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ORIGINAL ARTICLE

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Prognostic value of cytotoxic T-lymphocytes and CD40 in biopsies with early renal allograft rejection

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Abstract After renal transplantation, different immunological and non-immunological factors lead to long-term allograft deterioration. Acute rejection episodes are one risk factor for chronic renal allograft dysfunction (CRAD). Following the current Banff classification the histological grade in acute rejection episodes is of limited prognostic value, therefore, additional morphological surrogate markers would be helpful. We investigated the biopsies of 91 patients with early acute rejection episodes for the immunohistochemical expression of key molecules (perforin, granzyme B, TIA-1, CD40) in the T cell-mediated rejection process. Staining results were correlated to long-term allograft outcome. Patients with greater than 2% of granzyme B or greater than 25% of CD40-positive cells in the interstitial infiltrate showed significantly shorter allograft survival. Patients with a CD40-positive vascular rejection or greater than 2% of granzyme B-positive cells in the interstitial infiltrate were significantly correlated with an earlier onset of CRAD. Our findings provide potential morphological surrogate markers in biopsies with early acute rejection episodes after renal transplantation. These could become part of combined clinical and histological algorithms, allowing patient-specific risk estimation and customized therapy options to be made.

Keywords Renal transplantation · Acute rejection · Cytotoxic T-lymphocytes · CD40 · Immunohistochemistry

Introduction

The histological examination of a renal biopsy is crucial in the evaluation of renal allograft dysfunction and allows for definite discrimination between non-immunological (e.g. ischaemia-reperfusion injury, drug toxicity, infection) and immunological impairments (e.g. acute, chronic, cellular, humoral rejection) of the organ [1, 2]. Especially in the early phase after transplantation, prompt identification and adequate treatment of different mechanisms of allograft damage are crucial, since they are known to be risk factors for the development of long-term allograft dysfunction [3]. In this context, early acute rejection episodes can be a significant predictor of chronic renal allograft dysfunction (CRAD) and allograft loss [4, 5, 6, 7, 8, 9, 10]. However, the extent of the mononuclear cell infiltrate in the interstitium and the extent of tubulitis, the two morphological hallmarks of acute interstitial rejection, do not correlate with long-term deterioration of the allograft [2, 11]. Only the currently rarer, and more severe, vascular lesions are related to a worse outcome [2, 12, 13]. Therefore, additional morphological surrogate markers would be helpful to predict a worse long-term outcome after early rejection episodes, enabling timely, risk-adapted treatment to be carried out.

T-lymphocytes are a dominant and functionally decisive cell type in an acute cellular rejection episode. In this setting, cytotoxic T-lymphocytes (CTLs) play a pivotal role, since they can directly kill target cells (tubular epithelial and endothelial cells) in the allograft, by using cytoplasmatic cytotoxic effector molecules (perforin, granzyme B and cytotoxic granule membrane protein TIA-1) [14, 15, 16]. Numerous studies have shown that CTLs expressing cytotoxic effector molecules are increased in clinical and subclinical acute rejection episodes of renal allografts [17, 18, 19, 20, 21]. Not only in the graft, but also in the plasma and in the urine, increased amounts of cytotoxic effector molecules are detectable during acute rejection episodes, making these molecules useful as surveillance markers after renal transplantation [22, 23].

For the activation of allograft-specific T-lymphocytes, co-stimulatory molecules (CD28, CD80, CD86, CD40 and CD154) play a key role [12, 24, 25]. For example, the blockade of co-stimulatory signals induces prolonged graft survival and T-cell anergy in animal models [26, 27]. During the priming of CTLs, signalling by CD40 can mediate the expansion of allograft-specific CTLs [28, 29]. Several studies have demonstrated an increased expression of CD40 in tubular epithelial and endothelial cells, as well as interstitial infiltration by various CD40-expressing cells in acute and chronic rejection episodes [30, 31, 32, 33].

In order to investigate whether these pivotal molecules have any predictive value, we investigated biopsies from renal allografts with early acute rejection episodes for the immunohistochemical expression of perforin, granzyme B, TIA-1 and CD40. The results were statistically correlated with the long-term outcome of the transplanted organs.

