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ORIGINAL ARTICLE

Clinical relevance of preformed C4d-fixing and non-C4d-fixing HLA single antigen reactivity in renal allograft recipients

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Summary

Donor-specific alloantibodies (DSA), especially those fixing complement, may pose a particular immunologic risk to transplant recipients. To assess the clinical impact of C4d- or non-C4d-fixing (IgG) HLA sensitization, pretransplant sera obtained from 338 kidney allograft recipients prescreened by FlowPRA were retrospectively evaluated by Luminex single antigen (SA) testing using a novel fluorescent-labeled anti-C4d reagent for detection of antibody-triggered C4d deposition in addition to IgG binding. Recipients with [IgG]DSA ($n = 39$) showed a substantially higher rate of C4d positive rejection (33%) than 16 patients with [IgG] non-DSA (0%) or 283 antibody-negative patients (4%, multivariate analysis excluding retransplantation because of high co-linearity: $P < 0.0001$), and adversely affected 5-year death-censored graft survival (74% vs. 81% and 90%, respectively, multivariate model: $P < 0.05$). [C4d] DSA ($n = 21$) and [C4d] non-DSA ($n = 25$) increased rates of C4d positive rejections to a similar extent (24% and 28% vs. 4% in recipients without C4d-fixing reactivity; multivariate analysis: $P \leq 0.002$) with a trend towards adverse 5-year graft survival (76% and 76% vs. 90%; $P \leq 0.2$). In conclusion, Luminex-based characterization of HLA sensitization may be a useful strategy for risk stratification. Possibly as a result of intensified immunosuppression in presensitized recipients, identification of C4d-fixing DSA was not associated with a further increase of rejection and graft loss rates.

Introduction

Humoral presensitization is well known to pose a risk of allograft rejection and loss [1]. For many years, cell-based assays, i.e. standard complement-dependent cytotoxicity (CDC) testing and flow cytometry crossmatching (FCXM), have been the mainstay of serum analysis [2]. However, more recently, highly sensitive and specific solid phase HLA antibody tests have been developed in an attempt to accurately assess of individual immunologic risks in cytotoxic negative crossmatch (XM) kidney transplants. A critical advance has been the establishment of techniques using color-coded microspheres coated with defined single HLA or non-HLA antigens [3–5]. A major benefit of such technologies, which use flow cytometry or, more recently, the Luminex platform, may be that, without need for donor cells, they allow for identification and characterization of alloantibodies directed at individual donor antigens. However, even though many laboratories have now incorporated single antigen (SA) bead testing into routine, the actual clinical impact of preformed IgG donor-specific antibodies (DSA) uncovered by sensitive

solid phase assays, but escaping detection by CDC and/or FCXM, is still under discussion [6–11]. Considering the important role of the complement system as a key player in the process of allograft rejection [12,13], a clue to better distinguish between more and less harmful HLA presensitization could be to determine the ability of detected alloantibodies to activate the classical complement pathway [14,15]. For differentiation between complement- and noncomplement-fixing alloreactivities, we have established a protocol allowing for detection of human C4d complement split product deposition to FlowPRA screening beads coated with multiple HLA antigens ([C4d] FlowPRA screening) [16]. Applying this assay system, preformed C4d-fixing panel reactivity was found to be strongly associated with the occurrence of C4d positive antibody-mediated rejection (AMR) and inferior kidney allograft survival [17,18]. However, in this previous study, we did not specify targeted HLA antigens at a single HLA antigen level and were thus not able to evaluate if DSA were present or absent. Recently, Smith *et al.* [19] have adopted our flow cytometric protocol of [C4d] FlowPRA screening to Luminex-based SA testing for identification of C4d-fixing DSA. In a large cohort of heart transplant patients, they reported 80% graft loss rates for patients with preformed C4d-fixing DSA, suggesting that probing of C4d-fixing DSA may be useful to identify a patient subgroup at extensive immunologic risk [19].

In this study, we established a simplified Luminex SA protocol for detection of C4d-fixing alloreactivity using a novel fluorescent-labeled anti-C4d reagent for direct immunofluorescence. Re-testing a large cohort of kidney allograft recipients prescreened by FlowPRA [17], we applied standard Luminex SA testing for detection of alloreactive IgG and, in parallel, a modified technique for evaluation of the C4d-fixing ability of anti-HLA reactivities. The major objective of our study was to evaluate the actual clinical impact of IgG type and C4d-fixing Luminex SA reactivity with and without DSA in the setting of kidney allotransplantation.

