ORIGINAL ARTICLE

Messenger RNA for *FOXP3* in the Urine of Renal-Allograft Recipients

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ABSTRACT

BACKGROUND

The outcome of renal transplantation after an episode of acute rejection is difficult to predict, even with an allograft biopsy.

METHODS

We studied urine specimens from 36 subjects with acute rejection, 18 subjects with chronic allograft nephropathy, and 29 subjects with normal biopsy results. Levels of messenger RNA (mRNA) for *FOXP3*, a specification and functional factor for regulatory T lymphocytes, and mRNA for CD25, CD3ε, perforin, and 18S ribosomal RNA (rRNA) were measured with a kinetic, quantitative polymerase-chain-reaction assay. We examined associations of mRNA levels with acute rejection, rejection reversal, and graft failure.

RESULTS

The log-transformed mean (±SE) ratio of *FOXP3* mRNA copies to 18S ribosomal RNA copies was higher in urine from the group with acute rejection (3.8±0.5) than in the group with chronic allograft nephropathy (1.3±0.7) or the group with normal biopsy results (1.6±0.4) ($P<0.001$ by the Kruskal–Wallis test). *FOXP3* mRNA levels were inversely correlated with serum creatinine levels measured at the time of biopsy in the acute-rejection group (Spearman's correlation coefficient $=-0.38$, $P=0.02$) but not in the group with chronic allograft nephropathy or the group with normal biopsy results. Analyses of receiver-operating-characteristic curves demonstrated that reversal of acute rejection can be predicted with 90 percent sensitivity and 73 percent specificity with use of the optimal identified cutoff for *FOXP3* mRNA of 3.46 ($P=0.001$). *FOXP3* mRNA levels identified subjects at risk for graft failure within six months after the incident episode of acute rejection (relative risk for the lowest third of *FOXP3* mRNA levels, 6; $P=0.02$). None of the other mRNA levels were predictive of reversal of acute rejection or graft failure.

CONCLUSIONS

Measurement of *FOXP3* mRNA in urine may offer a noninvasive means of improving the prediction of outcome of acute rejection of renal transplants.

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N Engl J Med 2005;353:2342-51.

KIDNEY TRANSPLANTATION IS THE treatment of choice for most patients with end-stage renal disease (ESRD), but a shortage of organs limits its availability.[1] Renal-allograft failure is the fourth most common cause of ESRD in the United States[2] and contributes to the shortage of organs.

Acute rejection is an important risk factor for allograft failure.[3-6] The current approach to treatment of acute rejection is uniform, although it is well recognized that some rejection episodes are not fully reversible and lead to long-term graft dysfunction and failure, whereas others are easily treatable and benign.[6] The outcome of acute rejection is difficult to predict, and histologic features that are observed in allograft tissue obtained by core needle biopsy are currently the best predictors.[7,8] The invasive procedure of allograft biopsy, however, is associated with complications such as bleeding, arteriovenous fistula, and even graft loss.[9,10]

We previously reported a method using a quantitative polymerase chain reaction (PCR) to measure messenger RNA (mRNA) levels of immune products within urinary cells of renal-transplant recipients.[11-13] This noninvasive and nucleic acid–based technique allows for the early diagnosis of acute rejection by detection of increased levels of cytolytic T-cell products such as granzyme B and perforin,[11] integrins such as CD103,[12] and key chemokine and chemokine-receptor combinations such as gamma-inducible protein of 10kD (IP-10) and its receptor, CXCR3, which promote effector T-cell recruitment to a transplant.[13]

Recent studies have highlighted the role of a specialized subgroup of CD4+CD25+ T lymphocytes, termed regulatory T lymphocytes (Treg cells), in the suppression of autoimmunity.[14,15] Treg cells specifically express the X-linked forkhead/winged helix transcription factor, FOXP3,[16] and mutations in the human *FOXP3* gene result in an autoimmune disease characterized by polyendocrinopathy and enteropathy that is fatal in infancy.[17,18] In a similar manner, male mice with a loss-of-function mutation in the *Foxp3* gene (scurfy mice) or with a deficiency of the *Foxp3* gene generated by homologous recombination waste away and die within three to four weeks of life with multiorgan lymphocytic infiltrates; transfer of the *Foxp3* gene reverses this process.[16,19,20]

In view of the dominant role of Treg cells in the maintenance of self-tolerance[14,15] and in view of the cells' suppressive role in experimental models of transplantation tolerance,[21-23] we reasoned that measurement of *FOXP3* mRNA levels in urinary cells might provide insight into the immunologic events within a renal allograft undergoing acute rejection. We developed a kinetic, quantitative PCR assay and demonstrate that levels of *FOXP3* mRNA in urinary cells predict the reversibility of acute rejection and identify patients at high risk for graft loss after an episode of acute rejection.

