American Journal of Transplantation 2016; XX: 1–10
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doi: 10.1111/ajt.13848

Clinical Significance of Pretransplant Donor-Specific Antibodies in the Setting of Negative Cell-Based Flow Cytometry Crossmatching in Kidney Transplant Recipients

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Antibodies to donor-specific HLA antigens (donor-specific antibodies [DSA]) detected by single-antigen bead (SAB) analysis prior to kidney transplant have been associated with inferior graft outcomes. However, studies of pretransplant DSA, specifically in the setting of a negative flow cytometry crossmatch (FCXM) without desensitization therapy, are limited. Six hundred and sixty kidney and kidney–pancreas recipients with a negative pretransplant FCXM from September 2007 to August 2012 without desensitization therapy were analyzed with a median follow-up of 4.2 years. All patients underwent cell-based FCXM and SAB analysis on current and historic sera prior to transplantation. One hundred and sixty-two patients (24.5%) had DSA detected prior to transplant. One-year acute rejection rates were similar in DSA-positive versus DSA-negative patients (15.4% vs. 11.4%, respectively; p = 0.18) and were higher in those with DSA mean fluorescence intensity (MFI) greater than or equal to 3000 in multivariable analysis (p = 0.046). The estimated glomerular filtration rate (eGFR) at 3 and 4 years was lower in the DSA(+) versus the DSA(−) group (p = 0.050 at 3 years) without an impact on 5-year death-censored graft survival (89.0% vs. 90.6%, respectively; p = 0.53). Timing (current or historic) of DSA detection did not alter these findings. In conclusion, pretransplant DSA in the setting of a negative FCXM confers minimal immunologic risk in the intermediate term, does not necessitate desensitization therapy and should not represent a barrier to renal transplant.

Abbreviations: AR, acute rejection; CDC, complement-dependent cytometry crossmatch; cPRA, calculated panel-reactive antibody; DSA, donor-specific antibody; DSA(+), presence of pretransplant DSA; DSA(−), absence of pretransplant DSA; eGFR, estimated glomerular filtration rate; FCXM, flow cytometry crossmatch; MDRD, modification of renal disease; MFI, mean fluorescence intensity; SAB, single-antigen bead

Received 12 November 2015, revised 21 April 2016 and accepted for publication 22 April 2016

Introduction

The widespread utilization of single-antigen bead (SAB) assays prior to kidney transplantation has improved the detection of donor-specific antibody (DSA) in potential transplant recipients. Many studies have documented inferior renal graft outcomes in the presence of pretransplant DSA (1–15). However, the clinical importance of such DSA in the setting of a negative flow cytometry crossmatch (FCXM) at transplant remains controversial and the need for desensitization uncertain. There are limited published data regarding the impact of specific DSA characteristics such as class, strength or whether DSA persisted post-transplant on renal allograft outcomes when flow cytometry is negative. Additionally, the clinical significance of waxing/waning HLA antibodies pretransplant that are donor specific has not been reported in detail.

The use of desensitizing agents prior to transplant for those patients at increased immunologic risk based on pretransplant screening has permitted successful transplantation for those who may not otherwise have had access to this treatment, despite higher acute rejection rates and inferior graft survival compared to those without preformed DSA (16,17). Clinical experience has shown these treatments to be necessary for patients with high-risk immunologic screening assays (complement-dependent cytotoxicity crossmatch [CDC] and/or FCXM positivity); however, the necessity of these costly therapies in the setting of SAB positivity alone remains uncertain, with practices varying center by center (17,18).

Since September 2007, all renal transplant recipients at our center have undergone immunologic screening with

both SAB and FCXM assays prior to transplantation. The aim of this study was to evaluate the impact of pretransplant DSA in the setting of negative FCXM screening on intermediate-term renal allograft outcomes in patients not receiving desensitization therapy. To our knowledge, this is the largest study of outcomes following kidney transplantation in the setting of low-level pretransplant DSA with negative FCXM to date.

Materials and Methods

Patient population

This retrospective analysis included adult patients (≥18 years old) receiving a kidney or kidney–pancreas transplant at the University of Colorado Hospital between September 2007 and August 2012. Patients with positive FCXM and those who received desensitization therapy were excluded from analysis. The study was approved by the Colorado Multiple Institution Review Board.

All HLA testing, antibody analyses and crossmatches were performed by American Board of Histocompatibility and Immunogenetics (ABHI) board-certified technologists and reviewed by ABHI board-certified HLA specialists in an ASHI- and College of American Pathologists (CAP)-accredited laboratory (ClinImmune Labs, Aurora, CO) with 100% majority consensus on proficiency training for both antibody detection and flow crossmatching during the study period.

HLA typing and panel-reactive antibody (PRA) analysis

Recipients were typed by serology at HLA-A, -B, and -Cw and HLA-DR/DQ by sequence-specific oligonucleotide (SSO, LabType, One Lambda, Canoga Park, CA) with sequence specific primer (SSP) and sequencing used as necessary. Donors were typed by the organ recovery center and resolved in a similar manner by SSO, SSP, and sequencing as needed. The calculated PRA (cPRA) was determined using the Organ Procurement and Transplantation Network calculator (https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/) for any HLA-A, -B, -DR and -DQ antigens with an MFI greater than 2000 in the One Lambda single-antigen assay.

Pretransplant crossmatch

Cell-based FCXM was performed on current and historic (up to 6 months preceding transplant) recipient sera to detect antibodies against donor T or B cells. Briefly, mononuclear cells were isolated from whole blood (with RosetteSep, STEMCELL Technologies Inc., Vancouver, BC, Canada) or lymph nodes and treated with 0.5 mg/mL pronase for 30 min at 37°C, followed by a brief deoxyribonuclease treatment. The cells were then washed and incubated with patient sera (200 000 cells/20 μL serum and 30 μL RPMI) for 30 min at room temperature, followed by three washes and addition of an antibody cocktail containing CD3 PerCP-Cy5.5, CD19 PE, BD Biosciences and APC-AffiniPure F(ab)′$_2$ goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA) for 20 min and two more washes. Cell data events were acquired and analyzed with the FACSArray Bioanalyzer (Becton Dickinson, Franklin Lakes, NJ). A positive FCXM was validated at more than 2 standard deviations above the mean of negative control sera and defined as ≥330 MFI units for T cells and ≥1000 MFI for B cells. Positive FCXM sera were tested for DSA using LABScreen SAB (One Lambda). The dynamic range for both the T and B flow crossmatches was 50–20 000 MFI.

