Journal of Immunology Research

The Humoral Theory of Transplantation

Lead Guest Editor: Mepur H. Ravindranath
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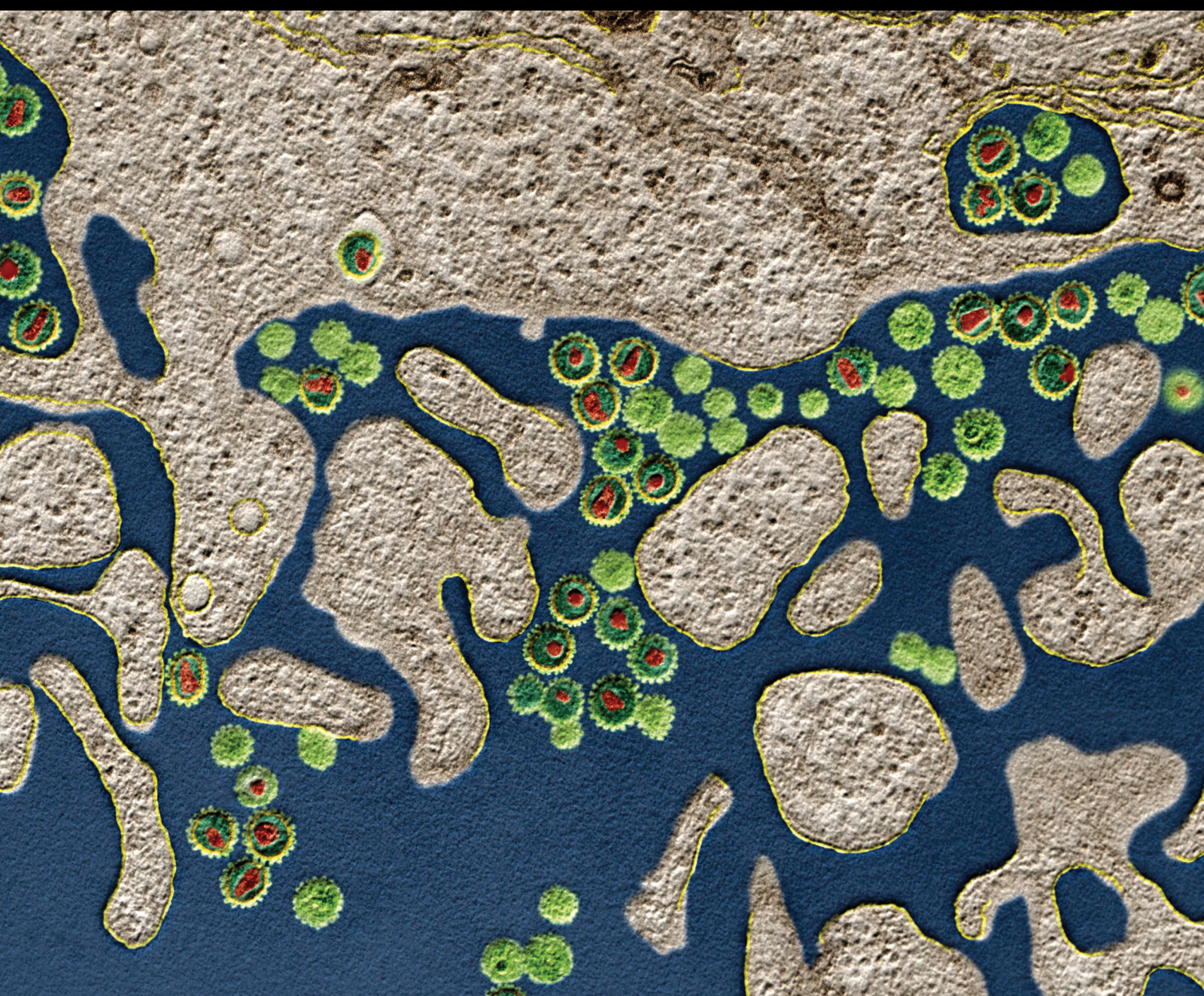

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Editorial Board

Contents

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Editorial

The Humoral Theory of Transplantation

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In 2003, after 45 years of research on the humoral theory of donor organ rejection, the late Professor Paul Ichiro Terasaki (09/10/1929–01/25/2016) proposed the Humoral Theory of Transplantation in the American Journal of Transplantation (3:665–673, 2003) and continued doing his research until his last breath. We respectfully dedicate this special issue in memory of Professor Paul I. Terasaki, the founding father of Humoral Theory of Transplant Immunology.

His theory not only impacts his contemporaries to develop better therapeutic strategies but also directs generations that follow him.

The graft of an organ from a living or deceased donor to a recipient causes many immunological reactions, which in many cases results in the failure of the allograft within the recipient's body. Whether grafts are destroyed by direct cytotoxicity, mediated by cellular immune components such as T cells, NK cells, or delayed-type hypersensitivity reactions, or by antibodies is the critical question. The ability of antibodies to destroy an organ within a few hours is well known; an example of this is the transfusion-related acute lung injury (TRALI). These antibodies can cause immediate rejection (even before closing the incision or soon afterwards) in hyper acute rejection, acute rejection (within a year), and chronic rejection (a year after posttransplant). The involved antibodies recognize either alloantigens such as cell surface MHC antigens (primarily donor-recipient mismatched HLA class I and class II antigens, known as donor specific antibody (DSA)) or normal or altered autoantigens such as endothelial antigens, angiotensin II type 1 receptor (AT1R), antiendothelin-I type A receptor (ET_AR), bioreactive C-terminal fragment of perlecan, vimentin, collagen V, K-a1 tubulin, and myosin to name a few. The primary objective of the proposed humoral theory is to provide a logical and rational course of clinical strategy for all allograft recipients. Antibody-mediated allograft deterioration is also considered an antibody-accelerated allograft senescence. The need to update clinical and experimental findings on antibody-mediated graft rejection and to explore the precise mechanisms underlying humoral rejection in designing therapeutic strategies to prevent allograft deterioration have prompted us to organize this issue.

We wish to profusely and individually thank every one of the contributors for positively responding to our request to memorialize the famous humoral theory of Dr. Paul Terasaki in the Journal of Immunology Research. The manuscripts received are categorized as research articles ($n = 9$), clinical studies ($n = 2$), and reviews ($n = 10$).

The special issue includes the following research articles: A. I. Sánchez-Fructuoso et al. (*Hospital Clinico San Carlos (IdiSS) Madrid, Spain*) documented that a shift in a single-nucleotide polymorphism (SNP: with a shift at position −308 from G to A) augmented the production of TNFα and is correlated with a significantly increased risk of acute rejection. This SNP is found to be a predictive biomarker of the efficacy of thymoglobulin, commonly used as an immunosuppressive regimen for lowering antibody production. Y.

Jiang et al. (*The First Affiliated Hospital, Zhejiang University, China*) examined the infiltration of CD20+ B cells and C4d in biopsies of patients ($n = 216$) with biopsy-proven acute cellular rejection (Banff I or II), in addition to serum creatinine and glomerular filtration rates to assess graft loss. In contrast to previous reports, they documented that the CD20+ patients (n-133) had significantly less graft loss and a better (but not a significant) survival rate and less steroid resistance than the CD20-negative group. It is concluded that the presence of CD20+ B cells in allografts is protective. J. C. Cicciarelli et al. (*USC Keck School of Medicine, Viracor-IBT Laboratories, MNTI Foundation, Los Angeles, CA*) analysed C1q and IgG subclasses in 73 renal allograft recipients for graft dysfunction with DSA. In analysing the graft biopsies for C4d, a remarkable difference was observed in cumulative DSA MFIs between the C4d+ group (12,500) and the CD4− group (<500). Among the C4d+ biopsy groups, 100% had DSA IgG, 85% had complement-fixing IgG, and 70% had C1q but did not observe any significant correlation between graft loss and C1q positivity. X. Zhao et al. (*Peking University People's Hospital, Beijing, China*) examined the association between anti-HLA DSA and prolonged isolated thrombocytopenia (PT) in a large cohort of unmanipulated haploidentical blood and bone marrow transplant (HBMT) patients ($n = 394$). The incidence of PT is significantly higher in patients with high MFIs (>1000) than those with low MFIs (<1000). Multivariate analysis revealed a significant correlation between high MFIs and hazard ratios of PT and, most importantly, a significant transplant-related mortality, thus emphasizing the need to include DSAs in the algorithm of unmanipulated HBMT. M. Toyoda et al. (*Cedars-Sinai Medical Center, Los Angeles, CA, USA*) report that IVIg plus rituximab, in combination with alemtuzumab and triple immunosuppression maintenance therapy, does not increase the risk of viral (CMV, EBV, and BKV) infections based on posttransplant viral infection status in 372 desensitized and 528 nonsensitized patients. Factors attributed to the low viremia include posttransplant antiviral prophylaxis, PCR monitoring the presence of memory T cells, viral specific antibodies, antiviral antibodies in IVIg, and NK cell-mediated ADCC in spite of the depletion of lymphocytes. M. Cioni et al. in Ginevri's group (*IRCCS Istituto G. Gaslini, Genova, Italy*) documented that the time interval from transplant to the occurrence of DSA may influence graft injury, using longitudinally collected sera of a cohort ($n = 114$) of pediatric renal graft recipients. De novo DSA developed within a year ($n = 15$), after a year ($n = 24$), and 24.6 months ($n = 39$) posttransplant. When comparing parameters such as C1q/C3d-binding, it was noted that only younger patients developed DSA earlier. Late antibody-mediated rejection occurred in 47% of the early group and in 58% of the late-onset group. Monitoring HLA antibodies throughout the posttransplant course was emphasized, despite its high costs and organizational challenges.

V. Jucaud (*Terasaki Foundation Laboratory, Los Angeles, CA, USA*) hypothesized that comparing the highest predicted binding affinity of nonself and self allo-HLA peptide for a transplant recipient's HLA-II antigens may distinguish immunogenic (which induces DSA formation) from nonimmunogenic mismatches. This hypothesis was tested on six renal-allograft recipients with HLA-II mismatches using different programs (HLA-matchmaker, PIRCHE-II, and an HLA-II immunogenicity algorithm). A significant association between DSA formation and the predicted HLA-DR presentation of nonself peptides was noted. It was shown that the methodology predicted DSA formation based on HLA mismatches, the recipients' HLA-DR phenotypes, and their identified permissible HLA mismatches to optimize HLA matching and to guide donor selection. K. Geneugelijk et al. in Spierings's group (*University Medical Center Utrecht, Utrecht, The Netherlands*) validated the computational methodology that they developed, which uses HLA haplotype frequency to allow epitope-based HLA matching from serological split level HLA typing. Their data documented that their computational approach is a powerful and reliable tool to estimate PIRCHE-II and epilet values when high-resolution HLA genotype data is not available.

The following two articles fall into the category of clinical studies: L. Zhu et al. (*Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China*) reported the incidence and patterns of early acute rejection episodes and a year of graft and patient survival outcomes of 33 renal allograft recipients after the 2nd, 3rd, and 4th kidney retransplants performed at their hospital. They documented a low incidence of early acute antibody-mediated rejection and satisfactory survival of the organ and patients after a year. The retransplant recipients had a high risk of developing early acute T cell-mediated rejection. The need for accurate diagnosis and reliable suppressive strategy was emphasized. N. Lachmann et al. (*Charite Universitatsmedizin Berlin, Berlin, Germany*) documented that 5-year graft survival, graft function, DSA levels, and serum creatinine levels can be improved with stepwise modification of the treatment regimens in a cohort ($n = 12$) with biopsy-proven antibody mediated rejection. The allograft recipients were initially administered (for 3 to 4 years) with rituximab/low-dose IVIg and plasmapheresis, then with bortezomib/low-dose IVIg (for a year) and later with bortezomib, high-dose IVIg, and plasmapheresis (> a year). These patients exhibited a significant reduction in serum creatinine and anti-HLA DSA after high-dose IVIg.

In addition, there are several review articles submitted for this special issue. E. J. Filippone and J. L. Farber (*Thomas Jefferson University, Philadelphia, USA*) reviewed the role of epitope analysis in optimizing HLA matching and assessed the pathogenicity of HLA antibodies in renal transplantation. E. Y. Cheng (*Terasaki Foundation Lab. & University of California, Los Angeles, CA, USA*) compared the humoral responses to allografts in liver and renal transplantation. In particular, the need for defining the histopathological characteristics of antibody-mediated liver graft rejection was emphasized and the question of whether all HLA antibodies are pathogenic in transplantation was addressed. A. Bharat and T. Mohanakumar (*NW Feinberg School of Medicine, Chicago, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA*) critically examined the role of humoral responses to tissue-restricted non-HLA self-antigens (such as AT1R, collagen V, and k-a1 tubulin) in

lung allograft survival in recipients' posttransplantation. C. Lefaucheur et al. (*INSERM, UMR-S970, Paris, France*) reviewed risk stratification of renal allograft rejection-based factors related to anti-HLA DSA antibodies, such as antibody strength, complement-binding capabilities, and IgG subclasses. They pointed out the identification of specific allograft injury patterns based on the nature of HLA-DSA, which may elucidate therapeutic strategies such B cell depletion or complement blockade with C5 inhibitors or C1 inhibitors. S. Sethi et al. with Jordan's group (*Cedars-Sinai Medical Center, Los Angeles, CA, USA*) reviewed the agents, IVIg, anti-CD20 antibodies (rituximab, obinutuzumab), proteasome inhibitors (bortezomib, carfilzomib), anti-IL-6R blocker (tocilizumab), IgG endopeptidase (Ides® produced by *Streptococcus pyogenes)*, blockers of B cell stimulator protein to B cell receptor (belimumab) blockers of C5 to inhibit the complement sequence (eculizumab), C1 esterase inhibitor, and belatacept (CTLA-Ig that can inhibit plasma cells and DSA generation) used in desensitization. S. Wang et al. in Zhu's group (*Fudan University, Shanghai, China*) summarized both antiendothelial alloantibodies (preformed and de novo antibodies against ABO blood groups, HLA, MICA) and autoantibodies (preformed and de novo antibodies against AT1R, ETAR, Perlecan, de novo against vimentin and preformed against endoglin, FLT3 ligand, and EDIL3). The alloantibodies are implicated in hyperacute and acute rejections and long-term graft injury, whereas the autoantibodies are implicated in acute and chronic graft injury. It is emphasized that endothelial cells are indispensable participants in the pathophysiology of antibody-mediated rejection, and therapies targeted at them show promise as an improvement over the prevailing immunosuppressive modalities. C. L. Butler et al. in Reed's group (*David Geffen School of Medicine, University of California, Los Angeles, CA*) identified microvasculature as the principal target of antibody-mediated injury and illustrates that both DSA and non-DSA are pathogenic through multiple mechanisms that have extensive crosstalk, such as T cell activation by antiendothelial antibodies. Since allograft rejection can occur throughout the lifetime of a transplanted organ, this study emphasized the need to delineate the crosstalk between HLA and non-HLA antibodies and their synergistic effects on graft injury and to assess their incidence among organ types. N. El-Awar et al. (*Terasaki Foundation Laboratory, Los Angeles, CA 90064, USA*) attributed the phenomenon of cross-reactivity in HLA antibodies to shared epitopes among HLA antigens and summarized the well-defined HLA-I unique epitopes, including cryptic epitopes, on β2-microglobulin-free HLA and HLA-II epitopes. It is suggested that the epitope-based matching for donor organs would minimize de novo DSA, improve allograft survival, and protect the allograft against chronic rejection. C. Süsal et al. (*University of Heidelberg, Heidelberg, D-69120, Germany*) reviewed the "Heidelberg algorithm," different measures of which may include monitoring donor-independent and donor-dependent antibodies to HLA class I and II, CDC T cell cross-matches in first transplants and B cell cross-matches in retransplant patients, having MFIs > 1000 using Luminex Labscreen Beadset in living donor organ recipients, having soluble CD30 $\geq$ 80 ng/ml, having good HLA match positivity in diseased donor organ recipients, acceptable mismatches from the Eurotransplant program, triple immunosuppressive regimens, protocol biopsies on days 7 and 90, DSA monitoring on days 0, 7, 30, 180 and every six months thereafter and running C1Q assays if DSA $\geq$ 3000 MFI. It is proposed that the algorithm has the potential to increase the number of transplantations in high-risk presensitized patients and diminishing (not totally eliminating) the impact of pre-existing antibodies on graft survival. It is hypothesized that T cell help from a preactivated immune system supports the deleterious impact of pretransplant DSA that would otherwise disappear in many graft recipients. M. H. Ravindranath (*Terasaki Foundation Laboratory, Los Angeles, CA 90064, USA*) reviewed the findings on the anti-HLA-E IgG2a mAbs (TFL-006 and TFl-007) and how they reacted with several HLA-Ia and HLA-Ib antigens, similar to that of therapeutic IVIg. In vitro, the mAbs mimicked IVIg in suppressing both antigen-specific activated T cells and anti-HLA Ab production by activated B cells, and they also expanded CD4+, CD25+, and $Foxp^3$+ Tregs, which are known to suppress T and B cells involved in antibody production. Therefore, it is proposed that the humanized version of mAbs would be useful in lowering Abs in allograft recipients in sensitized patients, promoting graft survival and preventing and controlling autoimmune diseases. M. Hamdorf et al. (*Terasaki Foundation Laboratory, Los Angeles, CA, USA*) discussed whether circulating miRNA, found in serum, plasma, and urine, can serve as an alternate to invasive biopsies and can function as an early noninvasive and robust diagnostic biomarker. This can help to develop further insight into pathways leading to the rejection process and to predict allograft rejection and failure.

In summary, this special issue encompasses advances in basic, clinical, and therapeutic aspects of the IgG antibodies existing in patients waiting for donor organs and for those who experience graft injury and loss after transplantation. The contributing authors highlighted the importance of identifying anti-HLA DSA that are pathogenic to allografts and the challenges encountered in monitoring the antibodies. Some investigators indicate that the role of antibodies may encompass cell mediated immune responses. Many authors consistently highlight the challenges encountered and the need for systematic, randomized controlled clinical trials for developing appropriate therapies to downregulate antibodies or antibody subclasses truly pathogenic to the allograft. As the late Professor Terasaki stated, "The purpose of a theory is to stimulate research proving its validity" (p.669, *AJT*, 2003, 3). We hope that this special issue stimulates further research to prove or disprove the validity of the humoral theory of rejection.

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Review Article

Clinical Relevance of HLA Antibodies in Kidney Transplantation: Recent Data from the Heidelberg Transplant Center and the Collaborative Transplant Study

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Herein, we summarize our recent findings from the international Collaborative Transplant Study (CTS) and Heidelberg Transplant Center regarding the role of HLA antibodies in kidney transplantation and their application into the clinical routine. Based on the antibody findings from the CTS serum study, an algorithm was developed in 2006 for the transplantation of high-risk sensitized patients at the Heidelberg Transplant Center which includes seven different pre- and posttransplant measures. Using this algorithm, the number of transplantations could be increased in high-risk presensitized patients and the previously existing impact of antibodies on graft survival could greatly be diminished but not totally eliminated. More recent findings led to the hypothesis that T cell help from a preactivated immune system supports the harmful effects of pretransplant donor-specific HLA antibodies that otherwise disappear in many cases after transplantation without any consequence.

1. Introduction

Although effective therapies to treat antibody-mediated rejection (AMR) still need to be developed, with his vision and strong determination, Paul Ichiro Terasaki was the driving force that convinced the transplant community to perform the necessary studies to comprehend the different aspects of humoral rejection in kidney transplantation. We dedicate this article therefore to this great scientist.

Thanks to the introduction of the single-antigen bead technique (SAB), which allows detection of HLA antibodies with high sensitivity, and improvement of pathological diagnosis, we widely understand today the role of donor-specific HLA antibodies (DSA) in the posttransplant phase. However, in which patients pretransplant DSA would exert their harmful effects is still not fully understood. Many patients were transplanted in the past in the presence of preexisting DSA; not all of them lost their grafts, even if the DSA was strong and complement-activating [1–3]. Pretransplant DSA disappear in many patients without any clinical consequence directly after transplantation, whereas in others, even weak pretransplant DSA persist and do harm in the subsequent course [3, 4].

2. Presensitization as a Major Problem

Kidney transplantation of presensitized patients with HLA antibodies in their serum is challenging mainly for two reasons. (1) To prevent a positive preoperative complement-dependent cytotoxicity (CDC) crossmatch result and diminish the harmful effects of pretransplant DSA, unacceptable HLA antigen mismatches are determined using sensitive assays and in the consequence many organ offers are excluded for these patients already at the virtual crossmatch level. Without further measures, presensitized patients accumulate on the kidney waiting list and face prolonged waiting times.

(2) Even when the pretransplant CDC crossmatch result is negative and the patient is successfully transplanted, long-term graft survival may be impaired in these patients, due to either persistence or reappearance of pretransplant DSA in the posttransplant phase or development of de novo DSA which can cause antibody-mediated tissue injury.

3. Heidelberg Algorithm for Transplantation of Presensitized High-Risk Patients

To overcome these two major problems, we introduced in April 2006 an algorithm for the transplantation of presensitized high-risk kidney transplant recipients at our center and adapted it further in 2007, 2009, and 2016 [3, 5–7]. A total of seven different measures are used in an integrated fashion to transplant these patients in a reasonable period of time with improved outcomes (Table 1). As shown in Figure 1(a), presensitized patients with ELISA-reactive HLA antibodies who were transplanted in the years 2000 to 2007 showed significantly lower graft survival rates than patients without ELISA-reactive HLA antibodies. This difference disappeared after the introduction of the Heidelberg Algorithm although more high-risk patients were transplanted (Figure 1(b)).

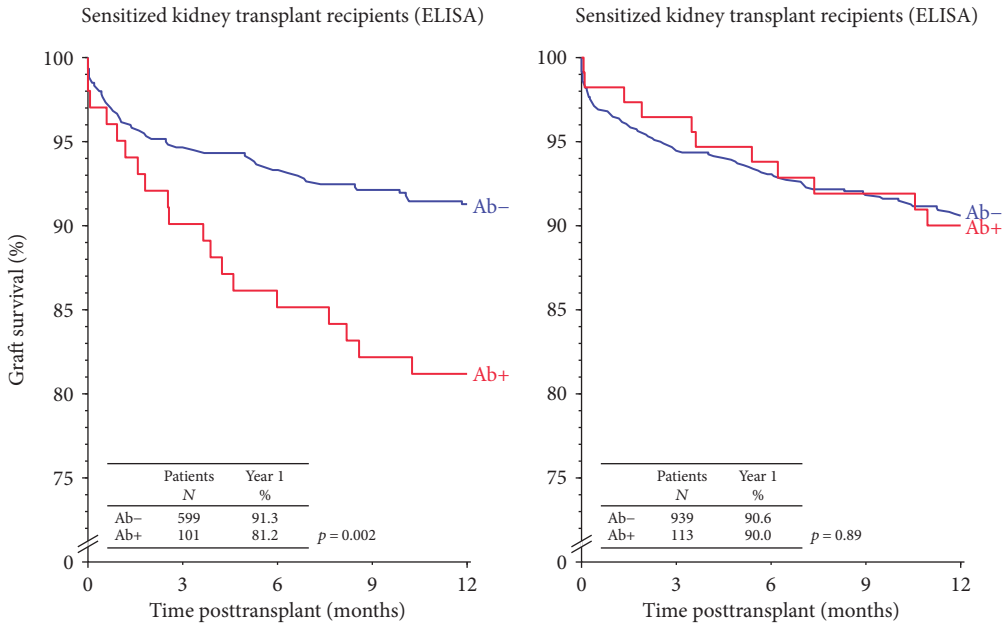
The most critical components of our integrative approach are the pretransplant identification of high-risk patients on the waiting list (measure 1) and the risk-stratified organ allocation (measures 2 and 3). For example, a patient who has a high cytotoxic PRA and/or is positive for both class I and II HLA antibodies in ELISA (measure 1) is at increased risk of graft loss. We reported in two independent series of 4136 and 5315 kidney transplant recipients on the increased risk of graft loss in the presence of pretransplant class I and class II HLA antibodies, as measured by ELISA [8, 9]. These patients may be successfully and timely transplanted when only a low number of HLA mismatches are present (measure 2) [9], and the transplantation is performed via the Eurotransplant Acceptable Mismatch program which allocates organs to highly immunized patients with high priority (measure 3) [10]. Since October 2016, pretransplant determination of the immune activation marker soluble CD30 (sCD30) in ELISA has also become an important component of pretransplant risk estimation in measure 1 of the Heidelberg Algorithm because pretransplant activation of the immune system, as measured by high sCD30 levels, was in two recent studies of 80 presensitized high-risk patients from Heidelberg and 385 presensitized patients from 13 transplant centers (corresponding to a series of some 1000 patients) found to be a substantial risk factor for graft loss in the presence of DSA (see below) [3, 7].

All as "high-risk"-categorized patients receive, during a deceased-donor organ offer process or in preparation for transplantation from a living donor, pre- and postoperative apheresis treatment (measures 4 and 5) to bring undetected antibody to a lower level and to prevent antibody-mediated allograft injury due to an early rebound of preexisting DSA. To prevent the development of de novo DSA, this is combined with the administration of anti-B cell antibody rituximab (measure 4). B cells are important antigen-presenting cells and are critical for T cell activation and the development of T cell memory during alloimmune responses. Despite a lack of effect against long-lived plasma cells, in some reports, anti-CD20 therapy was associated with a reduction of DSA reactivity. Rituximab may prevent the generation of antibody-producing cells from the naive B cell pool and may target short-lived plasma cells that express CD20 on their surface. In addition, anti-CD20 therapy may deplete B cell aggregates within allografts. Kohei et al. reported on 1.7% and 18.1% de novo DSA rates in patients, after ABO-incompatible or ABO-compatible kidney transplantation, indicating that targeting B cell immunity at the time of transplantation with rituximab may reduce antibody-mediated allograft injury during the subsequent course [11]. In our first series of 34 high-risk patients, severe cellular rejection was

Table 1: "Heidelberg Algorithm" (applied since April 2006).

(1) Pretransplant identification of high-risk patients
Donor-independent
(i) CDC-PRA-DTT ≥85% (current or historical)
(ii) HLA class I and II antibody positivity in ELISA
(iii) HLA class I positivity in ELISA (retransplant)
Donor-dependent
(i) Positive CDC B-cell crossmatch in retransplant recipients with HLA class II antibody positivity in ELISA
(ii) Positive CDC T-cell crossmatch
(iii) DSA ≥1,000 MFI (living donor; since April 2009)
(iv) DSA ≥1,000 MFI and sCD30 ≥ 80 ng/ml (since October 2016)
(2) Good HLA match in patients with HLA class I and class II antibody positivity in ELISA (deceased donor)
(i) CDC-PRA-DTT ≥10%: 0-1 HLA-A, -B, -DR mismatches
(ii) CDC-PRA-DTT <10%: 0-2 HLA-A, -B, -DR mismatches
(3) Acceptable Mismatch Program of Eurotransplant (deceased donor)
(i) CDC-PRA-DTT ≥85% (current or historical)
(4) Pretransplant treatment
(i) Single plasmapheresis (deceased donor)
(ii) Repeated immunoadsorption (living donor)
(iii) Triple immunosuppression (tacrolimus + enteric-coated mycophenolic sodium + methylprednisolone; in the case of living donor, together with the initiation of apheresis therapy)
(iv) Rituximab 375 mg/m^2 (when all crossmatches are negative)
(v) Thymoglobulin 1.5 mg/kg body weight preoperatively and a median of 2 times (range: 1–6) postoperatively (since April 2009; IL-2 receptor antagonist basiliximab before April 2009)
(5) Posttransplant treatment
(i) Repeated plasmapheresis (deceased donor)
(ii) Repeated immunoadsorption (living donor)
(6) Protocol biopsies
(i) On days 7 and 90 (since November 2007)
(7) Posttransplant monitoring of DSA
(i) On days 0, 7, 30, 180, and every 6 months thereafter
(ii) If deterioration of allograft function
(iii) C1q assay if DSA ≥3,000 MFI (since March 2016)

Adopted from [5]. CDC: complement-dependent cytotoxicity; PRA: panel reactive antibodies; DTT: dithiothreitol; DSA: donor-specific HLA antibodies; sCD30: soluble CD30.



(a) Heidelberg 2000–2007 (b) Heidelberg after 2007

Figure 1: Graft survival in patients with and without ELISA-reactive HLA antibodies who were transplanted at the Heidelberg Transplant Center between 2000 and 2007 (a) and after 2007 (b). Ab: ELISA-reactive HLA antibody.

infrequent under the usage of rituximab, even in the absence of thymoglobulin, while borderline changes were found frequently [5]. Since 2009, high-risk patients in addition receive T cell-eliminating induction therapy by thymoglobulin to target an early T cell response which would support de novo DSA and C1q-DSA development (see below). Protocol biopsies at days 7 and 90 (measure 6) and posttransplant antibody monitoring (measure 7) to diagnose AMR after successful kidney transplantation in its earlier stages complete the Heidelberg Algorithm.

Posttransplant antibody monitoring has recently been further refined with the introduction of the C1q assay. Patients with high mean fluorescence intensity (MFI) DSA of greater than 3000 are automatically tested since March 2016 for the presence of complement C1q component-binding DSA. We also consider posttransplant appearance of C1q-DSA a major risk factor for AMR-mediated graft loss during the further course (see below) [2–4].

Using this approach, even high-risk sensitized patients can be transplanted with graft survival rates that are not different from those of nonsensitized kidney recipients. In our initial analysis, 1-year graft survival, death-censored graft survival, and patient survival rates in 28 deceased donor kidney recipients were 92%, 96%, and 96%, respectively, and no graft loss or patient death was observed in the 6 living-donor kidney recipients [5]. AMR occurred in 1 living and 2 deceased donor kidney transplant recipients during the follow-up. However, the rate of cellular rejections (including borderline changes) in kidney graft biopsies and delayed graft function (DGF) were with 79% and 41%, respectively, quite high. We had previously reported that besides increased cold ischemia time, HLA antibodies and mild forms of AMR may also be involved in DGF [12]. To reduce this high rate of cellular rejection that may initiate AMR and DGF, interleukin-2 receptor antibody induction therapy was substituted by more potent thymoglobulin induction in high-risk sensitized patients from April 2009. This therapy is accompanied by rigorous infection prophylaxis by valganciclovir (when the donor is CMV-positive) and cotrimoxazole (in all patients).

4. Association of Posttransplant DSA with Graft Loss

We addressed the clinical value of posttransplant DSA monitoring which is the seventh measure of the Heidelberg Algorithm in three different cohorts: (1) in the CTS serum study, (2) in the Heidelberg pediatric cohort, and (3) in the Heidelberg presensitized high-risk population that was transplanted using the Heidelberg Algorithm.

4.1. CTS Data on the Impact of Posttransplant DSA. In the CTS serum study, we investigated a possible association of de novo development and persistence or loss of preexisting DSA with graft failure in 83 patients with failed kidney transplants and in 83 control patients without graft loss who were matched for eight different parameters, including the time after transplantation [4]. We chose this study design, because DSA determinations are costly and graft loss has increasingly become a rare event, and we wanted to include as many patients with graft loss into the analysis as possible. Eighty-three patients with graft loss correspond to a series of some 1000 transplant recipients. Antibody reactivity at five different cutoffs (500, 1000, 2000, 3000, and 5000 MFI) was evaluated systematically, and available recipient and donor DNA

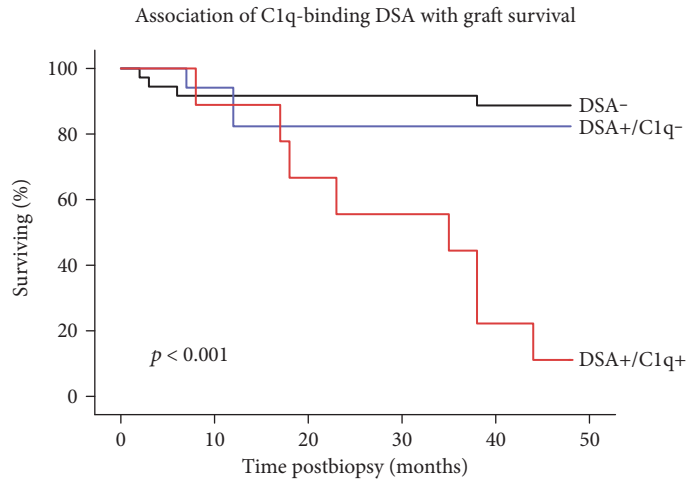


Figure 2: Postbiopsy kidney graft survival in pediatric patients stratified according to the donor-specific HLA antibody- (DSA-) C1q status at the time of indication biopsy. Patients with C1q-DSA positivity had a significantly inferior graft survival compared to patients without DSA ($p < 0.001$). Patients with DSA but without C1q positivity showed comparable graft survival to DSA-negative patients ($p = 0.55$) but significantly better graft survival than patients with C1q-DSA positivity ($p = 0.001$). Modified from [14].

allowed the precise determination of DSA against 10 different HLA loci.

In this study, the rate of de novo DSA and also non-DSA with ≥500 MFI was higher in the graft loss than in the nonrejector group (76% versus 40%, $p < 0.001$). Because of the low number of patients developing de novo DSA (22% of patients with graft loss), the DSA results did not reach statistical significance. At all cutoffs, there was a significantly higher rate of de novo non-DSA in patients with graft loss, which was explained rather by adsorption of DSA onto the graft than epitope sharing. Furthermore, the incidence of strong pretransplant DSA with 5000 MFI or higher that persist after transplantation was also higher in the graft loss group (10% versus 1%, $p = 0.034$).

The main problem in the clinical routine is that de novo DSA appear also in patients without immediate graft loss. When the C1q-binding ability of de novo or persistent DSA was analyzed in sera of patients with and without graft loss, none of the nonrejectors demonstrated C1q positivity, whereas 43% of patients with graft loss showed C1q-positive antibodies, although not necessarily donor-specific ($p < 0.001$). Overall, our data from this study indicated that the posttransplant presence of persisting or de novo HLA antibodies, especially if strong and C1q-binding, is associated with graft loss, even if the antibodies are not specific for mismatched donor HLA [4].

4.2. Evaluation of Posttransplant DSA Monitoring in Pediatric Patients with Indication Biopsy. Antibody effects appear to be stronger in pediatric than adult recipients [13]. Therefore, we found it important to investigate also in our pediatric cohort the diagnostic value of posttransplant DSA.

Sera of 62 patients who underwent clinically indicated graft biopsies were tested for DSA, and their association with specific histological lesions and subsequent graft outcome was analyzed [14]. Twenty-six patients (42%) were DSA-positive at the time of indication biopsy and nine (15%) of them were in addition C1q-positive. At 4 years after biopsy, the nine patients with C1q positivity showed a graft survival rate of 11%, which was strikingly lower than the 88% and 82% survival rates in DSA-negative and DSA-positive but C1q-negative patients, respectively ($p < 0.001$ and $p = 0.001$, resp.) (Figure 2). The majority (89%) of C1q-positive patients in this study were diagnosed with chronic active AMR. C1q-positive DSA (adjusted hazard ratio (HR) = 6.4), presence of transplant glomerulopathy (HR = 9.5), and estimated glomerular filtration rate (eGFR) at the time of indication biopsy (HR = 0.9) were risk factors for subsequent graft loss. Thus, the presence of C1q-positive DSA in the context of an indication biopsy identified a subgroup of pediatric renal transplant recipients with a markedly increased risk of subsequent graft loss. Because a fraction of DSA-positive patients escape rejection or graft dysfunction, the C1q assay appeared to increase the specificity of a positive DSA result regarding unfavorable transplant outcome.

