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Original contribution

Immunohistochemical markers of tissue injury in biopsies with transplant glomerulitis☆,☆☆

Ibrahim Batal MD[a,*], Jamil Azzi MD[b,c], Najib El-Haddad PhD[b,c], Leonardo V. Riella MD, PhD[b,c], John G. Lunz III PhD[a], Adriana Zeevi PhD[a], Eizaburo Sasatomi MD, PhD[a], Amit Basu MD[b,c], Henkie Tan MD, PhD[b,c], Ron Shapiro MD[b,c], Parmjeet Randhawa MD[a]

[a]*Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA*
[b]*Transplantation Research Center, Renal Division, Brigham and Women's Hospital, Boston, MA 02115, USA*
[c]*Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA*

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Summary Transplant glomerulitis is associated with suboptimal graft function. To understand its pathogenesis and to assess the parameters of potential prognostic value, we immunostained 25 paraffin-embedded allograft biopsies showing glomerulitis for markers of complement activation (C4d), cytotoxicity (Granzyme-B), apoptosis (Bcl-XL, Bcl-2, and Fas-L), and endothelial injury (von Willebrand factor). Staining was semiquantitatively assessed in different anatomical compartments, and comparison was made with 40 control allograft biopsies without glomerulitis. Biopsies with glomerulitis had more frequent incidence of "mixed" T-cell and antibody-mediated rejection compared with controls [8/25 (32%) versus 4/40 (10%), $P = .046$]. Furthermore, they had higher glomerular capillary-C4d scores (1.9 ± 1.1 versus 1.2 ± 1.2, $P = .015$), which tended to persist when biopsies showing transplant glomerulopathy were excluded. Higher glomerular capillary-C4d scores were observed in samples with versus without donor-specific antibody (2.5 ± 0.9 versus 1.2 ± 1.2, $P = .01$). Compared with controls, biopsies with glomerulitis had more intraglomerular (4.8 ± 4.5 versus 0.9± 0.8 cells/glomerulus, $P < .001$) and interstitial mainly peritubular capillary (6.1 ± 4.1 versus 3.2 ± 3.4 cells/hpf, $P = .002$) Granzyme-B$^+$ leukocytes. Higher mesangial–von Willebrand factor scores were noted in the glomerulitis group (1.8 ± 1.0 versus 0.8 ± 0.8, $P = .003$) and correlated with the percentage of inflamed glomeruli ($r = 0.54$, $P < .001$). Interstitial–von Willebrand factor was associated with a higher peritubular capillaritis score (interstitial–von Willebrand factor: 1.6 ± 1.2 versus no interstitial–von Willebrand factor: 0.6 ± 0.9, $P = .02$). Glomerular capillary–Bcl-XL was not associated with accommodation. Finally, no difference in Bcl-2 or Fas-L was observed upon comparing glomerulitis to controls. In conclusion, glomerular injury in transplant glomerulitis appears to be mediated by complement activation and cellular cytotoxicity. Mesangial– or interstitial–von Willebrand factor identified cases with more severe microcirculation injury.

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* Corresponding author. Currently at Brigham and Women's Hospital, Boston, MA 02115, USA.

E-mail address: ibatal@partners.org (I. Batal).

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1. Introduction

Transplant glomerulitis is characterized by intracapillary glomerular leukocytic inflammation in kidney allografts. It can be observed in association with antibody-mediated rejection (AMR) and/or T-cell–mediated rejection (TCMR) [1-3]. The presence and grade of glomerulitis correlate with proteinuria, peritubular capillaritis, peritubular capillary (PTC)-C4d staining, detection of circulating donor-specific antibody (DSA), development of chronic transplant glomerulopathy (TGP) and suboptimal graft survival [3-4]. The pathophysiology of transplant glomerulitis is not well understood. Hence, we performed a morphology-based study to understand the pathogenesis and potential prognostic factors of transplant glomerulitis. We immunostained a series of allograft biopsies with transplant glomerulitis for markers of complement activation (C4d), cellular cytotoxicity (Granzyme-B), apoptosis (Bcl-XL, Bcl-2, Fas-L), and endothelial injury (von Willebrand factor, or VWF). We compared the pattern of staining of glomerulitis samples with that of allograft biopsies without glomerulitis.

2. Material and methods

2.1. Biopsy material

We retrieved 111 renal allograft biopsies from 111 patients who received kidney allografts at the University of Pittsburgh Medical Center with (n = 67) and without (n = 44) transplant glomerulitis. All specimens were "for cause biopsies."

The detailed clinical, laboratory, and histologic characteristics of these patients have been published elsewhere [4]. Data collection procedures were approved by the University of Pittsburgh Institutional Review Board (IRB protocol #9030095). In the current study, we have characterized the immunohistochemical profile of the biopsy material.

Transplant glomerulitis was graded using the Banff 97 grading system (g0-g3: 0%, <25%, 25-75%, and >75% of glomeruli being affected, respectively) [5]. Affected glomeruli were defined by the presence of 5 or more leukocytes/glomerulus on periodic acid–Schiff–stained slides [4,6]. We found the latter grading system to be superior to grading based on the most inflamed glomerulus or the presence of capillary loop occlusion by inflammation [4]. In addition to transplant glomerulitis, histologic parameters were evaluated according to the Banff 97 criteria for renal allograft pathology [5], and samples were classified as negative, suspicious, or diagnostic for acute AMR based on Banff 2001 criteria [7].

Immunohistochemical studies were performed on biopsies with Banff grade g2 or g3 glomerulitis and compared with those of biopsies without glomerulitis (g = 0). Grade g1 samples were not analyzed because we did not encounter significant differences in any of the assessed clinicopathologic parameters when comparing g1 biopsies to biopsies without glomerulitis (g0) [4]. All biopsies with g2 or g3 glomerulitis and all biopsies without glomerulitis were included when sufficient remnant tissue material was available for analysis [glomerulitis (n = 25): g2 (n = 17) and g3 (n = 8), no glomerulitis (n = 40)].

2.2. Clinical parameters

Basic demographic and clinical parameters were retrieved from electronic medical records. In particular, percent of enzyme-linked immunosorbent assay panel reactive antibody (ELISA PRA) and detection of DSA were documented. Immune cell function values (ng ATP/mL whole blood) assessed using the Cylex ImmuKnow (Columbia, MD) assay were also recorded. Graft function was followed for a median of 600 days (interquartile range, 280-1160 days). The primary outcome parameter was graft failure defined as return to dialysis or transplant nephrectomy. The secondary outcome parameter was the development of TGP (cg $\geq$ 1) on follow-up, if it was absent (cg = 0) on the index biopsy.

2.3. Immunohistochemical stains

A panel of immunoperoxidase antibodies was performed on 4-micron formalin-fixed, paraffin-embedded renal allograft biopsies:

1) C4d: antigen retrieval was performed using Cell Conditioner 1 (CC1; Ventana Medical Systems, Tucson, AZ). Polyclonal mouse primary antibody (ALPCO Diagnostics, Windham, NH) was used (1:50 dilution, 44-minute incubation).
2) Granzyme-B: antigen retrieval was performed using CC1. Monoclonal mouse primary antibody (clone#B-7; Dako, Carpinteria, CA) was used (1:25 dilution, 40-minute incubation).
3) Bcl-XL: antigen retrieval was performed using microwave and incubation in Protein Block (Dako #X0909). Monoclonal mouse primary antibody (Invitrogen, Camarillo, CA) was used (1:50 dilution, 1-hour incubation).
4) Bcl-2: antigen retrieval was performed using CC1. Monoclonal mouse primary antibody (clone #124; Ventana Medical Systems) was used (1:50 dilution, 1-hour incubation).
5) Fas-L: antigen retrieval was performed by steaming for 20 minutes in EDTA buffer (PH 8.0) and incubation in Protein Block (Dako #X0909). Polyclonal rabbit primary antibody (Thermo Scientific, Fremont, CA) was used (1:100 dilution, 1-hour incubation).
6) VWF (factor VIII–related antigen): antigen retrieval was performed using CC1. Polyclonal primary rabbit antibody (Dako) was used (1:500 dilution, 8-minute incubation).

