Increased C4d in post-reperfusion biopsies and increased donor specific antibodies at one-week post transplant are risk factors for acute rejection in mild to moderately sensitized kidney transplant recipients

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Abstract

In order to define the intensity of immunosuppression, we examined risk factors for acute rejection in desensitization protocols that use baseline donor specific antibody levels measured as mean fluorescence intensity (MFI_max). The study included 146 patients transplanted with a negative flow crossmatch and a mean follow-up of 18 months with the majority (83%) followed for at least 1 year. At the time of transplant, mean calculated panel reactive antibody and MFI_max ranged from 10.3% to 57.2%, and 262 to 1691, respectively, between low and high-risk protocols. Mean MFI_max increased significantly from transplant to one-week and one-year. The incidence of acute rejection (mean 1.65 months) as a combination of clinical and subclinical rejection was 32% including 14% cellular, 12% antibody-mediated and 6% mixed rejection. In regression analyses, only C4d staining in post-reperfusion biopsies (hazard ratio 3.3, confidence interval 1.71 to 6.45) and increased donor specific antibodies at 1 week post-transplant were significant predictors of rejection. A rise in MFI_max by 500 was associated with a 2.8-fold risk of rejection. Thus, C4d staining in post-reperfusion biopsies and an early rise in donor specific antibodies after transplantation are risk factors for rejection in moderately sensitized patients.

Introduction

More than a third of patients on the active kidney transplant waitlist are sensitized, which means that they have a panel reactive antibodies (PRA) ≥10%. Nearly 8,000 of these patients are highly sensitized with a PRA ≥80%. While many die before receiving a
transplant, some undergo successful desensitization followed by kidney transplantation. Current preconditioning protocols combine anti-CD20 monoclonal antibody to deplete B cells, Bortezomib to eliminate plasma cells, and plasma exchange and IVIG to block or remove preformed donor specific antibodies (DSA) \(^1\text{-}^7\). Despite some success, desensitization protocols are limited by high acute rejection rates and suboptimal long-term outcomes \(^4\text{-}^8\). It is therefore important to determine novel rejection risk factors that could improve both short and long-term graft survival. Among these, the role of C4d staining in post-reperfusion biopsies and DSA monitoring in the early posttransplant period has yet to be defined. More specifically, it is unclear whether focally positive C4d staining in post-reperfusion biopsies is associated with poor graft outcomes. Similarly, the clinical relevance of an early rise in posttransplant DSA in moderately sensitized patients [flow crossmatch negative and DSA (+)] has to be determined \(^9\text{-}^{10}\).

We have defined preconditioning protocols that use pretransplant DSA measured by single antigen bead Luminex assay as mean fluorescence intensity (MFI\(_{\text{max}}\)) to characterize the intensity of immunosuppression \(^11\). These protocols are based on earlier observations that pretransplant anti-HLA antibodies \(\geq 100\) MFI carried a significant risk for antibody-mediated rejection (AMR) in both low and high-risk patients \(^12\text{-}^{13}\). The implementation of Luminex-based desensitization strategies in a pilot study of 48 patients, with peak PRA and DSA at 51\(\pm\)7% and 960\(\pm\)136 MFI\(_{\text{max}}\) was associated with acceptable clinical AMR and acute cellular rejection (ACR) rates (25% and 23% respectively) \(^11\). There were no graft losses or patient deaths at one year, and serum creatinine levels were comparable to nonsensitized patients transplanted in the same period \(^11\). We now report data on both traditional and novel risk factors associated with acute rejection in the first consecutive 146 patients undergoing desensitization. We examined the role of variables including age, gender, race, retransplant status, PRA, donor type, baseline DSA, the desensitization protocol, C4d staining in post-reperfusion biopsies and a change in DSA by one week post-transplant.

**Results**

**Baseline characteristics and immunological profiles (Tables 1, 2)**

All 146 patients that underwent desensitization and kidney transplantation between January 1\(^{\text{st}}\) 2009 and March 16\(^{\text{th}}\) 2011 were included in this study. There were 56, 13, 7, 21 and 49 patients in protocols D1 to D5 respectively. Mean age was 47\(\pm\)1 years and the majority were male (57.5%) and Caucasian (79%). Per design, all patients in protocols D1-3 received live donor transplants compared to deceased donor transplants in protocols D4 and D5. As anticipated, initial DSA values were significantly greater in protocol D3 (1862\(\pm\)460 MFI\(_{\text{max}}\)) compared to protocols D2 (973\(\pm\)175 MFI\(_{\text{max}}\)) and D1 (287\(\pm\)19 MFI\(_{\text{max}}\)) \((p<0.05)\). Desensitization was effective in reducing mean MFI\(_{\text{max}}\) levels in protocols D1-3 from enrollment (550\(\pm\)65) to the time of transplant (384\(\pm\)45, \(p=0.003\)). Not surprisingly, patients in protocol D5 had the highest mean PRA and DSA at transplant (57.2\(\%\pm\)5.7, \(p<0.001\) and 1691\(\pm\)144, \(p<0.001\) compared to all).
**Acute Rejection and kidney function at one year (Table 3)**

We next examined the one-year incidence of rejection, overall, and in each protocol. One hundred and twenty one patients (83% of all) were followed for at least one year at the time of these analyses. Mean follow-up time was 18±6.7 months. Mean time to acute rejection was 1.65±0.46 months. There was no graft loss or patient death during the study period. The overall incidence of acute rejection (clinical and subclinical) was 32% including 14% cellular, 12% antibody-mediated and 6% mixed rejection (Table 3). Of these, 14% were subclinical and were diagnosed by protocol biopsies. There was no difference in the incidence of rejection among protocols. At 12 months, mean eGFR and serum creatinine levels were 55±1.7 ml/min/1.73m² and 1.5±0.1 mg/dL respectively. There was no significant difference in kidney function among the 5 groups.

