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ORIGINAL ARTICLE

Antibody-mediated rejection despite inhibition of terminal complement

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Conflicts of interest
MDS has research contracts with Alexion Pharmaceuticals and Millennium Pharmaceuticals. LDC has research contracts with Alexion Pharmaceuticals. DBT receives royalties from One Lambda for C1q license. All other authors have no conflicts of interest.

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Summary

Terminal complement blockade has been shown to decrease the incidence of early acute antibody-mediated rejection (eAMR) in the first month after positive cross-match kidney transplant recipients, yet some patients still develop eAMR. The current study investigated possible mechanisms of eAMR despite eculizumab treatment. Of the 26 patients treated with eculizumab, two developed clinical eAMR and another patient developed histologic signs of eAMR without graft dysfunction ('subclinical eAMR'). Twenty-three did not have histologic injury on early surveillance biopsies. All 26 patients had therapeutic levels of eculizumab and showed complete blockade of complement in hemolytic assays. High levels of donor-specific alloantibody (DSA) including total IgG, IgG3, and C1q+ DSA were present in patients with and without eAMR, and none correlated well with eAMR. In contrast, IgM DSA was present in only four patients after transplantation: the two patients with clinical eAMR, one patient with subclinical AMR, and one patient without eAMR ($P = 0.006$ correlation with eAMR). Both clinical eAMR episodes were easily treated with plasma exchange which removed IgM more completely and rapidly than IgG, resulting in normalization of function and histology. These data suggest a possible role of antidonor IgM DSA in the pathogenesis of eAMR in patients treated with terminal complement blockade (ClinicalTrials.gov Identifier: NCT00670774).

Introduction

We recently demonstrated that terminal complement blockade with eculizumab, a humanized monoclonal antibody with high affinity for C5, decreased early acute antibody-mediated rejection (eAMR) in positive cross-match kidney transplantation (+XMKTx). The incidence of clinically significant eAMR in patients treated

post-transplant with eculizumab was 7.7% (2/26) compared with 41.2% (21/51, $P = 0.003$) in a historical control group treated with a similar plasma exchange (PE)-based protocol [1].

The goal of the current study was to investigate the possible causes of eAMR in the first month after +XMKTx in patients treated with eculizumab.

Patients and methods

Patient population

These studies were carried out using protocols approved by the Institutional Review Board of the Mayo Foundation and Clinic. Twenty-six consecutive +XMKTx patients provided written consent to participate in this study between 2008 and 2010 who received C5-inhibition (Eculizumab) as part of a PE-based desensitization protocol [1]. Study patients had samples taken at baseline, on the day of transplant and on postoperative days (POD) 7, 14, and 28. The primary endpoint of the original study and of this report was the incidence of biopsy-proven, clinically significant, eAMR in the first 28 days after transplantation.

Desensitization protocol

As described [1], recipients with baseline positive B cell flow cytometric cross-match (BFXM) channel shift greater than 200 and less than 450 were included in the eculizumab protocol. IgG DSA specificity was identified using LABscreen SAB (One Lambda, Canoga Park, CA) on a Luminex platform with levels expressed as the mean fluorescence intensity (MFI). The protocol included pretransplant PE if the BFXM was greater than 300 to reduce the pretransplant BFXM to less than 300; low-dose intravenous immune globulin (IVIG, 100 mg/kg) therapy was administered following PE; antithymocyte globulin and triple therapy of tacrolimus, mycophenolate mofetil, and prednisolone [2]. PE consisted 1.0 plasma volume exchanges performed using the COBE SPECTRA version 7.0 software (CaridianBCT, Lakewood, CO, USA) with acid citrate dextrose solution A (ACD-A) as the anticoagulant. Replacement fluid was 5% albumin ([3]). Eculizumab was given on day of transplant and day 1 (1200 mg and 600 mg, respectively) and weekly for the first 4 weeks and continued until the BFXM was less than 200. No protocol postoperative PE was performed after the first two patients; however, two patients did receive PE as treatment of eAMR.

