ORIGINAL ARTICLE

Pretransplant human leukocyte antigen antibodies detected by single-antigen bead assay are a risk factor for long-term kidney graft loss even in the absence of donor-specific antibodies

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SUMMARY

Clinical relevance of ELISA- and single-antigen bead assay (SAB)-detected pretransplant HLA antibodies (SAB-HLA-Ab) for kidney graft survival was evaluated retrospectively in 197 patients transplanted between 2002 and 2009 at the University Clinic Frankfurt. Having adjusted for retransplantation and delayed graft function, a significantly increased risk for death-censored graft loss was found in patients with pretransplant SAB-HLA-Ab [HR: 4.46; 95% confidence interval (CI): 1.47–13.48; $P = 0.008$]. The risk for increased graft loss was also significant in patients with pretransplant SAB-HLA-Ab but without SAB-detected donor-specific Ab (SAB-DSA) (HR: 4.91; 95% CI of 1.43–16.991; $P = 0.012$). ELISA was not sufficient to identify pretransplant immunized patients with an increased risk for graft loss. In immunized patients, graft loss was predominantly present in patients who received transplants with a mismatch on the HLA-DR locus. In conclusion, even if our study is limited due to small sample size, the results show an increased risk for long-term graft loss in patients with pretransplant SAB-HLA, even in the absence of DSA. SAB-HLA-Ab-positive patients, being negative in ELISA or CDC assay, might profit from a well-HLA-DR-matched graft and intensified immunosuppression.

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Key words
HLA class-I antibody, HLA class-II antibody, HLA-DR, renal transplantation, single-antigen bead assay

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Introduction

The single-antigen bead assay (SAB) is a highly sensitive technique for detecting and characterizing human leukocyte antigen (HLA) antibodies (Ab) [1]. Compared with complement-dependent lymphocyte cytotoxicity (CDC) and ELISA, this new technique has an extremely high resolution for detecting HLA class-I and class-II Ab in the patient's serum including donor-specific Ab (DSA). In patients awaiting kidney transplantation and in patients in the post-transplant phase, SAB-detected DSA (SAB-DSA) have been associated with an increased risk for acute and chronic antibody-mediated rejection (AMR) [2–5]. Thus, systematic post-transplant monitoring by SAB allows timely detection of *de novo* DSA and a timely initiation of rejection treatment [6–8]. Furthermore, pretransplant DSA information can be used for defining nonacceptable HLA antigen mismatches and to create desensitization plans in renal allograft recipients [9–11]. In the present study, we tested whether pretransplant SAB-DSA and non-DSA influence graft survival adversely.

Materials and methods

Patients

The study included a total of 197 patients who received deceased or living-donor kidneys transplanted between 2002 and 2009 at the University Clinic Frankfurt, Germany. All patients from whom a pretransplant serum 3–6 months prior to transplantation was available were included in this study. No other exclusions were made. Twenty-three of these patients received a combined pancreas and kidney transplant, and three patients received a combined liver and kidney transplant. Patients' consent for anonymous use of their clinical data was obtained. The investigations were performed according to the 2000 Declaration of Helsinki and the 2008 Declaration of Istanbul and adhered to the University Clinic Frankfurt guidelines. The patients were monitored regularly for the development of HLA-Ab using ELISA and CDC for 6.0 ± 4.5 years as mean ± standard deviation (SD) prior to transplantation and considered HLA-Ab positive when 1 quarterly screening by CDC or ELISA revealed panel reactive antibodies (PRA) > 5%. Immunosuppression of HLA class-I Ab-negative kidney transplant recipients was performed with cyclosporine A, mycophenolate mofetil (MMF), and steroids. In contrast, patients with preformed, CDC- and/or ELISA-detected HLA class-I Ab received intensified immunosuppression with antithymocyte globulin (ATG), tacrolimus, MMF, and steroids prophylactically. The median post-transplant follow-up of all patients was 17.5 months with an interquartile range of 6.8–34.0 months. Sixteen patients lost their grafts within 14.4 ± 13.3 months (mean ± SD). Pretransplant sera from all 197 patients were retrospectively analyzed by SAB.

Pretransplant antibody screening, differentiation, and cross-matching

The pretransplant HLA-Ab status was monitored quarterly, according to the standards of the European Federation of Immunogenetics. Routine monitoring for HLA class-I and HLA class-II Ab was performed by ELISA using the Lambda Antigen Tray Mixed Class-I and Class-II test (OneLambda, Canoga Park, CA, USA) for screening and the ELISA Lambda Antigen Tray Class-I or Class-II test (OneLambda) for HLA-Ab differentiation. In addition, twice yearly, CDC was performed for all patients on the kidney transplant waiting list and quarterly for all immunized patients using an in-house panel with unseparated T/B cells from at least 60 donors. Based on CDC and ELISA-PRA monitoring, between 2002 and 2009 unacceptable HLA-A and HLA-B antigens were excluded for donor selection in accordance with the Eurotransplant kidney allocation system.

