



## Clinical Implication of Non-Complement-Binding Donor-Specific Anti-HLA Antibodies in Heart Transplant Recipients -Risk Stratification by C1q-Binding Capacity

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### Abstract

**Background:** The development of de novo human leukocyte antigen (HLA) donor specific antibodies (DSA), detected by either cytotoxic or solid phase assays, was considered the major risk factor for cardiac graft failure in heart transplantation. However, it was shown that not all patients with persistent production of DSA suffered loss of their allografts. The ability to activate complement may be an important factor differentiating clinically relevant DSA from non-relevant DSA. Recently, a C1q-binding assay (C1qScreen; One Lambda, Inc. Canoga Park, CA) has been developed to identify complement-fixing HLA antibodies with high sensitivity and specificity. The aim of this study was to investigate the association between C1q-binding ability of HLA-DSA and the clinical outcomes post-transplant to identify clinically significant DSA after heart transplantation.

**Methods:** We enrolled 64 consecutive patients who received heart transplant between May 1999 and January 2015 in our institute. Sixty of 64 patients (93.7%) were screened for the presence of circulating DSA using Luminex Single Antigen Flow Bead assays between June 2014 and August 2015, and patients with post-transplant DSA with mean fluorescence intensity (MFI) >500 were selected to assess C1q fixation by C1q-binding assays. The clinical outcomes were compared with the results.

**Results:** Of 60 patients, twelve patients were considered as DSA positive (MFI >500, range 698-5952, class 1: 75% class 2: 17%, class1+2: 8%). All of these patients were identified as C1q negative. As the results, we divided into two groups; group C1q negative DSA (n=12) and group non-DSA (n=48). The rejection episodes, cardiac events, mortality, the development of cardiac graft vasculopathy and cardiac function were not statistically different between the two groups.

**Conclusion:** Patients producing C1q-negative DSA had good graft survival, which was comparable to that of DSA negative patients. Adding the assessment of the complement-binding capacity of DSA might redefine the traditional risk stratification of DSA positive patients after heart transplantation.

**Keywords:** Heart transplantation; Donor specific antibodies; Antibody mediated rejection; C1q; Complement-binding ability

### Introduction

Donor-specific anti-HLA antibodies (DSA) in heart transplantation (HTx) are associated with increased cardiac allograft injury and loss, including vascular injury and graft dysfunction [1-3]. The recent introduction of highly sensitive and specific techniques for the detection of anti-HLA antibodies, such as the Luminex single-antigen bead (SAB) assay, has increased our ability to identify sensitized patients and to define their immunological risk before and after transplantation. Although this new technology is highly sensitive, the clinical impact of DSA detected only by the solid phase assay is still controversial.

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Various studies have indicated an increased risk of antibody-mediated rejection (AMR) and an inferior outcome of cardiac transplant recipients who developed DSA that was detected only with the SAB assay and not with complement-dependent cytotoxicity (CDC) or flow cytometric assays [3-5]. However, it is also known that not all patients with persistent production of DSA suffer loss of their allografts, indicating that DSA are not equal in terms of their detrimental effects on allograft dysfunction probably due to AMR.

Since complement activation by antibodies determines the cytotoxic potential of these DSA, assessment of complement fixing ability may be important for the characterization of clinically relevant DSA.

A new solid-phase C1q-binding immunoassay (C1qScreen; One Lambda, Inc. Canoga Park, CA) has been developed to identify the C1q fixation capacity of anti-human leukocyte antigen (HLA) antibodies, which is the first step in the activation of the classical complement cascade. AC1q-positive de-novo DSA has been shown to be associated with an increased rate of AMR and transplant glomerulopathy in kidney transplantation [6-9]. However, the prevalence and clinical significance of DSA characterized by C1q-binding have not been well investigated in adult heart transplant patients.

The aim of this study was to investigate the association between the C1q-binding ability of DSA and clinical outcomes post-transplant, in order to elucidate clinical significance of DSA in heart transplantation.

