Rapid Communication

Early acute antibody-mediated rejection of a negative flow crossmatch
3rd kidney transplant with exclusive disparity at HLA-DP

Beata Mierzejewska a, Paul M. Schroder b, Caitlin E. Baum b, Annette Blair c, Connie Smith c, Rene J. Duquesnoy d, Marilyn Marrari d, Amira Gohara e, Deepak Malhotra e, Dinkar Kaw f, Michael A. Rees g, Stanislaw Stepkowski h

a, a.a., amino acid; Abs, antibodies; AMR, antibody-mediated rejection; cPRA, calculated panel reactive antibody; C’, complement; CDC, complement-dependent cytotoxicity; DSA, donor-specific alloantibodies; FXM, flow crossmatch; IgG, IgG mean fluorescence intensity; MCS, mean channel shift; MFI, median fluorescence intensity; MMF, mycophenolate mofetil; SAB, single antigen match; IgG MFI, IgG mean fluorescence intensity; MCS, mean channel shift; MFI, ment-dependent cytotoxicity; DSA, donor-specific alloantibodies; FXM, flow cross-rejection; cPRA, calculated panel reactive antibody; C’, complement; CDC, complement-dependent cytotoxicity (CDC) [2,4–6]. Some indicated that the presence of DSA with negative FXM or CDC suggested no risk of antibody-mediated AMR [2,4–6]. However, each of these studies described the effects of DSA in general and did not analyze the specific effects of DSA against HLA-DP. There are also conflicting reports regarding the impact of HLA-DP mismatches on kidney transplant survival. Indeed, patients with donors compatible at HLA A, B, C, DR and DQ have an 80% chance of being mismatched at DP [8]. Initial studies suggested that an exclusive mismatch of HLA-DP antigens alone had little impact on the survival of primary kidney transplants in non-sensitized recipients [9]. Other reports of high resolution DNA typing of 3600 first and 1300 repeat deceased donor kidney recipients revealed that the one-year transplant survival of first grafts was significantly higher without HLA mismatches than with two DPB mismatches [10]. Furthermore, re-transplant recipients with a calculated panel reactive antibody (cPRA) > 50% had a higher one-year graft survival in the absence of DPB mismatches [10]. Two case reports indicated selective disparity at DPB or DPA may be responsible for AMR of kidney re-transplants [11–13]. However, in each of these cases the FXM was positive. We present the case of a patient with early aggressive AMR of an exclusively DP-mismatched, FXM negative 3rd kidney transplant...
following previous immunization by two transplants. We used retrospective high-resolution typing, HLA Matchmaker, and the Luminex single antigen bead (SAB) assay to comprehensively analyze the immunization events.

2. Materials and methods

2.1. Flow crossmatch

A standard FXM method was used to analyze the patient's sera and single cell leukocyte preparations from donor peripheral blood by 3 color staining performed on a Coulter Epics XL (CD3-P, Fisher Scientific; CD19-PE-CY5 and IgG-FITC, Beckman Coulter). Serum samples were tested by FXM with pronase-treated donor cells using cutoff values of 40 mean channel shift (MCS) for T cells and 80 MCS for B cells. The FXM results were obtained as IgG mean fluorescence intensity (IgG MFI) values, which were re-calculated for MCS using the following formula: channel value = 256/log(10 + [IgG MFI]/1.024). The MCS was calculated by subtracting the negative control channel value from the serum sample channel value.

2.2. Single antigen bead assay

Patient sera were tested for class I (HLA A, B, and C) and class II (HLA DR, DQ, and DP) HLA Abs using SAB on a Luminex platform (LIFECODES LSA Single Antigen, Gen Probe and LABScreen Single Antigen, One Lambda). All tests were performed according to the manufacturer's protocol. Some sera were also tested for C1q-binding HLA Ab using commercially available kits (C1qScreen, One Lambda) and a modified wash technique. Ab specificity was analyzed using baseline normalized mean fluorescence intensity (MFI) values.