In addition, prior to transplantation, preformed lymphocytotoxic DSA and positive pretransplant cross-matches with unseparated or separated T cells and B cells resulted in exclusion from transplantation. Pretransplant cross-matches with unseparated T/B cells, and separated T cells were performed for all patients on the allocation list. Cross-matches with separated B cells were performed for patients positive for ELISA-detected HLA-DR or HLA-DQ Ab. Patients who were positive for HLA-DR or HLA-DQ DSA but negative in the pretransplant B-cell cross-match were not excluded from transplantation. For these cross-matches, the actual quarterly serum for non-immunized patients and fresh serum for immunized patients were used. Transplantation with repeated HLA-A, HLA-B, or HLA-DR mismatches was prohibited, if previous allografts were lost within 3 years after transplantation due to immunological reasons.

SAB on the luminex platform

Pretransplant sera (N = 197) were retrospectively examined by the LabScreen SAB assay according to the manufacturer's protocol (OneLambda). To abolish the prozone effect, all samples were frozen and heated to

56 °C for 10 min before testing [12]. As negative control, a commercially available test serum (OneLambda) was used. Patients were assigned SAB-detected HLA-Ab (SAB-HLA-Ab) positive, when one or more Ab for HLA-A, HLA-B, HLA-C, HLA-DRB1, or HLA-DQB1 were detected with a normalized mean fluorescence intensity (nMFI) ≥ 3000. DSA were assigned by comparing HLA-Ab specificities against the mismatched donor HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 antigens.

Statistical analysis

The cumulative probability of death-censored graft survival was estimated according to Kaplan and Meier, whereas the statistical comparison of patients with or without HLA-Ab was performed by multivariate Cox proportional hazard regression using retransplantation and delayed graft function (DGF) as potential confounders. Wald tests were used to assess significance if possible, that is, each group has events; otherwise, *P* values refer to the corresponding log-likelihood test. Furthermore, log-rank test was used for further explorative survival analysis. Stepwise multivariate logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence intervals (CI) associated with DGF. To analyze death-censored graft survival, patients who died with a functioning graft were censored at their death times. Continuous and normally distributed variables were compared between groups using unpaired *t*-tests. If continuous variables were not normally distributed, they were compared using the Mann–Whitney *U*-test. Categorical variables were compared by chi-square tests. Two-sided *P* values <0.05 were considered to be significant. Kaplan–Meier curves were drawn with GRAPHPAD PRISM 5.0 (La Jolla, California, USA). Chi-square tests, multivariate Cox regression analysis, and multivariate logistic regression analysis were performed using IBM SPSS 23 (Armonk, New York, USA) or BIAS software 11.0 (Frankfurt, Germany).

Results

Patient and transplant characteristics

With respect to the underlying renal disease, there were no significant differences between SAB-HLA-Ab-negative and SAB-HLA-Ab-positive patients. With a median follow-up of 17.5 months, 16 of 197 patients experienced graft loss and 12 of 197 patients died with a functioning graft. The mean recipient age was similar in SAB-HLA-Ab-negative and SAB-HLA-Ab-positive patients (50.7 ± 14.1 years vs. 52.7 ± 11.0 years). Compared with the SAB-HLA-Ab-negative group, the SAB-HLA-Ab-positive group consisted of a significantly higher number of retransplanted recipients (18.8% vs. 52.2%; $P < 0.001$) had a significantly longer time on dialysis (5.1 ± 3.6 years vs. 7.6 ± 5.1 years; $P < 0.001$) and were more likely to be CMV seropositive (53.9% vs. 80.6%; $P = 0.001$).

The rate of HLA-DR antigen mismatches (MM) was significantly lower among SAB-HLA-Ab-positive patients (1.13 ± 0.70 vs. 0.89 ± 0.67; $P = 0.024$), whereas for HLA-A and -B MM no significant differences were found. There was a higher incidence of DGF (14.8% vs. 26.08%; $P = 0.054$) in SAB-HLA-Ab-positive patients who also showed a slightly increased cold ischemia time (750.3 ± 449.2 min vs. 815.2 ± 352.2 min; $P = 0.088$) (Table 1). Multivariate logistic regression analysis with variables probably influencing DGF (cold ischemia time, donor age, retransplantation, HLA-Ab positivity) confirmed that in our cohort, immunization with HLA-Ab is a risk factor for DGF (adjusted OR 2.55, 95% CI 1.12–5.82, $P = 0.026$) [13].

Comparison of pretransplant ELISA- and SAB-detected HLA-Ab and their impact on death-censored graft survival

Five-year graft survival was analyzed according to the presence of HLA-Ab as defined by the two different solid-phase methods, ELISA and SAB. Therefore, the impact of HLA-Ab status was assessed in multivariate Cox regression adjusting for predictors of allograft survival, namely retransplantation and DGF [14–16]. Defining the patients' pretransplant Ab status by ELISA indicates a slightly but not significantly increased incidence of graft loss in patients immunized with HLA class-II Ab only ($P = 0.072$), whereas the exclusive immunization against neither HLA class-I ($n = 23$) nor both HLA class-I and HLA class-II antigens ($n = 15$) was associated with an increased risk for graft loss.

To assess whether or not SAB provides additional information on pretransplant immunization status, all patients were retrospectively tested by SAB. When patients without retransplantation and DGF were analyzed, the estimated 5-year graft survival in the 151 ELISA Ab-negative patients was 84.1% (95% CI 71–99%) as compared to the 91.0% rate in 128 SAB-Ab-negative patients (95% CI 82–100%). The 26 patients negative for ELISA-detected but positive for SAB-detected HLA-Ab had a strikingly low 5-year graft

Table 1. Patient and transplant characteristics.