Secondary antibodies: for C4d, Granzyme-B, Bcl-2, and VWF, affinity-purified biotinylated goat-antimouse

immunoglobulin G and immunoglobulin M or goat-anti-rabbit immunoglobulin G in phosphate buffer with ProClin 300 preservative (Sigma-Aldrich, St. Louis, MO) was used followed by streptavidin–horseradish peroxidase (Ventana Medical Systems). For Bcl-XL and Fas-L, universal Immpress (Vector Labs #MP-7500) was placed on the slides for 30 minutes at room temperature.

2.4. Positive controls for immunohistochemistry

A failed allograft kidney with AMR served as a positive control for C4d staining and showed diffuse linear to finely granular PTC-C4d. A lymph node with classical Hodgkin lymphoma served as a positive control for Bcl-XL and showed a homogenous cytoplasmic staining in Reed Sternberg and Hodgkin cells [8]. Sections from a normal tonsil served as positive controls for Granzyme-B, Bcl-2, Fas-L, and VWF. Granzyme-B generated a coarsely granular cytoplasmic signal in a subpopulation of leukocytes presumably representing cytotoxic T cells and natural killer cells. Cytoplasmic Bcl-2 staining was detected in many small lymphocytes located in the paracortical T zone as well as in the follicles (B zone). Cytoplasmic/membranous Fas-L staining could be demonstrated in plasma cells and occasional small lymphocytes as previously described [9]. VWF staining was observed in blood vessels including high endothelial venules.

2.5. Evaluation of immunohistochemical findings

All immunostains were systematically assessed in different anatomical compartments of the allograft kidney as follows:

1) Glomerular staining: the extent of glomerular capillary (GC), mesangial, and Bowman capsular staining was semiquantitatively graded as negative (0), minimal (1), segmental (2), and global (3) when staining was absent, and involved less than 10%, 10% to 50%, and more than 50% of the most intensely stained glomerulus, respectively. Podocyte staining, which was rarely detected and only with the anti–Bcl-2 antibody, was categorically recorded as present or absent.
2) Intraglomerular inflammatory cells: staining for Granzyme-B, Bcl-2, and Fas-L was quantified by averaging the number of stained leukocytes in the 5 most affected glomeruli. This number was also expressed as a percentage of the total number of intraglomerular leukocytes identified on a periodic acid–Schiff stain.
3) PTC staining: staining was semiquantitatively graded as negative (0), minimal (1), focal (2), and diffuse (3) when PTC staining was absent, and present in less than 10%, 10% to 50%, or more than 50% of the stained tissue available for evaluation, respectively.
4) Small artery staining: arterioles and arteries were grouped together. The staining was semiquantitatively assessed as negative (0), minimal (1), focal (2), and diffuse (3) when absent, and involved less than 10%, 10% to 50%, and more than 50% of the total number of the vessels, respectively.
5) Tubular-epithelial (TEP) staining: staining was semiquantitatively graded as negative (0), minimal (1), focal (2), and diffuse (3) when TEP staining was absent, and present in less than 10%, 10% to 50%, or more than 50% of the stained tissue available for evaluation, respectively.
6) Interstitial inflammatory cells: staining for Granzyme-B, Bcl-2, and Fas-L was quantified by averaging the number of stained leukocytes counted in the 5 most affected high-power fields (×40).

In addition to its extent, the intensity of staining was semiquantitatively graded (0-4). A composite staining score (0-12) was calculated for TEP by multiplying the extent (0-3) and the intensity (0-4) of the stain.

2.6. Statistics

Continuous and categorical data were compared using Mann-Whitney rank sum test and Fisher exact test, respectively. Correlation coefficient was performed using Spearman rank correlation. P values of .05 or less with 2-sided hypothesis testing were considered statistically significant.

3. Results

3.1. Histology findings

Biopsies with glomerulitis had more frequent incidence of mixed rejection (TCMR and AMR) compared with biopsies without glomerulitis (P = .046; Table 1). They also had higher peritubular capillaritis scores (P = .02) and tended to have higher TGP (cg) scores (P = .14) and mesangial matrix expansion (mm) scores (P = .06). As previously reported [4], patients with glomerulitis had higher incidence of graft failure (P = .001) and more frequent development of TGP on follow-up biopsies (P = .05; Table 1). Otherwise, no difference in the other Banff scores or in the posttransplantation time was detected (data not shown).

In all but one biopsy with glomerulitis, glomerular endocapillary inflammatory infiltrate was composed of a combination of mononuclear cells and neutrophils. Mononuclear cells predominated in all of these biopsies (range: 63%-98% of infiltrating leukocytes). Biopsies with glomerulitis containing more than 90% mononuclear cells had a more frequent incidence of TGP compared with those with 10% neutrophils or more but less than 90% mononuclear cells [5/14 (36%) versus 0/11 (0%), P = .046].

Table 1 Histologic assessment, detection of circulating DSA, and follow-up information for cases studied

	No glomerulitis (n = 40)	Glomerulitis (n = 25)
Mixed rejection (TCMR and AMR) [a]	4/40 (10%)	8/25 (32%)
TCMR	17/40 (43%)	7/25 (28%)
AMR (diagnostic/ suspicious)	6/40 (15%)	2/25 (8%)
Borderline changes suspicious for TCMR	13/40 (32%)	8/25 (32%)
Tubulitis (t)	1.8 ± 0.8	2.0 ± 0.9
Interstitial inflammation (i)	1.7 ± 0.7	1.9 ± 0.8
Intimal arteritis (v)	0.1 ± 0.4	0.1 ± 0.4
Peritubular capillaritis [b]	0.5 ± 0.8	1.2 ± 1.0
TGP (cg)	0.03 ± 0.16	0.36 ± 0.76
Mesangial matrix expansion (mm)	0.2 ± 0.5	0.7 ± 0.9
Interstitial fibrosis (ci)	1.0 ± 0.6	1.2 ± 0.6
Tubular atrophy (ct)	1.1 ± 0.5	1.2 ± 0.5
Arteriolar hyalinosis (ah)	0.9 ± 0.7	0.8 ± 0.8
Arterial fibrointimal thickening (cv)	0.9 ± 0.7	1.1 ± 0.8
DSA at the biopsy	4/32 (13%)	4/18 (22%)
DSA at anytime during the transplantation course	11/40 (28%)	9/25 (36%)
Development of TGP [c]	1/28 (4%; 682 d postbiopsy)	4/16 (25%; 256 ± 160 d postbiopsy)
2-y postbiopsy graft failure [d]	5/28 (18%)	11/16 (69%)

NOTE. Information on DSA at the time of biopsy was available on 50 occasions (18 with and 32 without glomerulitis). Follow-up information on development of TGP and 2-year postbiopsy graft failure was available for 44 patients (16 with and 28 without glomerulitis).

[a] Mixed rejection: $P = .046$ (glomerulitis *vs* no glomerulitis; Fisher exact test).

[b] Peritubular capillaritis: $P = .02$ (glomerulitis *vs* no glomerulitis; Mann-Whitney test).

[c] Development of TGP: $P = .05$ (glomerulitis *vs* no glomerulitis; Fisher exact test).

[d] Two-year postbiopsy graft failure: $P = .001$ (glomerulitis *vs* no glomerulitis; Fisher exact test).