## Methods

### Study population

In this population-based study, we enrolled all 60 consecutive patients who underwent heart transplantation at the National Cerebral and Cardiovascular Center, Japan, between May 1999 and January 2015, and who underwent the SAB assay of antibodies against HLA. In Japan, no patient with a positive prospective cytotoxic cross match against the donor T lymphocytes undergoes heart transplantation, according to the national rule for recipient selection. The stored patient sera that were sampled between June 2014 and August 2015 were retrospectively analyzed using a C1q-binding assay. All patients included in this study were followed for at least one year after HTx. This study was approved by the local Ethics Committee in our institute and was in compliance with local laws and regulations.

### Immunosuppression and follow-up

After transplantation, all patients received a standard triple-drug combination immunosuppressive therapy consisting of a calcineurin inhibitor (cyclosporine or tacrolimus), mycophenolate mofetil (MMF) and prednisolone (PSL). Induction therapy with monoclonal or polyclonal antibodies, such as the murine monoclonal anti-CD3 antibody (OKT3R) or an anti-IL-2 monoclonal antibody (basiliximab), was also used in patients with renal dysfunction in the perioperative period. Steroids were routinely withdrawn over time unless there was a major rejection event or there was granulocytopenia caused by other immunosuppressive drugs. Everolimus with reduced calcineurin inhibitor was used since 2007 after transplantation in patients with renal dysfunction and/or transplant cardiac allograft vasculopathy (CAV).

After transplant, endomyocardial biopsies were performed at 1, 2, 3, 5, 7 and 11 weeks, at 4, 5, 6, 9, and 12 months, then every 6 months for the next 4 years, following which they were performed

annually. Endomyocardial biopsies were also performed whenever acute cellular rejection (ACR) or AMR was clinically suspected. Histopathological results were based on the International Society for Heart and Lung Transplantation (ISHLT) standardized cardiac biopsy grading. Additionally, AMR was monitored by performing the flow cytometric panel reactive antibody (Flow PRA) test, or the SAB assay, to determine the presence of DSA, and by analyzing pathological findings that were monitored by immunohistochemical staining of endomyocardial biopsy tissue for analysis of parameters such as C4d staining and CD68 positive cells. Coronary angiography and intravascular ultrasonography (IVUS) were performed within the first to eleventh week to define a donor transmitted coronary arterial disease, at 12 months and on an annual basis thereafter after HTx. All the angiograms and IVUS findings were reviewed for evidence of CAV. Diagnosis of CAV was based on a minimum of 0.5 mm progression on a maximum intimal thickness (MIT) from the baseline on IVUS.

All outpatients were scheduled to be followed at least once a month after HTx to check their clinical condition by performing an electrocardiogram (ECG) and several laboratory tests including analysis of the trough level of immunosuppressive drugs. Echocardiography and coronary flow reserve (CFR) tests were conducted every 6 months in every patient.

### HLA typing

All recipients and donors were typed for HLA-A, HLA-B, HLA-C and HLA-DR using the Luminex assay system and HLA typing kits (WAKFlow HLA Typing kits, Wakunaga, Osaka, Japan). In Japan, prospective cytotoxic cross matching is performed by the Japan Organ Transplant Network to select recipients and any patient with a positive T lymphocyte cross match is not selected. Non-cytotoxic flow cytometric cross matching was also performed in our institution in every transplant patient.

### Detection and characterization of donor specific antibodies

Monitoring of de novo DSA production had been prospectively performed on the serum samples that were collected at regular intervals after transplantation. We performed a Flow PRA test every day after HTx for 2 weeks, then once a week thereafter until discharge, unless there was clinical suspicion for rejection. For outpatients, this test was performed at least once every 3 months in normal outpatients and every month in highly sensitized patients.

Every patient was screened using Flow PRA class 1 and class 2 Screening beads. To determine the donor specificity of detected antibodies, positive sera had been previously tested using SAB assays (LAB Screen Class 1 and 2 Single Antigen Beads, One Lambda, Inc.) according to the manufacturer's instructions. Analysis was performed using Fusion software (One Lambda, Inc.). Anti-HLA antibodies with mean fluorescence intensity (MFI) values greater than 500 at any point after HTx were considered positive. Transient DSA that temporarily appeared and then disappeared without any medical intervention were not considered to be positive DSA. Patients with positive DSA were selected for assessment of C1q fixation using C1q-binding assays (Figure 1). Non-HLA specific antibodies and IgG types were not analyzed in this study.