4.3. Impact of Posttransplant DSA in the Heidelberg High-Risk Collective. Our adult high-risk cohort which consists of patients who are transplanted via the Heidelberg Algorithm is a special population in which the antibody effects are expected to occur in an accelerated manner. Compared to that of the international CTS study, we have in this cohort low number of patients with graft loss but more precise information on individual patients. Recently, we analyzed 80 of these high-risk sensitized patients who were transplanted at our center from April 2006 to November 2011 with a minimum follow-up for all patients of 36 months [3].

Despite all measures, seven patients developed AMR and six of them lost their graft within the first 4 years after transplantation, and all six patients were positive for C1q-DSA (1 persistent, 4 de novo, and 1 persistent plus de novo C1q-DSA) (Figure 3(a)).

In contrast to this striking association between posttransplant C1q-DSA and AMR-related graft loss, the predictive

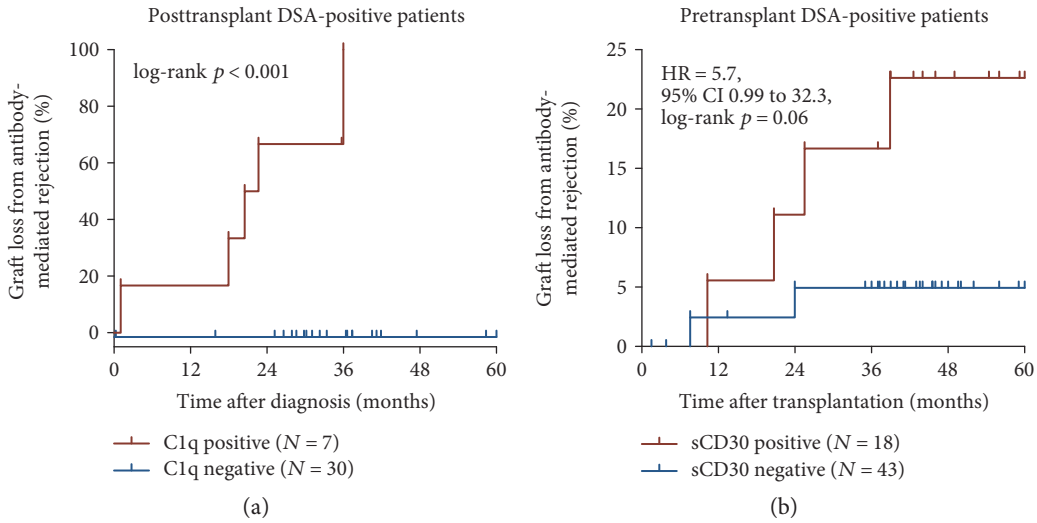


Figure 3: Graft loss from antibody-mediated rejection in high-risk sensitized patients with and without C1q-binding posttransplant donor-specific HLA antibodies (DSA) (a) and in patients who in addition to pretransplant DSA positivity had also increased levels of the immune activation marker sCD30 before transplantation (b).

value of pretransplant C1q-DSA was, even in this high-risk group, quite low. Of the 61 patients with pretransplant DSA (cutoff 500 MFI), 14 patients possessed C1q-DSA (cutoff 300 MFI). AMR rates and AMR-related graft loss in patients with pretransplant C1q-DSA were with 36% versus 28% and 14% versus 8%, respectively, not significantly different from the rates in patients with C1q-negative DSA. Interestingly, as many as 11 of 13 (85%) high-risk patients with pretransplant C1q-DSA and a posttransplant serum lost their C1q-DSA after surgery with an unremarkable clinical course, which is in line with the findings of Otten et al. and Loupy et al. that posttransplant but not pretransplant C1q-DSA predict AMR and AMR-related graft loss [1, 2].

5. Pretransplant DSA and sCD30

Earlier data from our group and others indicated that a preactivated immune system, as measured by sCD30, especially in combination with HLA antibodies is a good indicator of posttransplant rejection and graft loss [15–19]. Allostimulation results in the upregulation of the T cell activation marker CD30 on CD4 as well as CD8 memory T cells and increased release of the 88 kD sCD30 from these cells in an IFN-γ- and IL-2-dependent manner [20]. In the search for further biomarkers to improve risk estimation before transplantation as the basic component of Heidelberg Algorithm, we recently investigated a possible association of sCD30, DSA, and antibody-mediated graft loss in the group of 80 high-risk sensitized patients. The risk for AMR-related graft loss in 18 patients who had both, a positive pretransplant DSA value (cutoff 500 MFI) and a positive sCD30 value (cutoff 100 ng/mL), was 11 times higher than that in the remaining 62 patients (HR = 11.1, 95% CI 1.68 to 73.4, log-rank $P = 0.013$) and 5.7 times higher than that in DSA-positive but sCD30-negative patients (Figure 3(b)). Two patients who were sCD30-negative pretransplant and experienced AMR-related graft loss had a gap in immunosuppressive therapy and became sCD30-positive (posttransplant cutoff 40 ng/mL) prior to their AMR episode [3].

To substantiate this finding, we analyzed the combined impact of pretransplant DSA and the immune activation marker sCD30 on a larger cohort of 385 presensitized kidney transplant recipients from the CTS database who possessed ELISA- or CDC-reactive HLA antibodies in their serum [7]. In this study, a deleterious influence of pretransplant DSA (cutoff 1000 MFI) on 3-year graft survival was evident only in patients who were positive (≥80 ng/mL) for the immune activation marker sCD30. In the absence of sCD30 positivity, 3-year graft survival was almost identical in patients with or without DSA (83% and 84%, $P = 0.81$). In contrast, a strikingly lower 3-year graft survival rate of 62% was observed in patients who were both sCD30- and DSA-positive (HR 2.9, $P < 0.001$). Even in the presence of strong DSA with ≥5000 MFI, the 3-year graft survival rate was high if the recipients were sCD30-negative. An update of these results in 411 patients is shown in Figure 4.

However, our findings on the clinical relevance of SAB-detected pretransplant DSA and sCD30 are restricted to presensitized patients with CDC- or ELISA-reactive antibodies. We reported previously on the missing association of SAB-detected pretransplant DSA with graft loss in CDC- and ELISA-negative kidney graft recipients [21], which could partly be explained by false positive results due to reactivity with denatured antigen on the beads that can be observed in healthy individuals [22] as well as in kidney transplant recipients without history of an immunizing event [23].

We hypothesize that patients with pretransplant DSA and the activated immune system (as measured by pretransplant sCD30) require special attention after kidney transplantation. In these patients, a gap in immunosuppressive therapy may lead to persistence, reappearence, or de novo occurrence of strong, complement-activating DSA, resulting in severe AMR and, without immediate intervention, in AMR-related graft loss.

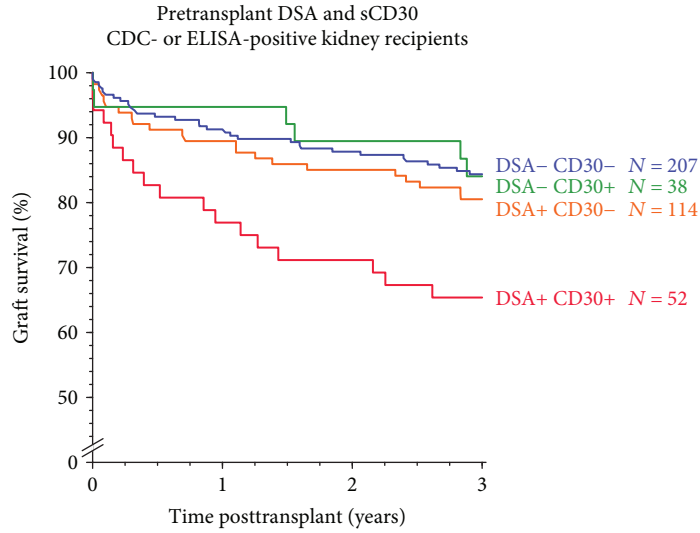


Figure 4: Impact of pretransplant sCD30 on graft survival in patients with and without pretransplant donor-specific HLA antibodies (DSA) in single-antigen bead testing. An update of the results from [7] is shown.

6. Conclusions

Integrated approaches are required for successful and timely transplantation of presensitized high-risk patients. Estimation of risk of graft failure prior to transplantation is important and requires further precision by introduction of additional biomarkers. Combination of DSA in presensitized patients with CDC- or ELISA-reactive antibodies with the immune activation marker sCD30 appears promising and deserves further evaluation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

HLA Class Ia and Ib Polyreactive Anti-HLA-E IgG2a Monoclonal Antibodies (TFL-006 and TFL-007) Suppress Anti-HLA IgG Production by CD19+ B Cells and Proliferation of CD4+ T Cells While Upregulating Tregs

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The anti-HLA-E IgG2a mAbs, TFL-006 and TFL-007, reacted with all HLA-I antigens, similar to the therapeutic preparations of IVIg. Indeed, IVIg lost its HLA reactivity, when its HLA-E reactivity was adsorbed out. US-FDA approved IVIg to reduce antibodies in autoimmune diseases. But the mechanism underlying IVIg-mediated antibody reduction could not be ascertained due to the presence of other polyclonal antibodies. In spite of it, the cost prohibitive high or low IVIg is administered to patients waiting for donor organ and for allograft recipients for lowering antiallograft antibodies. A mAb that could mimic IVIg in lowering Abs, with defined mechanism of action, would be highly beneficial for patients. Demonstrably, the anti-HLA-E mAbs mimicked several functions of IVIg relevant to suppressing the antiallograft Abs. The mAbs suppressed activated T cells and anti-HLA antibody production by activated B cells, which were dose-wise superior to IVIg. The anti-HLA-E mAb expanded CD4+, CD25+, and Foxp^3+ Tregs, which are known to suppress T and B cells involved in antibody production. These defined functions of the anti-HLA-E IgG2a mAbs at a level superior to IVIg encourage developing their humanized version to lower antibodies in allograft recipients, to promote graft survival, and to control autoimmune diseases.

1. Introduction

The humoral theory of transplantation recognizes that the high level of IgG Abs in patients waiting for donor organs and the Abs formed after transplantation are the causal factor in graft loss. Performing transplantation in patients with high levels of Abs (sensitized patients) is considered futile [1–4]. The de novo donor-specific Abs (DSA) formed against mismatched HLA molecules of different loci (HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ, and HLA-DP) are capable of damaging the allografts [1, 5–7]. DSA may cross-react with shared epitopes on other MHC molecules [8], to augment the levels of de novo nondonor-specific Abs (NDSA) [9–12]. In addition, compatible MHC molecules (e.g., HLA-Ib antigens) overexpressed upon inflammation may elicit antibodies and contribute to the pool of NDSA. Both DSA and NDSA are capable of binding and/or aggregating on the vascular endothelial lining, attracting complement components (C1q, C4d) which form complexes that cause vascular blockage leading to minimal graft function, rejection, and graft loss [11, 12]. The allograft recipients may also develop Abs against nonclassical HLA (HLA-E, HLA-F, and HLA-G) [13] and non-MHC autoantigens (e.g., AT1R, vimentin, collagen, myosins) that may or may not be released from the allograft. Interestingly, these Abs are also correlated with loss of function of the allograft [14–18]. Several therapies are contemplated, and a few were developed to lower these Ab levels.

Ab formation depends on both T and B cells to produce Abs against allo- or autoantigens. Therefore, aggressive suppressive strategies are developed to simultaneously deplete the T and B cells, in order to suppress the development of

Abs formed prior to (sensitization) or after transplantation (de novo Abs). One such aggressive immunotherapeutic strategy is induction therapy with rabbit or horse anti-human thymoglobulin, a polyreactive polyclonal mixture of nonspecific cytotoxic Abs capable of killing almost every immune cell, as documented by the list of immune cell surface antigens recognized [19].

An alternate strategy to suppress antibody formation was to transfuse polyclonal Abs purified from plasma pooled from thousands of donors, referred to as the intravenous immunoglobulin (IVIg), which either alone [20–23] or often in combination with plasmapheresis [24], or rituximab [25], a monoclonal Ab (mAb) that depletes CD20+ B cells [26]. IVIg is a complex entity consisting of polyreactive polyclonal IgG with a minor fraction of IgA Abs. Several immunosuppressive capabilities are attributed for IVIg, but its mechanism of action is far from clear, due to the polyclonality and polyreactivity of the mixture of Abs.

Most of the immunosuppressive therapies (IVIg, antithymoglobulin) involved in reducing antibody production were developed before the discovery of Tregs. It is well known now that Tregs are capable of controlling, depleting, or inhibiting CD4+ [27] and CD8+ [28, 29] T and B cells involved in antibody production [30, 31]. Tregs are also known to involve organ transplantation [32], and Tregs are found both in the recipients' lymphoid tissues posttransplantation and also at the graft sites [33].

While depleting T and B cells is important for preventing Ab formation before and after transplantation, such a therapy in combination with any therapy that induces and preserves the functionality of the tolerogenic Treg cells would be ideal and highly beneficial for allograft recipients [34], because these regulatory cells per se are potentially capable of suppressing Ab production. Although IVIg preparations were reported to suppress CD4+ T cells [35, 36], CD8+ T cells [37], and CD19+ B cells [38] and expand CD4+CD25 + Treg [39], the conflicting reports on the potential of IVIg to suppress T and B cells [40–43] cast doubt on the reliability of IVIg for depleting both T and B cells.

The polyclonality of the Abs in IVIg, or innate differences in the commercial preparations of IVIg [44], may account for the conflicting reports and doubts on its reliability. Therefore, an ideal therapeutic agent to achieve the combinatorial effect of depleting T and B cells and upregulating Treg could be a well-defined monoclonal Ab (mAb), which not only enables better understanding of its modus operandi but would also be the most cost-effective therapeutic agent than the high-dose IVIg. It could serve as an ideal and intensive therapeutic agent for desensitization and would minimize the formation of de novo DSA and NDSA to prevent Ab-mediated acute and/or chronic graft loss.

In this report, we review a series of experimental investigations made that show anti-HLA-E monoclonal IgG2a [45] binds to HLA-Ia and HLA-Ib molecules similar to IVIg preparations [46] and is simultaneously capable of depleting and arresting the functions of CD4+ T cells and CD8+ cytotoxic T cells [47], arresting anti-HLA Ab formation by CD19+/CD20− B cells [48] and upregulating the tolerogenic CD4+/CD25+/Foxp^3+ Treg (M. Taniguichi and M. H. Ravindranath, manuscript in preparation). The objective is to offer proof that anti-HLA IgG2a mAbs are functional mimics therapeutic IVIg in suppressing T and B cells, antibody production, and upregulation of Treg.

2. Experimental Approach and Observations

2.1. Monoclonal Anti-HLA-E mAbs Mimic Polyclonal IVIg In Vitro. The pool of Abs developed in sensitized patients are removed by plasmapheresis and substituted with plasma [49] or with IVIg [24] or IVIg alone [20–23] prior to obtaining a donor organ or transplantation. Though the mechanism is far from clear, one of the immunomodulatory roles of IVIg is to lower Abs. To further suppress the Ab production in these patients, rituximab was used as a combinatorial therapy [25]. Adverse events and the costs of therapies prohibit repeated administration of some of these IVIg-combinatorial therapies.

All the therapeutic preparations of IVIg examined revealed that they contain antibodies against all alleles of HLA-A, HLA-B, HLA-Cw, HLA-F, and HLA-G loci, and it reacted with all of the classical (HLA-Ia [HLA-A:31, HLA-B:50, and HLA-Cw:16]) and nonclassical (HLA-Ib [HLA-E: 2, HLA-F:1, and HLA-G:1) molecules in a Luminex single-antigen bead assay. When the HLA-E reactivity was specifically removed from IVIg, the entire HLA-Ia reactivity of IVIg disappeared, suggesting that the HLA-Ia reactivity of IVIg could be due to the cross-reactivity of anti-HLA-E Abs in IVIg with HLA-I antigens [46]. It is hypothesized that the anti-HLA-E reactivity of IVIg could be responsible for some of the immunomodulatory activities of IVIg.

It was hypothesized that a monoclonal Ab that could mimic the immunomodulatory functions of IVIg could serve as an ideal therapeutic agent. In this regard, a unique category of mAbs came to light while examining the HLA reactivity of more than 100 mAbs generated against β2-microglobulin-free heavy chain of HLA-E. A set of these mAbs (such as TFL-006 and TFL-007, both IgG2a mAbs) uniquely recognized a common epitope (amino acid sequence) (^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135}) shared by almost all alleles of HLA class I loci, and it reacted with all of the classical (HLA-Ia [HLA-A:31, HLA-B:50, and HLA-Cw:16]) and nonclassical (HLA-Ib [HLA-E: 2, HLA-F:1, and HLA-G:1]) molecules in a Luminex single-antigen bead assay (Table 1) [45, 46]. Strikingly, the HLA-I polyreactivity of the anti-HLA-E^R mAbs is identical to the HLA-Ia reactivity of different commercial preparations of IVIg. In studying their immunomodulatory capabilities, it was noted that these polyreactive HLA-E mAbs mimic some of the functions of IVIg critical for the suppression of the production of the anti-allograft Abs [47, 48].

Therefore, we tested whether HLA-Ia and Ib-reactive anti-HLA-E mAbs mimic some of the immunoregulatory functions of IVIg. This report reviews the different immunomodulatory functions of mAbs TFL-006 and TFL-007 that mimic IVIg, and it demonstrates that the performance of the mAb in vitro is much better than IVIg.

Table 1: Different therapeutic preparations of IVIg show extensive HLA-Ia and HLA-Ib reactivity, which is strikingly similar to the IgG2a monoclonal Abs developed in mice by immunizing with heavy chain of HLA-E^R [46, 47].

Different therapeutic preparations of IVIg versus different kinds of anti-HLA-E monoclonal antibodies	Reactivity of different HLA class I antigens					
	Classical HLA-Ia alleles			Nonclassical HLA-Ib		
	A	B	Cw	E	F	G
Maximum alleles tested	31	50	16	2	1	1
Therapeutic IVIg preparations						
IVIg (GlobEx, India)	20	39	16	2	1	1
IVIg (GammaStan, USA)	31	50	16	2	1	1
IVIg (Octagam, Mexico)	30	47	16	2	1	1
IVIg (Sandaglobulin, Euro)	30	47	16	2	1	1
Anti-HLA-Ib antibodies						
TFL-006 (IgG2a)	31	50	16	2	1	1
TFL-007 (IgG2a)	26	44	16	2	1	1
TFL-037 (IgG2b)	14	39	15	2	0	0
TFL-033 (IgG1)	0	0	0	2	0	0

2.2. mAb TFL-007 versus IVIg: Suppression of the Production of Anti-HLA Abs by B Cells In Vitro. To examine whether the anti-HLA-E IgG2a (TFL-007) suppresses the secretion of allo-HLA-II Ab by activated B^{memory} cells [48], a B cell population was separated from the peripheral blood mononuclear cells of a woman alloimmunized postpartum 23 years prior to testing, who had developed Abs directed against her husband's HLA class II antigen, after the first delivery (example number 1). In addition, we have also examined the efficacy of the mAb TFL-007 to suppress the anti-HLA-I Ab production by the immortalized B cells (hybridoma cell line, HML-416) from a woman similarly immunized postpartum by allo-HLA (example number 2).

In example number 1, the first daughter (current age 26) of the mother carried her father's nonmaternal allele, DRB1*01:01 which may have been responsible for the presence of anti-DRB1*01:01 IgG Ab in the mother. Feto-maternal transfer of HLA Abs and B cells is known to occur [50, 51]. Recent analysis of the maternal sera indicated that the high MFI of the anti-DRB1*01:01 persisted in the blood 26 years after alloimmunization, suggesting that the maternal B cells producing the Abs may be long-lived B^{mem} cells. The sera also reacted with lower MFI to DRB1*01:02, DRB1*04:04, DRB1*04:05, DRB1*14:02, and DRB1*04:01, which could be due to cross-reactivity of the primary allo-Abs anti-DRB1*01:01 IgG, as Cai et al. [8] construed for the presence of nondonor-specific anti-HLA-II Abs in allograft recipients.

The CD19+ B cells isolated from PBMC on day 0, consisted of naïve B cells (CD20+/CD27−/CD38+/−) (74.47%), B^{memory} cells (CD20+/CD27+/CD38−) (8.47%), and plasma cells (CD20−/CD27++/CD38++) (0.26%). These cells were activated in vitro with IL-2, IL-4, IL-6, IL-10, and IL-21 (at 1/4/4/2/2 ratio) and 1 μg/ml human CD40 Ab for 7 days (Figure 1), which resulted in an increase in the plasma cells from 0.26% to 36.25% on day 7.

Several microplate wells contained the primary alloAb (anti-DRB1*0101 IgG) with high MFI. The B cells from these wells were pooled and recultured in 4 wells on day 7 (Figure 1). On day 9, the cells were pooled and aliquoted into 3 wells and maintained without the cytokine combo or anti-CD40 mAb. These wells were exposed to medium or IVIg (1/100, 1.5 mg/ml) or mAb TFL-007s ("s" indicates the mAb purified from the supernatant) (1/100, 5 μg/ml) for 72 hours. IVIg protein concentration was 300-fold higher than that of purified TFL-007s (5 μg/ml) used in the treatment of B cells in culture.

The supernatants recovered from the respective wells were screened for the HLA-allo-Abs. The levels of allo-HLA Abs are compared between those recovered from the wells that contained only the medium with those that contained IVIg or mAb TFL-007. Since IVIG reacts with most HLA molecules nonspecifically, it could be construed that any detection IgG in samples containing IVIg is interfered, causing false positivity. However, we have compared the levels of allo-HLA-Abs found in the IVIg containing wells with that of the level observed in the medium only (without IVIg). Figure 2 reveals a critical finding for this investigation, that is, the level of allo-HLA Ab in the wells containing IVIg is significantly lower (28%, 46%, and 43% lower at 12, 24, and 72 hrs, respectively, than that of the Ab level in the well containing medium only. Indeed, IVIg (GamaSTAN) suppressed the secretion of the anti-DRB1*01:01 IgG at different time points but only marginally ($P < 0.04$). Suppression by TFL-007s was markedly different from that of IVIg. The mAb reduced the secretion of both primary (the anti-DRB1*01:01 IgG) and secondary Abs significantly ($P < 0.001$ to 0.0001) at levels higher than that of IVIg (Figure 2). The percentage difference at different time intervals of secretion of the anti-DRB1*01:01 IgG confirms the suppressive efficacy of TFL-007s. Indeed, it is mAb TFL-007s—not IVIg—that strongly suppressed the secretion of both primary and secondary allo-HLA-DRB IgG Abs (Figure 3) which was secreted by activated normal healthy human B^{mem} cells.

It is known that the extent of IVIg-mediated apoptosis of resting and activated human B cells was significantly lower than that observed with B cell hybridomas [42]. Therefore, we examined the ability of IVIg and TFL-007a ("a" indicates

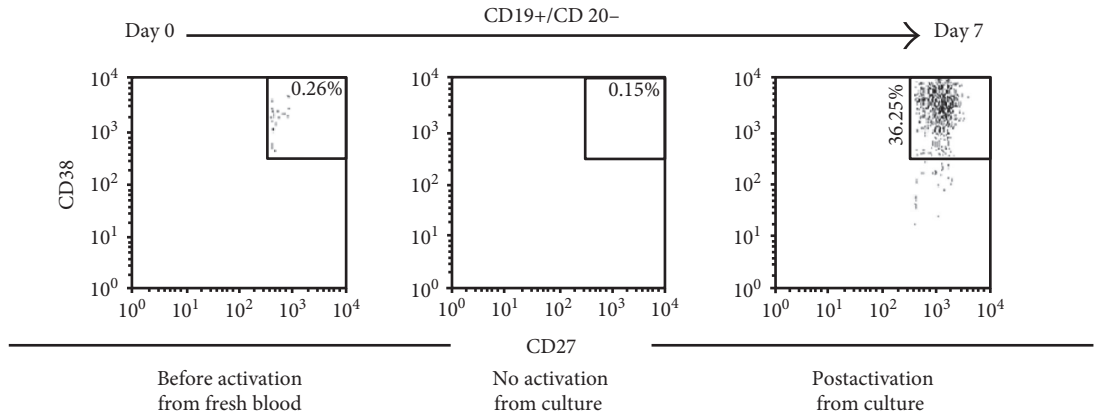

Figure 1: The B cells isolated from an alloimmunized woman's blood were activated in vitro by a selected battery of cytokines IL-2/IL-4/IL-6/IL-10/IL-21 (at 1/4/4/2/2 ratio) and 1 μg/ml human CD40 Ab for 7 days, which resulted in an increase in plasma cells (CD19+/CD20−/CD27++/CD38++) from 0.26% on day 0 to 36.25% on day 7 [48].

ascites purified mAb) to suppress the secretion of anti-HLA-I Abs secreted by the hybridoma (HML16) (example 2). HML-16 cell line produced anti-HLA-I Abs with high MFI against B*0702 > B*8101 > B*4201 > B*6701. Two different preparations of IVIg (GamaSTAN and Gamunex) were used with a media control. A high-dose IVIg, used in transplant patients, was used to suppress the Ab production by the hybridoma. IVIg-GamaSTAN (at dilutions 1/10, 1/20, and 1/40 with dosages 15 mg/ml, 7.5 mg/ml, and 3.75 mg/ml, respectively) and IVIg-Gamunex (at dilutions 1/10, 1/20, and 1/40, with dosages 10 mg/ml, 5 mg/ml, and 2.5 mg/ml, respectively) were used. Neither of the IVIgs suppressed the secretion of allo-HLA-B IgG by the hybridoma cells (Figures 4(a) and 4(c)). In striking contrast, the mAb TFL-007a significantly suppressed the secretion of both anti-HLA-B*0702 and anti-B*8101 IgG Abs (Figure 4(b) and 4(d)). Most importantly, TFL-007a showed dosimetric suppression of allo-HLA-I Abs. Indeed, anti-HLA-E mAb TFL-007a, in marked contrast to IVIg preparations, significantly suppressed the secretion of both allo-HLA-B Abs.

The comparison between the potential of therapeutic IVIg and monoclonal anti-HLA-E IgG2a (TFL-007) in suppressing the production of IgG Abs formed against (1) paternal-specific HLA-II antigen and cross-reactive HLA-II antigens by B cells (CD19+/CD20−/CD27+/CD38+) in a mother (example number 1) and (2) paternal HLA class I antigens by EBV immortalized B cells obtained from a mother (example number 2) confirmed that the immunosuppressive potential of anti-HLA-E IgG2a mAb is superior to IVIg. This exemplifies a cost-effective immunosuppressive therapeutic strategy by utilizing the mAb for desensitization of patients prior to transplantation as well as to suppress effectively both donor-specific and nondonor-specific HLA-I and HLA-II Abs.

2.3. TFL-007 or TFL-006 versus IVIg: Suppression Activated CD4+ and CD8+ T Cells In Vitro. The effects of different concentrations of IVIG on phytohemagglutinin (PHA) activated T lymphocytes were examined in vitro [52], and it was observed that IVIg controls the T lymphocyte activation, possibly by binding to the specific Fc-receptor expressed on the surface of activated T cells. Subsequent reports documented that IVIg suppresses cytokine-activated T lymphocytes [35], by apoptosis [38], by arresting the production of cytokine involved in the activation of T cells [53], and by suppressing proliferation of human (auto) antigen-specific T cells without inducing apoptosis [41]. The exact mechanism of suppression of T cell functions could not be defined unequivocally, as IVIg contains all of the subclasses of IgG Abs (Octogam: IgG1 65%, IgG2 30%, IgG3 3%, and IgG4 3%), their $F(ab)^2$ fragments and varying concentrations of IgA and lower amounts of IgM along with T-helper type 2 (Th2) cytokines and cytokine antagonists in different preparations of IVIg [54]. Furthermore, IVIg is prepared by purifying IgG from plasma, pooled from 10,000 to 60,000 donors, that contains several undefined antigen-specific and polyreactive Abs. In spite of the ambiguity, IVIg, in pre- and posttransplant patients, is considered to reduce T cell activation and proliferation (blastogenesis) in allograft recipient, and to suppress the production of anti-allograft Abs, biopsy-proven T cell-mediated allograft rejection and T-lymphoproliferative disorders developed posttransplantation [55, 56]. Since we observed that the performance of anti-HLA-E IgG2a mAb (TFL-007) was superior to IVIg in mimicking the suppression of anti-HLA body production by B^{memory} cells, we have also examined whether mAb TFL-007 and another closely related but similar IgG2a mAb TFL-006 are capable of suppressing blastogenesis and proliferation of CD4+ and CD8+ T lymphocytes [47], with appropriate controls.

For in vitro experimental purposes, T lymphocytes were recovered from the peripheral blood of mononuclear cells (PBMCs) of healthy human donors and isolated using Ficoll™-Hypaque (GE Healthcare BioSciences Corp., Piscataway, NJ, USA) and for isolating the lymphocytes LymphoKwick® (One Lambda) was used. The isolated lymphocytes were separated into two batches, one activated with phytohaemagglutinin (PHA) at a final concentration of 2·25 μl/ml and the other not activated (PHA-negative control). The CD4+ or CD8+ lymphoblasts were identified by

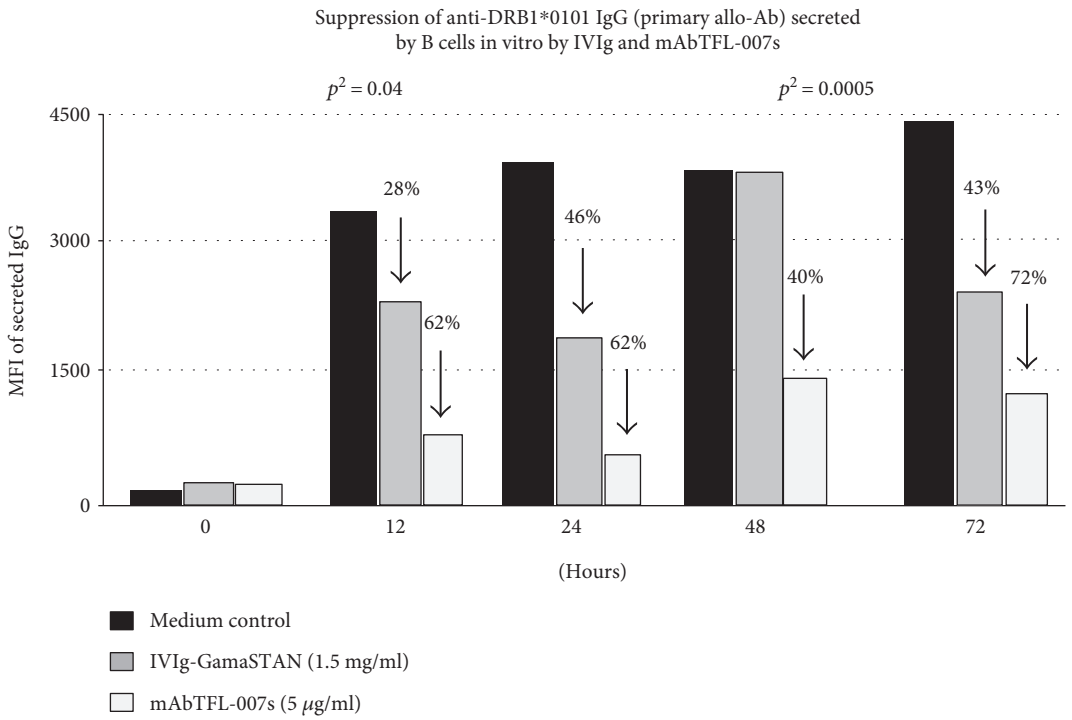

Figure 2: HLA molecular typing of an alloimmunized woman's family showed that the first child shared the father's HLA-II type (DRB1*0101 and DQA1*0101/DQB1*0501). Consequently, the mother had high levels of allo-Abs (based on mean fluorescent intensity observed with Luminex single-antigen bead assay) against both the DRB and DQ alleles, even 23 years after alloimmunization, indicating the presence of long lived B^{mem} cells. The allo-Abs with affinity for husband's HLA class II [primary alleles] are designated as "primary allo-Abs." The sera also contained "secondary allo-Abs" reacting to DRB1*0102, DRB1*0404, DRB1*0405, DRB1*1402, and DRB1*0401 (possibly those cross-reactive to the primary alleles). The B cells were isolated from the fresh peripheral blood of the mother. Using Ficoll-Paque PLUS, the peripheral blood mononuclear cells (PBMC) were isolated. The B cells (resting) were isolated from the PBMC by positive selection using CD19 Pan B Dynabeads® magnetic beads. B cells were detached by DETACHaBEAD® CD19. Purified human B cells were >95% CD19+, as determined by flow cytometry analysis. Purified B cells were plated at 0.2×10^6/200 μl/well in a sterile 96-well, round-bottom plate. B cells were cultured in Iscove's modified Dulbecco's medium, containing HEPES, L-glutamine, and sodium pyruvate, supplemented with 10% AB human serum, 5 μg/ml recombinant human (rh) insulin, 50 μg/ml rh transferring, 25 μg/ml gentamicin, and 50 μM 2-mercaptoethanol (2-ME). The resting B cells were activated with 25 ng/ml rh IL-2, 100 ng/ml rh IL-4, 100 ng/ml rh IL-6, 50 ng/ml rh IL-10, 50 ng/ml rh IL-21, and 1 μg/ml human CD40 Ab. On day 7 of the culture, 10 μl of culture supernatant from each well was analyzed for the presence of anti-HLA class II IgG allo-Abs. Cells from the wells that contained the HLA Abs were further harvested, washed three times, seeded into 4 wells, and activated as above. On days 8 and 9, the culture supernatants were tested for the secretion of allo-HLA Abs. The cells were pooled, washed (3×), and aliquoted into 3 wells: with medium alone; with GamaSTAN IVIg at 1/100 dilution, 1.5 mg/ml; and with mAb TFL-007s at 1/100 dilution containing 5 μg/ml. The cells were maintained in culture without any cytokine activators or anti-CD40 Ab for an additional 3 days, and 10 μl of culture supernatants from each well was analyzed for HLA allo-Abs at hours 0, 12, 24, 48, and 72. The figure shows the paired sample analysis of the triplicates at different hours. The paired sample two-tailed *t*-test was used to compare the results obtained at the stated hours with IVIg and TFL-007s against those for the control wells. The paired sample two-tailed *t*-test was carried out for IVIg and TFL-007s separately. Combined mean of the triplicate values obtained at 12 to 72 hrs for IVIg and TFL-007s was against the pooled values for the medium only of control wells (details in [48]).

the size (forward scatter) and by the granularity (side-scatter), using Flow cytometry. Blastogenesis of activated T cells and proliferation was monitored with CFSE, a cell-permeable dye [57]. IVIg- or mAb-mediated suppression of proliferation was recorded for cessation of mitosis, as measured by the successive twofold reductions in the CFS intensity after 72 h of treatment.