IgM DSA assay

Anti-HLA IgM was identified retrospectively using serum samples which were collected prior to desensitization with plasma exchange; prior to transplant (Day 0); and on POD 7, 14, and 28 in patients treated with eculizumab. This was carried out using a modification of the commercially available single antigen bead IgG detection assay (LABscreen SAB, One Lambda, Canoga Park, CA, USA). In brief, 20 µl serum and 3 µl single antigen beads of each class specificity were incubated for 30 min in the dark, washed, incubated with the 100 µl 1:100 secondary PE-conjugated anti-human IgM antibody [Goat Anti-Human IgM (µ) R-PE, Invitrogen Corporation, Camarillo, CA 93012, USA] for 30 min in the dark, washed and then resuspended before reading. IgG results were obtained at the same time points for these samples using the clinically approved protocol. The positive control anti-HLA IgM control serum was a kind gift of Karen Nelson, Puget Sound Blood Center. The positive control for the IgM assay produced an MFI of >8000 each assay, but did not detect IgG. An MFI >1000 was considered positive as in our IgG assay.

Dithiothreitol (DTT, Sigma, St Louis, MO, USA) was used to treat samples to cleave the disulfide bonds (ratio 1:1 sample volume to 0.01 M DTT, incubated at 37 °C for 15 min) to differentiate IgM specific binding from IgG. DTT treatment abrogated completely detection of IgM (data not shown). Assessment of IgG and IgM was carried out according to the technique described above for the undiluted and postdithiothreitol and postdilution samples for both isotypes.

Sera were tested initially on twelve patients at all time points where complete sera samples were available, but extended to six further patients at time of biopsy on day 7 and day 14 who had higher levels of IgG DSA. IgG3 HLA antibodies were tested in all patients by Terasaki Foundation using LABscreen SAB kits and a secondary anti-human IgG3 PE-conjugated antibody (IgG3 clone HP6050, Southern Biotech, Birmingham, AL, USA) [4].

C1q binding of DSA IgG

The Bio-C1q assay is a modification of the commercially available method and was performed as described in [5]. It detects both IgM and IgG. Briefly, 10 µl of serum, absorbed with beads (Spherotech, Lake Forest, IL, USA), was spiked with 10 µl (0.1 mg/ml) purified human C1q (Sigma) biotinylated in house (Bio-C1q) and incubated with 2.5 µl of LabScreen SAB for 30 min at room temperature (rt) followed by 10 µl of phycoerythrin-conjugated streptavidin. After an additional 20 min rt incubation, the beads were washed X2 with wash buffer and acquired on a Luminex instrument. Data were analyzed by HLA Fusion software. All reagents and software except absorption beads and Bio-C1q were obtained from One Lambda, Inc.

Efficacy of C5 inhibition

The efficacy of eculizumab therapy was assessed using two *in vitro* assays including: (i) serum drug levels using a validated enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab (PK levels); and (ii) activity as determined by the ability of the eculizumab-treated patient's serum to lyse chicken erythrocytes in a validated total human serum–complement hemolytic assay (PD assay) [6].

Criteria for AMR and histologic assessment

Renal allograft biopsies were obtained percutaneously using ultrasound guidance and submitted for routine light microscopy, immunofluorescence for C4d, and electron microscopy. All biopsies were reviewed by a pathologist, and AMR was diagnosed using standard Banff criteria [7] in combination with graft dysfunction (increase in serum creatinine $\geq$ 0.3 mg/dl over nadir).

Statistical analysis

Data were analyzed using JMP 9 (SAS, Cary, NC, USA). Continuous data were expressed as mean $\pm$ standard deviation and nominal data by counts and percentages. The data were compared using a Student's *t*-test for normally distributed data, otherwise nonparametric tests were applied. A P value of <0.05 was considered significant.

Results

Patient demographics have been described previously [1]. All 26 +XMKTx recipients received eculizumab for at least 1 month post-transplant – the duration of the primary endpoint of this study.