### Characterization of DSA by C1q assay

In patients with positive flow PRA, the ability of the DSA to fix complement was determined using SAB and C1q screen kits (One

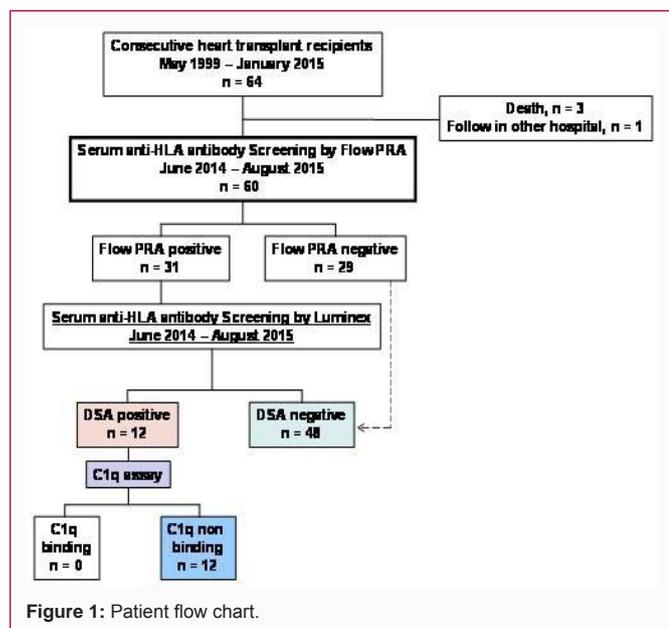


Table 1: Clinical and demographic Characteristics of the study population (n=60).

Variable	n=60
Age at HTx (yr), mean ± SD	36.1 ± 11.3
Sex (n)	
Male	47 (78.3%)
Female	13 (21.6)
Etiology (n)	
DCM	43 (80%)
dHCM	8 (13.3%)
ICM	2 (3.3%)
others	7 (11.6%)
LVAD before HTX (n)	54 (90%)
waiting period as status 1 (days)	851 ± 305
donor age (year)	40.4 ± 12.8
ischemic time (minutes)	196 ± 34
time after HTX (years)	6.7 ± 4.1

Data expressed with plus/minus sign are the mean ± SD, HTX: Heart Transplantation; DCM: Dilated Cardiomyopathy; dHCM: Dilated Hypertrophic Cardiomyopathy; ICM, Ischemic Cardiomyopathy; LVAD: Left Ventricular Assist Device

DSA status. Chi-square analysis or Student’s t-test were used for comparison of variables between groups, as appropriate. Statistical significance was set at p< 0.05. The Kaplan-Meier method was used to calculate cardiac event-free survival and pAMR event-free survival. Statistical comparisons of survival curves according to DSA status were made using Wilcoxon’s rank test. All data were analyzed using JMP version 12.0 (SAS Institute, Cary, NC, USA).

## Results

A flow chart of the study population is shown in Figure 1, and the demographics and characteristics of the patients are summarized in Table 1. Of 64 consecutive patients who received a HTx between May 1999 and January 2015, 47 (78.3%) were men, with a mean age of 36.1 ± 11.3 years (range, 17 years to 60 years) at transplant. The mean time of follow-up of patients was 6.7 ± 4.1 years (range 1.2 years to 16.3 years). Of the 64 patients, 60 (93.7%) were screened for HLA-antibodies using Flow PRA during the study period. Thirty-one (51.6%) patients were Flow PRA positive against donor HLA. Ultimately, 12 patients were considered as DSA positive. The DSA positive patients were screened with the C1q assay, and none of them were identified as C1q positive. No patient lost a graft during the study period, except for 3 patients who died and were excluded from the study (Figure 1).

