC cohort. Values of \( P < 0.05 \) were considered as significant. ROC curve analysis was performed on the STA and CAMR PBMC samples to determine the cut-off point of GrzB mRNA that yielded the highest combined sensitivity and specificity in diagnosing CAMR (see Figure 5 legend for explanation).

Given the sufficiently large sample size, multivariate analyses were performed to determine the influence of different clinical and demographic parameters on the expression of FOXP3, T-bet, and GrzB in the 164 STA patients. Note that the other groups were not included in this analysis due to their small sample size. Either a multivariate linear regression analysis was performed following log transformation of the data (FOXP3 and T-bet) or a multivariate generalized linear model was performed when log transformation did not succeed in normalizing the data (GrzB displayed a gamma distribution). If the hypothesis of linearity was not respected, then a polynomial function was used to model the relationship between the expression of the molecule measured and the clinical/demographic covariate.

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DISCLOSURES

None.

REFERENCES


34. Supplemental information for this article is available online at http://www.jasn.org/.