IgG Donor-Specific Anti-Human HLA Antibody Subclasses and Kidney Allograft Antibody-Mediated Injury

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ABSTRACT

Antibodies may have different pathogenicities according to IgG subclass. We investigated the association between IgG subclasses of circulating anti-human HLA antibodies and antibody-mediated kidney allograft injury. Among 635 consecutive kidney transplantations performed between 2008 and 2010, we enrolled 125 patients with donor-specific anti-human HLA antibodies (DSA) detected in the first year post-transplant. We assessed DSA characteristics, including specificity, HLA class specificity, mean fluorescence intensity (MFI), C1q-binding, and IgG subclass, and graft injury phenotype at the time of sera evaluation. Overall, 51 (40.8%) patients had acute antibody-mediated rejection (aABMR), 36 (28.8%) patients had subclinical ABMR (sABMR), and 38 (30.4%) patients were ABMR-free. The MFI of the immunodominant DSA (iDSA, the DSA with the highest MFI level) was 6724±464, and 41.6% of patients had iDSA showing C1q positivity. The distribution of iDSA IgG1–4 subclasses among the population was 75.2%, 44.0%, 28.0%, and 26.4%, respectively. An unsupervised principal component analysis integrating iDSA IgG subclasses revealed aABMR was mainly driven by IgG3 iDSA, whereas sABMR was driven by IgG4 iDSA. IgG3 iDSA was associated with a shorter time to rejection ($P<0.001$), increased microcirculation injury ($P=0.002$), and C4d capillary deposition ($P<0.001$). IgG4 iDSA was associated with later allograft injury with increased allograft glomerulopathy and interstitial fibrosis/tubular atrophy lesions ($P<0.001$ for all comparisons). Integrating iDSA HLA class specificity, MFI level, C1q-binding status, and IgG subclasses in a Cox survival model revealed IgG3 iDSA and C1q-binding iDSA were strongly and independently associated with allograft failure. These results suggest IgG iDSA subclasses identify distinct phenotypes of kidney allograft antibody-mediated injury.

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Antibody-mediated injury is the major determinant of kidney allograft failure[1,2] and represents a leading cause of ESRD in the United States and in Europe.[3,4] In recent years, the association between circulating donor-specific anti-human histocompatibility leucocyte antigen (anti-HLA DSA) and antibody-mediated rejection (ABMR) has been better characterized and has evolved from relation to causation.[5] Anti-HLA DSA may cause a wide spectrum of effects on the allograft, ranging from the absence of injury and no recognizable damage to indolent subclinical ABMR (sABMR) to full-blown acute ABMR (aABMR),[6] thus underlining the unmet need for the transplant community to identify distinct allograft phenotypes according to the characteristics of the recipient's

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anti-HLA DSA. Bridging the gap between the properties of circulating anti-HLA antibodies and the damage they induce in allografts could lead to a better understanding of the underlying humoral mechanisms and help to identify the harmful antibodies responsible for allograft loss.

In the last few years, the risk associated with anti-HLA DSA has progressively become better appreciated and, today, we are using some of their properties to improve the risk stratification for allograft loss. The strength of circulating anti-HLA DSA as expressed by mean fluorescence intensity (MFI)[7] and, more recently, their capacity to bind complement,[8–10] have been shown to be associated with poor allograft outcomes. However, these properties of circulating anti-HLA DSA may not distinguish among different rejection phenotypes. We recently observed that C1q-binding anti-HLA DSA were detected not only in recipients with aABMR but also in stable-state patients with sABMR on protocol biopsies and was associated with a high risk of allograft loss in both cases.[10]

Experimental data have suggested that antibodies exhibit different abilities to bind complement,[11,12] to recruit immune effector cells through the Fc receptor,[13] and to display different kinetics of appearance during the immune response[14] according to their IgG1–4 subclasses, revealing different pathogenicities of antibodies according to the profile of their subclasses. These properties have been translated into correlations between IgG subclass distribution and injury phenotypes in different pathologies, including in kidney diseases.[15–21]

To date, few studies have described the clinical relevance of IgG subclasses in solid organ transplantation. Post-transplant IgG3 DSA have been associated with poor allograft survival and chronic ABMR in liver recipients,[22] whereas no correlation was observed between the IgG subclass profile of pretransplant or post-transplant DSA and kidney transplant recipient outcome.[23,24] Moreover, there is no demonstration in the field of solid organ transplantation of the association of IgG1–4 profiles of circulating anti-HLA DSA with specific clinical and histologic phenotypes in the setting of antibody-mediated damage in allografts.

We hypothesized that anti-HLA DSA IgG subclasses might be associated with distinct clinical and histologic phenotypes of antibody-mediated injury. We conducted the present study to assess the distribution of IgG1–4 subclasses of circulating anti-HLA DSA and to define the spectrum of kidney antibody-mediated injury according to DSA IgG profiles. We thus aimed to integrate IgG subclasses in an extensive characterization of circulating anti-HLA DSA properties, including their strength and their complement-binding capacity.

RESULTS

Patient Characteristics

Among the 635 patients who underwent renal transplantation (356 at Necker Hospital and 279 at Saint-Louis Hospital) between January 2008 and January 2010 without pretransplant desensitization, 125 (19.7%) had circulating anti-HLA DSA within the first year after transplantation. The characteristics of our population at transplantation are summarized in Table 1.

Three distinct groups of patients were identified according to the clinical and histologic ABMR phenotypes: 51 (40.8%) patients had aABMR, 36 (28.8%) patients had sABMR, and 38 (30.4%) patients were ABMR-free. The baseline donor, recipient, and graft characteristics were similar across the three groups of patients (Table 1).

At the time of diagnosis, eGFR was 24.5±12.7 ml/min/1.73 m^2 in the aABMR group, 49.2±16.2 ml/min/1.73 m^2 in the sABMR group, and 57.5±19.6 ml/min/1.73 m^2 in the ABMR-free group ($P<0.001$).

Patients with aABMR had an increased microcirculation inflammation injury score (g+ptc score of 3.8±1.2) and a higher intimal arteritis score (0.6±1.0) compared with those of patients with sABMR (g+ptc score of 2.8±1.1 and intimal arteritis score of 0.1±0.5) and ABMR-free patients (g+ptc score of 0.2±0.5 and intimal arteritis score of 0) ($P<0.001$ and $P=0.03$, respectively). The patients with aABMR had a greater prevalence of C4d deposition in allograft peritubular capillaries (72.6%) than did the sABMR (27.8%) and ABMR-free patients (7.9%) ($P<0.001$). A description of the clinical and histologic characteristics of the three groups is provided in Supplemental Table 1.