IVIg or mAbs were added to the cells in culture by the addition of PHA over the course of two hours (the total volume was adjusted to 200 μl), based on a previous report that evaluated the effects of time differences in the addition of IVIg and other toxins after adding PHA [53]. After PHA treatment, the CD4+ T lymphoblast cell density increased five- to sixfold over the PHA-negative control (Figure 5(a)). Similarly, PHA-activated T lymphocytes proliferated dramatically (Figure 5(b)). On the other hand, IVIg strikingly suppressed the PHA-activated blastogenesis of lymphocytes (Figure 6). The density of the PHA-activated T cells decreased significantly in a dosimetric fashion, indicating that IVIg potentially suppresses PHA activated T-lymphoblasts. Similarly, CFSE profiles elucidated the cessation of activated T cell proliferation by IVIg (Figure 6).

The ability of the novel anti-HLA-E^R IgG2a mAb (TFL-007) to suppress the blastogenesis and proliferation

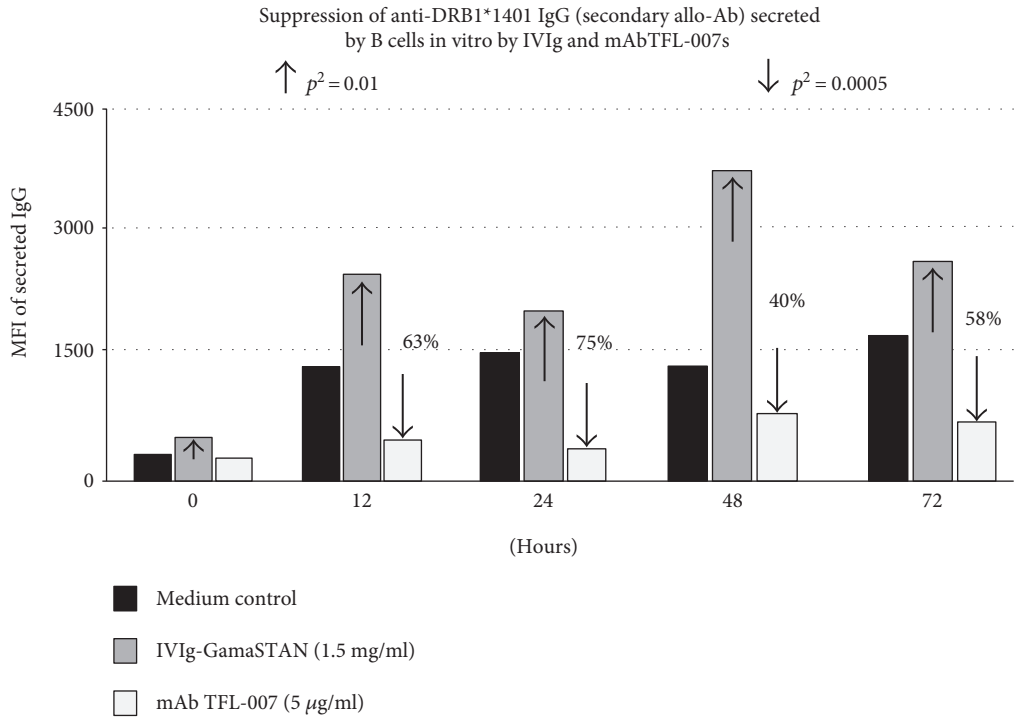

Figure 3: The anti-HLA-E mAb TFL-007s (conc. 5 μg/ml) but not IVIg (GamaStan, conc. 1.5 mg/ml) shows inhibition of the secondary allo-Ab anti-DRB1*1401 IgG. p^2 (two-tailed p value). The details of experimental protocol are identical to those of Figure 2.

of CD4+ and CD8+ T lymphocytes (Figure 7) is strikingly parallel to another anti-HLA-E IgG2a mAb (TFL-006) (Figure 8). At the same time, control antibodies such as anti-HLA-I reactive Abs and anti-HLA-E IgG2b mAb (TFL-037) are less reactive to HLA-I and nonreactive to HLA-F and HLA-G, and a HLA-E monospecific mAb, TFL-033, failed to suppress either blastogenesis or proliferation of PHA-activated T cells (Figure 8, Table 2).

Furthermore, in comparing the dose-dependent suppression of blastogenesis and proliferation of activated T cells of IVIg versus the mAb TFL-006 (both supernatant and that purified from the ascites), while bearing in mind the concentration of the agents used, one may witness the superiority of TFL mAbs over IVIg (Table 3), For additional details on lesser performance by IVIg, see the detailed figures in [47].

2.4. TFL-007 and TFL-006 versus IVIg: Upregulation of CD4+ CD25+ Foxp^3 Tregs In Vitro. The CD4+ CD25+ Foxp^3+ regulatory T cells (Tregs) are not only found in circulation [30] but also present at the site of the allograft [33]. They suppress Ab production by downregulating B memory and plasma cells [32] and depleting CD4+ [27] and CD8+ [28, 29] T cells that play a major role in graft rejection [34]. IVIg is known to upregulate Tregs [39]. The ability of anti-HLA-mAbs TFL-006 and TFL-007 to induce proliferation of CD4+CD25+Foxp^3+ Tregs obtained from normal and healthy donors was assessed (M. Taniguchi and M. H. Ravindranath, manuscript in preparation). In this study, we have compared the impact of IVIg with polyreactive anti-HLA-E mAb TFL-007 on untreated and PHA-treated isolated fractions of CD3+/CD4+ human T lymphocytes. A variety of cell surface markers, which include CD4, CD25 (IL-2Rα), CD45RA, and Foxp^3, were monitored using their respective monoclonal Abs. To illustrate this proof of principle, the effect of different commercial preparations of IVIg (Figure 9(a)) and anti-HLA-E mAb TFL-007 (Figure 9(b)) were studied (in triplicate) on the untreated T-regulatory cells (CD4+/CD25+/Foxp^3+) obtained from a healthy volunteer (TFL2). The mAb purified from ascites was used throughout. Figures 9(a) and 9(b) illustrate that the different commercial preparations of therapeutic IVIg at two different dilutions (1/10 and 1/80) failed to upregulate the Tregs, while mAb TFL-007a showed a significant increase in the number of cells compared to the controls. Further elaboration of the experiments will be available in a manuscript to be submitted shortly.

3. Discussion

3.1. The Enigma of IVIg: Problems and Solutions

3.1.1. Clinical Applications of IVIg. IVIg has been used for the treatment of several autoimmune ($n = 6$) and hematological diseases ($n = 18$), neuropathies ($n = 14$), cardiomyopathies, nephropathies ($n = 4$) including acute renal failure (ARF), congenital heart block, and eye and ear diseases, asthma and cystic fibrosis, recurrent pregnancy loss, diabetes mellitus, burns, chronic fatigue syndrome, and other syndromes, such as Rasmussen, Reiter, and Vogt-

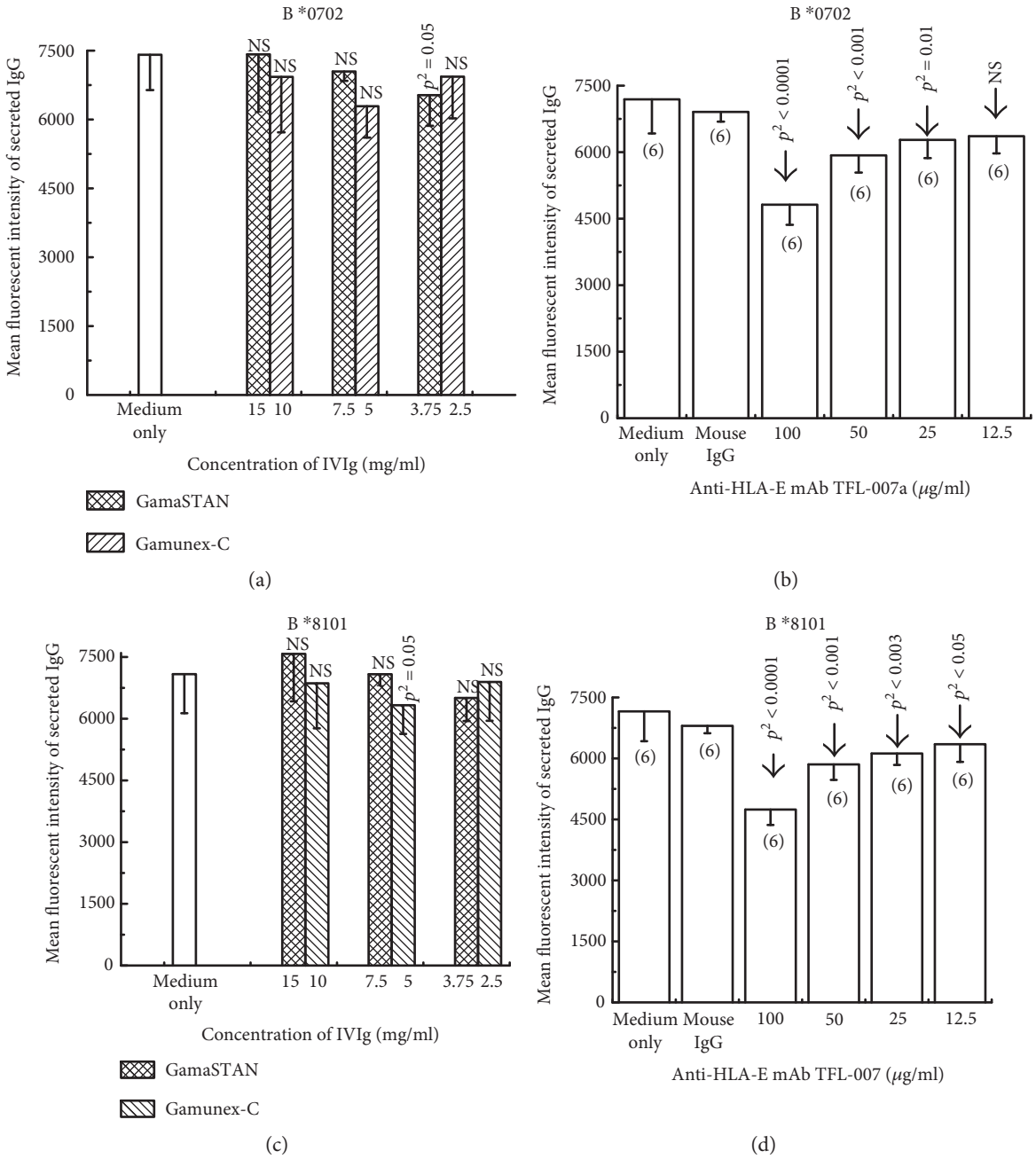

Figure 4: The human hybridoma cell line HML16 was generated from the resting B cells by EBV transformation; then the clone was fused with the murine, nonproducing myeloma cell line P3X63-Ag8.653 (ATCC® CRL 1580™). HML16 produced high MFI allo-Abs against B*0702, B*8101, B*6701, and B*4201 and low MFI allo-Abs against B*2708, B*2705, B*5501, B*5601, and B*8201. Figures are restricted to two of allo-HLA-I Abs, namely, B*0702 (a and b) and B*8101 (c and d). HML16 cells (cultured in RPMI-1640 + 20% heat-inactivated fetal bovine serum + 1 mM sodium pyruvate + L-glutamine-pen-strep solution + 50 μM 2-ME) were seeded at 1000/100 μl/well in a Falcon 96-well flat-plate and divided into 3 treatment groups: medium control, mouse IgG control (100 and 50 μg/ml), and TFL-007a. Medium control was compared with treatment by IVIg preparations (GamaSTAN and Gamunex-C; three subgroups for each), or four subgroups for TFL-007a were established for different doses except medium control. Six or more repetitions were performed with each subgroup (sample size is shown in (b) and (d)). The three subgroups of GamaSTAN-IVIg were at dilutions 1/10 (15 mg/ml), 1/20 (7.5 mg/ml), and 1/40 (3.75 mg/ml); the subgroups of Gamunex-C were at 1/10 (10 mg/ml), 1/20 (5 mg/ml), and 1/40 (2.5 mg/ml); and those of mAb TFL-007a were at 1/10 (100 μg/ml), 1/20 (50 μg/ml), 1/40 (25 μg/ml), and 1/80 (12.5 μg/ml). Twenty μl of culture supernatant from each well was analyzed for allo-HLA Abs at hours 0 and 72. The anti-HLA-E mAb TFL-007a (stock 627 μg/ml) at different concentrations (62.7, 32.35, 16.17, and 8.9 μg/ml) but not IVIg preparations (GamaStan (15, 7.5, and 3.75 mg/ml) and Gammunex (10, 5 and 2.5 mg/ml)) inhibited the production of anti-HLA Abs against B*0702 and B*8102 produced by EBV-immortalized B cell line, HML-16. Mean and SD of the hexaplicate samples are presented. The paired sample two-tailed *t*-test was carried out. Two-tailed *p* values are provided in the figure [49].

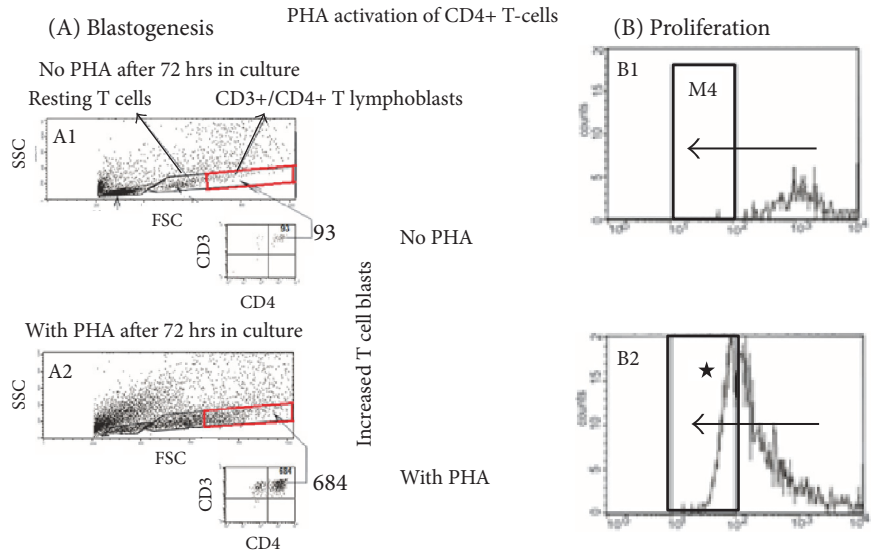

Figure 5: Phytohemagglutin (PHA) mediated activation of CD3+/CD4+ T lymphocytes. PHA induces blastogenesis and proliferation of the T cells. Experiments were done in triplicate (see [47]). Blastogenesis of was determined by counting the lymphoblasts, after culturing purified lymphocytes with or (as control) without PHA for 72 h. Proliferation was monitored by labelling the purified lymphocytes with the intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). After 72 h, the labelling of the cells was measured: PHA-treated T cells undergo four to six divisions. Using flow cytometry, the mitotic activity is measured by the successive twofold reductions in fluorescent intensity of the T cells placed in culture for 72 h. Experiments were done in triplicate [48]. In the no PHA box, the number of cells in the M4 column is highly negligible. The box "with PHA" shows increase in the cell numbers in M4 column (shown with ★).

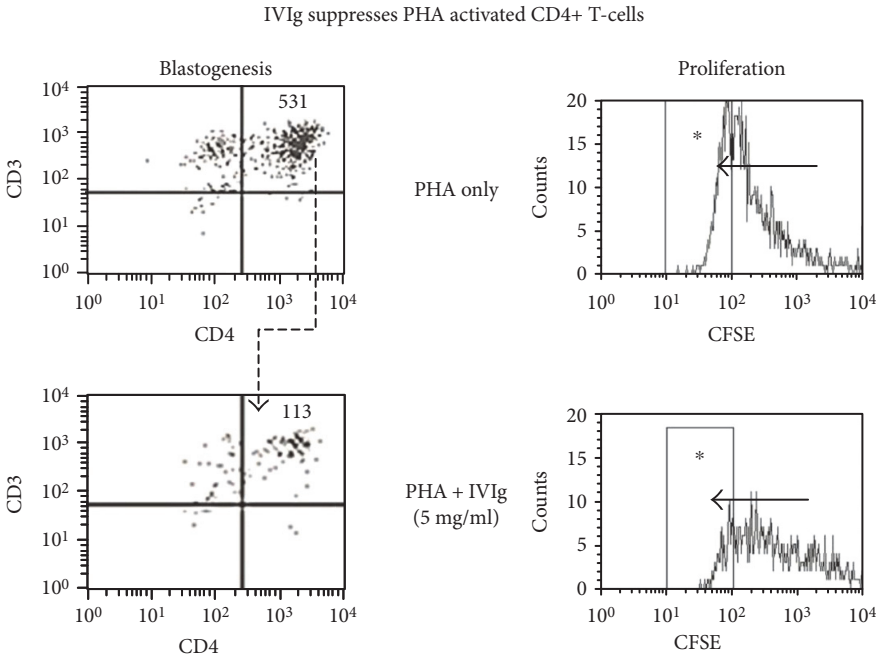

Figure 6: IVIg (Glob EX, VHB Life Sciences Limited, Mumbai, India) at a concentration of 5 mg/ml (at dilution 1/10) suppressed blastogenesis and proliferation of the PHA activated CD3+/CD4+ T lymphocytes. Experiments were done in triplicate (see [47]). When the cells divide, the CFSE is passed on to the progeny, as indicated in the upper box by the migration of staining from right to left (marked as ∗ in a rectangular area) at every sequential mitosis, with the number of cell divisions (mitosis 4 (M4)) determining the distance moved. Addition of IVIg (5 mg/ml) to wells with PHA suppressed the proliferation as indicated in the lower box within the rectangular area (marked as ∗). For details, see [47].

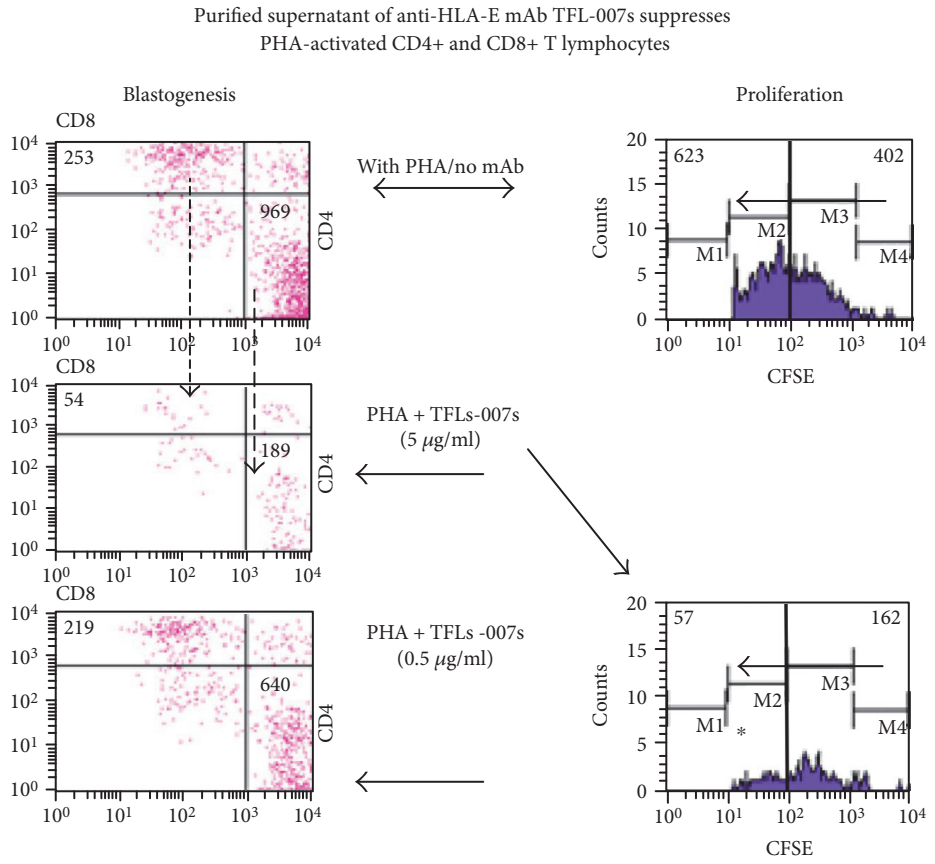

Figure 7: Dose-dependent inhibition of PHA-activated CD4+/CD8+ T cells in vitro with anti-HLA-E mAbs, TFL-007s ("s" for culture supernatants). The T cells were stained with PE-labeled anti-CD4 mAbs (*x*-axis) and PerCP-labeled anti-CD8 mAbs (*y*-axis). The profile is divided into three groups (only group 3 is represented in the figure; for details, see [47]) based on staining and size of cells to illustrate the differences in the CD4+ and CD8+ T cell populations and number of events. Group 1 comprises resting CD4+ and CD8+ lymphocytes, group 2 resting CD4+ and CD8+ lymphocytes, and group 3 CD4+ and CD8+ lymphoblasts. Flow cytometric profiles of PHA-treated CD4+ T cells (lower right of the boxes) and CD8+ T cells (upper left) from a normal non-alloimmunized donor (R) after treatment with mAb TFL-007s. The top row (treated only with PHA) shows the number of CD4+ T cells and the CD8+ T cells. The middle row (PHA and mAb TFL-007s at 1/10 dilution or 5 μg/ml) shows the number of both CD4+ ($p^2 < 0.001$) and CD8+ ($p^2 < 0.002$) T cells have decreased significantly. In comparison, the bottom row with the same treatment, but at 1/100 dilution (0.5 μg/ml), showed a dose-dependent decrease in the number of PHA-activated CD4+ ($p^2 < 0.004$) and CD8+ (not significant) T lymphocytes. Each block of figure represents one of the triplicate analyses (for further details, see [47]).

Koyanagi-Harada syndromes, and several viral infections including HIV [55]. IVIg is administered at a high dose (generally 1-2 gms per kg body weight) to decrease the severity of the immune response in patients with autoimmune diseases.

In spite of the extensive use of IVIg, the US Food and Drug Administration (FDA) has cautiously approved the use of IVIg for (1) Kawasaki disease, (2) immune-mediated thrombocytopenia, (3) primary immunodeficiencies, (4) hematopoietic stem cell transplantation (for those older than 20 yrs), (5) chronic B cell lymphocytic leukemia, and (6) pediatric HIV type 1 infection. Since 2004, the Canadian Blood Services and Canada's National Advisory committee on blood and blood products initiated and developed guidelines for the use of IVIg for sensitized patients who undergone solid organ transplantation [56]. In 2004, the US Medicare approved the Cedars-Sinai Hospital (Los Angeles, CA) IVIg protocol to minimize HLA Abs in patients waiting for donor kidneys so that such recipients could accept a living or deceased donor kidney.

3.1.2. Major Concerns about the Use of IVIg for Transplant Patients. The main concern for the cautious approval by FDA is mainly due to serious adverse side effects that occurred after infusion IVIg, such as anaphylactic shock, renal insufficiency, Steven-Johnson syndrome, aseptic meningitis, thromboembolic events, thrombosis, cytopenia, hemolysis, stroke, seizure, loss of consciousness, acute respiratory distress syndrome, pulmonary edema, acute bronchospasm, transfusion-associated lung injury, aseptic meningitis, delayed hemolytic reaction, acute myocardial infarction, and even acute renal failure [55, 56].

Thrombotic complications associated with the use of IVIg have been reported in twenty-nine cases including acute myocardial infarction, cerebral infarction, pulmonary

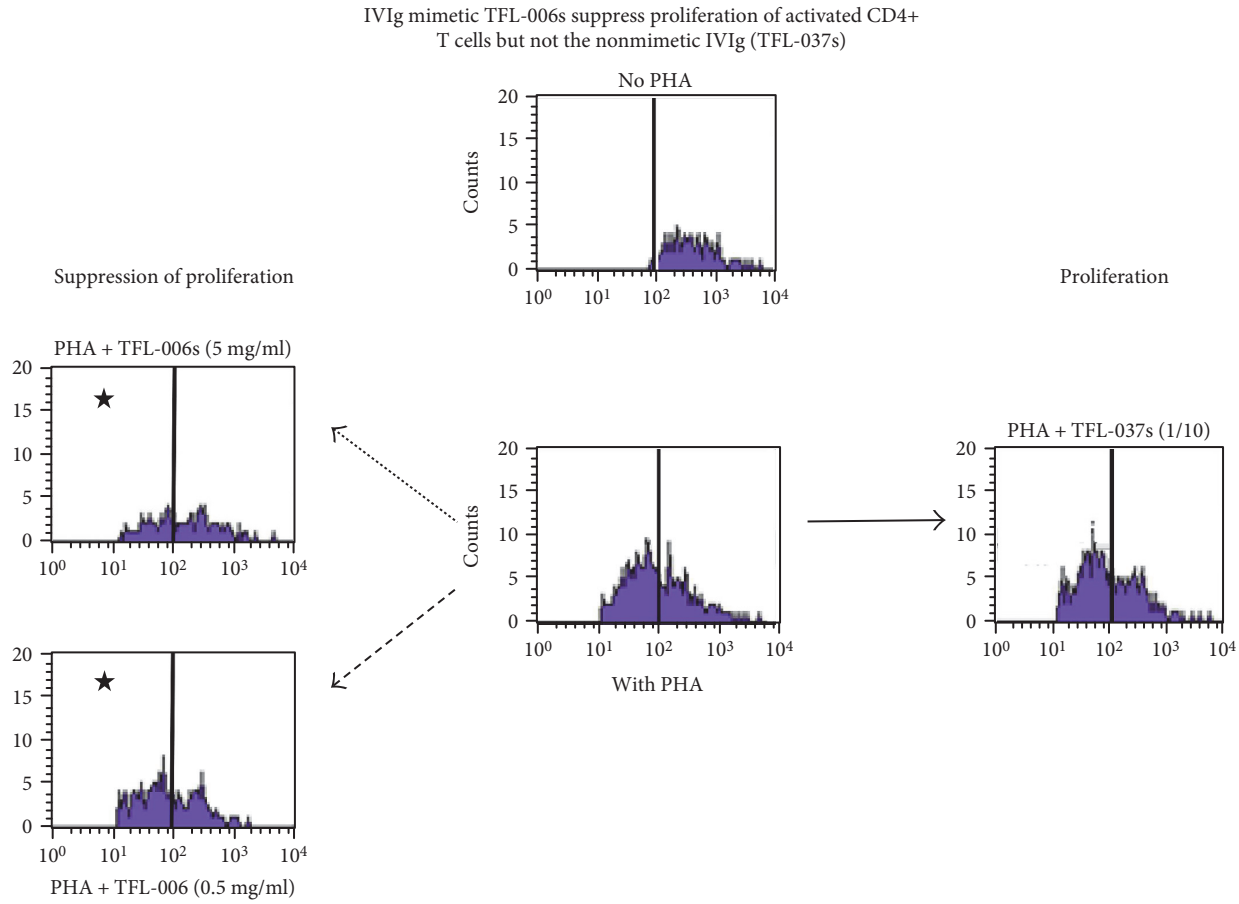

Figure 8: Suppression of proliferation of the PHA-activated CD4+ T cells by purified culture supernatant of anti-HLA-E mAb TFL-006s (at 5 μg/ml and at 0.5 μg/ml) but not by the control mAb TFL-037 (at 5 μg/ml) (for details, see [47]).

Table 2: The blastogenesis of PHA-activated CD4+ T cells was inhibited by anti-HLA-E mAbs TFL-006 and TFL-007 but not by negative control mAbs or by HLA-Ia mAbs or by nonpolyreactive HLA-E mAb TFL-037. Protein concentrations of culture supernatants are not given as they contain several exogenous and endogenous proteins. p^2 refers to two-tailed p value (for further details see [47]).

Negative control mAbs	Mean	SD	p^2
No PHA	264	14	
With PHA	969	117	0.0005*
PHA + Hu IgG1 control	1758	84	NS**
PHA + mAb 2124 (anti-HLA-A11/A43)	1758	84	NS**
PHA + mAb 9123 (anti-HLA-Ia+)	1435	276	NS**
Experimental anti-HLA-E mAbs			
PHA + mAb TFL-006 (1/100) 8.870 mg/ml	502	184	0.02**
PHA + mAb TFL-007 (1/100) 6.270 mg/ml	640	137	0.03**
PHA + mAb TFL-037 (1/100) 6.000 mg/ml	911	54	NS**

p^2 refers to 2-tailed p value. *The p value for "with PHA" is against "no PHA";**the other p values are against "PHA only."

embolism, deep venous thrombosis, hepatic veno-occlusive disease, and spinal cord ischemia. For renal toxicity alone, there are 32 reports published involving 78 patients in whom toxicity developed in association with IVIG treatment [58]. From June 1985 to November 1996, the FDA received 120 reports worldwide, 88 from the United States [59]. Between 1992 and 1998, 49 cases of ARF were reported to the French Regional Pharmacovigilance Center with marked creatinine increase after the initiation of IVIg therapy [60]. Transient renal failure mainly occurs when using sucrose-containing IVIg, owing to osmotic injury [61]. Specific adverse side effects were attributed to differences in osmolality, pH, and sugar and sodium content of the IVIg products.

Some of the adverse effects are attributed to HLA-II Abs in IVIg. There are several reports of occurrence of transfusion-related acute lung injury (TRALI) with one death after IVIG administration [62–67]. The mechanism underlying induction of TRALI by IVIg has been an enigma. Anti-HLA-II IgG observed in patients after plasma transfusion is implicated in TRALI [68]. The mechanism underlying the lung injury by IVIg has been clarified in humans [68]. The anti-HLA-II IgG binding to monocytes in patients with TRALI may induce the activation of neutrophils that may penetrate the endothelium of lungs, causing destruction of the endothelial cells [69]. Since the presence of HLA-II Abs in alloimmunized females led to the prevention of using blood from females for transfusion, avoidance has

Table 3: Comparison of the dose-dependent suppression of the blastogenesis of PHA activated CD4+/CD8− and CD4−/CD8+ T cells between anti-HLA-E mAb TFL-006 (supernatants (s) and purified ascites (a)) and IVIg, documenting the increased suppressive potential of TFL-mAb compared to that of an IVIg preparation. Protein concentrations of culture supernatants are not given as they contain several exogenous and endogenous proteins. p^2 refers to two-tailed p value (for further details, see [47]).

Treatments		Blastogenesis					
		CD3+/CD4+			CD3+/CD8+		
	Concentration	Mean	SD	p^2	Mean	SD	p^2
TFL-006 (IgG2a) culture supernatant							
No PHA		192	14		78	11	
PHA alone		1190	91	0.002	364	59	0.01*
PHA + murine IgG [1/100]		1033	92	NS	332	64	NS**
PHA + TFL-006s [1/10]	5.000 μg/ml	231	59	3*E*−04	70	25	0.007**
PHA + TFL-006s [1/20]	2.500 μg/ml	320	79	0.003	117	32	0.008**
PHA + TFL-006s [1/40]	1.250 μg/ml	575	63	0.004	204	20	0.03**
PHA + TFL-006s [1/80]	0.625 μg/ml	894	73	0.02	298	26	NS**
PHA + TFL-006s [1/160]	0.313 μg/ml	904	91	0.02	275	29	NS**
TFL-007 (IgG2a) ascite supernatant							
No PHA		190	3		70	3	
PHA alone		1243	106	0.003	403	31	0.003*
PHA + murine IgG [1/100]		1330	166	NS	422	37	NS**
PHA + TFL-006a [1/100]	8.870 μg/ml	478	193	0.008	176	75	0.02**
PHA + TFL-006a [1/200]	4.435 μg/ml	568	173	0.008	191	72	0.02**
PHA + TFL-006a [1/400]	2.218 μg/ml	588	195	0.01	207	67	0.02**
PHA + TFL-006a [1/800]	1.109 μg/ml	786	127	0.009	248	16	0.005**
PHA + TFL-006a [1/1600]	0.555 μg/ml	1499	158	NS	477	52	NS**
IVIg octagam (6 gm%) lot A913A6431							
No PHA		46	8		53	13	
PHA alone		1685	89	<0.0001	1951	171	<0.0001*
PHA + IVIg (1/10)	6.0 mg/ml	945	87	5*E*−04	1134	13	0.001**
PHA + IVIg (1/20)	3.0 mg/ml	1365	100	0.019	1717	198	NS**
PHA + IVIg (1/40)	1.5 mg/ml	1796	81	NS	2280	127	NS**

p^2 refers to 2-tailed p value; *the p value for "PHA alone versus no PHA"; **the other p values are against PHA alone.

become routine as a preventive measure against TRALI in several countries [62, 70]. It was reported that "this policy did indeed significantly reduce the incidence of TRALI both in large-scale surveillance studies and haemovigilance reports" [70].

All these observational studies warn against using IVIg, particularly high-dose IVIg, for patients waiting for donor organs and such use of IVIg should be preceded by titer tests of HLA Abs, because there is a distinct possibility that one of the active agents in IVIg is actually the anti-HLA Ab itself. Consequently, a balancing of the danger of TRALI must be carefully considered, and the effective action of the HLA Ab must be monitored when using IVIg. Therefore, failure of functional recovery by transplanted organs or their hyperacute rejection could be a consequence of IVIg used for desensitization.

The lead clinical investigators [20–25] used high-dose IVIg for desensitization therapy in patients waiting for organ donors or for lowering antiallograft Abs posttransplantation, changed from monotherapy of IVIg to combinatorial therapy with IVIg. There could be many reported and unreported reasons for the shift, but it could also be due to the increase of the desensitization efficiency, reduction of desensitization period (3 months to 1 month), and the cost. The combinational therapies involving IVIg is further improved for graft survival by expanding the use of IVIgs with anti-IL-6R Ab tocilizumab [71] and also with an IgG-degrading bacterial enzyme IdeS (IgG endopeptidase) [72].

In spite of these reports, others reported that IVIg failed to lower the mean percentage of pretransplant HLA Abs observed before IVIg infusion (85% before, 80% after IVIg administration) [73]. Paradoxically, in another patient cohort, an increase in the level of anti-HLA-I Abs was observed after IVIg treatment in 27% of the patients [74]. The investigators further validated that the calculated PRA did not reveal any significant changes in response to IVIg therapy. Most importantly, Marfo et al. [75] showed that IVIG together with rituximab treatment failed to reduce PRA levels or the mean fluorescent intensity of HLA Abs as measured in Luminex single-antigen bead assays in the patients.