Parameters	SAB-HLA-Ab-negative recipients (n = 128)	SAB-HLA-Ab-positive recipients (n = 69)	Significance (P)
Patient characteristics			
Recipient age (years, mean ± SD)	50.7 ± 14.1	52.7 ± 11.0	0.50*
Recipient female gender (%)	43 (33.6)	32 (46.4)	0.11†
Retransplants (%)	24 (18.8)	36 (52.2)	<0.001†
Time on dialysis (years; mean ± SD)	5.1 ± 3.6	7.6 ± 5.1	<0.001*
CMV-pos. recipient (%)	69/128 (53.9)	53/66 (80.3)	0.001†
CMV-pos. donor (%)	64/113 (56.6)	35/60 (58.3)	0.80†
CMV-neg. recipient/CMV-pos. donor (%)	27/113 (23.9)	9/60 (15.0)	0.16†
Transplant characteristics			
HLA-A + -B mismatch (mean ± SD)	2.14 ± 1.17	1.97 ± 1.18	0.29*
HLA-DR mismatch (mean ± SD)	1.13 ± 0.70	0.89 ± 0.67	0.024*
Donor age (mean ± SD)	52.4 ± 16.4	49.8 ± 15.0	0.18*
Living donor (%)	15 (12.1)	7 (10.2)	0.18†
Female donors (%)	64 (50.0)	33 (47.8)	0.73†
Cold ischemia time (min; mean ± SD)	750.3 ± 449.4 (n = 118)	815.2 ± 352.2 (n = 65)	0.088*
Warm ischemia time (minutes; mean ± SD)	49.4 ± 29.5 (n = 117)	56.0 ± 32.9 (n = 62)	0.21*
Delayed graft function (%)	19/128 (14.8)	18/69 (26.1)	0.054†
Time to graft loss (months; mean ± SD)	7.92 ± 6.62 (n = 5)	17.37 ± 15.37 (n = 11)	*0.36**
Graft loss (death-censored) (%)	5 (3.9)	11 (15.9)	0.003†

SAB, single-antigen bead assay; CMV, cytomegalovirus; pos., positive; neg., negative; SD, standard deviation.

*Mann–Whitney *U*-test.

†Chi-square test.

survival of 46.3%, as compared to the 90.4% rate in SAB-HLA-Ab-negative patients (Fig. 1). This finding was confirmed in the multivariate analysis (HR: 7.62; 95% CI of 2.3–25.5; $P < 0.001$). A higher risk for graft loss was also found in patients who were positive for ELISA and SAB-HLA-Ab, but the difference to ELISA-negative and SAB-Ab-negative patients did not reach statistical significance (HR: 2.55; 95% CI of 0.67–9.72; $P = 0.17$).

Comparison of transplant survival in patients with and without SAB-detected HLA-Ab

Compared with SAB-HLA-Ab-negative patients, adjusted statistical analysis revealed a significantly increased risk for graft loss in patients with exclusively SAB-HLA class-I Ab (graft survival: 68.8% vs. 89.5%; $P = 0.027$; HR: 3.35 with a 95% CI of 1.15–9.79) while simultaneous positivity for both SAB-HLA class-I and class-II Ab did not further affect the risk for graft loss (graft survival: 72.2% vs. 85.3%; $P = 0.25$; HR: 2.85 with a 95% CI of 0.89–9.14; Fig. 2a). The number of four exclusively SAB-HLA class-II Ab-positive patients was too small for a meaningful analysis, also because the cause for graft loss in the one of these patients was rather unclear. The patient suffered from diabetic nephropathy, coronary artery disease, mitral valve insufficiency grade II-III, and atrial fibrillation. Post-transplantation dialysis and a surgical intervention due to ureter complications were necessary. After intermittent recovery, CMV infection and volume overload, possibly due to heart insufficiency, occurred. GFR declined again. Renal biopsy revealed signs of chronic tubular interstitial damage and nephrosclerosis. Renal function did not recover, and chronic intermittent dialysis therapy was restarted. The data did not support the occurrence of AMR.

As retransplanted patients have an elevated immunological risk, a separate analysis was performed for this patient group. Adjusted analysis in retransplanted patients revealed a significantly increased risk for graft loss in patients with SAB-HLA class-I and/or SAB-HLA class-II Ab (graft survival: 61.1% vs. 100%; $P = 0.013$; log-likelihood test). This increased risk was especially seen in patients with both SAB-HLA class-I and class-II Ab (graft survival: 56.8% vs. 93%; $P = 0.075$; HR: 7.8 with a 95% CI of 0.81–74.9; Fig. S1). Importantly, a significantly increased risk for graft loss was also observed in first transplant recipients with pretransplant SAB-HLA class-I and/or SAB-HLA class-II Ab (graft survival: 79.5% vs. 93.6%; $P = 0.043$; HR: 3.45 with a 95% CI of 1.04–11.42). The impact of immunization with SAB-HLA