METHODS

STUDY COHORTS

We examined urine samples from 83 kidney-transplant recipients. In this group were 36 subjects with graft dysfunction (mean [±SD] creatinine level, 3.6±2.4 mg per deciliter [318.2±212.2 μmol per liter]) and biopsy-confirmed acute rejection (mean age, 41±12 years; 15 men and 21 women; 13 white, 12 black, and 11 with other racial or ethnic backgrounds; with 20 living and 16 deceased donors), 29 subjects with stable allograft function (mean creatinine level, 1.4±0.4 mg per deciliter [123.8±35.4 μmol per liter]) and normal allograft biopsy (mean age, 44±14 years; 15 men and 14 women; 12 white, 4 black, and 13 with other racial or ethnic backgrounds; with 26 living and 3 deceased donors), and 18 subjects with allograft dysfunction (mean creatinine level, 3.1±1.6 mg per deciliter [274.0±141.4 μmol per liter]) and biopsies classified as indicating chronic allograft nephropathy (mean age, 52±12 years; 9 men and 9 women; 9 white, 2 black, and 7 with other racial or ethnic backgrounds; with 5 living and 13 deceased donors).

Seventy-five of the 83 urine specimens were collected before the biopsy procedure, and 8 samples were obtained after the procedure. Formalin-fixed, paraffin-embedded renal-biopsy specimens were stained with hematoxylin and eosin, periodic acid–Schiff, and Masson's trichrome stains and were scored with the use of the Banff 97 classification[24] by a pathologist who was blinded to the results of molecular studies. Immunosuppression consisted of a calcineurin inhibitor–based regimen (cyclosporine or tacrolimus), with the administration of glucocorticoids, antilymphocyte antibodies (muromonab-CD3 [OKT3] or antithymocyte globulin), or both for the treatment of acute rejection.[11] The study was approved by the institutional review board at the Weill Medical College of Cornell University in New York, and each patient gave written informed consent.

QUANTITATION OF MRNA BY KINETIC, QUANTITATIVE PCR

Total RNA was isolated from urine-cell pellets, quantified and reverse transcribed to complementary DNA (cDNA).[11] We designed and synthesized oligonucleotide primers and fluorogenic probes for the measurement of mRNA levels of *FOXP3*, CD25, CD3ε, perforin, and 18S ribosomal RNA (rRNA) (Table 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org). PCR analysis was performed by a two-step process, a preamplification step followed by measurement of mRNA with an ABI Prism 7700 system (the PCR protocol is provided in the Supplementary Appendix). Transcript levels were calculated by a standard curve method,[12] and mRNA copy numbers were normalized with the use of 18S rRNA copy numbers (the number of mRNA copies in 1 μg of RNA divided by the number of 18S rRNA copies in 1 fg of RNA). When no detectable level of a transcript was found, a value equal to half the minimum observed 18S-normalized level was assigned. For an estimation of group means, this method is considered a reasonable substitute for the value of zero or the minimum detected value; moreover, the nonparametric statistical tests of group differences reported below are not affected by the choice of value.[25]

STATISTICAL ANALYSIS

The levels of mRNA for *FOXP3*, CD25, CD3ε, perforin, and 18S rRNA deviated from a normal distribution ($P<0.001$), but a log transformation substantially reduced the positive skew. We used the 18S-normalized level as the dependent variable in a Kruskal–Wallis test to identify any differences among the group with acute rejection, the group with chronic allograft nephropathy, and the group with normal biopsy results and then used Dunn's test for multiple comparisons. The Mann–Whitney test, equivalent to the Kruskal–Wallis test when applied to two groups, was used when mRNA levels were compared between two groups. Spearman's rank-order correlations were used to test for a monotonic association of the 18S-adjusted mRNA transcript levels with serum creatinine levels and time (in days) from kidney transplantation to biopsy. An episode of acute rejection was classified as reversible if the serum creatinine level returned to within 15 percent of the prerejection level within four weeks after the initiation of antirejection treatment. A second end point was the loss of the graft during the first six months after the diagnosis of acute rejection. We used receiver-operating-characteristic (ROC) curves to analyze mRNA levels in order to determine the cutoff points that yielded the highest combined sensitivity and specificity for predicting the outcome of an episode of acute rejection.

RESULTS

LEVELS OF *FOXP3* MRNA IN URINARY CELLS

The log-transformed mean (±SE) ratio of *FOXP3* mRNA copies to 18S-rRNA copies in urinary cells was 3.8±0.5 in the 36 subjects with acute rejection and was higher than the levels in both the 18 subjects with chronic allograft nephropathy (1.3±0.7) and the 29 subjects with normal biopsy results (1.6±0.4, $P<0.001$ by the Kruskal–Wallis test) (Fig. 1A). Among the three groups, the 18S-normalized, log-transformed mRNA levels of CD25 (6.9±0.4, 4.0±0.5, and 2.8±0.6, respectively; $P<0.001$), CD3ε (8.2±0.4, 4.3±0.5, and 1.6±0.5; $P<0.001$), and perforin (7.6±0.4, 4.5±0.4, and 2.8±0.4; $P<0.001$) were also highest in the acute-rejection cohort (Fig. 1B, 1C, and 1D).

FOXP3 MRNA LEVELS AND DISEASE SEVERITY

We observed a significant inverse relationship between the levels of *FOXP3* mRNA and serum creatinine measured during an episode of acute rejection (Spearman's correlation coefficient [r_s] = −0.38, $P=0.02$). By contrast, serum creatinine levels were not significantly related to mRNA levels of CD25 ($r_s=-0.01$, $P=0.93$), CD3ε ($r_s=-0.11$, $P=0.54$), or perforin ($r_s=-0.23$, $P=0.18$) in the acute-rejection group. Also, the mean (±SE) serum creatinine level in the 16 subjects with acute rejection of Banff grade IA (moderate tubulitis) did not differ significantly from that of the 20 subjects with grade IB (severe tubulitis) or more (3.3±0.6 mg per deciliter [291.7±53.0 μmol per liter] as compared with 3.8± 0.6 mg per deciliter [318.2±53.0 μmol per liter], $P=0.57$).