3.1.3. The Enigma of the Immunomodulatory Effects of IVIg. IVIg contains polyreactive natural Abs, which include IgG

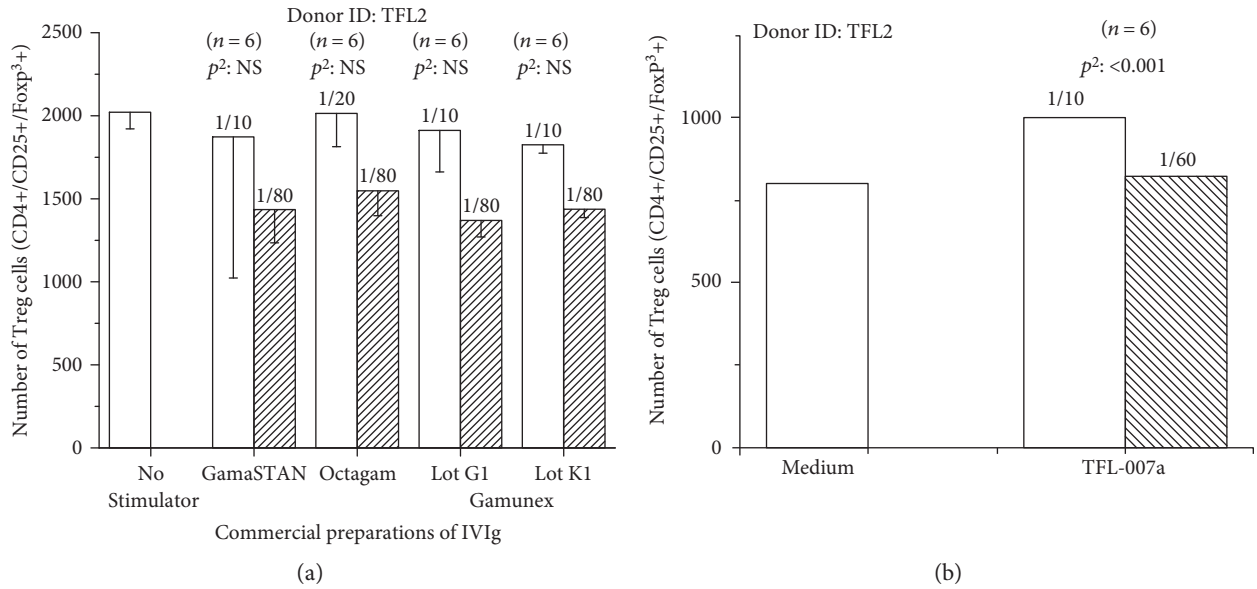

Figure 9: Effects of different commercial preparations of IVIg which include GamaSTAN™ S/D (15–18 gm%, lot 26NHCVI; Telacris Biotherapeutics Inc) at dilutions 1/10 (conc. 15 mg/ml) and 1/80 (conc. 1.2 mg/ml), Octagam® (6 gm%, lot A913A8431; Octapharma Pharmazeutika) at dilutions 1/20 (conc. 3 mg/ml) and 1/80 (conc. 0.75 mg/ml), and Gamunex®-C (10 gm%, lots 26NKLG1 and 26NKLK1, Telacris) at dilutions 1/10 (conc. 10 mg/ml) and 1/80 (conc. 8 mg/ml) (a) and mAb TFL-007a (at dilution 1/10, conc. 62.7 μg/ml; 1/80, conc. 7.84 μg/ml) (b) on PHA-untreated cells were compared with the effect of medium alone on the proliferation of Treg cells, defined as CD4+/CD25+/Foxp^3+. Note that IVIg preparations used in this study failed to upregulate Tregs in contrast to TFL-007a which significantly upregulates Treg cells. p^2 (two-tailed p value) (M. Taniguchi and M. H. Ravindranath, manuscript in preparation).

against endogenous and exogenous Abs, immunomodulating peptides, all the blood group antigens, and various cytokines. While few of the immunoregulatory mechanisms of action of IVIg have been proven, many proposed mechanisms still remain an enigma, due to polyreactivity, polyclonality, and diversity in the preparations of IVIg. Sapir and Shoenfeld [76] enlist these mechanisms as follows: (a) Fc-receptor blockade; (b) neutralization of pathogenic autoAbs via idiotypic and anti-idiotypic Abs; (c) effects on the Fas apoptotic pathway via agonistic and antagonistic anti-Fas autoAbs; (d) regulation of complement components; (e) modulation of cytokine secretion; (f) hindrance of natural-killer cell activity; (g) inhibition of matrix metalloproteinase-9; (h) suppression of NFkB activation and IkB degradation; (i) G1 cell cycle arrest; (j) prevention of tumor growth; (k) decrease in leukocyte recruitment; (l) attenuation of T cell stimulation; (m) effects on Ab kinetics; and (n) effects on dendritic cells. It is believed that various mechanisms of IVIg cooperate in a synergistic way.

We have reported earlier [47] that both blastogenesis and proliferation of activated T cells induce transitory expression of more than ten molecules enlisted earlier [47], which include IL-2R, Fc receptors for IgG, receptors of insulin, insulin-like growth factor, αfetoprotein, and transferin receptors, MICA, HLA-II, and β2-microglobulin-free heavy chain (HC) of HLA-I. It is often discussed that IVIg binds to the Fc-receptor for immunoregulation. Specifically, the inhibition of blastogenesis and proliferation of activated T and B cells by IVIg is attributed to Fc receptors for IgG (FcγRI (CD23), FcγRII (CD32), FcγRIII (CD16), and FcεR1 (CD64)) expressed on the immune cells upon activation [77]. It is far from clear as to how all of the four subclasses of IgG present in IVIg can simultaneously block the following Fc-receptors: FcγRI CD23, FcγRII/CD32, FcγRIII/CD16, and FcεR1/CD64, upregulated upon the activation of T and B cells. Paradoxically, another report [78] documented clearly that the inhibition of Ab production by B cells in vitro is brought about by F(ab')2 fragments of Abs but not by the Fc portion. The primary enigma revolves around the mechanism of action of IVIg, namely, its polyclonality that prevents specific recognition of the modus operandi of IVIg in lowering or depleting the Ab-producing B cells or the antigen-presenting T cells or the upregulation of Tregs.

Furthermore, it is highly paradoxical that IVIg is used to lower HLA Abs despite different formulations of IVIg per se which contains IgG Abs reacting to HLA-I (HLA-A, HLA-B, HLA-Cw, HLA-E, HLA-F, and HLA-G) [46] and HLA-II (DRB, DQA/DQB, and DPA/DPB) [79, 80]. Although most of the manufacturers have made efforts to prepare IVIg devoid of sucrose, which was considered as a major cause of adverse reactions, the aforementioned findings emphasize that the pharmaceutical manufacturers should document the level of HLA-II Abs as well as the levels of the soluble forms of HLA-Ia, HLA-Ib, and HLA-II antigens present in their therapeutic preparations of IVIg, particularly when they recommend IVIg for lowering HLA Abs pre- and posttransplantation.

In spite of these adverse effects and conflicting reports, the demand for therapeutic IVIg has steadily increased each year since 1992, which has resulted in product shortages and increased market prices [81]. However, there are intrinsic limitations with respect to the conventional production of

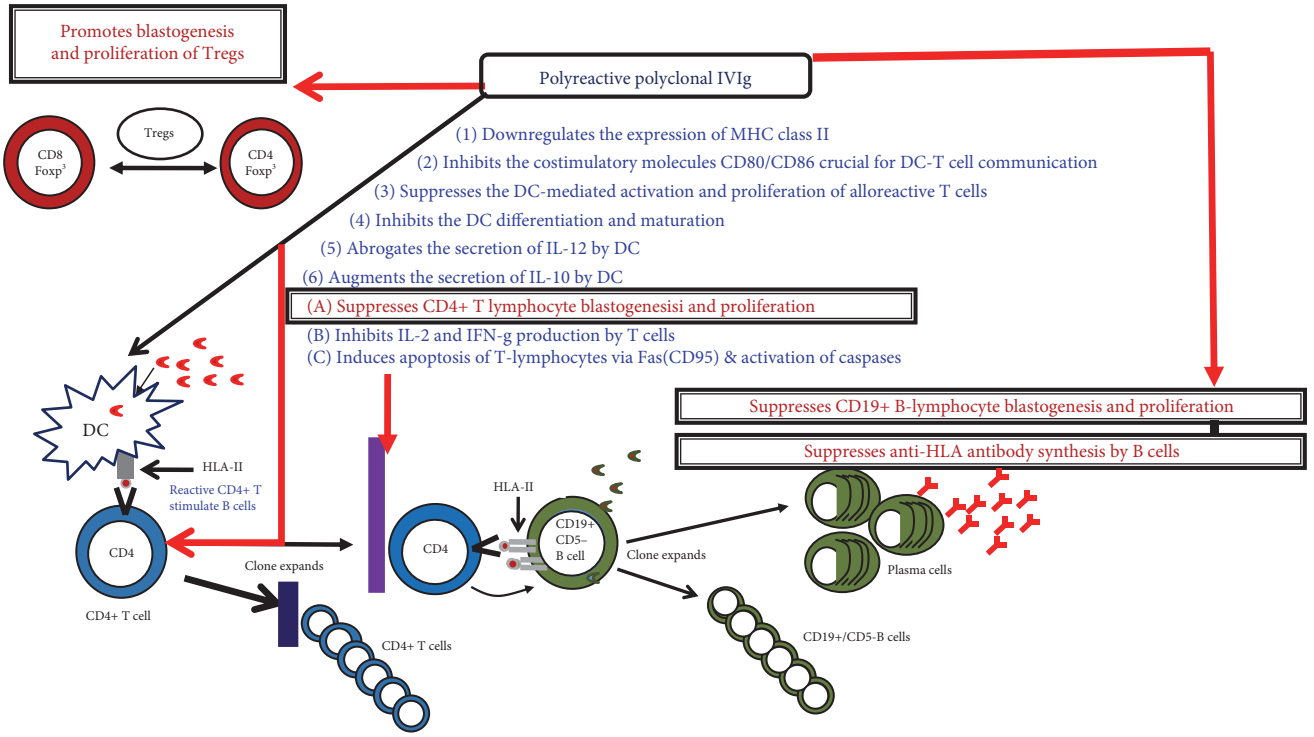

Figure 10: Different immunoregulatory roles attributed for the polyreactive polyclonal IVIg and proven functions of anti-HLA-E IgG2a monoclonal Abs. We have compared three functions of IVIg with that of anti-HLA-E IgG2a monoclonal Abs. We have monitored (1) suppression of CD19+ B lymphocyte blastogenesis, proliferation, and anti-HLA-I and anti-HLA-II IgG Abs, (2) suppression of blastogenesis and proliferation of CD4+ as well as CD8+ T lymphocytes, and (3) upregulation of blastogenesis and proliferation of Tregs. Our results indicate that the performance of anti-HLA-E mAbs TFL-006 and TFL-007 (the functions are recorded in red) is far superior to IVIg in achieving these functions [47, 48].

therapeutic IVIgs. The quantities of human plasma that can be collected from donors are limited. The only cost-effective, evidence-based immunoreactive and immunomodulatory strategy is to substitute IVIg with IVIg mimetics. The development of such IVIg substitutes or mimetics would stabilize and even reduce the use of donor plasma-derived IVIg, thereby securing such IVIg supplies for the most restricted and life-threatening immunodeficiency diseases.

3.2. Monoclonal Anti-HLA-E IgG2 as IVIg Mimetics. A reasonable alternative could be a monoclonal Ab that dosimetrically mimics one or more of the immunoregulatory mechanisms of IVIg (Figure 10) underlying suppression of antibody production but at the same time superior in its performance. This study evaluated and compared the immunomodulatory efficacy of HLA-I polyreactive anti-HLA-E monoclonal Abs TFL-007a and TFL-006a with that of a similarly polyreactive mixture of polyclonal Abs pooled from thousands of donor blood and purified as IVIg. Indeed, anti-HLA-E mAbs TFL-006 and TFL-007 are capable of performing three of the major immunoregulatory functions far more than IVIg. The three functions defined in this report are as follows:

(1) Suppression of CD19+ B lymphocyte blastogenesis, proliferation, and suppression of anti-HLA-I and anti-HLA-II IgG Abs

(2) Suppression of blastogenesis and proliferation of CD4+ as well as CD8+ T lymphocytes

(3) Expansion of CD4+, CD25+, and Foxp^3+ Tregs.

It is this report and earlier publications that document the experimental details that show TFL-006 and TFL-007, the IgG2 mAbs [45–48], bind to the surface antigens (HLA-Ia and HLA-Ib) on CD4+ T cells, CD8+ T cells, and CD19+ B cells to bring about the observed effects. The mAbs may bind to CD4+ T cells which are also positive for CD25 and Foxp^3 and perform a function quite opposite to what it does, when it binds to other activated CD4+ T cells, which are CD25 negative and Foxp^3 negative. The fundamental question is how does the mAbs distinguish CD4+/CD25− T cells from CD4+/CD25+/FoxP^3 cells to perform the opposite function. Can there be two different receptors or mechanisms of action for the mAbs?

We have discussed earlier as to how the mAbs TFL-006/TFL-007 may bind to HLA-E present on activated T and B cells [47, 48]. Of various molecules upregulated upon activation of T and B cells [47], two are probable targets for mAbs TFL-006 and TFL-007. They are

(1) expression of FcγReceptor IIa (CD32) [82],

(2) overexpression of heavy chain (HC) HLA-I molecules without β2-microglobulin called "open conformers" [83, 84].

The mAbs TFL-006 and TFL-007 are IgG2a, and at present, we are not certain whether receptors for IgG2a, namely, FcγRIIa (CD32), are specifically upregulated in the human T and B cells we have examined. It is known that IgG2a binds specifically to FcγRIIa (histidine at position 131), whereas IgG2a may bind to FcγRIIb in conjunction with FcγRI [85].

Alternately, the mAbs TFL-006 and TFL-007 may recognize a shared epitope on all HLA-I molecules. A typical structure of the intact HLA-I molecule consists of HC with β2-microglobulin. The shared epitope is masked by β2-microglobulin on HC of HLA-Ia and HLA-Ib and hence considered cryptic. Most interestingly, the HLA-I molecules are expressed uniquely as β2-microglobulin-free HLA class I α-chains on activated T and B cells [85–88], and furthermore, it is already known that β2-microglobulin-free HLA class I HC on activated T cells can serve as ligands for leukocyte receptors [89]. The HC contains three helical structures called α1, α2, and α3 helices. The α1 domain is most susceptible to structural changes, as the α2- and α3 domains contain disulfide bonds. Therefore, one may consider the α1 domain as a wobbling helical domain or simply a wobbler. The wobbling of the α1 domain may enable binding of the mAbs TFL-006/TFL-007 to their epitope on HLA-E that is shared with all other HLA-Ia molecules. The mAbs TFL-006/TFL-007 were raised by immunizing the folded β2m-free HLA-E^{R107}. The epitope of TFL-006 is shared by HLA-A/HLA-B/HLA-Cw/HLA-E/HLA-F and HLA-G, and the location of the sequence (shown in figures [90]) is cryptic in the β2-microglobulin-associated HC since β2-microglobulin masks the epitope. The epitope of the mAbs TFL-006 and TFL-007 is identified by inhibiting their binding to HLA-E-coated beads by the most common and accessible shared peptide sequences located on the α2 domain of HC of HLA-E which include ^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135} [47]. The ability of TFL-006 and TFL-007 to bind to regular HLA beads or acid-treated beads but not to iBeads, which are coated with β2-microglobulin-associated HCs of HLA-Ia, confirms that the epitope affinity of the mAbs TFL-006 and TFL-007 is β2-microglobulin-free HC of HLA-Ia. In view of its unique property of TFL-006 in recognizing β2-microglobulin-free HC of HLA-Ia, the works of Jucaud et al. consider it as a potential diagnostic tool to distinguish β2-microglobulin-associated HC of HLA-Ia from β2-microglobulin-free HC of HLA-Ia coated on Luminex single-antigen beads (One Lamda Inc/Thermofisher Inc) extensively used in monitoring HLA Abs in patients waiting for donor organs and those who underwent transplantation [91, 92]. Furthermore, the suppression of blastogenesis and proliferation by mAbs TFL-006 and TFL-007 but not by mAbs TFL-033 (IgG1) and TFL-037 (IgG2b), which do not bind to shared peptide sequences of the open conformers of HLA-I, further confirmed that the mAbs TFL-006 and TFL-007 bind to the epitopes exposed on the open conformers of HLA-E as well as other HLA-I molecules.

Furthermore, the above inferences are well supported by the fact that the HLA-I open conformers have extended cytoplasmic tails with the exposure of on otherwise cryptic tyrosyl residue at position 320 [93, 94] and serine at position 335, the sites for phosphorylation [95, 96]. Such elongation of the coiled cytoplasmic tail as in intact β2-microglobulin-associated HC may facilitate the wobbling of the α1 domain of HC and expose the HLA-I common and shared epitope for binding by TFL-006 and TFL-007. Therefore, it appears that the binding of TFL-006 or TFL-007 to the shared epitopes on the α1 domain on the open conformer may be involved in tyrosine and/or serine phosphorylation, which may lead to signal transduction to arrest proliferation and blastogenesis of T and B cells. At the same time, it may be involved in upregulation, depending on whether Tyrosine^{320} or Serine^{335} is involved. The TFL-006-/TFL-007-mediated phosphorylation of the cytoplasmic tails can induce dephosophorylation of T and B cells by activating phosphates, leading to the arrest of transcription factors and synthesis of proteins associated with proliferation. The reverse phenomenon may occur with CD4+/CD25+/Foxp^3+ Tregs. This may involve any or all HLA-I molecules or specifically HLA-E expressed on these activated T cells [97]. However, at present, simultaneous binding of mAb TFL-006 or TFL-007 to FcγRIIa (CD32) and β2-microglobulin-free HC of HLA-I (open conformer) or binding the F(ab') of mAb to the open conformers after Fc of mAb binding to FcγRIIa (CD32) cannot be ruled out. Such differential binding may account for the multifunctional capabilities of mAbs TFL-006 and TFL-007.

4. Conclusion

The observations reviewed in this report conclusively document that the HLA-I polyreactive anti-HLA-E IgG2a monoclonal Abs (TFL-006 and TFL-007) mimic not only the HLA-I reactivity of IVIg but also some of the critical functions such as (1) suppression of blastogenesis and proliferation of CD4+ T cells and CD8+ T cells, (2) effective inhibition of the production of anti-HLA-I and HLA-II Abs (such as "donor specific abs" formed against HLA-mismatched allografts and allo-HLA antibodies in transplant patients developed while waiting for a donor organ), and at the same time, (3) the upregulation of Tregs, which by themselves are capable of suppressing CD4+ T cells and CD8+ T cells and antibody production by B cells. Table 4 compares the unique features of the mAbs TFL-006 and TFL-007 with that of IVIg. The data presented in this review, as well as those presented earlier [45–48], indicate that the performance of the mAbs are indeed superior to IVIg, particularly in the following aspects:

(1) Unlike IVIg, the anti-HLA-E IgG2a mAb TFL-007 prevented anti-HLA Ab production by activated B cells. It is highly possible that TFL-007 can also suppress other antiallograft Abs produced by B cells.

(2) Both anti-HLA-E IgG2a mAbs TFL-006 and TFL-007 are capable of suppressing antispecific activated T cells but have a dosimetrically superior performance over suppression by IVIg. Such a therapeutic

Table 4: Comparison of the unique features of the mAbs TFL-006 and TFL-007 with that of IVIg.

Source, nature, and functions	Intravenous immunoglobulin (IVIg)	TFL-006 and TFL-007
Manufacturer	Several pharmaceutical firms	Terasaki Foundation Laboratory
Source	Purified from pooled plasma of 10,000 blood donors from humans in various countries	Immunized in mice with heavy chain of HLA-E^{R107} [45, 46]
		Murine (to be adapted for human use), not humanized
Nature of antibody	Human, polyclonal IgG with trace level of IgA	Murine, ascites purified monoclonal IgG
Subclass of IgG antibodies	IgG1, IgG2a, IgG3, IgG4	IgG2a only [45, 46]
Purity	Contains soluble HLA antigens and other non-IgG proteins, cytokines, and chemokines	100% purified protein of IgG2a [45, 46]
Antibody reactivity	HLA-A, HLA-B, HLA-Cw, HLA-E, HLA-F, HLA-G	HLA-A, HLA-B, HLA-Cw, HLA-E, HLA-F, HLA-G [45, 46]
	HLA-DR, HLA-DQA/DQB, HLA-DPA/DPB	None
	Fc-receptors: FcγI, FcγII, FcγIII, FcγIV (tested) [98]	FcgII (anticipated)
	Blood groups A, B, Rh [99–101]	Not applicable
	Escherichia coli bacterial antigens ranging from 94 to 238	Not applicable
	Antigens by different preparations of IVIg [102]	Not applicable
	Human albumin [46, 80]	Not applicable
	Phospholipids [100]	Not applicable
Stabilizer	Many	None
Protein concentration	Highly variable from 2 to 12%	Protein concentration can be adjusted to requirement
CD4+ T cell suppression	PHA or cytokine activated T cells [35, 36] by apoptosis [38]	PHA-activated T cells [47]
CD8+ T cell proliferation	By nonapoptosis (necrosis) [41], PHA-activated [36]	PHA-activated T cells [47]
B cell proliferation	Induce differentiation [42]	
	No effect on proliferation [43]	
Anti-HLA antibody suppression	PRA antibody reduction [44]	Reduction in the production of anti-HLA-I and anti-HLA-II IgG [48]
	Induce antibody secretion [42, 48]	
	Suppress selected HLA-II antibody production [48]	Suppress production of all HLA-II antibodies [48]
	Promote selected HLA-II antibody production [48]	
Expansion of Tregs	Promotes upregulation of Tregs [39]	Promotes upregulation of Tregs [M. Taniguchi and M. H. Ravindranath, manuscript in preparation]
Special application		to distinguish β2-microglobulin-associated HC of HLA-Ia from β2-microglobulin-free HC of HLA-Ia coated on Luminex single-antigen beads (One Lamda/Thermofisher Inc) [91, 92]

mAb is invaluable for preventing autoimmune diseases and lowering Abs in allograft recipients. A version of the mAb adapted for human use will fulfill the goal that is targeted by this review.

(3) Both anti-HLA-E IgG2a mAbs TFL-006 and TFL-007 have the unique potential to upregulate CD4+, CD25+, and $Foxp^3$+ T-regulatory cells, without any ambiguity, and have dosimetrically superior performance over therapeutic preparations of IVIg.

(4) Since the anti-HLA-E IgG2a mAbs TFL-006 and TFL-007 bind to β2-microglobulin-free heavy chains of HLA-I loci, it is an ideal diagnostic tool to monitor the contamination of β2-microglobulin-free HC of HLA-I loci in the Luminex HLA class I single-antigen beads; the presence of which can produce misleading results in monitoring transplant patients' Abs against intact HLA-I antigens (namely, β2-microglobulin-associated HC of HLA-I).

In view of the functional capabilities of both the anti-HLA-E IgG2a mAbs TFL-006 and TFL-007, there is a need to adapt both TFL-006 and TFL-007 for human use, in order to assess the therapeutic efficacy and potential of replacing IVIg for desensitization of organ transplant patients. Furthermore, these mAbs can be extended for other human

autoimmune diseases in which IVIg is used as a therapeutic agent.

Abbreviations

Abs: Antibodies
AT1R: Angiotensin receptor 1
DSA: Donor-specific antibodies
EBV: Epstein-Barr virus
FDA: Federal Drug Administration (US)
HC: Heavy chain
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigens
IL-6R: Interleukin-6 receptor
IVIg: Intravenous immunoglobulin
mAb: Monoclonal antibody
MFI: Mean fluorescent intensity
MHC: Major histocompatibility complex
NDSA: Nondonor-specific antibodies
PBMC: Peripheral blood mononuclear cells
PHA: Phytohemagglutinin
PRA: Panel reactive antibodies
TFL: Terasaki Foundation Laboratory
TRALI: Transfusion-related acute lung injury
Tregs: T-regulatory cells.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper. However, Terasaki Family Foundation, which funded the author's research projects through Terasaki Foundation Laboratory, a nonprofit organization, has US and European patents pending on the anti-HLA-E mAbs.

Acknowledgments

It is with immense pleasure and privilege that the author dedicates this review to his beloved mentor, the late Professor Paul Ichiro Terasaki, CEO and Director of Terasaki Foundation Laboratory and the proponent of the Humoral Theory of Transplantation, who passed away on January 25, 2016. The author would not have achieved the results or completed this study without the regular discussions, guidance, and constant encouragement and support of the late Professor Terasaki. He also wrote parts of the discussion in the original publications [46–49] and introduced to the author the adverse effects of IVIg, particularly about TRALI. All the HLA-Ia and HLA-Ib polyreactive TFL mAbs (TFL-006 and TFL-007) reported in this review as well as the monospecific mAb TFL-033 are under patent consideration (inventors: Professor Terasaki and the author) in US and Europe. The author thanks Dr. Mathew Everly, the current Director of Terasaki Foundation Laboratory, for encouraging his research presented in the review. His support is much appreciated. The author also wishes to thank Dr. Junchao Cai, his dear colleague at TFL, for the frequent discussions on the results, constant encouragement, and continued support for his research. The author wants to express his sincere thanks to Mrs. Judy Hopfield, the certified histocompatibility specialist who went through the manuscript line by line and edited it for its contents. Her outstanding and knowledgeable editing is much appreciated and a necessary prerequisite for TFL. The author also wants to express his special thanks to his endearing research associate Mr. Vadim Jucaud, another certified histocompatibility specialist, for his outstanding and constructive criticisms of the author's work and who assisted in all the experiments reported in the papers cited in this article.

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Review Article

HLA Epitopes: The Targets of Monoclonal and Alloantibodies Defined

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Sensitization to human leukocyte antigens (HLA) in organ transplant patients causes graft rejection, according to the humoral theory of transplantation. Sensitization is almost ubiquitous as anti-HLA antibodies are found in almost all sera of transplant recipients. Advances in testing assays and amino acid sequencing of HLA along with computer software contributed further to the understanding of antibody-antigen reactivity. It is commonly understood that antibodies bind to HLA antigens. With current knowledge of epitopes, it is more accurate to describe that antibodies bind to their target epitopes on the surface of HLA molecular chains. Epitopes are present on a single HLA (private epitope) or shared by multiple antigens (public epitope). The phenomenon of cross-reactivity in HLA testing, often explained as cross-reactive groups (CREGs) of antigens with antibody, can be clearly explained now by public epitopes. Since 2006, we defined and reported 194 HLA class I unique epitopes, including 56 cryptic epitopes on dissociated HLA class I heavy chains, 83 HLA class II epitopes, 60 epitopes on HLA-DRB1, 15 epitopes on HLA-DQB1, 3 epitopes on HLA-DQA1, 5 epitopes on HLA-DPB1, and 7 MICA epitopes. In this paper, we provide a summary of our findings.

1. Introduction

Sensitization to HLA antigens in organ transplant patients causes graft rejection, according to the humoral theory of transplantation [1]. Sensitization is almost ubiquitous as it is evident in the detection of anti-HLA antibodies in the sera of recipients—in one study, almost all patients waiting for regraft of a kidney transplant have anti-HLA antibodies [2]. Determining specificity of the anti-HLA antibody has advanced in recent years using recombinant HLA single antigens (SA) coated on color-coded Luminex beads [3]. The reactivity of anti-HLA antibodies with HLA antigens and the phenomenon of cross-reactivity has been the subject of investigation for decades. Amino acid sequences of the HLA molecules which greatly contributed to our understanding of antibody and antigen reactivity has been introduced since 1963 [4–12]. Antibodies are commonly described as binding to HLA antigens; however, it is more accurate to describe the reactivity of the antibody as binding to specific epitopes on the surface of HLA antigens—epitopes are conformational amino acid arrangements and are the targets of antibodies. Some epitopes are private, found exclusively on one antigen; others are public epitopes shared by two or more antigens. The phenomenon of cross-reactivity in HLA testing, often explained as cross-reactive groups (CREGs), of antigens with antibody can be clearly explained now by public epitopes—an antibody targeting a public epitope shows positive reaction with all antigens sharing the epitope.

Since 2006, we defined and reported on 194 HLA class I unique epitopes, including 138 epitopes on intact HLA class I (heavy chain + β2m + peptide), and 56 cryptic epitopes on dissociated HLA class I (heavy chain only) [13–18]. 110 epitopes on intact HLA class I were defined using murine monoclonal and human alloantibodies, and the remaining 28 epitopes were defined with naturally occurring anti-HLA antibodies. Naturally occurring (natural) HLA antibodies found in cord blood and healthy males were used to define the 56 cryptic epitopes on dissociated HLA class I. In addition, 83 HLA class II unique epitopes were defined and reported, including 60 epitopes on HLA-DRB1, 15 epitopes on HLA-DQB1, 3 epitopes on HLA-DQA1, and 5 epitopes

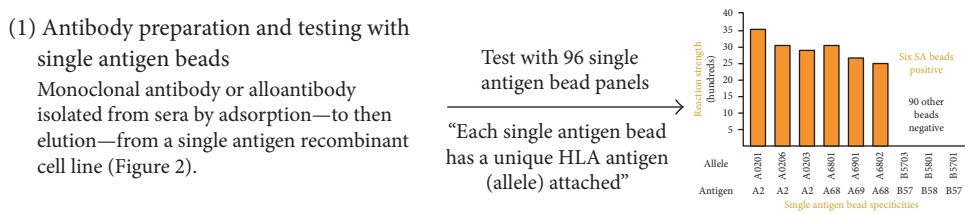


Figure 1: Main empirical testing steps to define HLA epitopes.

on HLA-DPB1 [15, 19–22]. All HLA-DRB1 epitopes were defined using solely amino acid sequence data, in contrast to HLA-DQA1, HLA-DQB1, and HLA-DPB1 epitopes that were defined using human alloantibodies. Lastly, we defined and reported on 7 MICA epitopes using human alloantibodies [15, 22]. In this paper, we provide a summary of our findings.

2. Materials and Methods

The principle we used to define HLA epitopes is summarized in (Figure 1). Briefly, if an antibody is determined to test positive with certain HLA antigens and negative with others, it is reasonable to assume that the antibody is targeting a specific epitope on the positive antigens. Epitopes are conformational arrangements of amino acids (aa) at sequence positions on the surface of antigens that must be within the binding span of the antibody. To define an epitope, a computer search, in published aa sequences of tested antigens, was performed to identify exclusively shared aa at one or more sequence positions among the positive antigens—these amino acids define the epitope.

Murine monoclonal antibodies or transplant recipient and healthy male HLA antibodies isolated from sera and cord blood by first adsorbing them onto appropriate recombinant HLA (rHLA) single antigen cells, then eluted with an acidic buffer (ImmunoPure IgG elution buffer, Pierce, Rockford, IL), and neutralized with 1 M TRIS-HCl pH 9.5 (Figure 2) were all tested with the single antigen beads (One Lambda Inc., Canoga Park, CA) to determine the specificity of the antibodies [14]. HLA class I SA beads treated with a buffer that dissociates the peptide and the beta-2-microglobulin (β2m) from the heavy chain of the intact HLA antigens on the beads [17] were used to reveal the specificity of antibodies targeting epitopes on dissociated class I heavy chains. MFI values of 1000 or above were considered positive except when the overall reactions of an eluted antibody were weak, a cutoff of MFI 400 was used.

Computer software was utilized to search for exclusive amino acids in the structure of antigens showing positive reactions with an antibody. Searches were performed within sequences of HLA class I heavy chains, MICA antigens, DR beta chains, DQ beta and alpha chains, and DPB chains. All amino acid sequences were obtained from the HLA Informatics Group at the Anthony Nolan website [23]. One or more amino acids found exclusively at the same sequence positions in the chains of positive antigens, but not in the sequence positions of negative antigens, were designated as the defining amino acids for an epitope. The defining amino acid(s) had to be within the antibody binding span [24, 25] — estimated at 494 Å2–750 Å2 area (Figure 3) and the aa(s) must be exposed at the surface of the antigen—exceptions are noted between parentheses (Table 1).

The efficacy of isolating HLA antibody from HLA sera with adsorption and elution assays, testing the eluted antibody with the SA beads to determine specificity and the definition of the epitope on the surface of positive antigens (corresponding to antibody specificity) are shown in (Figure 4). Alloserum with determined specificity A2, A68, A69, B57, and B58 was adsorbed separately with SA rHLA

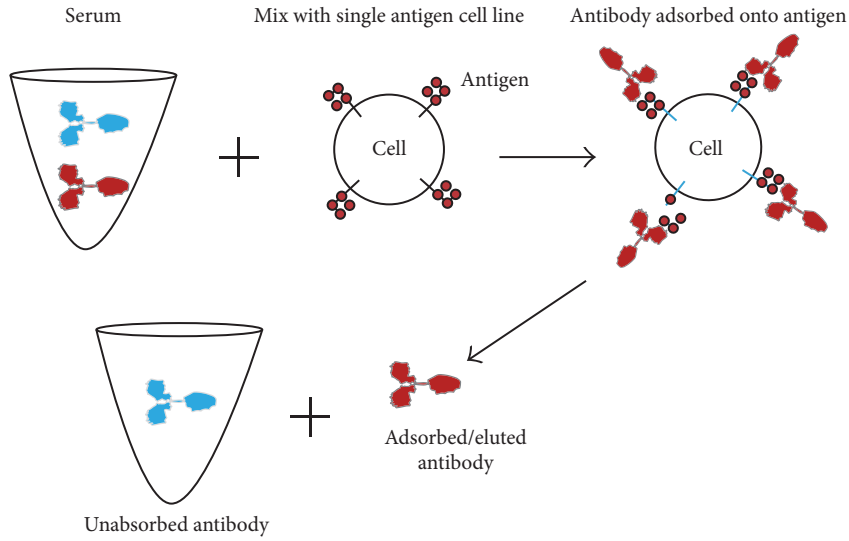


Figure 2: Alloantibody adsorption/elution with recombinant single antigen cell line. The antibody is eluted with an acidic buffer, and the eluate is neutralized with TRIS buffer.

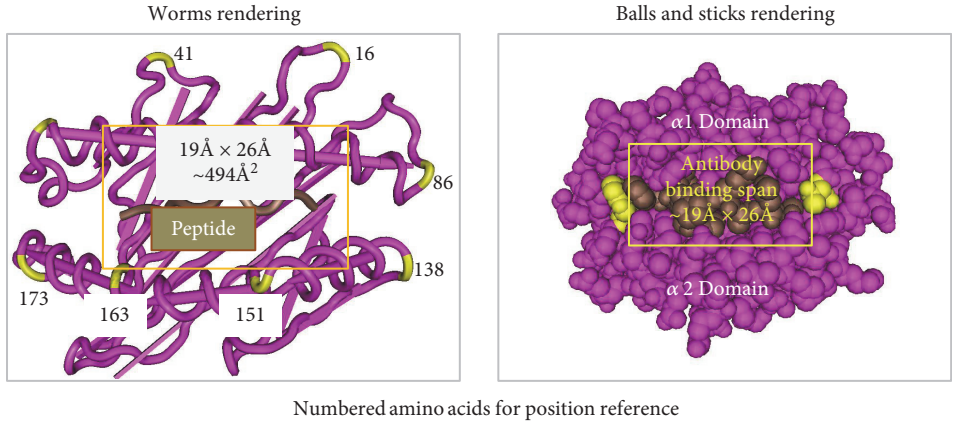


Figure 3: Top view of HLA class I heavy chain 1 and 2 domains. Rectangles show approximate binding span area of antibody.

A6901 and B5801 cells. Eluted antibodies tested with the SA beads showed specificity A2, A68, and A69 and A2, B57, and B58, respectively. HLA antigens A2, A68, and A69 share an epitope defined by glycine (G) at position 62; therefore, 62G defines the epitope. Similarly, HLA antigens A2, B57, and B58 share an epitope defined by threonine (T) at position 142 or histidine (H) at position 145; therefore 142T or 145H define the epitope.