**DSA and acute rejection (Tables 4-7, Figure 1)**

To determine the effects of transplantation on DSA, we first examined the changes in DSA over time. We evaluated MFI$_{\text{max}}$ and MFI$_{\text{sum}}$ per class, and for both class I and II, at the time of transplant, 1 week, 1 month, 3 months and 12 months. Table 4 and Figure 1 show that DSA increased after transplantation despite immunosuppression and desensitization strategies. Class II antibodies were the primary cause of this rise in DSA. Next, we discovered that the association between the rise in DSA and acute rejection was stronger at 1 week than at 1 month, 3 months and 12 months (Tables 5). We confirmed these findings by examining the predictive value of delta DSA at 1 week for any episode of acute rejection (Table 6). We noted that patients with delta MFI values higher than 500, 1,000 and 3,000 MFI, had a 1.9 to 10.8 times greater risk of subsequent rejection, regardless of class or type of DSA (Max or Sum). Notably, there was a linear relationship between MFI$_{\text{max}}$ and MFI$_{\text{sum}}$ at 1 week (MFI$_{\text{max}}$ = −84.95 + 0.37 MFI$_{\text{sum}}$, R$^2$=0.82, p < 0.001). Mean time to acute rejection in patients with a 1 week rise in MFI$_{\text{max}}$ > 500 and MFI$_{\text{sum}}$ > 500 was 1.45±0.4 and 1.60±0.4 months after transplant, respectively, suggesting a true predictive ability. Multivariate cox regression analyses confirmed the strong association of delta DSA at 1 week with acute rejection (Table 7). Separate multivariate regression analyses demonstrated a similar association between antibody-mediated rejection and delta DSA at 1 week (HR=3.7, 95% CI 1.69 to 8.10, p=0.002 for MFI$_{\text{max}}$ > 500), African American race (HR=13, 95% CI 3.9 to 42.9, p < 0.0001) and C4d staining in post-reperfusion biopsies (HR=7, 95% CI 3.0 to 16.6, p < 0.0001). Out of all these variables, only 1 week MFI$_{\text{max}}$ > 500 was associated with acute cellular rejection (HR=2.90, 95% CI 1.07 to 7.70, p=0.03), suggesting a cross talk between cellular and humoral immune response.

**Post-reperfusion C4d and acute rejection (Tables 7-8, Figures 2-3)**

We next examined the association between traditional and novel risk factors and acute rejection. Univariate analyses demonstrated that any C4d staining in post-reperfusion biopsies, a rise in DSA (MFI$_{\text{sum}}$ or Max) at one week > 500, African American race, young age and thymoglobulin induction were significantly associated with a greater risk of rejection (Table 7). Other covariates including PRA, retransplant status, live donor status, male gender, DSA at transplant and the desensitization protocol were not independently associated with rejection. Multivariate analyses including the 6 significant variables retained
only C4d staining, a rise in $MFI_{\text{sum}} > 500$ at 1 week and African American race as independent risk factors associated with acute rejection.

We next examined more closely the relationship between C4d staining in post-reperfusion biopsies and subsequent rejection. One hundred and seven patients (73.3%) underwent a post-reperfusion biopsy (Figure 2). Of these, 28 (26.1%) had positive C4d staining, which was focal in the majority of cases (85.7%). Five patients (17.8%) had Glomerulitis (Table 8). Forty-three (40%) samples were assessed on paraffin sections, which is known to give a weaker C4d signal compared to frozen sections. Post-reperfusion C4d staining was not significantly different among desensitization protocols. However, the incidence of subsequent acute rejection (50% vs. 26.5%, $p=0.03$) was greater in patients with positive C4 staining (Table 8). Specifically, these patients had a 6.8 times greater risk of earlier antibody-mediated or mixed rejection (95% CI 2.22 to 20.70, $p<0.0001$, Figure 3). Furthermore, C4d staining was associated with higher serum creatinine levels at one year ($1.7 \pm 0.2$ vs. $1.4 \pm 0.04$ mg/dL, $p=0.02$, eGFR $49 \pm 4.1$ vs. $56 \pm 1.9$ ml/min/1.73m$^2$, $p=0.1$).

To determine the role of cold ischemia on C4d staining, we performed 2 sets of studies. First, we examined C4d staining in a control group of 21 consecutive pre-implantation biopsies from deceased donors. Mean ($\pm$SD) cold ischemia time (CIT) for these kidneys was similar to sensitized deceased donor recipients in protocols D4 and D5 ($13.7 \pm 5.6$ vs. 14.3$\pm5.6$ hours, $p=0.8$). However, no pre-implantation biopsy stained positive for C4d compared to 22% in post-reperfusion biopsies from sensitized groups ($p=0.009$, Figure 2). Next, we compared C4d staining in live vs. deceased donor kidneys and did not find a significant difference (14% vs. 22%, $p=0.14$), suggesting that CIT is not directly involved in the pathogenesis of C4d deposition.