3.2. Endothelial C4d staining

Biopsies with glomerulitis had higher GC-C4d scores ($P = .015$; Table 2 and Fig. 1), which tended to persist even when samples showing concurrent TGP were excluded (1.7 ± 1.1 versus 1.2 ± 1.2, $P = .07$). Furthermore, samples with concurrent detectable DSA (n = 8) showed higher GC-C4d score (2.5 ± 0.9) compared with samples with negative DSA (n = 42, 1.2 ± 1.2, $P = .01$) even when samples showing TGP were excluded [DSA(+): 2.3 ± 1.0 (n = 6) versus DSA(−): 1.1 ± 1.1 (n = 39), $P = .03$]. In addition, GC-C4d scores correlated significantly with PTC-C4d scores ($r = 0.54$, $P < .001$), which tended to be higher in biopsies with glomerulitis (1.3 ± 1.1 versus 0.8 ± 1.0, $P = .08$; Table 2).

In patients with global GC-C4d, patients with glomerulitis had a higher incidence of graft failure compared with patients without glomerulitis [6/8 (75%) versus 0/7 (0%), $P = .006$]. In contrast, when only samples with glomerulitis were considered, patients with, compared with those without, global GC-C4d showed no significant difference in graft failure [6/8 (75%) versus 5/8 (63%)] or in the incidence of TGP on index biopsy or on follow-up (data not shown).

3.3. Glomerular and vascular Granzyme-B

Compared with biopsies lacking glomerulitis, those with glomerulitis had a higher absolute number ($P < .001$) and tended to have higher percentages ($P = .1$) of intraglomerular Granzyme-B$^+$ leukocytes (Table 2, Fig. 2). Furthermore, the percentage of intraglomerular Granzyme-B$^+$ leukocytes correlated significantly with immune cell function values ($r = 0.44$, $P = .02$). No GC, mesangial, Bowman capsular, podocytes, PTC, or arterial Granzyme-B staining was appreciated.

3.4. Tubulointerstitial Granzyme-B staining

TEP-Granzyme-B was negative except in a few samples that showed scattered and possibly artifactual staining at the edge of the biopsies (Table 2). Interestingly, Granzyme-B$^+$ leukocytes were mainly located in the peritubular capillaries and were more numerous in biopsies with glomerulitis ($P = .002$; Fig. 2). The number of these Granzyme-B+ leukocytes correlated with immune cell function values ($r = 0.5$, $P = .003$) and with Banff scores for peritubular capillaritis ($r = 0.5$, $P < .001$) but not with Banff scores for interstitial inflammation ($r = 0.1$, $P = .4$).

3.5. Glomerular and vascular BcL-XL staining

GC–Bcl-XL was absent in most samples with or without glomerulitis (Table 2). Global/segmental GC–Bcl-XL was observed in 4 samples [global (n = 1), segmental (n = 3)]. Such staining did not appear to be associated with accommodation; 3 (75%) of these biopsies had glomerulitis, 3 (75%) had documented TCMR [IA (n = 2), IB (n = 1)], and 1 (25%) was diagnostic for AMR. One biopsy had TGP on index biopsy, whereas another one showed TGP in a follow-up specimen. Two (50%) subjects developed graft failure 361 and 1455 days postbiopsy whereas a third one was lost to follow-up after 94 days with a serum creatinine of 5.5 mg/dL. Mesangial, Bowman capsular, or podocyte-Bcl-XL staining was never encountered, whereas minimal PTC and arterial Bcl-XL were rarely observed (Table 2). No intraglomerular Bcl-XL$^+$ leukocytes were seen.

Table 2 Immunohistochemical staining profile in different anatomical compartments in samples with and without glomerulitis

Immunostain	Component	Staining pattern and semiquantitative score [a]	No glomerulitis (n = 40)	Glomerulitis (n = 25)
C4d (complement activation marker)	GC C4d	Minimal	8/39 (21%)	9/25 (36%)
		Segmental	6/39 (15%)	2/25 (8%)
		Global	9/39 (23%)	12/25 (48%)
		Semiquantitative score [b]	1.2 ± 1.2	1.9 ± 1.1
	PTC C4d	Minimal	12/40 (30%)	8/25 (32%)
		Focal	6/40 (15%)	5/25 (20%)
		Diffuse	3/40 (7%)	5/25 (20%)
		Semiquantitative score	0.8 ± 1.0	1.3 ± 1.1
Granzyme-B (cytotoxicity marker)	Intraglomerular Granzyme-B$^+$ leukocytes	Average/glomerulus [c]	0.9 ± 0.8	4.8 ± 4.5
		%	42 ± 39	60 ± 46
	Tubular-epithelial Granzyme-B	Minimal	4/39 (10%)	2/20 (10%)
		Focal	1/39 (3%)	2/20 (10%)
	Interstitial Granzyme-B$^+$ l eukocytes [d]	Average/hpf [e]	3.2 ± 3.4	6.1± 4.1
Bcl-XL (antiapoptotic marker)	GC Bcl-XL	Minimal	3/38 (8%)	3/25 (12%)
		Segmental	1/38 (3%)	2/25 (8%)
		Global	0/38 (0%)	1/25 (4%)
		Semiquantitative score	0.1 ± 0.4	0.4 ± 0.8
	PTC Bcl-XL	Minimal	1/40 (2%)	0/25 (0%)
	Small arteries Bcl-XL	Minimal	1/40 (2%)	2/25 (8%)
	Tubular-epithelial Bcl-XL	Minimal	10/40 (25%)	7/25 (28%)
		Focal	15/40 (37%)	7/25 (28%)
		Diffuse	9/40 (23%)	9/25 (36%)
		Composite score	2.5 ± 2.1	3.4 ± 2.7
Bcl-2 (antiapoptotic marker)	Bowman capsule Bcl-2	Segmental	6/36 (17%)	4/21 (19%)
		Global	30/36 (83%)	16/21 (76%)
		Semiquantitative score	2.8 ± 0.4	2.7 ± 0.7
	Podocyte–Bcl-2 staining	Present	8/36 (22%)	3/21 (14%)
	Intraglomerular Bcl-2$^+$ leukocytes	Average/glomerulus [f]	1.4 ± 1.2	4.6 ± 4.1
		%	57 ± 51%	52 ± 43%
	Small arteries Bcl-2	Minimal	1/39 (3%)	0/22 (0%)
	Tubular-epithelial Bcl-2	Minimal	4/39 (10%)	3/22 (14%)
		Focal	23/39 (59%)	12/22 (54%)
		Diffuse	12/39 (31%)	6/22 (27%)
		Composite score	3.8 ± 2.2	3.4 ± 2.3
	Interstitial Bcl-2$^+$ leukocytes	Average/hpf	24 ± 17	26 ± 15
Fas-L (mediator of apoptosis)	GC Fas-L	Minimal	4/35 (11%)	4/24 (17%)
		Segmental	2/35 (6%)	1/24 (4%)
		Semiquantitative score	0.2 ± 0.5	0.3 ± 0.5
	Intraglomerular Fas-L$^+$ leukocytes	Average/glomerulus	0.0 ± 0.0	0.008 ± 0.04
	Tubular-epithelial Fas-L	Minimal	10/38 (26%)	5/24 (21%)
		Focal	8/38 (21%)	8/24 (33%)
		Diffuse	0/38 (0%)	1/24 (4%)
		Composite score	1.0 ± 1.4	1.3 ± 1.6
	Interstitial Fas-L$^+$ leukocytes	Average/hpf	1.0 ± 4.0	1.0 ± 5.0
VWF (marker of endothelial and endothelial injury)	GC-VWF	Minimal	3/19 (16%)	1/19 (5%)
		Segmental	7/19 (37%)	8/19 (42%)
		Global	9/19 (47%),	10/19 (53%)
		Semiquantitative score	2.3 ± 0.7	2.5± 0.6
	Mesangial-VWF	Minimal	7/19 (37%)	2/19 (10%)
		Segmental	4/19 (21%)	10/19 (53%)
		Global	0/19 (0%)	4/19 (21%)
		Semiquantitative score [g]	0.8 ± 0.8	1.8 ± 1.0

