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Tribbles-1 as a Novel Biomarker of Chronic Antibody-Mediated Rejection

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ABSTRACT

Diagnosis of the specific cause of late allograft injury is necessary if more personalized and efficient immunosuppressive regimens are to be introduced. This study sought previously unrecognized biomarkers for specific histologic diagnoses of late graft scarring by comparison of gene sets from published microarray studies. Tribbles-1 (TRIB1), a human homolog of *Drosophila* tribbles, was identified to be a potentially informative biomarker. For testing this, mRNA expression in 76 graft biopsies, 71 blood samples, and 11 urine samples were profiled from independent cohorts of renal transplant patients with different histologic diagnoses recruited at two European centers. TRIB1 but not TRIB2 or TRIB3 was found to be a potential blood and tissue biomarker of chronic antibody-mediated rejection, an active immune-mediated form of chronic allograft failure associated with a poor prognosis. TRIB1 mRNA levels in peripheral blood mononuclear cells discriminated patients with chronic antibody-mediated rejection from those with other types of late allograft injury with high sensitivity and specificity. TRIB1 was also upregulated in a rodent model of chronic cardiac vasculopathy, suggesting that this biomarker may be useful in other solid-organ transplants and across species. It was determined that TRIB1 is expressed primarily by antigen-presenting cells and activated endothelial cells. Overall, these data support the potential use of TRIB1 as a biomarker of chronic antibody-mediated allograft failure.

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Chronic allograft injury severely impedes successful kidney transplantation.[1,2] Deciphering the mechanisms of such late graft loss would enable more personalized treatment strategies but is hindered by the difficulty in assigning specific diagnoses. Recently, chronic allograft nephropathy (CAN), the nonspecific term used to describe all manners of late graft scarring, was ousted for the term "interstitial fibrosis and tubular atrophy" (IF/TA), to be used in cases in which no underlying cause can be identified.[3] IF/TA can be the result of mechanisms that are of unknown cause, non–immune mediated (*e.g.*, as a result of function overload[4] or calcineurin inhibitor toxicity (CNI tox)[5]) or immune mediated (*e.g.*, chronic antibody-mediated rejection [AMR][6]).

Distinguishing these different causes currently requires an invasive biopsy procedure. The discovery of novel and less invasive surrogate biomarkers of these different causes of late graft failure would facilitate the introduction of more targeted immu-

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Table 1. Studies used to identify common biomarkers of late graft injury[a]

Study	Subject	Sample	Gene List
Hotchkiss *et al.*,[13] 2006	CAN	Biopsies, Affymetrix	Selected upregulated genes in CAN (gene list published)
Flechner *et al.*,[12] 2004	Banff grade 3 *versus* Banff 1	Biopsies, Affymetrix	Genes up in Banff 3 *versus* Banff 01 (gene list available on the authors' website)
Scherer *et al.*,[11] 2003	CR	Biopsies, Affymetrix	Genes upregulated in biopsies at 6 mo that went on to develop CR at 1 yr *versus* biopsies at 6 mo with no CR at 1 yr (exhaustive gene list provided by the authors).
Donauer *et al.*,[10] 2003	CR	Biopsies, "homemade" according to Stanford protocol	Up in CR *versus* normal and polycystic kidneys (gene list published)

[a]CR, chronic rejection.

nosuppression and thereby improve long-term outcome. Such an approach has proved successful in the case of acute cellular rejection, for which urine levels of Granzyme B and FOXP3 transcripts have been shown to have diagnostic and prognostic value.[7–9] In the case of late graft injury, attention has been paid to genome-wide profiling in the search for biomarkers.[10–14] Although such studies have thrown light onto the gene pathways concerned, the analyses were performed on graft biopsies, meaning that the genes identified might not be applicable to a noninvasive approach. Moreover, differences in sample preparation, amplification, microarray type, and data analysis can lead to different pathologic signatures, which could undermine the relevance of the identified biomarkers to a wider context.

As a means to identify potentially robust and less invasive biomarkers of specific causes of late graft injury, we used a literature gene-set comparison approach followed by screening of genes of interest on biopsies and peripheral blood from patients with various diagnoses of late graft failure. Here we describe the exploration of one such gene of interest. The gene encodes tribbles homolog 1 (TRIB1; also known as C8FW, GIG2, and SKIP1), an intracellular human homolog of *Drosophila* tribbles, a family with three members. In the *Drosophila*, TRIB coordinate mitosis and morphogenesis.[15–17] In mammals, some evidence suggests TRIB1 to be a potent regulator of mitogen-activated protein kinase signaling[18] and to control the proliferation and chemotaxis of smooth muscle cells[19] as well as to modulate NF-IL-6–dependent gene expression after Toll-like receptor signaling.[20] The expression and role of endogenous TRIB1 in primary cells *in vivo* and thus its biologic relevance remain unknown.

Here we show that TRIB1 (but not TRIB2 or TRIB3) is significantly increased in both the blood and the graft but not the urine of kidney transplant patients who have chronic AMR *versus* other histologic and clinical diagnoses. These findings were reproduced in a rodent transplant model. TRIB1 is expressed primarily by antigen-presenting cells (APC) and, as such, is the first molecule of this type to be reported as a potential minimally invasive surrogate biomarker of chronic AMR.