Patients and methods

Patients

In this study, 338 consecutive adult renal transplant recipients (transplantation at the Medical University of Vienna between January 2001 and December 2002; 303 recipients of a deceased donor allograft) were included. Baseline characteristics have earlier been described in detail [17]. In brief, median recipient age was 52 years (interquartile range [IQR]: 41–60). Moreover, 119 (35%) recipients were female, 67 (20%) patients had a history of prior transplantation, and 87 (26%) were presensitized as suggested by a CDC-PRA ≥10%. Median HLA mismatch (A, B, DR) was three (IQR: 2–4), cold ischemia time 12 h (median, IQR: 7–17), and donor age 48 years (median, IQR: 39–59). The majority of study patients received calcineurin inhibitor-based maintenance immunosuppression. One hundred and seven patients (32%) received antibody induction with an anti-IL-2 receptor monoclonal antibody or a polyclonal anti-lymphocyte antibody. Twenty-eight broadly sensitized recipients of a deceased donor allograft were subjected to a previously detailed protocol of peritransplant immunoadsorption (IA) [20]. In 10 recipients subjected to this protocol, a positive current CDC-XM had been converted to negative by a single pretransplant IA session. All other recipients had a negative CDC-XM at the time of transplantation. Patient follow-up (until March 2008) was 77 months (median, IQR: 70–83).

Biopsies

Allograft biopsies were performed for renal dysfunction after exclusion of toxic calcineurin inhibitor levels or postrenal and prerenal causes of graft dysfunction. All biopsy specimens were prospectively stained for C4d applying a polyclonal rabbit anti-C4d reagent (BI-RC4D; Biomedica, Vienna, Austria) as previously described [21]. Biopsies were considered C4d positive in case of linear endothelial C4d deposition in at least a quarter of cortical peritubular capillaries. Cell-mediated rejection was diagnosed and classified according to the Banff '97 classification [22]. Morphologic lesions suggestive of antibody-mediated rejection (AMR) were classified according to a revision to the Banff '97 classification [23].

DyLight™ 549-conjugated C4d polyclonal antibody

Polyclonal anti-C4d antibody, BI-RC4D (Biomedica), was labeled with DyLight™ 549 applying a standard protocol. An IgG fraction devoid of nitrogen containing buffer substances was incubated for 1 h with DyLight™ 549 NHS Ester (Pierce Biotechnology, Rockford, IL, USA) at 22 °C in the dark. The reaction was stopped by incubation with an equal volume of 1 M Tris/HCl buffer (pH 6.8) for another hour, and conjugated antibody was finally dialysed against phosphate-buffered saline.

Staining efficiency of DyLight™ 549-labeled anti-C4d antibody was evaluated in direct comparison with the same anti-C4d reagent conjugated with fluorescein isothiocyanate (FITC) (Biomedica). Applying a previously detailed FlowPRA-based protocol [17], for in vitro C4d deposition, nonfluorescent FlowPRA HLA class I screening beads were incubated with patient sera known to contain C4d-fixing anti-HLA class I alloreactivity. C4d-coated beads were then incubated with saturating concentrations

of DyLight™ 549- or FITC-conjugated anti-C4d antibody. As assessed by flow cytometry, staining of beads with the two reagents revealed comparable mean fluorescence intensities (FI) and percent panel reactivities (data not shown).

Luminex-based detection of IgG and C4d-fixing HLA antibodies

For Luminex-based SA analysis of IgG and C4d-fixing anti-HLA alloreactivities, pretransplant sera obtained from 71 out of the 338 study patients, which had previously been shown to test positive by FlowPRA screening [17], were re-tested by LABScreen® Single Antigen assays (HLA class I: LABScreen® Single Antigen HLA Class I Antibody Detection Test – Combi; HLA class II: LABScreen® Single Antigen HLA Class II Antibody Detection Test – Group 1; One Lambda, Canoga Park, CA, USA). IgG anti-HLA reactivities ([IgG] Luminex) were evaluated according to the manufacturer's protocol. In brief, Luminex SA beads were incubated with undiluted sera in 96-well plates for 30 min at room temperature. After washing, phycoerythrin (PE)-conjugated goat anti-human IgG antibody (One Lambda) was added for another 30 min. For detection of C4d-fixation we adopted the protocol of [C4d] FlowPRA to Luminex application ([C4d] Luminex). Beads were incubated with undiluted serum for 30 min at 4 °C followed by 30 min incubation with an excess of nonbinding serum obtained from a nonsensitized healthy male volunteer as complement source. Beads were washed and incubated with DyLight™ 549-labeled pretitered anti-C4d polyclonal antibody at saturating concentration (30 min, 4 °C). For data acquisition, a LABScan™ 100 flow analyzer (Luminex Corporation, Austin, TX, USA) was used.