The overall median follow-up after transplantation was 56.6 months (range, 3.4–76.0). The median follow-up times were 51.7 months (range, 3.4–73.3) in patients with aABMR, 60.0 months (range, 13.0–76.0) in patients with sABMR, and 59.7 months (range, 22.1–73.1) in ABMR-free patients.

IgG DSA Subclass Distribution

The distribution of immunodominant DSA (iDSA; the DSA with the highest MFI level) IgG subclasses revealed that IgG1 was positive in 94 (75.2%) patients, IgG2 in 55 (44.0%) patients, IgG3 in 35 (28.0%) patients, and IgG4 in 33 (26.4%) patients. In total, 65 (52.0%) patients presented a mixture of IgG subclasses, with 14 (11.2%) being IgG1+2, 13 (10.4%) IgG1+2+3, nine (7.2%) IgG1+2+3+4, 17 (13.6%) IgG1+2+4, eight (6.4%) IgG1+3, two (1.6%) IgG1+4, and two (1.6%) IgG2+4. Overall, 21 (16.8%) iDSA samples that were positive in the Luminex-IgG assay were negative for IgG1–4 subclasses (mean pan-IgG MFI of 1784.2±372.1). Figure 1 presents in detail all of the iDSA IgG subclass combinations in the entire cohort. The subclass distribution was similar between preformed iDSA ($n=98$, 78.4%) and *de novo* iDSA ($n=27$, 21.6%) (Supplemental Table 6).

In total, 52 (41.6%) patients had C1q-binding iDSA, 49 (94.2%) being positive for IgG1, 40 (76.9%) for IgG2, 26 (50%) for IgG3, and 23 (44.2%) for IgG4. Forty-seven (90.4%) had multiple IgG subclasses. C1q-binding iDSA had the following subclass profiles: IgG1+2+3+4 ($n=8$, 15.4%), IgG1+2+3 ($n=10$, 19.2%), IgG1+2+4 ($n=13$, 25%), IgG1+2 ($n=9$, 17.3%), IgG1+3 ($n=5$, 9.6%), IgG1+4 ($n=2$, 3.8%), IgG1 alone ($n=2$, 3.8%), and IgG3 alone ($n=3$, 5.8%). In total,

Table 1. Baseline characteristics of the study population according to the clinical and histologic ABMR phenotypes

	All patients with DSA *n*=125	aABMR *n*=51	sABMR *n*=36	ABMR-free *n*=38	*P* Value
Recipient age – mean±SD	49.5±12.0	50.7±12.4	48.4±11.2	48.9±12.3	NS
Recipient male sex – *n* (%)	51 (40.8)	24 (47.1)	13 (36.1)	14 (36.8)	NS
Transplant number – *n* (%)					NS
1	52 (41.6)	22 (43.1)	13 (36.1)	17 (44.7)	
2	55 (44.0)	21 (41.2)	16 (44.4)	18 (47.4)	
3	17 (13.6)	7 (13.7)	7 (19.4)	3 (7.9)	
4	1 (0.8)	1 (2.0)	0	0	
Time since dialysis (year) – mean±SD	5.4±4.3	5.9±4.2	4.9±4.6	5.1±4.1	NS
Recipient blood type – *n* (%)					NS
A	60 (48.0)	22 (43.1)	20 (55.6)	18 (47.4)	
B	13 (10.4)	5 (9.8)	4 (11.1)	4 (10.5)	
AB	3 (2.4)	1 (2.0)	2 (5.6)	0	
O	49 (39.2)	23 (45.1)	10 (27.8)	16 (42.1)	
CKD – *n* (%)					NS
Glomerulopathy	39 (31.2)	15 (29.4)	11 (30.6)	13 (34.2)	
Vascular nephropathy	11 (8.8)	6 (11.8)	3 (8.3)	2 (5.3)	
Chronic interstitial nephropathy	23 (18.4)	8 (15.7)	7 (19.4)	8 (21.1)	
PKD	12 (9.6)	6 (11.8)	3 (8.3)	3 (7.9)	
Malformative uropathy	5 (4.0)	1 (2.0)	2 (5.6)	2 (5.3)	
Diabetes	8 (6.4)	5 (9.8)	1 (2.8)	2 (5.3)	
Not determined	27 (21.6)	10 (19.6)	9 (25.0)	8 (21.1)	
Donor age (year) – mean±SD	51.7±15.4	53.3±15.0	49.5±15.0	51.7±16.4	NS
Donor male sex – *n* (%)	72 (57.6)	25 (49.0)	23 (63.9)	24 (63.2)	NS
Donor type – *n* (%)					NS
Deceased SCD	69 (55.2)	25 (49.0)	24 (66.7)	20 (52.6)	
Deceased ECD	50 (40)	24 (47.1)	10 (27.8)	16 (42.1)	
Deceased DCD	1 (0.8)	0	1 (2.8)	0	
Living	5 (4.0)	2 (3.9)	1 (2.8)	2 (5.3)	
Cold ischemia time (hour) – mean±SD	20.1±7.9	20.5±7.6	21.5±9.3	18.2±6.4	NS
HLA mismatch – mean±SD					
A	0.8±0.7	0.8±0.7	0.7±0.7	0.9±0.7	NS
B	1.2±0.7	1.2±0.7	1.1±0.8	1.2±0.8	NS
DR	1.0±0.6	1.1±0.6	0.8±0.6	0.9±0.7	NS
Historical cytotoxic PRA (%) – mean±SD	25.4±32.1	31.5±34.7	26.7±32.8	16.1±25.8	0.06
Induction therapy – *n* (%)					NS
Basiliximab	41 (32.8)	18 (35.3)	12 (33.3)	11 (29.0)	
ATG	84 (67.2)	33 (64.7)	24 (66.7)	27 (71.0)	
CNI – *n* (%)					NS
Tacrolimus	101 (80.8)	44 (86.3)	27 (75.0)	30 (79.0)	
Cyclosporine	24 (19.2)	7 (13.7)	9 (25.0)	8 (21.1)	
IMPDHi – *n* (%)	125 (100)	51 (100)	36 (100)	38 (100)	—

PKD, polycystic kidney disease; SCD, standard criteria donor; ECD, expanded criteria donor; DCD, donation after circulatory death; PRA, panel reactive antibody; ATG, anti-thymocyte globulins; CNI, calcineurin inhibitors; IMPDHi, inosine monophosphate dehydrogenase inhibitors.

10 patients (19.2%) had a complement-binding subclass profile (IgG1 and/or IgG3), and 42 (80.8%) had a mixed profile (IgG1 and/or IgG3 plus IgG2 and/or IgG4).