RESULTS

Identification of TRIB1 mRNA as a Potential Biomarker of Chronic Graft Injury in Kidney Transplants

Comparison of the gene sets upregulated in late graft injury in four published studies concerning kidney transplantation (Table 1) led to the identification of TRIB1 as a potential biomarker, being present in two of four of the gene sets. TRIB1 mRNA was upregulated in biopsies classified as displaying

Table 2. Patients included in analysis of 6-mo protocol biopsies (Hanover Center)[a]

Parameter	Group		
	Normal	CNI Tox	IF/TA
n	6	7	7
Recipient age (yr; median [range])	60 (19 to 69)	56 (38 to 65)	48 (30 to 65)
Gender ratio (M:F)	3:3	4:3	5:2
Donor age (yr; median [range])	39 (25 to 69)	43 (31 to 56)	52 (49 to 77)
Time after transplantation (mo)	6	6	6
HLA incompatibilities (mean ± SD)	2.0 ± 1.6	2.1 ± 1.8	2.3 ± 1.4
% First transplantations	83	100	100
Cockroft creatinine clearance (ml/min; mean ± SD)	73 ± 33	48 ± 14	41 ± 14
Proteinuria (g/24 h; mean ± SD)	0.06 ± 0.03	0.04 ± 0.02	0.07 ± 0.04
Banff c grade (mean ± SD)	0.00 ± 0.00	0.00 ± 0.00	1.14 ± 0.38
Immunosuppression protocol	Antimetabolite: MMF (4/6) CNI: CsA (3/6), FK 506 (2/6)Rapamycin (0/6) Steroids (6/6)	Antimetabolite: MMF (5/7) CNI: CsA (6/7), FK 506 (1/7)Rapamycin (1/7) Steroids (5/7)	Antimetabolite: MMF (4/7) CNI: CsA (1/7), FK 506 (6/7) Rapamycin (0/7)Steroids (7/7)

[a]CsA, cyclosporin A; FK 506, Prograf; MMF, mycophenolate mofetil.

Table 3. Patients included in analysis of biopsies at ≥1 yr (Nantes Center)[a]

Parameter	Group				
	Normal	CNI Tox	IF/TA	TG (C4d$^-$, Anti-HLA$^-$)	TG (C4d$^+$, Anti-HLA$^+$; Chronic AMR)
n	7	7	9	11	18
Recipient age (yr; median [range])	35 (18 to 61)	47 (29 to 56)	53 (24 to 56)	62 (45 to 75)	47 (24 to 71)
Gender ratio (M:F)	6:1	4:3	4:5	4:7	10:9
Donor age (yr; median [range])	34 (12 to 74)	40 (16 to 48)	48 (23 to 64)	57 (8 to 65)	35 (14 to 75)
Time after transplantation (yr; median [range])	1 (1-yr protocol biopsies)	5 (1 to 11)	2 (1 to 22)	4 (1 to 14)	9 (1 to 18)
HLA incompatibilities (mean ± SD)	2.8 ± 1.7	2.7 ± 1.9	2.8 ± 1.2	4.3 ± 1.4	3.0 ± 1.6
% First transplantations[b]	100	57	100	91	68
Cockroft creatinine clearance (ml/min; mean ± SD)	75.6 ± 17.6	57.1 ± 31.9	56.8 ± 16.3	28.2 ± 17.3	29.1 ± 16.6
Proteinuria (g/24 h; median [range])	0.12 (0.05 to 0.43)	1.22 (0.14 to 4.47)[c]	0.14 (0.01 to 6.37)	1.00 (0.89 to 5.92)	1.88 (0.80 to 3.17)
C4d$^+$ biopsy	0/7	0/7	0/9	0/11	18/18
Banff c grade (mean ± SD)	0.0 ± 0.0	1.7 ± 0.8	1.3 ± 0.7	1.4 ± 0.7	2.1 ± 0.9
Immunosuppression protocol	Antimetabolite: Aza (0/7), MMF (6/7) CNI: CsA (0/7), FK 506 (6/7) Rapamycin (0/7) Steroids (1/7)	Antimetabolite: Aza (2/7), MMF (4/7) CNI: CsA (4/7), FK 506 (3/7) Rapamycin (0/7) Steroids (3/7)	Antimetabolite: Aza (2/9), MMF (6/9) CNI: CsA (1/9), FK 506 (4/9) Rapamycin (3/9) Steroids (4/9)	Antimetabolite: Aza (0/11), MMF (11/11) CNI: CsA (2/11), FK 506 (8/11) Rapamycin (1/11) Steroids (2/11)	Antimetabolite: Aza (2/18), MMF (9/18) CNI: CsA (8/18), FK 506 (6/18) Rapamycin (0/18) Steroids (4/18)

[a]Aza, azathioprine.
[b]All but one were from deceased donors.
[c]One patient had a proteinuria of 4.47 g/24 h at the time of biopsy, probably because of hyperfiltration (as a result of obesity [BMI >40]).

Banff grade 3 *versus* grade 0 in a study by Fleschner *et al.*[12] and was among the genes upregulated in 6-mo biopsies from kidneys that displayed CAN at 1 yr compared with 6-mo biopsies from kidneys that remained healthy at 1 yr, in a study by Scherer *et al.*[11] (exhaustive gene list provided by the authors).

Identification of TRIB1 mRNA as a Specific Intragraft Biomarker of Chronic AMR

Given the finding of TRIB1 mRNA upregulation in graft biopsies in two studies of the literature, differential TRIB1 expression was analyzed in biopsies with normal histology or different diagnoses of late injury (IF/TA of unknown etiology, CNI tox, transplant glomerulopathy [TG], and chronic AMR) from two independent cohorts of graft biopsies, one taken at an early time point (6-mo protocol biopsies) and the other at later time points (≥1 yr), from two European transplant centers (see the Concise Methods section and Tables 2 and 3 for definitions and clinical data). The rarity of biopsies diagnosed as chronic active cell-mediated rejection (C4d$^-$ chronic transplant arteriopathy) precluded their inclusion in the study. No differences were observed between the two cohorts; therefore, the data were pooled. As shown in Figure 1A, the quantity of TRIB1 mRNA was significantly higher in biopsies with chronic AMR *versus* normal histology ($P < 0.001$; Kruskal-Wallis test followed by a Dunn multiple comparison test) as well as *versus* other histologic diagnoses ($P < 0.0001$ to $P < 0.05$; nonparametric Mann-Whitney test). This was specific to TRIB1 because the other family members TRIB2 and TRIB3 showed no significant regulation (Supplemental Figure 1A). TRIB1 levels in "nontransplant" kidneys and transplant kidneys with normal histology were identical ($P = 0.95$; data not shown), indicating that TRIB1 is not upregulated as a result of the immunosuppressive regimen.