Pre- and posttransplant DSA screening

Kidney and kidney–pancreas transplant recipients on the waiting list were screened for pretransplant HLA antibodies monthly. Following transplant, DSA screening was performed at 1, 6 and 12 months and annually. Patients were also screened whenever clinically indicated (graft dysfunction). Patients were screened for both class I and class II antibodies using LABScreen Mixed beads (One Lambda). Positive tests were quantified using SAB (One Lambda) per the manufacturer's specifications. DSA were defined as an MFI greater than twofold above the negative control and an absolute MFI of greater than 500. All MFIs were normalized against negative control beads per the manufacturer's instructions. Positive beads were analyzed for mismatched donor antigens (or alleles) at HLA-A, -B and -DR loci to identify DSA. MFI values for each sample were recorded for each DSA. Additional donor and recipient molecular typing was performed to confirm donor specificity to HLA-C, DPB1, DQB1, and DQA1 when indicated.

Immunosuppression

In general, induction therapy with rabbit antithymocyte globulin (rATG, thymoglobulin) was utilized for living, nonrelated kidney and kidney–pancreas transplant recipients and those considered to be at higher immunologic risk (pretransplant cPRA greater than 20%, repeat transplant, African American race, cold ischemia time greater than 24 h). Otherwise, low-risk deceased donor and living-related donor recipients received no induction. IL2-RA (basiliximab) was used as induction therapy when part of clinical trial protocols. Most patients were placed on triple immunosuppression therapy, including tacrolimus, mycophenolate and steroids, with a minority receiving mammalian target of rapamycin inhibitor in place of mycophenolate. A smaller minority received experimental immunosuppressive regimens (sotrastaurin, tofacitinib, alefacept, and belatacept), as dictated by various clinical trial protocols (Table 1).

Clinical outcomes

Acute rejection (AR) was diagnosed clinically and pathologically in accordance with Banff '07 criteria (19). Clinical rejection was defined by acute kidney injury in the absence of other causes and a response to high-dose corticosteroid (500 mg daily for 3–5 days) and/or rATG 1.5 mg/kg/day for 5–7 days with reduction in serum creatinine to baseline. If screening for DSA was negative at the time of treatment, this was defined as clinically diagnosed T cell–mediated rejection. Mixed acute humoral/cellular rejection was treated with plasmapheresis/intravenous immunoglobulin, with or without concurrent thymoglobulin as indicated. C4d was detected using immunohistochemistry. Graft function was assessed by the estimated glomerular filtration rate (eGFR) calculated by the four-variable modification of renal disease (MDRD) equation (20). Graft failure was defined as a return to hemodialysis dependence or retransplant. Spot urine protein/creatinine ratios (mg/g) were used to estimate daily protein excretion. Proteinuria data were recorded at 1, 3, 6, and 12 months and then yearly. Four patients with recurrent primary focal segmental glomerulosclerosis (FSGS) were excluded from the proteinuria data analysis.

Properties of pretransplant DSA identified for analysis

DSA identified at any time pretransplant were included in the analysis. DSA groups based on class were described as class I, class II or mixed. Patients with pretransplant DSA (DSA(+)) were divided into three groups depending on the period the antibodies were last detected in relationship to transplant dates: (1) those with pretransplant DSA present more than 6 months prior to transplant and absent thereafter, (2) within 6 months of transplant but absent at final crossmatch and (3) those with DSA detected at final crossmatch. When assigning a time of detection category, the most recent DSA detected were used. For the DSA strength analysis, patients with pretransplant DSA were divided into three groups based on MFI strength: <1000, 1000–2999, and ≥3000. When assigning a strength category to patients with multiple DSA, the maximum MFI was used. Persistence of pretransplant DSA was defined as the presence of DSA at any time after renal transplant.

Table 1: Demographics by pretransplant DSA versus no pretransplant DSA

Variable	Total (N = 660)	Pretransplant DSA (N = 162)	No pretransplant DSA (N = 498)	p-value
Mean (SD) recipient age (years)[1]	48.6 (13.6)	47.3 (13.5)	49.1 (13.7)	0.14
Gender[1]				**0.043**
Male	378 (57%)	81 (50%)	297 (60%)	
Female	280 (43%)	80 (50%)	200 (40%)	
Ethnicity[1]				**0.017**
Caucasian	437 (66%)	91 (57%)	346 (70%)	
African American	69 (11%)	23 (14%)	46 (9%)	
Hispanic	124 (19%)	40 (25%)	84 (17%)	
Other	28 (4%)	7 (4%)	21 (4%)	
Diagnosis[1]				0.58
Diabetes	182 (28%)	45 (28%)	137 (27%)	
Hypertension	63 (10%)	20 (12%)	43 (9%)	
Glomerulonephritis	213 (32%)	46 (29%)	167 (34%)	
PKD	67 (10%)	16 (10%)	51 (10%)	
Other/unknown	133 (20%)	34 (21%)	99 (20%)	
Donor type				**<0.001**
Deceased	391 (59%)	115 (71%)	276 (55%)	
Living	269 (41%)	47 (29%)	222 (45%)	
Organ				0.35
Kidney	620 (94%)	155 (96%)	465 (93%)	
Kidney–pancreas	40 (6%)	7 (4%)	33 (7%)	
Retransplants	93 (14%)	34 (21%)	59 (12%)	**0.005**
Induction				**0.005**
No induction	235 (35%)	46 (28%)	189 (38%)	
rATG	395 (60%)	113 (70%)	282 (56%)	
IL-2	25 (4%)	1 (1%)	24 (5%)	
Other (OKT-3, TOL 101 alefacept)	5 (1%)	2 (1%)	3 (1%)	
Maintenance immunosuppression				0.79
CNI/mycophenolate/steroids	526 (80%)	132 (82%)	394 (79%)	
CNI/mTORi/steroids	64 (10%)	15 (9%)	49 (10%)	
Other	70 (10%)	15 (9%)	55 (11%)	
HLA mismatches				**<0.001**
0	49 (7%)	3 (2%)	46 (9%)	
1–3	202 (30%)	39 (24%)	163 (33%)	
4–6	416 (62%)	120 (74%)	289 (58%)	
% cPRA[2]				**<0.001**
0	486 (74%)	84 (52%)	402 (81%)	
1–19	47 (7%)	15 (9%)	32 (6%)	
20–79	77 (11%)	37 (23%)	40 (8%)	
80–100	50 (8%)	26 (16%)	24 (5%)	