For both [IgG] and [C4d] Luminex SA testing, thresholds for each anti-HLA reaction were determined according to average binding of negative control sera obtained from five nonsensitized healthy male volunteers. First, FI detected for each HLA-coated bead (HLAb) was corrected for binding to control no-antigen beads (NCb) according to the following formula: FI control serum HLAb/FI control serum NCb. For each bead, we then calculated the mean and standard deviation (SD) of corrected control serum reactivities. Analysing reactivities of patient sera, a test result was considered positive if (FI patient serum HLAb/FI patient serum NCb) was $>2 \times$ (mean of corrected binding of the five control sera plus three standard deviations).

Statistical methods

For univariate comparisons, chi-squared tests and Kruskal–Wallis tests were used, as appropriate. Multivariate logistic regression models were applied to determine the independent effects of pretransplant serology on C4d positive rejection rates. Results are presented as the odds ratio (OR) and the 95% confidence interval (CI). Kaplan–Meier analysis was used to calculate graft survival and the Mantel Cox Log-rank test was applied to compare survival between groups. A Cox proportional hazards model was applied to assess the effect of serologic results on graft survival adjusting for potential confounders. Results are given as the hazard ratio (HR) and the 95% CI. A two-sided *P*-value <0.05 was considered as statistically significant. Statistical calculations were performed using SPSS for Windows, version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Luminex single antigen detection of anti-HLA reactivity with or without C4d-fixing ability

Pretransplant sera obtained from 338 consecutive kidney transplant recipients were prescreened applying [IgG] FlowPRA HLA class I and/or II screening [17]. Recipients with flow panel reactivity ≥10% ($n = 71$) were re-evaluated by Luminex SA testing.

Evaluating 71 FlowPRA positive samples, a total of 9448 Luminex (HLA class I and II) single bead reactions were analysed. At a SA level, IgG binding was detected for 1960 single beads. Thirty-eight percent of IgG positive beads ($n = 753$) turned out to fix detectable amounts of C4d, whereas, this was only the case for 1.7% of the 7888 IgG negative beads ($n = 132$).

Applying Luminex SA testing, 55 of the 71 [IgG] FlowPRA positive patients (78%) were found to have detectable reactivity against one or more defined HLA class I and/or II antigens (Table 1). C4d-fixing Luminex SA

Table 1. Luminex SA testing of 71 [IgG] FlowPRA screening positive recipients.

	[IgG]FlowPRA positive ($n = 71/338$)	
Pretransplant serology	[IgG]Luminex	[C4d]Luminex
Patients with positive test result, *n*	55	46
Positive HLA class I SA beads, median *n* (IQR)	29 (15–44)	11 (3–28)
Positive HLA class II SA beads, median *n* (IQR)	13 (7–30)	9 (4–20)
Patients with DSA, *n*	39	21
DSA against 1 HLA antigen	31	16
DSA against 2 HLA antigens	4	5
DSA against 3 HLA antigens	3	0
DSA against 4 HLA antigens	1	0

DSA, donor-specific alloreactivity; IQR, interquartile range; SA, single antigen.

reactivity was uncovered in 46 of the [IgG] FlowPRA positive patients (65%). For only three of these patients no alloreactive IgG was detected. Median numbers of SA beads targeted by sera containing SA reactivity are listed in Table 1.

Comparing single bead reactivities with reported donor HLA antigens (HLA A, B, C, DR, DQ), 39 of the 55 [IgG] SA positive (71%) and 21 of the 46 [C4d] SA positive recipients (46%) were found to have detectable reactivity against one or more mismatched graft HLA-class I and/or II antigens ([IgG] or [C4d] DSA, respectively). In four of the 21 patients with [C4d] DSA no [IgG] DSA could be identified. In Table 1, numbers of donor antigens targeted by alloreactive sera are listed.

Pretransplant [IgG] Luminex single antigen reactivity and allograft outcomes

According to the results of two-step solid phase testing (FlowPRA prescreening and subsequent Luminex SA testing of FlowPRA positives), three patient groups were defined: [IgG] DSA patients (SA positive recipients with donor-specific reactivity, $n = 39$), [IgG] non-DSA patients (SA positive recipients without donor-specific reactivity, $n = 16$), and a [IgG] neg group (recipients negative by FlowPRA screening and a subgroup of 16 SA negative patients with ≥10% FlowPRA reactivity, where negative Luminex HLA antibody testing was considered to indicate false positive FlowPRA screening, $n = 283$).