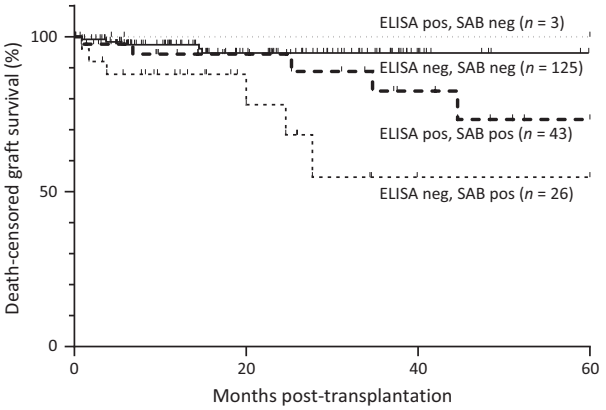

Figure 1 Relevance of SAB-detected HLA-Ab in patients negative or positive for ELISA-detected HLA-Ab for allograft survival. Kaplan–Meier graft survival in patients negative for HLA-Ab detected by ELISA and SAB (ELISA neg, SAB neg; slightly solid line), in patients negative for HLA-Ab detected by ELISA but positive for SAB-detected HLA-Ab (ELISA neg, SAB pos; slightly dotted line), in patients positive for HLA-Ab detected by ELISA and SAB (ELISA pos, SAB pos; strongly dashed line), and in patients positive for HLA-Ab detected by ELISA but negative for SAB-detected HLA-Ab (ELISA pos, SAB neg; slightly dashed line). In the ELISA-positive, SAB-HLA-Ab-negative group, the ELISA-detected specificities did not reach the cutoff value of 3000 nMFI. Adjusted statistical analysis revealed a significantly increased risk for graft loss in ELISA HLA-Ab-negative, but SAB-HLA-Ab-positive patients.

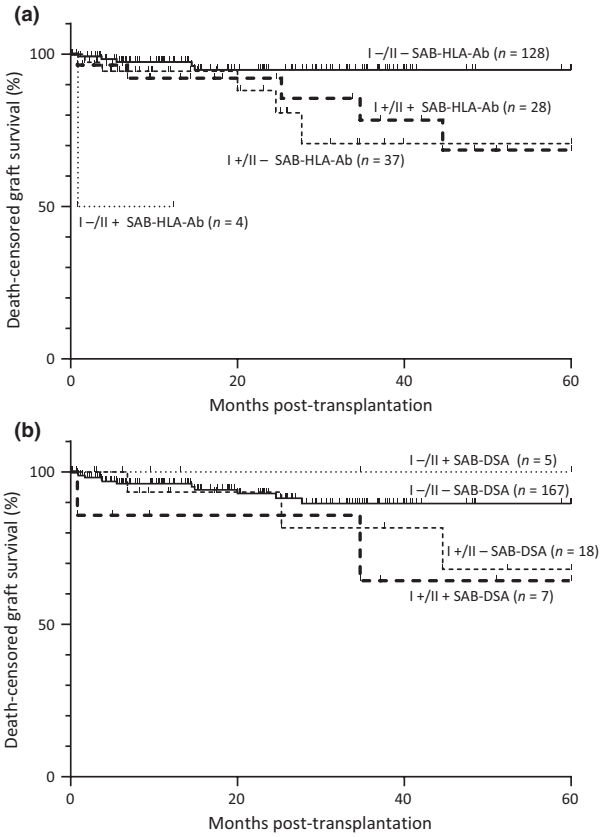

Figure 2 Impact of SAB-detected pretransplant HLA class-I and HLA class-II Ab and DSA on allograft survival. (a) Impact of SAB-detected pretransplant HLA class-I and HLA class-II Ab on death-censored graft survival. Kaplan–Meier graft survival of patients exclusively positive for SAB-HLA class-I Ab (I+/II- SAB-HLA-Ab; slightly dashed line), SAB-HLA class-II Ab (I-/II+ SAB-HLA-Ab; slightly dotted line), and patients positive for both SAB-HLA class-I and class-II Ab (HLA class-I+/II+ SAB-HLA-Ab; strongly dashed line). Results were compared to patients without SAB-Ab (I-/II- SAB-HLA-Ab; slightly, solid line). Adjusted statistical analysis revealed a significantly increased risk for graft loss in exclusively SAB-HLA class-I Ab-positive patients. (b) Impact of SAB-detected pretransplant HLA class-I and HLA class-II DSA on death-censored graft survival: Kaplan–Meier graft survival of patients with either exclusively HLA class-I SAB-DSA (I+/II-SAB-DSA; slightly dashed line), exclusively HLA class-II SAB-DSA (I-/II+ SAB-DSA; slightly dotted line) and HLA class-I plus class-II SAB-DSA (I+/II+ SAB-DSA; strongly dashed line), and patients without SAB-DSA (I-/II- SAB-DSA; slightly, solid line). For definition of SAB-HLA-Ab and SAB-DSA, a nMFI cutoff value ≥3000 was used.

class-I and/or SAB-HLA class-II Ab on patient death did not reach statistical significance (82.5% vs. 92.0%; $P = 0.152$; HR: 2.29 with a 95% CI of 0.74–7.10).

