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ORIGINAL ARTICLE

Donor-specific HLA antibodies: evaluating the risk for graft loss in renal transplant recipients with isotype switch from complement fixing IgG1/IgG3 to noncomplement fixing IgG2/IgG4 anti-HLA alloantibodies

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Summary

Human leukocyte antigen alloantibodies have a multitude of damaging effects on the allograft, both complement (C′) activation and Fc-independent ones. To date, the clinical significance of non-C′ fixing (NCF) HLA donor-specific antibodies (DSA) is still unclear. In this study, we investigated whether renal transplant recipients with NCF-DSA subclasses (IgG2/IgG4, IgA1/IgA2) are at higher risk of graft loss compared to patients with exclusively C′ fixing (IgG1/IgG3). Blood samples from 274 patients were analyzed for HLA IgG and IgA subclasses using a modified single-antigen bead assay. We identified 50 (18.2%) patients with circulating NCF antibodies either DSA (n = 17) or against third-party HLA (n = 33). NCF-DSAs were preferentially of IgG2/IgG4 isotype (11/17) and were mainly directed against HLA class II (13/17). NCF DSA were present as a mixture with strong C′ fixing IgG1/IgG3. Graft survival was similar between patients with exclusively C′ fixing antibodies and those with a mixture panel (log rang test P = 0.162), and also among patients with different immunoglobulin isotype and subclasses (long-rank test, P = 0.732). We conclude that expansion of DSA to NCF subclasses postrenal transplantation does not seem to be associated with worse graft survival as compared to the presence of exclusive C′ fixing subclasses.

Introduction

Monitoring of the development of anti-HLA class I and anti-HLA class II graft specific antibodies after transplantation is a significant area of interest within the transplant community. These antibodies attack the endothelium of the allograft and are strongly correlated with early graft failure or contribute to the slow damage and chronic allograft rejection [1,2]. There are many mechanisms of HLA antibody-mediated graft injury. According to experimental evidence, complement (C′) activation that is carried out by the Fc portion of the immunoglobulins as well as Fc-independent effects of MHC class I or class II antibodies may directly contribute to allograft injury [3].

To date, two different approaches have been used for the detection of clinically relevant immunoglobulin subclasses of anti-HLA antibodies: either identifying strong C′ fixing antibodies [4,5] or detecting anti-HLA antibody immunoglobulin subclasses [6,7]. Preformed C' fixing anti-HLA antibodies have been highly correlated with antibody-

mediated rejection and poor graft outcome by some researchers [8–10], but other studies did not reach that conclusion [4,11]. Moreover, there is evidence that C1q fixing anti-HLA class I and class II alloantibodies detected post-transplantation identify patients at risk of transplant glomerulopathy and graft loss [12].

Apart from strong C′ fixing antibodies, the maturation of immune response enables B cells to produce high-affinity antigen-specific weak or non-C′ fixing (NCF) antibodies to enhance the diversity of humoral immune response [13]. Previous studies revealed that a proportion of patients sensitized to HLAs had circulating NCF antibodies before and/or after transplantation. NCF anti-HLA class I and class II antibodies have been found to accumulate in rejected renal allografts [14] and are also present in the serum of renal retransplant candidates [15]. The assessment of the immunological risk of preformed NCF anti-HLA donor-specific antibodies (DSA) has given controversial results [16,17], while so far, there has not been a prospective study to identify the clinical relevance of these antibodies post-transplantation.

Materials and methods

Patients and study design

A blood sample was collected from 274 renal transplant recipients who were admitted to the Transplantation Unit of Laikon Hospital of Athens from January to December 2008 and analyzed for anti-HLA immunoglobulin subclasses. The patients had been transplanted without pretransplant donor-specific sensitization and were part of a larger cohort who were monitored for de novo development of IgGall (IgG1/IgG3/IgG2/IgG4) anti-HLA class I and class II antibodies with a standard IgGall SAB assay [18]. At the time of blood collection, ninety-four (n = 94) patients had developed de novo IgGall DSA (group A), eighty-eight (n = 88) patients had IgGall antibodies against third-party (TP) HLA (group B), and ninety-two (n = 92) patients had no IgGall anti-HLA antibodies (group C).