CNI, calcineurin inhibitor; cPRA, calculated panel-reactive antibody; DSA, donor-specific antibody; mTORi, mammalian target of rapamycin inhibitor; PKD, polycystic kidney disease; rATG, rabbit antithymocyte globulin; SD, standard deviation. Bold values = statistically significant at $p < 0.05$.

[1]660 transplants in 658 patients: 162 transplants with pretransplant DSA, one patient with two transplants in the study period; 498 transplants with no pretransplant DSA, one patient with two transplants in the study period.

[2]cPRA is at time of final crossmatch only.

Statistical methods

Patient characteristics in Table 1 were compared by DSA status using exact chi-squared and two-sample t-tests. Kaplan–Meier probabilities of graft and patient survival were plotted and compared by DSA status using log-rank tests. Cox's regression model was used to obtain estimates of hazard ratios for univariate analyses and also to compare DSA status allowing for age, gender, ethnicity, donor type, cPRA, number of HLA mismatches, induction therapy, and retransplant status. Development of posttransplant DSA was analyzed as a time-dependent covariate. Probability of AR was compared between DSA groups using logistic regression. Estimates of relative risk, adjusting for age, gender, ethnicity, donor type, cPRA, number of HLA mismatches, induction therapy and retransplant status, were calculated using Poisson regression with a robust variance estimator (21). A mixed-effects model with follow-up visit (1, 2, 3 or 4 years) as a class variable was used to analyze differences between groups for eGFR and proteinuria over time. The number of patients with eGFR and proteinuria at 5 years was too small for reliable estimates, so these outcomes were modeled between 1 and 4 years only. DSA groups were compared overall using a test of simple effects (22) in which the effect of DSA group was tested for each year posttransplant. Proteinuria was analyzed using a log transformation, as it was not normally distributed, and confidence intervals estimated from the models were back transformed to a linear scale. Last recorded eGFR and log proteinuria values were compared between groups using a general linear model for all available data to 5 years posttransplant. Study data were collected and managed using REDCap electronic data capture tools

hosted at the University of Colorado Denver. Initial data were extracted from the United Network for Organ Sharing database for our center; then, local data were supplemented to provide greater detail. All analysis was performed using SAS version 9.4 (SAS Inc., Cary, NC).

Results

Demographics and baseline characteristics

There were 690 kidney and kidney–pancreas recipients between September 1, 2007, and August 31, 2012. Seventeen patients who were FCXM positive, eight patients with a history of desensitization treatment and five patients with primary nonfunction were not included in the analysis, reducing the data set to 660 transplants. Two patients had two transplants in this period, resulting in 660 transplants in 658 patients with a median follow-up of 4.2 years. One hundred and sixty-two (24.5%) patients had pretransplant DSA detected at any time pretransplant, 68 of whom had more than one DSA. Baseline characteristics for all patients, DSA-positive (DSA(+)) and DSA-negative (DSA(−)) cohorts are shown in Table 1. DSA(+) patients were more likely to be female, African American or Hispanic; have a repeat transplant; receive a deceased donor allograft; have more HLA mismatches; have higher cPRA at transplant; and receive rATG induction at the time of transplant. The number of patients in each DSA(+) subgroup is shown in Table 2. Details for DSA(+) patients with 0% cPRA are available in Table S1.

Table 2: Number of patients by DSA group

DSA group	N (%)
DSA(+)	162 (24.6)
DSA(−)	498 (75.4)
Pretransplant DSA strength	
<1000 MFI	65 (9.9)
1000–2999 MFI	78 (11.8)
≥3000 MFI	19 (2.9)
DSA(−)	498 (75.4)
Pretransplant DSA class	
Class I	81 (12.3)
Class II	50 (7.6)
Mixed	31 (4.7)
DSA(−)	498 (75.4)
Timing of pretransplant DSA	
>6 months before transplant[1]	36 (5.5)
≤6 months before transplant[2]	95 (14.4)
At crossmatch[3]	31 (4.7)
DSA(−)	498 (75.4)
Persistence of pretransplant DSA	
Pretransplant and posttransplant DSA	112 (17.0)
Pretransplant DSA only	50 (7.6)
DSA(−)	498 (75.4)

DSA, donor-specific antibodies; MFI, mean fluorescence intensity.
[1]DSA detected more than 6 months prior to transplant and absent thereafter.
[2]DSA detected within 6 months of transplant and absent at final crossmatch.
[3]DSA detected at final crossmatch.

AR

One-year acute rejection (AR) rates were similar between DSA(+) and DSA(−) patients (15.4% vs. 11.4%, respectively; p = 0.18; Figure 1). Twenty-five DSA(+) patients experienced AR (11 cellular, 6 antibody mediated, 4 mixed and 4 clinically diagnosed). The proportion of cellular, clinical or mixed cellular/antibody-mediated rejection (AMR) AR was similar between DSA(+) and DSA(−) patients. However, 6/162 (3.7%) of DSA(+) patients had AMR versus 2/498 (0.4%) of DSA(−) patients (p = 0.023). The median time to AR for DSA(+) was 27 days (interquartile range [IQR] of 15–59 days) versus 25 days for DSA(−) (IQR of 12–114 days). Excluding 11 patients with isolated Cw or DP DSA did not significantly alter AR rates between groups.