Identification of the Three Distinct Clinical and Histologic Patterns According to the Characteristics of Circulating DSA

The characteristics of circulating anti-HLA iDSA: iDSA HLA class specificity, iDSA MFI, iDSA C1q-binding capacity, and iDSA IgG1–4 subclasses showed a markedly heterogeneous distribution in our cohort (Supplemental Figure 1). Based on this distribution, principal component analysis (PCA) identified the three distinct clinical and histologic patterns: aABMR, sABMR, and ABMR-free patients (Figure 2A). The horizontal axis distinguishes the ABMR-free pattern from antibody-mediated injury (aABMR and sABMR, which were mainly driven by iDSA MFI, iDSA C1q-binding capacity, and iDSA subclasses IgG1–4). The vertical axis segregates aABMR and sABMR patterns, with aABMR mainly driven by iDSA IgG3, whereas sABMR was mainly driven by iDSA IgG4 subclass and HLA class II iDSA (Figure 2B).

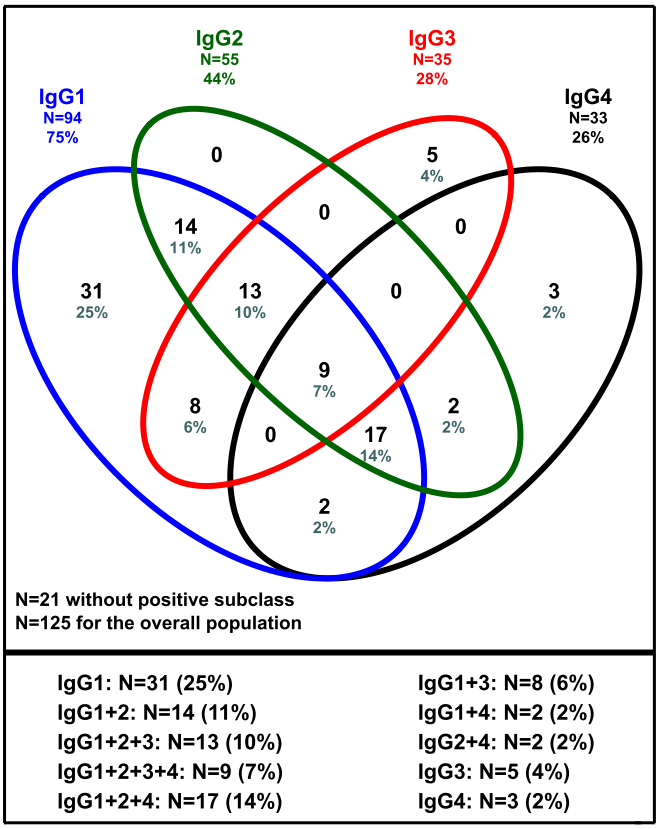

Figure 1. Distribution of IgG1–4 iDSA subclasses in the study population (Venn diagram).

Among the 51 patients with aABMR, the iDSA were HLA class I in 22 (43.1%) and HLA class II in 29 (56.9%) cases, and had a mean MFI of 7420±707 and a C1q-binding capacity in 31 (60.8%) cases. IgG1 was positive for 45 (88.2%), IgG2 for 27 (52.9%), IgG3 for 32 (62.8%), and IgG4 for eight (15.7%) iDSA.

Among the 36 patients with sABMR, the iDSA were HLA class I in six (16.7%) and HLA class II in 30 (83.3%) cases, and had a mean MFI of 9472±925 and a C1q-binding capacity in 20 (55.6%) cases. IgG1 was positive for 28 (77.8%), IgG2 for 25 (69.4%), IgG3 for three (8.3%), and IgG4 for 25 (69.4%) iDSA.

The iDSA of the 38 ABMR-free patients were HLA class I in 18 (47.4%) and HLA class II in 20 (52.6%) cases, and had a mean MFI of 3188±396 and a C1q-binding capacity in one (2.6%) case. IgG1 was positive for 21 (55.3%) and IgG2 for three (7.9%) iDSA. No IgG3 or IgG4 iDSA was found in ABMR-free patients.

IgG3 iDSA were more frequent in aABMR patients ($P<0.001$), whereas IgG4 iDSA were more frequent in the sABMR group ($P<0.001$), confirming the PCA analysis results.

The characteristics of circulating anti-HLA DSA in the three groups of patients are detailed in Table 2.

Evaluation of the Performance of IgG iDSA Subclasses to Identify Distinct Clinical and Histologic Allograft Injury Patterns

The detection of IgG3 iDSA and IgG4 iDSA showed an accurate discrimination of the three clinical and histologic phenotypes. The combination of IgG3 and/or IgG4 positivity showed a sensitivity of 67.8%, a specificity of 100%, a positive predictive value (PPV) of 100%, and a negative predictive value (NPV) of 57.6% to identify antibody-mediated injury (aABMR and sABMR). The sensitivity of IgG3 iDSA alone was 62.8%, the specificity 91.4%, the PPV 91.4%, and the NPV 62.8% to identify aABMR. IgG4 iDSA showed a sensitivity of 69.4%, a specificity of 91.0%, a PPV of 75.8%, and an NPV of 88.0% to identify sABMR.

Clinical and Histologic Characteristics of Patients with Antibody-Mediated Injury According to IgG iDSA Subclass Status

As IgG3 and IgG4 were the most discriminating IgG subclasses for identifying ABMR phenotypes, we studied their specific associations with allograft injury, allograft function, time to injury, and allograft loss.

Among the patients with antibody-mediated injury (aABMR and sABMR, n=87), we compared the clinical and histologic characteristics of patients with IgG3-positive iDSA to patients with IgG3-negative iDSA. The IgG subclass combinations in patients with IgG3-positive iDSA were IgG1+2+3+4: n=9 (25.7%), IgG1+2+3: n=13 (37.1%), IgG1+3: n=8 (22.9%), and IgG3: n=5 (14.3%), whereas those in patients with IgG3-negative iDSA were IgG1+2+4: n=17 (32.7%), IgG1+2: n=11 (21.2%), IgG1+4: n=2 (3.8%), IgG2+4: n=2 (3.8%), IgG4: n=3 (5.8%), IgG1: n=13 (25.0%), and no subclasses: n=4 (7.7%). IgG3 iDSA was associated with a shorter median time between transplantation and ABMR diagnosis ($P<0.001$), a lower eGFR at diagnosis (P=0.02), and a shorter median time to allograft failure ($P<0.001$). Histologically, individuals with IgG3 iDSA had higher scores of microvascular inflammation (P=0.002) and more frequent C4d deposition in allograft peritubular capillaries ($P<0.001$) (Table 3). Immunologically, IgG3 iDSA positivity was associated with the ability of iDSA to bind complement (P=0.02) (data not shown). In a sensitivity analysis, we confirmed that IgG3-positive iDSA remained associated with the same clinical and histologic characteristics independently of the IgG4 iDSA status (Supplemental Table 2).