The patients were transplanted between 1997 and February 2007, and the mean time of blood collection post-transplantation for this study was 8 ± 5.1 years. More specifically, post-transplantation time was 9.7 ± 6.3 years for patients with anti-HLA-DSA, 7.1 ± 4.7 years for patients with antibodies against TP-HLA and 7.3 ± 3.4 years for patients without antibodies. The immunosuppressive therapy consisted of corticosteroids, mycophenolate acid (MPA), or azathioprine (AZA) in few cases (n = 96), and a calcineurin inhibitor either cyclosporine A (n = 206) or tacrolimus (n = 68). All patients were followed up until December 2011 by serum creatinine levels and biopsies. Renal biopsies were performed following specific clinical indications such as deterioration of renal function estimated by serum creatinine clearance using Cockroft–Gault formula (decreased by 20%), proteinuria >0.5 g/day, or the presence of erythrocytes in urine. The biopsy findings graded according to Banff 2005 classification. All patients were informed about the details of the various procedures and asked for consent. The protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Anti-HLA antibody detection and assignment

Firstly, all sera were analyzed for anti-HLA class I and class II IgGall alloantibodies, using a commercial bead-based solid phase assay at generic level (LABScreen Mixed™12, lot 17, One Lambda Inc., Canoga Park, CA, USA). All patients' sera were tested according to the manufacturers' instructions and analyzed on a LABScan™ 100 Luminex flow analyzer. The definition of an anti-HLA IgGall alloantibody "positive" result in the generic bead-based assay was calculated with a cutoff value of "ratio 5," which is used in the standard laboratory protocol.

All sera were tested for specific IgGall anti-HLA class I (A, B, C) and class II (DR, DQ, DP) alloantibodies with a standard IgGall SAB assay (LABScreenR Single Antigen, One Lambda, Canoga Park-Inc). The method detects all C′ fixing and non-C′ fixing antibodies. Tests were performed according to the manufacturers' instructions and analyzed on a LABScan™ 100 Luminex flow analyzer. For the specific SAB analysis, reactions with normalized values of ≥1000 MFI (Mean Fluorescence Intensity) were considered as positive. The percentage of class I and II PRA was calculated virtually (vPRAs) using the antibody specificity by SAB analysis and the Eurotransplant program for virtual PRA calculations. For vPRAs calculations, a database from 4000 organ donors typed for HLA-A, B, C, DR, and DQ was used. (etrl@lumc.nl).

Anti-HLA antibody immunoglobulin subclass and isotype detection and assignment

In the second part of the study, all sera were analyzed to detect the various antibody immunoglobulin isotype and/or subclasses using a modified bead-based solid phase assay (Luminex) first on generic (LABScreen Mixed™12) and second on specific (LABScreenR Single Antigen, Class I and II) level as described in earlier time [15].

Non-C′ fixing IgG2 and IgG4 as well as IgA1 and IgA2 subclasses and C′ fixing (CF) IgG1 and IgG3 immunoglobulin isotypes were possible to be detected by replacement of the commercially used pan-anti-IgG secondary reporter antibody through secondary reporter antibodies specific against IgG1 (clone HP6001), and IgG3 (clone HP6050) for the detection of CF antibodies and against IgG2 (clone

31-7-4), IgG4 (clone HP6025), IgA1 (clone B3506B4), and IgA2 (clone 9604D2) to uncover the NCF antibody subclasses. All secondary antibodies were obtained from SouthernBiotech (Birmingham, AL, USA) and were R-phycoerythrin (R-PE) conjugated.

Due to the insufficient sensitivity of the bead-based test system, it was not possible to detect IgA isotype anti-HLA antibodies, which are usually present in a very low titer in the serum. Therefore, we calculated in the generic test, the positive results for IgA1-2 isotype antibodies with a decreased threshold value of "ratio 2" compared to the IgG1-4 subclass threshold of "ratio 5," which is used in the standard laboratory protocol.