Two patients developed acute clinical eAMR in the first month, and one had subclinical eAMR on a 1-week protocol biopsy. None of the other patients showed evidence of eAMR on biopsy (all patients underwent at least one protocol biopsy during the first month). Three patients developed graft dysfunction, and their biopsy did not demonstrate eAMR. Renal function in these patients improved without specific DSA-reduction therapy. No episodes of eAMR were diagnosed in months 1 to 12. All patients had functioning grafts at 1 year after transplantation.

AMR episodes

Two patients who met criteria for eAMR (Table 1) had graft dysfunction with the classic triad of AMR (one on postoperative day (POD) 7 and another on POD 14) that included: (i) a biopsy consistent with antibody-mediated damage on light microscopy (Banff 97 Level II or III AMR); (ii) C4d+ staining of the peritubular capillaries; and (iii) circulating DSA by both B flow cytometric cross-match and total IgG DSA.

The first patient that developed eAMR had a surveillance biopsy on day 4 which was C4d negative and showed only mild acute tubular injury without inflammation or other features of AMR. On day 7, the serum creatinine rose from 2.1 mg/dl to 2.7 mg/dl in 24 h, and the biopsy demonstrated glomerular thrombi, endothelial swelling, mesangiolysis, and glomerular neutrophil margination with acute tubular injury, and the peritubular capillaries showed diffuse dim staining for C4d. Electron microscopy showed endothelial swelling with loss of fenestration and peritubular epithelial cell swelling. Treatment consisted of PE (daily for 12 days), ongoing eculizumab (600 mg after each PE session), and low-dose IVIG. The serum creatinine decreased to 1.9 mg/dl by day 21. This patient, at 1 and 2 years of follow-up, had creatinine values of 1.7 mg/dl and 2.0 mg/dl, respectively (eGFR MDRD 34 ml/min and 28 ml/min).

The second patient with eAMR had a surveillance biopsy carried out on day 7 which did not show any evidence of tissue injury or inflammation by light microscopy,

Table 1. Post-transplant clinical characteristics of patients with AMR and those without AMR.

	N	Graft dysfunction*	DSA†	C4d deposition	Histologic injury
AMR	2	2	IgG and IgM	Yes	Yes‡
Subclinical AMR	1	No	IgG and IgM	Yes	Yes**
No AMR	23				
Normal histology low DSA§	15	2	IgG	11/15	None
Normal histology high DSA¶	8	1	IgG	7/8	None

*Increase in serum creatinine $\geq$ 0.3 mg/dl over baseline during the first month.
†DSA is a positive BFXM and/or DSA by solid phase assay that is either IgG or IgM.
‡Glomerular microthrombi, mesangiolysis, and/or peritubular capillaritis.
§Low DSA means highest post-transplant BFXM $<$ 360.
¶High DSA means highest post-transplant BFXM $\geq$ 360.
**Capillaritis only.

although there was diffuse C4d deposition in the peritubular capillaries. Electron microscopy did show segmental endothelial cell swelling of glomerular and peritubular capillaries. On POD 11, the serum creatinine rose to 1.3 mg/dl and on day 14, a biopsy was obtained that demonstrated glomeruli microthrombi, interstitial hemorrhage, glomerulitis and peritubular capillaritis in addition to diffuse C4d deposition in the peritubular capillaries. Electron microscopy showed diffuse swelling of endothelial cells in glomerular and peritubular capillaries (Fig. 1). Treatment consisted of PE (9 days), low-dose IVIG and continued eculizumab (600 mg after each PE). The 1-year and 2-year serum creatinine measurements were 1.5 mg/dl and 1.6 mg/dl (eGFR 38 ml/min and 35 ml/min), respectively.

A third patient met criteria for 'subclinical AMR' who had high levels of DSA (BFXM > 360), C4d+ staining and a biopsy consistent with antibody-mediated injury (peritubular capillaritis and glomerulitis on POD 7), but did not develop graft dysfunction during the first 28 days after transplantation, and did not receive additional therapy.