There was an increase in the AR rate with increasing pretransplant DSA strength and notably for those with MFI greater than 3000 (26.3% vs. 11.4%) in DSA(−) patients; however, this failed to reach statistical significance in univariate analysis (p = 0.24 for the group and p = 0.06 comparing >3000 MFI to DSA(−); Figure 1B).

There was no statistically significant association between 1-year AR rates and pretransplant HLA class (Figure 1C), although rates were higher in those with class II DSA. Rejection in patients with class II DSA was not attributable to a specific locus (DQ vs. DR vs. DP; data not shown). The timing of pretransplant DSA detection (>6 months and ≤6 months pretransplant vs. detection at final crossmatch) was not associated with significant differences in 1-year AR when compared to those without pretransplant DSA (p = 0.51; Figure 1D). Table S2 shows patient numbers with and without AR in each DSA subclass.

Posttransplant persistence (or recurrence, in circumstances of historical DSA) of DSA was identified in 112 patients. Posttransplant persistence and/or recurrence of pretransplant DSA was associated with significantly higher AR (p = 0.008; Figure 1E). However, the timing of posttransplant detection of recurrent pretransplant DSA (relative to the onset of AR) was not associated with AR incidence. The median time to detection of posttransplant DSA (by either protocol or indication screening) was 32 days (IQR of 27–181 days). AR within the first year posttransplant occurred in 23 patients with persistent/recurrent DSA(+). Of these 23, 10 patients had persistent/recurrent posttransplant DSA detected prior to the rejection episode, 10 had persistent/recurrent posttransplant DSA detected after the rejection episode and 3 had persistent/recurrent posttransplant DSA detected concurrent with rejection.

In multivariable analysis, the presence of pretransplant DSA (not otherwise characterized) was not associated with an increased risk of 1-year AR. However, pretransplant DSA MFI strength of greater than or equal to 3000 was associated with increased risk of 1-year AR

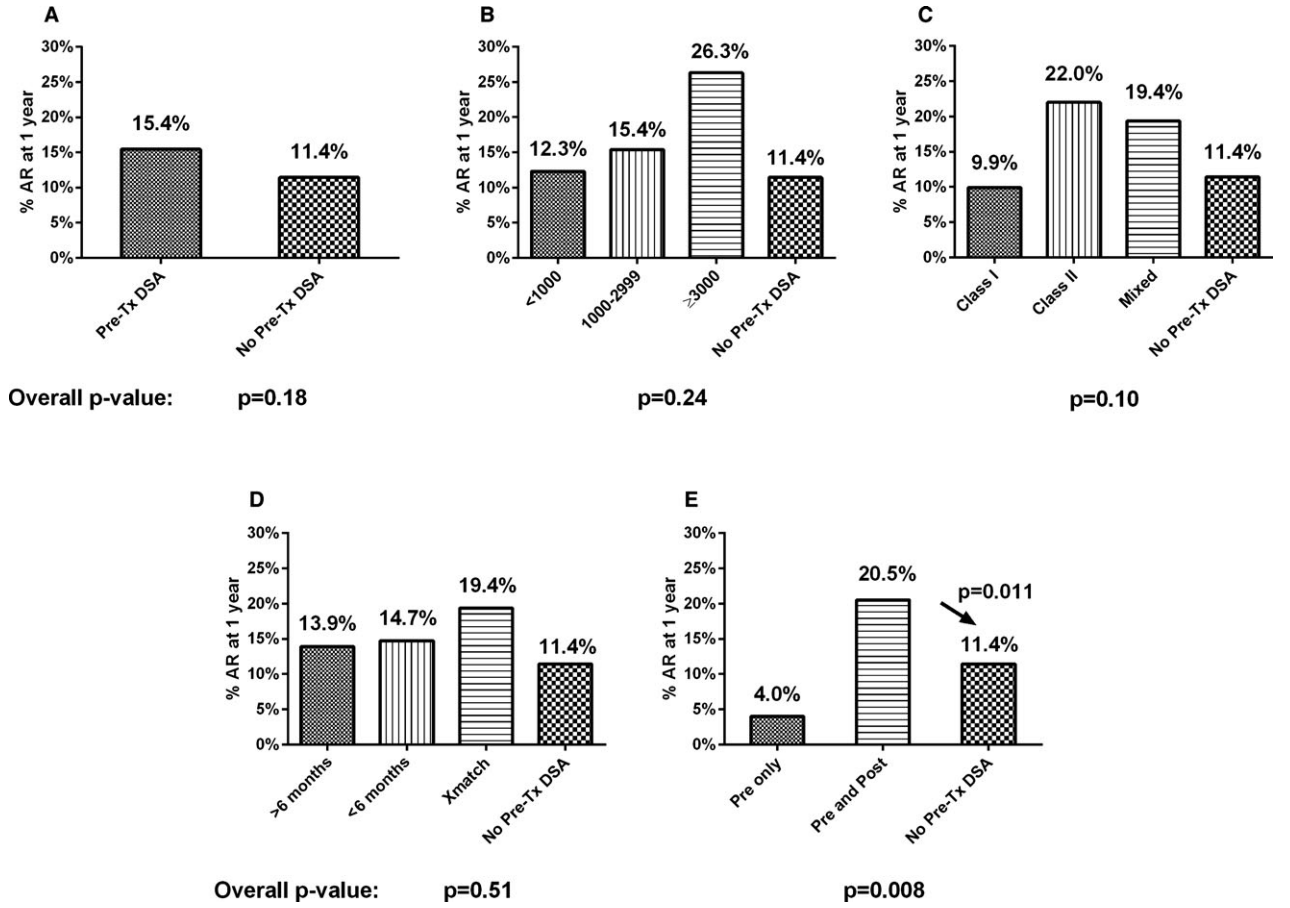

Figure 1: One-year incidence of AR based on (A) pretransplant DSA versus no pretransplant DSA, (B) strength of pretransplant DSA, (C) class of pretransplant DSA, (D) time of first pretransplant DSA detection relative to Xmatch, and (E) persistence of DSA posttransplant. AR, acute rejection; DSA, donor-specific antibodies; Post-Tx, posttransplant; Pre-Tx, pretransplant; Xmatch, crossmatch.