Impact of SAB-detected pretransplant DSA on graft survival

Compared with HLA-DSA-negative patients, the adjusted statistical analysis showed an increased risk for graft loss in patients with exclusively HLA class-I SAB-DSA, exclusively HLA class-II SAB-DSA, or with both HLA class-I and class-II SAB-DSA, but without reaching statistical significance (Fig. 2b). Also patients with HLA class-I and/or HLA class-II SAB-DSA demonstrated a lower death-censored graft survival than HLA-DSA-negative patients; however, the difference did not reach statistical significance (graft survival: 73.5% vs. 86.4%; adjusted analysis: $P = 0.20$; HR: 2.12 with a 95% CI of 0.68–6.57) (Fig. 3).

Transplant survival in immunized patients with and without pretransplant DSA

Compared with SAB-HLA-Ab-negative patients, SAB-HLA class-I Ab- and/or SAB-HLA class-II Ab-positive patients showed an inferior outcome (graft survival: 65.8% vs. 91.0%; $P = 0.008$; HR: 4.46 with a 95% CI of 1.47–13.48). Importantly, patients with SAB-HLA class-I and/or class-II Ab but without SAB-DSA displayed a significantly increased risk for graft loss (graft survival: 62.2 vs. 90.8%; $P = 0.012$; HR: 4.91 with a 95% CI of 1.43–16.91). Transplant survival in immunized patients without pretransplant SAB-DSA paralleled that of

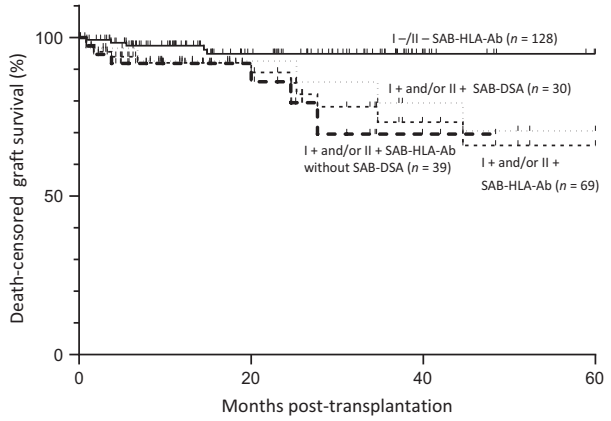

Figure 3 Comparison of SAB-detected pretransplant HLA class-I and class-II Ab with SAB-detected pretransplant HLA class-I and class-II DSA on graft survival. Kaplan–Meier graft survival in patients with HLA class-I and/or HLA class-II Ab including SAB-DSA (I+ and/or II+ SAB-HLA-Ab; slightly dashed line), with HLA class-I and/or HLA class-II SAB-DSA only (I+ and/or II+ SAB-DSA; slightly dotted line), and SAB-HLA class-I and/or class-II Ab-positive patients but without SAB-DSA (I+ and/or II+ SAB-HLA-Ab without SAB-DSA; strongly dashed line). Results were compared to patients without SAB-detected HLA-Ab (I-/II- SAB-HLA-Ab, black; slightly, solid line). Adjusted statistical analysis revealed a significantly increased risk for graft loss in SAB-HLA-Ab-positive and in SAB-HLA-Ab-positive/DSA-negative patients.

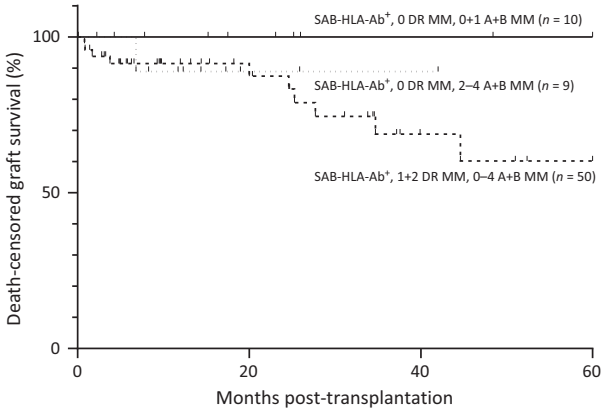

Figure 4 Significance of HLA-A, HLA-B, and HLA-DR mismatches on graft survival in patients immunized with SAB-detected, pretransplant HLA-Ab. Comparison of graft survival in transplants with 0 DR MM and 0-1 HLA-A, HLA-B MM (SAB-HLA-Ab$^+$, 0 DR MM, 0-1 HLA-A+B MM; slightly solid line), with 0 DR MM and ≥2 A+B MM (SAB-HLA-Ab$^+$, 0 DR MM, 2-4 A+B MM; slightly dotted line), and with HLA-DR MM (SAB-HLA-Ab$^+$, 1 + 2 DR MM; 0–4 A+B MM; slightly dashed line) in patients with SAB-HLA class-I and/or class-II Ab. SAB-Ab were defined with a cutoff value of nMFI ≥3000.

SAB-HLA-positive patients, suggesting that immunization against HLA antigens prior to transplantation per se is a risk factor for long-term graft survival (Fig. 3).