There was no correlation between the levels of *FOXP3* mRNA and serum creatinine that were measured in the group with chronic allograft nephropathy ($r_s=0.02$, $P=0.93$) or the group with normal biopsy results ($r_s=-0.08$, $P=0.67$).

FOXP3 MRNA LEVELS AND REVERSAL OF ACUTE REJECTION

Twenty-six of the 36 episodes of acute rejection qualified as successfully reversed; the remaining 10 did

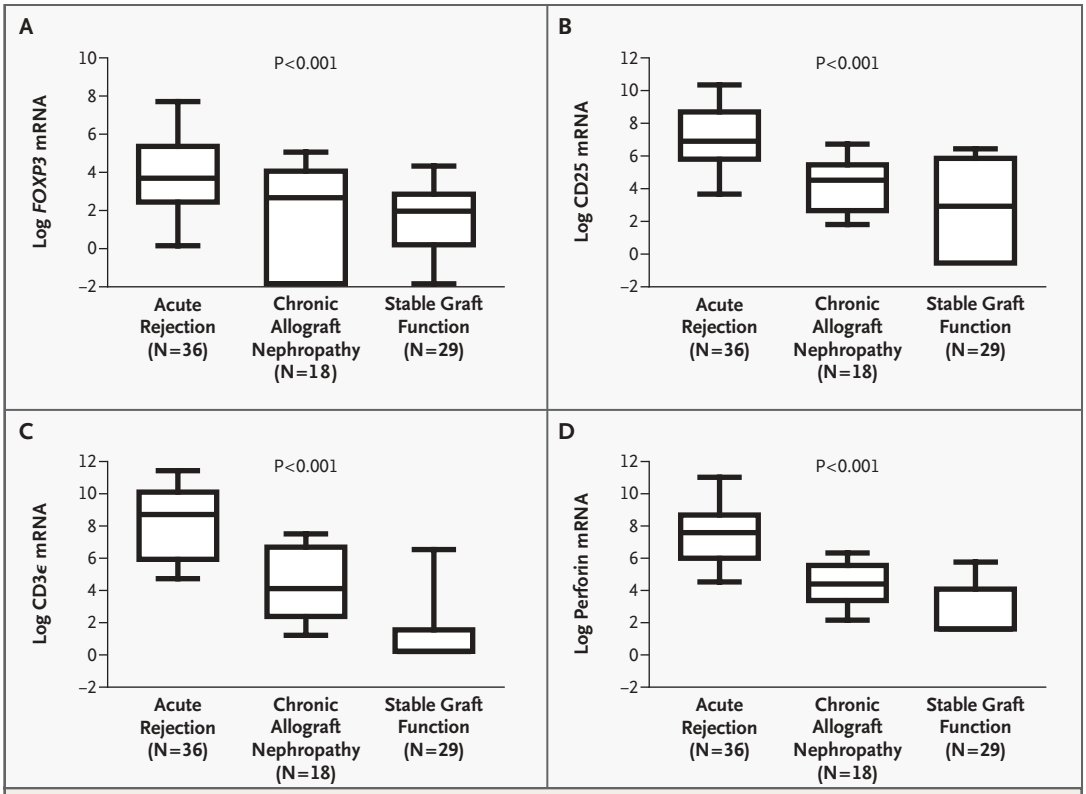

Figure 1. Levels of *FOXP3* mRNA in Urinary Cells.

Box plots show the 10th, 25th, 50th (median), 75th, and 90th percentile values for log-transformed ratios of mRNA copies to 18S rRNA copies for *FOXP3*, CD25, CD3ε, and perforin in urine samples obtained from 36 subjects with biopsy-confirmed acute rejection, 18 subjects with biopsy-confirmed chronic allograft nephropathy, and 29 subjects with stable graft function and normal biopsy results. The levels of mRNA for *FOXP3*, CD25, CD3ε, and perforin were higher in the urinary cells from subjects with acute rejection than in the subjects with chronic allograft nephropathy or normal biopsy results. P values are based on the Kruskal–Wallis test, with the log-transformed mRNA levels treated as the dependent variable. Dunn's multiple-comparison test showed that levels of *FOXP3* mRNA in the acute-rejection group were higher than those in both the group with chronic allograft nephropathy ($P<0.05$) and the group with normal biopsy results ($P<0.01$) (Panel A). CD25 mRNA levels were higher in the acute-rejection group than in both the group with chronic allograft nephropathy ($P<0.001$) and the group with normal biopsy results ($P<0.001$) (Panel B). CD3ε mRNA levels were higher in the acute-rejection group than in both the group with chronic allograft nephropathy ($P<0.01$) and the group with normal biopsy results ($P<0.001$); CD3ε mRNA levels were also higher in the group with chronic allograft nephropathy than in the group with normal biopsy results ($P<0.05$) (Panel C). Perforin mRNA levels were higher in the acute-rejection group than in both the group with chronic allograft nephropathy ($P<0.001$) and the group with normal biopsy results ($P<0.001$) (Panel D). In all cases, log-transformed levels, normalized for 18S rRNA, are shown.

not. Levels of *FOXP3* mRNA in urinary cells were significantly higher in the group with successful reversal than in the group without reversal (mean [±SE] level, 4.7±0.5 and 1.5±0.7, respectively; P=0.001) (Fig. 2A). In the two groups, the levels of mRNA for CD25 (7.3±0.4 and 6.0±0.9, P=0.22), CD3ε (8.5±0.5 and 7.4±0.8, P=0.35), and perforin (7.8±0.5 and 7.3±0.7, P=0.43) were not informative of outcome (Fig. 2B, 2C, and 2D).