TRIB1 levels were independent of recipient age ($r = -0.06$; NS), donor age ($r = -0.21$; NS), and number of donor–recipient HLA incompatibilities ($r = -0.12$; NS). Determination of the capacity of TRIB1 mRNA levels in graft biopsies to diagnose chronic AMR by receiver operating characteristic (ROC) curve analysis (Figure 1B) revealed a good discriminative power (area under the curve [AUC] of 0.7; 95% confidence interval 0.64 to 0.89) with a low sensitivity of 56% but a high specificity of 92% at a cutoff value of TRIB1 mRNA quantity of 1.31.

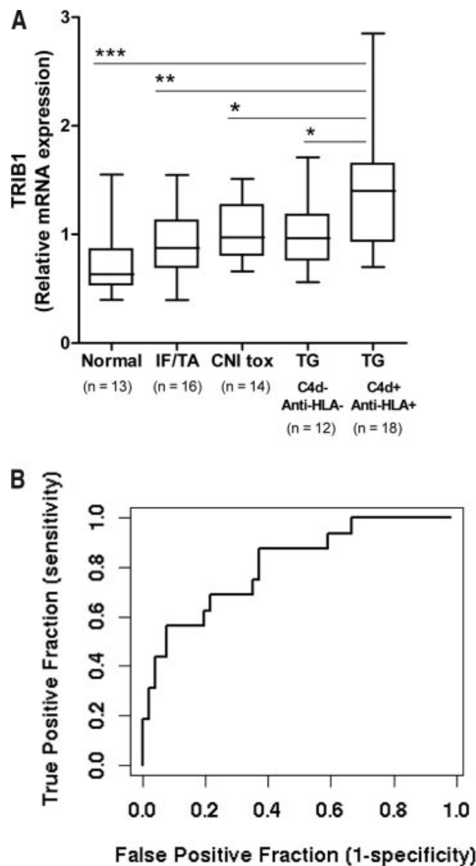

Figure 1. Differential TRIB1 mRNA expression in renal transplant biopsies and its capacity to diagnose chronic AMR. (A) TRIB1 mRNA transcription in biopsies displaying normal histology (Normal), IF/TA of unknown etiology, lesions evocative of CNI tox, TG (negative for C4d and anti-HLA), or chronic AMR (TG; positive for C4d and circulating anti-HLA). Results represent pooled data for 6-mo protocol biopsies and biopsies taken at ≥1 yr after transplantation (similar results were found for the two cohorts). Significantly higher levels of TRIB1 mRNA are present in chronic AMR than in normal histology ($P < 0.001$; Kruskal-Wallis test followed by a Dunn multiple comparison test) as well as *versus* other histologic diagnoses ($P < 0.0001$ to $P < 0.05$; nonparametric Mann-Whitney test). Statistical differences according to nonparametric Mann-Whitney tests are shown: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. TRIB1 mRNA was measured by quantitative RT-PCR, and expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) with results expressed in arbitrary units (see the Concise Methods section for details). (B) ROC curve analysis of TRIB1 mRNA in the combined 6-mo and ≥1 yr biopsy cohorts. The ROC curve measures the ability of TRIB1 mRNA quantity to classify correctly patients with and without chronic AMR. The ROC is represented as a graphical plot of the sensitivity *versus* (1 − specificity) for a binary classifier system because its discrimination threshold is varied. The sensitivity (also referred to as the "true-positive fraction") is how good the test is at picking out patients with chronic AMR. Specificity is the ability of the test to pick out patients who do not have chronic AMR. Thus, (1 − specificity) is also referred to as the "false-positive fraction." The accuracy of the test (*i.e.*, how well the test separates patients with and without chronic AMR) is measured by the area under the ROC curve, where an area of 1.0 represents a perfect test. Thus, the ROC curve should climb rapidly toward the upper left hand corner of the graph, meaning that the false-negative rate is high and the false-positive rate is low.

TRIB1 mRNA Is a Specific Peripheral Blood but not a Urine Biomarker of Chronic AMR

With the aim of less invasive detection, TRIB1 mRNA expression was next analyzed in peripheral blood mononuclear cells (PBMC) of healthy volunteers and patients under standard immunosuppression with either stable graft function or deteriorating graft function with biopsy-proven TG or chronic AMR (see Table 4 for clinical data). As shown in Figure 2A, the results paralleled those of the biopsies inasmuch as TRIB1 mRNA was significantly increased in the PBMC of patients with chronic AMR *versus* the healthy volunteers ($P < 0.0001$) as well as *versus* patients with stable graft function ($P < 0.01$) and patients with deteriorating graft function with biopsy-proven TG ($P < 0.05$). Again, this was specific to TRIB1 because TRIB2 and TRIB3 showed no regulation (Supplemental Figure 1B). TRIB1 levels were independent of recipient age ($r = -0.08$; NS), donor age ($r = -0.02$; NS), number of donor–recipient HLA incompatibilities ($r = -0.04$; NS), and time after transplantation ($r = -0.05$; NS). Moreover, TRIB1 levels were independent of cytomegalovirus status (data not shown). ROC curve analysis (Figure 2B) showed that measuring TRIB1 mRNA in the blood is a good discriminator of chronic AMR, with an AUC of 0.8 (95% confidence interval 0.67 to 0.91), a specificity of 88%, and a sensitivity of 75% (much higher than for the biopsies) at a cutoff value of 1.57. Thus, measuring TRIB1 in blood appears more informative than measuring TRIB1 in biopsies.

Finally, to determine whether TRIB1 mRNA could serve as a totally noninvasive biomarker, we analyzed its expression in urine pellets. Preliminary analyses in urine samples from patients with normal graft histology ($n = 4$) and patients with chronic AMR ($n = 7$) showed that this was not the case, with no significant differences between the two groups (Figure 2C).

