HLA-specific memory B cells : the missing link?

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Chapter 8

Comparison of HLA antibody specificities by plasma cells and memory B cells in repeat kidney transplant candidates

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Preliminary data

ABSTRACT

Kidney transplant recipients with pre-existing or *de novo* donor HLA specific antibodies pose a higher risk for developing antibody mediated rejection (ABMR) which remains to be the major cause of graft loss in kidney transplantation. Pretransplant risk assessment regarding the humoral alloimmune response in a transplant candidate is based on detection of donor HLA specific antibodies in serum that are mainly produced by bone marrow residing plasma cells. However, in patients with a history of alloimmunization, circulating memory B cells can also contribute to HLA antibody production upon re-challenge. In this regard, activated B cell culture supernatants can be used to screen for HLA antibodies produced by memory B cells. In this preliminary study, we aimed at comparing HLA antibody specificities in plasma samples and B cell culture supernatants from repeat transplant candidates (n=105). Our results showed that HLA antibodies detected in plasma samples and B cell culture supernatants display various specificity patterns. In addition to concurrent presence or absence of certain HLA antibody specificities in both sample sources, some HLA antibodies were detected only in plasma samples or solely in culture supernatants. Furthermore, in some cases where HLA antibodies were concurrently present in both plasma and culture supernatant, the hierarchy of HLA antibody specificities were reversed. These results suggest that identifying HLA antibody specificities produced by memory B cells in addition to serum antibody profiles in high risk transplant recipients may enable better risk estimation by providing a more complete picture of the humoral alloimmune response.

INTRODUCTION

In kidney transplantation, pre-existence or *de novo* development of donor directed HLA antibodies of IgG isotype is associated with (hyper)acute and chronic rejection (1). Serum samples are routinely screened for the presence of HLA antibodies before and after transplantation as the only measure of HLA-specific humoral alloimmune responses. Upon antigen recognition, naïve B cells can differentiate into short-lived plasmablasts and produce mainly low affinity antibodies of IgM isotype, or undergo germinal center reactions where somatic hypermutation, isotype switching and affinity maturation take place (2). B cells that have undergone germinal center reactions differentiate into memory B cells and/or plasma cells (3). While plasma cells mainly migrate to the bone marrow and become long-lived bone marrow resident cells that continuously produce antibodies, memory B cells circulate through the body and become an antibody secreting cell upon an antigenic re-challenge or possibly bystander activation during an infection (4, 5).

Which proportion of B cells going through germinal center reactions is committed to become plasma cells or memory B cells is unknown. Indeed, while for some viral antigens the correlation of serum antibodies *versus* antigen-specific memory B cell frequencies are known (6), it is not clear whether immunization against a certain HLA molecule will always result in generation of both memory B cells and plasma cells, and whether the ratio of these cells is different in individuals or even per HLA antigen.

In order to detect HLA antibodies deriving from memory B cells, *in vitro* polyclonal B cell activation is required. Our group was one of the pioneers in showing that memory B cells from pregnancy immunized women could be polyclonally activated *in vitro* to produce HLA antibodies (7). Later on, Han *et al*., showed that certain HLA antibody specificities derived from memory B cells were only present in B cell culture supernatants while being absent in serum samples from transplant recipients (8). Recently, Snanoudj *et al*., also showed that certain HLA antibodies were present in culture supernatants in a more restricted specificity pattern compared to serum antibodies (9). Altogether, these data suggest that B cell culture supernatants can be used to screen for HLA antibody specificities produced by HLA-specific memory B cells, and that the antibody specificity patterns detected are not identical to those from serum.

In this preliminary study, we aimed at comparing the HLA antibody repertoire of HLA-specific memory B cells and plasma cells in samples from repeat transplant candidates.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were collected prior to transplantation from repeat kidney transplant candidates (n=105) from 4 transplant centers in the Netherlands, and from healthy controls (n=102). Mononuclear cells were isolated by Ficoll Hypaque (Pharmacy LUMC, Leiden, the Netherlands) density gradient centrifugation and kept frozen in liquid nitrogen until further use. Samples were obtained with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands) under protocol number P13.025. (http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=4695).