Patients and methods

Patients

Ninety-nine renal allograft biopsies from 35 female and 64 male patients (aged 20–84years, mean 52.3 years) who had received transplants between 1989 and 1991 at the Medizinische Hochschule Hannover in Germany were examined; 15- or 16-gauge needle biopsies, taken within the first 3 months of transplantation and originally reported as acute rejection episodes, were obtained from the archives of the Institute of Pathology. The biopsy slides were reclassified according to the revised Banff '97 scheme [34]. Only biopsies that fulfilled the Banff criteria of adequacy (i.e. two biopsy cores containing at least a total of ten glomeruli and two arteries) were included for analysis. Since under the recent update of the Banff classification a C4d stain is recommended for all transplant biopsies, we included this procedure in our re-evaluation of our archival biopsies, using a commercially available polyclonal antiserum as recommended by the manufacturer (Biozol Diagnostica, Eching, Germany) [35]. C4d was regarded as positive if more than 25% of peri-tubular capillaries showed a linear staining pattern. For all patients, 10-year follow-up data of allograft function were available.

Immunohistochemistry

All further antibodies that were examined were monoclonal and commercially available (perforin, clone KM585, Hoelzel Diagnostics, Germany; granzyme B, clone GrB-7, Chemicon, USA; TIA-1, clone TIA-1, Coulter, USA; CD40, clone LOB-7/6, Serotec, UK). The staining was performed on formalin-fixed and paraffin-embedded renal core biopsies, following standard methods. In brief, after the paraffin had been removed, the slides were pretreated with heat by the microwave technique (perforin, granzyme B, TIA-1: citrate buffer, pH 6.0, 20 min at 100°C) or protease digestion (CD40:10 min at 37°C) followed by the blocking of endogenous peroxidase with 3% H_2O_2, as well as endogenous biotin by an avidin/biotin-blocking kit (Vector Laboratories, USA). The primary antibodies were incubated (dilutions: perforin 1:1000, granzyme B 1:75, TIA-1 1:1500, CD40 1:20) overnight at 4°C. A biotinylated secondary rabbit-anti-mouse antibody (1:1000, 60 min, room temperature; Zymed Laboratories, USA) for granzyme B, TIA-1, and CD40 and a biotinylated rabbit-anti-rat antibody (1:500, 60 min, room temperature; Immuno Research, Jackson Laboratories, USA) for perforin, were detected by a sensitive streptavidin–alkaline–phosphatase complex (1:200, 60 min, room temperature; NenLifeScience, USA). Fast red served as substrate, with haematoxylin for counterstaining. A cytoplasmic staining pattern was regarded as positive.

Evaluation

All markers except C4d were evaluated semi-quantitatively in the different morphological compartments of the renal cortex: tubules, interstitium, glomeruli and vessels. For interstitial CD40 the percentage of positive cells was averaged from three different high-power fields (HPFs) = ×400 magnification) of the most dense infiltrates. The percentage of CD40-expressing non-atrophic tubules was evaluated by the screening of the entire cortex present in a biopsy. For the glomeruli and vessels, only two categories were recorded, glomerulitis or endotheliitis, with CD40-positive cells present or absent.

For interstitial cytotoxic effector molecules (perforin, granzyme B and TIA-1) the percentage of positive CTLs was averaged from three different HPFs of the most dense infiltrates. For tubulitis in non-atrophic tubules, the

entire biopsy was screened and the tubular cross-section with the highest number of CTLs expressing the respective cytotoxic effector molecule was recorded. Similarly, for the glomeruli and the vessels two categories were evaluated, glomerulitis or endotheliitis with perforin, granzyme B or TIA 1-expressing cells present or absent.

Statistical analysis

The SPSS statistical package (version 10.0, SPSS, Chicago, Ill, USA) was used for all statistical analyses. For statistical correlation of each marker tested, the single results revealed in the tubular-interstitial compartments were divided into final expression groups (low, moderate or high expression) by their statistical distribution (Table 1). Kaplan–Meier analysis with log rank testing was used to assess the differences between the groups with regard to graft survival (endpoint = return to dialysis) and onset of CRAD (endpoint = continuous serum creatinine increase of >30% 6 months post-transplantation). For multivariate analysis (Cox regression), the effect of all univariate significant staining results, the C4d result, and the Banff '97 reclassification on the graft survival and onset of CRAD, were analysed. The analysis was performed with stepwise forward logistic regression (significance level for removing the variable from analysis = 0.1, for entering the variable = 0.05).