Similar results were obtained with cutoff values of 1000 nMFI. With a cutoff of 1000 nMFI, graft survival was significantly lower in patients with SAB-HLA-Ab (graft survival: 67.1% vs. 90.5%; $P = 0.027$; HR: 4.00 with a 95% CI of 1.17–13.64) and also in patients with SAB-HLA-Ab but without SAB-DSA (graft survival: 62.8% vs. 90.5%; $P = 0.025$; HR: 4.68 with a 95% CI of 1.22–17.99) compared with those with neither SAB-HLA class-I or class-II Ab as reference.

Influence of HLA mismatch (MM) on graft survival in immunized patients

The 5-year graft survival in the 58 transplants who had SAB-HLA class-I and/or class-II Ab and who received a kidney graft with two or more HLA-A, HLA-B, or HLA-DR mismatches was with 58.8% (95% CI 36.0–96.0%) strikingly low, whereas no graft loss was observed in the 11 patients who had SAB-Ab and who received a kidney graft with 0-1 HLA-A, HLA-B, or HLA-DR mismatches ($P = 0.060$). Importantly, 10 of the 11 immunized patients with graft loss had a HLA-DR mismatch (Fig. 4). Of these 10 patients, five patients were negative for pretransplant SAB-detected HLA-DR Ab. When patients negative for pretransplant SAB-Ab were analyzed, HLA-A, HLA-B, or HLA-DR mismatches were not associated with an increased risk for graft loss. These results underscore the fact that immunized patients benefit from well-HLA-matched grafts.

Influence of different SAB cutoff values in HLA-Ab-positive patients on 5-year graft survival

A detailed analysis on SAB cutoffs of 1000, 2000, and 3000 nMFI on 5-year graft survival is given in Table 2. Using adjusted cox regression analysis, patients with SAB-HLA class-I and/or class-II Ab revealed a significantly increased risk for graft loss when defining SAB-HLA-Ab positivity with cutoff values of 1000, 2000, and 3000 nMFI. With cutoff values of 1000 nMFI, 56% of patients revealed SAB-HLA-Ab and this group contained all patients with graft loss, whereas in the SAB-Ab-negative patients graft survival was 100%. With increasing cutoff values, the number of patients negative for SAB-HLA-Ab increased by 32% (from 87 patients to 128 patients).

Results of indication biopsies and proteinuria

In our cohort, 62 nonimmunized and 40 immunized patients were biopsied due to deterioration of glomerular filtration rate and/or proteinuria. Of the SAB-HLA-Ab-positive group, 18 patients were exclusively

Table 2. Different SAB cutoff values and their impact on 5-year graft survival in HLA-Ab-positive patients in adjusted statistical analysis.

Cutoff nMFI	SAB-HLA class-I and/or class-II Ab						
	Negative		Positive				
	Five-year graft survival	*N*	Five-year graft survival	*N*	HR	95% CI	*P*-value
1000	100	87	68.3	110	n.a.		<0.001
2000	90.3	117	70.8	80	3.40	1.11–10.25	0.030
3000	91.0	128	65.8	69	4.46	1.47–13.48	0.008

Adjusted statistical analysis of graft survival in patients with or without HLA-Ab was performed by Cox proportional hazard regression using retransplantation and delayed graft function (DGF) as potential confounders.

sensitized against HLA class-I antigens, three patients were exclusively sensitized against HLA class-II antigens, and 19 patients were sensitized against both HLA class-I and class-II antigens.

The incidence of transplant glomerulitis +/− C4d staining as indicator of acute AMR and of transplant glomerulopathy +/− C4d staining as indicator of chronic AMR was increased among patients with SAB-HLA-Ab; especially, patients with both SAB-HLA class-I and SAB-HLA class-II Ab revealed a significantly increased number of biopsies that were positive for glomerulitis (42% in SAB-HLA class-I Ab- and SAB-HLA class-II Ab-positive vs. 11% in SAB-Ab-negative patients, $P = 0.002$; Fig. 5a) and positive for C4d staining (37% in SAB-HLA class-I Ab- and SAB-HLA class-II Ab-positive vs. 6% in SAB-HLA-Ab-negative patients; $P = 0.005$; Fig. 5b). Patients with both SAB-HLA class-I and SAB-HLA class-II Ab also revealed a tendency for