When we compared patients with IgG4-positive iDSA (IgG1+2+3+4: n=9 [27.3%], IgG1+2+4 n=17 [51.5%], IgG1+4: n=2 [6.1%], IgG2+4: n=2 [6.1%], and IgG4: n=3 [9.1%]) to patients with IgG4 negative iDSA (IgG1+2+3: n=13 [24.1%], IgG1+2: n=11 [20.4%], IgG1+3: n=8 [14.8%], IgG1: n=13 [24.1%], IgG3: n=5 [9.3%], and no subclasses: n=4 [7.4%]), the detection of IgG4 iDSA was associated with later onset of injury (longer median time between transplantation and ABMR diagnosis [$P<0.001$] and better eGFR at diagnosis [$P<0.001$]). Histologically, patients with IgG4 iDSA exhibited more chronic lesions: allograft glomerulopathy ($P<0.001$)

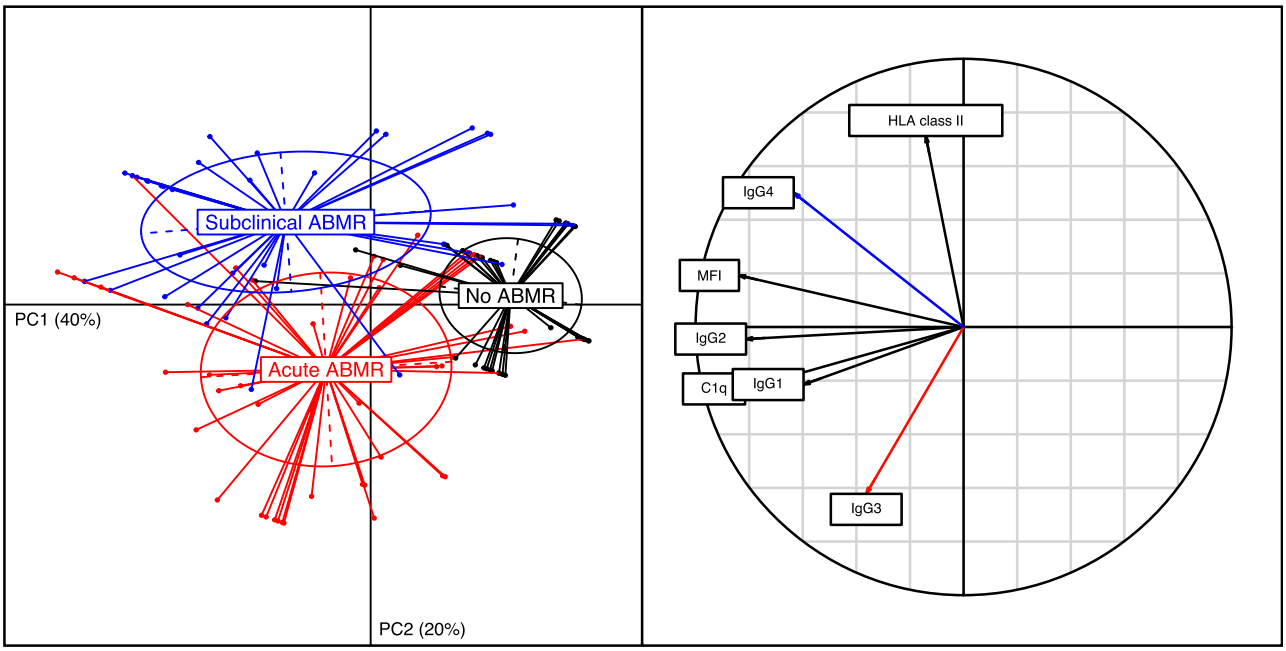

Figure 2. Identification of the three distinct rejection phenotypes according to the characteristics of the dominant donor-specific anti-HLA antibody (MFI, HLA class specificity, C1q-binding capacity, and IgG1–4).

Table 2. Characteristics of the donor-specific anti-HLA antibodies at the time of biopsy according to the clinical and histologic ABMR phenotypes

	aABMR *n*=51	sABMR *n*=36	ABMR-free *n*=38	*P* Value[+]	*P* Value[++]
Characteristics of all anti-HLA DSA					
Number – mean±SD.	2.5±1.5	2.3±1.4	1.8±1.2	NS	0.02
HLA class specificity – *n* (%)				0.02	<0.01
HLA class I	11 (21.6)	4 (11.1)	13 (34.2)		
HLA class II	14 (27.5)	21 (58.3)	15 (39.5)		
HLA class I + II	26 (51.0)	11 (30.6)	10 (26.3)		
Number of C1q-binding – mean±SD.	0.7±0.8	0.6±0.6	0.03±0.2	NS	<0.001
HLA class specificity of C1q-binding – *n* (%)				NS	NS
HLA class I	14 (45.2)	4 (20.0)	0		
HLA class II	16 (51.6)	15 (75.0)	1 (100)		
HLA class I+II	1 (3.2)	1 (5.0)	0		
Characteristics of iDSA					
HLA class specificity – *n* (%)				0.009	0.01
HLA class I	22 (43.1)	6 (16.7)	18 (47.4)		
HLA class II	29 (56.9)	30 (83.3)	20 (52.6)		
MFI – mean±SE.	7420±707	9472±925	3188±396	NS	<0.001
Preformed – *n* (%)	45 (88.2)	25 (69.4)	28 (73.7)	0.03	0.08
C1q binding – *n* (%)	31 (60.8)	20 (55.6)	1 (2.6)	NS	<0.001
IgG subclasses – *n* (%)					
IgG1	45 (88.2)	28 (77.8)	21 (55.3)	NS	0.002
IgG2	27 (52.9)	25 (69.4)	3 (7.9)	NS	<0.001
IgG3	32 (62.8)	3 (8.3)	0	<0.001	<0.001
IgG4	8 (15.7)	25 (69.4)	0	<0.001	<0.001
Multiple IgG subclasses – *n* (%)	35 (68.6)	27 (75.0)	3 (7.9)	NS	<0.001

P^{+} values are for the comparison between aABMR and sABMR.
P^{++} values are for the comparison among the three groups: aABMR, sABMR and ABMR-free.

and interstitial fibrosis/tubular atrophy (IF/TA) ($P<0.001$) (Table 4). IgG4-positive iDSA were more frequently HLA class II antibodies ($P=0.03$), had higher MFI ($P<0.001$), and were associated with IgG2 iDSA positivity ($P<0.001$) (data not shown). The sensitivity analysis showed that IgG4-positive iDSA remained associated with the same clinical and histologic characteristics independently of IgG3 iDSA positivity (Supplemental Table 3).

The clinical and histologic characteristics according to the IgG1 and IgG2 iDSA status are provided in Supplemental Tables 4 and 5.