(*continued on next page*)

Table 2 (*continued*)

Immunostain	Component	Staining pattern and semiquantitative score [a]	No glomerulitis (n = 40)	Glomerulitis (n = 25)
	PTC-VWF	Minimal	1/19 (5%)	0/20 (0%)
		Focal	6/19 (32%)	4/20 (20%)
		Diffuse	12/19 (63%)	16/20 (80%)
		Semiquantitative score	2.6 ± 0.6	2.8 ± 0.4
	Small arteries VWF	Diffuse	19/19 (100%)	20/20 (100%)

NOTE. The scoring system is explained in details in the "Material and methods" section. Tissue was not available for Granzyme-B, Bcl-2, Fas-L, and VWF immunostains in 5, 3, 1, and 5 samples with glomerulitis and 1, 1, 2, and 21 samples without glomerulitis, respectively. In the "glomerulitis" group, one section each stained for Granzyme-B, Bcl-2, and VWF immunostains did not contain glomeruli. In the "no glomerulitis" group, 1, 2, 2, 3, and 3 samples stained for C4d, Granzyme-B, Bcl-XL, Bcl-2, and Fas-L did not contain glomeruli, respectively. Abbreviation: hpf, high-power field.

[a] Staining patterns and scores: the percentage of biopsies in individual categories do not add to 100% because biopsies with negative staining have not been enumerated for the sake of simplicity.

[b] GC C4d score: $P = .015$ (glomerulitis *vs* no glomerulitis; Mann-Whitney test).

[c] Intraglomerular Granzyme-B$^+$ leukocytes: average/glomerulus: $P < .001$ and % showed a trend toward a difference: $P = .1$ (glomerulitis *vs* no glomerulitis; Mann-Whitney test).

[d] Interstitial Granzyme-B$^+$ are mainly found in the peritubular capillaries.

[e] Interstitial Granzyme-B$^+$ leukocytes: $P = .002$ (glomerulitis *vs* no glomerulitis; Mann-Whitney test).

[f] Intraglomerular Bcl-2$^+$ leukocytes: average/glomerulus: $P < .001$, whereas % was similar: $P = .9$ (glomerulitis *vs* no glomerulitis; Mann-Whitney test).

[g] Mesangial-VWF score: $P = .003$ (glomerulitis *vs* no glomerulitis; Mann-Whitney test).

3.6. Tubulointerstitial Bcl-XL staining

TEP–Bcl-XL composite scores did not differ in biopsies with and without glomerulitis (Table 2) and did not show any correlation with Banff scores for interstitial inflammation or fibrosis (data not shown). No Bcl-XL$^+$ leukocytes were observed.

3.7. Glomerular and vascular Bcl-2 staining

Bowman capsular Bcl-2 was frequently observed in biopsies with and without glomerulitis and was often classified as global (Table 2). In contrast, podocyte-Bcl-2 was infrequently observed in both groups. However, in biopsies that lacked glomerulitis but showed podocyte-Bcl-2 (n = 8), Bcl-2 staining was confined to rare cells, none had or subsequently developed TGP, and only 1 (12%) of the 8 patients had detectable proteinuria (100 mg/dL). In contrast, all patients with glomerulitis and podocyte-Bcl-2 (n = 3) had several positively stained cells, and all had focal segmental glomerulosclerosis and proteinuria [300 mg/dL (n = 2) and 100 mg/dL (n = 1)]. Two of these 3 patients had TGP on index biopsies, whereas the third patient subsequently developed TGP on follow-up (207 days postbiopsy). The absolute number but not the relative percentage of intraglomerular Bcl-2$^+$ leukocytes was higher in biopsies with glomerulitis (Table 2). This seems to reflect a general increase in inflammatory cells rather than a selective increase in Bcl-2$^+$ leukocytes. No convincing GC, mesangial, or PTC-Bcl-2 was detected in any sample. Minimal arterial Bcl-2 was rarely observed.

3.8. Tubulointerstitial Bcl-2 staining

TEP–Bcl-2 scores were similar in biopsies with and without glomerulitis (Table 2) and did not show any correlation with Banff scores for interstitial inflammation or fibrosis nor with TEP–Bcl-XL scores (data not shown). The average number of interstitial Bcl-2$^+$ leukocytes was similar in biopsies with and without glomerulitis (Table 2).

3.9. Glomerular and vascular Fas-L staining

Weak (intensity grade 1) and segmental GC-Fas-L was present in 3 samples, 1 with and 2 without glomerulitis (Table 2). All these 3 patients maintained their graft function until the end of follow-up, although 1 had TGP on index biopsy. One of the biopsies showed rare intraglomerular Fas-L$^+$ circulating inflammatory cells. Mesangial, Bowman capsular, podocyte, PTC, or arterial Fas-L staining was never observed.

3.10. Tubulointerstitial Fas-L staining

TEP–Fas-L composite scores did not differ in samples with compared with those without glomerulitis (Table 2). There was no correlation between composite TEP–Fas-L scores and Banff scores for interstitial inflammation or fibrosis (data not shown). However, TEP–Fas-L scores correlated weakly with TEP–Bcl-XL ($r = 0.27$, $P = .04$) and TEP–Bcl-2 ($r = 0.38$, $P = .004$) scores.

In addition to cytoplasmic/membranous TEP staining, all biopsies showed nuclear TEP–Fas-L. Seven biopsies (3 with and 4 without glomerulitis) showed occasional Fas-L$^+$ interstitial lymphoplasmacytic cells, which were often found in the interstitial space between tubules and were not associated with tubulitis.

3.11. Glomerular VWF staining

Glomerular staining for VWF was evaluable in 19 biopsies, each with and without glomerulitis. Several biopsies