We then evaluated the association between DSA (MFImax and $MFI_{\text{sum}}$ per class and total) at the time of transplant and C4d staining in post-reperfusion biopsies. Multivariate stepwise logistic regression analyses retained only class II $MFI_{\text{sum}}$ as a significant predictor of C4d staining. Specifically, the Odds Ratio for positive C4d was 1.0004, 95% CI 1.000 to 1.0007, $p=0.02$ when $MFI_{\text{sum}}$ was considered as a continuous variable and 6.2, 95% CI 1.54 to 24.80, $p=0.01$ when $MFI_{\text{sum}} > 3,000$, favoring an immunological explanation for the deposition of C4d in post-reperfusion biopsies.

**Discussion**

We examined the risk factors for rejection in 146 sensitized patients who underwent preconditioning based on DSA levels. All patients had a negative flow cytometry crossmatch. The overall incidence of clinical and subclinical rejection was 18% and 14% respectively, similar or more favorable than previously published data $^{4,14-16}$. The pathology was consistent with acute cellular, antibody mediated and mixed rejection in 14%, 12% and 6% of cases respectively. Rejection episodes occurred within a mean time of 1.65$\pm$0.46 months post-transplant. The evaluation of traditional and nontraditional covariates demonstrated that African American race, an increase in DSA by the first week post transplant and C4d staining (focal or diffuse) in the post-reperfusion biopsy were the most important predictors of rejection. The association was the strongest with antibody-
mediated rejection. These observations suggest that the treatment of these risk factors may prevent the incidence of rejection in sensitized patients.

Recent evidence suggests that both pre-transplant and de novo DSA are associated with poor allograft outcomes. Based on previous observations, we have defined preconditioning protocols that use DSA levels to characterize the intensity of immunosuppression. For example, mildly sensitized patients (MFI\textsubscript{max} between 100-500) only receive one week of tacrolimus and mycophenolic acid pre-transplant. While this cannot be considered as a “desensitization strategy” per se, the approach falls outside the standard-of-care and we have noticed a decline in rejection rates since the implementation of these protocols. Plasmapheresis and IVIG therapy are reserved for individuals with a DSA > 500 MFI\textsubscript{max} (live donors) or 1,000 MFI\textsubscript{max} (deceased donors). Univariate analyses confirmed some of the traditional risk factors including African-American race and younger age. The use of thymoglobulin was also associated with a greater risk of rejection, most likely because these patients were among the highest risk groups (deceased donor recipients) and did not have time for pretransplant plasmapheresis. More importantly, we analyzed and reported MFI max and sum, per class and total, and noted that all DSA increased after transplantation despite immunosuppression and desensitization. However, the rise was more pronounced for class II antibodies. We also observed that the association between the rise in DSA and acute rejection was stronger at 1 week than at 1 month, 3 months and 12 months. Last, we found that MFI\textsubscript{max} and MFI\textsubscript{sum} were linearly associated and both predicted acute rejection, suggesting that either one may be followed in the early post-transplant period. These observations are in agreement with recent data on the role of DSA in predicting acute rejection, and add new insights regarding the clinical value of early post-transplant DSA monitoring.

C4d is a split-product of the complement, covalently bound and stable. C4d staining usually indicates the activation of the complement cascade after the recognition of HLA antigens by the recipient’s anti-HLA antibodies. The presence of positive C4d staining in peritubular capillaries is therefore used as a surrogate marker of DSA participating in the process of rejection. The current definition of antibody-mediated rejection is based on the presence of circulating DSA, C4d staining and allograft pathology (ATN, capillary and/or glomerular inflammation, arterial injury). In the absence of renal pathology the term antibody-mediated changes may be used. In our study, 5 out of 28 cases had isolated glomerular inflammation and none had capillaritis. These suggest that antibody-mediated changes in post-reperfusion biopsies are early indicators of subsequent rejection in sensitized patients. Whether this is the beginning of a continuous rejection process or simply a biomarker is unknown. However, it is unlikely that C4d staining is a result of ischemia reperfusion injury since both live and deceased donor kidneys displayed comparable patterns of staining and pre-implantation biopsies with similar cold ischemia times were C4d negative.

In agreement with these findings, a study by Haas et al demonstrated that C4d staining in perioperative biopsies was not observed as a feature of ischemic or ischemia-reperfusion injury including in cadaveric grafts with cold ischemia times of as long as 41 hours. Similar to our findings, the investigators observed that 2 of 82 (2.4%) perioperative biopsies showed C4d staining in peritubular capillaries. In both cases, allografts underwent
subsequent antibody-mediated rejection on days 5 and 34. The authors concluded that C4d staining in peritubular capillaries might be seen as early as one hour after transplantation in some recipients with low levels of DSA. In a more recent study, 14 patients with antibody-mediated rejection had negative C4d staining in post-reperfusion biopsies. It is likely that these patients were not as sensitized as those in our or the Haas study. It is also possible that they developed de novo DSA, or that the C4d staining protocol was not the same. In fact, in our study, 21 patients with a negative initial C4d staining had a subsequent episode of rejection, suggesting that the studies are not contradictory but rather complementary.

