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ORIGINAL ARTICLE

Solid phase detection of C4d-fixing HLA antibodies to predict rejection in high immunological risk kidney transplant recipients

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Summary

Protocols for recipient desensitization may allow for successful kidney transplantation across major immunological barriers. Desensitized recipients, however, still face a considerable risk of antibody-mediated rejection (AMR), which underscores the need for risk stratification tools to individually tailor treatment. Here, we investigated whether solid phase detection of complement-fixing donor-specific antibodies (DSA) has the potential to improve AMR prediction in high-risk transplants. The study included 68 sensitized recipients of deceased donor kidney allografts who underwent peritransplant immunoadsorption for alloantibody depletion (median cytotoxic panel reactivity: 73%; crossmatch conversion: $n = 21$). Pre and post-transplant sera were subjected to detection of DSA-triggered C4d deposition ([C4d]DSA) applying single-antigen bead (SAB) technology. While standard crossmatch and [IgG]SAB testing failed to predict outcomes in our desensitized patients, detection of preformed [C4d]DSA ($n = 44$) was tightly associated with C4d-positive AMR [36% vs. 8%, $P = 0.01$; binary logistic regression: odds ratio: 10.1 (95% confidence interval: 1.6–64.2), $P = 0.01$]. Moreover, long-term death-censored graft survival tended to be worse among [C4d]DSA-positive recipients ($P = 0.07$). There were no associations with C4d-negative AMR or cellular rejection. [C4d]DSA detected 6 months post-transplantation were not related to clinical outcomes. Our data suggest that pretransplant SAB-based detection of complement-fixing DSA may be a valuable tool for risk stratification.

Introduction

Recipient sensitization to HLA antigens poses a risk of allograft rejection and reduces the chance of finding a matched donor [1,2]. Several desensitization protocols were shown to shorten waiting times and prevent antibody-mediated rejection (AMR) [3–7]. However, despite intense anti-humoral treatment, desensitized patients may still face a substantial risk of rejection [8].

One strategy to improve transplant outcomes may be the use of innovative diagnostic tools to guide organ allocation and anti-humoral treatment. In recent years, the serological repertoire has been refined by cell-independent single-antigen bead (SAB) assays for detailed analysis of individual HLA antibody patterns [9]. SAB-based detection of IgG-type donor-specific antibodies (DSA) may help predict crossmatch outcomes [10,11] and assess individual immunological risks [12–14]. However, the diagnostic value of

SAB tests in immunological high-risk recipients is controversially discussed [5,15,16]. In a recent study of sensitized kidney transplant recipients who underwent peritransplant immunoadsorption (IA) for HLA alloantibody depletion, we found that SAB-based DSA detection failed to predict immunological outcomes [5].

A promising strategy to enhance the predictive accuracy of SAB technology may be to assess the ability of DSA to trigger deleterious complement activation. In earlier work, we have established a flow cytometry-based assay which allows for solid phase detection of HLA antibody-triggered complement (split) product deposition [17]. This assay principle was recently applied to Luminex-based bead array technology for identification of complement-fixing DSA [18–21].

Here, we investigated whether SAB-based detection of C4d-fixing DSA ([C4d]DSA) has the potential to predict antibody/complement-triggered rejection in sensitized kidney transplant recipients subjected to IA-based desensitization. Our study included a cohort of 68 desensitized patients, in whom conventional antibody detection techniques, including complement-dependent cytotoxicity crossmatch (CDCXM) testing, have failed to predict immunological complications [5].

Patients and methods

Study patients

The study population consisted of 68 broadly sensitized recipients of deceased donor kidney allografts (transplantation at the Medical University Vienna between January 1999 and December 2008) who had been subjected to peritransplant IA for HLA antibody depletion [5], a protocol approved by the ethics committee of the Medical University Vienna (baseline data: see Table 1). Before desensitization, included subjects had 73% median complement-dependent cytotoxicity (CDC) reactivity [interquartile range (IQR): 56–86%]. Patients were followed up for 9 years (median, IQR: 6–11).