Based on these findings, we divided the patients into two groups; a DSA positive, C1q negative group (n=12; C1q negative DSA group) and a DSA negative group (n=48; non-DSA group). Comparison of the clinical information of the two groups is shown in Table 2. The follow-up period, rejection episodes, cardiac events, all-cause mortality, development of CAV and cardiac function were not statistically different between the two groups.

The characteristics of the DSA found in the serum samples of the 12 DSA positive patients are summarized in Table 3. Of these patients, 75% (n=9) had only class1 DSA; 8.3% (n=1) had only class 2 DSA; and 16.6% (n=2) had both class1 and class 2 DSA. Fifty percent (n=6) of the patients with DSA were treated with triple immune suppressive therapy (IST) including steroids, whereas only 10.4%

Lambda Inc.) according to the manufacturer’s instructions. The same SAB batch was used to detect pan-IgG and C1q-binding anti-HLA antibodies in any given serum. Although the C1q assay can detect IgG and non-IgG antibodies, all patients were primarily of the IgG subtype because the sera tested with the C1q assay were selected on the basis of the presence of IgG-DSA. Data were analyzed using MFI values, and the cut-off for a positive reaction was set as an MFI value greater than 500. Positive and negative control sera were included in each test.

## Allograft biopsies

Allograft biopsy surveillance was standardized and performed in all patients as follows. All endomyocardial biopsy specimens were graded based on the standard biopsy grading scheme, according to the 1990 ISHLT classification [10,11]. ISHLT grade 3A or higher rejection was usually treated with steroid pulse therapy. If there was resistance to pulse therapy, cytolytic therapy consisting of monoclonal or polyclonal antibodies was instituted. A rejection episode ≥ISHLT grade 2 that occurred any time during the follow up period was analyzed as a variable in this study. Diagnosis of AMR was made based on the 2011 ISHLT consensus as follows: histological evaluation of endothelial activation, intravascular macrophages and capillary destruction. Additionally, immunofluorescence (for C4d analysis) and immunoperoxidase (for CD68 analysis) staining of endomyocardial biopsy tissue were done for evaluation of AMR. Pathological AMR (pAMR) grading categories were: pAMR 0, negative; pAMR 1, (I+) immunohistological AMR alone, (H+) histological AMR alone; pAMR 2, pAMR that was both (H+) and (I+); and pAMR 3, severe pAMR [12]. pAMR >1 that occurred at any time during the follow-up period was analyzed as a variable in this study. pAMR >2 was treated with corticosteroids, plasmapheresis, intravenous immunoglobulin (IVIg), and rituximab (anti-B-cell antibodies).

## Statistical analysis

Descriptive statistics are presented as means ± standard deviation (SD), showing the median value (range) or number (percentage) for data with normal distribution or non-normal distribution, respectively. Patients were divided into groups according to

**Table 2:** Clinical information of the patients without, and with (C1q negative), donor-specific antibodies.

Virable	All n=60	DSA(C1q negative) n=12	No DSA n=48	p-value
pretransplant DSA	4	2	2	0.14
follow up period (years)	6.7 ± 4.1	6.8 ± 4.1	6.6 ± 4.5	0.9
Induction therapy OKT3	6	2	4	0.43
Basiliximab	24	7	17	0.2
Maintenance IST Tacrolimus/Everolimus	25	2	23	0.0037
Tacrolimus/MMF	14	3	11	
Everolimus/steroid	5	0	5	
CNI/MMF	4	0	4	
Other	12	7	5	
Triple IST therapy	12	6	6	0.0015
ACR ISHLT ≥ grade 2	21	5	16	0.699
PAMR ≥ grade 1	6	2	4	0.37
CAV	25	6	19	0.54
EF(%)	59.3 ± 5.5	59.0 ± 4.3	59.4 ± 5.8	0.74
BNP (pg/ml)	74.8 ± 51.5	84.3 ± 39.8	72.6 ± 54.0	0.21

DSA: Donor Specified Antibody; IST: Immunosuppressive Therapy; MMF: Mycophenolate Mofetil; CNI: Calcineurin Inhibitor; ACR: Acute Cellular Rejection; pAMR: Pathologic Antibody Mediated Rejection