For the identification of prevalence and specificity of the NCF immunoglobulin isotypes of anti-HLA alloantibodies, the sera were subsequently investigated using a modified SAB assay (Luminex). Similar to the generic assay, the modified IgA1-2 isotype SAB assays were considered more sensitive than the standard IgGall isotype assay and were calculated as "result positive" when reactions with normalized values of ≥500 MFI emerged.

Isotype controls

To verify the specificity of the replaced secondary reporter antibodies, they were tested in the Luminex assays with special isotype-control reagents. The applied controls were developed from Dechant *et al.* [19] and were for the first time used in 2004 in the context of the detection of NCF anti-HLA alloantibodies in sera of solid organ-transplanted patients [7]. These isotype controls consist of chimeric antibodies (chF3.3-X) directed against pan-anti-HLA class II antigens, differing only in their heavy chains—either against IgG1 and IgG3 (CFA) or IgG2, IgG4, IgA1, and IgA2 (NCF) isotypes and subclasses. The controls reacted positive with their correspondent secondary reporter antibodies and do not show any cross-reactivity.

Statistics

Continuous variables are presented with mean and standard deviation (SD). Quantitative variables are presented with absolute and relative frequencies. Chi-square and Fisher's exact tests were used for the comparison of proportions. Student's t-tests were computed for the comparison of mean values. Kaplan–Meier survival estimates for graft failure were graphed over the follow-up period. Log-rank test was used for the comparison of survival functions between different groups. Also, Cox regression analysis was used to obtain hazard ratios (HR) with 95% confidence intervals. *P* values reported are two-tailed. Statistical significance was set at 0.05, and analyses were conducted using SPSS statistical software (version 18.0).

Results

Detection of anti-HLA NCF antibody subclasses

Among the 274 patients: 50 patients (18.2%) had an expansion of humoral alloresponse to IgG2/IgG4 and/or IgA1/IgA2 anti-HLA antibodies, 134 patients (48.9%) had IgG anti-HLA antibodies without switch to NCF subtypes, and 90 patients (32.8%) had not detectable antibodies. The patient characteristics are shown on Table 1. No significant differences were found between patients with class switching to NCF antibodies and those without switching regarding recipient age, gender, donor status, first or second graft, immunosuppression, or follow-up time post-transplantation.

Weak or non-C′ fixing DSA subtypes were present in 17 of 50 patients, while 33 patients had NCF antibodies against TP-HLA. The distribution of NCF-DSA among the patients was as follow: 16/94 (17%) in patients with de novo IgGall DSA (group A), while the remaining 78 patients with DSA had exclusively C′ fixing DSA; 1/88 (1.1%) in patients with IgGall antibodies against TP-HLA (group B) these antibodies were of IgA subclasses; (group C) 0/92 (0.0%) in patients without IgGall anti-HLA antibodies. The presence of NCF-DSA was significantly correlated with high %vPRA values, ($P = 0.0001$) but not with HLA class I or class II sensitization or the degree of HLA-DR incompatibility. Concerning the distribution of NCF TP HLA antibodies ($n = 33$ cases) within the groups: 14/33 patients were from group A; 17/33 patients were from group B and 2/33 patients were from group C.

Isotypes and specificity of NCF anti-HLA antibody subclasses

Different Ig isotypes were observed between NCF anti-HLA-DSA and NCF antibodies specific to TP-HLA: anti-HLA-DSAs were more prominent within IgG2 and IgG4 subclasses (10/16, 62.5%), while antibodies specific to TP-HLA were more frequent within IgA1 and IgA2 subclasses (25/31, 80.6%) (Table 2). Interestingly, IgG2/4 isotypes occurred independently from IgA1/2. NCF anti-HLA-DSA and antibodies against TP-HLA differed regarding the HLA class specificity: The majority of DSAs were specific to HLA class II antigens (13/17, 76.5%) directed mainly against donor HLA-DQ, while the majority of antibodies against TP-HLA were specific to HLA class I antigens (29/33, 87.9%).

Immunoglobulin subclasses in patients with NCF anti-HLA-DSA

We further analyzed the sera from 17 patients with NCF anti-HLA-DSA for the presence of IgG1 and IgG3 strong C′ fixing subclasses**.** Table 3 shows the data from the 17 patients with expansion to IgG2/4 and/or IgA1/2 DSA. All

Table 1. Patients characteristics.