Recipient desensitization and immunosuppression

The protocol of peritransplant IA has been detailed elsewhere [5]. In brief, recipients with a CDC-panel reactivity $\geq 40\%$ were subjected to a single IA session immediately before transplantation. Patients with a positive CDCXM that could be rendered negative after IA treatment of 6 l plasma were allowed to proceed to transplantation. Following transplantation, all patients continued to receive IA every 1–3 days until stabilization of kidney function or graft loss, respectively. Recipients received pre-emptive therapy with a depleting anti-lymphocyte antibody or an IL-2 receptor antibody. Maintenance immunosuppression consisted of cyclosporin A or tacrolimus, mycophenolate mofetil or azathioprine, and steroids.

Serological analysis

According to our standard, nonenhanced CDCXM was performed against unseparated donor mononuclear cells

Table 1. Baseline recipient data in relation to the results of pretransplant [C4d] DSA detection.

Parameters	Overall cohort ($n = 68$)	Pretransplant [C4d]DSA		*P* value*
		Positive ($n = 44$)	Negative ($n = 24$)	
Baseline characteristics including classical risk factors for allosensitization				
Recipient age (years), median (IQR)	45 (37–54)	45 (37–52)	45 (37–56)	0.7
Female recipient gender, *n* (%)	26 (38)	15 (34)	11 (46)	0.3
Time on dialysis (years), median (IQR)	2.6 (1.9–4)	2.6 (1.8–4)	2.6 (2–4.2)	0.99
Recipients of a retransplant, *n* (%)	65 (96)	42 (96)	23 (96)	0.9
Number of prior transplants, median (IQR)	2 (2–3)	2 (2–3)	2 (2–3)	0.3
Prior pregnancies, *n* (% of female recipients)	15 (58)	8 (53)	7 (63)	0.7
Number of pregnancies, median (IQR)	1 (0–3)	1 (0–3)	1 (0–2)	0.96
Donor age (years), median (IQR)	42 (26–52)	41 (24–53)	44 (26–52)	0.9
Cold ischemia time (hours), median (IQR)	18 (13–22)	18 (13–20)	18 (12–23)	0.9
Immunological baseline data				
HLA mismatch in A,B, and DR, median (IQR)	3 (2–3)	3 (2–3)	3 (1–4)	0.2
CDC-panel reactivity (%), median (IQR)	73 (56–86)	70 (53–92)	77 (67–82)	0.6
CDCXM positives, *n* (%)	21 (31)	16 (36)	5 (21)	0.2
% virtual C4d-panel reactivity, median (IQR)	84 (68–97)	93 (75–98)	75 (39–91)	0.001
Pretransplant [IgG]DSA, *n* (%)	51 (75)	39 (89)	12 (50)	<0.001
[IgG]DSA number (DSA^+ samples), median (IQR)	2 (1–3)	2 (1–3)	1 (1–2)	0.8
MFI maximum of detected DSA, median (IQR)	2466 (1757–4277)	2623 (1808–4099)	2139 (1274–4887)	0.5

CDCXM, complement-dependent cytotoxicity crossmatch; DSA, donor-specific antibody; IQR, interquartile range.
**P* values are given for comparisons between [C4d]DSA-positive and -negative patient groups.

with dithiotreitol (DTT) to exclude IgM reactivities. For assessment of CDC-panel reactivity, sera were tested on a panel of mononuclear cells obtained from 30 to 50 phenotyped donors.

The HLA single-antigen reactivities were assessed on a Luminex platform applying LABScreen kits (One Lambda, Canoga Park, CA, USA). For detection of IgG-type DSA ([IgG]DSA) untreated sera were assayed according to the manufacturer's protocol. For selected experiments, sera were incubated with DTT (0.005 mol/l) to abrogate the prozone effect. For detection of IgM-type DSA the protocol was modified by using a phycoerythrin-conjugated anti-human IgM antibody (1:50 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) (22). For IgG and IgM detection, test thresholds were defined (i) according to a mean fluorescence intensity (MFI) above 500 and (ii) according to negative control bead binding and the results obtained with five nonbinding negative control sera, respectively [8].