The ROC curves (Fig. 3) show the fraction of true positive results (sensitivity) and false positive results (1−specificity) for various cutoff levels of mRNA for *FOXP3*, CD25, CD3ε, and perforin. The log-transformed threshold that gave the maximal sensitivity and specificity for *FOXP3* mRNA was 3.46; using the cutoff value of 3.46 derived from the data, the *FOXP3* mRNA level predicted rejection reversal with a sensitivity of 90 percent and a specificity of 73 percent (P=0.001) (Fig. 3A). The levels of mRNA for CD25, CD3ε, and perforin were not predictive of reversal of acute rejection (Fig. 3B, 3C, and 3D).

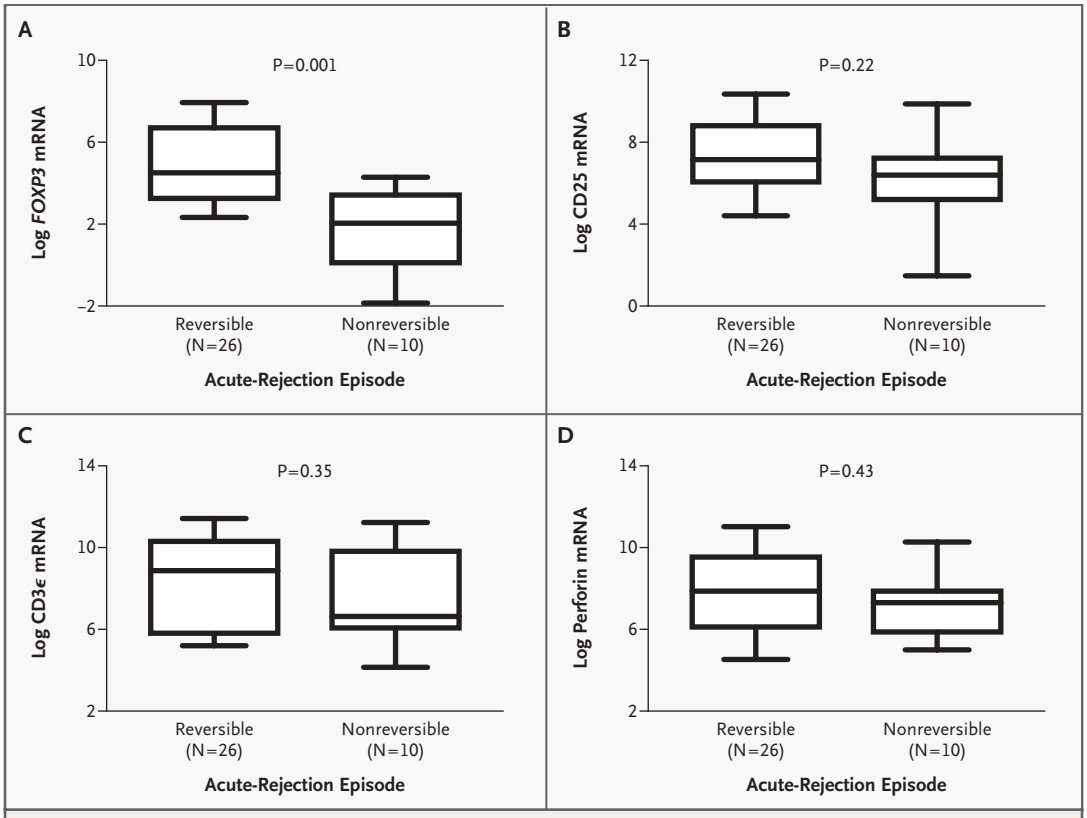

Figure 2. Correlation between Levels of *FOXP3* mRNA in Urinary Cells and Reversal of an Episode of Acute Rejection.

Box plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles for levels of mRNA for *FOXP3*, CD25, CD3ε, and perforin in urine samples obtained from 26 subjects with successful reversal of acute rejection (classified as reversible and defined by the return of serum creatinine levels to within 15 percent of prerejection levels within four weeks after the initiation of antirejection treatment) and 10 patients without reversal of acute rejection (nonreversible). The levels of mRNA for *FOXP3* but not for CD25, CD3ε, and perforin were significantly higher in subjects with reversible acute rejection than in subjects with nonreversible acute rejection. Two-tailed P values are based on the Mann–Whitney test. In all cases, log-transformed levels, normalized for 18S rRNA, are shown.