Kidney Allograft Survival According to the IgG iDSA Subclass Status

Patients with IgG3 iDSA showed a significantly lower allograft survival than patients without IgG3 iDSA ($P<0.001$) (Figure 3). The four-year kidney allograft survival was 92.8% in patients without IgG3 iDSA and 61.4% in patients with IgG3 iDSA. There were no differences in kidney allograft survival based on IgG1, IgG2, or IgG4 iDSA status.

The association of circulating iDSA characteristics with allograft loss in univariate and multivariate Cox regression analyses is shown in Table 5. Anti-HLA iDSA IgG3 positivity and C1-binding capacity were independently associated with allograft loss (adjusted hazard ratio [HR]=4.8, 95% confidence interval [95% CI], 1.7–13.3, $P=0.003$ and adjusted HR=3.6, 95% CI, 1.1–11.7, $P=0.03$, respectively).

Table 3. Clinical and histologic characteristics according to the IgG3 status in the recipients with antibody-mediated injury ($n=87$)

	IgG3 positive $n=35$	IgG3 negative $n=52$	*P* Value
Clinical characteristics			
Time to diagnosis* (d) – median (Q1–Q3)	35 (17–93)	356 (33–368)	<0.001
eGFR at biopsy (ml/min/1.73 m^2) – mean ±SD	28.6±13.9	38.8±20.5	0.02
Time to failure (d) – median (Q1–Q3)	628 (400–778)	1638 (1115–1692)	<0.001
Histologic characteristics (Banff scores)			
g + ptc score – mean±SD	3.9±1.3	3.0±1.1	0.002
i + t score – mean±SD	1.5±1.8	1.0±1.2	NS
v score – mean±SD	0.5±0.9	0.3±0.9	NS
cg score – mean±SD	0.4±0.9	0.4±0.6	NS
IF/TA score – mean±SD	0.7±0.9	1.3±1.1	<0.01
cv score – mean±SD	1.2±1.0	1.5±1.0	NS
C4d deposition – *n* (%)	28 (80.0)	19 (36.5)	<0.001

*Time between transplantation and allograft biopsy with concomitant anti-HLA antibodies assessment

Table 4. Clinical and histologic characteristics according to the IgG4 status in the recipients with antibody-mediated injury ($n=87$)

	IgG4 positive $n=33$	IgG4 negative $n=54$	*P* Value
Clinical characteristics			
Time to diagnosis* (d) – median (Q1–Q3)	365 (351–370)	33 (15–100)	<0.001
eGFR at biopsy (ml/min/1.73 m^2) – mean±SD	46.7±19.0	27.4±14.4	<0.001
Time to failure (d) – median (Q1–Q3)	1643 (1085–1673)	835 (628–1151)	NS
Histologic characteristics (Banff scores)			
g + ptc score – mean±SD	3.2±1.2	3.5±1.2	NS
i + t score – mean±SD	0.9±1.3	1.4±1.6	NS
v score – mean±SD	0.2±0.6	0.5±1.0	0.08
cg score – mean±SD	0.7±0.9	0.1±0.5	<0.001
IF/TA score – mean±SD	1.5±0.9	0.7±1.0	<0.001
cv score – mean±SD	1.7±0.9	1.2±1.1	0.06
C4d deposition – *n* (%)	11 (33.3)	36 (66.7)	0.002

*Time between transplantation and allograft biopsy with concomitant anti-HLA antibodies assessment.

DISCUSSION

In the present study, we have defined relevant associations between IgG DSA subclass profile and the clinical and histologic phenotypes of antibody-mediated injury in kidney allografts: aABMR, sABMR, and ABMR-free. We have shown for the first time in kidney transplant patients that circulating immunodominant DSA subtypes permit the identification of distinct patterns of antibody-mediated injury. The main finding is that IgG3 and IgG4 subclasses of the immunodominant DSA are not only highly associated with antibody-mediated damage but also correlated with its phenotypes, namely, aABMR, and sABMR, respectively. We also showed that the presence of IgG3 DSA was associated with a greater risk of graft loss.

Our data confirm previous studies showing that IgG1 represents the predominant anti-HLA DSA subclass reported in the solid organ transplantation literature, which is variably associated with the other IgG subclasses (IgG2, 3, and 4).[12,22–28] The initial appearance of strong complement-fixing IgG1 and IgG3 antibodies may expand to weak or noncomplement-binding IgG2 and IgG4 antibodies; therefore, various profiles of IgG subclasses are seen in transplant recipients.[29] Previous studies have failed to firmly identify a predictive subclass pattern due to the heterogeneous IgG subclass response and to several technical[30–32] and methodological limitations, mainly related to the small number of recipients included[26,33,34] or the lack of precise clinical and histologic phenotyping of the ABMR injury.[23,24]

The integration of different properties of circulating anti-HLA DSA, including HLA class specificity, strength, complement-binding capacity and IgG subclasses, allowed