[IgG] DSA and [IgG] non-DSA patients showed a higher rate of ≥10% pretransplant CDC-PRA reactivity and retransplantation than [IgG] neg patients (Table 2). On the basis of broad CDC-PRA reactivity, 21 (54%) of the [IgG] DSA and five (31%) of the [IgG] non-DSA recipients had been subjected to peritransplant IA for desensitization. Applying this protocol, among patients with [IgG] DSA, 10 patients had been transplanted after successful conversion of a positive CDC-XM. All other patients were CDC-XM negative already before initiation of IA treatment. Within the other two patient groups, all recipients had a negative current CDC crossmatch (Table 2).

Table 2. [IgG] Luminex SA test results – patient characteristics and graft outcomes.

Patient groups	[IgG]DSA ($n = 39$)	[IgG] non-DSA ($n = 16$)	[IgG]neg ($n = 283$)	*P*-value
Immunologic risk factors				
Retransplantation, *n* (%)	30 (77)	8 (50)	29 (10)	<0.001
HLA MM, median, IQR	3 (3–4)	2 (1–3)	3 (2–4)	0.02
Female gender, *n* (%)	16 (41)	9 (56)	94 (33)	0.13
CDC-PRA ≥10%, *n* (%)	31 (80)	7 (44)	49 (17)	<0.001
Peritransplant IA, *n* (%)	21 (54)	5 (31)	2 (1)	<0.001
CDC-XM conversion, *n*	10	0	0	
Clinical outcomes				
C4d positive rejection, *n* (%)	13 (33)	0 (0)	10 (4)	<0.0001
Cell-mediated rejection, *n* (%)	8 (21)	2 (13)	59 (21)	0.7
5-year censored survival	74%	81%	90%	0.004
5-year overall graft survival	69%	75%	79%	0.27

CDC, cell-dependent cytotoxicity; IA, immunoadsorption; PRA, panel reactive antibody; XM, crossmatch.

Twenty-three of the 338 patients (7%) were found to experience C4d positive acute graft dysfunction within the first 6 months. C4d-positive rejection usually occurred early after transplantation (median 14 days, IQR: 8–20 days). All but one of the C4d positive indication biopsies showed typical morphologic features suggestive of AMR. As shown in Table 2, as many as 13 of the 39 [IgG]DSA recipients (33%) developed C4d positive rejection. Conversely, among [IgG] non-DSA recipients, no episode of C4d positive rejection was reported. Among [IgG] neg recipients only 4% ($n = 10$) developed C4d positive rejection (Table 2). In univariate analysis, differences between patient groups were highly significant (Table 2). To test the independent effects of pretransplant serology, logistic regression analysis was applied. In this model, baseline variables imbalanced ($P < 0.2$) between patients with and without C4d deposition (cold ischemia time) or presumed to be potential risk factors for capillary C4d deposition (female gender, HLA mismatch) were considered. Retransplantation was not included because of its high co-linearity with serologic results ($P < 0.0001$). Multivariate analysis revealed a highly significant independent effect of [IgG] DSA (OR: 12.2 [95% CI: 4.6–33]; $P < 0.0001$).

Death-censored graft survival turned out to be worst in [IgG] DSA patients ($P = 0.004$; Fig. 1, Table 2). In a multivariate model (Cox regression analysis) including female gender, recipient age, donor age, cold ischemia time, donor type (living versus deceased donor), and HLA mismatch as potential confounders (retransplantation as a highly co-linear variable), the effect of [IgG]DSA on death-censored graft survival proved significant (HR: 2.3 [95% CI: 1.1–5.1]; $P = 0.036$).

In a subsequent subanalysis excluding 28 broadly sensitized patients subjected to desensitization by peritransplant IA (310 patients; 18 recipients with [IgG] DSA, 11 with [IgG] SA third party reactivity), we also found significantly higher AMR rates among patients with IgG DSA (28% vs. 0% in patients with [IgG] SA or 3% in patients without IgG reactivity; $P < 0.001$). Notably, in this analysis, no differences in graft survival rates were observed (89% vs. 91% vs. 90% 5-year death-censored graft survival).