Antibody-triggered C4d fixation to HLA-coated SAB was assessed following an earlier described protocol [13]. In brief, beads were incubated with serum for 30 min at 4 °C followed by 30 min incubation with alloantibody-negative serum obtained from a nonsensitized healthy male volunteer (complement source). Beads were washed and incubated for 30 min at 4 °C with DyLight 549-labeled anti-C4d polyclonal antibody (BI-RC4D; Biomedica, Vienna, Austria). For definition of test thresholds [5], MFI detected for each antigen-coated test bead was corrected for C4d binding to no-antigen control beads (normalized MFI: MFI test bead/MFI control bead). A test result was considered positive if the normalized MFI was >2× (mean of normalized MFI of five negative control sera plus three standard deviations). For our experiments, we calculated a median calculated cutoff score of 8 (IQR: 5–12).

Non-normalized MFI values obtained with [C4d]SAB testing were generally lower than those observed with [IgG] SAB tests. For our study samples, the median MFI detected on no-antigen control beads was 3 (IQR: 2–6). Moreover, median MFI values reported for five negative control sera detected on HLA-coated test beads were constantly below 5. A comparably lower level of absolute MFI was also observed for results above test thresholds. A representative test run of 44 different patient samples, which included a total of 8052 single bead reactions, revealed a median MFI of 215 (IQR: 75–405) for C4d-positive beads ($n = 1180$), while for negative single reactions a median MFI of 6 (IQR: 3–9) was computed.

Virtual HLA classes I and II [C4d] panel reactivities were calculated for a representative European donor population using a software tool from the Eurotransplant website (www.eurotransplant.eu).

Diagnosis and treatment of AMR

Forty-eight recipients were subjected to at least one indication biopsy, 38 within the first 3 months. Overall, 110 biopsies (one to five biopsies per patient) were performed. Biopsy specimens were stained for C4d on paraffin sections applying a polyclonal rabbit anti-C4d reagent (Biomedica). C4d positivity was defined as a linear staining along at least a quarter of peritubular capillaries (PTC) [22]. Acute and chronic active AMR were diagnosed and classified according to recent updates of the Banff classification [23,24]. We also documented specimens showing C4d deposits without any AMR-typical morphological features, as well as C4d-negative specimens showing morphological criteria suggesting acute [glomerulitis (Banff score, ag ≥ 1), ptc ≥ 1, severe intimal arteritis (v = 3), capillary microthrombi], and/or chronic AMR [chronic glomerulopathy (cg > 0)].

Episodes of acute C4d-positive AMR occurred early after transplantation [median of 14 days (IQR: 10–17 days)], and in most cases graft dysfunction could be reversed by continuation of serial IA treatment and, in case of concomitant cellular rejection, high-dose steroids and depleting antibody therapy. Patients with C4d-negative AMR features did not receive additional anti-humoral treatment. Four recipients with refractory early acute C4d-positive AMR, three with biopsy-proven thrombotic microangiopathy, one with mixed cellular and AMR, did not respond to treatment and lost their graft. Four patients lost their grafts because of chronic active C4d-positive AMR diagnosed after 16–76 months post-transplantation. In these patients no specific anti-humoral treatment was applied.

Statistical analyses

Chi-squared and Fisher's exact test were used to compare categories. For comparison of nonparametric data, the Mann–Whitney *U*-test was applied. Kaplan–Meier analysis was used to calculate patient and graft survival, and the Mantel Cox log-rank test was applied to compare cumulative survival between groups. We used binary logistic regression to examine the effect of preformed [C4d]DSA on C4d-positive AMR occurrence. Baseline variables were entered into the model if they were suggested to be risk factors of AMR (female gender, number of transplants, history of pregnancy, number of HLA mismatches, positive CDCXM, and number of [IgG]DSA). The results of the model are presented as the odds ratio and the 95% confidence interval (CI). We tested for interaction between baseline variables by means of multiplicative interaction terms and log likelihood ratio chi-squared tests. The linearity of the logit assumption was checked for continuous predictor variables and an analysis of residuals was performed. Regression diagnostics and overall model-fit were

performed according to standard procedures. To assess the goodness of fit we used the Hosmer-Lemeshow test. A two-sided *P*-value <0.05 was considered as statistically significant. Statistical calculations were performed using SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

The study population consisted of 68 broadly sensitized kidney transplant recipients subjected to peritransplant IA for HLA antibody elimination (baseline characteristics: see Table 1). Patients had 73% median CDC-panel reactivity (IQR: 56–86%). In 21 recipients a positive CDCXM was rendered negative by a single pretransplant IA session.