3. Results

3.1. Class I Epitopes on Intact Antigens. 138 unique epitopes were defined for one or a group of two or more intact HLA class I antigens. 110 unique epitopes were defined by using SA beads (Table 1, partial list; complete table in the supplemental information available online at https://doi.org/10.1155/2017/3406230) assays to test eluted alloantibodies that were adsorbed from human sera onto the surface of mammalian rHLA single antigen cells then eluted, and murine monoclonal antibodies to determine specificity of each antibody. Epitopes were defined by identifying exclusively unique amino acids among the positive antigens. Also defined were 28 unique epitopes targeted by naturally occurring anti-HLA antibodies found in sera of healthy males and in cord blood (Table 2). All epitopes were defined by identifying exclusively unique amino acids among the positive antigens. Here, we present partial lists in tables and example figures of epitopes—complete tables and other figures can be found in the supplemental information document.

The number of epitopes defined for each antigen, using human alloantibodies, varied from 4 to 23 (Table 3). In general, there was no correlation between the number of epitopes and the frequency of antigen in the population. For example, for HLA A2, the most frequent antigen ($f = 30.3\%$ to 54%), we defined 16 epitopes while for A25,

Table 1: HLA class I epitope—partial list. Full list of 110 epitopes in supplemental file.

Epitope number	Antigens that share epitope[a]	Amino acid(s) define epitope[b]	A/M	Adsorption rHLA cell line
1	A1, 36	44K/150V/158V/	M	N/A
6	A3	161D	M	N/A
4	A25, 26, 34, 43, 66	(9Y) + 149T/(74D) + 149T[b]	M	N/A
7	B7, 8, 13, 18, 27, 35, 37, 38, 39, 4005, 41, 42, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 59, 60, 61, 62, 64, 65, 67, 71, 72, 73, 75, 76, 77, 78, 81, 82	65Q[c]	M	N/A
8	B13	145L/41T + 46A	M	N/A
14	**A1**, 23, 2402, 80, **B76**	166D/167G	A	A2402
16	**A1**, 36, 11, 25, 26, 34, 43, 6601, 80, **B73**	[90D][c]	A	A8001
17	A2, **B57**, 58	62G	A	B5801
18	A2, 68, 69	142T/145H	A	A6901
40	Cw5, 8	177K	A	nn
222	A6602, **B7**, 13, 27, 47, 48, 60, 61, 73, 81, **Cw2**, Cw17	163E + 166E/ 163E + 167W	A4	Cw0202
223	B7, 13, 27, 47, 48, 60, 61, 81	76E + 163E	A	B0703
244	Cw2, 4, 5, 6, 15, 17, 18	77N + 80K	A	Cw1701
244	**B35**, 4005, 46, 49, 50, 51, 52, 53, 56, 57, 58, 62, 63, 71, 72, 75, 77, 78, **Cw9**, Cw10	163L + 167W	A	B62 (B1501)/B35
246	**B46**, 73, **Cw1**, 7, 8, 9, 10, 12, 14, 16	76V + 80N/73T + 76V + 79R	A/A	Cw1802/nn
249	A*2301, A*2402, A*2403, A*2501, A*3201, B*1513, B*1516, B*27052, B*3701, B*3801, B*4402, B*4403, B*4701, B*4901, B*5101, B*5102, B*5201, B*5202, B*5301, B*5701, B*5703, B*5801, B*5901 ***B*1301 & B*1302 Neg.***	82L + 145R / 83R + 145R	M	N/A
250	A*2301, A*2402, A*2403, A*3201, B*1301, B*1302, B*1513, B*1516, B*27052, B*3701, B*3801, B*4402, B*4403, B*4701, B*4901, B*5101, B*5102, B*5201, B*5202, B*5301, B*5701, B*5703, B*5801, B*5901 ***A2501 Neg.***	82L + 90A/83R + 90A	M	N/A

M designates murine monoclonal antibody, A designates alloantibody, and adsorption rHLA cell line indicates the cell line used to adsorb then elute the antibody; aa: amino acids; nn: not needed; N/A: not applicable; [a]serological antigens shown, alleles are shown when not all alleles of an antigen are positive (i.e., share epitope); [b]possible alternative epitopes are separated by "/"; plus sign "+" indicates two or more positions/aa needed to define the epitope; amino acids not exposed at the surface of the HLA molecule are between parentheses; [c]epitope also shared by C-locus antigens (not shown here) is between square brackets.

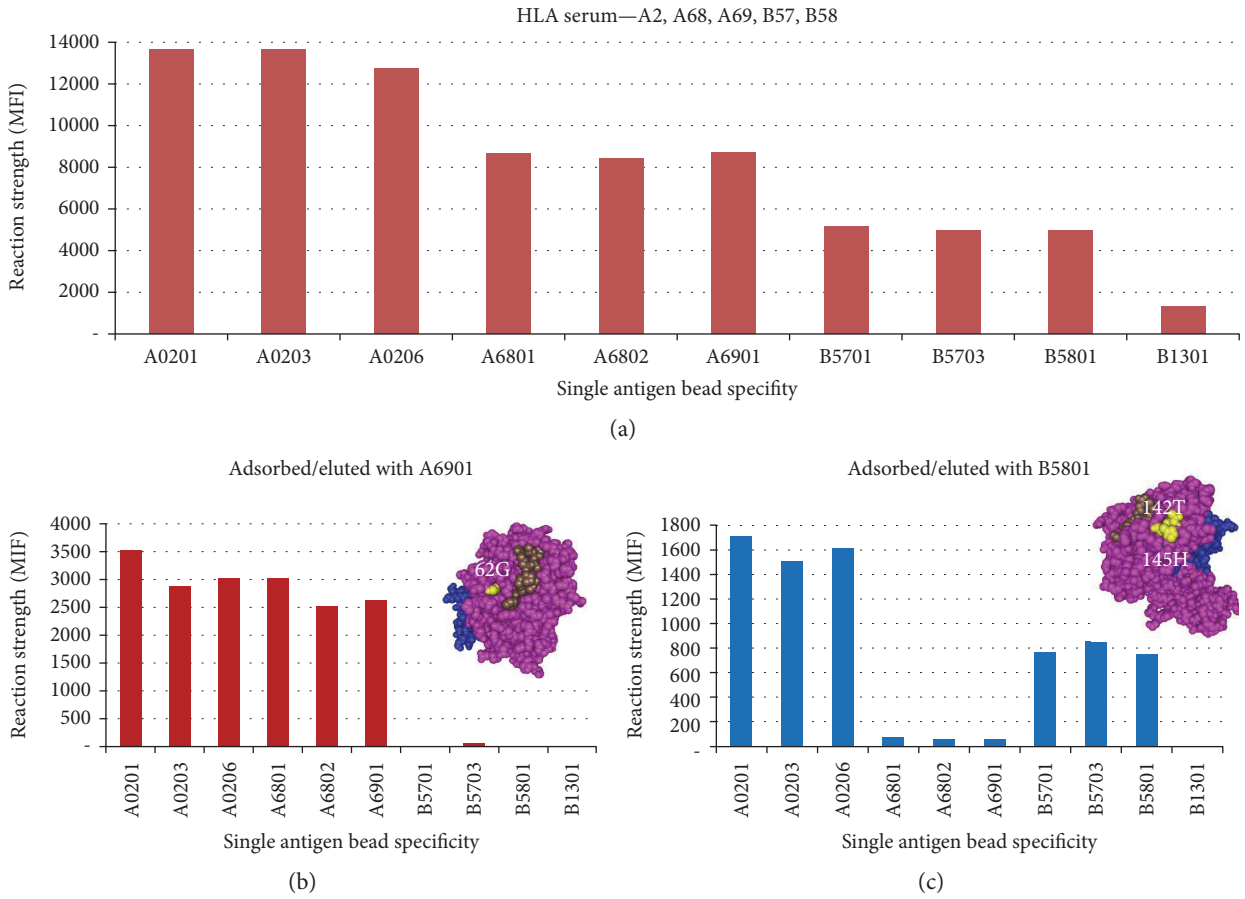


Figure 4: Alloserum with specificity A2, A68, A69, B57, and B58 (a). Antibody eluted from adsorption with A6901 recombinant cells has the specificity of A2, A68, and A69 (b). Antibody eluted from adsorption with B5801 recombinant cells has the specificity of A2, B57, and 58 (c).

Table 2: Epitopes on intact antigens targeted by naturally occurring antibodies—partial list. Complete list in supplemental file.

Epitope number	Dissociated antigen(s)	Epitope site	Epitope number	Dissociated antigen(s)	Epitope site
5059	A0101	158V + 163R	5073	B76	163L + 166D
201	A2	43Q + 62G	5075	Cw*0102, 0302, 0303, 0304, 1402, 1802	219W
3	A23, A24	65G	5076	Cw16	193L
31	A30, 31	56R	5077	Cw17	170G
5064	A3002	17S + 76E	5078	Cw7	273S
5066	A6602	149T	5081	Cw9, Cw10	163L + 173K
5068	A80	56E+	5085	B8	(67F) + 131R
406	B2705	65Q + 69A + 80T	5086	Cw6	80K + 90D + (114D)
236	B57, B58	43P + 62G			

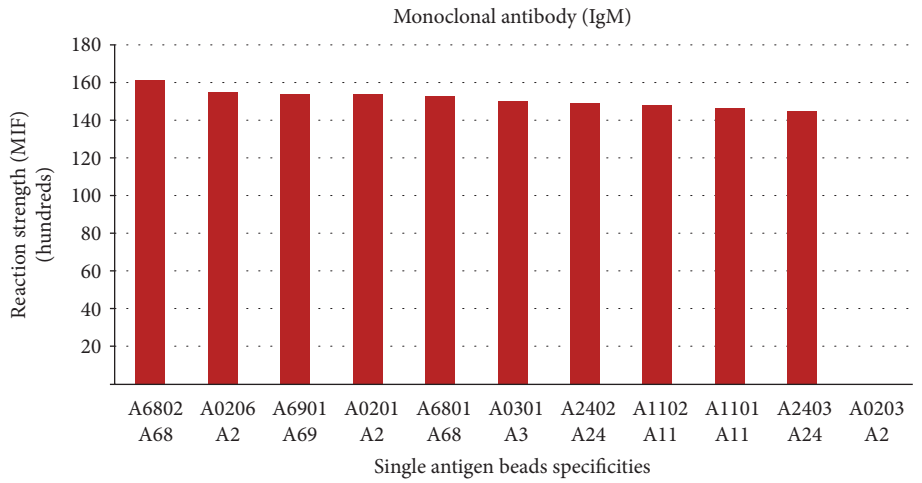
Plus sign "+" indicates two or more positions/aa needed to define the epitope; amino acids not exposed at the surface of the HLA molecule are between parentheses.

with a frequency of $f = 0.0\%$ to 6.1%, we defined 19 epitopes. Class I epitopes were found to be shared by antigens of the same locus or by inter-locus antigens—BC or ABC. Epitopes are defined by 1, 2, 3, or 4 aa's. For example, 17 A-locus epitopes were defined by 1 aa, two B-locus epitopes by 4 aa's, or two ABC loci epitopes defined by 2 aa's. In addition, amino acids and positions on the HLA class I heavy chain epitopes were found at varying frequencies in epitope definitions. The most frequent was position 163 located in the alpha 2 domain, and the aa threonine (T) was found to be the most frequent in our studies.

The following examples illustrate HLA class I epitopes for the A-locus, B-locus, and C-locus and AB-, BC-, and ABC-loci antigens. Illustration shows SA beads specificity, antigens sharing the epitopes, and their position on the HLA class I heavy chain.

Table 3: Partial list of epitopes on HLA class I antigens. Complete list in supplemental file.

Antigen	Number of epitopes	Epitope number																
A1	11	1	12	13	14	15	16	208	238	241	242	248						
A2	16	2	13	17	18	19	27	32	38	201	210	211	238	242	247	412	422	
A25	19	4	12	16	23	24	27	32	38	209	211	213	214	233	238	241	243	
		247	249	423														
A80	9	13	14	15	16	28	29	208	241	242	A80	9	13	14	15	16	28	29
B13	16	7	8	21	22	24	32	33	43	217	218	222	223	233	235	250	418	
B54	17	7	25	32	33	204	215	216	224	226	228	229	232	233	234	401	402	410
B76	13	7	14	22	25	33	43	211	216	218	227	233	240	403	B76	13	7	14
Cw1	5	32	205	232	246	421												
CW2	5	32	39	205	222	244												
CW4	4	32	205	232	244													
CW9	6	32	39	205	245	246	421											
CW10	6	32	39	205	245	246	421											



		aa position		
Antigen	Allele	149	150	151
A1	A*0101	A	V	H
A68	A*6802	A	A	H
A2	A*0206	A	A	H
A69	A*6901	A	A	H
A2	A*0201	A	A	H
A68	A*6801	A	A	H
A3	A*0301	A	A	H

		aa position		
Antigen	Allele	149	150	151
A1	A*0101	A	V	H
A24	A*2402	A	A	H
A11	A*1102	A	A	H
A11	A*1101	A	A	H
A24	A*2403	A	A	H
A2	A*0203	T	A	H

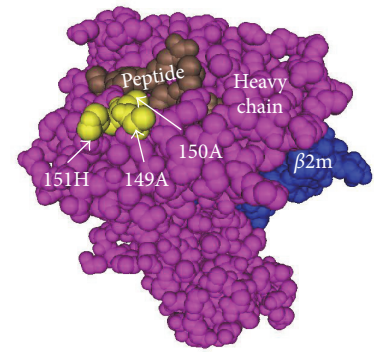
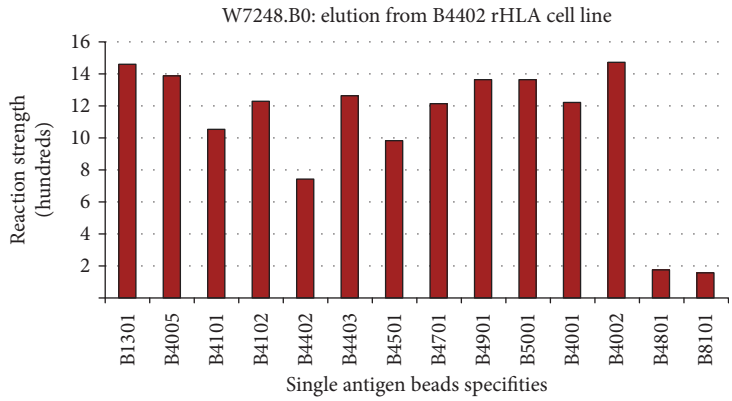


Figure 5: Epitope 422 shared by A-locus antigens A2, A3, A11, A24, A68, and A69 defined by the aa acid combination 149A + 150A + 151H. One amino acid substitution at position 149 (aa T substituted for aa a) could be the reason that A2 allele A*0203 is negative while alleles HLA A*0201 and A*0206 are positive.

A-locus: Epitope 422 is shared by A-locus antigens A2, A3, A11, A24, A68, and A69 defined by 149A + 150A + 151H combined. Three amino acids at three positions are necessary to define this epitope; indeed, HLA–A*02 : 01 and A*02 : 06 are positive while A*02 : 03 is negative. A*02 : 01 and A*02 : 06 share epitope 422 defined as 149A + 150A + 151H, while negative antigen A*02 : 03 does not share the epitope. A*02 : 03 has 150A + 151H but has



Antigen	Allele B0702	aa & position 41 A
B13	B1301	T
B4005	B4005	T
B41	B4101	T
B41	B4102	T
B44	B4402	T
B44	B4403	T
B45	B4501	T
B47	B4701	T
B49	B4901	T
B50	B5001	T
B60	B4001	T
B61	B4002	T
B48	B4801	A
B81	B8101	A

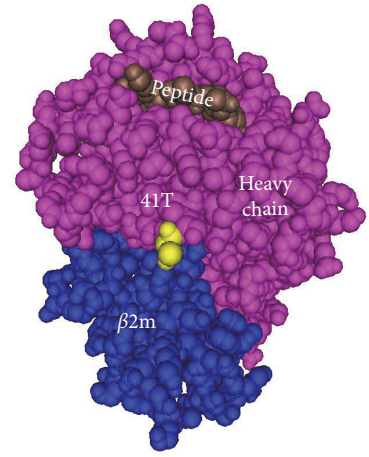
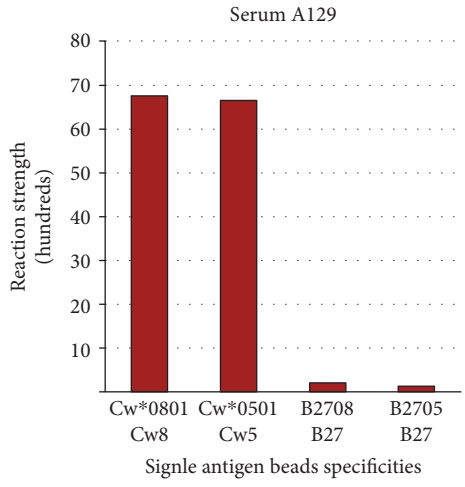


Figure 6: Epitope 21 shared by the B-locus antigens B13, B4005, B41, B44, B45, B47, B49, B50, B60, and B61 and defined by 41T.



	aa & positions 173	177	178
A*01011	E	E	T
B*07021	E	D	K
Cw*010201	E	E	T
Cw*080101	E	K	T
Cw*050101	E	K	T
B*27052	E	E	T
B*2708	E	E	T

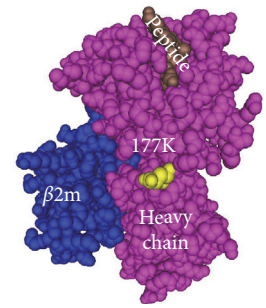
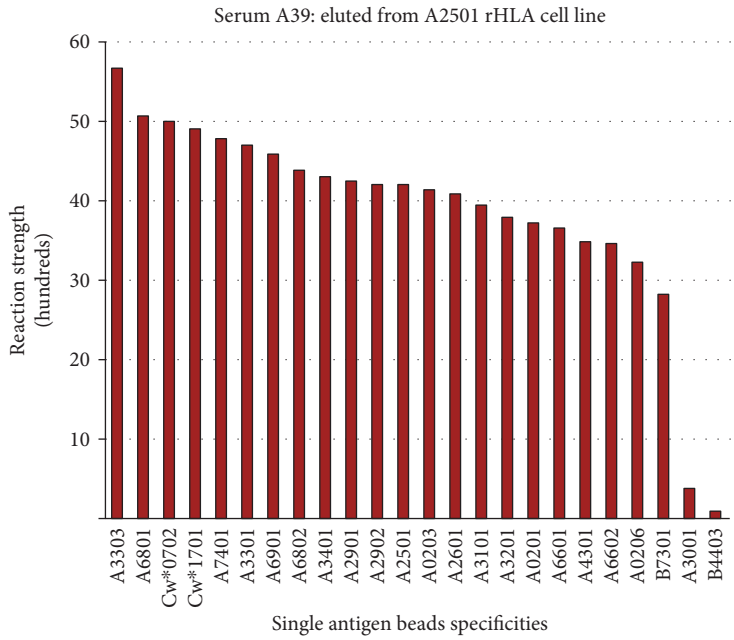


Figure 7: Epitope 40 shared by the C-locus antigens Cw*0801 and Cw*0501 and defined by 177K.

threonine (T) at position 149 instead of alanine (A)—one amino acid difference in the epitope renders the antibody to be nonreactive with A*02 : 03. The aa defining epitope 422 are exposed at the surface of the heavy chain and are within the binding span of the HLA antibody. The furthest amino acids are 7.88 Å apart (Figure 5).

B-locus: Epitope 21 is shared by B-locus antigens B13, B4005, B41, B44, B45, B47, B49, B50, B60 (B4001), and B61



	aa and position 253		aa and position 253
A*0101	E	A*0101	E
B*0702	E	B*0702	E
Cw*0102	E	Cw*0102	E
A*3303	Q	A*0203	Q
A*6801	Q	A*2601	Q
Cw*0702	Q	A*3101	Q
Cw*1701	Q	A*3201	Q
A*7401	Q	A*0201	Q
A*3301	Q	A*6601	Q
A*6901	Q	A*4301	Q
A*6802	Q	A*6602	Q
A*3401	Q	A*0206	Q
B*2901	Q	B*7301	Q
A*2902	Q	A*3001	E
B*2501	Q	B*4403	E

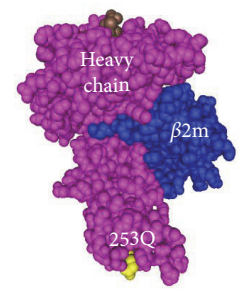


Figure 8: Epitope 38 shared by the ABC-loci antigens A2, A25, A26, A29, A31, A32, A33, A34, A43, A66, A68, A69, A74, B73, Cw7, and Cw17 and defined by the amino acid glutamine (Q) at position 253.

Table 4: Cryptic (C) epitopes on dissociated class I HLA antigen—partial list. Complete list in supplemental file.

Epitope number	Dissociated antigen(s)	Epitope site	Epitope number	Dissociated antigen(s)	Epitope site[c]
5006	A3002	(152R)	5033	Cw2	(211T)
5007	A31, A33	(73I)	5036	Cw17	(116F) + (143S)
5008	A3401	(63N) + (66K)	5038	Cw6	(9D) + (97W)
5009	A3402	(63N) + (66K) + (156L)	5039	Cw7	(66K) + (99S)
5010	A80	(31S)	5049	A6602	(114Q) + 163E
5027	B8	(9D)	5052	B76	(70N) + 166D
5031	B82	(24S) + (99F)			

[c]Amino acids and their positions on the HLA-dissociated antigens define each epitope. In intact antigens, these amino acids are not exposed at the surface (cryptic).

(B4002) and defined by 41T. Threonine (T) is exclusively unique to the antigens at position 41 located in the alpha 1 domain of the HLA class I heavy chain (Figure 6).

C-locus: Epitope 40 shared by the C-locus antigens Cw*0801 and Cw*0501 and defined by 177K located in the alpha 2 domain of the HLA heavy chain (Figure 7).

AB-Loci: Epitope 205 shared by the AB-loci A32, A74, B7, B8, B4005, B41, B42, B48, B60, B61, B73, and B81 and defined by 109L + 131R—the two positions are 11.8 Å apart and therefore within the binding span of the antibody. Also, the C-locus antigens Cw1, Cw2, Cw4, Cw5, Cw6, Cw7, Cw8, Cw9, Cw10, Cw12, Cw14, Cw15, Cw16, Cw17, and Cw18 share the same amino acids at positions 109 and131 but were not tested with the C-locus beads at the time of the study.

Epitope 24 is shared by the AB-loci Bw4-associated antigens A23, A24, A25, A32, B13, B2705, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63, and B77 and defined by either 82L or 83R located in the alpha 1 domain of the HLA class I heavy chain.

Epitope 423 is shared by the AB-loci Bw4-associated antigens A23, A25, A32, B2705, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63, and B77 (A24, B13 negative) and defined by 83R + 144Q + 145R. This epitope was defined using a monoclonal antibody and seems to be a variant of epitope 24 shared by all Bw4-associated antigens. Other variants of the BW4-associated antigens epitope (epitopes 249 and 250) also defined with monoclonal antibodies (Table 1).

BC-Loci: Epitope 246 is shared by BC-loci antigens B46, B73, Cw1, Cw7, Cw8, Cw9, Cw10, Cw12, Cw14, and Cw16

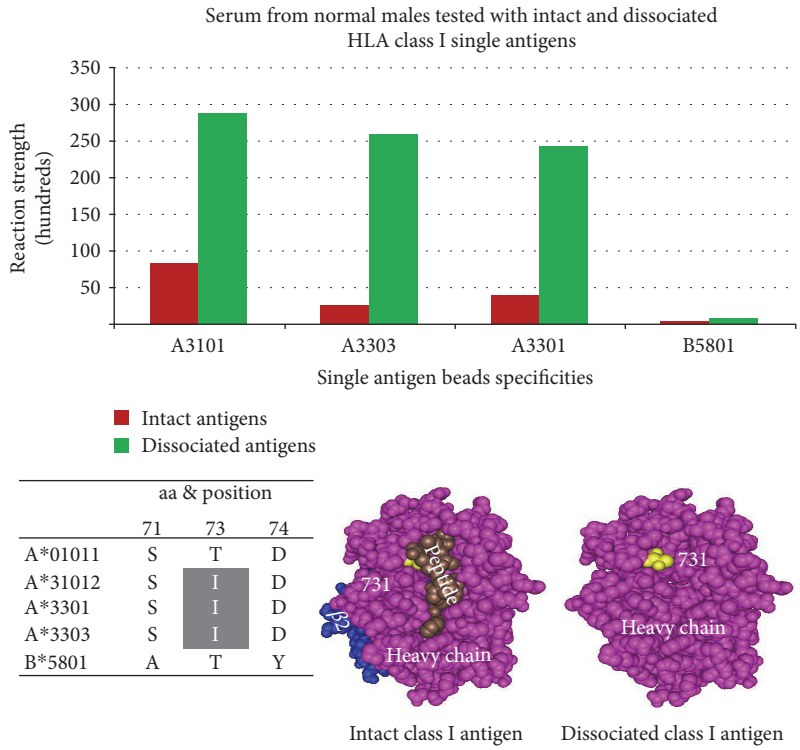


Figure 9: Epitope 5007 shared by the HLA class I A-locus antigens A31 and A33 and defined by isolucine (I) at position 73. The epitope is accessible on the dissociated antigens and show stronger reactivity when the peptide has been dissociated from the heavy chain. Position 73 is not exposed in an intact HLA class I antigen. After acid buffer treatment and neutralization of the eluate, epitope 5007 becomes exposed and reacts with the antibody 10-fold.

Table 5: MICA epitopes.

Epitope number	MICA antigens sharing epitope	aa/position define epitope[a]	rMICA cells used for adsorption/elution
6001	MICA*001, 012, 018	(24T)	MICA*018
6002	MICA*001, 002, 004, 007, 009, 012, 018, 027	91Q	ND
6003	MICA*004, 009	122V	ND
6004	MICA*027, 004, 009	(36Y)/129V/173E	MICA*004
6005	MICA*017	91R	ND
6006	MICA*004	181R	ND
6007	MICA*027	213I/251R	ND

ND: not done; amino acids not exposed on the surface of the MICA antigen are shown between parentheses; [a]possible alternative epitope definitions are separated by "/"; epitopes.

and defined by 76V + 80N. The two amino acids are 8.69 Å apart which is within the binding span of the HLA antibody.

ABC-loci: Epitope 38 is shared by the ABC-loci antigens A2, A25, A26, A29, A31, A32, A33, A34, A43, A66, A68, A69, A74, B73, Cw7, and Cw17 and defined by the amino acid glutamine (Q) at position 253 of the HLA class I heavy chain located in the alpha 3 domain proximal to the cell membrane (Figure 8).

3.2. Cryptic Epitopes on Dissociated HLA Class I Antigens. Naturally occurring anti HLA antibodies were detected in nonalloimmunized healthy males [26], and 96 of their target epitopes were defined [16]. 58 natural antibodies are only reactive with dissociated HLA class I antigens, heavy chain only (Table 4). 56 unique epitopes on dissociated HLA class I defined [16].

Epitope 5007 is shared by the HLA class I A-locus antigens A31 and A33 and defined by isoleucine (I) at the cryptic position 73. Antibody reactivity with the intact antigen is obstructed because position 73 is located under the peptide. It is slightly reactive with the intact HLA class I antigens. Reactivity increased by up to 10-fold with the dissociated antigens (heavy chain only)—when β2m and the peptide are dissociated from the heavy chain (Figure 9).

Epitope 5024 is shared by the HLA class I B-locus antigens B7, B42, B54, B55, B56, B67, B81, and B82 and defined by 66I + 70Q. Reactions strength of the antibody is stronger

MICA

Antigen	aa position 91
MICA*001	Q
MICA*002	Q
MICA*004	Q
MICA*007	Q
MICA*009	Q
MICA*012	Q
MICA*018	Q
MICA*027	Q
MICA*017	R

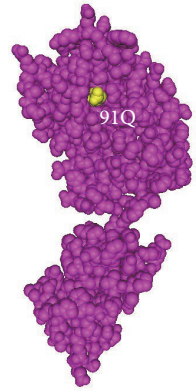


Figure 10: Epitope 6002 shared by MICA antigens MICA*001, 002, 004, 007, 009, 012, 018, and 027 and defined by glutamine (Q) at position 91.

HLA class II DR epitope

Antigen	aa pos. 77
DR1	T
DR103	T
DR10	T
DR11	T
DR12	T
DR15(2)	T
DR16(2)	T
DR4	T
DR4	T
DR13(6)	T
DR14(6)	T
DR7	T
DR8	T
DR9	T
DR18(3)	N
DR17(3)	N

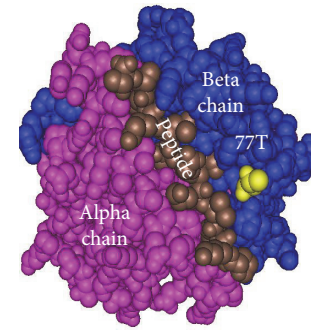


Figure 11: Epitope 1028 shared by class II DR antigens DR1, DR4, DR7, DR9, DR10, DR11, DR12, DR13, DR14, DR15, DR16, DR51, DR53, and DR103 and defined by threonine (T) at position 77.

Table 6: Partial list of HLA class II DR epitopes defined based on aa acid sequence of the beta chain of the antigens. Complete list in supplemental file.

Epitope number	DR antigens sharing epitope	Position/ amino acid
1001	DR7, DR9, DR53	4Q
1004	DR4, DR10	11V
1008	DR7	25Q
1017	DR11	58E
1018	DR7, DR8, DR11, DR12, DR13, DR16, DR51, DR103	70D
1028	DR1, DR4, DR7, DR9, DR10, DR11, DR12, DR13, DR14, DR15, DR16, DR51, DR53, DR103	77T
1029	DR7, DR9	78V
1039	DR1, DR7, DR9, DR15, DR16, DR51, DR52, DR53, DR103	140A
1032	DR7, DR8, DR9, DR10, DR11, DR12, DR13, DR14, DR17, DR18, DR52	96H

with the unobstructed epitope after dissociation of the peptide from the heavy chain.

Epitope 5037 is shared by the HLA C-locus antigens Cw4, Cw6, Cw17, and Cw18 and defined by 73A + 77N. Antibody reaction strength increases with the unobstructed epitope after removal of the peptide.

3.3. MICA Epitopes. MICA or MHC class I polypeptide-related sequence A antigens have similar aa structure as the HLA class I ABC heavy chains. However, MICA antigens are not associated with a peptide and beta 2 microglobulin. Seven epitopes were defined for MICA antigens (Table 5).

Epitope 6002 is shared by MICA antigens MICA*001, 002, 004, 007, 009, 012, 018, and 027 and defined by glutamine (Q) at position 91; therefore, 91Q defines the epitope (Figure 10).

Epitope 6004 is shared by MICA antigens MICA*04, 009, and 027 and defined by (36Y), 129V, or 173E.

3.4. Class II Epitopes

3.4.1. HLA-DRB1 Epitopes. Unlike class I epitopes, the 60 HLA class II B1 epitopes were defined based solely on amino acid sequence of the DR antigen beta chain where all epitopes can be defined by one single amino acid at one position (Table 6). The number of epitopes for each DR antigen was from 8 to 21 epitopes.

For example, epitope 1028 is shared by class II DR antigens DR1, DR4, DR7, DR9, DR10, DR11, DR12, DR13, DR14, DR15, DR16, DR51, DR53, and DR103 and defined by threonine (T) at position 77 (Figure 11).

3.4.2. HLA-DQA1 and HLA-DQB1 Epitopes. Eighteen HLA class II DQB1 and DQA1 epitopes are defined using the adsorption and elution assays described in the Materials and Methods above. Fifteen of the epitopes are located on the beta chain of the DQ antigen and three on the alpha chain (Table 7). The number of epitopes for DQB chains was 4–8 and only one for DQA chains (Table 8).

Sera from allosensitized patients can be expected to have anti-HLA antibodies to class I and II antigens. As illustrated in (Figure 12), this serum has antibodies directed against DR, DQ, and DP antigens. The serum was adsorbed with DQA1*02 : 01/DQB1*04 : 01 rHLA cells and the eluted antibody reacted with DQ4, DQ5, and DQ6 antigens which share epitope 2007 (Table 7).

One antigen mismatch can elicit an immune response to several epitopes on an HLA antigen. A serum from renal transplant patient with DQA1*02 : 01/DQB1*02 : 02 mismatch has two antibodies. One antibody targets epitope 2017 (defined by histidine (H) in position 52) on the DQA1*02 : 01 alpha chain and the other antibody targets

Table 7: Fifteen HLA class II DQβ epitopes and three DQα epitopes defined.

Epitope number[a]	DQ antigens sharing epitope	Position/amino acid[b]
2001	DQB2	28S/30S/37I/52L/55L
2002	DQB4	56L
2003	DQB4, DQB5, DQB6, DQB7, DQB8, DQB9	28T/46V/52P
2004	DQB5, DQB6	84E/85V/86A/89G/90I/221Q
2005	DQB7	45E
2006	DQB7, DQB8, DQB9	55P
2007	DQB4, DQB5, DQB6	52P + 55R
2008	DQB2, DQB5, DQB7, DQB8, DQB9	(9Y + 11F)
2009	DQB2, DQB4, DQB5, DQB6, DQB8, DQB9	34R + 45G
2010	DQB4, DQB5, DQB6, DQB8, DQB9	45G + 46V
2011	DQB5, DQB0601	38V + 46V
2012	DQB8, DQB9	45G + 55P
2013	DQB2, DQB4, DQB7, DQB8, DQB9	84Q/85L/86E/87L/89T/220H/221H
2014	DQB4, DQB7, DQB8, DQB9	77T + 84Q/77T + 85L/77T + 86E/77T + 87L/182N
2015	DQB5	70G + 71A/116I/125S
2017	DQA1*0201	47K/52H/54L
2018	DQA1*04/DQA1*05/DQA1*06	40G/47C
2019	DQA1*03	26S/47Q/56R/187T

[a]Epitope 2008 defined using mAb; [b]possible alternative epitopes are separated by "/"; epitopes that are defined by more than a single position/aa are separated by "+"; amino acids not exposed at the surface of the HLA molecule are between parentheses.

Table 8: Number and epitopes on HLA class II DQA1 and DQB1 antigens.

Antigen	Number of epitopes	Epitopes							
DQA1*0201	1	2017							
DQA1*03	1	2019							
DQA1*04	1	2018							
DQ2	4	2001	2008	2009	2013				
DQ4	7	2002	2003	2007	2009	2010	2013	2014	
DQ5	8	2003	2004	2007	2008	2009	2010	2011	2015
DQ0601	6	2003	2004	2007	2009	2010	2011		
DQ7	6	2003	2005	2006	2008	2013	2014		
DQ8	8	2003	2006	2008	2009	2010	2012	2013	2014
DQ9	8	2003	2006	2008	2009	2010	2012	2013	2014

epitope 2001 (defined by leucine (L) in position 52) on the DQB1*02 : 02 beta chain (Table 7). As shown in the table, alternative epitope definitions are separated by "/."