Successful reversal of acute rejection, as compared with unsuccessful reversal, was not predicted by the subjects' age (mean [±SD], 41±2.2 years and 40±4.6 years, respectively; P=0.68), sex (10 men and 16 women vs. 5 men and 5 women, P=0.71), race (10 white, 6 black, and 10 with other race or ethnic background vs. 3 white, 6 black, and 1 with other race or ethnic background; P=0.08), graft-donor source (15 living and 11 deceased vs. 5 living and 5 deceased, P=0.68), Banff histologic grade (11 with IA and 15 with >IA vs. 5 with IA and 5 with >IA, P=0.68), or initial antirejection treatment (24 with glucocorticoids and 2 with antilymphocyte antibodies vs. 7 with glucocorticoids and 3 with antilymphocyte antibodies, P=0.12). Among subjects with successful reversal, as compared with those with unsuccessful reversal, serum creatinine levels (median levels, 2.3 mg per deciliter and 6.5 mg per deciliter, respectively; P<0.001) and the time from kidney transplantation to the development of acute rejection (median time, 82 days and 523 days, respectively; P=0.008) were lower. In logistic-regression analyses predicting nonresponse, levels of *FOXP3* mRNA in urinary cells remained significant after statistical control for serum creatinine level (P=0.04) and the time from transplantation to rejection (P=0.02).

A linear combination of levels of *FOXP3* mRNA and creatinine was a better predictor of rejection reversal (90 percent sensitivity and 96 percent specificity) than *FOXP3* mRNA levels alone (90 percent sensitivity and 73 percent specificity) or serum creatinine levels alone (85 percent sensitivity and 90 percent specificity).

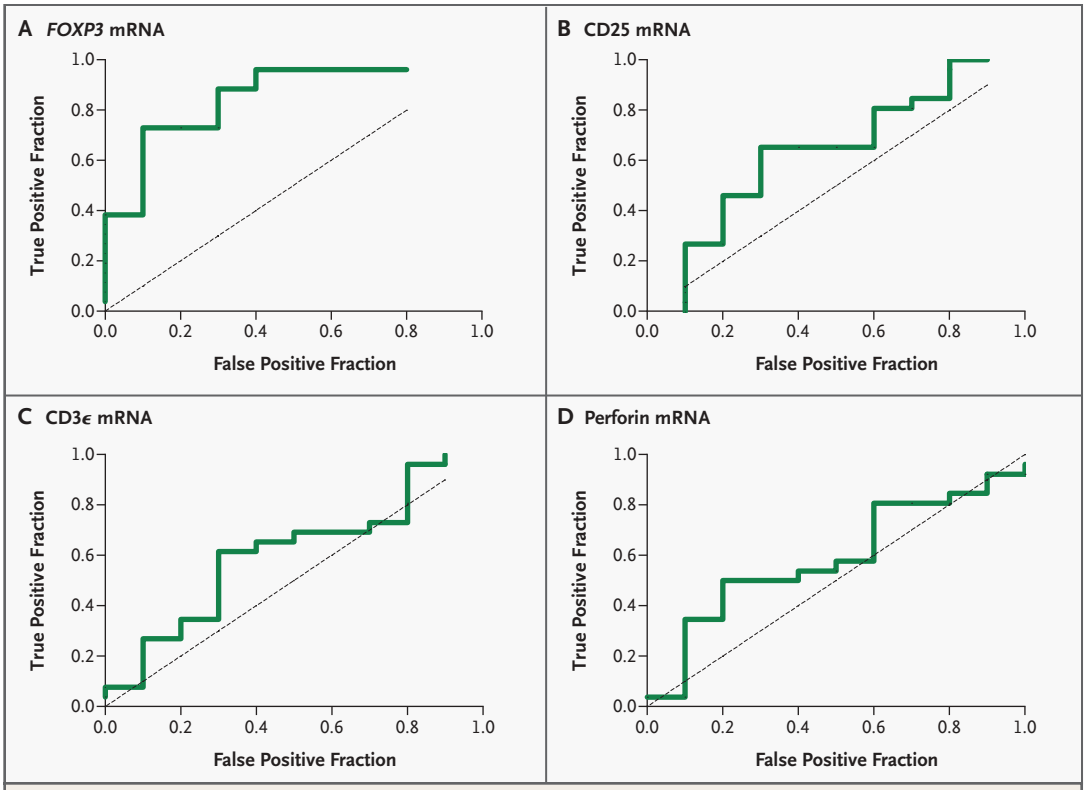

Figure 3. Receiver-Operating-Characteristic (ROC) Curves for mRNA Levels.

The fraction of true positive results (sensitivity) and false positive results (1–specificity) for levels of mRNA for *FOXP3*, CD25, CD3ε, and perforin, each normalized for 18S rRNA, as predictors of reversal of acute rejection are shown. The calculated area under the curve was 0.85 (95 percent confidence interval, 0.71 to 0.99) for *FOXP3* mRNA levels (Panel A) but only 0.63 (95 percent confidence interval, 0.42 to 0.84) for CD25 mRNA levels (Panel B), 0.60 (95 percent confidence interval, 0.39 to 0.81) for CD3ε mRNA levels (Panel C), and 0.58 (95 percent confidence interval, 0.38 to 0.79) for perforin mRNA levels (Panel D). A value of 0.5 is no better than that expected by chance (the null hypothesis), and a value of 1.0 reflects a perfect indicator. Of the four mRNA measures, only *FOXP3* predicts successful reversal significantly better than chance (P=0.001).