Forty-four recipients (65%) had pretransplant (and pre-IA) C4d-fixing DSA ([C4d]DSA). Twelve patients showed [C4d]DSA against both HLA class I and II antigens, 14 against HLA class I, and 18 against HLA class II antigen only.

As shown in Table 1, [C4d]DSA-positive and -negative patients did not differ regarding clinical baseline variables. Preformed [C4d]DSA did not significantly associate with

Table 2. Clinical outcomes in relation to the presence or absence of [C4d] DSA.

	Pretransplant [C4d]DSA		
Parameters	Positive (n = 44)	Negative (n = 24)	*P* value
C4d-positive AMR, *n* (%)	16 (36)	2 (8)	0.01
Acute, *n* (%)	13 (30)	2 (8)	0.04
Banff type I, *n*	6	1	
Banff type II, *n*	7	1	
Additional features of cellular rejection, *n*	1	2	
Chronic active, *n* (%)*	4 (9)	0	0.1
C4d without morphological AMR features†, *n* (%)	2 (5)	0 (0)	0.3
Morphological AMR features† without C4d, *n* (%)	7 (16)	4 (17)	0.9
T-cell-mediated rejection, *n* (%)	7 (16)	5 (21)	0.6
Banff type I, *n*	3	0	
Banff type II, *n*	4	5	
Delayed graft function‡, *n* (%)	19 (43)	11 (46)	0.7
% Death-censored graft survival§			0.07
1 year	79	87	
3 years	75	82	
5 years	72	82	
% Patient survival¶			0.7
1 year	98	92	
3 years	95	88	
5 years	88	83	
Serum creatinine (mg/dl)**, median (IQR)			
1 year	1.6 (1.3–2.7)	1.5 (1.2–1.8)	0.2
3 years	1.7 (1.2–5.0)	1.4 (1.1–4.2)	0.3
5 years	1.8 (1.3–5.0)	1.5 (1.1–5.0)	0.2
Urinary protein excretion (g/l)††, median (IQR)			
1 year	<0.05 (<0.05–0.1)	<0.05 (<0.05–0.05)	0.1
3 years	<0.05 (<0.05–0.2)	<0.05 (<0.05–0.1)	0.6
5 years	0.07 (<0.05–0.4)	<0.05 (<0.05–0.3)	0.3

AMR, antibody-mediated rejection; DSA, donor-specific antibodies; IQR, interquartile range.

*One of the patients with documented chronic active AMR was diagnosed for acute C4d-positive AMR early after transplantation. All patients diagnosed for chronic active AMR lost their graft upon follow-up.

†Morphological features of AMR included glomerulitis (Banff score ag $\geq$ 1), transplant glomerulopathy (cg $\geq$ 1), severe intimal arteritis (v = 3), ptc $\geq$ 1, and/or the finding of capillary microthrombi.

‡Delayed graft function was defined as the need for dialysis within the first post-transplant week.

§Causes of graft loss were acute and/or chronic active AMR (n = 8; seven of these losses were recorded in the [C4d]DSA-positive patient group), non-specified chronic graft dysfunction (n = 6), postoperative vascular thrombosis without rejection features (n = 5), or crescentic glomerulonephritis (n = 2).

¶Causes of death were unknown (n = 3), disseminated cancer (n = 2; gastric cancer and melanoma), accident (n = 2), postoperative hemorrhagic shock after bleeding of the peri-graft region (n = 1), intra-cerebral hemorrhage (n = 1), hyperkalemia (n = 1) or cardiovascular (n = 1).

**Recipients who returned to dialysis were assumed as having a serum creatinine of 5 mg/dl and included in nonparametric statistical analysis.

††Patients on dialysis were excluded from analysis of urinary protein excretion.