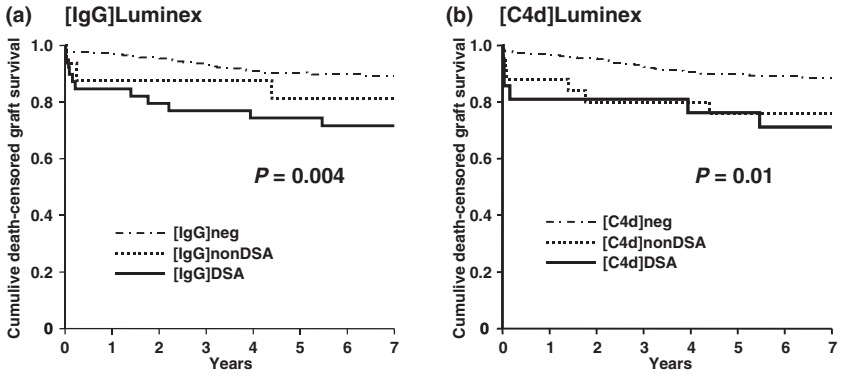

Figure 1 Luminex SA testing and Kaplan–Meier death-censored kidney graft survival. Patient groups were defined according to [IgG]Luminex (a) or [C4d]Luminex SA test results (b) as described in the text.

Pretransplant C4d-fixing Luminex HLA single antigen reactivity and allograft outcomes

For evaluation of the clinical value of [C4d] Luminex SA testing, groups of [C4d] DSA (n = 21) and [C4d] non-DSA patients (n = 25) were evaluated in comparison with a group ([C4d] neg) comprising recipients negative by FlowPRA prescreening or, if FlowPRA-positive, negative by [C4d] Luminex SA testing (n = 292).

As observed for [IgG] SA reactivity, the presence of [C4d] Luminex SA reactivity, with or without [C4d] DSA, was found to be tightly associated with current CDC-PRA reactivity and retransplantation (Table 3). Approximately 90% of patients with [C4d] DSA showed ≥10% CDC-PRA reactivity or had a history of prior transplantation. On the basis of broad CDC sensitization, a high proportion of [C4d] DSA patients, that is 12/21 (57%), had been subjected to peritransplant IA, whereby seven of them had been transplanted after conversion of a positive CDC-crossmatch (Table 3). Moreover, 13 (52%) [C4d] non-DSA patients were desensitized by IA (CDC-crossmatch conversion in three patients).

Table 3. [C4d]Luminex SA test results – patient characteristics and graft outcomes.

Patient groups	[C4d]DSA (n = 21)	[C4d] non-DSA (n = 25)	[C4d]neg (n = 292)	P-value
Immunologic risk factors				
Retransplantation, n (%)	18 (86)	20 (80)	29 (10)	<0.001
HLA MM, median, IQR	3 (2–4)	3 (2–3)	3 (2–4)	0.6
Female gender, n (%)	7 (33)	10 (40)	102 (35)	0.9
CDC-PRA ≥10%, n (%)	19 (91)	19 (76)	49 (17)	<0.001
Peritransplant IA, n (%)	12 (57)	13 (52)	3 (1)	<0.001
CDC-XM conversion, n	7	3	0	
Clinical outcomes				
C4d positive rejection, n (%)	5 (24)	7 (28)	11 (4)	<0.0001
Cell-mediated rejection, n (%)	4 (19)	6 (24)	59 (20)	0.9
5-year censored survival	76%	76%	90%	0.01
5-year overall graft survival	71%	68%	79%	0.28

CDC, cell-dependent cytotoxicity; IA, immunoadsorption; PRA, panel reactive antibody; XM, crossmatch.

C4d positive rejection was frequent among [C4d] Luminex positive patients either with (n = 5; 24%) or without [C4d] DSA (n = 7, 28%). Notably, for all seven rejecting patients within the [C4d] non-DSA group, noncomplement-fixing [IgG] DSA could be identified. As shown in Table 3, associations of detected [C4d]Luminex SA reactivity with C4d positive rejection were highly significant in univariate analysis ($P < 0.0001$). Applying multivariate analysis (logistic regression model) adjusting for the same confounders as described above for analysis of [IgG] Luminex results, both [C4d] DSA and [C4d] non-DSA were found to be independent predictors of C4d positive rejection ([C4d]DSA: OR 10.1 [95% CI: 3.2–31], $P < 0.0001$; [C4d] non-DSA: OR: 7.3 [2.1–25], $P = 0.002$).