**Table 3:** Characteristics of donor specific antibodies (DSA) found in patient serum samples.

Patient No.	DSA Target Antigen	SA beads		IST	induction	LCT/FCXM
		IgG(MFI)	C1q(MFI)			
1	A24, B51, DR9	11,898,991,288	5.1,3.2,0	CNI/Evelolimus	OKT3R	-/-
2	Cw14	746	15.6	Tacrolimus/MMF		-/-
3	B61, DR9, Cw10	14,099,871,247	11.9, 10.6, 12.6	Tacrolimus/MMF	OKT3R	-/+
4	Cw7	1844	4.9	Tacrolimus/MMF/Pred		-/-
5	Cw10	984	120.8	Tacrolimus/Evelolimus		-/-
6	B44	1353	93.1	Tacrolimus/Evelolimus/Pred	Basiliximab	-/-
7	A26	4,115	28.9	Tacrolimus/Evelolimus/Pred	Basiliximab	-/-
8	A31	857	65.7	Tacrolimus/Evelolimus/Pred	Basiliximab	-/-
9	Cw15	940	35.3	Tacrolimus/Evelolimus/Pred	Basiliximab	-/-
10	DQ7	6952	15.3	Tacrolimus/MMF/Pred	Basiliximab	-/-
11	A2, B61	1114, 1380	18.2, 111.3	Tacrolimus/MMF	Basiliximab	-/-
12	Cw10	698	47.6	Tacrolimus/Evelolimus	Basiliximab	-/-

DSA: Donor Specific Antibody; SA: singleantigen; MFI: Mean Fluorescence Intensity; IST: Immunosuppressive Therapy; MMF: Mycophenolate Mofetil; LCT: Lymphocyte Cytotoxicity Test; FCXM: Flow Cytometry Crossmatch

(n=5) of the patients in the non DSA group were treated with triple IST (p=0.0015), which indicated a tendency to perform strict IST in DSA positive patients. Induction therapy was performed in 75% (n=9) of the DSA positive patients (OKT3R, n=2; Basiliximab, n=7).

During the follow-up period, the composite endpoint of cardiac event (percutaneous coronary intervention (PCI), n=2; coronary artery bypass grafting (CABG), n=1) occurred in 3 patients in the non-DSA group, but did not occur in any of the patients in the C1q negative DSA group. There was no significant difference in cumulative cardiac event-free survival between the 2 groups (Wilcoxon test, p=0.42) (Figure 2A). Each patient had a history of refractory ACR or had been treated for pAMR2. One patient underwent PCI one month after HTx because of donor-transmitted disease.

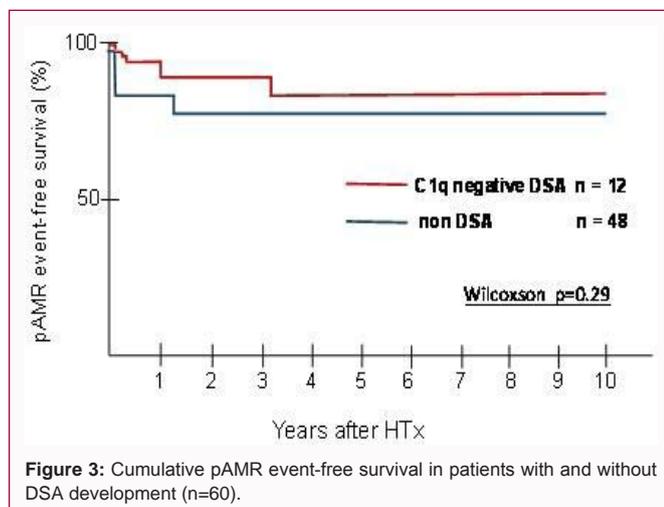
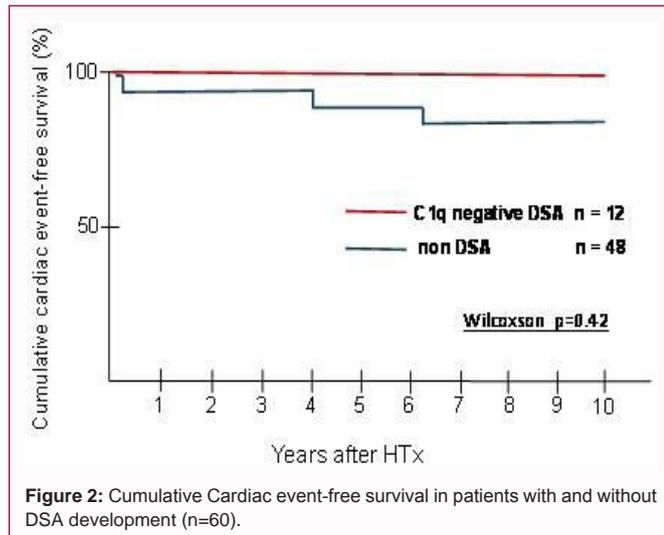
There was no significant difference in pAMR event free-survival between the two groups (Wilcoxon test, p=0.29) (Figure 2B). Two