TRIB1 Is a Blood and Intragraft Biomarker of Chronic AMR in the Rat

To determine the relevance of TRIB1 to chronic AMR in general (not limited to kidney transplantation and humans), we analyzed TRIB1 mRNA at day 100 after transplantation in the allografts and PBMC of rat recipients of MHC-mismatched cardiac allografts that were either tolerant (grafts displaying no histologic signs of rejection) or presenting a graft histology similar to that of chronic AMR in humans (grafts displaying histologic signs typical of chronic rejection including patches of mononuclear infiltrates within the myocardium, myocardial fibrosis, and varying degrees of chronic transplant vasculopathy, as well as presence of circulating and intragraft alloantibodies).[21] In accordance with the observations made in the human kidney transplant patients, TRIB1 mRNA was signifi-

Table 4. Patients included in analysis of PBMC[a]

Parameter	Group			
	Healthy Volunteers	Stable	TG (C4d$^-$, Anti-HLA$^-$)	TG (C4d$^+$, Anti-HLA$^+$; Chronic AMR)
n	17	27	7	20
Recipient age (yr; median [range])	48 (24 to 58)	54 (24 to 79)	63 (52 to 70)	50 (27 to 76)
Gender ratio (M:F)	8:9	16:11	4:3	9:11
Donor age (yr; median [range])	NA	27 (14 to 68)	62 (42 to 68)	37 (14 to 75)
Time after transplantation (yr; median [range])	NA	8 (3 to 22)	5 (2 to 7)	8 (1 to 25)
HLA incompatibilities (mean ± SD)	NA	3.3 ± 1.1	4.1 ± 1.6	3.0 ± 1.9
% First transplantations[b]	NA	100	86	60
Cockroft creatinine clearance (ml/min; mean ± SD)	NA	69.4 ± 31.4	19.4 ± 8.7	30.7 ± 15.6
Proteinuria (g/24 h; median [range])	NA	0.14 (0.00 to 1.17)[b]	1.39 (0.42 to 2.98)	3.22 (0.13 to 11.52)
C4d$^+$ biopsy	NA	NA (biopsy not available)	No	Yes
Banff c grade (mean ± SD)	NA	NA (biopsy not available)	2.3 ± 0.6	1.7 ± 1.2
Immunosuppression protocol	None	Antimetabolite: Aza (6/27), MMF (17/27) CNI: CsA (22/27), FK 506 (5/27) Steroids (4/27)	Antimetabolite: Aza (0/7), MMF (3/7) CNI: CsA (0/7), FK 506 (7/7) Steroids (3/7)	Antimetabolite: Aza (1/20), MMF (12/20) CNI: CsA (4/20), FK 506 (13/20) Steroids (11/20)

[a]NA, not applicable.
[b]One STA patient had a proteinuria >1 g/24 h (1.17), which was likely due to hyperfiltration as a result of obesity (BMI 46.6); she had excellent clearance (106 ml/min).

cantly increased in both the heart allograft and the PBMC of the rat recipients whose grafts displayed signs of chronic transplant vasculopathy *versus* those that showed no signs of rejection 100 d after transplantation ($P < 0.05$; Figure 3), further confirming the relevance of TRIB1 to chronic AMR in a rodent model and, additionally, in cardiac allotransplantation.

TRIB1 mRNA Is Primarily a Marker of APC and Endothelial Cells

Given the specific regulation of TRIB1 described in the previous section and the lack of knowledge concerning endogenous tribbles expression even under normal conditions, we next analyzed TRIB1, 2, and 3 mRNA in various immune compartments and peripheral blood cell subtypes of healthy volunteers. The results presented in Figure 4 show that each family member has specific tissue- and cell type–specific expression with TRIB1 displaying a profile distinct from that of TRIB2 and 3, which themselves display relatively similar expression profiles. In particular, within the immune system (Figure 4A), TRIB1 is expressed to the greatest extent in peripheral blood lymphocytes, whereas TRIB2 and TRIB3 are expressed predominantly in the spleen. Within the PBMC, TRIB1 is expressed primarily by monocytes and B cells (Figure 4B), whereas TRIB2 and 3 are expressed almost exclusively by lymphocytes: T and B cells in the case of TRIB2 and B cells only in the case of TRIB3. Analysis of peripheral blood monocytes and monocyte-derived dendritic cells (DC) showed that TRIB1 is a marker of activated monocytes and certain types of mature DC (Figure 4C), whereas TRIB2 is expressed only by immature DC, and TRIB3 shows little if any regulation in these cell types. Finally, given that the increased TRIB1 mRNA levels in biopsies with chronic AMR could be the result of chronic endothelial cell (EC) activation by anti-HLA antibodies, complement, and proinflammatory mediators, we studied TRIB1 transcription in resting *versus* activated human renal artery–derived EC.

The data in Figure 4D show that TRIB1 is indeed a marker of activated EC, being upregulated upon activation with the proinflammatory cytokine TNF-α. This was not the case for TRIB2, which is downregulated upon activation and TRIB3, which showed no regulation under these conditions. Basal TRIB1 levels were approximately five-fold lower in EC than in monocytes (data not shown). Taken together, these data show that TRIB1 is primarily a marker of APC and EC, where it is regulated differentially according to cell type and activation status as well as with respect to its fellow family members.

DISCUSSION

In this study, we set out to discover novel minimally invasive biomarkers of more precise histologic diagnoses of late graft scarring. Using a literature gene-set comparison approach for late graft injury, we identified TRIB1, a human homolog of