Eculizumab levels

Serum eculizumab drug levels were therapeutic in all patients, and the hemolytic assay was blocked, indicating effective complement blockade. There was no significant difference in either drug level or hemolytic score between the three patients with rejection and the 23 patients without rejection (see Fig. 2).

DSA levels and AMR

We previously have shown that high DSA levels after +XMKTx correlate highly with the development of acute AMR in patients not treated with eculizumab [1]. In the current study, DSA levels were allowed to increase without PE. Eculizumab treatment did not appear to prevent the

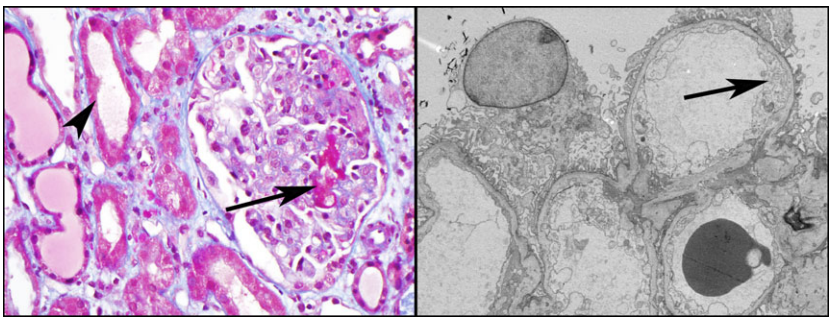

Figure 1 Histologic evidence of early AMR in eculizumab-treated patients with DSA IgM. By light microscopy, a glomerulus shows a thrombus (arrow) and acute tubular injury (arrowhead) (left panel). By electron microscopy, glomerular endothelial cells are enlarged (arrow) and show loss of fenestrations and microvillous change.

	PD assay (%hemolysis)				PK Reported (ug/ml)			
	Week 0	Week 1	Week 2	Week 4	Week 0	Week 1	Week 2	Week 4
AMR	95 ± 2.8*	9 ± 12.7	1 ± 1.4	28 ± 39.6	0 ± 0	110.1 ± 24.4	162.5 ± 53.8	154.8 ± 157.8
No AMR	84.5 ± 23.2	3.6 ± 3.4	2.4 ± 3.3	2.1 ± 3.5	1.8 ± 4.8	127 ± 61.1	129.7 ± 46.2	151.6 ± 46.5

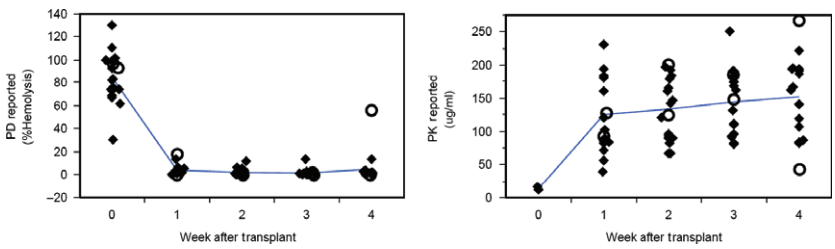

Figure 2 Eculizumab levels and complement blockade. Hemolytic assays showed complete blockade (left panel), and eculizumab levels were therapeutic (right panel) in all patients including at the time of AMR in the two patients who developed eAMR (open circles) and nonrejectors (closed diamonds). Actual levels expressed as means ± standard deviation.