Polyclonal activation of B cells

Polyclonal B cell activation was carried out by stimulating peripheral blood mononuclear cells (PBMCs) with an activation cocktail consisting of 2.5 µg/ml Toll-like receptor (TLR) 7/8 agonist (Resiquimod-R848) (Sigma) and 1000 IL-2 IU/ml (Proleukin, Novartis, Amsterdam, the Netherlands) (10). PBMC cultures were carried out in 10% FBS/IMDM for 6 days by seeding $2x10^6$ cells/well in 24-well flat-bottomed plates (Corning Inc., Corning, NY, USA) at 37^0C in 5% CO_2 humidified incubator. At day 6, supernatants were collected and 10-fold concentrated using 0.5 ml centrifugal filter units (Amicon Ultra, Merck Millipore Ltd. Cork, Ireland), and frozen at -20^0C until further use. To determine the extent of B cell activation, total Immunoglobulin G (IgG) ELISPOT assays were performed as previously described (11).

Flow cytometry

Flow cytometry was performed according to standard protocols using the following antibodies (clone): CD19 (J3119), CD27 (1A4CD27) (both from Beckman Coulter, Woerden, the Netherlands) and CD38 (HIT2) (eBioscience, San Diego, CA, USA).

HLA antibody detection

Serum/plasma samples and concentrated B cell culture supernatants were screened for the presence of HLA antibodies by Luminex using Lifecodes Lifescreen Deluxe kit (Immucor Transplant Diagnostics, Stamford, USA). Positive samples were further tested using LabScreen HLA class I and class II single antigen beads (One Lambda, Canoga, USA) to identify HLA antibody specificities.

Statistical analysis

The Mann-Whitney *U* test was used for comparisons of the results between groups, $p< 0.05$ was considered statistically significant.

RESULTS

Characterization of in vitro polyclonal activation of PBMC samples from patients and controls

PBMC samples were collected from repeat transplant candidates at the day of transplantation (n=38; 36%), one day before the transplantation (n=53; 51%) or at various time points before the transplantation (n=14, 13%; median: 24 days, range: 3-660 days). Samples were obtained after the first transplantation in 76 patients, after the second transplantation in 22 patients and following third transplantation in 7 patients.

Upon polyclonal activation, the median percentage of B cells in patient samples increased from 4% (range: 0.1-25%) before activation to a median of 45% (range: 0.3-73%) (Figure 1A). In addition, the percentage of $CD27^{+}CD38^{hi}$ within the $CD19^{+}$ B cells (plasmablasts) increased from a median of 0.3% (range: 0-6.3%) before activation to 42% (range: 0.4-85%) at day 6 of the culture (Figure 1B). Polyclonal activation of B cells from patients resulted in a median of 64 IgG spots per 1000 plated cells (range: 0-317 SFU/1000 cells) (Figure 1C).