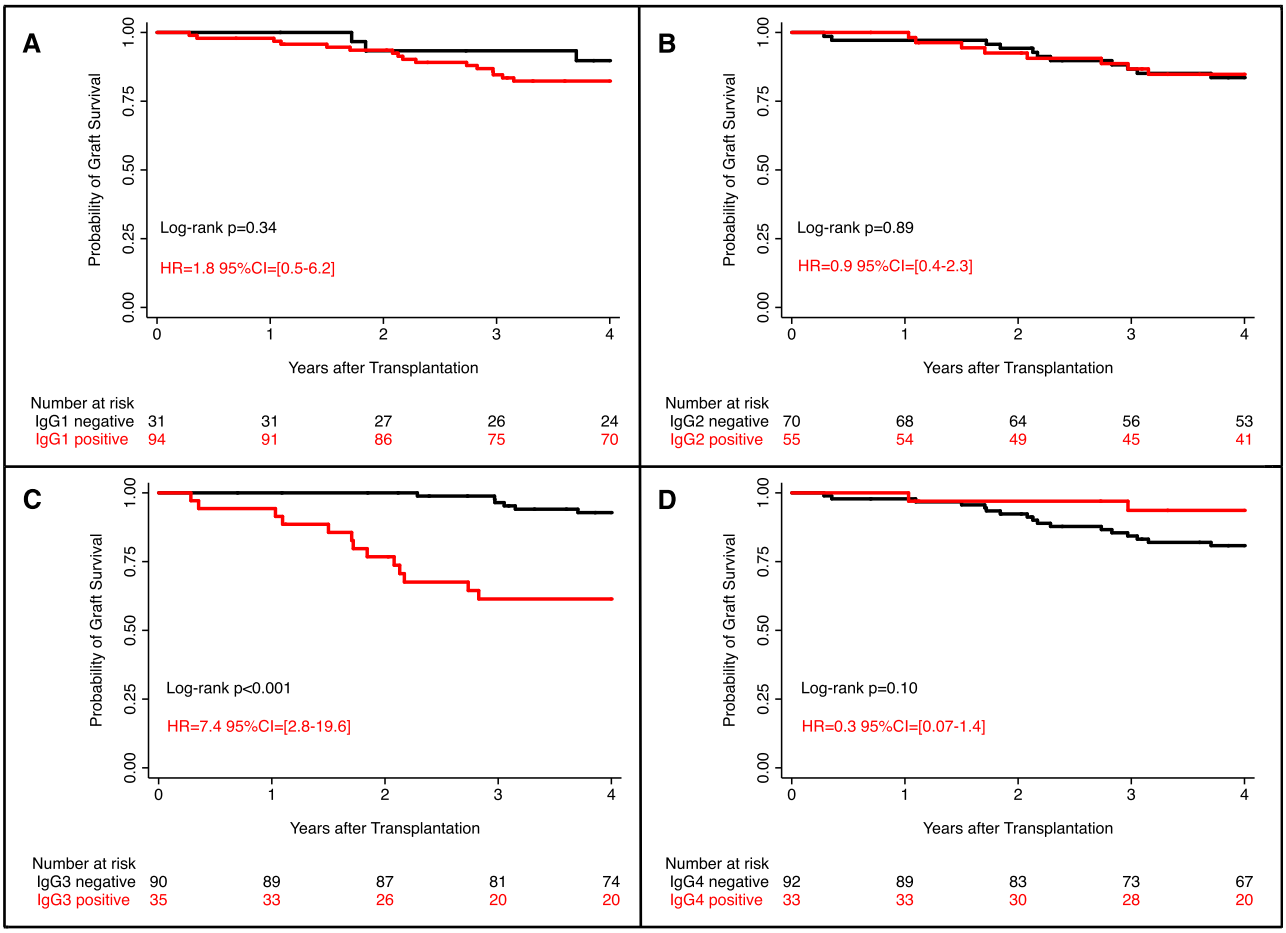

Figure 3. Kaplan–Meier curves for death-censored kidney allograft survival according to iDSA IgG1 (A), IgG2 (B), IgG3 (C), and IgG4 (D) subclass status.

us to identify three distinct clinical and histologic patterns of antibody-mediated injury, as recognized by the latest Banff classification: aABMR, sABMR, and ABMR-free.[35] The unsupervised PCA allowed us to hierarchically rank the variables that characterize these different patterns of antibody-mediated injury. Combining iDSA HLA class specificity, iDSA MFI, iDSA C1q-binding capacity, and iDSA IgG1–4 subclasses, we distinguished between ABMR-free and antibody-mediated injury. Not surprisingly, all IgG subclasses are involved in separating ABMR-free from antibody-mediated injury, as recipients with antibody-mediated injury had multiple IgG DSA subclasses, whereas ABMR-free patients had mostly IgG1 alone or no subclass detected. We also confirmed previous studies showing that DSA without any positive IgG subclass exhibit low pan-IgG MFI.[23] Among the patients with antibody-mediated injury, only the presence of IgG3 and/or IgG4 DSA was informative in distinguishing aABMR from sABMR.

Our study demonstrated that the presence of IgG3 and IgG4 performed well, with high specificity and high PPV, in identifying the three clinically and histologically distinct phenotypes of antibody-mediated injury. From a clinical perspective, the IgG subtype of circulating DSA may promote the need for allograft biopsy and may enhance the diagnosis of the type of antibody-mediated injury. Furthermore, based on the histologic findings, early and targeted treatment may be initiated before chronic irreversible damages occur. The identification of specific injury phenotypes related to IgG subclasses could provide a rationale to develop more specific therapeutic approaches such as B cell depletion with rituximab in patients with IgG4-associated ABMR.[36] Furthermore, the distinct immunologic effect of IgG DSA subclasses may explain why the current therapies used in the treatment of ABMR, including complement-targeting therapy,[37–39] can have varying results.

Our study provides new insight into the relationships between circulating anti-HLA DSA subtypes and kidney allograft injury and survival. Similarly to previous studies,[12,23,24,40] noncomplement-fixing IgG2 and/or IgG4 DSA alone had a low prevalence in our cohort (4.0%). In contrast, 44.0% of our patients exhibited a mixture of complement-fixing and noncomplement-fixing DSA. Until now, the expansion to noncomplement-fixing DSA was considered potentially beneficial by "blocking" the binding of complement-fixing DSA, which are considered more harmful.[29] In neonatal membranous glomerulopathy, only the presence of IgG4 alone was associated with protection.[41,42] However, in the

Table 5. Characteristics of circulating iDSA associated with kidney allograft survival

	N	Failures	HR	95% CI	*P* Value
Univariate Cox regression	125	19			
MFI					
continuous	125	19	1	0.99–1	0.16
C1q-binding					
No	73	4	1		
Yes	52	15	6.2	2.1–18.8	0.001
HLA class specificity					
HLA class I	46	5	1		
HLA class II	79	14	1.7	0.6–4.7	0.31
IgG1					
No	31	3	1		
Yes	94	16	1.8	0.5–6.2	0.35
IgG2					
No	70	11	1		
Yes	55	8	0.9	0.4–2.3	0.89
IgG3					
No	90	6	1		
Yes	35	13	7.4	2.8–19.6	<0.001
IgG4					
No	92	17	1		
Yes	33	2	0.3	0.07–1.4	0.12
Multivariate Cox regression	125	19			
C1q-binding					
No	73	4	1		
Yes	52	15	3.6	1.1–11.7	0.03
IgG3					
No	90	6	1		
Yes	35	13	4.8	1.7–13.3	0.003

presence of a mixture of complement- and noncomplement-fixing DSA, renal allograft survival was equally affected.[23,24]

In total, 91.4% (32/35) of the IgG3-positive DSA recipients had aABMR, which is characterized by intense microvascular inflammation and higher C4d deposition in allografts. This result supports the notion that this IgG subtype is associated with a greater ability to bind Fc receptor on monocytes, macrophages, and natural killer cells,[13] which are the main effector cells involved in ABMR.[43] We also showed that the presence of the IgG3 DSA subclass was associated with a greater risk of graft loss, independently of the DSA capacity to bind complement, suggesting the contribution of multiple effector mechanisms leading to graft loss.