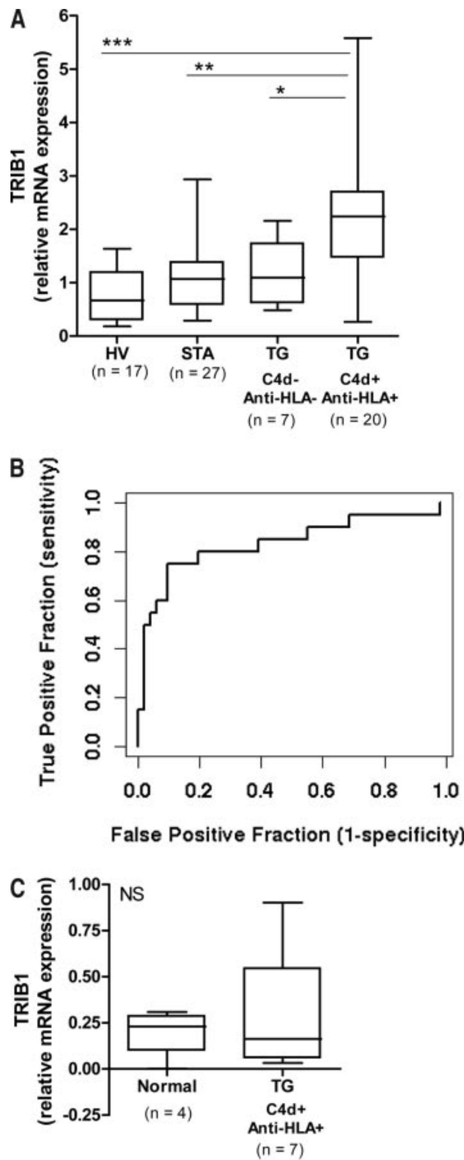

Figure 2. Differential TRIB1 mRNA profiles in the PBMC of renal transplant patients. (A) TRIB1 mRNA transcription in PBMC from healthy volunteers (HV), patients with stable graft function under standard immunosuppression (STA), deteriorating graft function under standard immunosuppression with biopsy-proven TG (negative for C4d and anti-HLA), and deteriorating graft function under standard immunosuppression with biopsy-proven chronic AMR (TG, positive for C4d and anti-HLA). Statistical differences according to nonparametric Mann-Whitney tests are shown; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. TRIB1 mRNA was measured by quantitative RT-PCR, and 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 the Concise Methods section for details). (B) ROC curve analysis of TRIB1 mRNA in the PBMC (see Figure 1 legend for explanation). (C) TRIB1 mRNA quantification in urine from renal transplant patients with normal graft histology and biopsy-proven chronic AMR. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the housekeeping gene 18S, with results expressed in arbitrary units (see the Concise Methods section for details).

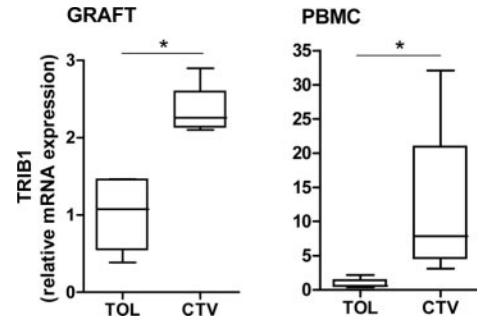

Figure 3. Differential TRIB1 mRNA profiles in the PBMC and allografts of rat recipients of MHC-mismatched heart grafts. TRIB1 mRNA transcription at day 100 after transplantation in the allografts and PBMC of rat recipients of MHC-mismatched cardiac transplants displaying normal histology after a 20-d course of the deoxyspergualin analogue LF015-095 (TOL) or displaying signs of chronic transplant vasculopathy (CTV) after pretransplantation DST as described previously.[21] Note that the results are expressed in arbitrary units and the *y* axes of the two graphs are not comparable because different reference samples were used for each sample type. Rat Trb1 mRNA was measured by quantitative RT-PCR using custom-made primers and probes, and expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the housekeeping gene HPRT (see the Concise Methods section for details).

Drosophila tribbles,[15–17] as a potentially informative biomarker. TRIB1 is a scarcely characterized member of the tribbles family (see references[22,23] for review) that has been shown to be a potent regulator of cell signaling[18] in various cells lines. We thus explored the potential of TRIB1 as a tissue, peripheral blood, and urine biomarker by measuring its mRNA profiles in graft biopsies, blood, and urine from healthy volunteers and kidney transplant recipients with different histologic and/or clinical diagnoses. We also used a bedside-to-bench approach by analyzing its expression in a rodent model of chronic transplant vasculopathy with alloantibodies and complement activation.

Overall, we found that TRIB1 mRNA but not that of its fellow family members was specifically increased in graft biopsies and blood but not urine from patients with chronic AMR, a state of active, immunologically driven, late graft injury that was recently individualized as a specific entity.[3] Moreover, TRIB1 mRNA in the blood was more specific and sensitive for diagnosing chronic AMR than TRIB1 mRNA in biopsies. These results were reproduced in a rodent cardiac transplant model, where, again, TRIB1 mRNA was specifically increased in both the blood and grafts of rats with chronic transplant vasculopathy, suggesting that this biomarker could potentially be informative in heart transplantation and that this rodent model may be useful for further analyses of TRIB1 in transplantation. Although currently limited by a lack of available tools, functional analyses of TRIB1 in this rodent transplant model would be very informative because they could help to determine whether TRIB1 is a potential therapeutic target. Along these lines, we are developing TRIB1 knockout mice so as to address these issues. Moreover, examining TRIB1 in var-

