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ORIGINAL ARTICLE

DQ molecules are the principal stimulators of *de novo* donor-specific antibodies in nonsensitized pediatric recipients receiving a first kidney transplant

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All the authors have read and approved the manuscript and have no conflicts of interest to declare.

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Summary

Data on the different HLA-antibody (Ab) categories in pediatric kidney recipients developing *de novo* donor-specific Abs (DSA) after transplantation are scarce. We retrospectively evaluated 82 consecutive nonsensitized pediatric recipients of a first kidney graft for *de novo* HLA Ab occurrence and antigen specificity. At a median follow-up of 6 years, 29% of patients developed *de novo* DSA, while 45% had *de novo* non-DSA. DSA appeared at 25-month median time post-transplant and were mostly directed toward HLA-DQ antigens. Considering each HLA antigen, the estimated rate of DQ DSA (7.55 per 100 person-years) was much higher than the rates observed for non-DQ DSA. The HLA-DQ Ab recognized determinants of the DQβ chain in 70% of cases, α chain in 25% of cases, and both chains in one patient. Non-DSA peaked earlier than DSA, and were largely directed against HLA class I specificities that belonged to HLA-A- and HLA-B-related cross-reacting epitope groups (CREG) in 56% of cases. Our results indicate a need for evaluating HLA-DQ compatibilities in kidney allocation, in order to minimize post-transplant development of *de novo* DSA, known to be responsible for antibody-mediated rejection and graft loss.

Introduction

Humoral alloimmunity has been recently recognized as the principal cause of chronic antibody-mediated rejection (CAMR), a major obstacle to long-term graft survival [1–5]. The emerging role of *de novo* post-transplant HLA DSA in the pathogenesis of late allograft damage [6–15] has prompted recommendations on post-transplant HLA Ab

monitoring as a tool to identify patients at risk for antibody-mediated rejection and eventual graft loss.

Recently, many groups have reported that HLA-DQ Abs represent the most frequent DSA category developing *de novo* in kidney transplant (KTx) recipients and suggested a role for HLA-DQ Abs in mediating graft damage [11–15]. These observations have brought to the forefront the discussion about the clinical relevance of matching the HLA-DQ antigen, in addition to HLA-A, HLA-B, and HLA-DR loci, in kidney graft allocation.

In this study, we have sequentially analyzed the incidence and specificity of the different *de novo* HLA Abs developing in a cohort of nonsensitized pediatric KTx recipients receiving a first allograft. Our aim was to identify a possible immunological hierarchy dictating HLA-alloantibody induction, while excluding any bias due to the influence of previously failed grafts and/or pretransplant non-DSA (NDSA) on *de novo* HLA DSA development.

Patients and methods

Patients

Eighty-two consecutive pediatric patients, referred between March 2003 and December 2010 to the Genoa Pediatric Kidney Transplant Program for first allografting, were included in this study. Pretransplant patients' sera were screened three-monthly for the presence of panel-reactive anti-HLA antibodies (PRA), using the NIH prolonged incubation complement-dependent cytotoxicity (CDC) technique against a selected panel of lymphocytes, and yearly on a Luminex platform by a bead-based screening assay, complemented by single antigen assay when needed. Patients with a positive historic HLA Ab, no longer detectable during the pretransplant follow-up screening, were excluded. Thus, only patients whose sera resulted negative when collected while on the waiting list and at transplantation were enrolled. Grafts were performed after a negative T-cell cross-match by the standard complement-dependent cytotoxicity method.

After transplantation, monitoring of *de novo* HLA Ab development was carried out utilizing patients sera collected three-monthly in the first post-transplant year and annually thereafter until the last follow-up.

Our standard of care for this cohort of low immunological risk patients consisted of induction with basiliximab, and a triple-drug immunosuppressive regimen including a calcineurine inhibitor, mycophenolate mofetil, and prednisone. Biopsy-proven acute cellular rejection episodes were treated with pulse intravenous methylprednisolone. Patients developing late antibody-mediated rejection, as evidenced by circulating HLA DSA and histological features of antibody-mediated tissue and vascular injuries, were treated with a protocol including a combination of plasmapheresis, i.v. human Ig and anti-CD20 monoclonal antibody. The follow-up period for study patients was between 2.9 and 10.5 years, with a median of 6.1 years. Demographic details are given in Table 1. This study was conducted according to Institutional Review Board guidelines.