	All patients $N = 274$	(a) Patients with antibodies and expansion to NCF subtypes $N = 50$	(b) Patients with antibodies w/o expansion to NCF subtypes $N = 134$	(c) Patients w/o antibodies $N = 90$	P*
Follow-up time in years (mean ± SD)		2.98 ± 2.30	3.62 ± 2.65	4.09 ± 2.43	0.042†
Recipient age (mean ± SD)		45 ± 13	45 ± 12	46 ± 14	NS
Recipient gender					
Male	178	27 (54.0%)	87 (64.9%)	64 (71.1%)	NS
Female	96	23 (46.0%)	47 (35.1%)	26 (28.9%)	
Donor status					
LD	150	24 (48.0%)	68 (50.7%)	58 (64.4%)	NS
DD	124	26 (52.0%)	66 (49.3%)	32 (35.6%)	
Transplantation					
Re-transplantation	11	5 (10.0%)	6 (4.5%)	0 (0.0%)	0.001†
Primary transplantation	263	45 (90.0%)	128 (95.5%)	90 (100.0%)	
Immunosuppression					
MPA-based therapy	178	31 (62.0%)	83 (61.9%)	64 (71.1%)	NS
AZA-based therapy	96	19 (38.0%)	51 (38.1%)	26 (28.9%)	
With CsA	206	40 (80.0%)	106 (79.1%)	60 (66.7%)	
With Tac	68	10 (20.0%)	28 (20.9%)	30 (33.3%)	

NCF, non-C′ fixing; SD, standard deviation; w/o, without; NS, nonsignificant; LD, living donor; DD, diseased donor; MPA, mycophenolate acid; AZA, azathioprine; CsA, Cyclosporine A; Tac, tacrolimus.
*Chi-squared test (Yate s corrected).
†*avsc* (only the significant p are shown).

Table 2. Distribution of different NCF immunoglobulin subclasses between anti-HLA-DSA and anti-TP-HLA antibodies. NCF antibody specificity to HLA class I and /or class II is also shown. Anti-HLA-DQ DSAs were present in 12/13 (92.3%) patients.

	Anti-HLA NCF antibody specificity					
	Anti-HLA-DSA ($N = 17$)			Anti-HLA TP ($N = 33$)		
Immunoglobulin subclass	Anti-HLA-A, B, C	Anti-HLA-DR, DQ, DP	Both	Anti-HLA-A, B, C	Anti-HLA-DR, DQ, DP	Both
IgG2/4 ($N = 16$)	0	10	0	4	2	0
IgA1/A2 ($N = 31$)	4	2	0	23	0	2
Both ($N = 3$)	0	1	0	2	0	0

HLA, human leukocyte antigen; NCF, non-C′ fixing; DSA, donor-specific antibody; TP, third party.

except one patient had a mixture panel of IgG1/IgG3 strong C′ fixing and NCF anti-HLA-DSA. IgG1 was the most frequent immunoglobulin subclass (94.1%) followed by IgG2/IgG4 (64.7%), IgA1/IgA2 (41.2%), and IgG3 DSA (17.6%). DSA of IgG2/4 subclasses appeared independently from IgA1/2 DSA. The most frequent antibody pattern (10/17) was that of the IgG1 and IgG2/4 anti-HLA-DSA. In 14/16 patients with a mixture panel, the strong C′ fixing and NCF-DSA the antibodies had the same anti-HLA-DQ specificity.

Graft biopsy results

During the follow-up, 85 biopsy samples were analyzed: 16 samples from patients with expansion to NCF anti-HLA antibodies either DSA or against TP-HLA, 48 samples from patients with anti-HLA antibodies without expansion to NCF subclasses, and 21 from patients without antibodies. Histopathological findings consistent with acute antibody-mediated rejection (AAMR) were found in seven cases that were equally distributed between patients with DSA without expansion to NCF subclasses (10.8%) and those with DSA of a mixture panel (22.2%) (P=NS). Chronic alloimmune injury was diagnosed in 52 cases. Twenty-one of 52 biopsies (40.4%) were positive for capillary C4d deposition: five of nine biopsies (55.5%) from patients with DSA of a mixture panel and 9 of 37 biopsies (24.3%) from patients with DSA of exclusively C′ fixing antibodies (P = NS) (Table 4).