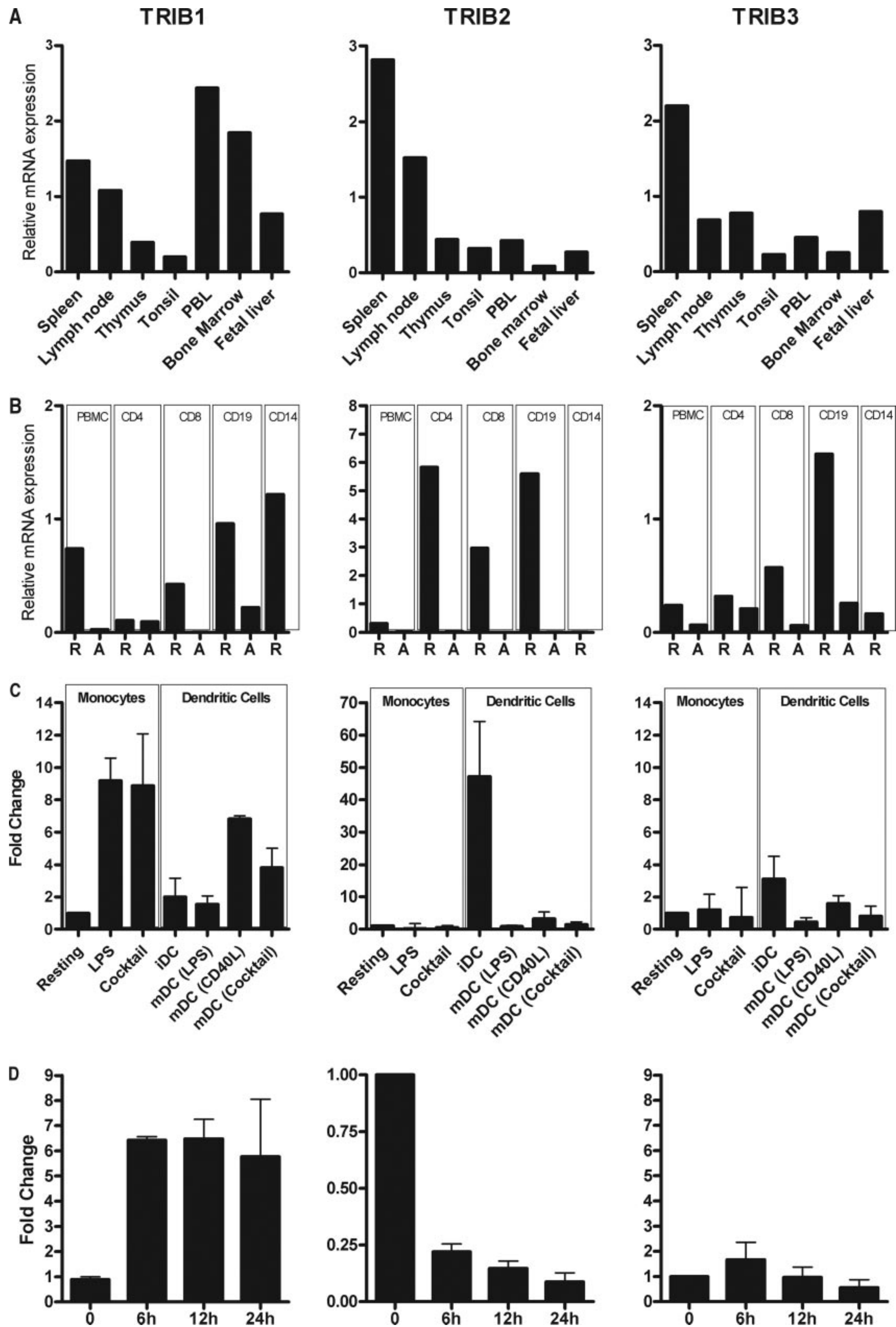

Figure 4. Human tribbles mRNA expression within the immune system and various cell subtypes of healthy individuals. (A and B) TRIB1 (left), TRIB2 (middle), and TRIB3 (right) mRNA in various immune compartments (A) and resting (R) and activated (A) peripheral blood cell (PBL) subtypes of healthy volunteers (B; commercially available cDNA sets derived from a pool of healthy human donors [see the Concise Methods section]; note that activated monocytes were not provided with the set). Results are expressed in arbitrary units. (C)

ious cell types in rat allografts or blood samples and any differences in mitogen-activated protein kinase signaling pathways downstream of TRIB1 could provide mechanistic insights of relevance in chronic AMR.

The finding that TRIB1 transcript accumulation was significantly higher in chronic AMR than all of the other diagnoses studied, including in C4d$^-$ and anti–HLA$^-$ TG, suggests TRIB1 to be a marker of an active immune response. Although TG is thought to be closely associated with AMR,[24,25] the exact pathogenesis of C4d$^-$ and anti–HLA$^-$ TG is unclear. Several explanations were put forward at the 2005 Banff meeting,[3] including residual injury from previous episodes of AMR, T cell–mediated responses, or nonalloimmune causes. Our results suggest this pathology to be indeed different from the C4d$^+$, anti–HLA$^+$ equivalent. The expression of TRIB1 mRNA in APC and, in particular, activated monocytes and DC lend further credence to its implication in an active immune response. Moreover, we found TRIB1 to be upregulated in the blood of patients with ANCA-associated vasculitis (J.A.-C., unpublished observations), further supporting TRIB1 as a biomarker of ongoing immune activation and an antibody response.

Several hypotheses could explain the increase in TRIB1 in the graft. First, our finding of TRIB1 expression by activated EC suggests that this increase could be the result of intragraft EC activation through antibody deposition and complement activation in chronic AMR *versus* other types of late graft injury. The increase could also arise from an increase in TRIB1-expressing infiltrating APC within the grafts displaying chronic AMR. Although a specific increase in infiltrating APC in chronic AMR has not been formally reported to date, this is likely given that an increased monocyte infiltrate has been reported in C4d$^+$ cases of acute rejection,[26] and a recent report showed that C4d$^+$ biopsies have a higher monocyte/T cell ratio than C4d$^-$ ones.[27] Although an increase in B cell infiltration has been reported in chronic AMR, resulting from a phenomenon known as lymphoid neogenesis,[28] it is unlikely that TRIB1 is increased as a result of this because TRIB3, which is expressed primarily by B cells, was not increased and activated B cells downregulate their TRIB1 mRNA.

In the PBMC, despite being expressed primarily in monocytes and B cells, it is unlikely that the increase in TRIB1 in the blood stems from an increase in either of these cell types because TRIB1 mRNA did not correlate tightly with blood monocyte numbers (Spearman $r = 0.37$); TRIB1 is downregulated by activated B cells; TRIB3 was not increased in chronic AMR despite its exclusive expression by B cells; and we previously reported no significant differences in B cell numbers between patients with chronic AMR, patients with stable graft function, and healthy volunteers.[29] It is possible, therefore, that the value of TRIB1 in PBMC is related to the level of activation of monocyte or DC subsets, reflecting rather an increase in the proportion of activated monocytes or DC. Another possibility is that TRIB1 is increased as a result of an increase in circulating TRIB1-expressing EC or their progenitors. It indeed was reported previously that patients with vascular rejection have increased numbers of circulating EC.[30] Confirmation of these hypotheses would require an adequate antibody to detect endogenous TRIB1, which is currently unavailable.