The majority (25/33, 75.8%) of IgG4-containing DSA was present in patients with features of sABMR. Furthermore, patients with IgG4 DSA exhibited a specific phenotype of antibody-mediated injury that was characterized by the predominance of chronic features as represented by transplant glomerulopathy and IF/TA, supporting experimental data suggesting that complement fixation may not be required for chronic ABMR.[44,45]

This study has some limitations. On the one hand, the DSA characterization and injury phenotyping are limited to the first year after transplantation and therefore do not capture late ABMR. On the other hand, in this cohort, we were able to correlate the interplay between the IgG subtype and the early phenotype of graft injury without the background of chronic allograft features. Furthermore, future analyses should not only include the categorical variables of the presence or absence of IgG subtypes DSA but also integrate the strength of binding (MFI) and the kinetics of IgG subtypes. Nevertheless, the IgG subtype reagents must be optimized before this method can be reliably applied in clinical practice.

Our study provides a step forward in the development of risk stratification models based on the integration of the properties of anti-HLA antibodies (HLA class specificity, strength, complement-binding ability, IgG subclasses) and the related allograft injuries. Further studies are needed to determine the potential benefit and cost-effectiveness of implementing IgG subclasses assessment to the current transplant medicine practice.

In conclusion, advances in the characterization of circulating anti-HLA DSA have allowed us to identify distinct clinical and histologic patterns of antibody-mediated injury in kidney allografts. This study demonstrates for the first time, in a reasonably large and well phenotyped cohort of kidney recipients, the clinical relevance of IgG DSA subclasses and their association with the phenotype of antibody-mediated injury.

CONCISE METHODS

Study Population

We considered all consecutive patients who underwent kidney transplantation at Necker Hospital and Saint-Louis Hospital (Paris, France) between January 2008 and January 2010 (n=647). All transplantations were compatible with the ABO blood group with a negative T cell and B cell complement-dependent cytotoxicity crossmatch. We excluded patients who received pretransplant desensitization protocols (n=12).

We included all patients with circulating anti-HLA DSA detected in the first year after transplantation (n=125). All enrolled patients were tested for anti-HLA DSA characteristics: DSA specificity, DSA HLA class specificity, DSA MFI level, DSA C1q-binding capacity, and DSA IgG1–4 subclasses, at the time of rejection in patients who experienced an acute rejection during the first year post-transplant or at 1-year post-transplant in patients without acute rejection. The kidney allograft injury phenotype was assessed at the time of sera evaluation. The study design is detailed in Figure 4.

Clinical Data

Clinical data from Necker Hospital regarding donor and recipient were extracted from the DIVAT clinical prospective cohort (official website: www.divat.fr). Data from Saint-Louis Hospital were excerpted from the French national registry agency (Agence de la Biomédecine) database CRISTAL (official website: www.sipg.sante.fr/portail/). Each patient from the present study provided written informed consent to be included in the DIVAT and CRISTAL database networks. These registries are approved by the National French Commission for bioinformatics data and patient liberty (DIVAT: CNIL, Registration number: 1016618,

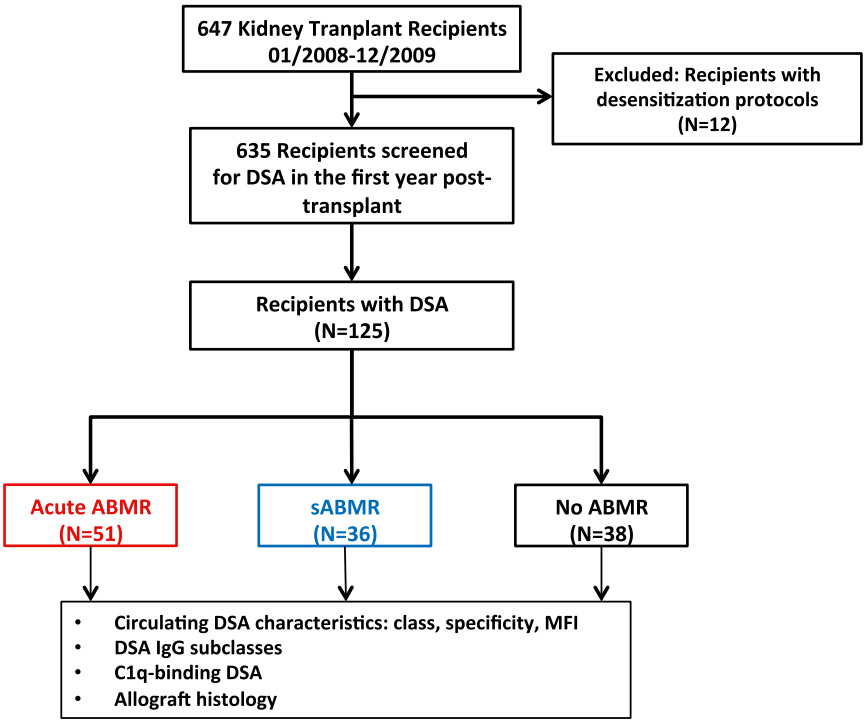

Figure 4. Flow chart of the study population.

validated June 8, 2004; CRISTAL: CNIL, Registration number: 363505, validated April 3, 1996). Coding was used to ensure strict donor and recipient anonymity. The data are computerized in real time and at each transplant anniversary and are submitted for an annual audit. All data were retrieved on July 31, 2014. The study was approved by the Institutional Review Boards of Necker Hospital and Saint-Louis Hospital.

Delayed graft function was defined as a dialysis requirement during the first week after transplant (except for a single dialysis session for hyperkalemia or volume overload). Renal function was assessed by the eGFR with the abbreviated Modification of Diet in Renal Disease (MDRD) formula[46] at the time of biopsy, at 1 year post-transplant and at the final follow-up.

The immunosuppression protocols and treatment of allograft rejections after transplantation were similar between the centers. The protocols and treatments are described in the Methods section in the Supplemental Appendix.

Kidney Allograft Histology

We included the biopsies performed at the time of rejection in patients who experienced an acute rejection during the first year post-transplant or at 1-year post-transplant in patients without acute rejection.

Renal tissue from the biopsies was fixed in acetic-formol-absolute alcohol fixative and stained with Masson's trichrome and periodic acid–Schiff. All of the graft biopsies were scored and graded from 0 to 3 according to the updated Banff criteria[35,47–49] for the following histologic factors: glomerulitis (g), tubulitis (t), mononuclear cell interstitial inflammation (i), intimal arteritis (v), peritubular capillaritis (ptc), allograft glomerulopathy (cg), IF/TA, arteriolar hyaline thickening (ah), and vascular fibrous intimal thickening (cv). C4d staining was performed by immunochemical analysis on paraffin sections using polyclonal human anti-C4d antibodies (Biomedica Gruppe).

All graft biopsies were scored and graded by trained pathologists (J-P.D.v.H., J.V.) who were unaware of the patients' clinical and immunologic status.