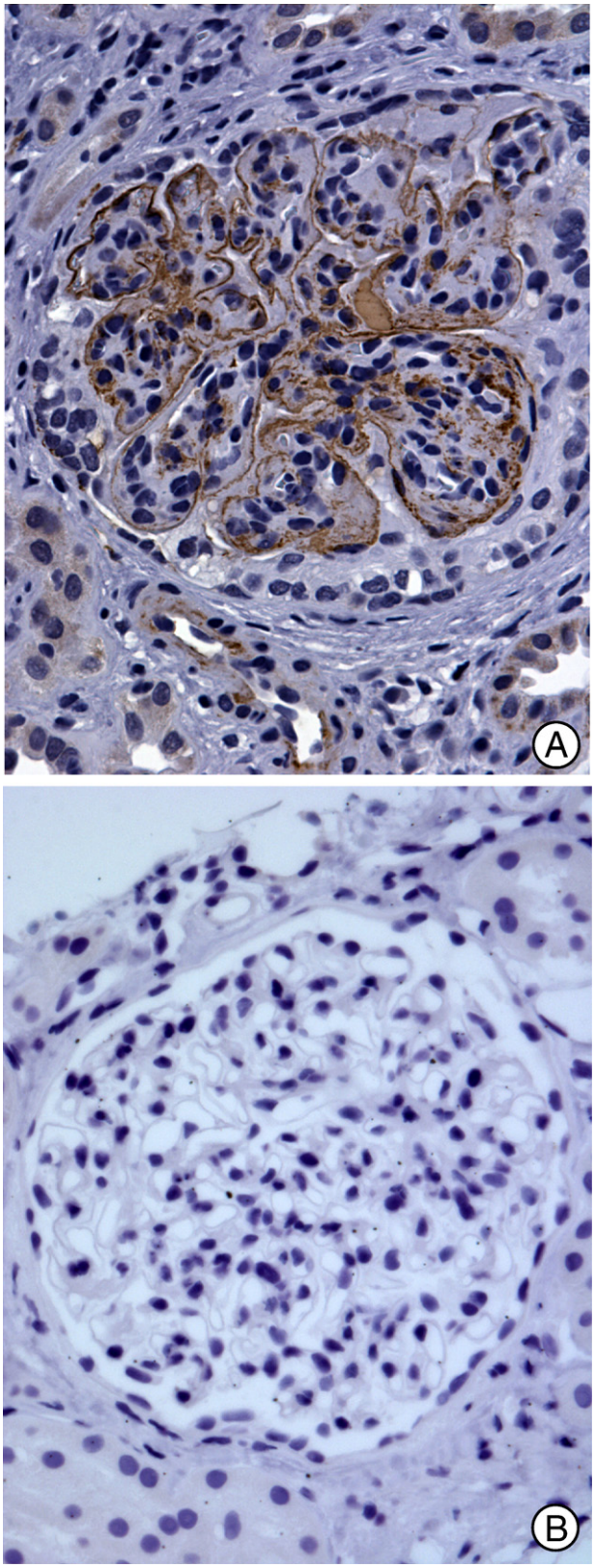

Fig. 1 A, Strong GC staining for C4d in a biopsy with severe transplant glomerulitis and podocyte hyperplasia without crescent formation. Electron microscopy showed no evidence of immune complex glomerulonephritis. B, A second biopsy without glomerulitis shows no GC staining (C4d immunostain, original magnification ×400).

could not be studied because the tissue had been exhausted during the course of immunohistochemical staining performed earlier. There was no significant difference in GC-VWF scores between the 2 aforementioned groups (Table 2).

In contrast, mesangial-VWF score was higher in biopsies with glomerulitis (P = .003) even when samples showing concurrent TGP were excluded [glomerulitis (n = 15) 1.7 ± 1.0 versus without glomerulitis (n = 18) 0.8 ± 0.8, P = .02]. Mesangial-VWF scores correlated with glomerulitis grade (r = 0.57, P = .02) and with the percentage of inflamed glomeruli (r = 0.54, P < .001). Global/segmental mesangial-VWF was observed in 14 biopsies with glomerulitis (4 global and 10 segmental) (Fig. 3) and 4 biopsies without glomerulitis (all segmental). Compared with samples with negative/minimal mesangial-VWF, samples with global/segmental mesangial-VWF showed a trend toward worse 2-year graft survival [6/11 (55%) versus 2/13 (15%), P = .08] and numerically but not statistically higher frequency of index TGP [4/18 (22%) versus 1/20 (5%)] and subsequent development of TGP [3/11 (27%) versus 1/15 (7%)], respectively.

3.12. PTC-VWF staining

Diffuse PTC-VWF was detected in 80% versus 63% of samples with versus without glomerulitis, respectively. No significant difference in PTC-VWF score was detected upon comparing the 2 aforementioned groups (Table 2). PTC-VWF staining did not significantly correlate with PTC-C4d scores (data not shown), nor did it show significant association with AMR and TCMR (Table 3).

The clinical significance of PTC-VWF scores was then compared with that of PTC-C4d scores by studying the associations with peritubular capillaritis, ELISA PRA, and DSA. As expected, PTC-C4d scores showed significant correlations with the percent of ELISA PRA class I (r = 0.3, P = .03), ELISA PRA class II (r = 0.4, P = .01), and peritubular capillaritis scores (r = 0.3, P = .04). In contrast, PTC-VWF failed to correlate with any of the aforementioned parameters (data not shown). Furthermore, the detection of DSA was associated with higher semiquantitative scores of PTC-C4d but not PTC-VWF (Table 4).

Finally, 8 samples showed spilling of VWF staining into the interstitium (5 with and 3 without glomerulitis; Fig. 4). All these samples had diffuse PTC-VWF, whose intensity was graded as grade 4 in 6 (75%) and grade 3 in 2 (25%) of these biopsies. Samples with spilling of VWF into the interstitium had significantly higher incidence of AMR (P = .03) and had numerical higher incidence of graft failure compared with samples without interstitial-VWF (Table 3). In addition, biopsies with interstitial-VWF had higher peritubular capillaritis scores (1.6 ± 1.2 versus 0.6 ± 0.9, P = .02) but not tubulitis or interstitial inflammation scores compared with samples without interstitial-VWF.