Death-censored graft survival rates were significantly lower in [C4d] Luminex SA positive patients than in [C4d] SA negative patients, however, without a difference between patients with and without [C4d] DSA (Fig. 1, Table 3). A multivariate model (Cox regression analysis) excluding retransplantation because of its high co-linearity with serologic results, revealed a trend towards decreased death-censored survival for both [C4d] DSA and [C4d] non-DSA patients ([C4d] DSA: HR 2.4 [95% CI: 0.9–6.0]; $P = 0.07$; [C4d] non-DSA: HR 1.9 [0.7–4.9]; $P = 0.2$).

In a subanalysis excluding the 28 patients subjected to peritransplant IA (310 patients; nine patients with [C4d] DSA, 12 with [C4d] SA reactivity), we observed a trend towards higher rates of C4d-positive dysfunction among patients with [C4d] DSA (11%) and [C4d] SA reactivity (17%; patients without C4d-fixing reactivity: 4%), but no difference with respect to 5-year graft survival (89% and 92% vs. 90%).

Finally, the data were analysed to compare between patients with C4d-fixing DSA ($n = 21$; four of them were [IgG] DSA negative) and patients with non-C4d-fixing [IgG] DSA ($n = 22$). Both subgroups had significantly higher AMR rates [five of 21 (24%) and nine of 22 recipients (41%), respectively] than the group of patients without preformed donor-specific reactivity (3%; $P < 0.001$]. Moreover, recipients with preformed DSA, either with or without C4d-fixing capability, had inferior death-censored graft survival (76% vs. 68%, respectively, vs. 90% 5-year graft survival in DSA-negative patients; $P < 0.001$). Notably, for patients with C4d-fixing DSA, there was apparently no evidence for a further increase of AMR or graft loss rates.

Discussion

In this study, we have set up a new protocol of Luminex-based detection of C4d-fixing HLA reactivity applying a fluorescent-labeled anti-C4d antibody for direct IF. At the level of single HLA antigens, the Luminex platform enabled us to distinguish between donor-specific and non-donor-specific HLA antibodies, with or without complement-fixing ability. Our initial hypothesis was that, in extension of our previous observation of inferior graft outcomes in patients with preformed C4d-fixing panel reactivity [17], assessment of C4d-fixing DSA could further distinguish a patient subgroup at particular immunologic risk. Indeed, applying a similar protocol of modified Luminex-based SA detection using an anti-C4d monoclonal antibody for indirect staining; Smith *et al.* [19] have demonstrated a substantial clinical impact of preformed C4d-fixing DSA in a cohort of heart allograft recipients. They reported 20% 1-year graft survival rates for recipients having preformed C4d-fixing DSA, whereas, in absence of DSA, C4d-fixing reactivity did not considerably affect survival [19].

Using our Luminex-based technique for in vitro C4d detection, we observed a tight association between C4d fixation to Luminex beads and the presence of bound IgG, a finding, which is in accordance with our previous results obtained with FlowPRA [17]. Luminex-based analysis of patient sera revealed that <2% of IgG negative single beads were capable to fix C4d. Accordingly, only few patients had C4d reactivity in absence of detectable alloreactive IgG. The occasional finding of C4d positive but IgG negative results may reflect false positive reactions, but could also have its explanation in complement fixation by alloreactive IgM or subthreshold levels of IgG escaping detection.

A major finding of our study was that identification of [IgG] DSA predicted a high rate of C4d positive graft dysfunction and inferior long-term graft survival, an effect which proved significant in multivariate models. In contrast, for patients with non-DSA IgG reactivity, no case of C4d-positive graft dysfunction and survival rates similar to test negative recipients were found. Few previous studies have addressed the impact of [IgG] DSA detected by Luminex on clinical outcomes. Two smaller studies have found increased rejection rates for patients having Luminex [IgG] DSA [6,9], whereas in a third study no such effect was reported [8]. Importantly, reporting overall rejection rates, none of these studies have provided a separate analysis of AMR. Discrepant results among trials may at least in part be caused by inclusion also of antibody-independent rejection episodes. Of note, in our study, cell-mediated rejection, which, in our cohort, was much more common than humoral rejection, was not associated with pretransplant serology. Our finding of a strong association between preformed [IgG] DSA and occurrence of AMR is also supported by two recent studies applying ELISA- or FlowPRA-based DSA detection, where DSA were found to predict AMR and adverse kidney allograft survival [7,24].

In line with our previous results obtained with [C4d] FlowPRA screening [17], in this study, preformed C4d-fixing Luminex SA alloreactivity in general, with or without detectable C4d-fixing DSA, was found to be associated with the occurrence of C4d positive rejection and adverse graft survival. Regarding our observation of a considerable number of C4d-positive rejections also in the group of [C4d]non-DSA patients, it is important to note that in all these cases, (noncomplement-fixing) [IgG] DSA could be identified. This finding strongly reinforces specific diagnostic value of DSA detection for prediction of AMR.