HLA class I and HLA class II typing

Genomic DNA was extracted from whole blood samples using an automated DNA extractor. Recipient low-resolution HLA-A*, HLA-B*, HLA-DRB1*, HLA-DQB1* typing was performed with a microarray bead-based technique (Lambda Array Beads Multi-Analyte System LABMAS, One Lambda, Canoga Park, CA, USA). HLA typing of donors was performed by both serology (GTI, Waukesha, WI, USA; Canoga Park, CA, USA) and polymerase chain reaction-sequence-specific primers (PCR-SSP) (ABDR SSP Combi Tray, Olerup SSP, Saltsjöbaden, Sweden). When recipient sera were found to display *de novo* antibodies directed against histocompatibility antigens, such as HLA class I C and /or one of the following class II such as DQB1*, DQA1*, or DP alleles, both recipient and donor were retrospectively typed for the relevant loci at high resolution with sequence-based typing (SBT). We used locus-specific primers (Atria Genetics, San Francisco, CA, USA). DNA sequences were obtained after processing with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed with Assign 3.5+ software (Conexio Genomics, Applecross, WA, Australia).

HLA antibody detection with microarray bead-based assay

The presence of pre- and *de novo* post-transplant HLA IgG antibodies in patients' sera, collected as specified above, was tested with a bead-based detection assay [16]. Briefly,

Table 1. Demographics of the 82 patients analyzed in the study.

Recipient age (years, median, and range)	14 (2–27)
Recipient sex (F/M)	46/36
Kidney donor (living/cadaver)	12/70
Donor age (years, median, and range)	13.5 (1–55)
Donor sex (F/M)	51/31
HLA-A, HLA-B, HLA-DR, HLA-DQ mismatches (mean ± SD)	3.87 ± 1.46
A locus mismatches (mean ± SD)	1.00 ± 0.61
B locus mismatches (mean ± SD)	1.33 ± 0.63
DR locus mismatches (mean ± SD)	0.79 ± 0.54
DQ locus mismatches (mean ± SD)	0.74 ± 0.62
Follow-up (years, median, and range)	6.1 (2.9–10.5)
Delayed graft function (n of affected patients)	13
T-cell-mediated rejection ≥1A BANFF	7
<1 year after transplantation	4
>1 year after transplantation	3

we used the LABScreen Mixed kit (One Lambda Inc.), which simultaneously detects class I and class II antibodies with microbeads coated with purified class I and class II HLA antigens [17]. Results above a cutoff value of 3.0 ratio between sample and negative control were considered positive. The Single Antigen kit (One Lambda) was used to identify HLA specificities; results above an MFI cutoff value of 1.000 were considered positive. The tests were carried out according to the manufacturer's instructions, and the analysis was performed with One Lambda software (HLA Visual Version 2.2). With this assay, antibodies directed against histocompatibility antigens, such as HLA class I Cw and HLA class II DR, DP, and DQ antigens are also amenable to testing.

Statistical analysis

Rates of HLA Ab development according to the specific HLA antigen mismatch were computed as number of patients with development of antigen-specific DSA overtime, expressed as events per 100 person-years, together with their 95% confidence intervals (95% CI). Mismatch versus no mismatch comparisons were performed with the log-rank test. Mismatch rates of different HLA classes were compared informally by inspection of the estimated rates and 95% CIs. Statistical analysis was performed using the Stata 13 (StataCorp LP, College Station, TX, USA).

Results

De novo HLA antibody development

Fifty-two patients of 82 (63%) developed *de novo* HLA antibodies; 24 had DSA (29%), with nine showing positivity for both DSA and NDSA, while the remaining 28 developed NDSA only (34%).

Among the DSA-positive patients, 12 developed anti-class I HLA Abs and 20 anti-class II (including eight recipients with both class I and class II HLA Abs). In particular, 10 patients developed anti-HLA Abs to A locus, eight to B, one to DR, and 20 to DQ. In the NDSA-positive cohort, 31 patients developed anti-class I HLA Abs and 12 anti-class II (including six recipients with both class I and class II HLA Abs). In particular, nine recipients developed anti-HLA Abs to A locus, 15 to B, eight to C, three to DR, six to DQ, and two to DP. DSA appeared at a median of 25 months post-transplantation (range 3–93 months), whereas NDSA was observed at a median of 20 months (range 1–92 months). The probability of DSA and NDSA development after transplantation had different kinetics, a higher percentage of NDSA was observed in the first year (17%) and found to decline thereafter, whereas DSA development peaked at the third year (12.7%). In DSA patients, the cumulative incidence of HLA class II Abs was consistently higher than class I Abs throughout the whole follow-up (Fig. 1).