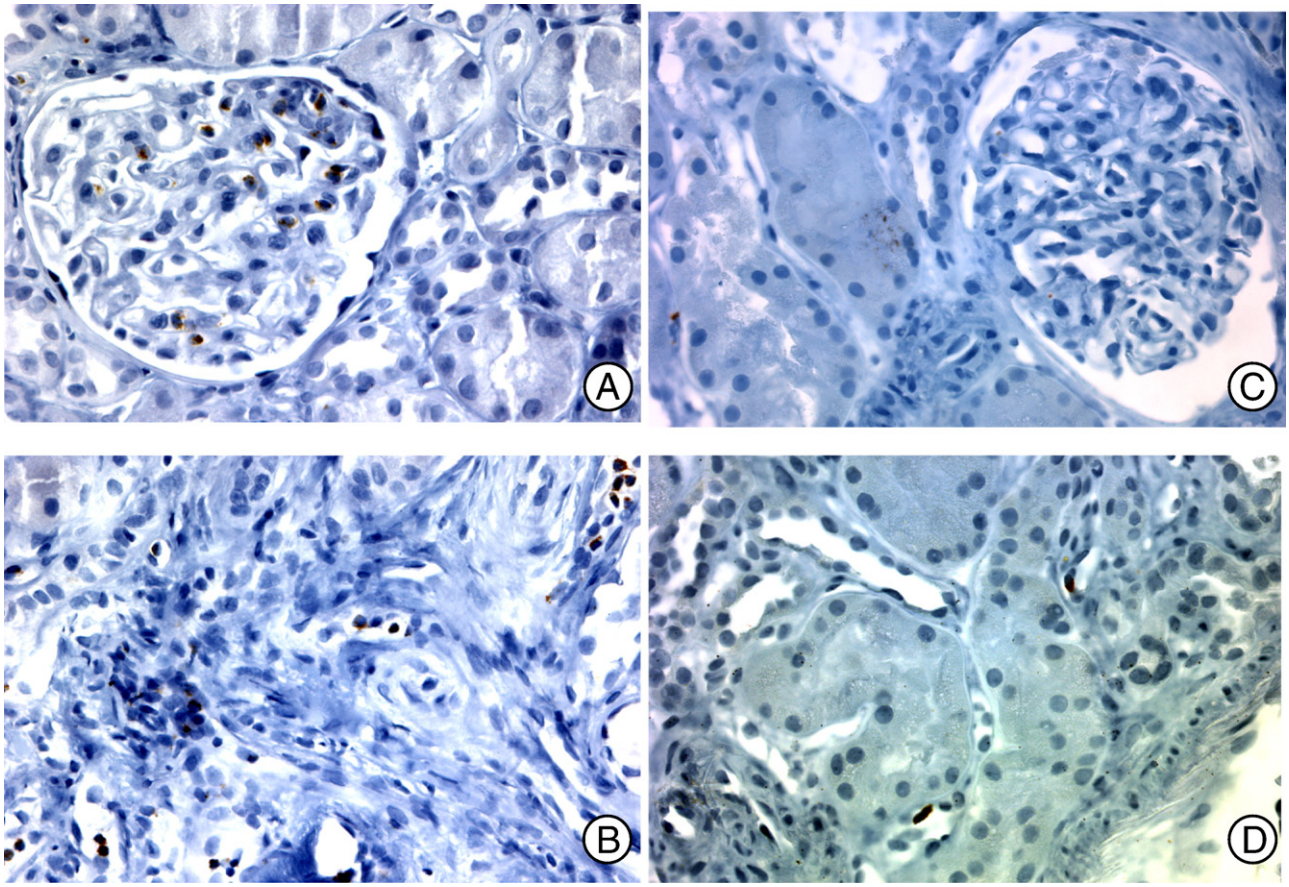

Fig. 2 A, A biopsy with transplant glomerulitis showing that a large number of intraglomerular leukocytes are granzyme-B$^+$. B, The same biopsy as in panel A showing numerous granzyme-B$^+$ cells in the peritubular capillaries and an occasional cell in the interstitial compartment. C, A biopsy without glomerulitis showing absence of granzyme-B$^+$ cells in the glomeruli. D, The same biopsy as in panel C showing sparse granzyme-B$^+$ cells in the peritubular capillaries (C4d immunostain, original magnification ×400). A color version of this figure can be viewed in the online version of this article on the journal's website at http://www.humanpathol.com).

4. Discussion

Transplant glomerulitis is a manifestation of allograft injury and is characterized by glomerular endocapillary inflammatory infiltrate. It was initially attributed to cytomegalovirus infection [10]. Later on, with the declining incidence of cytomegalovirus, transplant glomerulitis has been increasingly linked to acute rejection [5,7]. We recently

Table 3 The diagnostic significance of PTC-VWF staining

PTC-VWF		AMR (diagnostic/suspicious)	TCMR	Graft failure within 2 y
Histologic patterns	Minimal (n = 1)	0/1 (0%)	0/1 (0%) Borderline (n = 1)	0/1 (0%)
	Focal (n = 10)	2/10 (20%)	4/10 (40%) [1B (n = 3), 1A (n = 1)] Borderline (n = 6)	3/7 (43%)
	Diffuse (n = 28)	11/28 (39%)	20/28 (71%) [2B (n = 1), 2A (n = 2), 1B (n = 6), 1A (n = 11)] Borderline (n = 8)	7/17 (41%)
VWF spill	No interstitial-VWF (n = 31)	8/31 (26%)	19/31 (61%) [2B (n = 1), 1B (n = 4), 1A (n = 14)] Borderline (n = 12)	6/19 (32%)
	Interstitial-VWF (n = 8)	5/7 (63%) [a]	5/8 (63%) [2A (n = 2), 1B (n = 3)] Borderline (n = 3)	4/6 (67%)

NOTE. Follow-up information on 2-year postbiopsy graft failure was available for 25 patients (1 with minimal VWF, 7 with focal VWF, and 17 with diffuse VWF including 6 with spilling of VWF into the interstitium). Abbreviations: AMR, antibody-mediated rejection; TCMR, T-cell mediated rejection; PTC, peritubular capillary; VWF, Von Willebrand factor.

[a] $P = .03$ (Diagnostic/suspicious for AMR); interstitial *vs* no interstitial-VWF (Fisher exact test).

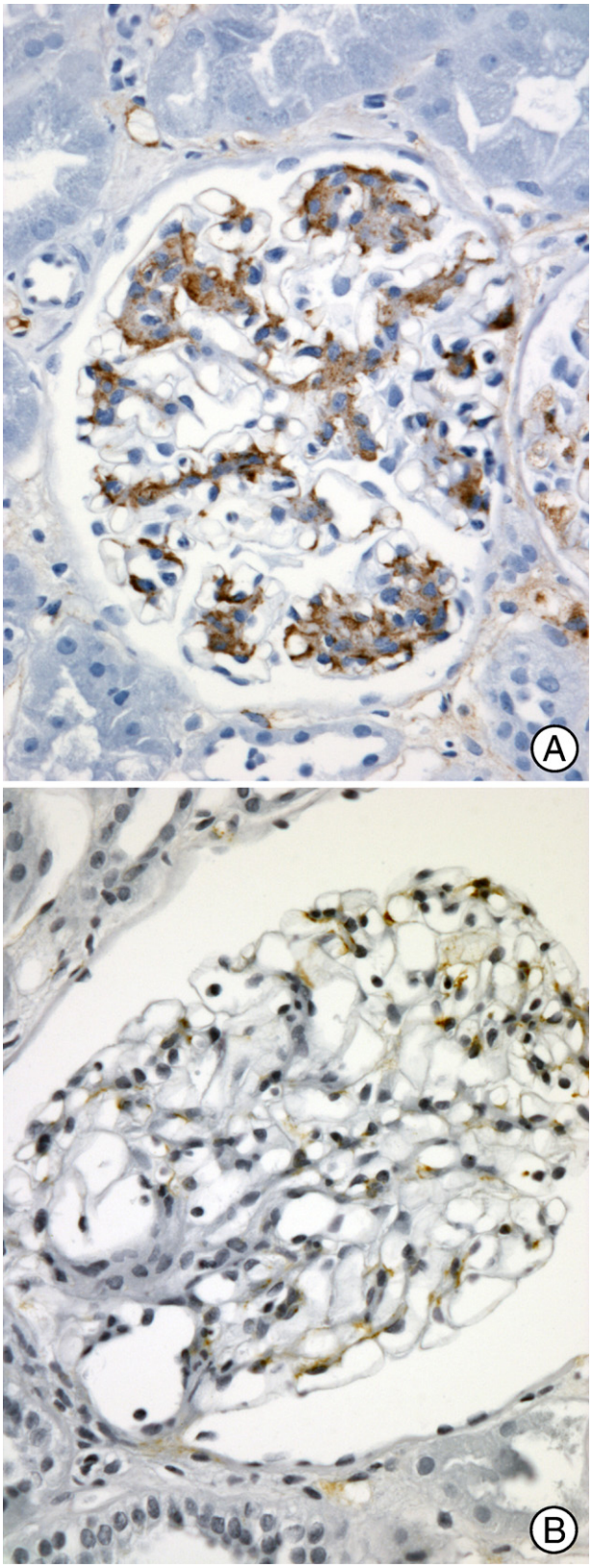

Fig. 3 A, A biopsy with transplant glomerulitis showing staining for VWF in glomerular capillary wall and the mesangium. B, A second biopsy with glomerulitis: VWF staining is seen only in the glomerular capillary loops (VWF immunostain, original magnification ×400).

Table 4 Relationship between PTC C4d and VWF scores and the presence of circulating donor-specific antibodies

		PTC-VWF score (0-3)	PTC-C4d (0-3)
DSA at biopsy	DSA+ (n = 6)	2.8 ± 0.4	2.3 ± 0.8 [a]
	DSA− (n = 24)	2.7 ± 0.5	1.0 ± 1.0
DSA anytime	DSA+ (n = 13)	2.7 ± 0.5	1.9 ± 1.0 [b]
	DSA− (n = 26)	2.7 ± 0.5	0.7 ± 0.9

NOTE> PTC-VW and PTC-C4d staining were semiquantitatively graded as negative (0), minimal (1), focal (2), and diffuse (3) when PTC staining was absent, and present in less than 10%, 10% to 50%, or more than 50% of the stained tissue available for evaluation, respectively. Abbreviations: PTC, peritubular capillary; VWF, Von Willebrand factor; DSA, circulating donor-specific antibody.

[a] $P = .009$ PTC-C4d scores: DSA + *vs* DSA-ve at the time of biopsy (Mann-Whitney).