FOXP3 mRNA LEVELS AND ALLOGRAFT FAILURE

Ten of the 36 subjects with acute rejection lost their grafts within six months after the incident episode of acute rejection, and 9 of those 10 subjects did not respond to the initial antirejection therapy. Renal-allograft recipients with a failed allograft within six months after the episode of acute rejection had significantly lower *FOXP3* mRNA levels in their urinary cells than the 26 subjects who had a functioning allograft (2.0±0.8 and 4.5±0.5, respectively; P=0.01). In the two groups, the levels of mRNA for CD25 (6.6±0.7 and 7.1±0.5, P=0.33), CD3ε (7.9±0.7 and 8.3±0.5, P=0.76), and perforin (7.8±0.6 and 7.6±0.5, P=0.90) did not predict allograft loss.

The rate of and relative risk of graft failure within six months after an episode of acute rejection, for thirds of each mRNA measure, are shown in Figure 4. At the highest third of *FOXP3* mRNA levels, the graft failure rate was 8 percent; at the middle third, the graft failure rate was 25 percent and the relative risk was 3; and at the lowest third, the graft failure rate was 50 percent and the relative risk was 6 (P=0.02 by the chi-square test for linear trend) (Fig. 4A). In contrast, the rate of graft failure after an episode of acute rejection did not differ significantly across the thirds of mRNA levels for CD25, CD3ε, and perforin (Fig. 4B, 4C, and 4D).

Graft failure as compared with graft success was not predicted by the subjects' age (mean [±SD] ages, 39±4.2 years and 42±2.4 years, respectively; P=0.52), sex (4 men and 6 women vs. 11 men and 15 women, P=0.90), race (3 white, 6 black, and 1 with other race or ethnic background vs. 10 white, 6 black, and 10 with other race or ethnic back-

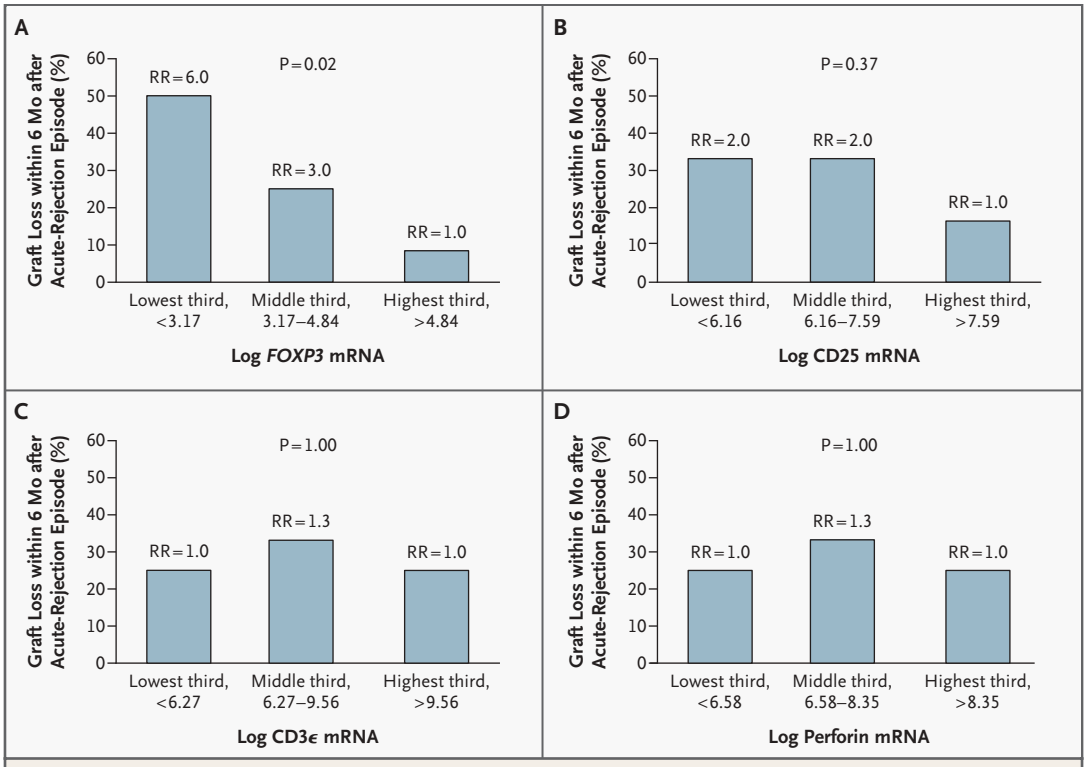

Figure 4. Relative Risk (RR) of Graft Failure after an Episode of Acute Rejection.

The rates and relative risks of graft failure within six months after an episode of acute rejection for thirds of *FOXP3*, CD25, CD3ϵ, and perforin mRNA levels are shown. The graft-failure rate was 50 percent and the relative risk was 6 for the lowest third of *FOXP3* mRNA levels; 25 percent and 3, respectively, for the middle third; and 8 percent for the highest third (P=0.02 by the chi-square test for linear trend) (Panel A). There was no relationship between graft failure after an episode of acute rejection and the thirds of mRNA levels for CD25, CD3ϵ, and perforin (Panels B, C, and D).

ground; P=0.08), graft-donor source (5 living and 5 deceased vs. 15 living and 11 deceased, P=0.68), Banff histologic grade (5 with IA and 5 with >IA vs. 11 with IA and 15 with >IA, P=0.68), or initial antirejection treatment (24 with glucocorticoids and 2 with antilymphocyte antibodies vs. 7 with glucocorticoids and 3 with antilymphocyte antibodies, P=0.12). In subjects with graft failure, as compared with subjects with graft success, serum creatinine levels (median levels, 6.5 mg per deciliter [574.6 μmol per liter] and 2.3 mg per deciliter [203.3 μmol per liter], respectively; P<0.001) and the time from kidney transplantation to the development of acute rejection (median time, 562 days and 82 days; P=0.003) were significantly greater. In a logistic-regression analysis, *FOXP3* mRNA levels became nonsignificant after control for serum creatinine levels (P=0.13) or time between transplantation and rejection (P=0.09).