None of the patients developed an acute AMR. No CAMR events were observed in the NDSA cohort, while a high incidence of CAMR (14/24 patients—58%) was observed in DSA-positive patients at a median time of 1.4 years (range 0–3.5) from the detection of HLA Abs. These patients displayed anti-DQ Abs alone in seven cases, anti-class I Abs alone in two cases, and both anti-class I and II Abs in the remaining five cases. In the latter patients, class II Abs consisted of anti-DQ Abs, associated with anti-DR in one case.

Role of HLA mismatches on antibody induction

We further analyzed the influence of donor/recipient antigen mismatches for each HLA locus on *de novo* antibody induction. The mean allele mismatch number, shown in Table 1, was significantly lower for DR and DQ loci than A and B loci (DQ vs. B $P < 0.0001$; DQ vs. A $P < 0.01$; DR vs. B $P < 0.0001$; DR vs. A $P < 0.05$). Despite a better donor/recipient match at class II antigens, development of *de novo* DQ DSA following allograft in DQ mismatched pairs was more frequent than any other DSA specific for other mismatched HLA antigens. In detail, considering each HLA antigen, the estimated rate of DQ DSA (7.55 per 100 person-years, $P < 0.001$ vs. no DQ DSA mismatch) was much higher than the rates observed for DSA directed to A, B, and DR loci (2.56, 1.83, and 0.26 per 100 person-years, respectively) (Table 2).

On the contrary, HLA class I, and in particular HLA-B, mismatches were more often responsible for NDSA development (estimated rates for HLA-B, HLA-A, HLA-DR,

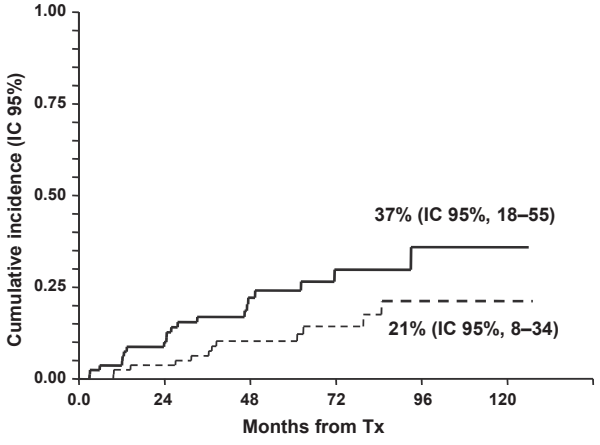

Figure 1 Cumulative incidence of HLA class I and class II DSA. Class II DSA: continuous line; class I DSA: dashed line. 95% confidence interval is also reported.

Table 2. Estimated rates of DSA and NDSA development according to each specific HLA locus mismatch. Data are reported as rate per 100 person-years and 95% confidence interval.

	DSA			NDSA		
Evaluated patients (n = 82)	Number of Ab-positive patients	Estimated rate (95% CI)	P value	Number of Ab-positive patients*	Estimated rate (95% CI)	P value
HLA-A Abs in HLA-A-mismatched patients (n = 67)	10	2.56 (1.38–4.76)	0.12	8	1.98 (0.99–3.96)	0.57
HLA-B Abs in HLA-B-mismatched patients (n = 75)	8	1.83 (0.91–3.65)	0.38	13	3.03 (1.76–5.21)	0.53
HLA-DR Abs in HLA-DR-mismatched patients (n = 60)	1	0.26 (0.04–1.84)	0.55	2	0.52 (0.13–2.11)	0.80
HLA-DQ Abs in HLA-DQ-mismatched patients (n = 55)	20	7.55 (4.87–11.71)	<0.001	5	1.48 (0.61–3.55)	0.38

*One of nine patients developing HLA-A Abs had no A locus mismatch; two of 15 patients developing HLA-B Abs had no B locus mismatch; one of three patients developing HLA-DR Abs had no DR locus mismatch; one of six patients developing HLA-DQ Abs had no DQ locus mismatch.

HLA-DQ NDSA: 3.03, 1.98, 0.52, and 1.48 per 100 person-years, respectively) (Table 2).