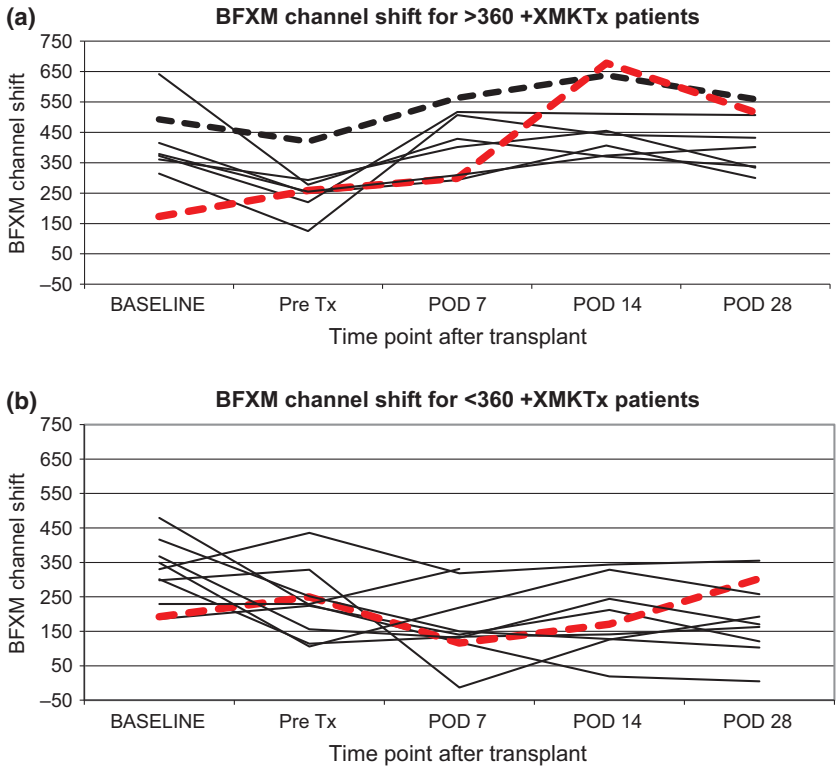

Figure 3 Eculizumab does not prevent the development of high levels of alloantibody after transplantation. (a) BFXM channel shift of patients following treatment with eculizumab with a high DSA level (BFX > 360) in the first month after transplant. Rejecter in red-dashed line (- - - -); continuous lines are nonrejecters (——); black dotted line is the subclinical rejection patient (·····). (b) BFXM following transplant with BFXM < 360. Patient with rejection had indistinguishable levels from rest of cohort. Rejecter in red dashed line (- - - -); continuous lines are nonrejecters (——).

development of high levels of DSA in that in the first month 38.5% (10/26) of eculizumab-treated patients develop a BFXM channel shift >360 – a level associated with AMR in 100% of historical controls (see Fig. 3). Two of the three cases of AMR (one clinical and one subclinical) had a BFXM > 360 and in the other, the highest BFXM was 303. This patient's highest IgG DSA was against Cw7 which can be poorly expressed on lymphocytes. Thus, eight patients treated with eculizumab who did not develop rejection would have been predicted to have AMR based on historical data. In addition, all 10 of the biopsies were C4d+ (with high levels of DSA – IgG DSA SAB, median 10 453, Q1 3480-Q3 11 710; BFXM median 445, Q1 409-Q3 544) consistent with antibody-dependent complement activation of the allograft.

A more detailed analysis of DSA levels by Ig type is presented in Fig. 4. This shows that high levels of DSA were present at some time point in the first month after transplantation in both rejecting and nonrejecting patients. Specifically, high levels of total IgG (as commonly measured in the LABscreen SAB assay), IgG3, and C1q+ binding were detected in both groups, and none were associated with the development of eAMR (comparison was not significant with $P = 0.279$; $P = 0.799$ and $P = 0.711$, respectively).

In contrast, IgM DSA was detected in only four patients after transplantation including: the two patients with eAMR (maximum MFI levels of 1721 against HLA-B8 and 8816 against HLA-DR7), the patient with subclinical eAMR (maximum MFI = 1997 against HLA-DQ8), and a patient who had normal biopsies throughout (maximum MFI = 2320 against HLA-DR53). Comparison between rejecters and nonrejecters with respect to IgM DSA MFI was significantly higher ($P = 0.006$). Interestingly, this fourth patient also had persistently high DSA that necessitated continued treatment with eculizumab for 1 year according to study protocol. This patient developed transplant glomerulopathy on a 7-month biopsy, and his allograft failed at 15 months after transplantation.