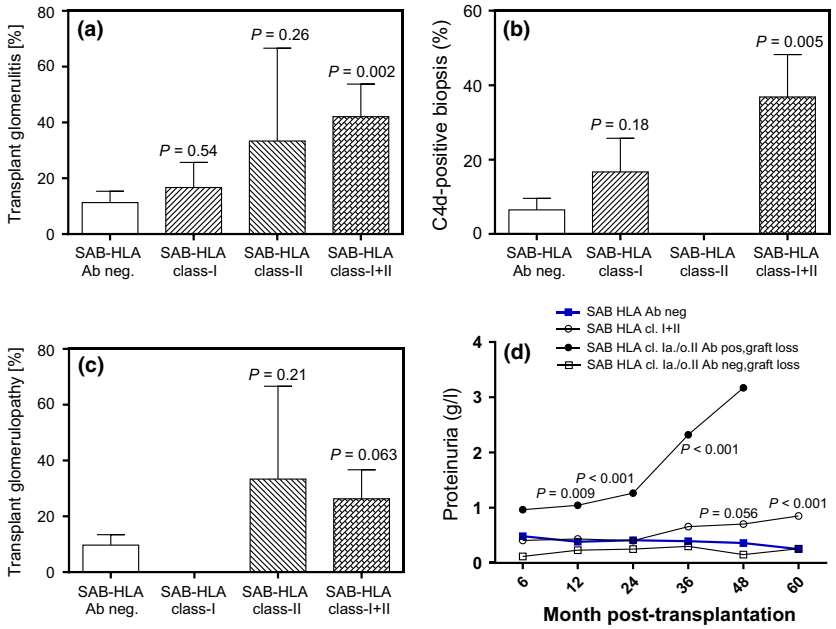

Figure 5 Association of the presence of SAB-HLA class-I Ab and SAB-HLA class-II Ab detected by SAB with glomerulitis, and C4d positivity, glomerulopathy, and proteinuria. Results of indication biopsies: Percentage of (a) glomerulitis-positive biopsies, (b) C4d-positive biopsies, (c) glomerulopathy-positive biopsies from patients negative or positive exclusively for SAB-HLA class-I or SAB-HLA class-II Ab, or for both SAB-HLA class-I and class-II Ab. Investigated biopsies in the control group: 62; patients exclusively positive for SAB-HLA class-I Ab: 18; patients exclusively positive for SAB-HLA class-II Ab: 3; and patients positive for both SAB-HLA class-I and class-II Ab: 19. Significance was determined with unpaired *t*-test. (d) Proteinuria, detected in patients negative or positive for both SAB-HLA class-I and class-II Ab and in patients with graft loss and positive or negative for both SAB-HLA class-I and/or SAB-HLA class-II Ab. (Significance was determined by unpaired *t*-test.)

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an increased number of biopsies positive for glomerulopathy (26% in SAB-HLA class-I Ab- and class-II Ab- vs. 10% in SAB-HLA-Ab-negative patients, $P = 0.06$; Fig. 5c). This effect diminished among patients who had exclusively SAB-HLA class-I Ab or SAB-HLA class-II Ab. Multivariate logistic regression analysis showed that patients with a positive C4d score, positive glomerulitis score, or positive glomerulopathy score had a 3.8 increased risk of graft failure (95% CI 1.20–11.82, $P = 0.022$). Although patients at high immunological risk were regularly treated with calcineurin inhibitors (CNI), no histological signs for CNI toxicity were seen in the indication biopsies. In addition, no signs for an increase in pyelonephritis or infection-associated allograft disorder were found.

Proteinuria

In our analysis, patients, positive for both SAB-HLA class-I and class-II Ab, revealed a significantly increased risk to develop proteinuria. Within the first 2 years after transplantation, urinary protein concentrations in patients with and without SAB-HLA-Ab were identical. Within and after the third year, however, urinary protein values in the urine increased steadily in patients with both SAB-HLA class-I and class-II Ab until the results reached significance in the fifth year. This increase in proteinuria was not seen in patients, who were exclusively positive for pretransplant SAB-HLA class-I Ab or SAB-HLA class-II Ab. Importantly, proteinuria was significantly increased among patients positive for SAB-HLA class-I and/or class-II Ab who experienced graft loss, whereas SAB-HLA-Ab-negative patients with graft loss did not develop proteinuria (Fig. 5d).

Comparison of HLA-Ab specificities detected by ELISA and SAB

On average, 68.1% of ELISA-detected HLA class-I Ab and 79.5% of ELISA-detected HLA class-II Ab specificities were detected by SAB. In patients, in whom the ELISA assay allowed specification of HLA-Ab, ELISA detected 22.1% of SAB-HLA class-I Ab and 39.0% of SAB-HLA class-II Ab specificities. 98.5% of HLA class-I Ab specificities and 95.5% of HLA class-II Ab specificities detected by ELISA revealed SAB nMFI values >4000. On average, patients with polyspecific results in the ELISA showed 30 SAB-detected HLA class-I and 16 different SAB-detected HLA class-II (DR, DQ) Ab specificities.

In retransplanted patients, evaluation of immunization due to mismatched HLA antigens from previous transplantations displayed SAB-HLA-Ab against 59.1 ± 37.4% of mismatched HLA antigens, and against 19.8 ± 23.7% of mismatched HLA antigens, when detected by ELISA (for more details, see Figs S2 and S3).

Natural Ab

According to the results of Gombos *et al.* and Morales-Buenrostro *et al.*, presence of natural Ab with specificities HLA-A*24:02, HLA-A*30:02, HLA-A*31:01, HLA-B*08:01, HLA-B*15:12, HLA-B*37:01, HLA-B*44:02, HLA-B*82:01, HLA-C*05:01, HLA-C*17:01, and HLA-DQB1*03:01 was analyzed. On average, in SAB-HLA-Ab-positive patients, 22.4% of HLA-Ab represented 'natural Ab'. Furthermore, 93% of 'natural' Ab reacting against split antigens (HLA-A*24:02, HLA-A*30:02, HLA-A*31:01, HLA-B*15:12, HLA-B*44:02) also reacted against other split antigens of the same serotype. Also in one case, the natural Ab was detectable by the ELISA. Thus, we decided not to exclude natural Ab for definition of immunized patients.

Discussion

Pretransplant DSA detected by SAB represent a risk factor for graft survival [17–20]. We asked whether pretransplant immunization even in the absence of SAB-DSA is associated with a higher prevalence of kidney graft loss.