Definition of Antibody-Mediated Injury Phenotypes

Kidney allograft rejection was diagnosed according to the most recent international Banff classification.[35] aABMR was defined by acute renal failure (serum creatinine increase >15% compared with the baseline values after transplantation) or delayed graft function and (*1*) histologic evidence of acute tissue injury, including one or more of the following: microvascular inflammation (g>0 and/or ptc>0), intimal or transmural arteritis (v>0), acute thrombotic microangiopathy in the absence of any other cause or acute tubular injury in the absence of any other apparent cause, and (*2*) evidence of current antibody interaction with vascular endothelium with linear C4d staining in peritubular capillaries (C4d>0) or moderate microvascular inflammation (g+ptc≥2).

sABMR was defined by stable renal function and histologic evidence of acute tissue injury and evidence of current antibody interaction with vascular endothelium as previously defined on a 1-year protocol biopsy. Stable renal function was defined as variability in serum creatinine not exceeding 15% of the baseline values within a period of 21 days before the biopsy.[50]

The absence of ABMR (ABMR-free) was defined by stable renal function as previously described and the absence of glomerulitis, peritubular capillaritis, intimal arteritis, thrombotic microangiopathy, and allograft glomerulopathy on a 1-year protocol biopsy.

Detection and Characterization of Donor-Specific Antibodies

Detection of Donor-Specific Antibodies in the Overall Cohort (n=635)

All kidney transplant recipients were tested for circulating donor-specific anti-HLA-A, -B, -Cw, -DR, -DQ, and -DP antibodies in serum samples obtained 1 year after transplantation or during an episode of acute rejection within 1 year after transplantation. Single-antigen flow bead assays were used (One Lambda, Inc., Canoga Park, CA) on a Luminex platform. All beads showing a normalized MFI greater than 500 were considered positive. The iDSA was considered to have the highest MFI.

HLA typing of the recipients was performed by molecular biology (Innolipa HLA typing kit; Innogenetics, Belgium). For all kidney transplant donors, tissue typing was initially performed using the microlymphocytotoxicity technique with One Lambda Inc. tissue typing trays at transplantation and was confirmed by molecular biology.

Characterization of Donor-Specific Antibodies in the Study Population (n=*125*)

Serum samples from patients with circulating anti-HLA DSA were analyzed in a blinded fashion at the University of Pittsburgh for the presence of C1q-binding anti-HLA DSA and for the presence of IgG1–4 subclasses.

The presence of C1q-fixing anti-HLA donor-specific antibodies was assessed using single-antigen flow bead assays according to the manufacturer's protocol (C1q Screen TM, One Lambda, Inc.).[10]

The IgG subclass assay was performed as reported previously.[23] The standard single-antigen assay was modified, replacing the phycoerythrin-conjugated anti-pan human IgG reporter antibody with monoclonal antibodies specific for IgG1–4 subclasses (IgG1 clone HP6001, IgG2 clone 31–7-4, IgG3 clone HP6050, IgG4 clone HP6025; Southern Biotech). In brief, 10 μl of patient serum was mixed with 2.5 μl of beads (LABScreen, One Lambda, Inc.) for 30 minutes in the dark at room temperature. The beads were washed with 175 μL 1× wash buffer (One Lambda, Inc.), then centrifuged for 5 minutes at 1800 g. After discarding the supernatant, two more washes with 200 μL 1× buffer were performed. Then, 25 μl of each appropriately diluted phycoerythrin-labeled anti-IgG1–4 subclass reporter antibody was added (concentrations: anti-IgG1=5 μg/ml; anti-IgG2=5 μg/ml; anti-IgG3=20 μg/ml; anti-IgG4=20 μg/ml) and incubated for 30 minutes in the dark at room temperature. After three wash steps, 70 μl of phosphate-buffered saline was added, and the data were acquired on the Luminex 100 analyzer.

The background reactivity was evaluated by testing four negative control sera from healthy, non-sensitized anti-HLA antibody-negative males. We determined for each IgG subclass antibody and each bead the mean of the four control sera + five standard deviations and if it was below 500 MFI the IgG1-4 reactivity was considered positive if the normalized MFI was ≥500. For rare beads less than 5% the positive value was considered >500 MFI based on the mean of control sera + five standard deviations.

For each patient, we evaluated the number, HLA class specificity, MFI, and the C1q-binding capacity of all detected anti-HLA DSA, and for iDSA, we also considered the IgG1–4 subclasses. The specificity of the iDSA based on pan-IgG reactivity was applied to the other tests including C1q binding and IgG subtype analysis.

Pretransplant (day 0) sera were retrospectively assessed by single-antigen flow bead assays (One Lambda, Inc.) on a Luminex platform to identify the preformed anti-HLA DSA.

One Lambda donated reagents but was not otherwise involved in either the conduct of the study or the preparation of the manuscript.

Statistical Analysis

Mean±SD values and frequency are provided for the description of the continuous and categorical variables, respectively, with the exception of MFI, for which we used the mean±SE because of its wide distribution. The means and the proportion were compared using *t*-test and the chi-squared test (or Kruskal–Wallis and Fisher's exact test if appropriate, respectively). A Bonferroni correction for several tests was used when performing two-by-two comparisons.

To address the hypothesis that DSA characteristics are associated with distinct rejection profiles, we performed unsupervised methods, such as hierarchical cluster analysis and a PCA, based on a combination of the following anti-HLA iDSA characteristics: iDSA HLA class specificity, iDSA MFI, iDSA C1q-binding ability, and iDSA IgG1–4 subclasses. PCA analyses produced two main results: a correlation circle and a projection of the individuals. The correlation circle allows for a graphical examination of the relationships among the parameters and the graphical parameter contribution of the axes (positive or negative contribution: vector direction; strength of the contribution: vector length when projected on the axis). The projection of the individuals allows for the graphical localization of aABMR, sABMR, and ABMR-free patients on the two axes that were previously defined using the correlation circle.

Kidney allograft survival was assessed by the Kaplan–Meier method and compared among the groups with the log-rank test. A follow-up was calculated using a reverse Kaplan–Meier estimation. Kidney allograft survival was calculated from the date of transplantation to the date of allograft loss. In the case of death with a functioning graft, graft survival was censored at the time of death. Cox proportional-hazards models were used to estimate the HRs and 95% CIs for kidney allograft loss.

The association of immunologic factors (iDSA HLA class specificity, iDSA MFI, iDSA C1q-binding capacity, and iDSA IgG1–4 subclasses) with allograft loss was assessed with univariate Cox regression analyses. A *P* value threshold of <0.05 was used to select factors that were then entered into a single multivariate Cox model.

Values of $P<0.05$ were considered statistically significant, and all tests were two-sided. All analyses were performed using STATA version 12 and R version 2.15.2 (R Development Core Team; http://www.r-project.org). Hierarchical cluster analysis and dendrograms were performed with the hcluster module of the amap package of R, and PCA was performed with the dudi.pca module of the ade4 package (version 1.5–1) of R.

DISCLOSURES

None.

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