In the rat model used, in which the results reflected those in the renal transplant patients, the lumens of the blood vessels within hearts displaying chronic transplant vasculopathy (CTV) were obstructed by numerous CD68$^+$ monocyte/macrophage infiltrates, which was not the case in the tolerant animals in which the phenomenon of EC accommodation was observed.[21] As for the renal transplant patients, no significant differences were found in the blood cell numbers of the two groups of rats (unpublished), suggesting rather an increase in activated *versus* resting monocytes or an increase in circulating TRIB1-expressing EC.

In conclusion, we have identified TRIB1 mRNA as a potential minimally invasive biomarker of chronic AMR because it is upregulated in the peripheral blood, where it displays high sensitivity and specificity. Although it is unlikely that TRIB1 is a stand-alone biomarker for chronic AMR, so far we have been unable to identify other molecules that show the same expression profile, even among well-characterized markers such as Granzyme B (data not shown); however, in the event of other biomarkers' being identified, TRIB1 could form part of a panel of genes, as in the case of the Cardiac Allograft Rejection Gene Expression Observation (CARGO) study.[31] Moreover, this study focused on chronic injury, but TRIB1 could also be increased in acute rejection. TRIB1 differs from the other minimally invasive biomarkers of transplant rejection described to date that are of T/NK cell origin,[7–9,32] in that it is expressed primarily by APC as well as EC. As such, our findings point toward a possible but as yet unconfirmed role for APC in this pathology. These data now need to be confirmed in longitudinal and multicenter analyses. Such analyses could contribute to the development of a blood test to diagnose chronic AMR without the need for C4d staining on invasive biopsies or to identify patients who may be experiencing chronic AMR and require a biopsy. This would facilitate a more rapid introduction of targeted immunosuppressive regimens to improve long-term graft outcomes.

Fold change in TRIB1, TRIB2, and TRIB3 mRNA in resting and activated monocytes (24 h with LPS or a proinflammatory cocktail [see the Concise Methods section for details]) and in immature DC (iDC) and mature DC (mDC) after 48 h of activation with LPS, CD40L, or the same proinflammatory cocktail ($n = 3$). D. Fold change in TRIB1, TRIB2, and TRIB3 mRNA in resting *versus* activated (TNF-α for 6, 12, and 24 h) human renal aorta–derived EC ($n \geq 3$). TRIB1 mRNA was measured by quantitative RT-PCR, and expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to a housekeeping gene HPRT (see the Concise Methods section for details).

CONCISE METHODS

Gene-Set Comparison

With the aim of identifying robust biomarkers of late graft injury, we compared the gene sets from four different published microarray studies of late graft injury in kidney transplant biopsies using different platform technologies[10–13] (see Table 1). All genes (>3000 in total) were converted into a single identifier, the "gene ID," using the gene ID conversion tool available on the DAVID Bioinformatic Resources 2006 website (http://david.abcc.ncifcrf.gov/home.jsp) and repeats of the same ID identified by sorting in Microsoft Excel.

Patients

The study was performed on 76 biopsies, 71 blood samples, and 11 urine samples. All patients and healthy volunteers who participated in this study gave informed consent, and the study was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biologic Risks at both participating centers (Nantes, France, and Hannover, Germany).

6-Mo Protocol Biopsies. Six-month protocol biopsies (Protocol Biopsy Program, Hannover, Germany[33,34]) were classified according to the updated Banff classification criteria[3,35] as showing normal histology ($n = 6$), lesions of IF/TA of unknown etiology ($n = 7$), or CNI tox ($n = 7$). The demographic and clinical data for these patients are provided in Table 2.

Biopsies at ≥1 Yr. Biopsies at ≥1 yr (Nantes CHU) were classified according to the updated Banff classification criteria[3,35] as displaying normal histology ($n = 7$), lesions of IF/TA of unknown etiology ($n = 9$), lesions of CNI tox ($n = 7$), TG ($C4d^-$ and anti-HLA^-; $n = 11$), or chronic AMR (defined by the diagnostic triad of circulating anti-HLA antibody[36,37] associated with TG and deposition of the complement split product C4d in graft biopsies[36,38] (see also reference[3]; $n = 18$). None of the chronic AMR biopsies displayed signs of acute AMR such as vascular necrosis, thrombosis, or edema. The biopsies showed a variable degree of capillaritis and glomerulitis with lymphocyte and macrophage elements. The demographic and clinical data for all of these patients are provided in Table 3.

PBMC. PBMC (Nantes CHU) were prepared from the blood of healthy volunteers ($n = 17$) and 54 kidney transplant patients whose statuses were defined on a histologic and/or clinical basis (see next). The demographic and clinical data for these patients are provided in Table 4.

- Standard Immunosuppression with Stable Graft Function: Patients under standard immunosuppression with stable graft function ($n = 27$; creatinemia <150 μmol/L and proteinuria <1 g/24 h for at least 3 yr including over the two previous follow-up appointments with <20% change between the two time points [6 mo]) and no circulating anti-HLA antibodies at the time of the blood sample. No biopsies were available for these patients because they presented no deterioration of graft function (certain cDNA samples were prepared by TcLand S.A., Nantes, France).
- TG: Patients under standard immunosuppression with biopsy-proven TG ($n = 7$; basement membrane duplication negative for both C4d and circulating anti-HLA).
- Chronic AMR: Patients under standard immunosuppression with biopsy-proven chronic AMR ($n = 20$) according to the already described definition. Both TG and chronic AMR were diagnosed on biopsies performed in the context of a progressive deterioration of renal function (blood creatinemia ≥150 μmol/L and/or proteinuria ≥1 g/24 h). Sixteen patients overlapped between the PBMC and the Nantes biopsy cohorts (blood samples were unavailable for certain patients included in the biopsy cohort and *vice versa*).

Urine. Urine (Nantes CHU) consisted of kidney transplant patients with normal histology ($n = 4$) or chronic AMR ($n = 7$), all of whom were included in the Nantes biopsy cohort.