In contrast to a recently published retrospective heart transplant study [19], we were unable to detect a difference in graft outcomes between [C4d] DSA and [C4d] non-DSA recipients. Importantly, there was also no major difference between patients with C4d-fixing and patients with non-C4d-fixing DSA. Indeed, our analysis, which is limited by its retrospective design, did not provide convincing evidence for a major diagnostic advantage of [C4d] Luminex SA testing. A possible explanation for this difference to the heart study [19] could be that many of our [C4d] (and [IgG]) Luminex positive recipients had been subjected to antibody depletion by peritransplant IA, which, according to our local practice, was applied on the basis of broad CDC-PRA reactivity. This and similar protocols have earlier been shown to effectively prevent rejection in broadly sensitized recipients of a deceased donor allograft with or without a positive CDC crossmatch [20,25]. Notably, a substantial number of patients with C4d-fixing DSA have had a positive current CDC crossmatch convertible by a single pretransplant IA

session. Our assumption of a considerable improvement of outcomes in DSA-positive patients by peritransplant IA may be in accordance with a recent study by Akalin *et al.* [26], who demonstrated that peritransplant antibody depletion may substantially decrease the risk of acute antibody-mediated rejection in sensitized patients with high levels of Luminex DSA.

Of note, a separate analysis excluding broadly sensitized patients subjected to peritransplant IA, still failed to reveal differences in AMR rates between patients with [C4d]DSA and recipients with [C4d]SA third party reactivity only. Moreover, exclusion of IA patients did not considerably affect the results of our comparison of C4d-fixing versus non-C4d-fixing DSA. A drawback of such subanalyses, however, may be the evident selection bias of excluding patients with a particularly high immunologic risk (high rate of retransplantation and broad CDC-PRA reactivity), who, according to our routine, had been subjected to IA treatment. Moreover, exclusion of a considerable number of presensitized patients resulted in marked decrease in group sizes and low end point frequencies. This may impede a valid interpretation of statistical results.

In summary, this study reinforces specific diagnostic relevance of solid phase detection of IgG type DSA and supports its use as a tool for pretransplant risk stratification. Despite its inherent limitations (retrospective design; amelioration of outcomes by anti-humoral treatment), the results of the present study advocate the use of (Luminex-based) solid-phase antibody testing for specification of HLA reactivity patterns and, most importantly, identification of donor-specific reactivities. One rationale for the implementation of this technique in clinical routine may be that, in the context of cell-based assays, solid phase DSA detection could help identify patients at increased immunologic risk and guide the implementation of specific anti-humoral desensitization strategies. However, our data also clearly demonstrate that preformed (donor-specific) alloreactivity detected by sensitive Luminex testing does not inevitably cause rejection and inferior graft performance. Using 'supersensitive' antibody testing, it will be a major challenge to define valid detection thresholds, to reliably identify false positive results, and to develop strategies to more precisely predict the actual clinical impact of individual reactivity patterns. In this respect, one potential improvement could be the *in vitro* detection of complement fixation, which may serve as a surrogate of the ability of preformed antibody to trigger complement activation (and C4d deposition) in the microvasculature of the transplanted organ. Using a novel protocol of [C4d] Luminex testing we describe tight associations of preformed C4d-fixing HLA panel reactivity with the occurrence of AMR. However, presumably as a result of amelioration of transplant outcomes by targeted recipient desensitization, for recipients having C4d-fixing DSA, we were unable to demonstrate a further increase of rejection and graft loss rates. Nevertheless, the results of this study which included the development of a Luminex-based technique for direct C4d staining could provide a valuable basis for future (prospective) studies clarifying the actual value of this innovative assay principle.

Authorship

MW, GAB: designed and performed research; and wrote the paper. GB, ME, HR: collected data and performed research. GFK, GFF: collected and analysed data.