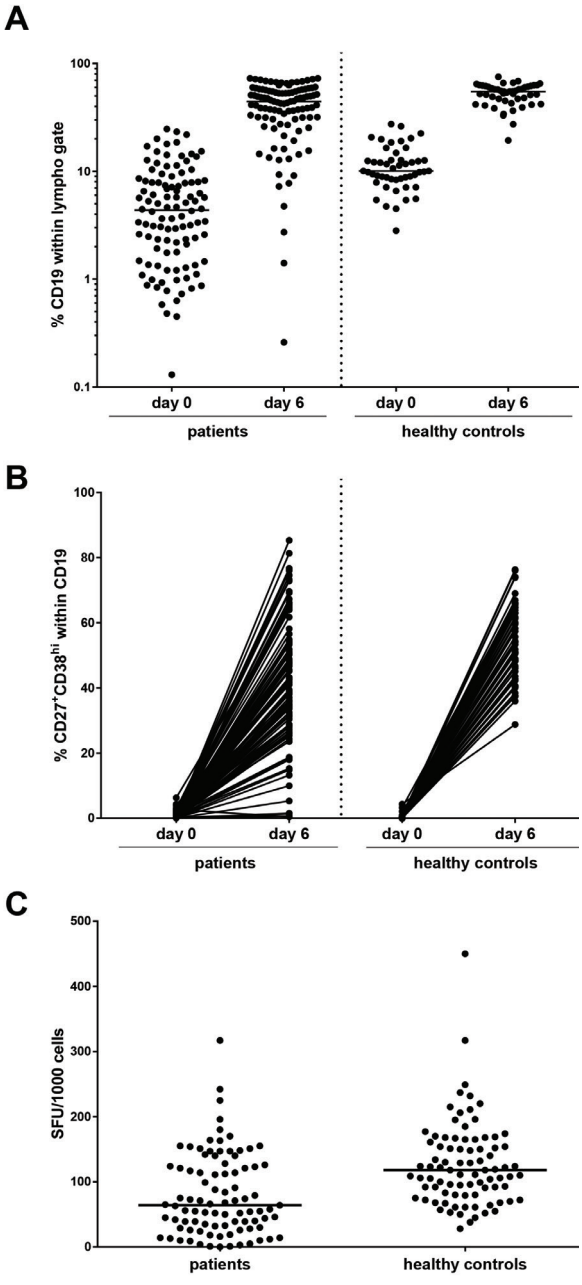

Figure 1. Characterization of polyclonal activation in patient samples. *(A) Patients have significantly lower percentage of $CD19^{+}$ cells before and after polyclonal activation, (B) lower percentage of $CD19^{+}CD27^{+}CD38^{hi}$ cells upon activation and (C) lower number of IgG spots compared to healthy controls.*

The percentage of $CD19^+$ B cells in patient samples was significantly lower than that of healthy controls before (median: 10%; range: 3-27%, $p<0.0001$) and after (median: 55%; range: 19-75%, p=0.001) polyclonal activation (Figure 1A). Likewise, at day 6 of the pre-culture phase, the percentage of $CD27^+CD38^{hi}$ B cells was significantly lower in patients (median 42%, range: 0.4-85%) compared to healthy controls (median: 54%; range: 29-76%, $p<0.0001$) (Figure 1B). In accordance with these findings, significantly lower spot counts were observed in samples derived from patients (median SFU: 64; range: 0-317) compared to healthy controls (median SFU: 118; range: 28-450 SFU/1000 cells) upon polyclonal activation ($p<0.0001$) (Figure 1C).

Distribution of HLA antibody specificities in culture supernatants and plasma samples

HLA antibody analysis in 10-fold concentrated supernatants revealed that 24/105 (23%) patients had evidence for circulating memory B cells directed at the mismatched HLA of the previous donors whereas no HLA antibodies were detected in supernatants of 81/105 (77%) patients. Among those with detectable supernatant HLA antibodies (n=24), 6 (25%) samples had HLA class I antibodies only, 11 (46%) had class II antibodies only and 7 (29%) samples had antibodies directed at both HLA class I and II. While 23% of the patients had HLA antibodies in their culture supernatants, serum HLA antibodies were present in 52% (n=55) of the patients. HLA antibodies detected in plasma samples and 10-fold concentrated B cell culture supernatants displayed various patterns. As exemplified in Figure 2, some HLA antibody