Results

Histomorphological and clinical findings

Reclassification of the 99 biopsies revealed borderline changes in 31 cases and acute rejection in 60, when the revised Banff '97 classification was applied. Of the 60 cases with acute rejection, 23 were grade Ia, three grade Ib, 19 grade IIa, 12 grade IIb and three were grade III. In eight cases, either no changes consistent with an acute rejection episode or insufficient material according to the Banff '97 scheme, were present. These cases were excluded from all further statistical analyses. Twenty-three cases were C4d positive (23.2%). Fifty-six patients (56.6%) lost their graft within 0.3–111 months (mean 28.8) after transplantation. Twenty-eight patients (28.3%) developed CRAD within 6–96 months (mean 46.1) after transplantation. Twenty-four patients (24.2%) died within the 10-year follow-up period, eight of these with a functioning graft.

Immunohistochemical findings

Expression of CD40 in tubular epithelial cells was found in 93% of the cases with acute rejection and in 67% of the borderline cases. CD40-positive endotheliitis was perceived in 81% of the cases with vascular rejection. No distinct correlation of the average CD40 positivity with the histological grade of tubulo-interstitial rejection was present (Table 2). More cases in higher grades with vascular rejection were CD40 positive (Table 2).

For perforin, granzyme B and TIA-1, different numbers and percentages of positive CTLs were stained in the different morphological compartments of the renal cortex (Table 2). In general, more TIA 1-positive CTLs than perforin or granzyme B-positive cells were detectable. Within each morphological grade of rejection a wide range of the single results was present. By averaging the percent positivity for each effector molecule we revealed a trend of increasing positivity parallel to the severity of histological rejection grades of both tubulo-interstitial and vascular rejection episodes (Table 2).

Table 1 Statistically determined cut-off levels for perforin, granzyme B, TIA-1 and CD40 tubulo-interstitial expression groups

Marker	Interstitial compartment Percentage of stained cells within the interstitial infiltrate	Tubular compartment Maximum number of positive cells per tubular cross-section
Granzyme B		
Low	0–1%	0
Moderate	2–3%	1
High	>3%	>1
Perforin		
Low	0–1%	0
Moderate	2–5%	1–2
High	>5%	>2
TIA-1		
Low	1–8%	0
Moderate	9–14%	1–2
High	>14%	>2
		Percentage of positive tubular cross-sections
CD40		
Low	<5%	<5%
Moderate	5–25%	5–25%
High	>25%	>25%

Table 2 Correlation of immunohistochemical positivity with grade of rejection (Banff '97). *Interstitial* percentage of positive cells within the interstitial infiltrate per high-power field (×400 magnification), *tubular* maximum number of positive cells per tubular cross-section; for CD40 the percentage of positive tubules, respectively, *vascular* positive cells in arterial cross-sections (given as a fraction of cases with sufficient arteries), *glomerular* positive cells in glomeruli (given as a fraction of cases with sufficient glomeruli), *NA* not applicable

Expression pattern	Banff '97					
	Borderline $n=31$	Ia $n=23$	Ib $n=3$	IIa $n=19$	IIb $n=12$	III $n=3$
Interstitial CD40	6.8% (0–28)	27.5% (4–53)	24.6% (2–55)	24.7% (3–57)	38.8% (3–75)	49.0% (22–76)
Tubular CD40	7.0% (0–35)	9.4% (0–45)	22.3% (0–36)	5.1% (0–20)	15.9% (1–75)	23.0% (4–40)
Vascular CD40	NA	NA	NA	3/5	8/9	2/2
Glomerular CD40	NA	NA	NA	3/5	6/9	0/2
Interstitial perforin	4.6% (0–33)	4.8% (0–12)	5.3% (1–11)	6.8% (0–15)	7.0% (1–14)	5% (5)
Tubular perforin	0.5 (0–4)	1.1 (0–3)	2 (1–3)	2.5 (0–5)	2.3 (0–6)	6.5 (5–8)
Vascular perforin	NA	NA	NA	4/8	9/10	1/2
Glomerular perforin	NA	NA	NA	5/8	6/10	0/2
Interstitial granzyme B	2% (0–10)	2.9% (0–5)	5.6% (1–8)	5.1% (0–12)	5.6% (0–15)	14% (3–25)
Tubular granzyme B	0.2 (0–2)	0.6 (0–2)	2.6 (2–4)	1.2 (0–3)	2.2 (0.6)	6 (6)
Vascular granzyme B	NA	NA	NA	4/7	7/9	2/2
Glomerular granzyme B	NA	NA	NA	5/7	5/9	2/2
Interstitial TIA-1	15.9% (5–32)	11.5% (3–25)	14.0% (8–22)	17.5% (4–31)	15.9% (2–85)	27% (13–41)
Tubular TIA-1	1.0 (0–6)	1.6 (0–5)	4.3 (3–5)	2.8 (0–6)	1.9 (0–6)	6 (5–7)
Vascular TIA-1	NA	NA	NA	7/10	9/12	2/2
Glomerular TIA-1	NA	NA	NA	9/10	11/12	2/2