Although the observational design and the relatively small sample size of our study imply limitations as confounders cannot be fully controlled, our results indicate that patients with pretransplant SAB-detected HLA-Ab and especially HLA class-I Ab reveal a significantly increased risk for chronic graft loss. In addition, pretransplant immunized patients with SAB-HLA-Ab, but without SAB-DSA, had a significantly reduced graft survival, indicating that pretransplant sensitization against HLA antigens per se increases the risk for graft loss. These results are consistent with those reported by Susal *et al.* [21,22], who found in two independent series of some 5000 kidney transplant recipients from the CTS that presensitization against both HLA class-I and class-II antigens resulted in a significantly increased risk for graft loss.

There are several possible explanations for an increased risk for graft loss in the absence of SAB-detected DSA. First, public epitopes presented by different HLA antigens may cause a widespread binding of

the epitope-specific Ab to different SAB leading to decreased MFI values for a single-bead species [23]. Secondly, denaturation of HLA antigens on the SAB may cause low affinities of HLA-Ab with reduced nMFI values [24]. More conceivably, immunized patients with an activated immune system might have an increased risk to develop *de novo* DSA which can result in reduced graft survival [25]. This hypothesis is supported by the presence of graft loss only in pretransplant immunized patients with an increased number of HLA mismatches, and especially in transplants with a HLA-DR mismatch, a finding which is in accordance with other studies [10,20].

The deteriorating effect of HLA-DR mismatches on kidney transplantation outcome may be related to an increased renal expression of HLA class-II antigens during graft injury [26,27]. Mismatched HLA-DR grafts particularly elicit $CD4^+$T-cell responses, which modulate immune responses [28–30] that may trigger allograft rejection. Furthermore, clinical analyses show that HLA-DR mismatches are related to an increased risk of acute rejection, to an intensified rejection treatment, and to high-dose maintenance immunosuppression [31–35].

Although we did not determine the development of *de novo* HLA-Ab during rejection episodes, signs of acute and/or chronic AMR detected in indication biopsies and increased proteinuria especially in patients positive for both HLA class-I and HLA class-II Ab suggest presence of DSA or *de novo* DSA after transplantation [36–39].

In particular, we asked whether SAB, compared with ELISA, provides additional information on the pretransplant immunization status with significance for transplant survival. We found that 26 patients with negative ELISA results displayed HLA class-I and/or HLA class-II Ab in SAB testing. These additionally identified patients exhibited an increased risk for graft loss. Hence, nonimmunized patients as determined by SAB provided a better long-term graft survival, compared with ELISA-negative patients.

In our transplant center, patients with preformed, CDC- and/or ELISA-detected HLA class-I Ab received intensified immunosuppression with tacrolimus, MMF, ATG, and steroids, prophylactically. Strikingly, these patients demonstrated no increased risk for graft loss. On the contrary, the patients, who were positive for SAB-detected HLA-Ab but negative in the ELISA, revealed a significant risk for graft loss. Potentially, these patients would also have benefited from an intensified immunosuppressive therapy.

Although SAB technology may be superior to ELISA, this technology might reveal limitations with significant impact on allograft allocation decisions for renal transplant patients and transplantation outcome. Currently, it is not clear which cutoff values should be used to define unacceptable HLA-Ab specificities. In our study, we used a cutoff value of 3000 nMFI, which represents a robust cutoff for pretransplant and post-transplant DSA and association with an increased risk for AMR and a positive cytotoxic cross-match [40–44]. Lower cutoff values might increase the sensitivity but might also decrease the specificity for detection of patients with an increased risk for graft loss. Due to the high sensitivity of the assay, more immunized patients with higher PRA values would be on the kidney waiting list. These high PRA values could prolong the time on the waiting list, which is on average associated with an increased mortality for patients with ESRD.

In conclusion, compared with ELISA, SAB demonstrates a higher sensitivity for detection of transplant-relevant HLA-Ab, which leads to identification of additional patients with pretransplant, non-DSA, and DSA. These patients revealed an increased risk for AMR and for graft loss. Our results indicate that transplant recipients with pretransplant, SAB-detected HLA-Ab require increased immunological surveillance and may profit from intensified immunosuppressive therapy to overcome the increased risk for allograft rejection. One additional important strategy to overcome the increased rejection rate is the allocation of well-HLA-matched grafts to immunized patients with higher priority [21,45].

Authorship

RR: designed study, collected and analyzed data and wrote the manuscript. CS and EH: designed study and analyzed data. SK and SQ: collected data. AS and LH: collected data and contributed important laboratory examinations. MB, AA-V, SB, CB and SG: contributed important clinical examinations. ES and HG: designed study. CS: designed study and performed study. IAH: designed study, performed study and wrote the manuscript.

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Conflict of interest

The authors of this manuscript have no conflict of interests to disclose.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Death censored graft survival in non-immunized and immunized re-transplanted patients.

Figure S2. Frequency of HLA-A, -B and -C, Ab detected by SAB and ELISA in the 69 immunized patients.

Figure S3. Frequency of HLA-DR, and DQ Ab detected by SAB and ELISA in the 69 immunized patients.

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