HLA antibody characteristics

Within the 24 KTx recipients positive for post-transplant DSA, 22 patients displayed mismatches for class I (mostly for both A and B antigens) and class II (both DR and DQ in 20/22 cases), whereas the remaining two patients expressed class I mismatches only. Among the 22 patients mismatched both for class I and class II antigens, 14 patients (64%) developed class II Abs only, six patients both class I and II Abs (27%), and only two patients class I Abs alone (9%). Among the HLA-DR full-matched donor–recipient pairs, two recipients of 22 were found to develop anti-DQ DSA. The two patients exclusively expressing class I mismatches developed, as expected, class I Abs only. All class II Abs showed specificity for HLA-DQ antigens; in one case, a concomitant presence of HLA-DR Abs was also detected.

Concerning DQ chain (alpha and beta) Ab specificities, HLA-DQB1* Abs were the most represented category (70%), whereas HLA-DQA1* Abs were found in 25% of cases. One patient displayed both HLA-DQA1* and HLA-DQB1* Abs. DQ Abs more frequently showed specificity for DQA1*05 (reactivity against *0501 and *0505 alleles), likely in relation to the fact that these alpha chains are associated in DQ molecules with DQB1*02 and DQB1*03 (*07). Indeed, Abs directed to these two DQB1* specificities were also highly represented (Fig. 2a). Regarding Abs to HLA class I, HLA-A2 was the prevalent Ab specificity, as expected (Fig. 2b). The majority of patients belonging to the NDSA group (25/37; 68%) developed class I-specific antibodies, six patients (16%) expressed class II Abs, whereas the remaining 6 (16%) developed both HLA class I and class II Abs. Among class I-specific Abs, the majority of NDSA (56%) recognized antigens belonging to cross-reactive HLA-A and HLA-B epitope groups (CREG), related to donor-mismatched HLA antigens (Fig. 3b). Epitopes belonging to HLA locus B CREG were the most represented, with the two antigens B63 and B76, belonging to B5 CREG-5C, showing the highest percentage (Fig. 3a).

Conversely, Abs directed to DRB1* and DQB1*/A1* epitopes shared with mismatched donor antigens represented only 26% of class II-specific NDSA (Fig. 3d). Also in the case of class II-specific NDSA, anti-HLA-DQ represented the majority of Abs (76%; anti-DQA1*: 50% and anti-DQB1*: 26%). The remaining 24% consisted of HLA-DRB1* Abs (Fig. 3d). Among the recognized single class II specificities, DQA1*05 and DQB1*03 were the most represented, together with DRB1*04 (Fig. 2c). In the NDSA group, Abs to HLA-C (8/37 patients; 22%) and to HLA-DP (2/37, 5%) were also observed (Fig. 3a and c).

Discussion

Sensitization to HLA antigens occurs mainly through blood transfusions, pregnancy, and previous organ transplants. When present before kidney transplantation, HLA antibodies limit access to transplantation because of cross-match incompatibility and negatively impact on graft survival [1,18–20]. When formed *de novo* post-transplant, they may be responsible for CAMR development and progression to graft loss [5–15]. The role of *de novo* HLA Abs in mediating alloimmune reactions and graft damage needs to be further investigated in nonsensitized KTx recipients, through an in-depth characterization of DSA. This could lead to the identification of tools able to prevent DSA development, and to a more precise definition of "pathogenetic" DSA, in order to focus HLA Ab monitoring and pre-emptive treatment measures [21,22].

Our study focused on the evaluation of the hierarchy of HLA Ab development in a cohort of 82 nonsensitized pediatric recipients at first KTx, followed longitudinally throughout their post-transplant course. In this population, we had previously observed a significant prevalence of