Effect of anti-HLA antibody subclass on graft survival

At the end of the study, 53 from 274 patients (19.3%) had lost the graft: 36 patients with anti-HLA-DSA, 14 patients

Table 3. The presence of strong C fixing (IgG1/3) and weak or non-C fixing (IgG2/4,IgA1/2) DSA (gray boxes**)** in 17 patients is shown. In 16/17 cases strong C′ fixing IgG1/3 DSAs were present. IgG2/4 DSA appear independently from IgA1/2 DSA. Biopsy results and graft failure were not correlated with any antibody pattern.

	DSA subclasses						
Patients	IgG3	IgG1	IgA1	IgG2	IgG4	IgA2	Biopsy results Graft outcome
MHL01							FG
ATH02							FG
KOL04							CAMR C4d+ Graft Failure
MAR05							CAMR C4d+ FG
BAK06							AAMR C4d+ FG
MARK07							CAMR C4d− FG
ROUD08							FG
LAIN14							FG
BOUL15							CAMR C4d+ Graft Failure
STR16							FG
NT03							AAMR C4d+ CAMR C4d+ FG
MAND09							CAMR C4d+ FG
TEN11							FG
ANTR13							FG
FOU17							CAMR C4d− Graft Failure
VELIS10							FG
KAR12							CAMR C4d+ Graft Failure

AAMR, acute antibody-mediated rejection; CAMR, chronic antibody-mediated rejection; FG, functioning graft.

Table 4. Histopathological results from 85 renal graft biopsies.

			Acute alloimmune injury		Chronic alloimmune injury		
Anti-HLA antibodies		Biopsies (N = 85)	AAMR (n = 7) n (%)	CMR (n = 12) n (%)	C4d pos (n = 21) n (%)	C4d neg (n = 31) n (%)	Nonimmune injury (n = 14) n (%)
C′ fixing only	DSA	37	4 (10.8)	4 (10.8)	9 (24.3)	15 (40.5)	5 (14.7)
	TP-HLA	11	1 (9.1)	1 (9.1)	0 (0.0)	4 (36.4)	5 (45.5)
Mixture Panel	DSA	9	2 (22.2)	0 (0.0)	5 (55.5)	2 (22.2)	0 (0.0)
	TP-HLA	7	0 (0.0)	3 (42.8)	2 (28.6)	2 (28.6)	0 (0.0)
W/o antibodies		21	0 (0.0)	4 (19.0)	5 (23.8)	8 (38.0)	4 (19.0)

HLA, human leukocyte antigen; AAMR, acute antibody-mediated rejection (C4d capillary deposition was present in all AAMR cases); CMR, cell mediated rejection; DSA, donor-specific antibody; TP, third party; Mixture panel, C′ fixing and NCF (noncomplement fixing) antibodies; w/o, without.

with antibodies against TP-HLA, and three patients without antibodies. Within the remaining 221 patients with a functioning graft, 134 patients displayed anti-HLA antibodies and 87 patients were tested negative for anti-HLA antibodies. Anti-HLA NCF antibodies were equally distributed between patients with antibodies who lost the graft (12/50, 24.0%) and those with antibodies and functioning graft (38/134, 28.4%).