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References

1. Cecka JM, Zhang Q, Reed EF. Preformed cytotoxic antibodies in potential allograft recipients: recent data. *Hum Immunol* 2005; **66**: 343.
2. Gebel HM, Bray RA, Nickerson P. Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: contraindication vs. risk. *Am J Transplant* 2003; **3**: 1488.
3. Pei R, Wang G, Tarsitani C, *et al.* Simultaneous HLA Class I and Class II antibodies screening with flow cytometry. *Hum Immunol* 1998; **59**: 313.
4. Pei R, Lee JH, Shih NJ, Chen M, Terasaki PI. Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. *Transplantation* 2003; **75**: 43.
5. Zou Y, Stastny P, Süsal C, Döhler B, Opelz G. Antibodies against MICA antigens and kidney-transplant rejection. *N Engl J Med* 2007; **357**: 1293.
6. Gibney EM, Cagle LR, Freed B, Warnell SE, Chan L, Wiseman AC. Detection of donor-specific antibodies using HLA-coated microspheres: another tool for kidney transplant risk stratification. *Nephrol Dial Transplant* 2006; **21**: 2625.
7. Bielmann D, Honger G, Lutz D, Mihatsch MJ, Steiger J, Schaub S. Pretransplant risk assessment in renal allograft recipients using virtual crossmatching. *Am J Transplant* 2007; **7**: 626.
8. Gupta A, Iveson V, Varagunam M, Bodger S, Sinnott P, Thuraisingham RC. Pretransplant donor-specific antibodies in cytotoxic negative crossmatch kidney transplants: are they relevant? *Transplantation* 2008; **85**: 1200.

9. van den Berg-Loonen EM, Billen EV, Voorter CE, *et al.* Clinical relevance of pretransplant donor-directed antibodies detected by single antigen beads in highly sensitized renal transplant patients. *Transplantation* 2008; **85**: 1086.
10. Vaughan R, Shaw O. How much donor human leukocyte antigen-specific antibody is too much for a renal transplant? *Transplantation* 2008; **85**: 1081.
11. Li X, Ishida H, Yamaguchi Y, Tanabe K. Poor graft outcome in recipients with de novo donor-specific anti-HLA antibodies after living related kidney transplantation. *Transpl Int* 2008; **21**: 1145.
12. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *J Am Soc Nephrol* 2007; **18**: 1046.
13. Wasowska BA, Lee CY, Halushka MK, Baldwin WM III. New concepts of complement in allorecognition and graft rejection. *Cell Immunol* 2007; **248**: 18.
14. Lederer SR, Schneeberger H, Albert E, *et al.* Early renal graft dysfunction. The role of preformed antibodies to DR-typed lymphoblastoid cell lines. *Transplantation* 1996; **61**: 313.
15. Böhmig GA, Bartel G, Wahrmann M. Antibodies, isotypes and complement in allograft rejection. *Curr Opin Organ Transplant* 2008; **13**: 411.
16. Wahrmann M, Exner M, Regele H, *et al.* Flow cytometry based detection of HLA alloantibody mediated classical complement activation. *J Immunol Methods* 2003; **275**: 149.
17. Wahrmann M, Exner M, Schillinger M, *et al.* Pivotal role of complement-fixing HLA alloantibodies in presensitized kidney allograft recipients. *Am J Transplant* 2006; **6**(5 Pt 1): 1033.
18. Bartel G, Wahrmann M, Exner M, *et al.* In vitro detection of C4d-fixing HLA alloantibodies: associations with capillary C4d deposition in kidney allografts. *Am J Transplant* 2008; **8**: 41.
19. Smith JD, Hamour IM, Banner NR, Rose ML. C4d fixing, luminex binding antibodies – a new tool for prediction of graft failure after heart transplantation. *Am J Transplant* 2007; **7**: 2809.
20. Lorenz M, Regele H, Schillinger M, *et al.* Peritransplant immunoadsorption: a strategy enabling transplantation in highly sensitized crossmatch-positive cadaveric kidney allograft recipients. *Transplantation* 2005; **79**: 696.
21. Regele H, Exner M, Watschinger B, *et al.* Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. *Nephrol Dial Transplant* 2001; **16**: 2058.
22. Racusen LC, Solez K, Colvin RB, *et al.* The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; **55**: 713.
23. 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.
24. Lefaucheur C, Suberbielle-Boissel C, Hill GS, *et al.* Clinical relevance of preformed HLA donor-specific antibodies in kidney transplantation. *Am J Transplant* 2008; **8**: 324.
25. Beimler JH, Morath C, Schmidt J, *et al.* Successful deceased-donor kidney transplantation in crossmatch-positive patients with peritransplant plasma exchange and Rituximab. *Transplantation* 2009; **87**: 668.
26. Akalin E, Dinavahi R, Friedlander R, *et al.* Addition of plasmapheresis decreases the incidence of acute antibody-mediated rejection in sensitized patients with strong donor-specific antibodies. *Clin J Am Soc Nephrol* 2008; **3**: 1160.