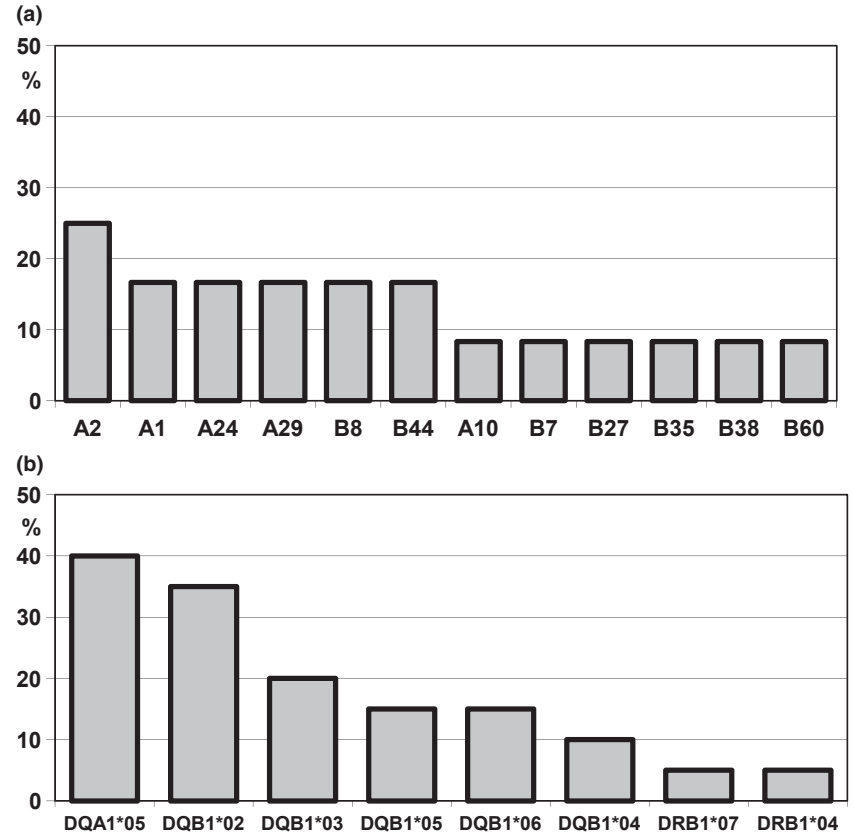

Figure 2 DSA antigen specificities identified in the study cohort. (a) Percent frequencies of HLA-DQA1*, HLA-DQB1*, and HLA-DRB1* two-digit alleles recognized by *de novo* class II DSA present in 20 pediatric kidney recipients. (b) Percent frequencies of HLA-A and HLA-B antigens recognized by *de novo* class I DSA present in 12 pediatric kidney recipients.

DSA directed to HLA class II antigens that were associated with CAMR development [12], and here we intended to verify whether further analysis supported a change in our organ allocation algorithm.

The analysis of *de novo* class II antibody specificities demonstrated that they all recognized DQ antigens, with only a negligible proportion directed to DR molecules, even though the numbers of DR and DQ mismatches were comparable. The polymorphism of both alpha and beta chains in complete DQ molecules, contrary to HLA-DR antigens, which, like class I antigens, are polymorphic in the beta chain only, could be responsible for this higher prevalence and strength of the DQ antibody category. In this regard, it is worth underlining that antibody responses against DQ alpha chains were consistently found in our patients, as already demonstrated by Deng *et al.* [23]. Despite a better donor/recipient match at class II antigens, the majority of DSA were directed to class II antigens, differently from NDSA, which were characterized by a preponderance of HLA class I-directed Abs. In addition to the higher polymorphism of the DQ locus, another possible explanation for class II antibody prevalence in DSA group may reside in the fact that class II antigens are better presented via the indirect pathway, which is known to contribute to late host alloresponses, rather than the direct pathway, which is largely active in the early period after transplantation [24,25].

The hypothesis of a humoral alloresponse persisting throughout the post-transplant period via an indirect pathway might explain the observation that nonsensitized patients developing *de novo* DSA, differently from preformed DSA, are less likely to undergo antibody-mediated acute rejection. Rather, the presence of *de novo* DSA has been preferentially associated with more indolent microvascular lesions and a slower progression to graft loss [26].

The observation that NDSA peaked earlier than DSA, and that, in patients displaying both HLA antibody categories, NDSA often preceded DSA may suggest that NDSA detection could be considered as a warning for the subsequent DSA appearance. This is in line with the observation that, in sensitized patients, the presence of pretransplant NDSA has been found to be a risk factor for subsequent post-transplant *de novo* DSA development [15].

The finding that *de novo* DSA are able to mediate kidney graft damage and that the majority of DSA in our low