2.3. HLA typing

The 4-digit HLA types for the patient as well as donors 1 and 2 were determined by Sanger sequence-based typing using Life Technology SeqCore kits (Invitrogen). The 2-digit HLA typing for donor 3 was performed for HLA A, B, C, DR, and DQ using sequence-specific oligonucleotide probes (PCR-SSOP-Luminex; GenProbe). This typing was converted to 4-digits using HLAMatchmaker and haplotype frequency data (based on the National Marrow Donor Program and the Allele Frequency Net Database). The 4-digit typing of HLA-DP for donor 3 was performed using PCR-SSOP (LabType SSO DPA1/DPB1, One Lambda).

2.4. HLAMatchmaker

The HLAMatchmaker program was used to determine HLA Ab-binding to eplets (structurally-defined epitopes consisting of polymorphic amino acids (a.a.) located within a 3-Å radius on the Ab-accessible surface of the HLA molecules). Each eplet represents a potential Ab-binding site on a 4-digit HLA allele. HLAMatchmaker determines self and non-self eplets for each HLA allele in the Luminex SAB HLA panel. Furthermore, eplets on HLA alleles which yield negative reactivities are considered acceptable eplet mismatches and are eliminated from the analysis. The remaining eplets on HLA alleles with positive reactivities are analyzed to explain the Ab reactivity patterns. HLAMatchmaker analyses are performed using raw MFI values, and the Excel spreadsheet-based program is accessible at http://www.HLAMatchmaker.net.

3. Results

3.1. Clinical case description

The patient is a 24 years old Caucasian female with end-stage renal disease secondary to reflux nephropathy and recurrent pyelonephritis. At age 9, she received a living-donor transplant (cPRA = 0%; negative cytotoxic crossmatch) which was mismatched at HLA A/B/C/DR/DQ/DP (Table 1; a timeline of events is shown in Fig. 1). Immunosuppression composed of cyclosporine/mycophenolate mofetil (MMF) for 5 years followed by rapamycin/MMF protected the transplant for 7 years. Over the next 3 years, kidney function continued to deteriorate, the patient was placed on dialysis, and the kidney was removed after the 2nd transplant. The 2nd deceased-donor transplant was mismatched only at DPB1 and DRB3 (Table 1). Despite a negative FXM and potent quadruple therapy (alemtuzumab, tacrolimus, mycophenolic acid, and methylprednisolone), the transplant failed within one year due to acute and chronic rejection and was removed 14 months after transplantation. As a result of the two failed transplants and over 30 blood transfusions, the patient had cPRA of 100%.

The 3rd deceased-donor transplant was matched at HLA A/B/C/DR/DQ but mismatched for DPA1 and DPB1 (Table 1). Immunosuppression consisted of alemtuzumab induction, 5 days of steroids, and maintenance with tacrolimus and mycophenolate sodium. Despite the fact that just prior to the 3rd transplant there were DSA against DP (Table 3; results explained in Section 3.3), the FXM results with both T (MCS = 0) and B (MCS = 62) cells were negative (Table 2). This observation suggests that even relatively stringent FXM analysis may fail to reveal a potential danger from

Table 1

<table>
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<th>B</th>
<th>C</th>
<th>DRB1</th>
<th>DRB3</th>
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<td>05:01</td>
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</tr>
</tbody>
</table>

a Bold typeface indicates HLA type disparity from that of the recipient.
b HLA allele typing for recipient and donors 1 and 2 was performed by a Sanger sequencing method.
c DRB4*1:FDX includes DRB4*1:01, DRB4*1:03, DRB4*1:04, DRB4*1:05, DRB4*1:06, and DRB4*1:07.
d DPA1*02:AB includes DPA1*02:01 and DPA1*02:02, which have identical epitopes when analyzed by HLAMatchmaker (http://www.hlamatchmaker.net).
e 4-Digit A, B, C, DR, and DQ typing for donor 3 was obtained by converting medium resolution PCR typing (PCR-SSOP LUMINEX) using HLAMatchmaker; DP typing was performed by PCR-SSOP.
DP-directed DSA, perhaps due to the low cell surface expression of HLA-DP antigens [14]. Indeed, within 11 days post-transplant, the patient had elevated creatinine (3 mg/dL) and biopsy-confirmed C4d+ acute AMR (Banff 2a; Fig. 2A). Immediate therapy with 5 doses of plasmapheresis and 10-day thymoglobulin, followed by subsequent rounds of plasmapheresis, IVIG and rituximab failed to reverse C4d+ AMR, and the graft was lost 6 months after transplantation.