Our study has several limitations including the sample size and the non-uniform use of frozen sections for C4d staining on post-reperfusion biopsies. Furthermore, we had no access to post-reperfusion biopsies in patients without pre-transplant DSA. Similarly, pending the normalization and validation of Luminex assays across transplant centers, it is difficult to compare a MFI of < 1000 between institutions. In fact, the FDA recognizes the Luminex assay only as a qualitative assay and not a quantitative test. At our center, the evaluation of 66 positive T and B cell flow crossmatch (FXM) results as part of the optimization of our assays showed that a mean channel shift of 204 by FXM corresponded to a mean DSA of 1524 $MFI_{\text{max}}$. While these numbers are clinically relevant at our center, they may not be the same at other institutions. Similarly, we have developed our protocols based on immunodominant DSA ($MFI_{\text{max}}$). However, there is no evidence that lower levels of DSA or non-HLA DSA are not clinically relevant. Last it is important to note intra-assay variability of Luminex readings, even if the significant changes that we observed in one week DSA were beyond this variability. Additionally, run-to-run variability of the assay may be >1,000 MFI. Repeated testing of the same serum on different days is not the same as testing 2 sera from the same individual on differing days. In sum, while we recognize the limitations of MFI values and the biological relevance of DSA monitoring, we believe that significant changes in DSA levels may have a role in the follow-up of sensitized kidney transplant recipients.

In conclusion, post-reperfusion C4d staining and increased DSA at one week are novel risk factors for rejection in sensitized patients, suggesting a clinical role for posttransplant DSA monitoring. Further studies are needed to determine if immunosuppressive strategies addressing these conditions can prevent rejection in sensitized patients.

**Methods**

**Patients and desensitization protocols**

The details of Luminex-based desensitization protocols at our Institution were reported earlier. Briefly, these protocols that were implemented on January 1st 2009 use the immunodominant DSA mean fluorescence intensity ($MFI_{\text{max}}$) measured by single antigen bead (SAB) Luminex assay (One Lambda) to stratify kidney transplant recipients in 5 desensitization protocols (D1 to D5) as outlined in Table 1. In this manuscript we report the outcomes of the first 146 consecutive patients, all of whom had a negative flow cytometry crossmatch at the time of transplant. The UW Institutional Review Board and Human Subjects Committee approved our study number M2010-1296. The desensitization protocols
used are as follows. Protocol D1 is defined as low intensity while protocols D2-5 are defined as high intensity since they involve plasmapheresis (in all) and Thymoglobin induction (D4 and D5).

**Protocol D1 (MFI\textsubscript{max} 100-500)**

Live donor kidney transplant recipients receive tacrolimus, 2mg twice daily, and mycophenolate sodium, 720 mg twice daily (Myfortic\textsuperscript{®} Novartis, East Hanover, NJ) for 1 week prior to transplant. Induction therapy is basiliximab (Simulect, Novartis, East Hanover, NJ), 20 mg on day 0 and day 4).

**Protocol D2 (MFI\textsubscript{max} 501-1000)**

Live donor kidney transplant recipients receive tacrolimus, 2mg twice daily, starting 1 week pre-transplant, plasma exchange and intravenous Immunoglobulin (IVIG, Gammagard SD, Baxter Healthcare), 100 mg/kg (PE/IVIG), and mycophenolate mofetil (Cellcept, Roche), 1 gram IV after PE/IVIG on pre-transplant days 3 and 1. Induction is with basiliximab. PE/IVIG treatments are repeated on postoperative days 1 and 3.

**Protocol D3 (MFI\textsubscript{max} 1001-3000)**

Live donor kidney transplant recipients receive 2 additional sessions of PE/IVIG on pre-transplant days 7 and 5.

**Protocol D4 (MFI\textsubscript{max} 501-1000)**

Deceased donor kidney transplant recipients receive induction with rabbit antithymocyte globulin (rATG; Thymoglobulin, Genzyme, Cambridge, MA) 5-7 mg/kg. Deceased donors with MFI 100-500 are not part of desensitization protocols and are treated with standard immunosuppression including basiliximab induction and triple therapy with tacrolimus, MPA and steroids.

**Protocol D5 (MFI\textsubscript{max} 1001-3000)**

Deceased donor kidney transplant recipients receive one treatment of PE/IVIG pre-transplant and induction with thymoglobulin 5-7 mg/kg. PE/IVIG treatments are continued on postoperative days 1 and 3.

All patients receive steroid induction with dexamethasone 100 mg IV intraoperatively, 50 mg IV on postoperative day 1 and steroids are tapered to prednisone 30 mg daily at time of discharge. Intravenous mycophenolate mofetil, 1 g BID, is given for 48 hours, and then is converted to Myfortic 720 mg BID. All patients who receive plasma exchange (protocols D2, D3, D5) are also treated with IVIG 100 mg/kg weekly for 4 weeks beginning 1 week after they have completed their plasma exchange.

**Maintenance Immunosuppression and viral prophylaxis**

Maintenance immunosuppression consists of a three-drug regimen of prednisone, tacrolimus and mycophenolate sodium. No corticosteroid withdrawal, avoidance or minimization is pursued in these patients. Prednisone dose at 1-month post transplant is 5 to 10 mg per day.
Tacrolimus target level is 8-11 ng/mL at discharge. Patients received 3 months of treatment with acyclovir if donor/recipient CMV combination was −/− and 3 months of treatment with valganciclovir if either donor or recipient had positive CMV serology.