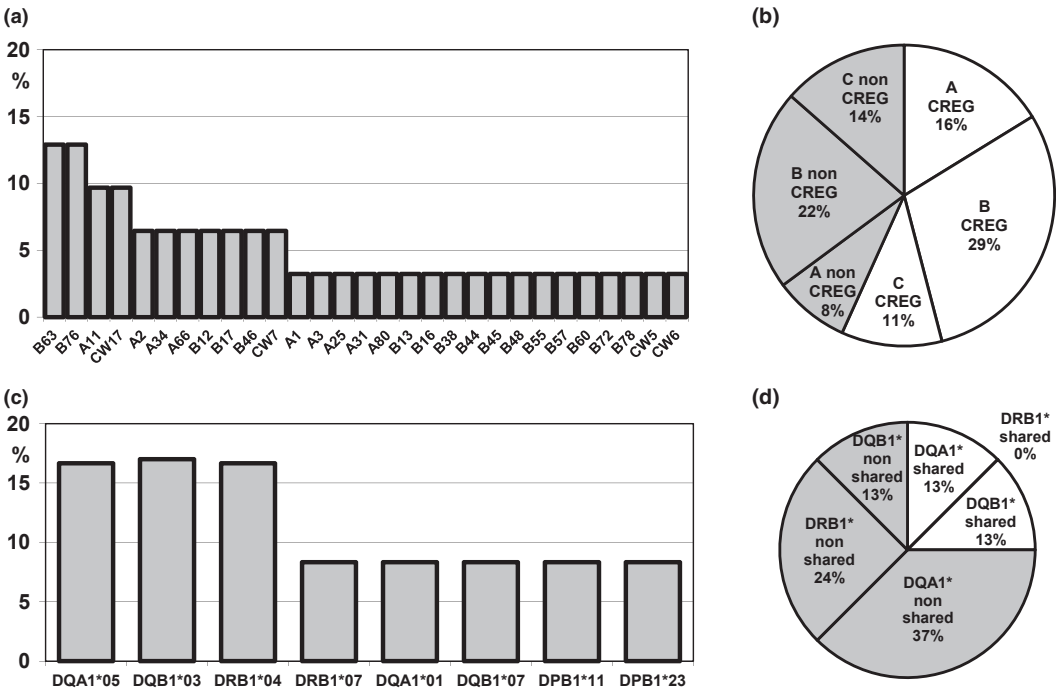

Figure 3 NDSA antigen specificities identified in the study cohort. (a) Percent frequencies of HLA-A, HLA-B, and HLA-C antigens recognized by *de novo* class I NDSA present in 31 pediatric kidney recipients. (b) Percent number of CREG and non-CREG antigens recognized at HLA-A, HLA-B, and HLA-C loci by *de novo* class I NDSA present in 31 pediatric kidney recipients. (c) Percent frequencies of HLA-DQA1*, HLA-DQB1*, HLA-DRB1*, and HLA-DPB1* two-digit alleles recognized by *de novo* class II NDSA present in 12 pediatric kidney recipients. (d) Percent number of shared and nonshared epitopes recognized at HLA-DQA1*, HLA-DQB1*, HLA-DRB1* loci by *de novo* class II NDSA present in 12 pediatric kidney recipients.

immunological risk population are specific for DQ antigens [11–15] put these molecules at the vertex of an immune stimulatory hierarchy. Moreover, our data demonstrated that *de novo* anti-DQ Abs, as observed for anti-class I, are able, even when present alone, to mediate kidney damage, thus suggesting a considerable harmful role of this antibody category. Accordingly, we believe, in line with other recent reports [11–15], that DQ locus typing should be introduced as a matching parameter in organ allocation algorithms. Indeed, although the well-known strong association between HLA-DR and HLA-DQ specificities may result in a good DQ matching in the presence of a good DR matching, a proportion of patients presents rare association variants or absence of any linkage disequilibrium. In this regard, one of the two fully matched DR patients developing anti-DQ Abs developed CAMR. Moreover, in the case of patients found positive for anti-DQ Abs in pretransplant sera, donor typing for DQA1* and DQB1* alleles should be assessed to avoid prohibited antigens. On the basis of preliminary evidence on the potential impact of DQ matching [12], in 2011, we introduced a prospective donor HLA-DQB1* typing, and from that period, we exclude from transplant patients with DSA directed against antigens of the DQ locus. Further, as suggested by a very recent paper by Wiebe *et al.* [27], class II matching could even be brought to an higher level such as epitope matching, in order to further minimize *de novo* DSA development. It is hoped that these measures decrease the incidence rate of DSA, ultimately contributing to the improvement in graft survival.

Being aware that the alloresponse is regulated as a system in equilibrium, optimization of DQ matching, while hindering anti-DQ DSA development, could theoretically lead to higher anti-class I DSA production. However, this eventuality could be better managed, as it has been shown that anti-class I DSA are more easily removed than class II DSA [28].

Authorship

FG, AN and MCa: designed the study. AN, FG and PC: performed manuscript writing. ATa, MCi, MR, CB and RB: carried out data production and collection. MCi and CK: analyzed the data. IF, ATr and AM: involved in patient care. GG, PD, DP and CK: involved in manuscript critical reading.

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