Table 3: Multivariable analysis of rejection in the first year posttransplant (N = 82) (adjusted for age, gender, ethnicity, donor type, PRA, HLA mismatches, induction therapy, and retransplant) and separate models for each type of DSA occurrence (pretransplant yes/no, strength, class, timing, and persistence posttransplant)

Variable	Relative risk (95% CI)	p-value
Pretransplant DSA versus no pretransplant DSA	1.15 (0.73–1.83)	0.55
Maximum pretransplant DSA strength <3000 versus no pretransplant DSA	1.04 (0.63–1.71)	0.89
Maximum pretransplant DSA strength ≥3000 versus no pretransplant DSA	2.26 (1.01–5.05)	**0.046**
Pretransplant DSA class I versus no pretransplant DSA	0.75 (0.37–1.53)	0.43
Pretransplant DSA class II or mixed versus no pretransplant DSA	1.56 (0.93–2.63)	0.09
Pretransplant DSA >6 months before crossmatch versus no pretransplant DSA	1.16 (0.48–2.79)	0.75
Pretransplant DSA <6 months before crossmatch versus no pretransplant DSA	1.06 (0.59–1.89)	0.85
Pretransplant DSA at crossmatch versus no pretransplant DSA	1.45 (0.70–3.01)	0.32
Persistent DSA posttransplant versus no pretransplant DSA	1.45 (0.90–2.33)	0.12
Pretransplant DSA only versus no pretransplant DSA	0.37 (0.09–1.51)	0.17

CI, confidence interval; DSA, donor-specific antibodies; PRA, calculated panel-reactive antibody. Bold values = statistically significant at $p < 0.05$.

(relative risk = 2.26, p = 0.046), suggesting a confounding effect of other covariates. Similar multivariable analysis for HLA class, timing of detection or persistence/recurrence of pretransplant DSA did not show a significantly increased risk of 1-year AR for the DSA(+) versus DSA(−) groups (Table 3).

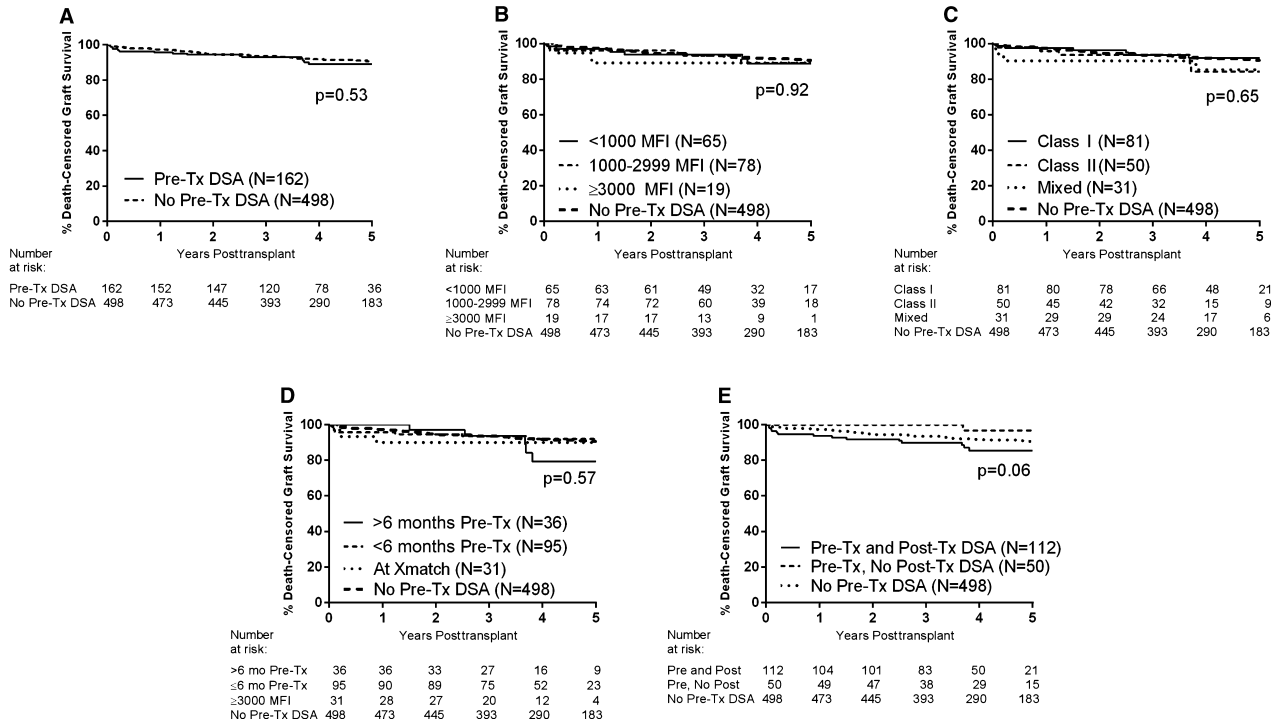

Figure 2: Five-year death-censored graft survival by (A) pretransplant DSA versus no pretransplant DSA, (B) strength of pretransplant DSA, (C) class of pretransplant DSA, (D) timing of pretransplant DSA, and (E) persistence of DSA posttransplant. DSA, donor-specific antibodies; MFI, mean fluorescence intensity; Post-Tx, posttransplant; Pre-Tx, pretransplant; Xmatch, crossmatch.

Graft survival

Figure 2 shows the 5-year Kaplan–Meier death-censored graft survival in patients with and without pretransplant DSA and in those with DSA as categorized by strength, class, timing, and persistence posttransplant. Five-year death-censored graft survival was similar when comparing DSA(+) patients (89.0%) vs. DSA(−) patients (90.6%, p = 0.53; Figure 2A) and when comparing various DSA strengths: <1000 (88.7%), 1000–3000 (89.2%), ≥3000 (89.2%), and no DSA (90.6%) (p = 0.92; Figure 2B).