**Single antigen bead (SAB) Luminex assay**

The immunodominant DSA (MFI\textsubscript{max}) was determined using the Luminex assay as described previously \textsuperscript{11,13}. Briefly, we analyzed HLA antibodies by LabScreen® Single Antigen Class I and II bead kits from One Lambda, Inc (OLI, Canoga Park, CA, USA). The resultant Luminex output files were analyzed with HLA Visual® analysis software (OLI). Normalized MFI of beads representing antigens mismatched to donor antigens including HLA A, B, DRB, DQA1, DQB1 and DPB1 were identified and recorded. High definition allele testing was performed on the donor to confirm allele specificity. MFI\textsubscript{max} was defined as the highest MFI value for either classes I or II. MFI\textsubscript{sum} was the sum of relevant DSA per class and was determined for class I, class II and both class I and II antibodies. Day to day variability of bead MFI values was assessed using 100 independent determinations of antibody MFI values using same antibody-positive serum sample. For beads with an average MFI = 478, the standard deviation (SE) was 87.8 (8.8) and CV was 18.4. For beads with an average MFI= 1,249, the standard deviation (SE) was 231 (23.1) and CV was 18.5.

**Diagnosis of Rejection**

Protocol biopsies were performed at baseline, 30 minutes after reperfusion, at months 1 and 12. In addition, indication biopsies were performed when clinically indicated. Fresh core kidney biopsies were fixed in 10% neutral buffered formalin for at least 6 hours for paraffin embedding. Two to three microns in thickness sections were obtained and four serial sections per biopsy were stained with hematoxylin-eosin, Masson’s Trichrome, periodic acid-Schiff (PAS), and argyrophilic impregnations (PAMM). Biopsies were interpreted for adequacy and rejection scores according to the Banff 97 criteria updated in 2010 \textsuperscript{19}. Immunolabeling for the complement fraction C4d was performed on 4 acetone-fixed frozen sections and labeled with a 1:600 dilution of an anti-C4d monoclonal antibody (Biogenesis, Kingston, NH). On post-reperfusion biopsies C4d was performed on formalin fixed paraffin-embedded tissue using anti-C4d rabbit polyclonal antibody. C4d results were interpreted as negative (<1%), minimally positive (1-10%) focally (10-50%) and diffusely positive (more than 50% of PTC labeled). Clinical acute antibody mediated rejection was diagnosed in patients that fulfilled the following criteria: allograft dysfunction, evidence of allograft injury (ATN, peritubular capillaritis and/or vascular fibrinoid necrosis), PTC diffusely positive for C4d, and evidence of circulating DSA. Clinical acute cellular rejection was diagnosed when the indication biopsy showed evidence of interstitial mononuclear inflammation along with tubulitis and/or endarteritis. Subclinical rejection was defined when the above pathological findings were present in a protocol biopsy. To control for the potential role of ischemia on C4d staining, we included a control group of 21 consecutive deceased donor pre-implantation biopsies in our studies.

**Statistical Analyses**

We assessed baseline characteristics and outcomes between desensitization protocols among the first 146 patients transplanted since January 1\textsuperscript{st} 2009. Data are described as mean
Continuous numerical data were compared using Student’s t test or the one-way analysis of variance (ANOVA) when appropriate. Post-transplant changes in DSA were examined using a paired t-test. Categorical data were analyzed using Fisher’s exact test or the chi-square test when appropriate. Kaplan-Meier survival analyses were used to compare rejection rates. Univariate and multivariable stepwise Cox regression analyses were performed to determine the risk factors associated with acute rejection. P values ≤0.05 were deemed statistically significant.

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References

1. Djamali A, Muth B, Torrealba J, et al. Bortezomib as a rescue therapy for hyperacute and multi-drug resistant mixed acute rejection after kidney transplantation. Clin Transpl. 2009;485–490. [PubMed: 20524320]

2. Sberro-Soussan R, Zuber J, Suberbielle-Boissel C, et al. Bortezomib as the Sole Post-Renal Transplantation Desensitization Agent Does Not Decrease Donor-Specific Anti-HLA Antibodies. Am J Transplant. 2010; 10:681–686. [PubMed: 20121729]

3. Jordan SC, Vo AA, Peng A, et al. Intravenous gammaglobulin (IVIG): a novel approach to improve transplant rates and outcomes in highly HLA-sensitized patients. Am J Transplant. 2006; 6:459–466. [PubMed: 16468954]

4. Stegall MD, Gloor J, Winters JL, et al. A comparison of plasmapheresis versus high-dose IVIG desensitization in renal allograft recipients with high levels of donor specific alloantibody. Am J Transplant. 2006; 6:346–351. [PubMed: 16426319]

5. Thielke JJ, West-Thielke PM, Herren HL, et al. Living donor kidney transplantation across positive crossmatch: the University of Illinois at Chicago experience. Transplantation. 2009; 87:268–273. [PubMed: 19155983]

6. Montgomery RA, Lonze BE, King KE, et al. Desensitization in HLA-incompatible kidney recipients and survival. The New England journal of medicine. 2011; 365:318–326. [PubMed: 21793744]

7. Magee CC, Felgueiras J, Tineckam K, et al. Renal transplantation in patients with positive lymphocytotoxicity crossmatches: one center’s experience. Transplantation. 2008; 86:96–103. [PubMed: 18622824]

8. Trivedi HL, Terasaki PI, Feroz A, et al. Abrogation of anti-HLA antibodies via proteasome inhibition. Transplantation. 2009; 87:1555–1561. [PubMed: 19461494]

9. Burns JM, Cornell LD, Perry DK, et al. Alloantibody levels and acute humoral rejection early after positive crossmatch kidney transplantation. American journal of transplantation. 2008; 8:2684–2694. [PubMed: 18976305]