A linear combination of levels of *FOXP3* mRNA and creatinine was a better predictor of graft failure (90 percent sensitivity and 92 percent specificity) than were either *FOXP3* mRNA levels alone (80 percent sensitivity and 69 percent specificity) or serum creatinine levels alone (85 percent sensitivity and 90 percent specificity).

FOXP3 mRNA Levels and Time to Acute Rejection

Late acute rejection (acute rejection occurring at least three months after transplantation) results in an outcome that is inferior to that of early acute rejection.[26,27] We found a strong inverse relationship between levels of *FOXP3* mRNA in urinary cells and the time from kidney transplantation to the development of acute rejection ($r_s=-0.42$, P=0.01) (Fig. 5A). Levels of *FOXP3* mRNA in urinary cells were lower in 11 urine specimens from patients with late acute rejection than in 25 specimens from patients with early acute rejection (mean [±SE] lev-

el, 2.5±0.6 and 4.7±0.5; P=0.009). CD25 mRNA levels also showed an inverse relation (r_s=−0.45, P=0.006) (Fig. 5B), and the levels were lower during late rejection than during early acute rejection (5.8±0.8 and 7.4±0.4, P=0.07). There was no inverse relation between the time from kidney transplantation to the development of acute rejection and the mRNA levels of CD3ε (r_s=−0.26, P=0.12) (Fig. 5C) or perforin (r_s=−0.02, P=0.91) (Fig. 5D). There was also no correlation between the serum creatinine levels at the time of acute rejection and the time from kidney transplantation to the development of acute rejection (r_s=0.23, P=0.17).

DISCUSSION

Previous studies have associated acute allograft rejection with cytotoxic T cells and have shown that monitoring the levels of these cells in blood, urine, or both is helpful in the treatment of renal-allograft recipients.[11,28,29] Our study suggests that levels of *FOXP3* mRNA in urinary cells may serve as a mechanistically informative biomarker of acute-rejection outcome.

Depletion or dysfunction of Treg cells can result in autoimmune disease; tolerance to experimental allografts, on the other hand, is associated with graft infiltration by Treg cells.[14-16,21-23] An attractive hypothesis is that the Treg cells prevent the emergence of effector T cells, and that the absence of disease or tolerance is due to the lack of an immune response. An alternative hypothesis is that Treg cells play a "damage control" role rather than a preventive one.[30] We suggest that the host antidonor immune repertoire during acute rejection includes the activation of graft-destructive effector cells as well as graft-protective Treg cells. We observed that levels of *FOXP3* mRNA in urinary cells, a defining functional marker of Treg cells, and mRNA for perforin, a cytotoxic effector, are both expressed in a heightened fashion during acute rejection and that high levels of *FOXP3* mRNA are associated with reversible acute rejection and a lower risk of graft failure. These findings are consistent with the hypothesis that Treg cells serve to limit antiallograft immunity and that the lack of counterregulation by Treg cells during an episode of acute rejection results in unrestrained effector-cell activity, impaired allograft function, and even graft failure.

Histologic analysis of renal allografts is considered to be the best predictor of acute rejection.[7,8] However, it has long been recognized that cellular interstitial infiltration is not invariably associated with allograft dysfunction or failure.[31] In the current study, Banff grades of acute rejection did not predict rejection outcome, and the serum creatinine levels did not vary across Banff grades. We suggest that graft-infiltrating cells comprise both graft-destructive cells such as cytotoxic T cells and graft-protective *FOXP3*-expressing Treg cells and that graft dysfunction and response to therapy may be predicted more accurately when the heterogeneous nature of the cellular components is better resolved.

Elevated levels of serum creatinine are an established risk factor for renal allograft failure[32] and in our studies were shown to be a strong predictor of the outcome of acute rejection. Do levels of *FOXP3* mRNA in urinary cells provide information above and beyond that provided by serum creatinine levels? In our study, levels of serum creatinine and *FOXP3* mRNA in urinary cells were independent predictors of reversal of acute rejection. Moreover, rejection reversal and graft loss were predicted with a higher degree of accuracy with the use of levels of both *FOXP3* mRNA and creatinine than with either one alone. However, since our estimates of sensitivity and specificity were calculated from the same sample that was used to select the cutoff points, the estimates are upwardly biased and need to be reevaluated in an independent sample.

In both adult and pediatric recipients of renal allografts, an episode of late acute rejection is associated with a lower rate of graft survival than an episode of early acute rejection.[26,27] Our finding of an inverse relationship between levels of *FOXP3* mRNA in urinary cells and the time to acute rejection suggests a cellular mechanism for the hitherto unexplained poor outcome associated with late acute rejection.