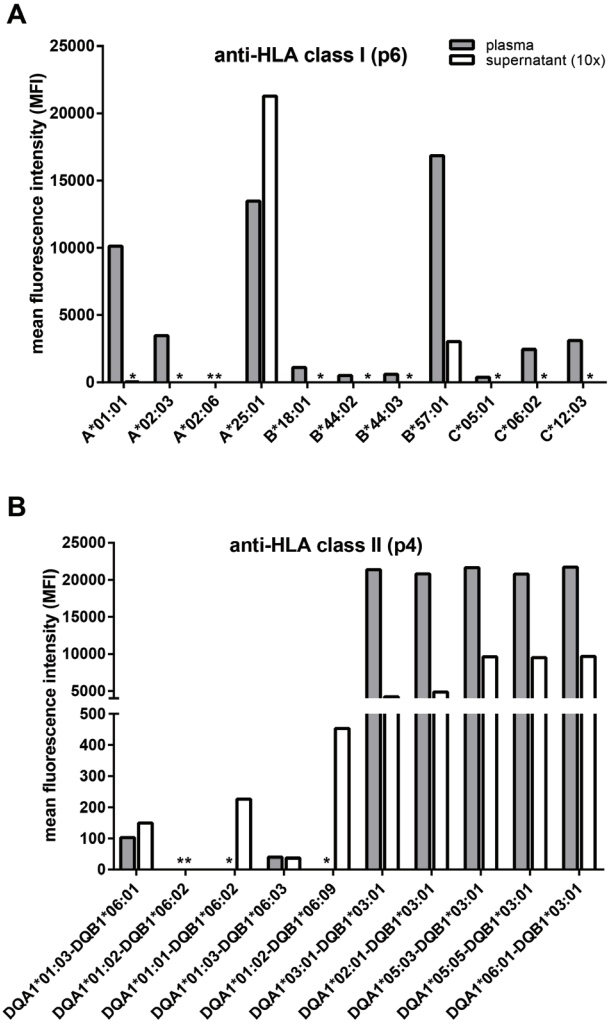

Figure 2. HLA antibody patterns in plasma and (10x) culture supernatants. *Distribution of HLA antibodies directed to previous graft donor mismatch (A) HLA class I (p6) and (B) HLA class II (p4) in 2 different repeat transplant candidates.*

specificities were present only in plasma samples whereas some were present both in the plasma and culture supernatants. Interestingly, as shown for anti HLA-A*25:01 and B*57:01 in figure 2A, the hierarchy of the HLA antibody specificities were reversed in culture supernatants in comparison to plasma samples. Furthermore, while there were no antibodies detected against certain HLA mismatches both in the plasma and culture supernatants, some HLA antibody specificities (e.g. DQA1*01:01/DQB1*06:02 or DQA1*01:02/DQB1*06:09) were found only in B cell culture supernatants (Figure 2B).

Emergence of HLA antibody specificities in culture supernatants upon further concentration

The fact that supernatant HLA antibodies were detected in only 44% of patients with serum antibodies (n=24/55) leaves the possibility that IgG levels in the supernatants are too low for proper HLA antibody detection. We therefore compared the concentration of IgG in culture supernatants to plasma samples obtained at the same bleeding date in a smaller group of transplant recipients (n=28). Healthy individuals with (n=4) or without (n=4) a history of alloimmunization were included as controls. Similar to the larger cohort, 50% of the patients in this group (n=14/28) had HLA antibodies in plasma and among those with plasma HLA antibodies 42% (n=6/14) of the patients had detectable HLA antibodies in B cell culture supernatants (Table 1). As summarized in Table 1, supernatant IgG concentrations of patient samples were significantly lower than that of healthy controls while plasma IgG concentrations did not differ between these two groups.