10. Kimball PM, Baker MA, Wagner MB, et al. Surveillance of alloantibodies after transplantation identifies the risk of chronic rejection. Kidney international. 2011; 79:1131–1137. [PubMed: 21270760]

11. Niederhaus SV, Muth B, Lorentzen DF, et al. Luminox-Based Desensitization Protocols: The University of Wisconsin Initial Experience. Transplantation. 2011; 1:12–17. [PubMed: 21512428]

12. Muth B, Samaniego-Picota M, Lorentzen D, et al. Risk-Factors for AMR and Decreased Kidney Function in Sensitized Kidney Transplant Recipients Followed-Up for a Median of 2 Years. American Journal of Transplantation. 2010; 2010 WTC Abstract Book: Poster 608.
13. Singh N, Djamali A, Lorentzen D, et al. Pretransplant Donor-Specific Antibodies Detected by Single-Antigen Bead Flow Cytometry Are Associated With Inferior Kidney Transplant Outcomes. Transplantation. 2010; 90:1079–1084. [PubMed: 21293194]

14. Jordan SC, Pescoitz MD. Presensitization: the problem and its management. Clin J Am Soc Nephrol. 2006; 1:421–432. [PubMed: 17699241]

15. Loupy A, Suberbielle-Boissel C, Hill GS, et al. Outcome of subclinical antibody-mediated rejection in kidney transplant recipients with preformed donor-specific antibodies. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2009; 9:2561–2570.

16. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012; 12:1157–1167.

17. Amico P, Honger G, Mayr M, et al. Clinical relevance of pretransplant donor-specific HLA antibodies detected by single-antigen flow-beads. Transplantation. 2009; 87:1681–1688. [PubMed: 19502960]

18. Lefaucheur C, Loupy A, Hill GS, et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. Journal of the American Society of Nephrology : JASN. 2010; 21:1398–1406. [PubMed: 20634297]

19. Sis B, Mengel M, Haas M, et al. Banff ’09 Meeting Report: Antibody Mediated Graft Deterioration and Implementation of Banff Working Groups. Am J Transplant. 2010

20. Haas M, Ratner LE, Montgomery RA. C4d staining of perioperative renal transplant biopsies. Transplantation. 2002; 74:711–717. [PubMed: 12352891]

21. David-Neto E, David DS, Ginani GF, et al. C4d staining in post-reperfusion renal biopsy is not useful for the early detection of antibody-mediated rejection when CDC crossmatching is negative. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2011; 26:1388–1392.

22. Archdeacon P, Chan M, Neuland C, et al. Summary of FDA antibody-mediated rejection workshop. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2011; 11:896–906.
Figure 1. Changes in DSA after transplantation
Panel a. The bar graph displays mean DSA levels (MFI\textsubscript{max}) early post transplant in all desensitization protocols.
Panel b. The graph shows mean DSA levels in all patients throughout the first posttransplant year. DSA increased with time despite immunosuppression and desensitization. MFI\textsubscript{sum} class II was the primary cause of the rise in DSA.
Figure 2. Post-reperfusion biopsy and C4d staining

Panel A. Percent patients in each protocol that underwent a post-reperfusion biopsy (blue bar) and % patients in each protocol that had positive C4d staining in the reperfusion biopsy (red bar). Actual numbers are displayed within the bars.

Panel B. C4d staining by immunoperoxidase in a post-reperfusion biopsy (a) and in a pre-implantation control biopsy (b). Only some of the post-reperfusion biopsies were positive for C4d.
Figure 3. Kaplan-Meier survival curve for rejection free probability in patients with or without post-reperfusion C4d staining
C4d (+) patients were at significantly greater risk for antibody-mediated or mixed rejection.
Table 1

MFI-Based Desensitization Protocols

| Protocol | Donor type | MFI<sub>max</sub> | Induction | PE + IVIG Pre and post transplant | TAC + MPA 1 week pre-Transplant |
|----------|------------|-----------------|-----------|---------------------------------|---------------------------------|
| D1       | LD         | 101-500         | Basiliximab | −                               | +                               |
| D2       | LD         | 501-1000        | Basiliximab | 2 pre, 2 post                  | −                               |
| D3       | LD         | 1001-3000       | Basiliximab | 4 pre, 2 post                  | −                               |
| D4       | DD         | 501-1000        | Thymoglobulin | −                               | −                               |
| D5       | DD         | 1001-3000       | Thymoglobulin | 1 pre, 2 post                  | −                               |

LD, live donor; DD, deceased donor; PE + IVIG, plasma exchange and intravenous immunoglobulins (100mg/kg); TAC + MPA, Tacrolimus and Mycophenolic Acid
Table 2
Baseline characteristics

|                  | All  | D1   | D2   | D3   | D4   | D5   | p     |
|------------------|------|------|------|------|------|------|-------|
| N                | 146  | 56   | 13   | 7    | 21   | 49   | N/A   |
| Age (years)      | 47±1 | 48.9±1.7 | 50±2.7 | 42.1±4.1 | 44.8±2.4 | 45.2±1.9 | 0.3   |
| Caucasian (%)    | 79   | 91   | 84.6 | 100  | 55   | 73.5 | 0.004 |
| Male (%)         | 57.5 | 62.5 | 53.8 | 57.1 | 62   | 51   | 0.7   |
| Live donor (%)   | 52   | 100  | 100  | 100  | 0    | 0    | <0.0001 |
| PRA (%)          | 35±3 | 10.3±2.7 | 34.9±11.3 | 46.4±14.2 | 43.3±8.3 | 57.2±5.7 | <0.001 |
| Initial MFI<sub>max</sub> | 550±53 | 287±19 | 973±175 | 1862±460 | N/A | N/A | <0.05 |
| MFI<sub>max</sub> at Transplant | 880±72 | 262±18 | 732±120 | 720±115 | 766±79 | 1691±144 | <0.001 |