The class of pretransplant DSA also did not correlate with 5-year death-censored graft survival when compared to the DSA(−) group (91.9% in class I vs. 84.2% in class II vs. 85.3% in mixed class I + II vs. 90.6% in the DSA (−) group, respectively; p = 0.65; Figure 2C), nor did the timing of pretransplant DSA detection (79.3% vs. 92.0% vs. 90.0% vs. 90.6% for DSA detected more than 6 months and less than or equal to 6 months pretransplant, detection at final crossmatch and no DSA, respectively; p = 0.57; Figure 2D). Those with persistence/recurrence of pretransplant DSA experienced a nonsignificant decrease in 5-year death-censored graft survival when compared to those with pretransplant DSA only versus DSA(−) (85.5% vs. 96.8% vs. 90.6%, respectively; p = 0.06; Figure 2E).

In multivariable analysis, the presence of pretransplant DSA (not otherwise characterized) was not associated with an increased risk of 5-year graft loss (hazard ratio = 0.97, p = 0.93; Table 4). Similar multivariable analysis for DSA strength, HLA class, timing of detection or persistence/recurrence did not show a significantly increased risk of 5-year graft loss for the DSA(+) group versus the DSA(−) group (Table 4).

Glomerular filtration rate (GFR) and proteinuria

Graft function was estimated by the MDRD GFR at 1, 2, 3, and 4 years posttransplant. Comparisons between time points with mixed-effects models showed lower eGFR in the DSA(+) cohort (overall) compared with the DSA(−) group at year 3 (54.0 mL/min vs. 58.3 mL/min, p = 0.050) and nonsignificant reduction at year 4 (52.5 mL/min vs. 57.1 mL/min, p = 0.12; Figure 3A). Similar analyses for DSA characteristics, including HLA class, timing of detection and persistence/recurrence of pretransplant DSA, did not show a significant risk of lower eGFR for overall comparisons between the DSA(+) and DSA(−) cohorts. There was a significant difference in eGFR between subgroups comparing maximum MFI strength at 3 years posttransplant (p = 0.028; Figure 3B) but not at 4 years posttransplant. However, only 9 of the initial 19 patients in this subgroup had data at this time point.

Table 4: Multivariable analysis of 5-year death-censored graft survival (adjusted for age, gender, ethnicity, donor type, PRA, HLA mismatches, induction therapy, and retransplant)

Variable	Hazard ratio (95% CI)	p-value
Pretransplant DSA versus no pretransplant DSA	0.97 (0.51–1.86)	0.93
Maximum pretransplant DSA strength <3000 versus no pretransplant DSA	0.95 (0.48–1.87)	0.88
Maximum pretransplant DSA strength ≥3000 versus no pretransplant DSA	1.15 (0.26–5.07)	0.85
Pretransplant DSA class I versus no pretransplant DSA	0.75 (0.31–1.81)	0.52
Pretransplant DSA class II or mixed versus no pretransplant DSA	1.25 (0.57–2.74)	0.59
Pretransplant DSA >6 months before crossmatch versus no pretransplant DSA	1.59 (0.61–4.11)	0.34
Pretransplant DSA <6 months before crossmatch versus no pretransplant DSA	0.66 (0.28–1.58)	0.35
Pretransplant DSA at crossmatch versus no pretransplant DSA	1.39 (0.41–4.75)	0.60
Persistent DSA posttransplant versus pretransplant DSA only or none	1.33 (0.67–2.63)	0.41

Separate models were used for each type of DSA occurrence (pretransplant yes/no, strength, class, timing, and persistence posttransplant). CI, confidence interval; DSA, donor-specific antibodies; PRA, calculated panel-reactive antibody.

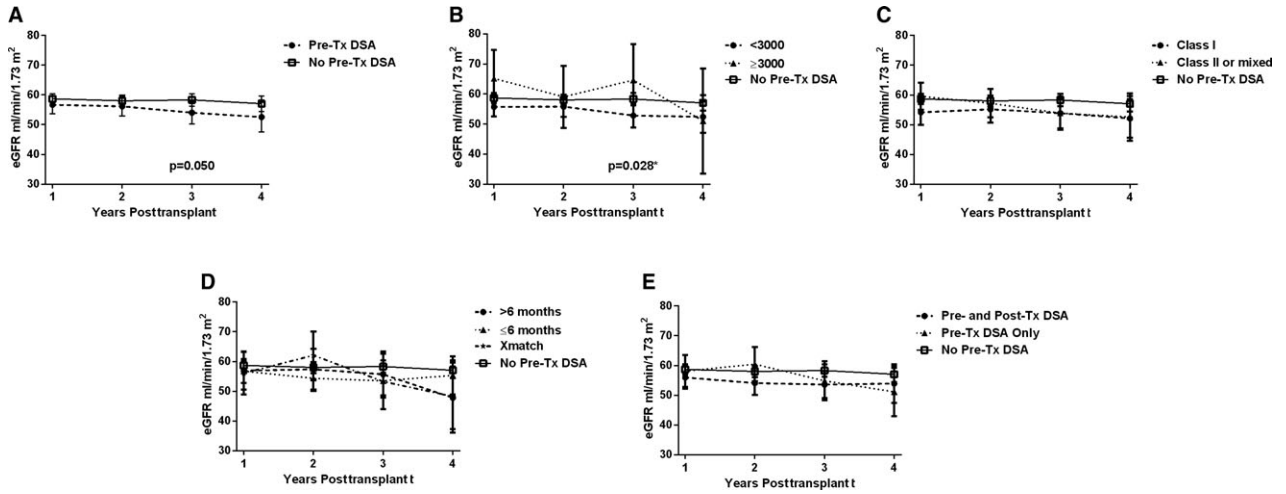

Figure 3: eGFR (± 95% CI) from 1 to 4 years posttransplant, (A) pretransplant DSA versus no pretransplant DSA, (B) by strength of pretransplant DSA, (C) by class of pretransplant DSA, (D) by timing of pretransplant DSA, and (E) by persistence of pretransplant DSA. Overall comparisons between DSA(+) subgroups and DSA(−) subgroups (Figures 3B–E) were only statistically significant for strength of DSA at 3 years posttransplant, but patients with MFI greater than or equal to 3000 did not have significantly lower eGFR than those without DSA. *Based on nine patients at 3 years with MFI greater than or equal to 3000 and eGFR data. CI, confidence interval; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; MFI, mean fluorescence intensity; Post-Tx, posttransplant; Pre-Tx, pretransplant; Xmatch, crossmatch.