3.2. SAB analysis after the 3rd transplant

SAB analysis of serum collected 5 weeks after the 3rd transplant confirmed potent IgG DSA reactivity by a bead coated with donor DPA1⁄02:01 and DPB1⁄01:01 (9282 MFI, One Lambda; Fig. 2B top left panel; 16291 MFI, GenProbe; Table 3). Analysis of IgG subclasses using the One Lambda assay revealed that following the 1st transplant both IgG1 (2211 MFI) and IgG2 (4608 MFI) DSA were present, but after the 3rd transplant DSA were entirely complement (C')-binding IgG1 (7580 MFI; Fig. 2B top right and bottom left panels); both IgG3 and IgG4 were negative (not shown). This was supported by the C1q-SAB assay, as DSA had negative results prior to, and very potent C1q-binding activity (22647 MFI) after, the 3rd transplant (Fig. 2B bottom right panel). Luminex testing of sera at multiple dilutions (including 1:8 and 1:16) gave negative results, demonstrating the lack of a prozone effect (data not shown).

Table 3
Reactivities against the 3rd donor mismatched HLA-DP alleles (DPA1⁄02:01/02:02, DPB1⁄01:01, DPB1⁄04:02) after the 1st transplant as well as before and after the 3rd transplant.

<table>
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<th>#</th>
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<th>HLA-DPB1⁄</th>
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<th>Prior Tx 3</th>
<th>After Tx 3</th>
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<td>DPA1</td>
<td>DPB1</td>
<td>MFI</td>
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<td>02:01 self</td>
<td>0</td>
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<tr>
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<td>04:01 self</td>
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<tr>
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<td>01:01 donor</td>
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<tr>
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<tr>
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<td>04:01 self</td>
<td>265</td>
<td>None</td>
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<tr>
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<td>04:02 donor</td>
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<tr>
<td>9</td>
<td>01:03 self</td>
<td>11:01 1st donor</td>
<td>7841</td>
<td>N/A</td>
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N/A – not applicable.
None – lack of reactivity.
Row 1 to 8 – Gen Probe SAB assay (LIFECODES LSA Single Antigen).
Row 9 – One Lambda SAB assay (LABScreen Single Antigen).
Bold – indicates an allele or eplet of the 3rd donor and the associated positive MFI values.

a DPA1⁄02:01 and DPA1⁄02:02 have identical allogeneic eplet profiles in HLA Matchmaker and thus DPA1⁄02:02 is considered to be an informative donor allele.

b MFIs are presented as baseline normalized values.
3.3. Retrospective SAB analysis

Retrospective SAB analyses of sera after the 1st, prior to the 3rd, and after the 3rd transplant revealed broad immunization, including Abs against DPA1\(^{02:01}\) and DPB1\(^{01:01}\) of the 3rd donor (Table 3). The DSA kinetics were displayed by informative beads coated with self and donor chains in various combinations in order to elucidate the Abs associated with rejection of the 3rd transplant. Although self-beads (Table 3 rows 1 and 2) showed no reactivity, two beads that corresponded to the 3rd transplant DPA1\(^{02:01}\)/DPB1\(^{01:01}\) alleles measuring IgG (upper left panel), IgG1 (upper right panel), IgG2 (lower left panel) and C1q-binding (lower right panel). (C) Kinetics of anti-DP response directed against DPA1\(^{02:01}\) (upper left panel), DPA1\(^{02:02}\) (upper middle panel), DPA1\(^{04:01}\) (upper right panel), DPB1\(^{01:01}\) (lower left panel), DPB1\(^{11:01}\) (lower middle panel) and DPB1\(^{03:01}\) (lower right panel). Each panel represents a time course of median fluorescence intensity (MFI) of serum Abs binding to the indicated HLA. The beads selected for the analysis of a DPA1 chain (DPA1\(^{02:01}\), DPA1\(^{02:02}\), or DPA1\(^{04:01}\)) were combined with the recipient self-DPB1\(^{04:01}\). In a similar fashion, beads selected for the analysis of a DPB1 chain (DPB1\(^{01:01}\), DPB1\(^{11:01}\), or DPB1\(^{03:01}\)) were paired with the recipient self-DPA1\(^{01:03}\) chain. Consequently, each panel in 1C represents the kinetics of Ab response to the DPA1 or DPB1 chain. Each dot represents one serum time point and is representative of multiple tests of serum from that time point.