Patient #1 had increased levels of DSA IgM (anti-B8 and anti-Cw7) at day 7 correlating with eAMR. Plasma exchange started on Day 7 rapidly reversed the eAMR episode and led to reduced IgM DSA (Fig. 5a). IgG DSA continued to rise despite the resolution of AMR both clinically

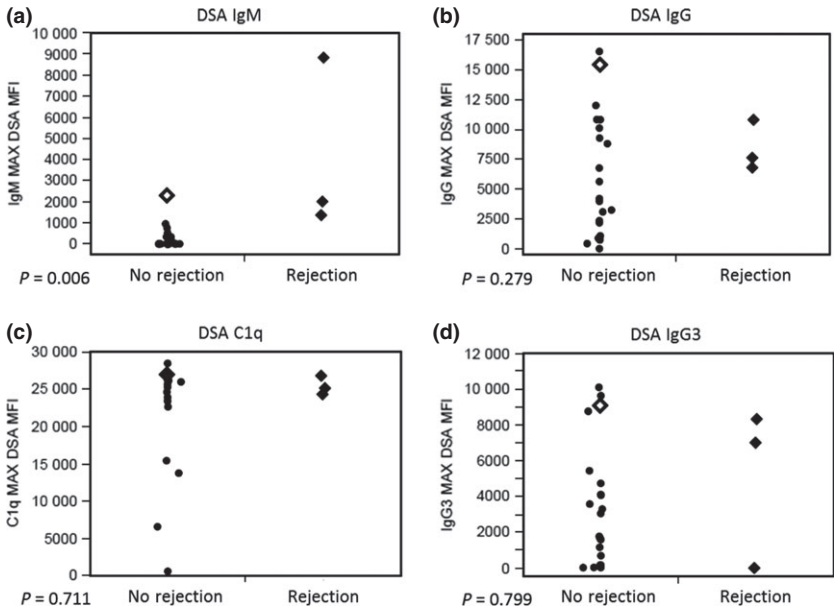

Figure 4 Comparison of levels of antidonor antibody during the first month post-transplantation demonstrates significantly higher levels in rejecters for DSA IgM (a), but no difference in DSA IgG (b); DSA C1q (c); or DSA IgG3 (d). Patients with rejection are with full diamonds, the one patient with no rejection, but accelerated transplant glomerulopathy is in open diamond, and the nonrejecters are in closed circles.

and on follow-up biopsies. Patient #2 showed an increase in DSA IgM at day 7 correlating with early peritubular capillaritis and then subsequent developed clinical AMR on day 14. Again, the eAMR episode was easily treated with plasma exchange and IgM was quickly reduced while IgG DSA continued to rise (Fig. 5b). Figure 5c shows DSA levels typical of a patient with persistently high IgG DSA (MFI > 8000) without IgM DSA who did not develop eAMR.

Discussion

The fact that terminal complement inhibition significantly reduces the rate of acute clinical AMR in +XMKTx patients with high levels of DSA and evidence of complement deposition in the allograft suggests that most cases of AMR are C5-dependent [1]. However, despite C5 inhibition being therapeutic and functionally adequate, 2 +XMKTx recipients who received eculizumab developed clear-cut, clinically significant AMR and another developed subclinical AMR. All three of these patients had IgM DSA detected, and only one of 23 patients without AMR had IgM DSA. That the two episodes of eAMR were reversed easily with PE which more effectively removed IgM without reducing IgG levels further supports the concept that IgM DSA might be a possible causative agent in early AMR independent of C5. Importantly, high levels of total IgG DSA and complement-binding DSA (IgG3 subclass and C1q+) were commonly present in recipients who did not have eAMR further supporting the concept that complement-binding IgG was not the mediator of eAMR in eculizumab-treated patients.

We are not suggesting that IgM is the only possible mechanism of AMR in eculizumab-treated patients. Further studies of larger numbers of patients might reveal other possible mechanisms. For example, it is possible that high levels of DSA might be sufficient to cause forme fruste AMR in some patients via either direct endothelial cell activation or via infiltration of cells via FcγR binding to IgG bound to the allograft. Yet our data from the current study did not support either of these mechanisms as a cause of early clinical AMR.