A mechanistic hypothesis engendered by our study is that drugs that enhance the generation of Treg cells, or the administration of Treg cells themselves, may improve the outcome of acute rejection. Cyclosporine and tacrolimus both inhibit the production of interleukin-2, an essential growth factor for Treg cells,[33] but induce the production of transforming growth factor β1,[34,35] an inducer of *FOXP3* and a promoter of the development of CD4+CD25+ Treg cells.[36,37] Sirolimus (rapamycin) has been shown to promote the expansion of murine Treg cells in vitro,[38] and glucocorticoids have been reported to increase the expression of *FOXP3* mRNA in human CD4+ cells.[39] However, the in vivo effects

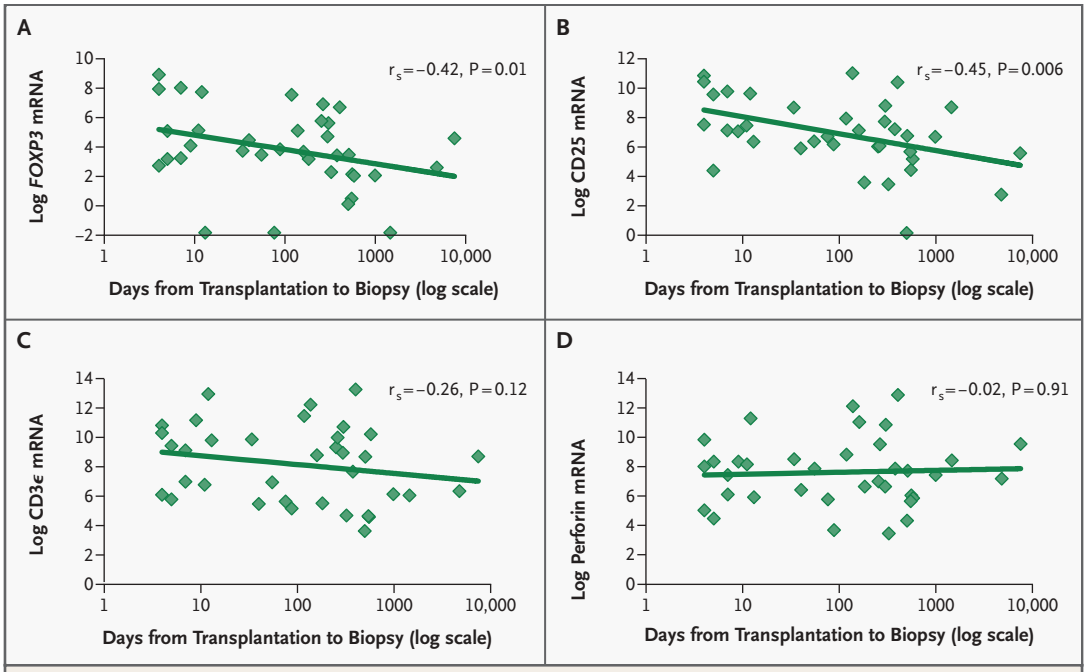

Figure 5. Levels of mRNA in Urinary Cells and the Time from Kidney Transplantation to the Development of Acute Rejection.

The relationship between levels of mRNA in urine samples and the time from kidney transplantation to the development of biopsy-confirmed acute rejection is shown, along with Spearman's rank-order correlation (r_s). A significant inverse relationship between the levels of *FOXP3* mRNA and CD25 mRNA in urinary cells and the time from kidney transplantation to the development of acute rejection was found (Panels A and B). There was no such relationship between levels of CD3ε mRNA or perforin mRNA in urinary cells and the time from kidney transplantation to the development of acute rejection (Panels C and D). Log-transformed mRNA levels, normalized for 18S rRNA, are shown. The numbers of days from renal transplantation to the development of acute rejection are shown on a log scale.

of drugs on the induction, expansion, and function of Treg cells in allograft recipients remain to be fully characterized.

How might *FOXP3*-expressing Treg cells exert their salutary activity during an episode of acute rejection? Treg cells have been shown to dampen or suppress local host immune responses by acting on antigen-presenting cells, directly modulating effector-cell functions, or both.[14,15] Mechanisms of immunosuppression by *FOXP3*-expressing Treg cells include direct cell contact, cytokine signaling, and inhibition of transcription of genes central to effector functions.[40] The role of these mechanisms in mitigating the acute-rejection response remains undetermined.

In sum, our study suggests that levels of *FOXP3* mRNA in urinary cells may serve as a mechanistically informative biomarker of acute-rejection outcome, with lower levels associated with irreversible acute rejection and even graft failure. In addition to suggesting a robust cellular mechanism for the clinically important differences in the outcome of acute-rejection episodes, the strategy we present here may ultimately lead to individualized treatment of renal-allograft recipients and inform antirejection therapy, including the consideration of infusion of Treg cells to treat acute rejection of allografts.

Supported in part by awards (AI51652 and AI60706, to Dr. Suthanthiran, and AI54720, to Dr. Hancock) from the National Institutes of Health, from the International Society of Nephrology (to Dr. Naqvi), and from the American Society of Transplantation (to Dr. Lee).

A U.S. patent entitled "Methods of Evaluating Transplant Rejection" (6187534) was issued on February 13, 2001; Dr. Suthanthiran is one of the inventors. The patent is owned jointly by Harvard Medical School, Cornell University, and the Beth Israel–Deaconess Medical Center, Boston.

We are indebted to Dr. Phyllis August for her careful review of this article and to Linda Stackhouse for her assistance in the preparation of the manuscript.

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