Fig. 2. Kinetics of immunization against donor DP alleles. (A) Histology of the 3rd transplant during rejection. H&E staining of a biopsy during the 3rd kidney rejection showing moderate tubulitis, focal calcifications, and necrosis with large lymphocytic infiltrates and focal hemorrhage to the interstitium graded as Banff 2a and with a black arrow indicating vessels with transmural inflammation and swollen endothelial cells (upper panel) and C4d-positive staining during the 3rd kidney rejection with a black arrow indicating C4d-positive in peritubular capillaries (lower panel). (B) Kinetics of DSA response directed against a bead coated with the 3rd transplant DPA1\(^{02:01}\) and DPB1\(^{01:01}\) alleles measuring IgG (upper left panel), IgG1 (upper right panel), IgG2 (lower left panel) and C1q-binding (lower right panel). (C) Kinetics of anti-DP response directed against DPA1\(^{02:01}\) (upper left panel), DPA1\(^{02:02}\) (upper middle panel), DPA1\(^{04:01}\) (upper right panel), DPB1\(^{01:01}\) (lower left panel), DPB1\(^{11:01}\) (lower middle panel) and DPB1\(^{03:01}\) (lower right panel). Each panel represents a time course of median fluorescence intensity (MFI) of serum Abs binding to the indicated HLA. The beads selected for the analysis of a DPA1 chain (DPA1\(^{02:01}\), DPA1\(^{02:02}\), or DPA1\(^{04:01}\)) were combined with the recipient self-DPB1\(^{04:01}\). In a similar fashion, beads selected for the analysis of a DPB1 chain (DPB1\(^{01:01}\), DPB1\(^{11:01}\), or DPB1\(^{03:01}\)) were paired with the recipient self-DPA1\(^{01:03}\) chain. Consequently, each panel in 1C represents the kinetics of Ab response to the DPA1 or DPB1 chain. Each dot represents one serum time point and is representative of multiple tests of serum from that time point.
developed by exposure to the 3rd transplant DPB1*01:01 (7658 MFI) which shares eplets (Table 4) with the 1st transplant DPB1*11:01 (7841 MFI; Table 3 row 9). Thus, DPA1 and DPB1 immunization by the 1st transplant was associated with an acute AMR of the 3rd kidney transplant. Notably, the patient had previously been exposed to 3rd donor DPB1*04:02 and did not develop reactivity, whereas the patient had never been exposed to 3rd donor DPB1*01:01 and yet immediately developed potent reactivity. As such, prospective HLA typing of all HLA alleles and subsequent eplet analysis should be considered for all sensitized transplant patients in order to distinguish acceptable versus unacceptable alleles.

3.4. Eplet-defined analysis by HLAMatchmaker

To follow the DSA kinetics prior to and for 6 months after the 3rd transplant, we analyzed Ab binding to a single bead with donor DPB1*02:01 (Fig. 2C; top left) or DPB1*02:02 (Fig. 2C; top middle) combined with self-DPA1*04:01. Since these two DPB1 alleles have identical allogeneic eplet profiles in HLAMatchmaker (111R, 51RA, 83A, and 127P; Table 4), they both displayed no reactivity after the 1st transplant (312/265 MFI). Then, their reactivity increased prior to the third transplant (3125/3138 MFI) during the rejection of the 3rd transplant and became very high to the third transplant (9 time points), all three beads had almost identical patterns of Ab binding. From this, we concluded that the 111R eplet dominated the immunization phase. During the rejection process and for 6 months following the 3rd transplant (9 time points), all three beads had almost identical patterns, suggesting that an aggressive AMR had resulted in active spreading of reactivity, producing Abs reactive not only to the 111R eplet but also to the 51RA, 83A, and/or 127P eplets (Fig. 2C; top panels). We also analyzed the Ab reactivity to a bead with the 3rd donor DPB1*01:01/self-DPA1 (Fig. 2C; lower left panel) and found that it was almost identical to the pattern of a bead with the 1st donor DPB1*11:01/self-DPA1 (Fig. 2C; lower middle panel). Because two eplets (35YA and 84DEAV) are shared by these DPB1 alleles, we initially suspected that both eplets dominated immunization after the 1st transplant and actively participated in acute AMR of the 3rd transplant. However, since the same pattern of Ab binding was also detected using a bead with DPB1*03:01/self-DPA1 (Fig. 2C; lower left panel) containing only the 84DEAV eplet, we concluded that the 84DEAV eplet played the crucial role in DPB DSA immunity. From these analyses, we have concluded that eplet 111R controlled an immunization process against DPB1*02:01 and eplet 84DEAV against DPB1*11:01 of the 1st donor. As many as four eplets on DPB1*02:01 and two shared eplets on DPB1*01:01 were associated with the AMR after the 3rd transplant. These results suggest that eplet spreading in DPB1*02:01 together with eplet sharing between DPB1*11:01 and DPB1*01:01 may contribute to AMR by pre-transplant FXM-undetectable DSA leading to a robust acute AMR.