Kaplan–Meier survival curves were graphed over the follow-up period in three groups of patient: patients with a mixture panel of strong C′ fixing and weak or non-C′ fixing antibodies (NCFA) either DSA or against TP-HLA, patients with exclusively C′ fixing antibodies (CFA) DSA or against TP-HLA, and patients without antibodies (no Abs). From this analysis, it was found that graft survival was comparable between patients with a mixture panel of antibodies and those with exclusively C′ fixing antibodies (log-rank test, P = 0.162) (Fig. 1). In addition, graft survival was compared within the group of patients with DSA between patients with exclusively strong C′ fixing DSA and those with mixture panel (C′ fixing and weak or NCF) DSA. It was found that graft survival was comparable between patients with a mixture panel of DSA and patients with exclusively strong C′ fixing DSA (P = 0.371). Furthermore, no significant difference in graft survival was found among patients with DSA of IgA1/2 isotypes, patients with DSA of IgG2/4 isotypes, and those with exclusively C′ fixing DSA (long-rank test, P = 0.732) (data not shown). Among the

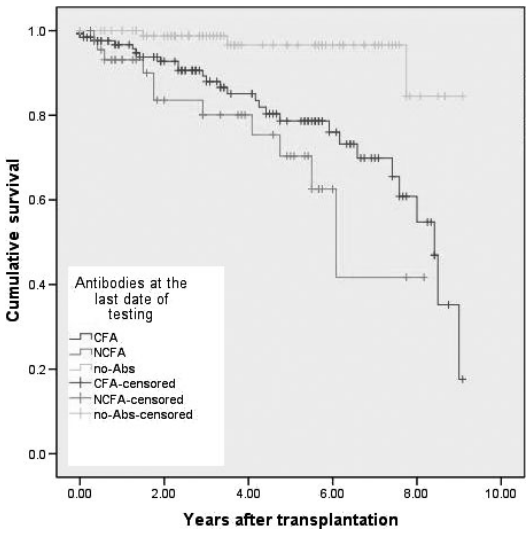

Figure 1 Kaplan–Meier survival curves were graphed over the follow-up period. The patients are divided into three groups: patients with a mixture panel of C′ fixing and non-C′ fixing antibodies (NCFA), patients with exclusively C′ fixing antibodies (CFA) and patients without antibodies (no Abs). The graft survival in patients without antibodies was significantly higher compared to those with a mixture panel (log rang test, $P < 0.001$) and those with exclusively CFA (log rang test, $P = 0.002$). No difference in graft survival was found between the two groups with antibodies (log rang test, $P = 0.162$).

Table 5. Cox regression analysis was used to obtain hazard ratios (HR) with 95% confidence intervals. Patients with exclusively strong C fixing anti-HLA antibodies either DSA or against TP- HLA had greater hazard for graft failure in comparison with patients without antibodies. No other difference was found between the groups.

	Hazard ratio	95.0% confidence interval	*P* value
Non -HLA	1.00*		
DSA – exclusively C′ fixing subtypes	4.81	1.65–14.03	0.004
DSA, mixture panel	2.84	0.74–10.97	0.129
TP-HLA antibodies-exclusively C′ fixing subtypes	4.36	1.42–13.38	0.010
TP-HLA antibodies, mixture panel	3.33	0.74–15.02	0.117

HLA, human leukocyte antigen; DSA, donor-specific antibody; TP, third party.
*Indicates reference category.

patients with antibodies against TP-HLA, no significant difference was found in graft survival between those with exclusively C′ fixing antibodies and patients with expansion to non-C′ fixing subtypes ($P = 0.573$) (data not shown). Finally, Cox regression analysis was used to define variables predictive of graft failure. The analysis revealed that only patients with exclusively C′ fixing antibodies either DSA or against TP-HLA had greater hazard for graft failure as compared to patients without antibodies ($P = 0.004$ and $P = 0.010$, respectively) (Table 5).

Discussion

The subject that we confronted in this study was to define the immunological risk of graft failure in renal transplant recipients with maturation of humoral alloresponse to non-complement fixing (NCF) anti-HLA antibodies. Until today, few retrospective studies post-transplantation identified NCF anti-HLA antibody subclasses in patients with specific clinicopathological conditions as chronic rejection or C4d-positive biopsies [11,20]. The aim of this study was to investigate whether monitoring renal transplant recipients for NCF anti-HLA subtypes may identify clinically relevant and harmful antibodies. For NCF isotype detection, we used a modified solid phase assay which allowed the detection and specification of anti-HLA IgG2/IgG4 and IgA1/IgA2 antibody isotypes. The patients had been tested previously with a standard pan-IgG SAB assay and were separated into three groups: patients with de novo IgG anti-HLA-DSA, patients with IgG alloantibodies against third-party HLA, and patients without IgG antibodies. The allograft survival was compared among patients with and without anti-HLA immunoglobulin switch to NCF subclasses and patients without alloantibodies.