Table 1: Demographics of patients and controls

	Patients	Controls	p value
n	28	8	
Gender			
female	14	4	
male	14	4	
Age (years)	44 (20-69)	47 (39-62)	0.4
Number of previous tx (n)			
1	19	n/a	
2	7		
3	2		
Immunosuppressive treatment (n)			
Yes	21	n/a	
No	7		
Supernatant IgG concentration (10x) (µg/ml)	19 (0-144)	94 (14-135)	0.001
Total IgG SFU (1000 cells/well)	66 (0-242)	127 (64-249)	0.02
%CD19 (day 0)	2 (0-23)	12 (7-20)	0.0003
%CD19 (day 6)	40 (0-72)	55 (47-63)	0.03
Supernatant HLA antibody status (n)			
only class I +	3	0	
only class II +	2	1	
both +	1	3	
both -	22	4	
Plasma IgG concentration (µg/ml)	4284 (884-15519)	7291 (3043-8420)	0.08
Plasma HLA antibody status (n)			
only class I +	4	0	
only class II +	6	0	
both +	4	4	
both -	14	4	

To determine whether HLA antibody concentration was affecting the ability to detect the antibodies, we diluted the plasma samples from 8 patients to the level of IgG present in 6-day culture supernatants. As shown in Table 2, all HLA class I antibodies disappeared when the plasma IgG levels were as low as that of supernatants whereas the majority of HLA class II positivity persisted despite the dilution of plasma samples. To formally prove that low IgG concentrations in the supernatants were causing the lack of HLA antibody detection, we aimed to increase the IgG content in culture supernatants further by culturing for a longer period

and more extensively concentrating the supernatants. To this aim, we polyclonally activated 2 patient samples (p25 and p26) for 6, 10 and 20 days and followed the IgG concentration in the supernatants, as well as the total IgG producing cell counts at each time point. As expected, longer culture periods resulted in higher IgG concentrations. However, total IgG producing B cell counts became undetectable at day 20 as evident by lack of spots in ELISPOT assays, most likely due to cell death. We next concentrated the culture supernatants obtained at day 10 (IgG p25: 9 µg/ml and p26: 21 µg/ml) up to 356 µg/ml (p25) and 1153 µg/ml (p26) and tested for the presence of HLA antibodies.

Table 2: Presence of HLA antibodies in culture supernatants and plasma samples

	supernatant (10x)		plasma (neat)		plasma (diluted)		
patient #	class 1	class 2	class 1	class 2	class 1	class 2	dilution
p1	+	-	+	+	-	-	1:146
p2	+	-	+	-	-	-	1:232
p3	+	-	+	-	-	-	1:299
p4	-	+	+	+	-	+	1:295
p5	-	+	-	+	-	+	1:61
p6	+	+	+	+	-	+	1:699
p25	-	-	+	-	-	-	1:307
p26	-	-	-	+	-	+	1:87

Interestingly, although IgG levels in these culture supernatants were still lower than in the corresponding neat plasma samples, we found that certain HLA antibodies (directed to Bw4 epitopes) detected in neat plasma samples were emerging in supernatant sample p25 upon further concentration. However, some strong HLA antibodies present in plasma samples such as HLA-B*07:02 and B*82:01 did not appear in the supernatant at all despite further concentration (Figure 3A). Similarly, in sample p26, while some HLA-DR and HLA-DP antibody specificities emerged in supernatants upon further concentration, HLA-DQ antibodies were equally detectable in both diluted plasma samples and further concentrated supernatants (Figure 3B).