(16.6%) patients in the C1q negative DSA group, and 4 (8.3%) patients in the non-DSA group experienced pAMR >grade 1.

## Discussion

In this study, the association of C1q binding status with anti-HLA antibody and clinical outcomes of HTx was investigated. We found that patients with C1q-negative DSA had good graft survival and AMR-free survival, which were comparable to those of non-DSA patients. In other words, patients with DSA showed a better outcome if their anti-HLA antibodies did not show a complement-binding capacity in the C1q assay.

An updated consensus for diagnosing AMR was published in 2011, which specifically detailed histological AMR features based on hematoxylin eosin (H-E) staining, immunofluorescence staining, or both. Clinical cardiac symptoms and hemodynamic status are no longer needed for diagnosing AMR because of recent studies that



demonstrated an increased development of CAV and inferior survival in patients with asymptomatic biopsy-proven AMR [1,12-14]. However, apart from the fact that this updated method of diagnosing AMR recognizes the importance of diagnosing pathological asymptomatic AMR before it becomes clinically symptomatic, [15,16] the issues of invasiveness of the biopsy procedures, timing intervals, and the differences in immunofluorescence staining methods among institutions have not been solved.

Recent studies have indicated a correlation between the serological existence of DSA detected after heart transplantation and developing AMR, CAV, graft injury and graft loss [17,18]. Due to the limited value of histological change for the diagnosis of late AMR and its invasiveness, and to complications accompanying repeated biopsy procedures, DSA identified by using sensitive solid-phase immunoassays have come to play a more important role as a non-invasive, repeatable diagnostic method for predicting prognosis after HTx.

Besides the clinical value of monitoring serum DSA after heart transplantation, it is also known that not all patients with persistent production of DSA show a worse outcome. Since the solid phase IgG-SAB assay detects both complement-fixing and non-complement-fixing DSA, we further investigated this characteristic of DSA in our patients to clarify risk stratifications among patients with DSA.

In present study, all of the patients with DSA turned out to be C1q-negative. Since there were no patients with positive C1q DSA, we compared the prognosis of the patients with non-complement DSA with that of non-DSA patients to examine the prognosis and clinical implication of possessing non-complement DSA. We found that there were no significant differences in patient survival between C1q-negative DSA and non-DSA patients. These results were consistent with the hypothesis that if these antibodies do not fix complement, and thus do not initiate the complement cascade, these antibodies may not cause graft injury [19,20]. To our knowledge, there have been no reports in the adult cardiac transplant literature of systematic studies that evaluated the prognosis of patients producing C1q-negative DSA. However, in a previous study of kidney transplantation, the percentage of DSA-positive patients that were C1q-negative was reported as 60% [21] and, consistent with our study, their graft prognosis was far better than that of the C1q-positive DSA patients [8,22,23].

One of the possible reasons as to why there were no C1q-positive patients in our study is that recipients with a positive CDC assay are excluded in donor matching in Japan. As a result, highly sensitized patients with an extremely high titer of DSA were not included from the start of this study.