Proteinuria analyses were performed at 1, 2, 3, and 4 years posttransplant using mixed-effects models to compare between time points. Four patients with a history of recurrent FSGS were excluded from the analysis. There was no significant difference in proteinuria over time between the DSA(+) cohort (not otherwise characterized) and the DSA(−) cohort (Figure 4A). There was a significant difference in proteinuria when comparing the subgroups with a maximum DSA strength of less than 3000 versus greater than or equal to 3000 versus DSA(−) ($p = 0.045$) at year 3 posttransplant; however, only 5 of the initial 19 patients with a maximum MFI strength greater than or equal to 3000 had data available at this time point. Similar analysis for DSA characteristics, including HLA class, timing of detection or persistence/recurrence of pretransplant DSA, did not show a significant risk of proteinuria (Table S3 shows the last available eGFR and proteinuria 1–5 years posttransplant).

Using a cutoff of 1000 DSA for defining the presence of pretransplant DSA and categorizing as 1000 to <2000, 2000 to <3000 and ≥3000 MFI did not show any differences in results or interpretation from the analysis using a cutoff of 500 MFI (data not shown).

Discussion

In this article, we describe the characteristics and clinical impact of pretransplant DSA in 660 consecutively transplanted kidney or kidney–pancreas recipients with a negative pretransplant FCXM in the absence of desensitization therapy. We found an incidence of

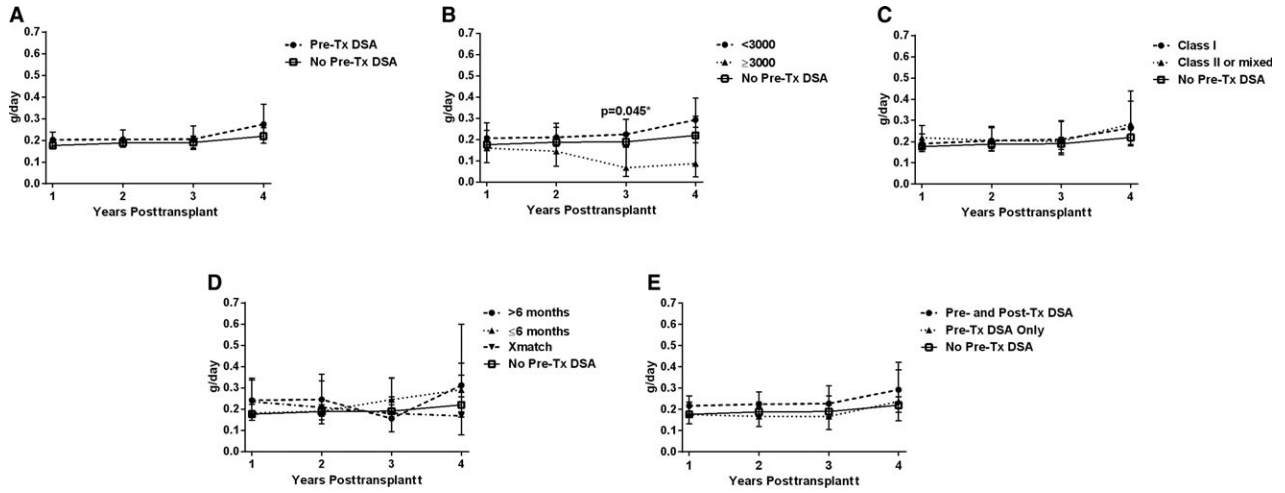

Figure 4: Proteinuria (± 95% CI) from 1 to 4 years posttransplant, (A) pretransplant DSA versus no pretransplant DSA, (B) by strength of pretransplant DSA, (C) by class of pretransplant DSA, (D) by timing of pretransplant DSA, and (E) by persistence of pretransplant DSA. Only the comparison between DSA strength in DSA(+) subgroups versus DSA(−) subgroups at 3 years posttransplant was statistically significant; however, patients in the ≥3000 subgroup had lower levels of proteinuria. *Based on five patients at 3 years with MFI greater than or equal to 3000 and proteinuria recorded. CI, confidence interval; DSA, donor-specific antibodies; MFI, mean fluorescence intensity; Post-Tx, posttransplant; Pre-Tx, pretransplant; Xmatch, crossmatch.

pretransplant DSA at any point prior to transplant in 25% of recipients. Taken in aggregate, pretransplant DSA with a negative FCXM did not significantly impact AR rates, graft function (GFR or proteinuria) or intermediate-term graft survival. While pretransplant DSA with MFI greater than or equal to 3000 (detected at any time prior to transplant) was associated with a significantly higher AR risk in multivariable analysis, this did not translate into inferior graft survival. To our knowledge, this study is the first to evaluate the clinical significance of historically identified FCXM-negative DSA and is the largest and most comprehensive study to date evaluating DSA in the setting of a negative FCXM. These data have important implications for pretransplant risk stratification and the utilization of desensitization therapies.