The efficacy of adsorption and elution assays is demonstrated where one serum with DQ specificity, including DQA1*02 : 01, underwent four separate adsorptions and elutions with rHLA DQ cells. Two of the cells have the relevant DQA1*02 : 01 chain, and the eluted antibodies show positive reactions with all heterodimers that contain the DQA1*02 : 01chain (red and green bars). However, eluents from adsorptions with irrelevant cells (no DQA1*02 : 01 chain) showed negative reactions (yellow and blue bars) (Figure 13).

The following examples illustrate HLA-DQA1 and HLA-DQB1 epitopes. Illustration shows SA beads specificity, antigens sharing the epitopes and their position on the DQA1 and DQB1 chains.

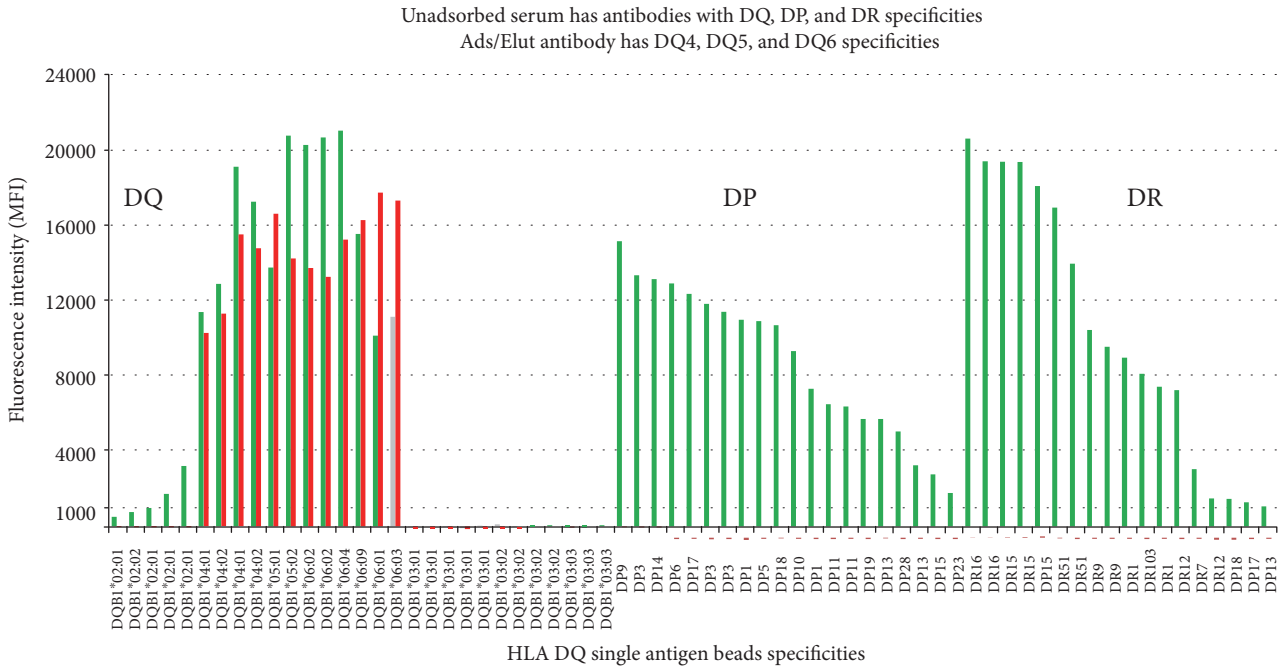
HLA-DQA1 epitopes: epitope 2018 is shared by the alpha chains of the DQ4, DQ5, and DQ6 antigens and defined by glutamine (Q) at position 53.

HLA-DQB1 epitopes: epitope 2002 is shared exclusively by the beta chains of the DQ4 antigen and defined by leucine (L) in position 56.

Epitope 2010 is shared by the beta chains of DQ antigens DQ4, DQ5, DQ6, DQ8, and DQ9 and defined by 45G + 46V.

Epitope 2022 is exclusive to DQB1*05 : 01 chain on the DQ5 antigen and defined by 125S + 126Q.

Epitope 2006 is shared by DQB1*03 : 01 (DQ7), DQB1*03 : 02 (DQ8), and DQB1*03 : 03 (DQ9) and



HLA DQ allele	Position/amino acid		HLA DQ allele	Position/amino acid	
	52	55		52	55
DQB1*05:01	P	R	DQB1*05:01	P	R
DQB1*04:01	P	R	DQB1*06:04	P	R
DQB1*04:02	P	R	DQB1*06:09	P	R
DQB1*05:01	P	R	DQB1*02:01	L	L
DQB1*05:02	P	R	DQB1*02:02	L	L
DQB1*06:01	P	R	DQB1*03:01	P	P
DQB1*06:02	P	R	DQB1*03:02	P	P
DQB1*06:03	P	R	DQB1*03:03	P	P

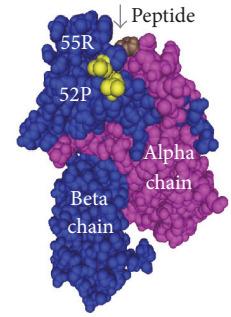


Figure 12: Unabsorbed serum has antibodies with specificity to DR, DQ, and DP antigens (green bars). After adsorbing the serum with rHLA DQ cells, the eluted antibody shows specificity to DQ antigens only (red bars).

defined by proline (P) at position 55 on the beta chains of the DQ antigens.

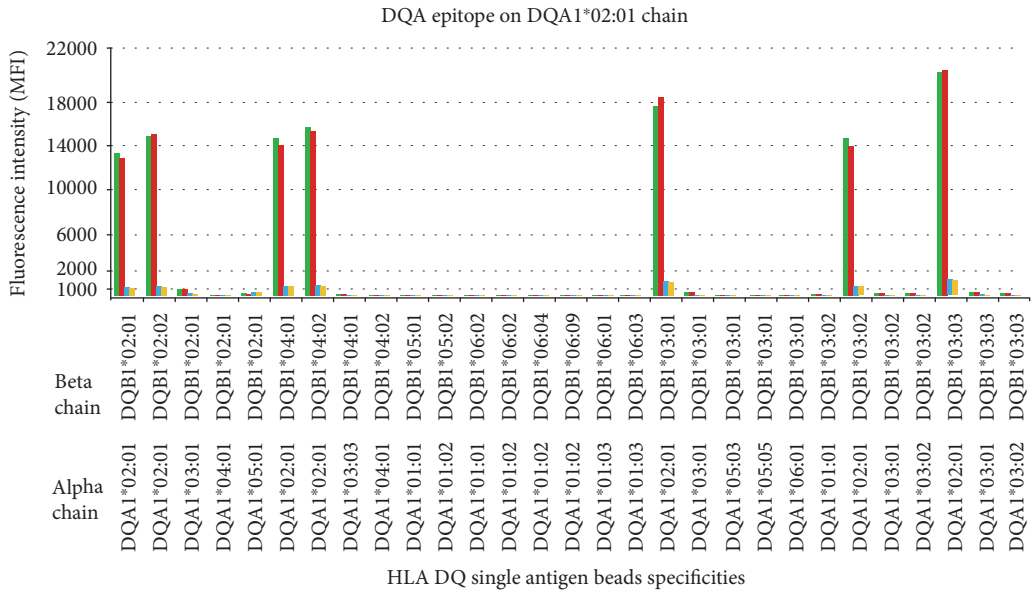
3.4.3. HLA-DPB1 Epitopes. Five HLA class II DPB epitopes were defined. Four of the epitopes required 3-4 amino acids for definition, and one was defined by a single amino acid/position (Table 9).

Epitope 4001 is shared by the HLA class II DPB1 chains of the DP antigens DPB1*01 : 01, DPB1*03 : 01, DPB1*05 : 01, DPB1*09 : 01, DPB1*10 : 01, DPB1*11 : 01, DPB1*13 : 01, DPB1*14 : 01, DPB1*17 : 01, and DPB1*19 : 01 and defined by 84D + 85E + 86A + 87V. All four amino acids needed to define the epitope. Negative antigens that did not share epitope 4001 are shown as gray bars (Figure 14).

HLA class II DP epitope 4003 is shared by DPB1 chains DPB1*02 : 01, DPB1*04 : 02, DPB1*10 : 01, and DPB1*18 : 01 (red bars) and defined by 84D + 85E + 86A + 87V. Negative antigens that did not share epitope 4003 are shown in gray bars (Figure 15).

4. Discussion

Cross-reactivity of antibodies with HLA antigens has been investigated for decades [4, 5, 7–9, 27]. Studies to identify HLA epitope, the target of antibodies, started more than 50 years ago [10], and numerous other studies followed since then [5, 6, 11, 28–34]. The amino acid structure of HLA antigen chains was reported for the HLA A2 in 1987 [12], and now, complete sequences of all known HLA antigen chains are readily available online [23]. Single antigens expressed on a mammalian cell line allowed us to simplify adsorption/elution assays and isolate one antibody from multispecific allosera, with multiple antibodies, and test the isolated antibody with the single antigen beads to more accurately determine antibody specificity. Isolated antibodies tested with large panels of HLA class I or class II single antigen beads were shown to be positive with certain antigens of the bead panels and negative with others. It is, therefore, reasonable to assume that the positive antigens share a public



HLA DQ allele	Position/ amino acid 52	HLA DQ allele	Position/ amino acid 52
DQA1*01:01	S	DQA1*01:01	S
DQA1*02:01	H	DQA1*03:03	R
DQA1*01:01	S	DQA1*04:01	R
DQA1*01:02	S	DQA1*05:01	R
DQA1*01:03	S	DQA1*05:03	R
DQA1*03:01	R	DQA1*05:05	R
DQA1*03:02	R	DQA1*06:01	R

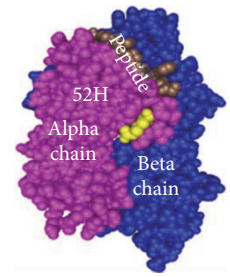


Figure 13: Several DQ antigens (heterodimers) with the DQA1*02 : 01 chain are shown below. They all share epitope 2017 which is defined by histidine (H) in position 52 of DQA1*02 : 01 chain. Eluted antibodies from relevant DQ antigens are positive (red and green bars). Eluted antibodies from irrelevant (no DQA1*02 : 01) are negative (yellow and blue bars).

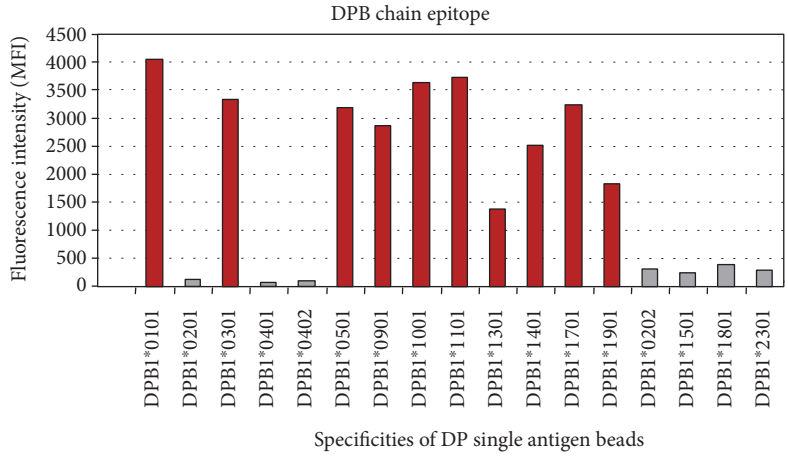
Table 9: HLA class II DP epitopes.

Epitope number	DP antigens sharing epitope	Position/amino acid[a]
4001	DPB1*0101, DPB1*0301, DPB1*0501, DPB1*0901, DPB1*1001, DPB1*1101, DPB1*1301, DPB1*1401, DPB1*1701, DPB1*1901	84D + 85E + 86A + −87V
4002	DPB1*0301, DPB1*0901, DPB1*1401, DPB1*1701	55D + 56E + -57D
4003	DPB1*0201, DPB1*0402, DPB1*1001, DPB1*1801	55D + 56E + -57E
4004	DPB1*1101, DPB1*1501	(33Q)
4005	DPB1*0201, DPB1*0401, DPB1*0402	84G + 85G + 86P + 87M

[a]Epitopes defined by more than a single position/aa are separated by "+"; amino acids not exposed at the surface of the HLA molecule are between parentheses.

epitope which can easily be confirmed by looking at the amino acid sequences of these antigens.

HLA single antigen bead assays are simplified assigning anti-HLA antibody specificities by simply listing all antigens that are positive with the serum or antibody. Because a positive antibody-antigen reaction indicates binding of antibody to the single antigen on the bead, we postulate that the antibody must be specific to the antigen. However, the single antigen beads assay often reveals more antibody specificities than other antibody detection assays. This is clearly seen when an immunological response to a mismatched antigen produces antibody specificity to nondonor antigens and in some cases unexpectedly to rare antigens. HLA antigens share public epitopes; therefore, the extra antibody specificity



Allele	Amino acids and position			
	84	85	86	87
DPB1*01:01	D	E	A	V
DPB1*01:01	D	E	A	V
DPB1*03:01	D	E	A	V
DPB1*05:01	D	E	A	V
DPB1*09:01	D	E	A	V
DPB1*10:01	D	E	A	V
DPB1*11:01	D	E	A	V
DPB1*13:01	D	E	A	V
DPB1*14:01	D	E	A	V
DPB1*17:01	D	E	A	V
DPB1*19:01	D	E	A	V
DPB1*02:01	G	G	P	M
DPB1*04:01	G	G	P	M
DPB1*04:02	G	G	P	M

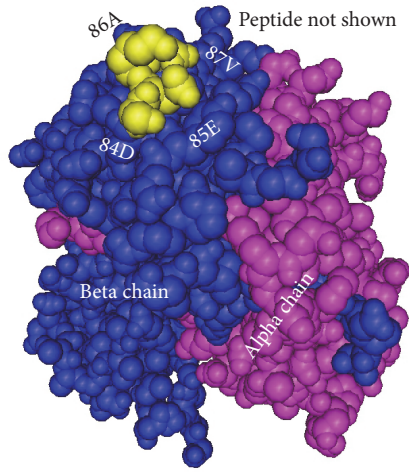


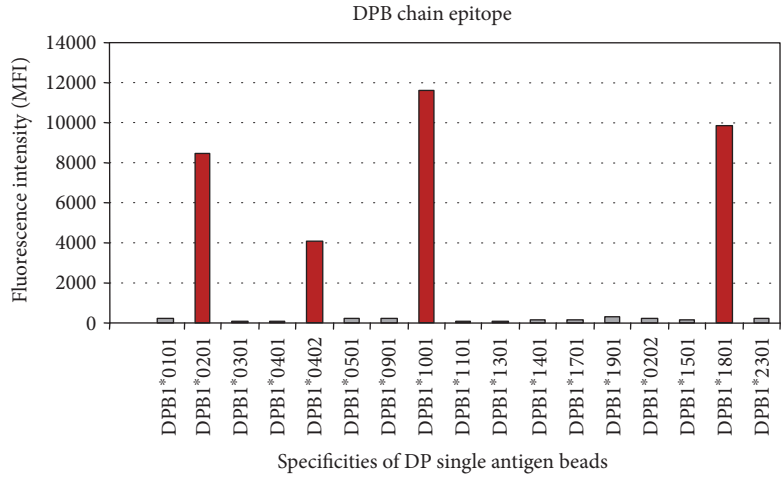
Figure 14: HLA class II DP epitope 4001 shared by DPB chains DPB1*0101, DPB1*0301, DPB1*0501, DPB1*0901, DPB1*1001, DPB1*1101, DPB1*1301, DPB1*1401, DPB1*1701, and DPB1*1901 (red bars) and defined by 84D + 85E + 86A + 87V. Negative antigens that did not share epitope 4001 are shown in (gray bars).

of non-donor-specific and rare antigens can now be explained as antibody binding to public epitopes located on the positive antigens. Defining epitopes of HLA gives us better understanding of the breadth of non-donor-specific specificities found in sera. For example, specificity of antibodies to rare antigens like A80 and B76 were unexpectedly higher than the antigens' frequency (<0.5%) in the general population. The two antigens have 9 and 13 epitopes, respectively (Table 3).

HLA epitopes were defined using computer software by searching, in published sequences of class I and class II antigens, for exclusive amino acids at the same position(s) that are shared by all positive-reacting antigens. Amino acid sequences and the 3D structures of available HLA antigens, used to ensure that aa's are exposed on the surface of the antigens, helped in defining close to 300 epitopes. Assay-positive antigens that share epitopes, defined by exclusively shared aa's, correspond to the antibody specificities. Although it is beyond the scope of assays used in our studies to determine the exact conformational arrangement of each epitope and all amino acids that constitute the epitope, the defining amino acids must be a focal part of the epitope. Public epitopes found exclusively on positive antigens and not on negative antigens are likely not coincidences. For several epitopes defined in our studies, the difference of one aa among alleles of the same antigen, at least one amino acid position can determine whether the allele is positive or negative with the antibody (Figure 5).

We have demonstrated that some antibodies target an epitope on one single antigen (private epitope) or an epitope on a group of two or more antigens (public epitopes). Furthermore, in anti-DQ antisera, immunological responses can produce antibodies to epitopes located on either or both polymorphic chains of the DQ antigens.

The usefulness of epitopes beyond determining correct antibody specificity in sera of transplant patients has been the subject of study recently. Reports on matching donors and recipients or selecting organ donors based on epitope matching are numerous. For example, Duquesnoy reported



Allele	Amino acids and position					
	53	54	55	56	57	58
DPB1*01:01	R	P	A	A	E	Y
DPB1*02:01	R	P	D	E	E	Y
DPB1*04:02	R	P	D	E	E	Y
DPB1*10:01	R	P	D	E	E	Y
DPB1*18:01	R	P	D	E	E	Y
DPB1*01:01	R	P	A	A	P	Y
DPB1*03:01	R	P	D	E	D	Y
DPB1*04:01	R	P	A	A	E	Y
DPB1*05:01	R	P	E	A	E	Y
DPB1*09:01	R	P	D	E	D	Y
DPB1*11:01	R	P	A	A	E	Y
DPB1*13:01	R	P	A	A	E	Y
DPB1*14:01	R	P	D	E	D	Y
DPB1*17:01	R	P	D	E	D	Y
DPB1*19:01	R	P	E	A	E	Y

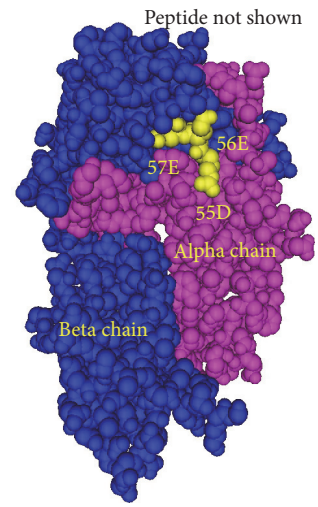


Figure 15: HLA class II DP epitope 4003 shared by DPB chains DPB1*0201, DPB1*0402, DPB1*1001, and DPB1*1801 (red bars) and defined by 84D + 85E + 86A + 87V. Negative antigens that did not share epitope 4003 are shown in (gray bars).

on HLA epitope-based matching for organ transplantation [35, 36]. Wiebe reported on epitope matching to minimize de novo donor-specific antibodies to improve transplantation outcome [37] and Walton et al. reported on the usefulness of matching at the epitope level which protects against chronic lung allograft dysfunction [38].

Conflicts of Interest

Nadim El-Awar has no conflict of interest regarding the publication of this paper (retired from One Lambda Inc., or now Thermo Fisher Scientific, September 11, 2014). Vadim Jucaud has no conflict of interest regarding the publication of this paper. Anh Nguyen has no conflict of interest regarding the publication of this paper.

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Research Article

Donor-Specific Anti-Human Leukocyte Antigen Antibodies Predict Prolonged Isolated Thrombocytopenia and Inferior Outcomes of Haploidentical Hematopoietic Stem Cell Transplantation

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Prolonged isolated thrombocytopenia (PT) after allogeneic stem cell transplantation (allo-SCT) has a great impact on transplant outcome. In this study, we performed a retrospective analysis to investigate the association of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSAs) with PT in 394 patients who underwent unmanipulated haploidentical blood and marrow transplantation (HBMT). For HLA antibody positive samples with a median fluorescent intensity (MFI) > 500, DSAs were further examined. A total of 390 patients (99.0%) achieved sustained myeloid engraftment. Of the 394 cases tested, 45 (11.4%) were DSA positive. The cumulative incidence of PT in this cohort of patients was $9.9 \pm 1.5\%$. The incidence of PT was higher in patients with a MFI ≥ 1000 compared with those with a MFI < 1000 ($16.8 \pm 6.4\%$ versus $7.4 \pm 1.4\%$, $P = 0.05$). Multivariate analysis showed that the presence of DSAs (MFI ≥ 1000) was correlated to PT (hazard ratio (HR) 3.262; 95% confidence interval (CI), 1.339–7.946; $P = 0.009$) and transplant-related mortality (HR 2.320; 95% CI, 1.169–4.426; $P = 0.044$). Our results, for the first time, suggest an association of DSAs with PT after unmanipulated HBMT. It would help screen out the suitable donor and guide intervention. This indicated that DSAs should be incorporated in the algorithm for unmanipulated HBMT.

1. Introduction

For patients with hematologic malignancies, allogeneic stem cell transplantation (allo-SCT) is a kind of curative treatment [1–3]. Recently, haploidentical SCT provides alternative treatment options for patients lacking human-leukocyte antigen- (HLA-) matched related or unrelated donors. However, prolonged isolated thrombocytopenia (PT), which is defined as the engraftment of all peripheral blood cell lines other than a platelet (PLT) count $\geq 20 \times 10^9$/L or dependence on PLT transfusions for more than 90 days after allo-SCT, has a great impact on transplant outcomes, especially in haploidentical SCT settings. The incidence of PT is around 5 to 37% after transplantation [4–6]. In our center, we established an unmanipulated haploidentical blood and marrow transplantation (HBMT) protocol that has a lower incidence of graft failure compared to other haploidentical transplant modalities [7], but PT still significantly increases the risk of transplant-related mortality (TRM) [4–6, 8]. Although the impaired PLT production and accelerated peripheral destruction are known to be the major causes of PT [4–6], there still might be other undiscovered factors that remain to be clarified [9].

Donor-specific antibodies (DSAs) are the anti-human leukocyte antigen (HLA) antibodies that specifically respond

to the mismatched antigen of donor [10–12]. Several researchers, including us, have confirmed the effects of DSAs on graft failure (GF), including graft rejection (GR) and poor graft function (PGF), in patients who underwent haploidentical SCT either with T cell depletion or with T cell replete [13–15]. However, there is no data on the relationship of DSAs with PT after haploidentical SCT. Here, we performed a retrospective analysis to investigate the association of DSAs with the occurrence of PT in patients who underwent unmanipulated HBMT.

2. Materials and Methods

2.1. Patients. The consecutive patients who received unmanipulated HBMT from March 2010 to March 2014 at Peking University Institute of Hematology were enrolled in this study. All cases underwent DSA examination and had the complete data of DSA before transplantation. The transplant protocol was approved by the Institutional Review Board of Peking University People's Hospital, and the IRB approval number is 2012-27. The clinical trial registration number is NCT01617473. All patients signed informed consent forms. This study was conducted in accordance with the Declaration of Helsinki. The characteristics of patients and donors were shown in Table 1.

2.2. Transplant Protocol. The unmanipulated HBMT was performed as previously described [16, 17]. Patients were conditioned with busulfan (BU, 0.8 mg/kg iv, q6h), cyclophosphamide (CTX, 1.8 g/m^2/d for 2 days), and antithymocyte globulin (rabbit ATG, Sang Stat, Lyon, France) (2.5 mg/kg/d iv for 4 days) or total body irradiation (TBI, 7.7 Gy), CTX, and ATG. All patients received G-CSF-mobilized bone marrow (BM) and peripheral blood stem cell transfusion. Cyclosporine A, mycophenolate mofetil, and short-term methotrexate were used for prophylaxis of graft-versus-host disease (GVHD).

2.3. Anti-HLA Antibody and DSA Examination. The patients and donors underwent HLA allele typing of at least the A, B, and DRB1 loci routinely. The examination was performed as previously [15]. In brief, patient plasma/serum was screened for class I and class II HLA antibodies with a LABScreen Mixed Kit (One Lambda, Canoga Park, CA, USA). The samples were incubated with mixed HLA class I- and class II-coated microspheres for 30 min in the dark and then washed before being incubated with anti-human immunoglobulin G-conjugated fluorescein isothiocyanate as described above for the first incubation. Finally, the samples were examined by a Luminex 200 flow analyzer (Luminex, Austin, TX, USA), and the data were analyzed with the HLA Fusion 3.2 software (One Lambda). The MFI of anti-HLA antibodies was obtained using the formula: sample beads − negative control beads. The samples with a MFI > 500 were further tested for the specificity of the antibody (DSA), using a LABScreen Single Antigen Kit (One Lambda).

2.4. Definitions of Engraftment. Neutrophil engraftment was defined as achieving an ANC more than 0.5×10^9/L for three consecutive days, and platelet recovery was defined as

Table 1: Patient and donor characteristics.

Characteristics	$n = 394$
Median age (range), years	26 (2–58)
Male sex, *n* (%)	231 (58.6%)
Diagnosis	
AML, *n* (%)	160 (40.6%)
ALL, *n* (%)	133 (33.8%)
CML, *n* (%)	22 (5.6%)
MDS, *n* (%)	41 (10.4%)
Others, *n* (%)	38 (9.6%)
Disease status, SR (%)	301 (76.4%)
Conditioning regimen	
MA, *n* (%)	394 (100%)
Number of HLA-A, B, DR mismatched, *n* (%)	
0	3 (0.8%)
1	22 (5.6%)
2	87 (22.1%)
3	282 (71.6%)
Donor-recipient sex match, *n* (%)	
Male-male	149 (37.8%)
Male-female	102 (25.9%)
Female-male	84 (21.3%)
Female-female	59 (15%)
Donor-recipient relationship, *n* (%)	
Father-child	150 (38.1%)
Mother-child	57 (14.5%)
Sibling-sibling	117 (29.%)
Child-parent	58 (14.7%)
Others	12 (3.0%)
ABO matched, *n* (%)	
Matched	224 (56.9%)
Major mismatched	74 (18.8%)
Minor mismatched	22 (5.6%)
Bidirect mismatched	74 (18.8%)
Cell compositions in allografts, median (range)	
Infused nuclear cells, 10^8/kg	8.23 (1.78–23.69)
Infused CD34+ cells, 10^6/kg	2.61 (0.39–16.82)
Infused lymphocytes, 10^8/kg	2.93 (0.16–9.49)
Infused CD3+ cells, 10^8/kg	2.0 (0.1–5.93)
Infused CD4+ cells, 10^8/kg	1.1 (0.15–3.94)
Infused CD8+ cells, 10^8/kg	0.69 (0.05–2.47)
Infused CD14+ cells, 10^8/kg	1.48 (0.19–6.13)
aGVHD	
Grade 0-I aGVHD	259 (65.7%)
Grade II–IV aGVHD	135 (34.3%)
cGVHD	
No cGVHD	266 (67.5%)
cGVHD	128 (32.5%)

Table 2: The HLA-antibodies and DSA in different statuses of engraftment.

	Good engraftment	PT	PGF	GR
With positive anti-HLA antibody (*n*, %)	27/295 (9.2%)	6/32 (18.8%)	12/39 (30.8%)	4/4 (100%)
Median DSA MFI	3096	9374	4843	17214
Range of DSA MFI	504–12969	1403–18950	600–11736	3793–19948

achieving a platelet count more than 20×10^9/L without platelet transfusions for seven consecutive days. Chimerism analysis was performed by DNA fingerprinting for short-tandem repeats in blood samples and/or chromosome fluorescence in situ hybridization of BM samples [18]. Full donor chimerism was defined as ≥95% leukocytes of donor origin in peripheral blood samples. Mixed chimerism was defined as more than 5% but less than 95% leukocytes of donor origin.

Primary GF consists of GR and PGF. GR is the failure to engraft neutrophils by day +28 for three consecutive days and the absence of donor hematopoiesis. PGF was defined as the presence of three cytopenic counts (ANC $\leq 0.5 \times 10^9$/L, platelet $\leq 20 \times 10^9$/L, and hemoglobin (HGB) ≤80 g/L) beyond day +28 with a complete donor chimerism in the absence of severe GVHD or hematological relapse. PT was defined as the engraftment of all peripheral blood cell lines other than a PLT count $\geq 20 \times 10^9$/L or dependence on PLT transfusions for more than 90 days after allo-HSCT in the presence of complete donor chimerism. Patients with evidence of PGF or hematologic relapse within 90 days after transplantation were excluded.

2.5. Statistical Analysis. The reference date of June 30th, 2016, was used to define the end of follow-up. The median follow-up was 796 (range: 25–2309) days. The Fisher exact test or Wilcoxon test was used for two-group comparisons. Disease-free survival (DFS), TRM, and overall survival (OS) were calculated according to Kaplan-Meier statistics. Death before +90 d, GR, and PGF were considered a competing risk for PT. The difference of PT between groups was tested according to Gray's method, using R software for statistical computing. A two-sided P value of 0.05 was considered significant. The log-rank test was used for comparisons of Kaplan-Meier curves. Potential prognostic factors for PT, OS, DFS, relapse, and TRM were examined using Cox proportional hazards models. SPSS 22.0 software was used (Mathsoft, Seattle, WA, USA) for statistical analysis.

3. Results

3.1. The General Clinical Characteristics of Patients. Total of 394 patients were enrolled in this study. The median age of patients was 26 years (range, 2–58 years). All patients received a myeloablative conditioning regimen. The median dose of infused $CD34^+$ cells was 2.61 $(0.39–16.82) \times 10^6$/kg. Except for patient age ($P = 0.020$), cases with PT and those without PT had equivalent patient and donor characteristics (Table 1).

3.2. Engraftment. Three hundred ninety patients achieved myeloid engraftment except for 4 patients (1.0%). The median time to neutrophil engraftment was 13 days (range, 8–27 days). Up to the follow-up, the incidence of platelet engraftment was 91.9% and the median time to platelet engraftment was 17 days (range, 6–265 days). 32 patients did not meet the criteria of platelet engraftment. Total of 4 patients developed GR and 39 patients (9.9%) were PGF. Among the patients who met the criteria of PGF, 9 patients died before +90 days and 16 patients later met the criteria of PT. In patients who met the criteria of PT, 16 patients only met the criteria of PT but not PGF. Thus, the cumulative incidence of PT in this cohort of patients was 9.9 ± 1.5%.

3.3. HLA Antibodies and DSA. Among all the patients, there were 99 (25.1%) with positive anti-HLA antibody, consisted of 48 males and 51 females. Of these positive patients, 63 (16.0%) had antibodies against HLA class I antigens and 57 (14.5%) had antibodies against HLA class II antigens. 31 (7.9%) patients had HLA antibodies against both classes I and II. In all HLA antibody positive patients, 45 (45.5%) had positive DSA. Of the 394 cases tested, 45 (11.4%) were DSA positive. Because we previously showed that high antibody titers of DSAs (MFI ≥ 10,000) were correlated to primary GR ($P < 0.001$) and that low antibody titers of DSAs (MFI ≥ 2000) were strongly associated with primary PGF ($P = 0.005$) [15], here, we analyzed the impacts of DSAs (MFI ≥ 1000) and DSAs (MFI ≥ 2000) on PT. Of the DSA positive patients, the MFI of 37 (9.4%) patients were more than 1000 and the MFI of 31 (7.9%) patients were more than 2000. The detailed information about HLA antibodies and DSA was shown in Table 2.

3.4. Relationship of DSAs with PT after Unmanipulated HBMT. The percentages of cases with positive anti-HLA antibody were 9.2% (27/295) in patients with good engraftment, 18.8% (6/32) in patients with PT, 30.8% (12/39) in patients with PGF, and 100% in patients with GR (Table 2). By using a competing risk analysis, the incidence of PT was higher in patient with a MFI ≥ 1000 compared with those with a MFI < 1000 (16.8 ± 6.4% versus 7.4 ± 1.4%, $P = 0.05$). For the competing events including death before +90 d, GR, and PGF, the incidence of PT was higher in patients with a MFI ≥ 1000 compared with those with a MFI < 1000 (29.9 ± 7.7% versus 6.5 ± 1.3%, $P < 0.001$). Univariate analysis showed that factors including elder ($P = 0.020$), infused lower dose of $CD34^+$ cells ($P = 0.061$), positive HLA antibody ($P = 0.092$), and HLA class II antibody ($P = 0.056$) and DSAs (MFI ≥ 1000, $P = 0.058$) were associated with PT after unmanipulated HBMT (Table 3). However, multivariate analysis showed that the presence of DSAs (MFI ≥ 1000) (hazard ratio (HR) 3.262; 95% confidence interval (CI), 1.339–7.946; $P = 0.009$) was independently associated with PT. It also

Table 3: Patient characteristics in groups with and without PT.

	PT	Without PT	*P* value
N	32	362	
Patient age, median (range)	36 (3–54)	26 (2–58)	**0.020**
Patient sex, male, *n* (%)	17 (53.1)	214 (59.1)	0.320
Diagnosis, *n* (%)			0.510
AML	13 (40.6)	147 (40.6)	
ALL	14 (43.8)	119 (32.9)	
CML	1 (3.1)	21 (5.8)	
MDS	3 (9.4)	38 (10.5)	
Others	1 (3.1)	37 (10.2)	
Disease risk, *n* (%)			
SR	22 (68.8)	279 (77.1)	0.288
HR	10 (31.3)	83 (22.9)	
HLA incompatibility, *n* (%)			0.533
0 locus	0	3 (0.8)	
1 locus	2 (6.3)	20 (5.5)	
2 loci	4 (12.5)	83 (22.9)	
3 loci	26 (81.3)	256 (70.7)	
Donor-patient relation, *n* (%)			0.260
Sibling donor	9 (28.1)	108 (29.8)	
Father donor	4 (12.5)	53 (14.6)	
Mother donor	9 (28.1)	141 (39.0)	
Children donor	9 (28.1)	49 (13.5)	
Others	1 (3.1)	11 (3.0)	
Donor-patient sex match, number (%)			0.722
Male to male	11 (34.4)	138 (38.1)	
Male to female	11 (34.4)	91 (25.1)	
Female to male	6 (18.8)	78 (21.5)	
Female to female	4 (12.5)	55 (15.2)	
ABO matched, *n* (%)			0.213
Matched	15 (46.9)	209 (57.7)	
Major mismatched	8 (25.0)	66 (18.2)	
Minor mismatched	5 (15.6)	69 (19.1)	
Bidirect mismatched	4 (12.5)	18 (5.0)	
Cell compositions in allografts, *n* (%)			
Infused nuclear cells ≥ median	15 (46.9)	181 (50.0)	0.735
Infused $CD34^+$ cells ≥ median	11 (34.4)	187 (51.7)	**0.061**
Infused lymphocytes ≥ median	13 (48.1)	160 (50.3)	0.829
Infused $CD3^+$ cells ≥ median	14 (51.9)	160 (50.3)	0.878
Infused $CD4^+$ cells ≥ median	16 (59.3)	159 (50.0)	0.356
Infused $CD8^+$ cells ≥ median	16 (59.3)	157 (49.4)	0.324
Infused $CD14^+$ cells ≥ median	16 (59.3)	157 (49.4)	0.324
Grade II–IV aGVHD	12 (44.4)	105 (33.0)	0.229
cGVHD	8 (25.0)	120 (33.2)	0.320
HLA antibody positive	12 (37.5)	87 (24.0)	**0.092**
HLA-I antibody positive	6 (18.8)	57 (17.9)	0.579
HLA-II antibody positive	8 (25.0)	49 (13.5)	**0.056**
HLA-DP antibody positive	3 (9.4)	29 (8.0)	0.732
DSA positive	6 (18.8)	39 (10.8)	0.174

Table 3: Continued.