[b] $P = .001$ PTC-C4d scores: DSA + *vs* DSA-ve any time posttransplantation (Mann-Whitney).

showed that higher grades of transplant glomerulitis are associated with proteinuria, peritubular capillaritis, PTC-C4d, suboptimal response to antirejection therapy, subsequent development of TGP and DSA, and worse graft survival [4]. In the current study, we noted that samples with glomerulitis had a more frequent incidence of mixed rejection (TCMR and AMR) compared with samples lacking glomerulitis.

Studies on the immunopathologic profile of transplant glomerulitis are limited. Magil and coworkers [1] showed that intraglomerular lymphocytes are more prominent in TCMR, whereas monocytes predominate in AMR. We sought to further understand the pathogenesis of transplant glomerulitis using a battery of immunoperoxidase stains. A systematic discussion of our findings with appropriate references to the literature follows.

The first point of interest to us was whether complement activation participates in the development of transplant glomerulitis. Using polyclonal C4d staining on paraffin-embedded tissue, some [11] but not all [12] investigators suggested that GC-C4d deposition is specific for TGP. In our experience, although GC-C4d is frequently observed in biopsies with TGP, it can also be detected in other glomerular lesions (eg, ischemic glomerulopathy, lupus nephritis, membranous nephropathy, and antiglomerular basement membrane glomerulonephritis) [13-14]. In the current study, higher GC-C4d scores were observed in biopsies with versus without glomerulitis. This difference tended to persist even when samples showing TGP were excluded. Furthermore, GC-C4d scores correlated with PTC-C4d scores and with detectable DSA. Nevertheless, global GC-C4d by itself failed to predict significantly worse prognosis in biopsies with glomerulitis. These data suggest that glomerular endothelial injury in transplant glomerulitis is at least partly provoked by antibody-mediated activation of the complement pathway.

VWF is a large protein stored in the platelets as well as in the endothelial Weibel-Palade bodies. Reidy et al [15] described an increase in the expression of endothelial-VWF staining after endothelial injury. Accumulating data suggest

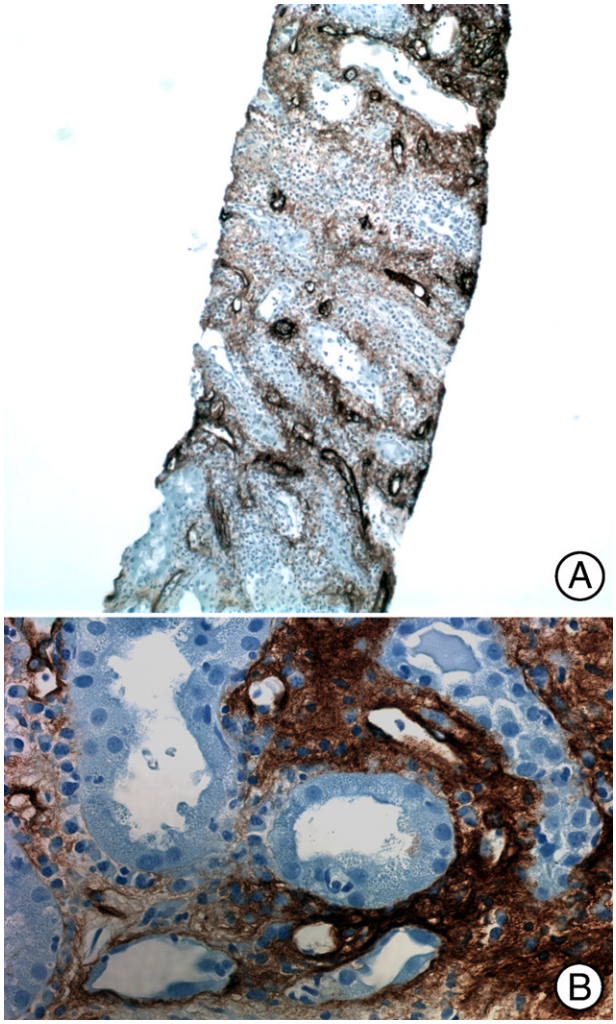

Fig. 4 Diffuse PTC-VWF staining with spill into the interstitium in a patient showing transplant glomerulitis. The patient had severe acute allograft injury associated with mixed TCMR (Banff grade 2A) and acute AMR (A: VWF immunostain, original magnification ×100; B: VWF immunostain, original magnification ×400).

that endothelial transcripts are more sensitive markers for AMR than PTC-C4d and that VWF is the strongest individual endothelial transcript up-regulated in AMR [16-17]. This prompted us to evaluate the expression of VWF in parallel with that of C4d. In contrast to PTC-C4d, PTC-VWF scores failed to correlate with DSA or peritubular capillaritis. Nevertheless, an extreme pattern of VWF staining characterized by diffuse strong PTC-VWF and spilling of VWF into the interstitium was identified in 8 biopsies. This pattern was associated with a higher peritubular capillaritis score and higher incidence of AMR and tended to have more frequent incidence of graft failure when compared with samples without interstitial-VWF. Similarly, higher mesangial, but not GC-VWF scores, were observed in samples with glomerulitis. Such mesangial-VWF scores correlated with glomerulitis score and tended to be associated with worse prognosis. One prior study has described prominent mesangial-VWF in a subset of renal allograft biopsies with intimal arteritis and intraglomerular monocyte infiltration [18]. In primates, mesangial-VWF has been noted in association with chronic rejection and TGP [19]. We contend that severe microcirculation endothelial injury leads to interstitial and/or mesangial-VWF staining by spilling of stored VWF from the endothelium or by extravasation of platelets from the circulation.

Granzyme-B is a protease that can be stored and released from the granules of $CD8^+$ cytotoxic lymphocytes and natural killer cells. It is believed to play a major role in cell-mediated killing. A recent study demonstrated an increase in Granzyme-B mRNA in patients with TCMR as well as in patients with chronic AMR [20]. Another study described an association between Granzyme-B and TGP [21]. In the current study, we assessed the potential participation of the granzyme-perforin pathway in the pathology of transplant glomerulitis. Compared with biopsies lacking glomerulitis, those with glomerulitis had increased absolute number ($P < .001$) and tended to have higher percentages ($P = .1$) of intraglomerular Granzyme-B^+ leukocytes suggesting a total and a selective increase in Granzyme-B^+ leukocytes. This was accompanied by a higher number of interstitial, mainly PTC, Granzyme-B^+ cells. The latter correlated with the score of peritubular capillaritis but not interstitial inflammation. Furthermore, higher numbers of intraglomerular and interstitial Granzyme-B^+ leukocytes correlated significantly with higher immune cell function values. Nevertheless, the possibility that the observed increase in Granzyme-B^+ leukocytes is the result rather than the cause of tissue injury cannot be excluded by our data.

The number of apoptotic cells is increased during acute rejection [22]. It was, therefore, of interest to study apoptotic-related markers in biopsies with transplant glomerulitis. We evaluated the Fas–Fas-L and Bcl-2 family pathways, which are associated with programmed cell death. Fas-L can induce apoptosis in a target cell upon binding to Fas-receptor. In the current study, only rare intraglomerular Fas-L^+ leukocytes were appreciated. This finding argues against a major role of intraglomerular leukocyte–Fas-L in the pathogenesis of transplant glomerulitis. However, it is known that immunohistochemistry is not as sensitive as molecular techniques to detect Fas-L expression. On the other hand, we detected segmental GC–Fas-L only in 3 patients, none of whom developed graft failure on follow-up. The sample size is too small to draw a meaningful conclusion about a potential graft-protecting effect of endothelial Fas-L expression. With regard to TEP–Fas-L staining, Fas-L score correlated weakly with each of Bcl-Xl and Bcl-2 scores but was similar in biopsies with versus without glomerulitis. In addition to TEP cytoplasmic/membranous staining, our biopsy material frequently showed nuclear TEP–Fas-L staining, the significance of which is not clear.