HLA mismatch or CDC-panel reactivity. [C4d]DSA-positive recipients, however, had significantly higher [C4d]-panel reactivity and were more often [IgG]DSA-positive (Table 1). Remarkably, five patients were found to be [C4d]DSA-positive but [IgG]DSA-negative. While none of these subjects had IgM-type DSA, one of them turned [IgG]DSA-positive after serum treatment with DTT, suggesting interference by the prozone effect in this specific case (data not shown). There was also a considerable disagreement between the results of CDCXM and [C4d]DSA detection. Five patients were CDCXM- and [IgG]DSA-positive but [C4d]DSA-negative. Such assay discrepancies were not because of differences regarding [IgG]DSA results. CDCXM-positive/[C4d]DSA-positive and CDCXM-positive/[C4d]DSA-negative samples showed a comparable sum of [IgG]DSA MFI values [median 5349 (IQR: 3234–7614) vs. 6515 (992–19 991); $P > 0.99$] or [IgG]DSA number [median of 2 (range 1–5) in either group], respectively.

Impact of pretransplant C4d-fixing DSA on kidney transplant outcomes

In contrast to current CDCXM and/or preformed [IgG]DSA [5], preformed [C4d]DSA were tightly associated with clinical C4d-positive AMR (Table 2). While 16 (36%) of the [C4d]DSA-positive recipients developed C4d-positive acute and/or chronic active AMR, this was observed for only two (8%) of the [C4d]DSA-negative patients ($P = 0.01$). Associations of preformed [C4d]DSA with C4d-positive AMR were found for both CDCXM-positive (occurrence of AMR episodes in 4 of 16 [C4d]DSA-positive versus none of 5 [C4d]DSA-negative subjects; $P = 0.21$) and CDCXM-negative patients (AMR in 12 of 28 [C4d]DSA-positive versus two of 19 [C4d]DSA-negative subjects; $P = 0.017$). In multivariate analysis, detection of preformed [C4d]DSA turned out to be an independent risk factor for C4d-positive AMR (odds ratio: 10.1; 95% CI 1.6–64.2, $P = 0.01$).

Acute C4d-positive AMR occurred significantly more often among [C4d]DSA-positive patients (13 of the 15 documented cases), and all four recipients diagnosed with chronic active AMR (one case preceded by an episode of acute C4d-positive AMR) were [C4d]DSA-positive before transplantation (Table 2).

Despite a strong relationship between preformed [C4d]DSA and C4d-positive AMR, a considerable number of [C4d]DSA-positive patients ($n = 28$) did not experience clinical AMR. In an attempt to define distinct qualitative properties of C4d-fixing reactivity that could further improve risk assessment, we performed a subsequent analysis focusing on the group of [C4d]DSA-positive recipients. As shown in Table 3, [C4d]DSA-positive patients with or without C4d-positive AMR did not differ regarding [IgG]DSA number or binding strength, virtual [C4d]-panel reactivity, number and HLA class specificity of [C4d]DSA, C4d binding intensity, or the extent of [C4d]DSA depletion upon pre-operative IA, respectively.

We identified two recipients in whom biopsies revealed capillary C4d deposits without any morphological AMR features. They both tested [C4d]DSA-positive (Table 2). In addition, 11 (17%) recipients were diagnosed as having clinical C4d-negative AMR. Remarkably, this type of rejection was not associated with preformed [C4d]DSA (Table 2). Similarly, comparative analyses did not reveal significant differences regarding T-cell-mediated rejection rates, delayed graft function, or allograft function, protein

Table 3. Alloreactivity patterns among [C4d]DSA-positive recipients in relation to acute and/or chronic C4d-positive AMR.

	[C4d]DSA-positive recipients ($n = 44$)		
Parameters	C4d-positive AMR ($n = 16$)	No C4d-positive AMR ($n = 28$)	*P* value
Pretransplant virtual C4d-panel reactivity	94 (80–98)	89 (68–99)	0.4
Pretransplant [C4d]DSA			
Number of targeted antigens, median (IQR)	1 (1–3)	1 (1–2)	>0.99
C4d binding intensity (MFI*), median (IQR)	129 (22–382)	311 (80–848)	0.08
HLA class I reactivity, *n* (%)	8 (50)	18 (64)	0.4
HLA class II reactivity, *n* (%)	12 (75)	18 (64)	0.5
Persistence of [C4d]DSA after pretransplant IA†, *n* (%)	1/3 (33)†	6/14 (43)†	>0.99
CDCXM positives, *n* (%)	4 (25)	12 (43)	0.2
Pretransplant [IgG]DSA, *n* (%)	14 (88)	25 (89)	0.9
[IgG]DSA number (DSA^+ samples), median (IQR)	2 (1–3)	2 (1–4)	>0.99
MFI maximum of detected DSA, median (IQR)	2772 (1860–3891)	2473 (1800–4822)	0.7