* Initial MFI<sub>max</sub> and MFI<sub>max</sub> at transplant is the same for protocols D4 and D5 (deceased donor recipients).
### Table 3

One-year rejection rates and kidney function per protocol

|                      | All | D1 | D2 | D3 | D4 | D5 | p   |
|----------------------|-----|----|----|----|----|----|-----|
| **Donor Type**       |     |    |    |    |    |    |     |
| Live                 |     |    |    |    |    |    |     |
| Deceased             |     |    |    |    |    |    |     |
| **Sample Size**      | 146 | 56 | 13 | 7  | 21 | 49 |     |
| **Number of patients followed ≥ 12M** | 121 | 45 | 12 | 7  | 16 | 41 |     |
| **Acute Rejection (Clinical)** | 18% | 21% | 31% | 14% | 19% | 12% | 0.5 |
| **Acute Rejection (Clinical and Subclinical)** | 32% | 31% | 31% | 14% | 24% | 40% | 0.4 |
| **Acute Cellular Rejection** | 14% | 20% | 23% | 0%  | 5%  | 10% | 0.2 |
| **Antibody-Mediated Rejection** | 12% | 7%  | 0%  | 0%  | 14% | 20% | 0.1 |
| **Mixed Rejection**   | 6%  | 4%  | 8%  | 14% | 5%  | 10% | 0.6 |
| **12M eGFR (ml/min/1.73m²)** | 55±1.7 | 52.2±2 | 56.2±5.1 | 58.7±4.3 | 54.4±4.9 | 57.4±4 | 0.7 |
| **12M Creatinine (mg/dL)** | 1.5±0.1 | 1.5±0.1 | 1.3±0.1 | 1.3±0.1 | 1.6±0.2 | 1.4±0.1 | 0.6 |
Table 4

Change in DSA after Transplant

| Time    | DSA | HLA Class | Mean MFI ± SEM | * p   |
|---------|-----|-----------|----------------|-------|
|         |     | I         | II             |       |
|         |     | I or II   | I and II       |       |
| Transplant | Max | 443 ± 62  | 625 ± 78       |       |
|          |     | 860 ± 83  |                |       |
|          | Sum | 610 ± 78  | 973 ± 104      |       |
|          |     | 1583 ± 154|                |       |
| 1 week   | Max | 509 ± 69  | 908 ± 153      | 0.4   |
|          |     | 1150 ± 165|                | 0.05  |
|          | Sum | 827 ± 99  | 1735 ± 318     | 0.05  |
|          |     | 2562 ± 344|                | 0.005 |
| 1 month  | Max | 600 ± 125 | 1049 ± 212     | 0.26  |
|          |     | 1361 ± 247|                | 0.11  |
|          | Sum | 1001 ± 209| 1655 ± 260     | 0.11  |
|          |     | 2657 ± 398|                | 0.03  |
| 3 months | Max | 627 ± 120 | 1043 ± 220     | 0.32  |
|          |     | 1411 ± 273|                | 0.11  |
|          | Sum | 960 ± 225 | 1446 ± 253     | 0.30  |
|          |     | 2384 ± 372|                | 0.13  |
| 12 months| Max | 533 ± 135 | 1602 ± 321     | 0.82  |
|          |     | 1854 ± 372|                | 0.004 |
|          | Sum | 692 ± 220 | 2428 ± 471     | 0.97  |
|          |     | 3120 ± 573|                | 0.004 |

* p: Paired samples t-test compared to the time of transplant
Table 5

Association of DSA and acute rejection

| Time    | DSA | HLA Class | HR     | 95% CI       | *p   |
|---------|-----|-----------|--------|--------------|------|
|         | Max | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I or II   | -      | -            | -    |
|         | Sum | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I and II  | -      | -            | -    |
| Transplant | Max | I         | 1.0007 | 1.0004 to 1.001 | <0.0001 |
|         |     | II        | 1.0002 | 1.0001 to 1.0003 | 0.001 |
|         |     | I or II   | 1.0002 | 1.0001 to 1.0003 | <0.0001 |
|         | Sum | I         | 1.0005 | 1.0003 to 1.0007 | <0.0001 |
|         |     | II        | 1.0001 | 1.0000 to 1.0001 | 0.0001 |
|         |     | I and II  | 1.0001 | 1.0000 to 1.0001 | <0.0001 |
| 1 week  | Max | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I or II   | -      | -            | -    |
|         | Sum | I         | 1.0001 | 1.0000 to 1.0001 | 0.02 |
|         |     | II        | -      | -            | -    |
|         |     | I and II  | 1.0000 | 1.0000 to 1.0001 | 0.03 |
| 1 month | Max | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I or II   | -      | -            | -    |
|         | Sum | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I and II  | -      | -            | -    |
| 3 months| Max | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I or II   | -      | -            | -    |
|         | Sum | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I and II  | -      | -            | -    |
| 12 months| Max | I         | -      | -            | -    |
|         |     | II        | -      | -            | -    |
|         |     | I or II   | -      | -            | -    |
|         | Sum | I         | 1.0000 | 1.0000 to 1.0001 | 0.03 |
|         |     | II        | 1.0000 | 1.0000 to 1.0001 | 0.04 |