Several small studies have reported clinical outcomes in patients with pretransplant DSA in the setting of a negative FCXM with variable conclusions (1–7). The results of these studies were pooled in a recent meta-analysis (23), which included a total of 145 patients with pretransplant DSA and reported an increased incidence of AMR (25% vs. 11%) and a significant decrease in graft survival in the presence of pretransplant DSA versus those without. These data are limited, however, by small individual sample sizes, variable follow-up periods (ranging from 8 to 60 months) and inconsistent use of desensitization therapies. In addition to these limitations, this analysis was weighted heavily by the study by Willicombe et al (5) due to a sample size of 480 patients (44 with DSA). This study did not include B cell FCXM in the pretransplant screening process, and a majority of class II DSA was of strength likely to result in positive FCXM. By comparison, our study analyzed a significantly larger cohort (n = 660, 162 with DSA), and all patients included in the current study underwent consistent T cell and B cell FCXM screening prior to transplant, with none receiving desensitization therapy. This enables more accurate associations between nondesensitized FCXM-negative pretransplant DSA and graft outcomes, as well as additional analyses of pretransplant DSA subgroups that may be considered at higher or lower risk for poor outcomes.

Our ability to draw firm conclusions on the association between pretransplant DSA and longer-term graft outcomes is limited by the intermediate follow-up period (median of 4.2 years) of this study. However, proteinuria is associated with an increased risk of graft failure in patients with DSA and may be considered an early marker for eventual graft loss (7,24). We report similar degrees of proteinuria in patients with and without pretransplant DSA throughout the duration of our study. Interestingly, we also found a lower GFR in the DSA(+) group starting from 3 years posttransplant; however, this was of borderline significance at 3 years and did not reach statistical significance at 4 years. While this did not translate to inferior graft survival at 5 years, this finding warrants further study and follow-up, as GFR may also be considered a surrogate for future graft survival.

Attempts have been made in various studies to identify specific characteristics of pretransplant DSA associated with inferior graft outcomes. However, only a handful of studies have reported these findings in the setting

of a negative flow cytometry crossmatch. When considering the class of pretransplant DSA, Willicombe et al (5) report worse AMR-free survival in patients with class II or mixed-class antibodies compared to those without pretransplant DSA. Patients with reported AMR had higher MFI strength (mean 4089 vs. 2328 in those without, $p = 0.046$). While our data also associated a higher DSA MFI (≥3000) with increased AR risk in multivariable analysis, we did not find any significant association between DSA class and clinical outcome.

The clinical impact of posttransplant recurrence or persistence of pretransplant DSA on renal allograft outcomes has also been documented in a few studies with inconsistent findings. Patel et al (1) reported AMR in 50% (8/16 patients) of their patient cohort with recurrent pretransplant DSA. In contrast, Gupta et al (2) did not report increased risk of AMR or worse graft survival in their study population with recurrent pretransplant DSA ($n = 4$). Likewise, our study did not demonstrate an increased risk of 1-year AR nor worse 5-year graft survival after multivariable analysis in a considerably larger cohort ($n = 112$). The timing of the posttransplant persistence/recurrence of the DSA detection relative to AR onset was also not predictive of AR.

To our knowledge, there are no studies on the impact of pretransplant DSA strength or the effect of waxing/waning DSA (the timing of detection) in the setting of negative FCXM. Our study shows an increased 1-year risk of AR in patients with pretransplant DSA MFI greater than or equal to 3000 without an impact on 5-year death-censored graft survival. We did not find a significant association between DSA detection time relative to transplant and graft outcomes. In contrast, Lefaucheur et al (13) showed an increased risk of AMR when DSA is present both historically and at the time of final crossmatch compared to historical DSA only (40% vs. 27%); however, the patients in this study did not undergo FCXM testing.

Our study identified pretransplant DSA in 25% of FCXM-negative patients included in our cohort. This incidence has been variable (5–33%) in reports (1–7) and is due to multiple factors, including the use of different MFI cutoffs for HLA antibody positivity, the sensitivity and specificity of different antibody detection methods and the DSA HLA loci included in the analysis. Our incidence of 25% is higher than a number of reports of pretransplant DSA formation, likely due to our threshold of 500 MFI for positive results, and our comprehensive evaluation of mismatches at HLA-DP, -DQ, and -Cw loci. We believe that an MFI cutoff of 500 is justified, as MFIs as low as 100 have been reported as pathogenic (25). Increasing the cutoff to 1000 in a sensitivity analysis did not change our findings.

Strengths of this study include the number of patients with pretransplant DSA (162) not undergoing desensitization therapy and the consistent use of pretransplant FCXM screening. Our ability to analyze pre- and posttransplant production of DSA in patients receiving similar immunosuppression regimens consistent with modern treatment protocols and robust HLA antibody analyses with molecular typing for allele-level antibodies against HLA-A, -B, -C, -DRB1, -DPB1, DQB1- and DQA1 antigens supports the conclusions reached in this study. The main limitations to this study are the retrospective design, leading to potential inconsistencies in the capturing of clinical events, and a median follow-up of 4.2 years, which limits our ability to draw firm conclusions on longer-term graft survival. Protocol biopsies were not performed during the study inclusion dates, and we are unable to report subclinical pathology in this cohort. Despite the use of high-resolution typing to confirm donor specificity, the presence of denatured HLA antigens and the combinations of class II alpha and beta chains introduce the possibility of occasional false positivity.

In summary, the data presented in this report suggest that pretransplant DSA carries minimal clinical risk when associated with negative FCXM in a cohort of patients not receiving desensitization therapy. While acknowledging that further follow-up is required in order to draw firm conclusions regarding the risk of longer-term graft attrition, we do not recommend withholding transplant for patients falling into this category of pretransplant immunologic testing and suggest reserving desensitization therapy for those patients exhibiting higher degrees of donor-specific immunologic risk.

Acknowledgments

The authors would like to acknowledge the staff of ClinImmune Labs for assistance with the HLA analysis. The REDCap database is supported by National Institutes of Health (NIH)/National Center for Advancing Translational Sciences Colorado Clinical and Translational Science Award Grant UL1 TR001082. The contents of this article do not necessarily represent official NIH views.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1: Donor-specific antibody (DSA) specificity, mean fluorescence intensity (MFI) and point of assay (POA) for 84 patients with 0% panel-reactive antibody (PRA).

Table S2: Raw data on confirmed 1-year rejection.

Table S3: Last estimated glomerular filtration rate (eGFR) and proteinuria in the period 1–5 years posttransplant.