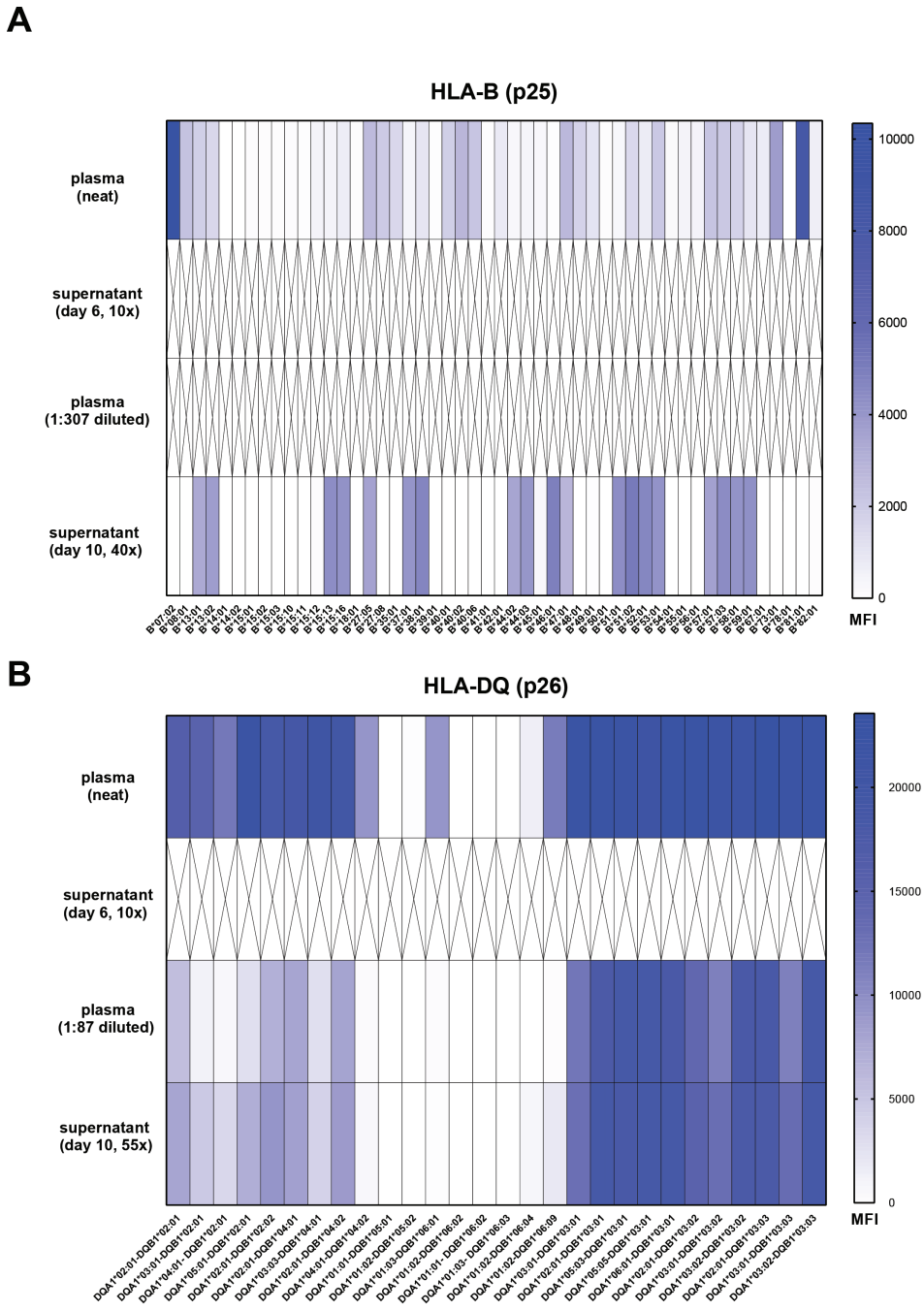

Figure 3. Emergence of HLA antibodies in culture supernatants upon further concentration. *(A) HLA-B antibody specificities as detected by Luminex single antigen bead assays in neat and diluted plasma samples in comparison to concentrated culture supernatants from patient p25. (B) HLA-DQ directed antibody specificities in samples from patient p26.*

DISCUSSION

In clinical kidney transplantation, rapid donor-specific antibody production deriving from an anamnestic response of memory B cells may contribute to antibody mediated rejection (ABMR) episodes (12, 13). Repeat transplant candidates, patients undergoing desensitization therapy or women receiving grafts from their spouses or children comprise a high risk patient group for developing ABMR since they may harbor silent HLA-specific memory B cells which can differentiate into antibody producing plasma cells upon re-challenge (14).