To confirm this hypothesis, we retrospectively examined the sera of recipient and donor candidates who had been judged as positive CDC by the Japan Organ Transplant Network and for whom HTx was therefore not performed, in order to check C1q binding ability. These data confirmed that positive CDC results in an extremely high MFI level of IgG DSA with positive C1q.

Some studies have reported a correlation between C1q-binding ability and the MFI level of IgG DSA. Thus, it has been reported that the MFI levels of IgG DSA in the C1q-positive group were significantly higher than those in the C1q-negative group [7,24-26]. Zeevi et al. [26] reported a significant relationship between C1q-binding activity and antibody strength as measured by the MFI titer, in analysis of over 800 serum samples. Thammancichanond et al. [25] also reported that C1q binding ability correlated with the strength of DSA, which was measured as the MFI level; 11 of the 12 patients with C1q-positive DSA in that study had an MFI level of IgG DSA >8,000. Schaub et al. [24] reported an IgG-DSA cut-off value of 14154 in the prediction of C1q positivity. On the other hand, other reports have warned that C1q binding does not always correlate with the results of the conventional SAB assay [6,7,21,26,27]. The complement cascade, which is initiated by the binding of C1q, requires C1q binding sites on at least two antibodies in close steric proximity. Thus, C1q binding depends on both the density of DSA and their IgG subclass [28,29]. Since the standard SAB assay detects all types of IgG-subclass, the proportion of the IgG subtypes included in each serum might explain the discrepancies between the DSA-MFI titer and C1q positivity.

In our study, the MFI value of IgG DSA ranged from 698 to 6952, and the mean MFI value was  $1880 \pm 1839$ , which was a significantly lower titer compared to other previous reports. The lower MFI value of IgG DSA in our study might be due to the method of selecting the heart donors as already described, as well as the immunosuppressive protocol of our institution.

Regarding IST, in our study patients with DSA were more likely to be on triple immunosuppression with tacrolimus, mycophenolate mofetil, and prednisone than on double immunosuppression, as a

precaution against the development of AMR. Although we usually hesitate to decrease the strength of IST in DSA-positive patients, the favorable result of this study suggested that it might be possible to reconsider the risk and the appropriate IST for each C1q-negative DSA patient, based on the assumption of a harmless effect of C1q-negative DSA. A decrease in IST may avoid unwanted events such as infectious disease, malignancy and kidney dysfunction, which can derive from the immunosuppressive therapy itself or from an over-immunosuppressive regimen [30]. However, from a different point of view, it could also be said that the intense immunosuppressive therapy of the DSA positive patients in this study might have the advantage of leading to insufficient production of DSA for the development of AMR-related events. For this reason, careful observation of the MFI level of IgG DSA is still recommended when trying to wean DSA positive/C1q negative patients off strict immunosuppressive therapy [31].

Nevertheless, some studies have reported an unfavorable prognosis in kidney transplanted patients that show long-term persistence of C1q-negative DSA [24], and the clinical roles and the effects of possessing non-complement-binding DSA are still controversial [25,32]. An important point that needs to be kept in mind is that a negative C1q result does not mean that the detected DSA will remain that way forever. This is because a low titer of DSA might significantly increase due to a strong and durable immune memory response in sensitized situations, and a negative C1q DSA might change to C1q-positive. Although our patients with C1q negative DSA showed good survival in the follow up period (mean follow up period,  $6.7 \pm 4.1$  years), it is still necessary to closely observe C1q negative patients with a high DSA-MFI, balancing the risk and benefit of reducing IST, and also the cost and benefit of the C1q assay and IgG subclass analysis.

There are several limitations of this study. First, we could not evaluate the prognosis of positive DSA patients with positive C1q since there was no such study participant. Second, the impact of non-HLA specific antibodies and IgG types were not considered in this study. Since our study was limited to a small cohort, continuous and additional studies are necessary to further confirm the prognosis of patients producing C1q-negative DSA.

In conclusion, this study demonstrated that patients producing non-complement binding DSA had good graft survival, which was comparable to that of DSA negative patients. Therefore, addition of the assessment of the complement-binding capacity of DSA to the diagnosis of DSA patients might redefine the traditional risk stratification of DSA positive patients after HTx.

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