*p value for Stepwise Cox Regression Analysis evaluating MFI as a continuous variable and risk factor for acute rejection
## Table 6

Association of a rise in DSA at 1 week and acute rejection

| DSA            | Rise in MFI | Regression Analysis |
|----------------|-------------|---------------------|
|                | HR          | 95% CI              | p       |
| Max class I    |             |                     |         |
| > 500          | 3.8         | 2.01 to 7.41        | 0.0001  |
| > 1,000        | 3.6         | 1.64 to 7.71        | 0.001   |
| > 3,000        | -           | -                   | -       |
| Max class II   |             |                     |         |
| > 500          | 1.9         | 1.01 to 3.69        | 0.04    |
| > 1,000        | 2.5         | 1.20 to 5.10        | 0.01    |
| > 3,000        | 5.6         | 2.17 to 14.44       | 0.0004  |
| Sum class I    |             |                     |         |
| > 500          | 3.2         | 1.80 to 6.10        | 0.0001  |
| > 1,000        | 4.1         | 2.01 to 8.20        | 0.0001  |
| > 3,000        | 10.8        | 3.61 to 32.12       | <0.0001 |
| Sum class II   |             |                     |         |
| > 500          | 2.2         | 1.25 to 3.88        | 0.006   |
| > 1,000        | 1.9         | 1.02 to 3.45        | 0.04    |
| > 3,000        | 4.9         | 1.9 to 12.6         | 0.001   |
| Max class I or II |           |                     |         |
| > 500          | 2.8         | 1.47 to 5.22        | 0.001   |
| > 1,000        | 3.3         | 1.60 to 6.73        | 0.001   |
| > 3,000        | 9.4         | 3.13 to 28.43       | 0.0001  |
| Sum class I and II |         |                     |         |
| > 500          | 3           | 1.75 to 5.4         | 0.0001  |
| > 1,000        | 3           | 1.75 to 5.5         | 0.0001  |
| > 3,000        | 3.7         | 1.93 to 6.90        | 0.0001  |
Table 7
Risk factors for acute rejection in Luminex-based desensitization protocols

|                       | Univariate |          |          |         |          | Multivariate |          |          |
|-----------------------|------------|----------|----------|---------|----------|--------------|----------|----------|
|                       | HR | 95% CI  | p    | HR    | 95% CI | p     |
| Reperfusion C4d (+)   | 3.67 | 1.89 to 7.12 | 0.0001 | 3.3   | 1.71 to 6.45 | 0.0004 |
| ↑ MFI<sub>max</sub> at 1wk > 500 | 2.8 | 1.47 to 5.22 | 0.001  | -     | -       | -           |
| ↑ MFI<sub>sum</sub> at 1wk > 500 | 3   | 1.75 to 5.4 | 0.0001 | 2.6   | 1.43 to 4.93 | 0.003  |
| Black race            | 2.79 | 1.22 to 6.39 | 0.01   | 3.7   | 1.71 to 6.47 | 0.005  |
| Age                   | 0.97 | 0.94 to 0.99 | 0.01   | -     | -       | -           |
| ATG induction         | 2.16 | 1.18 to 3.9  | 0.01   | -     | -       | -           |
| PRA                   | -   | -         | -      | -     | -       | -           |
| Retransplant status   | -   | -         | -      | -     | -       | -           |
| Live donor            | -   | -         | -      | -     | -       | -           |
| Male                  | -   | -         | -      | -     | -       | -           |
| MFI<sub>max</sub> prior to therapy | - | -         | -      | -     | -       | -           |
| MFI<sub>max</sub> at Transplant | - | -         | -      | -     | -       | -           |
| D1                    | -   | -         | -      | -     | -       | -           |
| D2                    | -   | -         | -      | -     | -       | -           |
| D3                    | -   | -         | -      | -     | -       | -           |
| D4                    | -   | -         | -      | -     | -       | -           |
| D5                    | -   | -         | -      | -     | -       | -           |
### Table 8

C4d staining in post-reperfusion biopsies and subsequent rejection in the first year.

| Pathology                  | C4d (−) | C4d (+) | p     |
|----------------------------|---------|---------|-------|
| Sample size (%)            | 79 (73.8) | 28 (26.1) |       |
| **Pathology**              |         |         |       |
| Focal C4d (%)              | N/A     | 24 (85.7) |       |
| Diffuse C4d (%)            | N/A     | 2 (7)    |       |
| Glomerulitis (%)           | 0       | 5 (17.8) |       |
| Capillaritis (%)           | 0       | 0       |       |
| **Subsequent Rejection**   |         |         |       |
| Any Acute Rejection (%)    | 21 (26.5) | 14 (50)  | 0.03  |
| Acute AMR (%)              | 3 (3.8)  | 8 (28.6)  | 0.0008|
| Acute Cellular Rejection (%)| 14 (17.7) | 2 (7.1) | 0.2   |
| Acute Mixed Rejection (%)  | 4 (5)    | 4 (14.3) | 0.2   |

One hundred and seven out of all 146 (73.3%) patients underwent post-reperfusion biopsies.