Rat Heart Transplant Model of Chronic Rejection

The rat transplantation model used has been described previously.[21,39,40] Briefly, the model used was that of heart allotransplantation from LEW.1W donors to MHC-mismatched LEW.1A recipients receiving either a donor-specific blood transfusion (DST) 7 and 14 d before transplantation or a 20-d course of the deoxyspergualin analogue LF015-095. In this model, at day 100 after transplantation, the grafts of DST-treated animals showed clear-cut histologic signs of chronic transplant vasculopathy,[21,41] whereas those of LF015-095–treated animals did not and thus were tolerant.[21,40] As previously reported,[21,40] this tolerance was donor specific. Analyses were performed on DST-treated ($n = 4$) and LF-treated recipients ($n = 4$) at day 100 after transplantation.

Biopsies, PBMC, and Urine

Human kidney transplant biopsies were taken with a 16- or 18-G needle and either stored in RNAlater (QIAGEN, Courtaboeuf, France) or embedded in Tissue Tek (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. Rat heart transplant tissue was processed as described previously.[21] Peripheral blood from healthy volunteers, patients, and rats was collected in EDTA Vacutainers, and PBMC were separated by density centrifugation using Lymphosep, lymphocyte separation media (Bio West, Nuaille, France). PBMC were stored in TRIzol (Invitrogen, Cergy Pontoise, France) at −80°C until use. Human urine samples (≥50 ml) were collected in sterile containers and treated within 2 h.

Preparation and Activation of Human PBMC and DC and Human Renal Artery–Derived EC

Monocytes were prepared by positive selection using MACS anti-CD14 antibody-conjugated magnetic microbeads (Miltenyi Biotech, Paris, France; purity >97%). For monocyte-derived DC differentiation, $CD14^+$ cells were cultured as described previously[42] except that at day 5, cells were washed and cultured for an additional 48 h in medium alone; 1 μg/ml LPS; 1 μg/ml shCD40L (Amgen, Thousand Oaks, CA); or a proinflammatory cocktail consisting of 10 ng/ml recombinant human TNF-α, 20 ng/ml recombinant human IL-6, 10 ng/ml recombinant human IL-1α (all from R&D Systems, Abingdon, UK), and 1 μg/ml PGE2 (Sigma-Aldrich, Saint-Quentin Fallavier,

France). For monocyte activation tests, $CD14^+$ cells were cultured (1×10^6 cells/ml) as previously for 24 h alone or in the presence of 1 μg/ml LPS or the previous proinflammatory cocktail. Human renal artery–derived EC were cultured and activated with recombinant human TNF-α as described previously[43] for 6, 12, and 24 h. At least 10^6 monocytes, DC, and EC were washed and resuspended in the appropriate volume of TRIzol reagent and stored at -80°C until use.

RNA Extraction and Preparation of cDNA

RNA extraction from all biopsies was performed using the QIAgen RNA microextraction kit (QIAGEN) with on-column DNase treatment according to the manufacturer's instructions. In the case of Tissue Tek–embedded biopsies, the biopsies were recovered before homogenization by a rapid passage in ice-cold RNase-free water. Homogenization was performed with a PT 3100 Polytron Rotor-Stator (Kinematica, AG, Luzern, Switzerland). RNA quality and quantity were determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA). The total quantity of RNA extracted per biopsy ranged from approximately 50 ng to 2 μg. RNA was reverse-transcribed into cDNA using an RT-Omniscript kit (QIAGEN) according to the manufacturer's instructions. RNA was extracted from cardiac allografts as described previously.[21] RNA was extracted from human and rat PBMC, human monocytes, DC, and EC using the TRIzol method (Invitrogen) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment (Roche, Indianapolis, IN). RNA concentration was calculated using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and RNA quality was determined by running 1 μg on an agarose gel. RNA was reverse-transcribed into cDNA using polydT oligonucleotide and Maloney leukemia virus reverse transcription (Invitrogen). RNA was extracted from human urine samples as described previously[32] and reverse-transcribed into cDNA using an RT-Omniscript kit (QIAGEN) according to the manufacturer's instructions.

Human Immune System and Blood Fractions cDNA

Human Immune System and Blood Fraction MTC panels containing cDNA from a pool of donors together with a positive and negative control were purchased from Ozyme (Saint-Quentin en Yvelines, France). The Immune System panel included cDNA from human peripheral blood leukocytes, bone marrow, spleen, lymph node, fetal liver, thymus, and tonsil. The Blood Fractions panel included cDNA from resting and activated human PBMC, CD4, CD8, and CD19 cells as well as resting CD14 cells (activated CD14 cells not provided).

Real-Time Quantitative PCR

Real-time quantitative PCR was performed in an Applied Biosystems GenAmp 7700 sequence detection system (Applied Biosystems, Foster City, CA) using a commercially available primer and probe set for human TRIB1, TRIB2, and TRIB3 (Applied Biosystems; Hs00179769_m1, Hs00222224_m1, and Hs01082394_m1). For measurement of rat Trib1, Applied Biosystems File Builder software was used to design a primer and probe set to cover the interexonic regions of the homologous sections of the rat and mouse mRNA sequences (NM_144549.2 and XM-343250.3; forward primer sequence GCCCACTGCCACCAGT, reverse primer sequence GGGCAGCCATGTTTATCTGACA, probe sequence ACCCAGCTTAGACTGGAAAG). Specificity was checked using BLAST on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Hypoxanthine phosphoribosyl transferase (human Hs99999909_m1; rat Rn01527838_g1) or 18S (Hs99999901_s1; for urine samples only) were used as endogenous controls to normalize RNA starting quantity. Relative expression between a given sample and a reference sample was calculated according to the $2^{-\Delta\Delta Ct}$ method,[44] where the reference represents one-fold expression, as described previously.[45]

Statistical Analyses

The nonparametric Mann-Whitney test was used for comparison between two groups, and the nonparametric Kruskal-Wallis test was performed for comparison of more than two groups. The nonparametric Spearman test was used for correlations. Values of $P < 0.05$ were considered as significant. ROC curve analysis was performed to determine the cutoff point of TRIB1 mRNA in biopsies and blood that yielded the highest combined sensitivity and specificity in diagnosing chronic AMR (see Figure 1 legend for explanation).

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DISCLOSURES

J.A.C., M.G., C.B., S.B., and J.P.S. hold a patent concerning TRIB1 for the diagnosis of chronic rejection in transplantation.

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