Non-C′ fixing anti-HLA antibodies were found in a small percentage of the patients (18.2%) post-transplantation and were directed either against donor HLA (6.2%) or against TP-HLA (12%). As the patients were not sensitized against the donor before transplantation, the development of de novo anti-HLA-DSA and the expansion to NCF subtypes must have occurred under the current immunosuppression. It is questionable why some patients switch anti-HLA immunoglobulins from C′ fixing to NCF, and others do not. In this study, a high vPRA value was the only parameter that was found to be associated with NCF antibody detection. We postulate that high vPRA value represents high numbers of activated B-cell clones with different antibody specificity, and in that situation, there is a higher possibility for class switch recombination. Alternatively, these patients could be high responders in terms of immune activation. No other significant correlation was found between the presence of NCF anti-HLA antibodies and the time post-transplantation, previous transplantation, specific immunosuppressive protocol, donor type, recipient gender, sensitization against HLA class I or class II, or the HLA-DR incompatibility with the graft.

Interestingly, IgG2/IgG4 isotype predominated within NCF anti-HLA-DSA, while IgA1/IgA2 isotype predominated within NCF TP-HLA antibodies. High prevalence of donor-specific IgG2/4 alloantibodies had been reported previously in sensitized patients awaiting for

re-transplantation [7,15]. It is not finally clarified why anti-HLA-DSA when switch to NCF subclasses are found mainly within the IgG2/IgG4 isotype. Immunoglobulin class switch recombination is crucial for the generation of antibody diversity and is regulated by cytokines produced by T helper cells, dendritic cells, and CD40 and/or TLR stimulation of B cells. This procedure is one-way street and in man leads from the expression of IgG3 and IgG1 strong C′ fixing subclasses to IgA1, IgG2, IgG4, and IgA2 weak or NCF immunoglobulin subclasses [13,21]. It is not known whether immunosuppression that interferes with cytokine production may affect the isotype in which B cells will switch. Additionally, it is known that the class switch to IgA is enhanced by TGFβ [22], which is produced locally during kidney allograft rejection [23]. In this regard, it would have been interesting to know whether patients with anti-HLA antibodies of the IgA isotype might have undergone less often a transplantectomy in contrast to those without. Furthermore, discrepancy between NCF-DSA and TP-HLA antibodies was observed regarding the antibody specificity. IgG2/IgG4 DSAs were preferentially directed against anti-HLA class II and particularly anti-HLA-DQ antibodies, while IgA1/IgA2 TP-HLA antibodies were preferentially directed against HLA class I. In a previous study, IgG4 antibodies were found to be directed preferentially against HLA class II antigens in patients waiting for re-transplantation [15]. The discrepancy in antibody specificity between different isotypes in this study is probably due to the selection of patients with specific characteristics. Therefore, we can assume that analysis of a different cohort might lead to a different distribution of anti-HLA antibody specificity between IgG2/IgG4 and IgA1/IgA2 isotypes.

In this study, NCF anti-HLA-DSA subclasses were present as mixture with strong C′ fixing IgG1/IgG3 anti-HLA-DSA. IgG1 was the predominant subclass in the cases with mixture of C′ fixing and non-C′ fixing subclasses. There was no difference regarding the risk of graft loss between patients with mixture panel of anti-HLA-DSA and patients with exclusively strong C′ fixing DSA. These results are in line with the results of Hoenger *et al.* [24], who found that in 90% of patients with pretransplant anti-HLA-DSA, the antibodies were composed of isolated strong or a mixture of strong and weak/noncomplement fixing subclasses. In the same study, the outcome between the two groups was similar regarding the incidence of antibody-mediated rejection. Recently, another group found similar graft survival between patients who were transplanted with negative cross-match but preformed C1q fixing DSA and those with preformed non-C′ fixing DSA [25].