Correlation to renal allograft survival

Univariate analysis (Table 3, left column) for cases with >2% of granzyme B-positive CTLs in the interstitial infiltrate (Fig. 1a) demonstrates significantly ($P=0.0002$) shorter allograft survival (Fig. 2). Significantly shorter allograft survival was also demonstrated for cases with >two TIA 1-positive CTLs per tubular cross-section ($P=0.0005$), for cases with >25% of CD40-positive cells in the interstitial infiltrate ($P=0.0006$) and vascular rejection with CD40-positive cells ($P=0.0084$). All other evaluated staining patterns, the C4d staining result, and the Banff '97 grades, showed no significant correlation with allograft survival.

In multivariate analysis (Table 4), only the presence of >25% CD40-positive or >2% of granzyme B-positive cells in the interstitial infiltrate were significantly correlated with shorter allograft survival.

Table 3 Univariate analysis of immunohistochemical findings and Banff '97 grade with renal allograft survival and manifestation of chronic renal allograft dysfunction. *Interstitial* percentage of positive cells within the interstitial infiltrate per high power field (×400 magnification), *tubular* maximum number of positive cells per tubular cross-section; for CD40 the percentage of positive tubules, respectively, *vascular* positive cells in arterial cross-sections, *glomerular* positive cells in glomeruli, *NS* not significant

[a] **< 0.01 significant**; 0.01–0.1 borderline significant; > 0.1 NS

Key molecule	Renal allograft survival P^a	Key molecule	Chronic renal allograft dysfunction P^a
Interstitial granzyme B	**0.0002**	**Tubular TIA-1**	**0.0005**
Tubular TIA-1	**0.0005**	**Interstitial granzyme B**	**0.0014**
Interstitial CD40	**0.0006**	**Vascular CD40**	**0.0048**
Vascular CD40	**0.0084**	Tubular perforin	0.0342
Tubular granzyme B	0.0175	Glomerular CD40	0.0979
Tubular perforin	0.0490	Tubular granzyme B	NS
Interstitial TIA-1	NS	Interstitial CD40	NS
Vascular TIA-1	NS	Glomerular granzyme B	NS
Tubular CD40	NS	Vascular perforin	NS
Interstitial perforin	NS	Vascular TIA-1	NS
Glomerular granzyme B	NS	Vascular Granzyme B	NS
Glomerular CD40	NS	Glomerular TIA-1	NS
Vascular granzyme B	NS	Glomerular perforin	NS
Vascular perforin	NS	Interstitial TIA-1	NS
Glomerular TIA-1	NS	Interstitial perforin	NS
Glomerular perforin	NS	Tubular CD40	NS
C4d	NS	C4d	NS
Banff '97	NS	Banff '97	0.0672