AMR, antibody-mediated rejection; CDCXM, complement-dependent cytotoxicity crossmatch; DSA, donor-specific antibodies; IA, immunoadsorption; IQR, interquartile range; MFI, mean fluorescence intensity.

*For each patient the sum of MFI of detected DSA was calculated.

†Serum samples obtained after processing of 6 l patient plasma were available for 17 initially [C4d]DSA-positive patients.

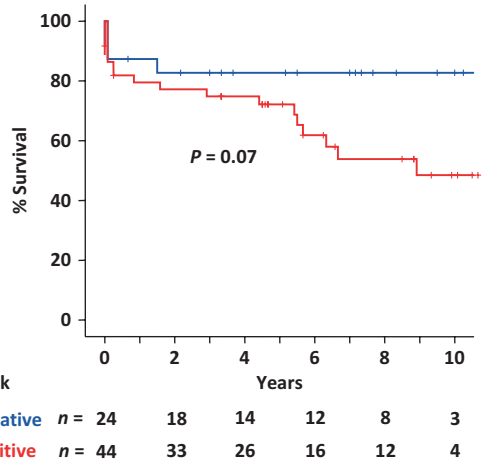

Figure 1 Pretransplant C4d-fixing donor-specific antibodies (DSA) and kidney allograft survival. Kaplan–Meier analysis of death-censored graft survival is shown in relation to the presence of preformed [C4d]DSA. The Mantel Cox log-rank test was used to compare survival rates between groups.

excretion, and patient survival at years 1, 3, and 5, respectively (Table 2). However, as shown in Table 2 and Fig. 1, there was a trend towards worse death-censored graft survival among [C4d]DSA-positive patients.

Impact of post-transplant C4d-fixing DSA on kidney transplant outcomes

For 51 patients with a functioning allograft, serum samples collected 6 months after transplantation were available for retrospective analysis. Of 35 pretransplant [C4d]DSA-positive patients, 21 turned [C4d]DSA-negative, while 14 subjects showed persistent [C4d]DSA. *De novo* C4d-fixing reactivity to beads bearing donor HLA antigens was found in six of the 16 initially [C4d]DSA-negative patients. There were no differences between post-transplant [C4d]DSA-positive and [C4d]DSA-negative recipients regarding rates of C4d-positive or C4d-negative AMR, cellular rejection, delayed graft function, serum creatinine, protein excretion, or graft and patient survival, respectively (Table 4). Remarkably, none of the six patients who developed *de novo* C4d-fixation experienced clinical AMR (not shown).

Discussion

In search of risk stratification tools to predict AMR in high immunological risk transplant recipients, we here examined the predictive value of SAB-based detection of C4d-fixing HLA antibodies in the context of apheresis-based desensitization. A key finding was that, in contrast to standard CDC or SAB assays, [C4d]DSA detection was associated with antibody/complement-triggered rejection. Observed associations remained significant also in a multivariate model and were independent of the results of conventional serological assays including CDCXM. Our data are supportive of the idea that *in vitro* complement detection is a valuable tool to identify harmful (complement-activating) alloantibodies [18–20]. Based on our study results, it is tempting to speculate that *in vitro* C4d detection could be a useful marker to identify recipients who may benefit from additional preemptive treatment, such as measures targeting complement activation [25].

In line with earlier studies [10], we found only weak concordance between SAB and CDCXM results. Assay

Table 4. Clinical outcomes in relation to the presence or absence of post-transplant [C4d]DSA detected after 6 months.