Currently, humoral immune responses in the setting of clinical kidney transplantation are only measured by means of serum HLA antibodies which are mainly produced by bone-marrow residing plasma cells. However, exposure to foreign HLA via blood transfusions, pregnancies or previous transplantations may result in the formation of dormant memory B cells which become antibody secreting cells after re-encounter with the immunizing antigen (4).

HLA antibody specificities deriving from memory B cells can be detected in culture supernatants of activated B cells. While in the study of Han *et al*., IgG isotype of HLA antibodies in 8-14 day culture supernatants were found in 13 of 16 transplant patients tested covering 50 HLA specificities, the majority (70%) of these antibody specificities overlapped with those in found in serum and only 4 (8%) of them were detected solely in culture supernatants. Interestingly, in addition to IgG antibodies, the authors detected 24 DSA of IgM isotype with almost half of them (45%) present only in B cell culture supernatants. Recently, in 10-day B cell culture supernatants that were 30-fold concentrated, Snanoudj *et al*., found HLA-specific antibodies in 18 out of 39 (50%) transplant recipients among whom 5 (13%) patients had HLA antibody specificities detected only in B cell culture supernatants (9). The differences among these studies may lie on the different B cell activation cocktails used as well as the differences in concentrations of B cell supernatants, which may result in appearance of some HLA specificities otherwise undetected. So far there is no established way on how much to concentrate B cell culture supernatants. As a consequence, the detectability of HLA specificities in culture supernatants is not standardized and may be related to the concentration of IgG in the culture supernatant.

Here we show that HLA antibodies derived through polyclonal activation of memory B cells were detected in 23% of 10-fold concentrated culture supernatants from repeat transplant candidates whereas approximately 52% of the serum samples from these recipients were positive for HLA antibodies. One explanation for this discrepancy between the incidence of HLA antibodies in serum and culture supernatants from patients could be the significantly lower amount of IgG in patient samples compared to controls. Appearance of HLA antibodies in samples with higher IgG concentrations (Figure 3) suggests that relatively lower IgG amounts may have affected the detectability of the HLA-antibodies in our culture supernatants. An alternative explanation for this discrepancy is that immunization against a certain HLA which results in HLA-specific plasma cell formation, leading to serum HLA antibodies, might not

always be associated with concurrent HLA-specific memory B cell generation. Interestingly, we observed persistence of HLA class II antibodies in diluted plasma samples whereas HLA class I antibodies were undetectable. This may possibly explain the predominance of HLA class II antibodies in patients who do not respond well to ABMR treatment modalities involving removal of DSA and elimination of B cells/plasma cells (15, 16).

In order to accurately compare the HLA antibody specificities deriving from plasma cells in serum and reactivation of memory B cells in culture supernatants, ideally similar IgG concentrations in both sources need to be achieved. We are currently working on isolating IgG from culture supernatants by Protein G affinity purification to reach an IgG concentration that allows for a more standardized HLA antibody comparison in serum and culture supernatants. Presence of serum DSA has long been a barrier for transplantation of highly immunized patients. With the advances in immunosuppressive agents and HLA antibody testing, a considerable percentage of these patients can now be transplanted following desensitization treatments that lead to a decrease or complete disappearance of DSA titers. Some patients, even without DSA following desensitization, can manifest with ABMR early in the post transplantation period (13, 17). Identification of HLA antibody specificities with a memory profile in the absence or presence of serum antibodies in patients with DSA before transplantation may help clinicians to discriminate the ones that are more prone to develop ABMR post transplant. It is clear that not every highly immunized patient can benefit from desensitization treatments. Determination of the discrepancies in HLA antibody specificities deriving from memory B cells and plasma cells in immunized transplant recipients may allow for developing strategies to exclude some of the so called unacceptable HLA antibody specificities from the antibody profile of patients with very broad immunization.

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