4. Discussion

There are conflicting reports discussing the importance of DP disparity in AMR, and some emphasize the role of DP-specific Abs in re-transplants; however, in each of these reports DSA were present to such an extent that FXM were positive [9–12]. The clinical relevance of DP-directed Abs and their pathogenicity became more apparent after a recent description of two patients with DPB disparity who developed recurrent acute AMR and graft failure [11]. Recent evidence showed that even low levels of pre-transplant C-binding DSA were associated with early AMR, although DSA against HLA-DP were not studied [15]. Others found that sensitization to DP is caused by transplants and blood transfusions as well as cross-reactivities with other antigens (for example, DR antigens [16]) by sharing common eplets [13,17,18]. Another mechanism of sensitization, epitope (eplet) spreading, is an expansion of the immune response from an inducing epitope (eplet) to non-cross-reactive epitopes (epitets) [19,20] and has been shown
to have a boosting effect, especially with recurring rejections [21]. The 84DEAV eplet was well-defined by monoclonal Abs [22,23] documenting the kinetics of immunization at the eplet level and revealing that spreading of reactive eplets (as observed with DPB1∗01:01) as well as sharing eplets on immunizing and retransplant alleles (DPB1∗11:01 and DPB1∗01:01) can alone be responsible for sensitization and acute AMR. Since DSA may bind to epitopes composed by a.a. of two class II HLA chains [26,27], we suggest that the most informative beads were those coated with donor DPB1∗02:01/DPB1∗01:01 alleles.

In summary, we present the case of a highly-sensitized patient which demonstrates that pre-transplant DSA against repeated mismatched HLA-DP are associated with early AMR, even in the absence of FXM positivity. Accordingly, we recommend that the presence of DP-directed Abs in sensitized patients should require the identification of both patient and donor DPA and DPB before transplantation. Furthermore, eplet-based analysis of immunization history combined with the high resolution HLA typing of sensitized patients and donors can allow clinicians to evaluate the immunogenicity of disparate donor HLA alleles and thus the relative risk of AMR. Therefore, sensitized patients with relevant donor specific HLA-DP Abs ought to be thoroughly analyzed before transplantation, with a consideration for desensitization, and should have close monitoring of DSA after transplantation.

Contributions to the paper

Beata Mierzejewska performed SAB assays and analyses, performed HLA-Matchmaker analyses, prepared figures and tables, edited paper; Paul Schroder prepared figures and tables, edited paper; Caitlin Baum prepared figures and tables, edited paper; Annette Blair performed SAB assays and analyses; Connie Smith performed SAB assays and analyses; Rene J. Duquesnoy performed HLA-Matchmaker analyses; Marilyn Marrari performed HLA-Matchmaker analyses; Amira Gohara performed pathology examination and analyses of biopsy results; Deepak Malhotra performed clinical therapy of the patient and analyses of clinical results; Dinkar Kaw performed clinical therapy of the patient and analyses of clinical results; Robert Liwski performed C1q measurements and analyses; Michael A. Rees performed kidney transplantation, clinical therapy of the patient and analyses of clinical results; Dinkar Stepkowski performed analyses of tissue typing results, wrote paper.

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References