We also are not suggesting that IgM is the major cause of AMR in +XMKTx recipients not treated with eculizumab. In the absence of terminal complement blockade, it is likely that IgG DSA is the major cause and its blockade is the major reason for the reduced incidence of AMR in eculizumab-treated patients. However, IgM DSA may be capable of causing eAMR in specific situations. Techniques to identify IgM have varied over time, and the resulting conclusions from these studies have ranged from the irrelevance of IgM to a protective mechanism to being associated with poor outcomes [8–12]. More recently, DSA IgM binding complement was associated with AMR without DSA IgG in cardiac transplantation [13]. One of the factors that may have contributed to this confusion is that different methods have been used to identify IgM and few have been able to identify specificities clearly [14–18].

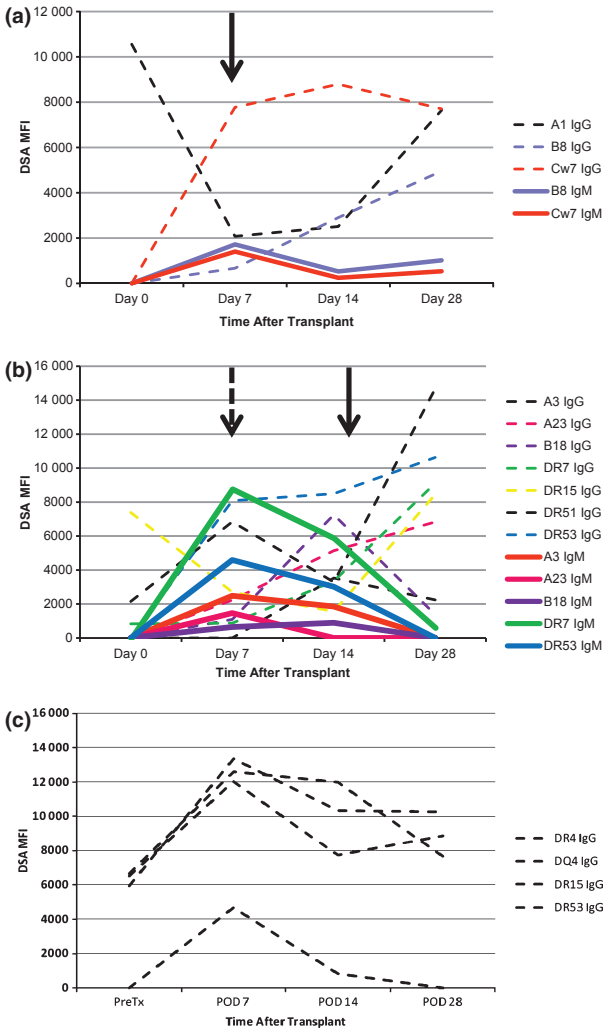

Figure 5 Anti-HLA antibody patterns in patients who developed early AMR and one without AMR. (a) AMR Patient #1 DSA Levels. Increased levels of DSA IgM (anti-B8 and anti-Cw7) at day 7 correlating with early AMR (solid arrow). Plasma exchange started on Day 7 rapidly reversed the AMR episode and led to reduced IgM DSA but IgG DSA continued to rise. (b) AMR Patient #2 DSA Levels. Increase in DSA IgM at day 7 correlating with early peritubular capillaritis (dashed arrow) and then subsequent early AMR (solid arrow) in patient 2. Again, the AMR episode was easily treated with plasma exchange and IgM quickly was reduced while IgG DSA continued to rise. (c) High levels of IgG DSA without IgM DSA in a patient who did not develop AMR. Typical DSA pattern in a patient with persistently high IgG DSA and C4d deposition on surveillance biopsy who did not develop AMR and did not have IgM DSA.