	PT	Without PT	*P* value
MFI > 1000	6 (18.6)	31 (8.6)	**0.058**
MFI > 2000	4 (12.5)	27 (7.5)	0.310

AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CML: chronic myeloid leukemia; MDS: myelodysplastic syndrome; SR: standard risk; HR: high risk; aGVHD: acute graft-versus-host disease; cGVHD: chronic graft-versus-host disease; HLA-I/II: class I/II HLA antibody; HLA-DP: class I and II HLA antibody double positive; DSA: donor-specific antibody; DSA-DP: class I and II DSA double positive; MFI: median fluorescent intensity. Bold means variates included in the multivariate analysis since their *P* value < 0.1 in the univariate analysis.

Table 4: Multivariate analysis of factors associated with transplant outcomes.

	HR	95% CI	*P* value
Prolonged isolated thrombocytopenia			
Infused CD34+ cells ≥ median	0.481	0.232–1.001	0.050
DSA			
MFI ≥ 1000	3.262	1.339–7.946	0.009
TRM			
Disease status	1.753	(0.985–3.118)	0.056
Infused CD4+ cells ≥ median	1.899	(1.079–3.340)	0.026
DSA			
MFI ≥ 1000	2.320	(1.169–4.426)	0.044
Relapse			
Disease status	3.882	(2.103–7.166)	<0.001
HLA-DP	2.552	(1.139–5.742)	0.0230
DFS			
Disease status	2.667	(1.761–4.038)	<0.001
cGVHD	0.622	(0.423–0.916)	<0.016
OS			
Disease status	2.504	(1.643–3.817)	<0.001
cGVHD	0.612	(0.413–0.908)	0.015
DSA			
MFI ≥ 1000	1.220	(0.998–2.138)	0.082

seemed that an infused lower dose of $CD34^+$ cells might predict a higher incidence of PT (HR 0.481; 95% CI, 0.232–1.001; $P = 0.05$, Table 4).

3.5. Relationship of DSAs with Transplant Outcomes. To further investigate the relationship of DSAs with other transplant outcomes including TRM, relapse, DFS, and OS, we also took the above factors into the univariate and multivariate analyses. In the univariate analysis, DSAs (MFI ≥ 1000, $P = 0.076$), grade II–IV acute GVHD ($P = 0.033$), infused $CD4^+$ ($P = 0.024$) and $CD3^+$ cells ($P = 0.088$), donor-recipient relationship ($P = 0.097$), and disease status at the transplantation ($P = 0.029$) were associated with TRM. The results of multivariate analysis showed that an infused higher dose of $CD4^+$ cells and DSAs (MFI ≥ 1000) were the independent risk factors of TRM (Table 4). For relapse, disease status ($P < 0.001$), sex match ($P = 0.09$), and HLA-DP ($P = 0.024$) were brought into multivariate analysis. Finally, patients in high risk and HLA-DP were related to leukemia relapse (Table 4). For DFS, chronic GVHD ($P = 0.011$), disease status ($P < 0.001$), and sex match ($P = 0.094$) were conducted into multivariate analysis. As our expected, patients in high risk and with chronic GVHD were associated with DFS (Table 4). It showed that with chronic GVHD ($P = 0.006$), disease status ($P < 0.001$) and DSAs (MFI ≥ 1000, $P = 0.09$) were related to OS in univariate and multivariate analyses though DSAs (MFI ≥ 1000) did not show a statistical significance (Table 4).

3.6. Effects of PT on Transplant Outcomes. Compared to cases with PT, patients without PT had lower incidence of TRM (16.3 ± 0.02% versus 32.5 ± 0.09%, $P = 0.043$), similar incidence of relapse (12.3 ± 0.02% versus 14.4 ± 0.07%, $P = 0.677$), and higher probabilities of DFS (74.7 ± 0.02% versus 56.3 ± 0.88%, $P = 0.046$) and OS (75.2 ± 0.02% versus 56.3 ± 0.88%, $P = 0.047$) (Figure 1). Multivariate analysis showed that the onset of PT was independently associated with TRM (HR, 2.717; 95% CI, 1.343–5.498; $P = 0.005$). Besides, patients with PT seemed also to have a worse DFS (HR, 1.695; 95% CI, 0.984–2.921; $P = 0.057$) and OS (HR, 1.629; 95% CI, 0.928–2.858; $P = 0.089$).

4. Discussion

The association of DSAs with graft failure has been demonstrated by other researchers and us in either HLA-matched unrelated donor transplant, umbilical cord blood transplantation, or haploidentical transplantation [10–12, 15, 19]. In this study, we, for the first time, found an association of the presence of DSAs with PT after unmanipulated HBMT, indicating that DSAs may be involved in the pathogenesis of this complication. In addition, patients with the onset of PT-experienced inferior transplant outcomes provide further evidence suggesting that the presence of DSAs should be incorporated in the donor selection algorithm [20, 21].

The definition of a threshold for DSAs, according to MFI, is a premise for analyzing the association of DSAs with PT after transplantation. In previous studies, several cutoff values, such as MFI >500, 1000, 1500, 2000, and 5000, have been defined as DSA positive that was associated with graft failure in different transplant modalities with different conditioning regimens and GVHD prophylaxis [10, 11, 14, 15, 22]. In this study, the cutoff value of DSA MFI was 1000, which was associated with the onset of PT after unmanipulated HBMT. Our previous observation indicated that patients with DSA MFI ≥ 10000 experienced graft rejection and cases with DSA MFI ≥ 2000 experienced PGF. Our results suggest that high, intermediate, and low antibody titers of DSAs may lead to GR, PGF, and PT, respectively [15]. The fact that patients with PT experienced higher cumulative incidence of

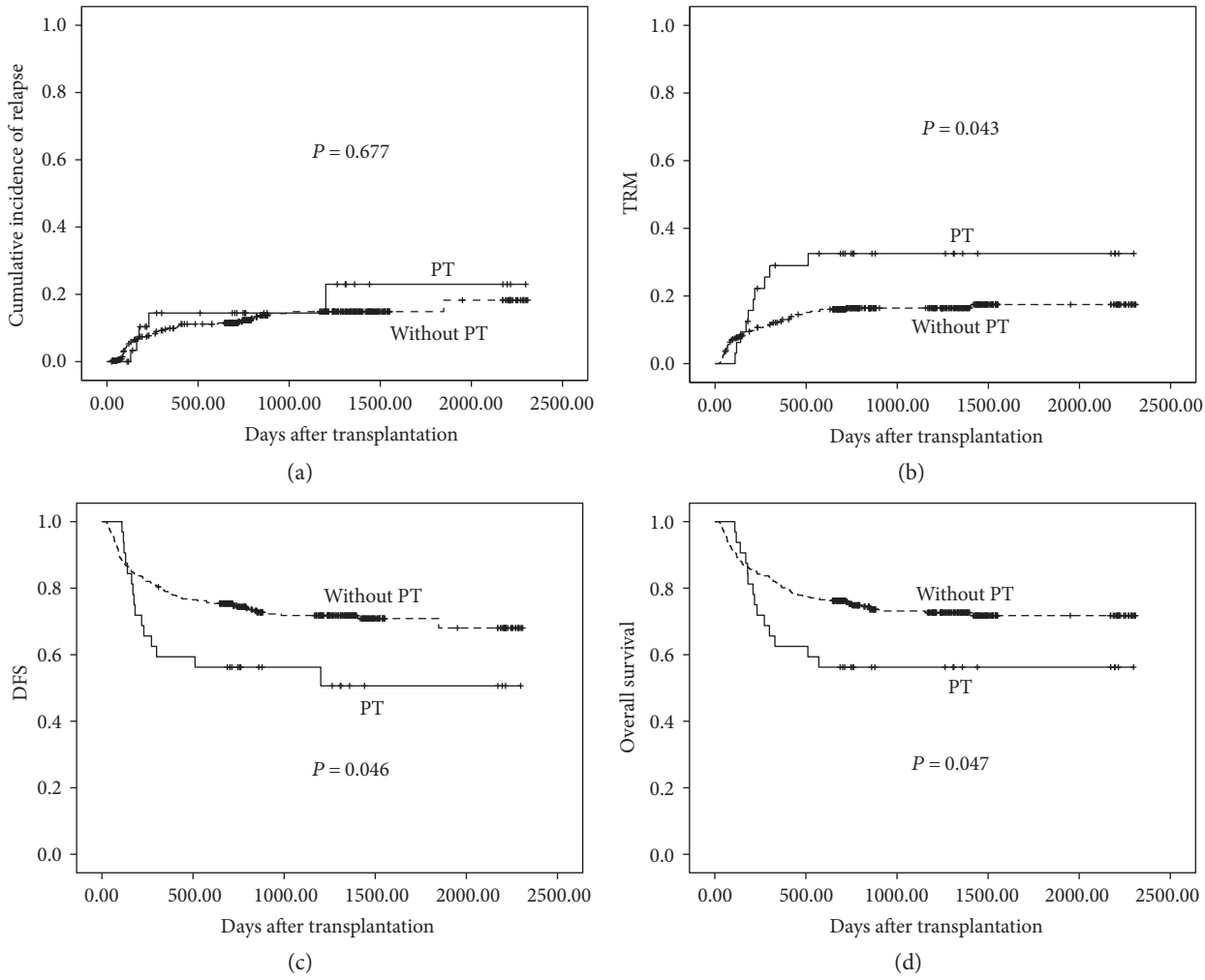

Figure 1: PT and transplant outcomes after haploidentical transplantation. (a) Cumulative incidence of relapse (CIR) and (b) TRM. (c) Disease-free survival. (d) Overall survival.

TRM and inferior DFS and OS supports the logical theory that the presence of DSAs results in PT, which may contribute to inferior survival.

Barge et al. [23] have found that DSAs may kill donor cells through antibody-dependent cell-mediated cytotoxicity (ADCC). In vitro experiment performed by us showed that DSAs could induce apoptosis of $CD34^+$ cells and endothelial progenitor cells (EPCs) in the allografts (unpublished data). Kong et al. [24] from our center reported that the PT patients exhibited remarkable decreases in cellular elements of the vascular microenvironment of the bone marrow, including EPCs and perivascular cells, compared to the cases with good graft function after transplantation and the healthy donors, respectively. A multivariate analysis revealed that EPCs were an independent risk factor for PT. Their data suggested that an impaired BM vascular microenvironment may contribute to the occurrence of PT after allo-SCT [24]. It is reasonable that DSAs may lead to BM vascular microenvironment impairment via inducing apoptosis of EPCs, although further studies are needed. Our group also demonstrated that the recruitment of $CD8^+$ T cells into BM might explain the suppression of megakaryocyte apoptosis through the elevated expression of $CX3CR1^+$ in PT after allo-SCT [25]. These results indicate that an immune-mediated mechanism may contribute to the pathogenesis of PT after unmanipulated HBMT.

The present study had several limitations. First, our study is a single-center retrospective study. A prospective study with a training group and validation group is needed. Second, the present study only investigated the unmanipulated HBMT modality with an ATG conditioning regimen. Therefore, our results should be further confirmed in a multicenter clinical trial and in T cell-depleted haploidentical SCT modalities or in T cell-replete haploidentical SCT settings with postcyclophosphamide [26].

In conclusion, our results not only, for the first time, suggested an association of DSAs with PT after unmanipulated HBMT but also confirmed the effects of PT on inferior transplant outcomes. In addition, our study is clinically relevant and provides further evidence that DSAs should be incorporated in the algorithm for unmanipulated HBMT.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Yingjun Chang designed the study. Yingjun Chang and Xiaosu Zhao collected the data. Yingjun Chang and Xiaosu Zhao analyzed the data and wrote the manuscript. All authors contributed to the interpretation of the data, preparation of the manuscript, and approval of the final version.

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Review Article

Not All Antibodies Are Created Equal: Factors That Influence Antibody Mediated Rejection

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Consistent with Dr. Paul Terasaki's "humoral theory of rejection" numerous studies have shown that HLA antibodies can cause acute and chronic antibody mediated rejection (AMR) and decreased graft survival. New evidence also supports a role for antibodies to non-HLA antigens in AMR and allograft injury. Despite the remarkable efforts by leaders in the field who pioneered single antigen bead technology for detection of donor specific antibodies, a considerable amount of work is still needed to better define the antibody attributes that are associated with AMR pathology. This review highlights what is currently known about the clinical context of pre and posttransplant antibodies, antibody characteristics that influence AMR, and the paths after donor specific antibody production (no rejection, subclinical rejection, and clinical dysfunction with AMR).

1. Introduction

Antibody mediated rejection (AMR) is a major contributor to rejection risk and allograft loss in solid organ transplantation [1, 2]. AMR diagnostic criteria were first established in cardiac [3] and renal [4] transplantation and have recently been described for pancreas [5] and lung transplantation [6] and, although historically controversial, are proposed for liver [7] and intestinal [8] allografts as well. AMR incidence is approximately 10–20% in cardiac [9], 5–8% in renal [10], 4–25% in lung [11, 12], and 24% in liver [13] transplant. Central features of AMR pathology include endothelial cell (EC) swelling, microvascular inflammation, and intravascular CD68+ macrophages with or without complement deposition. Antibodies, most notably those specific for human leukocyte antigen (HLA), mediate effector functions that manifest in the histopathology of AMR. HLA are the most polymorphic genes in the human genome and as such result in the development of alloantibodies when an exact match is not found, as the donor allograft contains foreign HLA. The presence of HLA donor specific antibodies (DSA) is highly indicative of AMR [14, 15]. The advent of HLA DSA detection methods [16–18] has led to studies identifying the correlation of HLA DSA with more severe AMR, yet we still are unable to fully predict how harmful or "pathogenic" DSA will be. Further complicating matters is the recent association of non-HLA antibodies with allograft rejection. A greater understanding of all the factors (donor and recipient characteristics and antibody attributes) that contribute to rejection is needed to enhance the predictive performance of risk assessments and better determine which patients are at an increased risk for AMR. This review will address the clinical context of pre- and posttransplant antibodies, HLA and non-HLA antibody characteristics that influence AMR, and the three outcomes (stable function, subclinical dysfunction, and clinical dysfunction with AMR) mediated by these antibodies.

2. HLA Antibodies

2.1. Pretransplant. Pretransplant sensitization can occur from prior transplants, blood transfusions, pregnancy, and mechanical assist devices (in heart failure) resulting in autoantibody formation. Allosensitization affects approximately 6–9% of cardiac transplant candidates [19, 20] and 23% of renal transplant candidates prior to transplantation [21]. Patients who are presensitized have a significantly increased risk of developing AMR within the first three years after cardiac transplantation compared to those who are not sensitized [22].

Preformed HLA antibodies were also associated with AMR in kidney transplantation [23–25]. In a multicenter prospective clinical study, kidney allograft recipients with HLA antibodies were associated with an increased risk for graft failure 1 year after transplant [26]. In liver transplantation, preformed DSA has been associated with an increased risk of AMR [7, 27]. Roux et al. found that preformed HLA DSA was associated with AMR, chronic dysfunction, and graft loss in a lung transplant cohort with 2-year follow-up [28].

2.2. Posttransplant. After transplantation, 24% of renal allograft recipients will develop de novo HLA DSA within ten years [29] and approximately 25% of cardiac allograft recipients will develop de novo HLA DSA within thirteen years [30]. Nearly one-third of low risk patients (first transplant, no DSA) develop de novo DSA by 12 years after transplant [31]. De novo DSA development rates are 25–50% after lung transplantation [6]. Additionally, long-term posttransplant follow-up of renal allograft recipients revealed a significant decline in the ten-year graft survival rate for recipients that developed de novo antibodies to HLA [32] compared to those that did not. Smith et al. reported that de novo and persistent DSA postcardiac transplant were associated with poor long-term patient survival [30]. De novo DSA in liver transplantation is also associated with AMR [27, 33]. Many studies in lung transplant do not address the temporal timing of DSA potentially because AMR has only recently been recognized in lung transplantation and the presence of circulating DSA (regardless of preformed or de novo) is a key diagnostic standard [6]. However, multiple studies have found an associated risk of AMR in patients with DSA in lung transplantation [12, 34].

3. Non-HLA Antibodies

3.1. Pretransplant. Non-HLA antigens have been shown to be expressed intracellularly, on the EC cell surface and to apoptotic cells [35]. Non-HLA antibodies can occur independently or can occur concurrently with HLA DSA within patients, sometimes creating a synergistic effect on the allograft [36]. Additionally, antibodies specific for angiotensin II type 1 receptor (AT_1R) can precede de novo HLA DSA [36]. AT_1R antibodies are autoantibodies. Pretransplant AT_1R antibodies have been associated with AMR in kidney [37, 38] and heart [39] transplant recipients. MICA is a polymorphic nonclassical class I antigen that is closely linked to the HLA-B locus and is upregulated on endothelial and epithelial cells during cellular stress. Antibodies to MICA have been found in the serum of renal [40] transplant recipients and were associated with humoral rejection and graft loss. Perlecan is a heparin sulfate proteoglycan that is cleaved by cathepsin-L to form a C-terminal fragment called LG3, as it contains three laminin-like globular domains. Pretransplant LG3 antibodies have been found to be associated with acute vascular rejection independent of HLA DSA in kidney transplant recipients [41]. Patients with preformed collagen V and Kα-tubulin antibodies were at an increased risk of developing HLA DSA, and bronchiolitis obliterans syndrome (BOS) [42], a manifestation of chronic rejection.

3.2. Posttransplant. AT_1R antibodies are associated with AMR in cardiac [39] and renal [43–45] transplantation. Others have reported that concomitant HLA and AT_1R antibodies in renal and cardiac transplantation increased the risk of AMR and decreased graft survival [36, 46]. Endothelin type A receptor (ET_AR) antibodies have also been reported in renal [47] and cardiac [39] transplantation. Antibodies against AT_1R and ET_AR have also been shown to be increased in lung allograft recipients with cystic fibrosis [48]. Antibodies to MICA have been found in the serum of renal [40] and cardiac [49] transplant recipients, although conflicting evidence exists about the independent pathogenic role of MICA in chronic rejection [50, 51]. Posttransplant LG3 antibodies have been found in renal transplant recipients [41, 52]. Collagen V (Col V) is an extracellular matrix protein expressed on the lung interstitium and lung epithelial cells. Col V antibodies have been found in sera from lung allograft recipients diagnosed with bronchiolitis obliterans syndrome (BOS), a manifestation of chronic rejection [53, 54]. Additionally, Col V autoantibodies are associated with AMR and cardiac allograft vasculopathy (CAV) in cardiac transplant recipients [55] and transplant glomerulopathy in renal allograft recipients [56].

4. Three Paths after DSA

Patients with DSA do not represent a uniform category. Patients are either transplanted with no allosensitization, with HLA antibodies but no donor specific antibodies (3rd party), or with preformed DSA. Additionally, patients with a history of sensitization may never have circulating DSA detected in screening protocols, even though they have formed T and B cell alloimmune memory. Despite strong evidence that DSA are associated with increased rejection incidence and reduced graft survival, it is unknown why a subset of patients with DSA does *not* experience poorer graft outcomes in these studies [31, 57, 58]. This creates uncertainty about how to manage patients who exhibit DSA on routine monitoring but have no clinical signs of graft dysfunction or whether a preformed HLA DSA of a certain strength or titer can be safely crossed. Extraordinarily high levels of DSA (>10,000 MFI in our experience), especially to HLA class I antigens, have been shown to be cytotoxic and place patients at risk of hyperacute rejection via complement activation; such strong DSA are typically avoided with the exception of liver transplantation [59, 60]. Transplant recipients with DSA can exhibit overt rejection (acute or chronic) with clinical dysfunction, indolent dysfunction (slow decline in graft function) with subclinical rejection on protocol biopsy, or stable function and normal biopsy (Figure 1).

4.1. Clinical Dysfunction with AMR. Evidence of clinical allograft dysfunction is an important consideration in diagnosis of symptomatic (clinical) AMR. Nearly half of patients transplanted with preformed DSA experienced AMR, compared

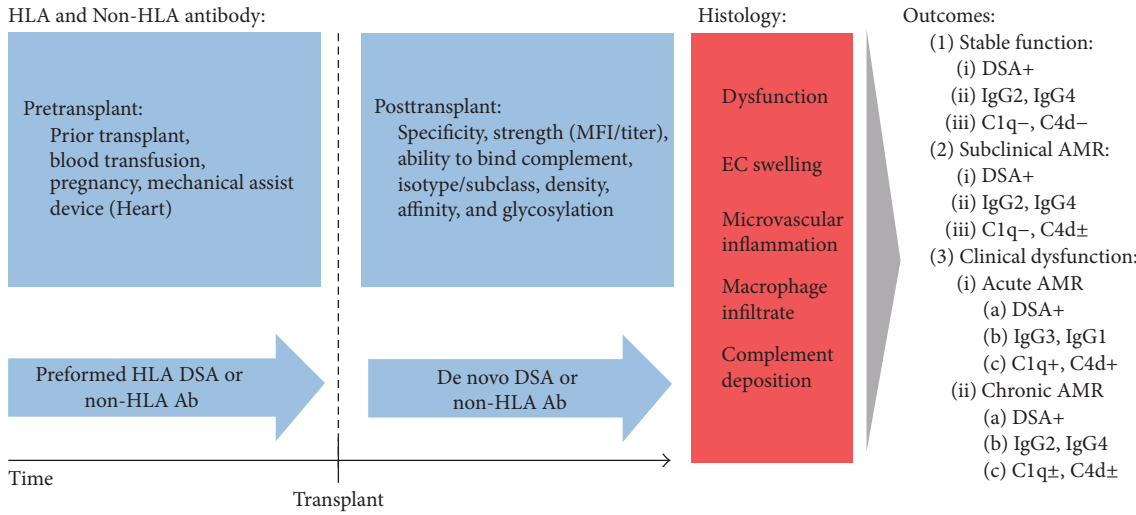

Figure 1: Factors influencing AMR. Schematic of the antibody components that influence AMR's pathogenesis. Depicted are the antibody factors (blue) that influence AMR pathology (shown in red). Antibody factors influencing AMR include sensitization pretransplant and antibody attributes such as specificity, ability to bind complement, isotype/subclass, strength (MFI/titer), density, affinity, and glycosylation. AMR histology (red) includes graft dysfunction, endothelial cell (EC) swelling, microvascular inflammation, and macrophage infiltrate and can occur with or without complement deposition. The three outcomes after DSA include stable function, subclinical AMR and clinical dysfunction with AMR (either acute or chronic). Stable function in the presence of DSA is typically seen in those patients with IgG2/IgG4 antibodies that do not show signs of complement binding antibodies (C1q−, C4d−). Subclinical AMR is typically seen in those patients with IgG2/IgG4 antibodies that may show signs of complement binding antibodies (C1q−, C4d±). Clinical dysfunction with AMR can be grouped into acute or chronic AMR. Acute AMR is typically seen in those patients with IgG3/IgG1 antibodies that are complement binding antibodies (C1q+, C4d+). Chronic AMR is typically seen in those patients with IgG2/IgG4 antibodies that may or may not include complement binding antibodies (C1q±, C4d±).

with less than 1% in those without pretransplant DSA [61]. Of renal transplant recipients with preformed DSA who developed AMR, the majority were flow crossmatch positive [61–63]. De novo DSA is often observed at the same time as clinical dysfunction [32], and the vast majority of patients presenting with allograft functional impairment and dnDSA were nonadherent [32]. Thus patients are more likely to develop AMR if their DSA is strong enough to cause a positive flow crossmatch and more likely to experience graft dysfunction if they were medication nonadherent. In the long-term, patients experiencing clinical dysfunction with AMR have the worst 5-year graft survival compared with TCMR or no rejection [58].

4.2. Subclinical AMR. Studies evaluating protocol biopsies have reported a high incidence of subclinical AMR that is likely missed by monitoring strategies that biopsy only for cause. One-year surveillance biopsies in DSA+ patients with stable function nonetheless often revealed C4d deposition and peritubular capillaritis [32], indicative of "smoldering" inflammation not present in patients without DSA. Similarly, Loupy et al. showed that 14% of clinically stable renal transplant recipients had evidence of subclinical AMR on one-year surveillance biopsy [57]. The majority of these patients had performed DSA. Importantly, these studies have demonstrated that patients with subclinical AMR (i.e., no acute dysfunction) fare significantly worse than their DSA negative counterparts [57], with faster decline in GFR of renal allografts [31, 57, 61] and higher rates of CAV in cardiac allografts [64–66]. Renal transplant recipients with subclinical AMR who received treatment with plasmapheresis unfortunately had comparable outcomes to those who were not untreated, and both had a significant decrement in 5-year survival compared with AMR-free controls [61]. Similarly, half of cardiac transplants that failed more than one year after transplant due to chronic rejection had a history of subclinical AMR [67]. While patients with clinically symptomatic AMR fare worse than those with subclinical AMR, both groups have significantly reduced 10-year outcomes compared with stable, DSA negative patients [31].

4.3. DSA with Stable Function and No Rejection. Intriguingly, up to half of patients with preformed DSA did *not* have rejection, subclinical AMR, or otherwise, at the time of one-year biopsy [57, 68]. Approximately 20% of stable patients with no evidence of rejection on protocol biopsy also had DSA. Thus, a critical, yet unanswered, question is which patient, donor, and antibody characteristics might protect from rejection and graft dysfunction in the presence of DSA, a question which is addressed in part in the next section.

5. Mechanisms of Antibody Mediated Graft Injury

5.1. HLA Antibodies. Antibodies mediate allograft injury and contribute to graft pathology through three main types of

effector functions: EC activation, complement activation, and leukocyte interaction/activation. Alterations in these effector functions modulate rejection severity. Antibody characteristics, such as titer, isotype/subclass, glycosylation, and affinity, can influence these effector functions. The interface between the allograft and its recipient is the thin layer of donor EC lining the walls of the blood vessels supplying nutrients to the allograft. Gene profiling studies of renal [69–71] and cardiac biopsies [72] undergoing AMR identified EC activation as a significant contributor to graft pathology. Crosslinking of HLA expressed on the surface of EC by DSA triggers a series of intracellular signaling events and activation of immune responses, which are manifested in the histopathological findings in AMR pathology [73]. DSA binding to HLA induces EC activation, resulting in P-selectin expression and mammalian target of rapamycin (mTOR) dependent cellular migration, proliferation, and protein synthesis [74–77]. Positive staining of phosphorylated mTOR signaling proteins including S6 kinase and S6 ribosomal protein in the capillary EC of endomyocardial biopsies strongly correlated with diagnosis of AMR [78, 79]. EC activation facilitates chemokine expression leading to leukocyte recruitment to inflammatory sites [80]. In addition, increased EC and smooth muscle proliferation results in a thickening of the tunica intima [81], a hallmark of chronic AMR in all solid organ transplant patients [3]. Antibody titer affects EC signal transduction and subsequent EC activation, as increasing quantities of HLA antibody result in augmented FGFR expression and cellular proliferation [82], whereas decreased antibody titer results in upregulation of prosurvival genes and antiapoptotic proteins in EC [83]. An additional antibody-independent factor that influences HLA-mediated signaling is the density of HLA molecule expression on the EC surface. HLA antigen expression on graft endothelium is increased during allograft rejection in response to IFNγ and induces CIITA activation and subsequent HLA Class II expression [84–87]. The density of HLA on the surface of EC directly affects the degree of DSA binding to the graft and downstream effector functions.

Although complement deposition is no longer necessary for AMR diagnosis, complement binding DSA increases a patient's risk for kidney allograft loss five years after transplant [88] and complement binding antibodies were more predictive, than HLA DSA alone, of an increased risk for AMR and decreased graft survival in cardiac transplant ten years after transplant [89]. Antibody isotype and subclass play a significant role in induction of the classical complement pathway, with IgM, IgG3, and IgG1 having the highest degree of complement activation [90]. Antibody affinity mediated by IgG hexamers has been shown to be more efficient than isolated IgG molecules at activating the complement cascade [91]. Complement binding is also increased when there is an increase in the amount of antibody bound to cells [92, 93]. High panel reactive antibodies (PRA) are associated with increased complement activation [92]. Lastly, polymorphisms within the complement genetic locus could potentially affect the degree of complement activation [94, 95], whereas differential expression of complement regulatory proteins by the donor tissue could also affect the response of endothelium to complement components [96]. With respect to the downstream effects of complement activation, Janewit et al. demonstrated that complement activation and deposition on EC resulted in noncanonical NFκB activation [97] whereas Cravedi et al. highlighted a role for complement activation in promotion of a Th1 response during alloimmune reactions [98]. Taken together, this information highlights the potential contributions of DSA on complement activation and promotion of alloimmunity.

Leukocyte recruitment and activation are a common histological feature of AMR. Macrophage infiltration is observed in heart [3] and renal [99] AMR and predicts a worse outcome [100]. Neutrophil recruitment is seen in lung transplantation and intragraft natural killer (NK) cells have been identified by molecular microscopy techniques in renal [71] and cardiac biopsies [72] diagnosed with AMR. IgG subclass dictates Fc receptor binding affinity [101, 102], thereby influencing leukocyte recruitment. Several studies have attempted to characterize the repertoire of DSA immunoglobulin subclasses in transplant recipients and correlate them with allograft outcomes. Their results have suggested that IgG3 DSA are a driver of acute AMR [103, 104], while IgG4 correlates more closely with subclinical AMR [105] and chronic rejection [106, 107]. Moreover, different terminal moieties in the Fc glycan of IgG have been demonstrated to change the inflammatory nature of antibodies. Sialylated IgG promotes a more tolerant environment, whereas glycans with terminal galactose residues are affiliated with a proinflammatory response [108]. Altered P-selectin expression allows for an increase in leukocyte recruitment [77 depending on subclass, by engaging FcγRs, 109], a common histological feature across solid organ transplant [3, 6, 109]. DSA also facilitated NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) in an IFNγ and cell-contact dependent manner [110]. Collectively, the effector functions of DSA, while multifactorial themselves, are even more complex and multilayered when antibody characteristics are altered.

5.2. Non-HLA Antibodies. There is less mechanistic data for non-HLA antibodies in the pathogenesis of AMR. However, non-HLA antibodies can also mediate EC activation and complement activation and leukocyte interaction/activation. Non-HLA antibodies that activate EC can increase the expression of HLA class I and II and have been shown to develop independently or in conjunction with HLA DSA [36, 111]. AT_1R antibodies mediate endothelial cell activation and vasoconstriction by binding to the second extracellular loop of the AT_1R protein and act as an angiotensin II agonist promoting downstream activation of AP-1 and NF-κB [112]. AT_1R and ET_AR antibodies frequently occur together in patient sera [111]; but there are no studies linking their pathologic mechanism. DSA bound to EC are also capable of activating the classical complement pathway, resulting in detection of C4d deposition along the capillary walls within allograft biopsies [113–115]. C4d deposition in graft histology has only been detected in a subset of patients with AT_1R antibodies suggesting that the mechanism of injury for AT_1R antibodies is not the complement pathway [43, 44, 112]. However, other non-HLA antibodies such as MICA

can activate complement [116]. Evidence suggests that Col V antibodies increase IL-17 and IFNγ secreting T cells [117]. LG3 antibodies promote the migration of smooth muscle cells or mesenchymal stem cells to cause vascular injury [118].

Experimental models and clinical experience demonstrate that anti-donor HLA and non-HLA antibodies exhibit pathogenic functions through multiple mechanisms that likely have extensive crosstalk. AMR manifests as a broad spectrum both histologically and symptomatically. Across solid organs, the microvasculature is the principal target of antibody mediated injury. A single uniform approach to prevent graft injury and loss in the setting of donor specific antibodies will probably not be effective for all patients, and personalized therapies tailored to address unique patient and donor features will be needed to protect from AMR and chronic rejection. Non-HLA antibodies have also been associated with TCMR in renal transplantation [119] suggesting additional mechanisms of action that promote distinct graft pathology phenotypes compared to HLA DSA. While these histopathological features are diagnostically useful, they are an in situ readout of the downstream effects of DSA-mediated effector functions. Recent work is uncovering additional mechanisms by which DSA can mediate immune activation. Further studies are needed to delineate the crosstalk between HLA and non-HLA antibodies and their synergistic effect on graft injury and to assess their incidence across different organ types.

6. Conclusions and Future Directions

Collectively, data on antibody pathogenicity defined by the antibody specificity, isotype, and ability to activate EC and complement can lead to different effector functions that mediate different pathological outcomes. Further studies to clarify which HLA and non-HLA antibody attributes (strength, subclasses, glycosylation, and affinity) contribute to subclinical, acute, and chronic AMR would be useful in order to identify biomarkers of different outcomes. Employment of newer techniques, such as the "molecular microscope," can provide additional insight into the active transcriptome in the graft tissue, allowing a measurement of the local inflammation and the transcriptome signature for AMR [68]. Clinical research to determine how effective these parameters are at risk stratifying patients is needed. Enhanced understanding of the HLA and non-HLA mechanisms in allograft injury is needed to help identify additional therapeutic targets and further understand the potential synergistic relationship between them. Allograft rejection can occur throughout the lifetime of a transplanted organ and as such further understanding of the sensitization and pathologic mechanisms is needed to better risk stratify patients and achieve the goal of increasing long-term survival.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Carrie L. Butler, Nicole M. Valenzuela, and Kimberly A. Thomas contributed equally to this work.

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Research Article

De Novo Donor-Specific HLA Antibodies Developing Early or Late after Transplant Are Associated with the Same Risk of Graft Damage and Loss in Nonsensitized Kidney Recipients

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De novo posttransplant donor-specific HLA-antibody (*dn*DSA) detection is now recognized as a tool to identify patients at risk for antibody-mediated rejection (AMR) and graft loss. It is still unclear whether the time interval from transplant to DSA occurrence influences graft damage. Utilizing sera collected longitudinally, we evaluated 114 consecutive primary pediatric kidney recipients grafted between 2002 and 2013 for *dn*DSA occurrence by Luminex platform. *dn*DSAs occurred in 39 patients at a median time of 24.6 months. In 15 patients, *dn*DSAs developed within 1 year (*early-onset* group), while the other 24 seroconverted after the first posttransplant year (*late-onset* group). The two groups were comparable when considering patient- and transplant-related factors, as well as DSA biological properties, including C1q and C3d complement-binding ability. Only recipient age at transplant significantly differed in the two cohorts, with younger patients showing earlier *dn*DSA development. Late AMR was diagnosed in 47% of the *early* group and in 58% of the *late* group. Graft loss occurred in 3/15 (20%) and 4/24 (17%) patients in *early-* and *late-onset* groups, respectively (p = ns). In our pediatric kidney recipients, *dn*DSAs predict AMR and graft loss irrespective of the time elapsed between transplantation and antibody occurrence.