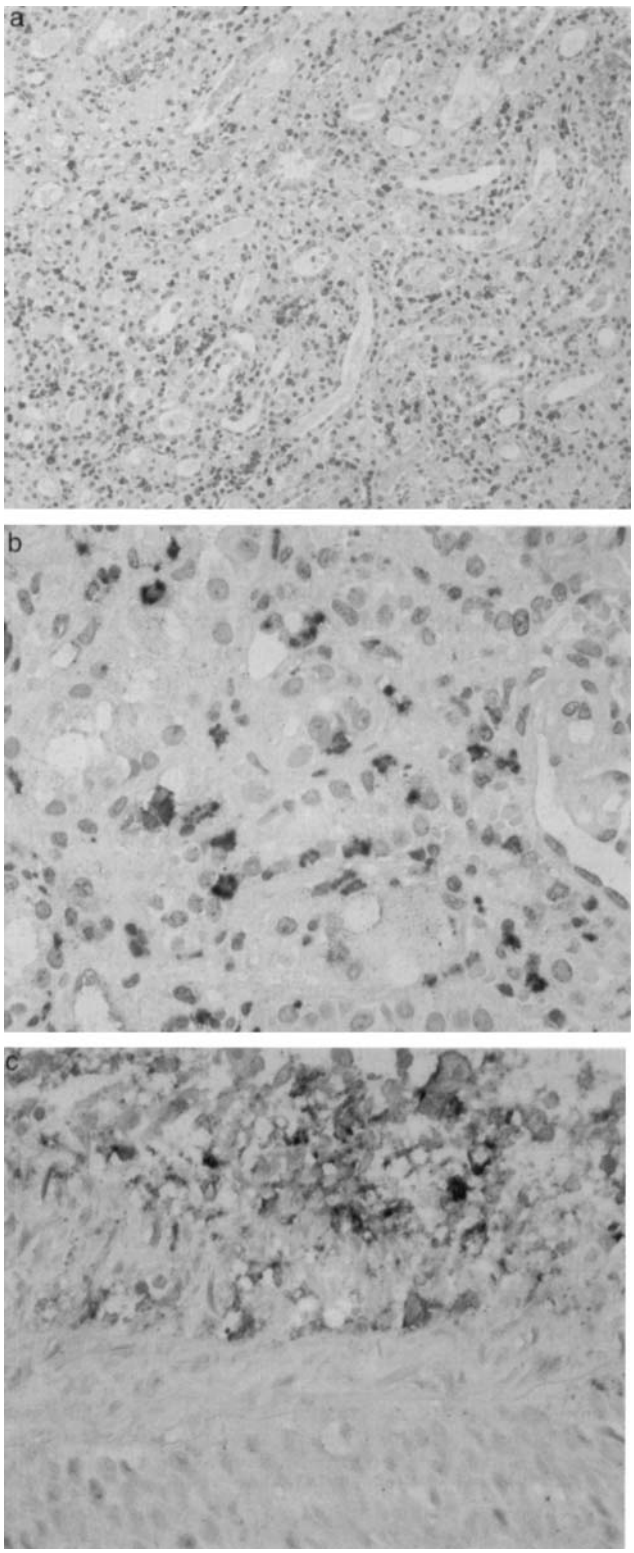

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Fig. 1 a Biopsy with acute interstitial rejection (grade Ia) depicting that >2% of the infiltrating cells in the interstitium are positive for granzyme B (×250). **b**Tubulitis with more than two TIA 1-positive cells in a tubular cross-section (×630). **c** Acute vascular rejection with CD40-positive cells in the subendothelial space (*bottom* smooth muscle cells of media; *top/luminal* subendothelial infiltrating cells) (×630)

Correlation to chronic renal allograft dysfunction

Univariate correlation (Table 3, right column) of cases with >two TIA 1-positive CTLs per tubular cross-section (Fig. 1b) predicts significantly ($P=0.0005$) earlier onset of CRAD. More than 2% of granzyme B-positive CTLs in the interstitial infiltrate ($P=0.0014$) and CD40-positive endotheliitis ($P=0.0048$) are also significantly correlated with earlier onset of CRAD. Correlation of borderline significance is demonstrated between the Banff '97 grade and the development of CRAD, while no significant correlation was found for C4d and CRAD.

In multivariate analysis (Table 4), CD40-positive vascular rejection (Fig. 1c) and the detection of >2% granzyme B-positive CTLs in the interstitium correlates with early-onset CRAD.

Discussion

Since the introduction of modern immunosuppressive drugs, a continuous increase in short-term renal allograft survival has been observed, whereas no noticeable improvement in long-term outcome has occurred [36]. A main cause for long-term graft failure is chronic rejection, which is frequently the final product of prior acute rejection episodes [10]. Currently, most early acute

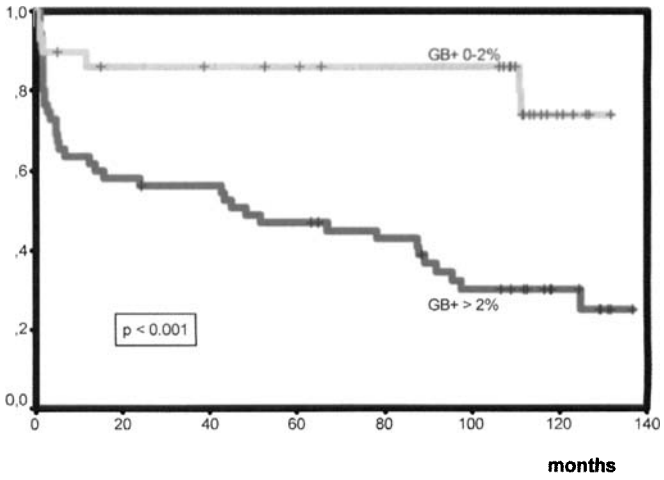

Fig. 2 Kaplan–Meier graph for allograft survival in patients with greater than and less than 2% granzyme B-positive cells in the interstitial infiltrate