Parameters	Post-transplant [C4d]DSA		*P* value
	Positive (n = 22)	Negative (n = 29)	
C4d-positive AMR, *n* (%)	5 (23)	9 (31)	0.5
Acute, *n* (%)	3 (14)	8 (28)	0.2
Chronic active, *n* (%)	2 (9)	1 (4)	0.4
C4d without morphological AMR features*, *n* (%)	0 (0)	2 (7)	0.2
Morphological AMR features* without C4d, *n* (%)	4 (19)	6 (21)	0.8
T-cell-mediated rejection, *n* (%)	2 (9)	7 (24)	0.2
Delayed graft function†, *n* (%)	9 (41)	17 (59)	0.2
5-year death-censored graft survival, %	85	85	0.4
5-year patient survival, %	91	86	0.5
5-year serum creatinine (mg/dl)‡, median (IQR)	1.6 (1.3–4.8)	1.7 (1.2–2.4)	0.7
5-year urinary protein excretion (g/l)§, median (IQR)	0.07 (0.05–0.2)	0.06 (<0.05–0.5)	0.8

AMR, antibody-mediated rejection; DSA, donor-specific antibodies; IQR, interquartile range.
*Morphological evidence of AMR was defined as described in Table 2.
†Delayed graft function was defined as the need for dialysis within the first post-transplant week.
‡Patients who returned to dialysis were assumed as having a serum creatinine of 5 mg/dl for inclusion in nonparametric statistical analysis.
§Recipients on dialysis were excluded from analysis of protein excretion.

discrepancies may be explained by false-positive CDC results because of non-HLA antibody or autoantibody binding [26]. Conversely, the use of unseparated lymphocytes for CDC testing may have led to false-negative results, e.g. because of impaired detection of HLA class II reactivities on B cells. Moreover, interpreting test results, methodological differences regarding detection of complement activation have to be taken into account (SAB-based analysis of an early step of human classical complement activation versus detection of rabbit complement-triggered cytolysis via the final common path). Finally, one may argue that solid phase complement-fixation assays using bead array technology do not account for interactions of multiple DSA against different HLA antigens expressed on target cells. Our finding that CDCXM-positive but [C4d]DSA-negative samples did not differ from dual-positive samples regarding MFI sum or number of [IgG]DSA, however, may argue against a primary role of such technical drawbacks.

In line with our previous data [13], many [C4d]DSA-negative subjects tested positive with [IgG]SAB testing, which may reflect detection of less relevant noncomplement-activating reactivities. However, there were also some patients who were [IgG] or [IgM]DSA-negative but [C4d]DSA-positive. Considering that complement-fixation may not only be a function of IgG binding strength but also of IgG subclass patterns [27], it is tempting to speculate that very low levels of complement-fixing IgG subclasses, undetectable by conventional [IgG]SAB technology, may have been sufficient to trigger significant C4d deposition. One alternative explanation could be false-low or -negative [IgG]SAB results because of the prozone effect [28,29]. In a recent study, Schnaidt *et al.* [29] found that this *in vitro* artifact may be caused by blockade of IgG detection by high levels of C1 attached to densely bound antibody. Even though speculative, this could at least partly explain earlier data suggesting a limited predictive value of standard SAB-based IgG alloantibody detection just among immunized recipients with excessive levels of preformed antibody [5,15]. In a re-evaluation of sera with DTT to counteract the prozone effect, one of the [C4d]DSA-positive/[IgG]DSA-negative samples was indeed found to contain strong IgG-type donor reactivity.

[C4d]DSA-positive and [C4d]DSA-negative recipients considerably differed with respect to capillary C4d deposits in indication biopsies, a well-established marker of deleterious intragraft complement activation [30]. In contrast, no difference was observed regarding C4d-negative AMR, suggesting that our assay principle may not be helpful in predicting complement-independent rejection processes. There are now several reports suggesting that C4d-negative rejection may pose a considerable risk of chronic injury and subsequent graft loss [31–34]. Moreover, experimental studies have revealed distinct candidate mechanisms underlying complement-independent graft injury, including direct effects of alloantibody binding to endothelial cells or damage through interaction with NK cells [35,36].

An important observation was that a considerable proportion of patients having preformed [C4d]DSA did not experience clinical rejection. Without protocol biopsies, a role of subclinical rejection in our patients remains speculative. In search of serological parameters for further improvement of risk stratification, we were unable to identify additional serological parameters synergizing the prognostic relevance of [C4d]DSA detection.