One final objective of this study was to evaluate whether expression of antiapoptotic markers could identify a state of

accommodation. In vitro experiments suggest that low-dose class I DSA can up-regulate Bcl-XL [23], which can down-regulate caspases and promote endothelial cell survival [24-26]. In our biopsy material, GC–Bcl-XL was only occasionally observed in a setting that was not clinically or histologically consistent with accommodation. TEP–Bcl-XL scores were similar in samples with or without glomerulitis and did not correlate with interstitial inflammation or fibrosis. Taken together, our findings do not justify performing routine Bcl-XL staining in renal allograft biopsies. On reviewing a previous study where GC–Bcl-XL was linked to accommodation [26], only 1 of 3 of patients with GC–Bcl-XL had a functioning graft after a follow-up of 33 months, whereas one developed graft failure at 44 months and another lacked long follow-up (7 months).

Our experience with Bcl-2 as an antiapoptotic marker was also disappointing. Using formalin-fixed tissue, we could not detect convincing endothelial staining. The frequency of Bcl-2$^+$ leukocytes was similar in the presence or absence of glomerulitis. Three samples with glomerulitis that also showed podocyte-Bcl-2 had concurrent focal segmental glomerulosclerosis and had or subsequently developed TGP on follow-up. Nakopoulou et al [27] described podocyte–Bcl-2 near glomerular sclerotic lesions and proposed a possible association with chronic rejection. Suzuki et al [28] found TEP–Bcl-2 to be protective against ischemia/reperfusion injury and subsequent development of interstitial fibrosis. However, we did not find a difference in TEP–Bcl-2 scores in samples with versus without glomerulitis, and we did not observe any correlation between TEP–Bcl-2 scores and the Banff scores for interstitial inflammation or fibrosis.

In conclusion, our study demonstrates that biopsies with glomerulitis are associated with a more frequent incidence of "mixed" rejection (TCMR and AMR) compared with biopsies without glomerulitis. Complement deposition in glomerular capillaries correlates with DSA and appears to play a role in endothelial glomerular injury. In addition, increased Granzyme-B$^+$ leukocytes in microcirculation suggest that cell-mediated cytotoxicity may also participate in endothelial damage. VWF expression was associated with severe microcirculatory injury, but larger studies are needed to confirm its role as a potential prognostic marker. No immunohistochemical markers of accommodation could be validated.

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References

[1] Magil AB. Infiltrating cell types in transplant glomerulitis: relationship to peritubular capillary C4d deposition. Am J Kidney Dis 2005;45:1084-9.

[2] Magil AB, Tinckam K. Monocytes and peritubular capillary C4d deposition in acute renal allograft rejection. Kidney Int 2003;63:1888-93.

[3] Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. Kidney Int 2005;68:1866-74.

[4] Batal I, Lunz JG, Aggarwal N, et al. A critical appraisal of methods to grade transplant glomerulitis in renal allograft biopsies. Am J Transplant 2010;10:2442-52.

[5] Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int 1999;55:713-23.

[6] Churg J, Sobin LH. Classification of glomerular diseases. In: Churg J, Sobin LH, editors. Renal disease; classification and atlas of glomerular diseases. 1st ed. Tokyo, Japan: Igaku-Shoin Ltd; 1982. p. 6-7.

[7] Racusen LC, Colvin RB, Solez K, et al. Antibody-mediated rejection criteria—an addition to the Banff 97 classification of renal allograft rejection. Am J Transplant 2003;3:708-14.

[8] Kim LH, Nadarajah VS, Peh SC, et al. Expression of Bcl-2 family members and presence of Epstein-Barr virus in the regulation of cell growth and death in classical Hodgkin's lymphoma. Histopathology 2004;44:257-67.

[9] Strater J, Mariani SM, Walczak H, et al. CD95 ligand (CD95L) in normal human lymphoid tissues: a subset of plasma cells are prominent producers of CD95L. Am J Pathol 1999:193-201.

[10] Richardson WP, Colvin RB, Cheeseman SH, et al. Glomerulopathy associated with cytomegalovirus viremia in renal allografts. N Engl J Med 1981;305:57-63.

[11] Sijpkens YW, Joosten SA, Wong MC, et al. Immunologic risk factors and glomerular C4d deposits in chronic transplant glomerulopathy. Kidney Int 2004;65:2409-18.

[12] Seemayer CA, Gaspert A, Nickeleit V, et al. C4d staining of renal allograft biopsies: a comparative analysis of different staining techniques. Nephrol Dial Transplant 2007;22:568-76.

[13] Batal I, Girnita A, Zeevi A, et al. Clinical significance of the distribution of C4d deposits in different anatomic compartments of the allograft kidney. Mod Pathol 2008;21:1490-8.

[14] Batal I, Chalasani G, Wu C, et al. Deposition of complement product C4d in anti-glomerular basement membrane glomerulonephritis. Am J Kidney Dis 2009;53:1098-101.

[15] Reidy MA, Chopek M, Chao S, et al. Injury induces increase of von Willebrand factor in rat endothelial cells. Am J Pathol 1989;134:857-64.

[16] Sis B, Jhangri GS, Bunnag S, et al. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. Am J Transplant 2009;9:2312-23.

[17] Sis B, Halloran PF. Endothelial transcripts uncover a previously unknown phenotype: C4d-negative antibody-mediated rejection. Curr Opin Organ Transplant 2010;15:42-8.

[18] Ozdemir BH, Sar A, Haberal M. The importance of glomerular deposits of von Willebrand factor in human renal allografts. Ren Fail 2006;28:315-3121.

[19] Lagoo AS, Buckley PJ, Burchell LJ, et al. Increased glomerular deposits of von Willebrand factor in chronic, but not acute, rejection of primate renal allografts. Transplantation 2000;70:877-86.

[20] Ashton-Chess J, Dugast E, Colvin RB, et al. Regulatory, effector, and cytotoxic T cell profiles in long-term kidney transplant patients. J Am Soc Nephrol 2009;20:1113-22.

[21] Homs S, Mansour H, Desvaux D, et al. Predominant Th1 and cytotoxic phenotype in biopsies from renal transplant recipients with transplant glomerulopathy. Am J Transplant 2009;9:1230-6.

[22] Porter CJ, Ronan JE, Cassidy MJ. fas-fas-ligand antigen expression and its relationship to increased apoptosis in acute renal transplant rejection. Transplantation 2000;69:1091-4.

[23] Jin YP, Fishbein MC, Said JW, et al. Anti-HLA class I antibody–mediated activation of the PI3K/Akt signaling pathway and induction

of Bcl-2 and Bcl-xL expression in endothelial cells. Hum Immunol 2004;65:291-302.

[24] Bach FH, Ferran C, Candinas D, et al. Accommodation of xenografts: expression of "protective genes" in endothelial and smooth muscle cells. Transplant Proc 1997;29:56-8.

[25] Bach FH, Ferran C, Hechenleitner P, et al. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med 1997; 3:196-204.

[26] Salama AD, Delikouras A, Pusey CD, et al. Transplant accommodation in highly sensitized patients: a potential role for Bcl-xL and alloantibody. Am J Transplant 2001;1:260-9.

[27] Nakopoulou L, Stefananki K, Papadakis J, et al. Expression of bcl-2 oncoprotein in various types of glomerulonephritis and renal allografts. Nephrol Dial Transplant 1996;11:997-1002.

[28] Suzuki C, Isaka Y, Shimizu S, et al. Bcl-2 protects tubular epithelial cells from ischemia reperfusion injury by inhibiting apoptosis. Cell Transplant 2008;17:223-9.