We were not able to find any NCF isotype association with worse graft survival. A study from Arnold *et al.* [26] in renal transplant candidates waiting for re-transplantation showed that patients with IgA HLA antibodies had a shorter back to waiting list time regardless the IgG status. According to our results, we cannot support that patients with IgA1/IgA2 anti-HLA antibodies either DSA or against TP-HLA had a worse outcome compared to those with other C′ fixing or NCF isotypes. Previous studies showed that IgG2/IgG4 DSAs were not harmful antibodies. In one study, anti-HLA-DQ DSA before transplantation and negative CDC cross-match characterized patients with rejection-free post-transplant course [17]. Furthermore, the investigation of eluates of explanted kidneys after nephrectomy showed no difference in graft survival between patients with anti-HLA class II DSA of IgG2/IgG4 and recipients without antibodies [14].

Biopsy results in this study revealed equal distribution of AAMR or chronic alloimmune injury between patients with exclusively C′ fixing anti-HLA-DSA and patients with mixture panel anti-HLA-DSA. Even though the number of patients who underwent a biopsy was low, it was observed a trend for higher incidence of C4d capillary deposition in biopsies from patients with chronic alloimmune injury and mixture panel of antibodies.

Considering the important role of complement activation induced by anti-HLA antibodies in alloantibody-dependent graft damage, most of the published studies focus on C′ fixing anti-HLA antibodies, which discriminate from the supposed less harmful non-C′ fixing antibodies. A sensitive C1q SAB assay that detects low-level C1q fixing antibodies has been used in patients with C4d+ and C4d− biopsies, which revealed that the presence of C1q fixing DSA identify patients with risk for transplant glomerulopathy and graft loss [12]. Similar results were showed by another study among 193 consecutive renal transplants, according to which 43% of the patients with de novo DSA had C1q fixing antibodies. These antibodies were associated with greater risk of acute rejection and allograft loss [5]. In contrast, we found that all (94) patients with de novo DSA tested by IgG all assay had C′ fixing antibodies either exclusively or as mixture with NCF antibodies, and there was no difference in graft survival between the two groups. This discrepancy may be due to different approach as focus on C1q fixing antibodies or the analysis of Ig subtypes and the different study population.

Apart from complement activation, the antibodies may possess also other Fc-independent effector functions. Experimental data suggest that complement fixation may not be a requisite for disease in chronic antibody-mediated rejection [27]. Donor-specific antibodies targeting MHC class I and class II molecules can elicit the key features of transplant vasculopathy by modulating endothelial gene expression and function, augmenting cellular immunity or inducing an endothelial pro-inflammatory phenotype that may have important clinical consequences [28]. A previous study by Kaneku *et al.* [20] supports that the percentage of patients

with multiple IgG subclasses is significantly higher in chronic rejection patients compared to those without chronic rejection. In our prospective study, we were not able to find any difference regarding the risk of graft failure between patients with spreading to multiple immunoglobulin subclasses and those without. Perhaps, the prospective type of our study and the small number of patients with donor-specific anti-HLA NCF subclasses were insufficient for more clear conclusions. Taking under consideration the slow endothelial damage from noncomplement fixing antibodies, we may need more time to evaluate the additional effect of different NCF anti-HLA antibodies on graft failure.

In conclusion, monitoring of renal transplant recipients for non-C′ fixing anti-HLA subclasses identifies patients with expansion of humoral alloresponse to IgG2/IgG4 and IgA1/IgA2 immunoglobulin isotypes. However, the results presented here show that expansion to NCF anti-HLA antibodies postrenal transplantation is not likely to provide a significant value for worse graft survival comparing to patients with exclusively C′ fixing antibodies. An extended follow-up time period may be needed to achieve more accurate information concerning the role of non-C′ fixing alloantibodies during the transplantation course.

Authorship

MLA: Performed the experiments, provided the data, and participated in writing the paper. I-SN: Collected and analyzed data, performed the study, and wrote the paper. IIND: Provided intellectual content of critical importance to the work designed and revised the paper. BMS: Participated in research design and in revising the paper. JNB: Provided clinical and histopathological data. AGI: Designed the research, contributed important reagents, and wrote the paper.

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