Remarkably, in patients with a functioning graft, post-transplant assaying for C4d fixation did not reveal any associations with clinical outcomes. It was also a striking finding that, as earlier observed for [IgG]DSA detection [5], none of the patients with *de novo* C4d fixation were diagnosed as having clinical AMR. Numerous experimental and clinical studies have suggested that in recipients with functioning allografts the presence of circulating DSA may reflect an early stage of rejection preceding graft injury and dysfunction occurring at a later time [1,2,37]. However, there is also some evidence suggesting that in selected recipients such reactivity might also occur without affecting long-term clinical outcomes [38,39]. This phenomenon was described to be more common among high-risk patients subjected to recipient desensitization, and it was speculated that this could reflect a role of transplant accommodation triggered by transient lowering of alloantibody levels [40–42].

A major limitation of our study is the lack of protocol biopsies. Accordingly, in our patients, the significance of subclinical AMR and its relation to preformed [C4d]DSA will remain unanswered. Indeed, recent studies have shown the frequent occurrence of subclinical AMR features in high-risk recipients, even after intense pre-emptive antihumoral therapy, and there is evidence suggesting a tight association between subclinical microcirculation injury, subsequent chronic injury and inferior long-term graft survival [43–46].

Our present study, included a distinct cohort of broadly sensitized patients subjected to desensitization, in whom conventional serological assays failed to predict rejection. Previous studies investigating the impact of complement-fixation assays in 'standard' patients have revealed controversial results. Hönger *et al.* [47] reported that detecting the C4d-fixing ability of 'low level' [IgG]DSA failed to enhance the predictive accuracy of SAB tests in CDCXM-negative patients. In contrast, in a study of heart allograft recipients, such reactivity was found to strongly associate with graft survival [18].

In our present study, *in vitro* complement activation was determined by detecting the early complement

activation step of C4 cleavage and C4 split product deposition. An alternative solid phase test principle, also first described by our working group [17,27], may be the detection of C1q deposition, a critical initial complement activation step preceding C4 activation. C1q detection has recently been modified for Luminex-based single-antigen testing using exogenous recombinant C1q [21], and detection of C1q-fixing DSA on SAB was found to be predictive of rejection and graft loss [20,48]. An advantage over C4d detection may be that the C1q assay may in general produce higher levels of fluorescence intensity [21]. Nevertheless, considering a direct functional interrelationship between C1 attachment and subsequent C4 activation, one may expect a tight concordance between the results of C1q- and C4d-fixation SAB assays. Indeed, applying modified FlowPRA screening in a cohort of 66 presensitized transplant candidates, we have earlier described highly significant correlations between C4d- and C1q-fixing panel reactivities [27]. However, correlations between the results of C1q and C4d SAB tests in relation to clinical outcomes or crossmatch results, respectively, have not yet been systematically evaluated. Considering qualitative differences between IgG1 and IgG3 subclasses regarding C1q binding and C4 activation [49], one may speculate that differential patterns of IgG subclasses could cause discrepant assay results and in such cases the combined use of different complement-fixation assays could improve the predictive accuracy of SAB testing.

Our present study was restricted to the detection of anti-HLA alloantibodies. Accordingly, additional clinical relevance of (complement-fixing) non-HLA reactivity in our patients cannot be excluded. For assessment of such reactivity, several assay kits are meanwhile available, including Luminex-based detection of alloantibodies against major histocompatibility complex class I-related chain A antigens [50]. Future studies will have to clarify whether detecting their complement-activating ability has the potential to improve the predictive accuracy of pretransplant serology.

In summary, our study demonstrates that detection of complement-fixing DSA may help identify patients at particular risk for complement-mediated AMR. Future trials will be needed to clarify whether adjustments of desensitization protocols on the basis of SAB-based complement detection, e.g. by implementing measures that directly block complement-triggered injury [25], could further improve transplant outcomes.

Authorship

GB, MW, HR, and GAB: designed study, performed study, collected data, analyzed data, wrote the paper. ES, ŽK, WHH, FM, and GJZ: analyzed data, wrote the paper. CW and MH: analyzed data.

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