Immunoglobulin M commonly has been associated with early B cell responses, but IgM DSA production can be long-lived similar to that of IgG production and can be produced in a memory B cell response [19,20]. In the current study, 15.4% (4/26) had IgM DSA and all had the same specificity as the IgG DSA. The coexistence of IgM and IgG to the same specificity has been previously reported [21]. More recently, primary renal allograft recipients with both IgM and IgG3 DSA have significantly higher rates of allograft loss and worse rejection [22].

One patient in this study had increased IgM after transplantation, but did not develop eAMR. The reason for the lack of AMR in this patient is unclear, but underlies the fact that not all IgM may be capable of causing eAMR. While this patient went on to develop chronic AMR by 1 year, the current study cannot clearly address the role of IgM in chronic injury because C5-independent processes may contribute to chronic injury.

We previously have shown that a BFXM channel shift >360 post-transplant was associated with AMR with a sensitivity of 100% and a specificity of 95.4% in +XMKTx who develop AMR [23]. Thus, we would have expected almost all of the eculizumab-treated patients in this study who developed high DSA (approximately 1/3 of all transplanted) to have developed AMR, yet only one did. The other case had a low BFXM, but high DSA by single antigen beads against an HLA-Cw7 which is known to be variably expressed on lymphocytes. The IgG3 DSA was not detectable for this allele on solid phase assay, but had high levels of C1q binding. Interestingly, in this case, the increase in IgM DSA preceded the development of IgG DSA, and the episode of AMR occurred when the IgM DSA was rising.

It is possible that DSA can injure the allograft via complement-independent mechanisms. For example, anti-HLA antibody can directly activate endothelial cells *in vitro* without complement and NK cells may attach to membrane-bound IgG via their Fcγ receptor. However, these mechanisms do not explain why some patients with high levels of IgG DSA developed AMR and others did not [24].

Immunoglobulin M might cause AMR via several mechanisms. For example, IgM has a higher affinity for C3 (not blocked by eculizumab) and this may significantly increase the local effect of C3 and its breakdown products [25]. The increased number of C3 binding sites compared with IgG may lead to increased inflammation and tissue damage sufficient to cause histologic changes of AMR and graft dysfunction and altered monocyte infiltration [26,27]. C3a, an anaphylatoxin, increases intra-endothelial cell dilatation, and this increase also might enhance the pro-thrombotic process. The exposure of intra-endothelial gap junctions leads to intravascular coagulation on endothelial surfaces due to the increased expression of tissue factor and plasminogen activator inhibitor type I via complement activation [3]. C3a also can cause neutrophil adhesion, chemotaxis and cellular allograft infiltrate [28]. Local tissue injury leads to increased C3 production, further increasing the local inflammation and cellular infiltrate [29]. C3b produced binds to endothelium and attracts leukocytes which express complement receptors [30]. C3b also amplifies

the alternate complement pathway, as C3b bound to tissue is not inhibited by regulatory proteins [31]. The increase in C3b activates C3 convertase, as C3b becomes an active protein – thus increasing the presence of C3a in the allograft [32]. Furthermore, IgM mannose-binding lectin (MBL) complexes can initiate cell injury. Without the presence of C1q, it has been demonstrated that binding of MBL can initiate cell injury and lead to increased C3 deposition. This would increase the cell injury through the C3 pathway described above, in addition to the classical pathway [33].

We conclude that these data, while preliminary, suggest a possible role of IgM DSA in the pathogenesis of AMR in patients treated with terminal complement blockade. These data also suggest a testable hypothesis in that monitoring IgM DSA, and preemptive PE might further reduce AMR in eculizumab-treated patients.

Authorship

AB, LDC, PGD and MDS: participated in research design. AB, LDC, SR, JLW, PGD and MDS: participated in the writing of the paper. AB, DBT, FS, MJE, MJG, NAH, SR, JLW and MDS: participated in the performance of the research. DBT, MJE, NA and MJG: contributed new reagents or analytic tools. AB, DBT, FS, MJE, LDC, PGD and MDS: participated in data analysis.

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