Table 4 Significant findings for renal allograft survival and manifestation of chronic renal allograft dysfunction after multivariate analysis (Cox regression; all other tested factors were not significant in the multivariate stepwise Cox analysis)

Findings	
Renal allograft survival	
>25% CD40 + interstitial cells	0.001
>2% Granzyme B + interstitial cells	0.003
Chronic renal allograft dysfunction	
CD40 + vascular rejection	0.017
>2% Granzyme B + interstitial cells	0.032

rejection episodes can be treated successfully; however, a group of these patients, nonetheless, develop chronic allograft dysfunction. Therefore, the establishment of morphological surrogate markers in early acute rejection episodes, still the most frequent indication for biopsy, would be very helpful in identifying these "high risk" patients, allowing adequate treatment to be carried out, and, simultaneously, preventing over-treatment of "low risk" patients. Furthermore, since the currently applied Banff classification defines borderline changes of uncertain significance, and the recommended morphological grading of more frequent acute tubulo-interstitial rejection episodes is without any prognostic value, there is obvious need to gain additional information from allograft biopsies [2, 11, 34].

Our results suggest that, in particular, granzyme B and CD40, as T cell-related markers that play a pivotal role in the process of acute cellular rejection, can predict an unfavourable long-term course. Biopsies with greater than 2% granzyme B or greater than 25% CD40-positive interstitial cells are significantly correlated with shorter allograft survival in the univariate and multivariate analyses. Furthermore, such low numbers (>2%) of granzyme B-positive interstitial CTLs also significantly correlated with earlier onset CRAD in univariate and multivariate analyses. These findings can signify a more intense T cell-mediated immune response in the early post-transplantation phase [16, 25]. Since our immunohistochemical findings are independent of the histological grade of rejection according to the Banff '97 scheme, they should allow for better discrimination between patients with early acute rejection episodes who are likely to respond to treatment, and those with a likely partial response. The latter group might have a propensity for ongoing, subclinical, immunological damage that would contribute to decreased graft survival [20]. Additionally, in cases with CD40-positive vascular rejection, CRAD is of significantly earlier onset, both on univariate and multivariate analyses. Although more cases with morphologically higher grades of vascular rejection are CD40 positive, the immunohistochemical findings statistically overrule the histological findings with respect to the long-term survival of the allograft. A possible explanation for a predictive value of CD40 might be that CD40, besides its essential role in specific T-cell priming, also has the capability to stimulate B-cells and, therefore, the production of allograft-specific, or even endothelial-specific antibodies [25, 33]. Those alloantibodies, together with CD40-induced inflammatory mediators (cytokines, chemokines and growth factors) are known to be responsible for the manifestation of allograft vasculopathy, a morphological hallmark of chronic renal allograft rejection [13, 25, 31]. Therefore, CD40 might play a dual role in allograft rejection, on the one hand promoting the expansion of allograft-specific CTLs in acute cellular rejection episodes, and on the other hand inducing B-cell responses in chronic humoral-mediated rejection processes [25, 31, 33]. However, within our collective we were not able to demonstrate any significant statistical correlation (Spearman rho test; data not shown) between C4d as a putative marker of humoral rejection and CD40 vasculopathy.

Early immunological events are not always crucial for the long-term survival of a renal transplant. Numerous later, non-immunological factors also impair allograft outcome. Nevertheless, the immunohistochemical finding for granzyme B (>2% positive cells in the interstitial infiltrate) was, in our patients, a predictor for a late event (allograft loss) as well as an earlier event (onset of CRAD) in the course after renal transplantation. Obviously, no single clinical, biochemical or immunohistochemical/morphological marker will be sufficient to predict, completely, the long-term course of a renal allograft. Furthermore, biopsy findings, morphological as well as semi-quantitative immunohistochemical, might be hampered by limited reproducibility [37]. Therefore, the challenge lies, predominantly, in the developing of algorithms, which integrate clinical, biochemical and biopsy findings so that the treatment of each individual patient can be better managed. These results might provide potential immunohistochemical candidates for the generation of individual risk profiles.

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