1. Introduction

Humoral alloimmunity leading to chronic antibody-mediated rejection (AMR) has been recognized as a major obstacle to long-term kidney graft (KTx) survival [1–5]. In addition to sensitized patients who suffer from poorer kidney graft outcome due to a higher incidence of AMR, a number of pretransplant HLA-antibody-negative kidney recipients, usually considered at low immunological risk, will also develop chronic allograft dysfunction and, ultimately, graft loss [6–16]. A positive association between the presence of de novo posttransplant donor-specific HLA antibodies (*dn*DSAs) after transplantation and poor transplant outcome has been demonstrated also in this patient category.

This observation has prompted recommendations on posttransplant HLA-antibody monitoring as a tool to identify patients at risk for antibody-mediated rejection and graft loss [6–16].

It has also been shown that DSA development continues as an active process even many years after transplantation [12, 14–17], and although DSAs may be detected also in patients with long-term functioning allografts, persistent kidney loss due to antibody-mediated injury is observed throughout the whole posttransplant period [5]. Time to DSA development has been suggested as a variable that could impact transplant outcome, with *early*-onset DSAs being associated with lower graft survival [7], but data on this clinical scenario are not conclusive [9, 17].

We conducted a longitudinal analysis on a pediatric cohort of pretransplant HLA-antibody-negative, first kidney recipients sequentially monitored for posttransplant DSA onset and alloantibody biological properties, in order to evaluate whether the timing of *dn*DSA appearance could influence AMR development and graft outcome.

2. Patients and Methods

2.1. Patients. Between July 2002 and March 2013, 125 consecutive patients were referred to the Genoa Pediatric Kidney Transplant Program for first allografting. Pretransplant patient sera were screened periodically for the presence of panel reactive anti-HLA antibodies by complement dependent cytotoxicity technique and by a bead-based assay [12]. All grafts were performed after a negative T cell crossmatch. Our standard of care for low immunological risk kidney transplant patients consisted of induction with basiliximab and a triple drug immunosuppressive regimen including a calcineurin inhibitor (cyclosporin A or tacrolimus), mycophenolate mofetil, and prednisone. Biopsy-proven acute cellular rejection episodes were treated with pulse intravenous methylprednisolone. Patients developing late AMR, as evidenced by circulating HLA DSAs and histological features of antibody-mediated tissue and vascular injuries, were treated with a protocol including a combination of plasmaphereses, i.v. human Ig, and anti-CD20 monoclonal antibody. Graft function was estimated by calculating eGFR using the Schwartz [18] or MDRD [19] formula, when appropriate.

Graft biopsies were performed for clinical indication (graft function decline and/or proteinuria); since 2010, DSA positivity was also included among indications. Rejections were histologically graded following the Banff 97 criteria with updates. Banff 2009 and Banff 2013 criteria were employed for classifying C4d positive and negative AMR [20, 21]. All biopsies performed before 2014 were regraded according to the Banff 2013 criteria. C4d staining was performed on frozen sections by indirect immunofluorescence.

This study was approved by the Institutional Review Board of the Fondazione Ca' Granda, Ospedale Maggiore Policlinico, Milano (867/2014).

2.2. Detection and Characterization of HLA Antibodies. Recipients of first graft who were found positive for the presence of anti-HLA antibodies in current and/or historical pretransplant sera ($n = 11$) were not included, resulting in a total of 114 nonsensitized first kidney allograft pediatric recipients monitored for *dn*DSA (Table 1). Sera for HLA-antibody monitoring were collected at transplantation, every three months in the first posttransplant year and annually thereafter. Samples obtained before 01/2010 belonged to a unique source of sera analyzed retrospectively for HLA antibodies, while from 02/2010 all samples were collected and analyzed prospectively [12]. An average of >8 samples per patient were analyzed. Complement-binding activity was analyzed on sera collected at DSA appearance and at biopsy or at follow-up.

HLA typing of kidney graft recipients and donors was performed as previously described [22]. Anti-HLA class I and class II IgG antibodies were tested with a bead-based detection assay after serum EDTA treatment, to avoid underestimation of antibody MFI strength [23–25]. We used the LABScreen Mixed kit and the Single-Antigen Bead (SAB) assays (One Lambda Inc., CA, USA) to identify HLA class I and class II specificities [12, 22]. Screening assay results above a cut-off value of 3.0 ratio between the sample and negative control were considered positive. Single-antigen results above a MFI cut-off value of 1.000 were considered positive. Heat inactivated patient sera were tested with C1qScreen™ (One Lambda) for identification of complement-binding antibodies, as described [26]. Antibody positivity was assigned at >500 MFI. Serum samples were analyzed in a blinded fashion for the presence of C3d-binding DSA with the single-antigen flow bead technology, according to the manufacturer's protocol (Immucor Lifecode Transplant Diagnostics Nijlen, Belgium). Positivity was assigned as previously detailed [27].

2.3. Statistical Analysis. Data were described as the mean and standard deviation (SD) or median and range if continuous and as count and percent if categorical. To determine differences among patient groups, categorical variables were compared by chi-squared analysis, continuous variables with t-tests, and, if skewed, nonparametric tests (Kruskal-Wallis one-way analysis of variance, Mann–Whitney U test). p values < 0.05 were considered statistically significant. Event-free survival was estimated with the Kaplan-Meier method and was compared between risk groups with the log-rank test. For graft failure, censoring event was death with functioning graft. For AMR, censoring event was graft failure. Patients who did not experience graft failure or AMR were censored at the end of the follow-up. Stata 13 (Stata Corporation, College Station, TX, USA) or the NCSS System (NCSS, Cary, NC) was used for computation.

3. Results

3.1. Clinical and Immunological Characteristics of the Patients according to Time of dnDSA Development. The cohort median follow-up was 6.7 years (range 2.0–12.6). Antibody identification was based on longitudinal analysis of collected sera in both retrospective and prospective sample series. Among the 114 patients analyzed, 39 patients (34%) developed *dn*DSAs at a median time of 24.6 months (range 3–115

Table 1: Clinical features of the patients analysed and according to the date of de novo donor specific HLA antibody (*dn*DSA) onset.

Variables	All patients ($n = 114$)	All patients with *dn*DSAs ($n = 39$)	Patients with *dn*DSAs occurring within year 1 ($n = 15$)	Patients with *dn*DSAs occurring beyond year 1 ($n = 24$)	p value
Characteristics at Tx					
Recipient					
Male sex	69 (60.5%)	27 (69%)	12 (80%)	15 (62.5%)	0.30
Age	13.4	13.20	10.27	15.03	**<0.05**
Donor					
Male sex	70 (61.4%)	25 (64%)	11 (73%)	14 (58%)	0.50
Age	17.2	13.15	11.53	14.17	0.46
Deceased	97 (85%)	36 (92%)	14 (93%)	22 (92%)	1
Transplantation					
Number of total HLA A, B mismatches	2.36	2.56	2.47	2.63	0.70
Number of total HLA DR, DQ mismatches	1.61	1.85	2.00	1.75	0.42
Number of total HLA A, B, DR, DQ mismatches	3.97	4.41	4.47	4.38	0.81
Characteristics after Tx					
CyA in maintenance IS	66 (58%)	30 (77%)	10 (67%)	20 (83%)	0.27
Delayed graft function	13 (11%)	4 (10%)	1 (7%)	3 (12%)	0.50
Acute T cell-mediated rejection*	18 (16%)	8 (20%)	2 (13%)	6 (25%)	0.45
eGFR < 60 at 1 year	14 (12%)	7 (18%)	1 (7%)	6 (25%)	0.21
AMR	21 (18%)	21 (54%)	7 (47%)	14 (58%)	0.52

*Including borderline changes.

Tx: transplantation; CyA: cyclosporin A; IS: maintenance immunosuppression; eGFR: estimated glomerular filtration rate (ml/min/1.73 m^2).

Table 2: Antibody characteristics in 39 de novo donor specific HLA antibody (*dn*DSA) positive patients.

Variables	All patients with *dn*DSAs ($n = 39$)	Patients with *dn*DSAs occurring within year 1 ($n = 15$)	Patients with *dn*DSAs occurring beyond year 1 ($n = 24$)	p value
*dn*DSA specificities, nr/patient*	1.97 ± 1.29	1.87 ± 1.25	2.04 ± 1.33	0.68
Persistent** *dn*DSAs	35 (90%)	13 (87%)	22 (92%)	0.63
HLA class I *dn*DSAs	8 (21%)	2 (13%)	6 (25%)	0.45
HLA class II *dn*DSAs	18 (46%)	8 (53%)	10 (42%)	0.52
HLA class I and II *dn*DSAs	13 (33%)	5 (33%)	8 (33%)	1.00
HLA-A *dn*DSAs	16 (41%)	5 (33%)	11 (61%)	0.51
HLA-B *dn*DSAs	12 (31%)	3 (20%)	9 (37%)	0.30
HLA-C *dn*DSAs	7 (18%)	3 (20%)	4 (17%)	1.00
HLA-DR *dn*DSAs	6 (15%)	3 (20%)	3 (12%)	0.66
HLA-DQ *dn*DSAs	28 (72%)	11 (73%)	17 (71%)	1.00
HLA-DP *dn*DSAs	1 (3%)	0	1 (4%)	1.00
Immunodominant *dn*DSAs				
MFI at onset*	9501 ± 7198	10483 ± 7020	8888 ± 7387	0.51
MFI at biopsy or peak*	12043 ± 7842	12061 ± 6683	12031 ± 8626	0.99
C1q positivityof *dn*DSAs				
At *dn*DSA onset	25 (64%)	12 (80%)	13 (54%)	0.17
At biopsy or MFI peak	29 (74%)	12 (80%)	17 (71%)	0.71
C3d positivityof *dn*DSAs				
At *dn*DSA onset	9 (23%)	3 (20%)	6 (25%)	1.00
At biopsy or MFI peak	16 (41%)	6 (40%)	10 (42%)	1.00

Percentages are calculated on the total number of patients from each group indicated at the top of the respective columns.
All data are reported as absolute numbers, unless otherwise specified; * data reported as mean ± sd.
** DSA persistence was defined as positivity of the immunodominant DSA in all analyzed samples after first positivity.
MFI: mean fluorescence intensity.

months). The mean number of DSA specificities found per patient was 1.97 (±1.29).

*dn*DSA-positive KTx recipients were stratified in two groups, based on time to DSA appearance. We considered patients with antibody occurrence within the first 12 months (the period of greater immunosuppression reduction) as those more prone to mount an immune response to the graft (*early-onset* group, $n = 15$) and patients with antibody occurrence beyond the first posttransplant year as the *late-onset* group ($n = 24$) (Table 1). The median time of DSA appearance from transplantation was 9 months (range 3–12) in the early group and 47 months (range 17–115) in the late group. The two groups were comparable when considering patient- and transplant-related factors, such as recipient sex, living versus deceased donor graft source, cyclosporine or tacrolimus administration, delayed graft function, 1-year estimated glomerular filtration rate (eGFR), HLA class I and II mismatches, and incidence of T cell mediated rejection (TCMR) and late AMR. Only recipient age at transplant was found to be significantly different in the two cohorts, with younger patients showing earlier *dn*DSA development (Table 1).

Patients belonging to the two groups did not display any difference in all analyzed HLA-antibody characteristics, including HLA class and locus specificity, persistence, MFI, and C1q and C3d complement fraction binding ability (Table 2). Antibodies detected in *dn*DSA-positive patients recognized a total of 78 HLA antigen specificities. In the two patient groups, HLA class I and class II specificities were equally distributed, and a similar pattern was observed when the analysis was carried out for each HLA antigen locus (Table 3). As observed in the whole cohort, DQ *dn*DSAs were the most represented antibodies in both groups. Regarding DSA biological properties, such as MFI and C1q- and C3d-binding ability, no significant differences were observed in the two groups (Table 3). However, *dn*DSAs differed for their complement-binding capability, as, with the exception of HLA A2, all C3d-positive DSAs recognized HLA class II and, in particular, DQ antigens, while C1q-positive DSAs were homogeneously distributed between the two classes (Figure 1). All C3d-binding DSAs were also found to bind C1q.

3.2. Time to dnDSA Emergence and Correlation with AMR and Clinical Outcome. AMR was diagnosed in 21 patients at a median follow-up of 4.8 years from kidney transplantation and was observed only in patients positive for *dn*DSAs (Table 1). Considering BANFF 2013 criteria for classification of AMR, 10 were acute active, and 11 were chronic active. The distribution of acute active and chronic active AMR did not

Table 3: Characteristics of 78 de novo donor specific HLA antibodies (*dn*DSAs) detected in 39 DSA positive kidney recipients.

Variables	Total number of *dn*DSAs ($n = 78$)	*dn*DSAs occurring within year 1 ($n = 26$)	*dn*DSAs occurring beyond year 1 ($n = 52$)	p value
HLA class I *dn*DSAs	40 (51%)	11 (42%)	29 (56%)	0.34
HLA class II *dn*DSAs	38 (49%)	15 (58%)	23 (44%)	0.34
HLA class I *dn*DSAs, MFI*	4678 ± 4516	4838 ± 4717	4618 ±4521	0.89
HLA class II *dn*DSAs, MFI*	12033 ± 8410	10629 ± 7568	12949 ±8960	0.41
HLA-A *dn*DSAs**	18 (23%)	5 (19%)	13 (25%)	0.78
HLA-B *dn*DSAs**	15 (19%)	3 (11%)	12 (23%)	0.36
HLA-C *dn*DSAs	7 (9%)	3 (11%)	4 (7%)	0.68
HLA-DR *dn*DSAs**	7 (9%)	4 (15%)	3 (6%)	0.21
HLA-DQ *dn*DSAs**	30 (38%)	11 (42%)	19 (36%)	0.63
HLA-DP *dn*DSAs	1 (1%)	0	1 (2%)	1.00
C1q binding of *dn*DSAs	44 (56%)	17 (65%)	27 (52%)	0.33
HLA class I *dn*DSAs	20 (26%)	7 (27%)	13 (25%)	1.00
HLA class II *dn*DSAs	24 (31%)	10 (38%)	14 (27%)	0.31
C3d binding of *dn*DSAs	18 (23%)	7 (27%)	11 (21%)	0.58
HLA class I *dn*DSAs	1 (1%)	1 (4%)	0	0.33
HLA class II *dn*DSAs	17 (22%)	6 (23%)	11 (21%)	1.00

Percentages are calculated on the total number of antibodies from each group indicated at the top of the respective columns. All data are reported as absolute numbers, unless otherwise specified.
*Data reported as mean ± sd.
**The number of antibodies detailed in this table is higher than that reported in Table 2, as some patients have multiple DSAs at this locus.
MFI: mean fluorescence intensity.

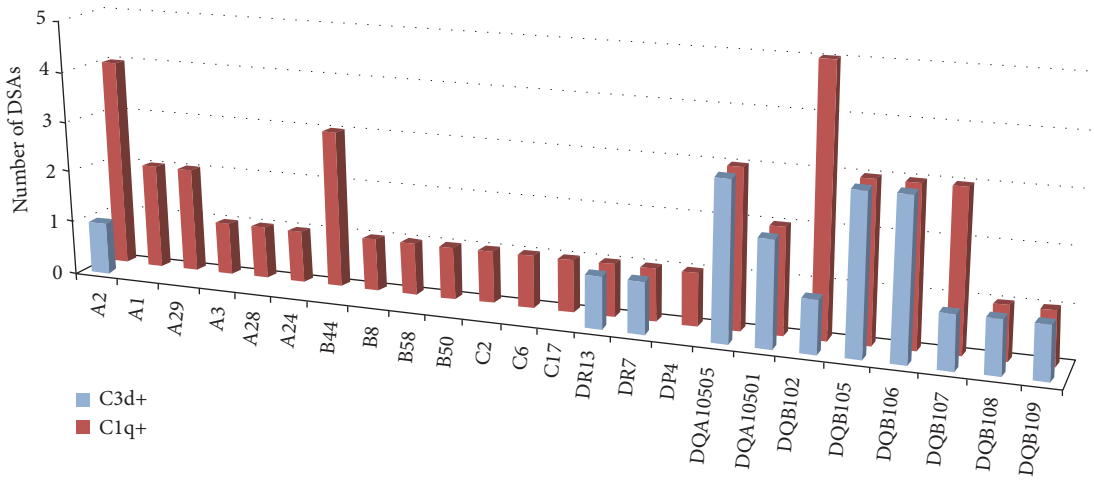

Figure 1: HLA antigens recognized by C3d and/or C1q positive DSAs in the 39 *dn*DSA-positive patients. A total of 78 *dn*DSAs were identified in the 39 kidney recipients. Of those, 44 bound C1q and 18 displayed binding ability for C3d.

differ between the *early-* and *late-onset groups*. To evaluate the damaging effect of *dn*DSAs on the kidney graft, we analyzed the rate of AMR-free survival from the time of DSA onset. The interval from *dn*DSA development to AMR was 2.5 years (range 1.0–4.9) in the *early-onset* group, compared to 1.1 years (range 0.1–4.6; $p = 0.08$) in the *late-onset* group. AMR-free survival did not differ between *early-* and *late-onset groups* (Figure 2(a)).

The histological findings were investigated in graft biopsies obtained from 30 out of 35 patients with persistent *dn*DSAs (Figure 3); for the remaining 5 graft recipients, no biopsies were available, as the patients refused the procedure due to stable good allograft function. The histological findings were analyzed both individually (interstitial inflammation-*i*-, tubulitis-*t*-, *ptc*, glomerulitis-*g*-, interstitial fibrosis-*c*-, tubular atrophy-*ct*-, transplant

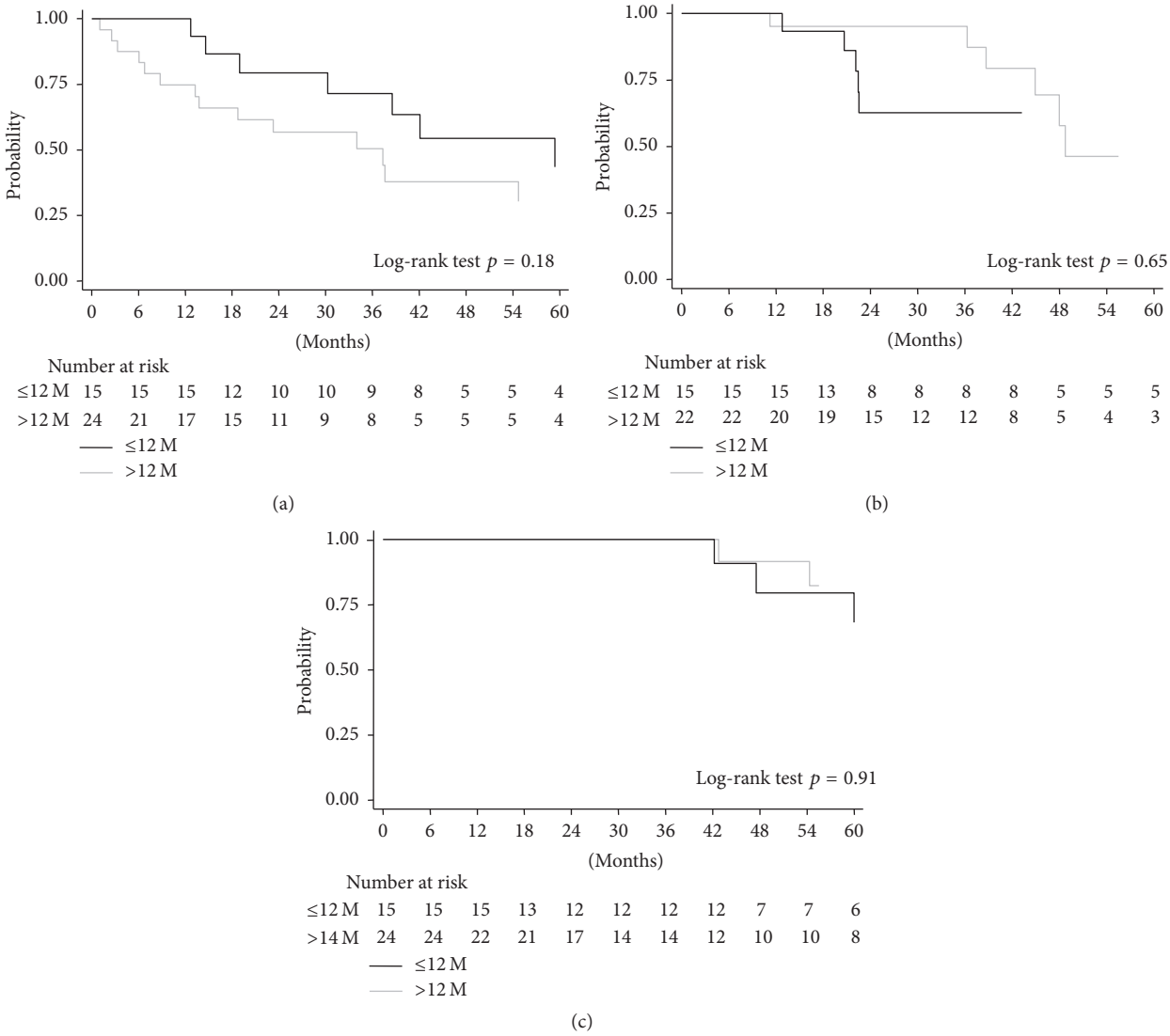

Figure 2: Risk of developing late antibody-mediated rejection (AMR), renal function decline, and graft loss, in the 39 patients who developed de novo donor-specific antibodies (*dn*DSAs), according to the time to HLA-antibody occurrence. (a) AMR-free allograft survival in kidney graft recipients, stratified by early or late development of *dn*DSAs; (b) renal graft function decline (eGFR ≤ 50 ml/min/1.73 m^2) in kidney graft recipients, stratified by early or late development of *dn*DSAs; (c) allograft survival in kidney graft recipients, stratified by early or late development of *dn*DSAs. The statistical difference between Kaplan-Meier survival curves was evaluated by the log-rank test and differences with p values < 0.05 were considered statistically significant.

glomerulopathy-*cg*-, chronic vascular changes-*cv*-, and intimal arteritis-*v*-) and in functional clusters (*ptc* + *g* referring to microcirculation inflammation, *ptc* + *g* + *cg* to microcirculation lesions, *i* + *t* to tubulointerstitial inflammation, and *ci* + *ct* to tubulointerstitial scarring). No significant differences were observed between the two groups (Figure 3).

We then evaluated the impact of *early*- versus *late-onset* *dn*DSAs on graft loss. In the whole cohort of 114 patients, 9 grafts were lost, among which 7 grafts were lost due to AMR and 2 to focal glomerulosclerosis recurrence. The latter 2 patients were *dn*DSA negative. Among the 7 graft losses due to AMR, 3 were observed in the *early-onset* group and 4 in the *late-onset* *dn*DSA group. The median time interval from *dn*DSA onset to graft loss was 4.0 years (range 3.5–5.0) in the *early-onset* group, compared to 5.5 years (range 3.6–6.5) in the *late-onset* group (p = ns) (Figure 2(c)). As the number of graft losses in our cohort was limited, eGFR ≤ 50 ml/min/1.73 m^2 was alternatively employed as an outcome end-point. Also in this case, no difference was observed between the *early-onset* and *late-onset* groups (Figure 2(b)).

4. Discussion

The problem of clarifying whether HLA antibodies developing at different posttransplant intervals could have different cytotoxic capabilities and graft tissue damage potential has

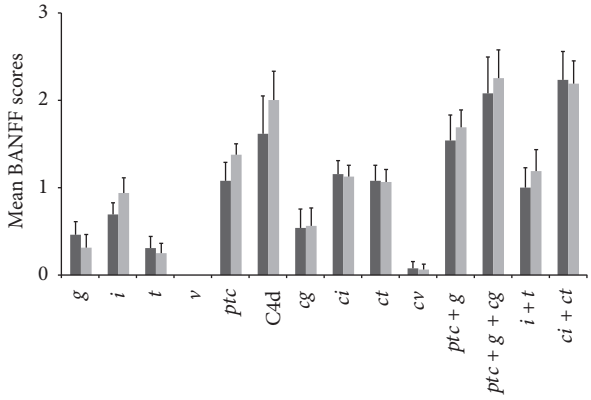

Figure 3: Histological analysis in 30 graft biopsies obtained from 13 recipients displaying *early-onset dn*DSAs (dark grey bars) and 17 recipients positive for *late-onset dn*DSAs (light grey bars). The biopsy findings were analyzed both individually (*i, t, ptc, g, ci, ct, cg, cv, v*) and in functional clusters (*ptc* + *g* referring to microcirculation inflammation, *ptc* + *g* + *cg* to microcirculation lesions, *i* + *t* to tubulointerstitial inflammation, and *ci* + *ct* to tubulointerstitial scarring). Data are presented as the mean ± standard error. For each parameter, no significant difference was observed between the two groups.

relevance in view of the need to establish the optimal terms of posttransplant DSA surveillance strategy, particularly concerning monitoring length.

Our study, carried out in a homogeneous patient population not including sensitized recipients, demonstrates that the time interval to AMR development and graft loss, evaluated from the first *dn*DSA appearance, does not differ in the *early-* and *late-onset* HLA-antibody groups. In previous studies, it had been shown that DSAs developing within the first year after transplantation resulted in early graft failure, whereas *late-onset* DSAs, although also detrimental, seemed to require a longer time to finally cause graft damage and loss [7, 9]. These latter observations likely reflected the presence of a proportion of sensitized patients, in whom rapid development of DSA-mediated tissue damage could have been sustained by the presence of a cytokine inflammatory milieu [28, 29] and further amplified by a parallel action of non-DSAs specific for mismatched cross-reactive epitopes [8]. In our cohort of pediatric recipients, a model intrinsically free of relevant comorbidities, a thorough and prolonged posttransplant antibody monitoring permitted an accurate estimate of the interval between DSA onset and graft function deterioration, thus allowing assessment of the actual damaging potential of *dn*DSAs emerging in the late posttransplant period. Through this longitudinal detection approach, we demonstrated that DSAs in the two patient groups displayed equivalent damaging capabilities. Indeed, *early-* and *late-onset dn*DSAs did not differ in the biological properties, such as high MFI values and complement-binding ability, recently demonstrated to be the main determinants of antibody-mediated graft damage and loss [26, 27, 30–32]. In particular, all of the graft losses in both groups were observed in patients displaying DSAs capable of C3d binding, as a result of progressive acquisition over time of C1q- and C3d-fixing ability, paralleled by an increase in MFI values [27]. The size of our pediatric cohort, smaller than average adult series, may have partly influenced our statistical findings and limited our ability to dissect the respective role of complement-binding activity and MFI on graft outcome. While Lee et al. observed an earlier production of HLA class I DSAs [7], we found that HLA class I and class II *dn*DSAs were comparably represented in both *early-* and *late-onset* groups. This apparent discrepancy could be in part explained by the fact that our study exclusively analyzed nonsensitized recipients. Indeed, in a first set alloresponse condition, the ubiquitous cellular expression of class I HLA antigens within the kidney graft tissue may be balanced by the greater stimulating capability of the highly polymorphic class II molecules, in particular HLA DQ antigens [11–15, 22]. Moreover, comparing C1q- and C3d-binding capabilities in class I and class II *dn*DSAs, we demonstrated in both patient groups that C3d binding was almost exclusively a property of class II, whereas C1q binding was expressed in a similar percentage by both classes. This finding gives additional strength to previous data demonstrating that, in nonsensitized low-risk kidney recipients, class II specific and, in particular, anti-DQ de novo antibodies are the principal effectors of graft loss in all posttransplant phases [11–15, 22, 27, 30]. The equivalency of *early* and *late dn*DSA damaging capacity was further supported by the observation that the two study groups displayed a similar histological pattern of tissue graft damage. In this regard, it is worth underlining that, in our cohort represented by recipients of grafts from young donors, the susceptibility to HLA-antibody mediated insult is only marginally influenced by organ ageing [33].

At present, the reasons for *dn*DSA production in some patients and not in others, as well as the biological factors influencing their development at different times after transplant, in the presence of the same degree of HLA mismatching and the same immunosuppressive regimen, are not completely understood. In this study, younger recipient age appeared to favor an earlier *dn*DSA production, likely reflecting a propensity to display a stronger alloreactivity, that suggests a note of caution in immunosuppressive therapy minimization in the pediatric setting.

5. Conclusions

Based on our findings, management of patients found positive for *dn*DSAs at late phases of posttransplant follow-up should not differ from that applied in the early-onset *dn*DSA patient group.

Thus, monitoring of HLA antibodies throughout the entire posttransplant course is recommended, despite high costs and organization difficulties, in order to identify patients at risk for AMR and graft loss.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Michela Cioni and Arcangelo Nocera equally share first authorship; Patrizia Comoli and Fabrizio Ginevri equally share senior authorship.

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Research Article

The Immunogenicity of HLA Class II Mismatches: The Predicted Presentation of Nonself Allo-HLA-Derived Peptide by the HLA-DR Phenotype of the Recipient Is Associated with the Formation of DSA

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The identification of permissible HLA class II mismatches can prevent DSA in mismatched transplantation. The HLA-DR phenotype of recipients contributes to DSA formation by presenting allo-HLA-derived peptides to T-helper cells, which induces the differentiation of B cells into plasma cells. Comparing the binding affinity of self and nonself allo-HLA-derived peptides for recipients' HLA class II antigens may distinguish immunogenic HLA mismatches from nonimmunogenic ones. The binding affinities of allo-HLA-derived peptides to recipients' HLA-DR and HLA-DQ antigens were predicted using the NetMHCIIpan 3.1 server. HLA class II mismatches were classified based on whether they induced DSA and whether self or nonself peptide was predicted to bind with highest affinity to recipients' HLA-DR and HLA-DQ. Other mismatch characteristics (eplet, hydrophobic, electrostatic, and amino acid mismatch scores and PIRCHE-II) were evaluated. A significant association occurred between DSA formation and the predicted HLA-DR presentation of nonself peptides ($P = 0.0169$; accuracy = 80%; sensitivity = 88%; specificity = 63%). In contrast, mismatch characteristics did not differ significantly between mismatches that induced DSA and the ones that did not, except for PIRCHE-II ($P = 0.0094$). This methodology predicts DSA formation based on HLA mismatches and recipients' HLA-DR phenotype and may identify permissible HLA mismatches to help optimize HLA matching and guide donor selection.

1. Introduction

Human Leukocyte Antigen (HLA) incompatible transplantation frequently leads to the formation of HLA donor-specific antibodies (DSAs). DSAs are now considered to be the main cause of allograft loss [1, 2], since they are associated with an increased risk for antibody-mediated rejection (AMR), poor long-term survival, and chronic rejection [1, 3–5]. To that end, the identification of permissible HLA mismatches—not inducing antibody formation—has been essential for mismatched transplantation, as it is clear that not all HLA mismatches are immunogenic and lead to the formation of DSA [6, 7].

The development of DSA requires the activation of the transplant recipient's adaptive immune system against allogeneic HLA (allo-HLA), a process known as the indirect allorecognition pathway (reviewed in [8, 9]). This alloreactive response involves both B and T cell compartments of the immune system, particularly the cognate CD4+ T cell helper function, to produce long-lasting IgG alloantibodies [8, 10, 11]. Indeed, the indirect allorecognition initiates when the B cell receptors (BCR) of allospecific B cells bind to their cognate allo-HLA. This binding induces B cells to internalize and process the allo-HLA into allo-HLA-derived peptides (allo-HLApep). Only certain allo-HLApep are then loaded onto the recipient's HLA class II molecules and presented on the B cell surface. The presented allo-HLApep can be recognized by the T cell receptors (TCR) of the recipient's allo-HLA-specific follicular helper CD4+ T cells (allo-T_{FH}); these cells have undergone thymic selection, which creates a pool of allo-T_{FH} that expresses TCR with a low affinity for "self" peptides/HLA class II complex and a high affinity for "nonself" ones [12, 13]. Only the costimulatory signal provided by allo-T_{FH} induces B cell proliferation and differentiation into memory B cells

and IgG-secreting plasma cells, both specific for the allo-HLA [9, 14, 15].

The immunogenic potential, or immunogenicity, of allo-HLA molecules remains to be fully elucidated. Nonetheless, allo-HLA molecules have to carry two important immunogenic domains to induce a humoral immune response [16]. The first domain is a B cell epitope that is specific for a unique BCR expressed on the surface of allospecific B cells. The second domain, a T cell epitope recognized by allo-T_{FH}, is the allo-HLApep presented by the recipient's HLA class II antigens on the surface of allospecific B cells. Several computational approaches have been developed to characterize and clarify the immunogenicity of allo-HLA (reviewed in [17]).

B cell epitopes (or HLA antibody epitopes) can be identified using the Duquesnoy HLAmatchmaker method, which is a quantitative tool used to distinguish polymorphic eplets of donor/recipient HLA mismatches in order to identify actual and potential antigenic determinant specificities of human allo-HLA and murine monoclonal antibodies [18–20]. HLAmatchmaker evaluates the eplet mismatch load of a transplant, and both transplant survival and DSA formation were correlated with high eplet mismatch load [20–22]. This is supported by Dankers et al., who reported that DSA formation correlated with the number of amino acid triplet differences [23]. Moreover, characterizing the frequencies of epitope-specific allo-HLA antibodies provides a measure of the relative immunogenicity of HLA eplet mismatches [24]. Similarly, the group of Komoliaptsis V. have shown that the number and physiochemical properties (hydrophobicity and electrostatic charge difference) of amino acid mismatches were correlated with the presence and level of DSA [25–28]. While the relative immunogenicity of HLA antigens was assessed using the antigenicity of HLA epitopes, it does not fully reveal their immunogenicity [7].

On the other hand, the HLA class II phenotype, HLA-DR in particular [29, 30], of the recipient contributes to the immunogenicity of HLA mismatches by presenting allo-HLA-derived T cell epitopes and thus influencing CD4+ T cell and B cell interactions [31]. Potential T cell epitopes can be identified using an Internet-based prediction tool (NetMHCIIpan 3.1, developed by Nielsen M. and his team) to determine the binding affinity of nonamer cores from any protein sequence [32], with the highest performance comparable to other available methods [33]. The group of Spierings E. developed an allogeneic HLA-derived, predicted indirectly recognizable HLA epitopes, HLA class II-presented (PIRCHE-II) method to identify the number of nonself peptides with high predicted binding affinities (IC_{50} < 1000 nM) for a specific HLA class II antigen [34]. The allogeneic HLA-derived PIRCHE-II number was correlated with the development of HLA antibodies in kidney and pancreas transplantation [34, 35], as well as during pregnancy [36], suggesting that allo-HLA-derived T cell epitopes are critical for evaluating the immunogenicity of HLA.

The immunogenicity of HLA mismatches, assessed using a B cell epitope or a T cell epitope approach, had limitations. Indeed, in many mismatches with low eplet mismatch load, physiochemical property differences, or PIRCHE-II numbers, DSA can be formed and vice versa. The overlap in the distribution of the aforementioned HLA mismatch characteristics between DSA producer and DSA nonproducers suggests that other factors are involved in the immunogenicity of HLA mismatches [37].

To my knowledge, the self component of HLA mismatches has not been investigated before, yet it may be a factor influencing the immunogenicity of allo-HLA. Because HLA molecules have both unique and shared sequences of amino acids, allospecific B cells, which processed an allo-HLA, can present self and/or nonself allo-HLApep depending on their binding affinity for the recipient's HLA class II expressed. The thymic selection of T cells creates a repertoire of allo-T_{FH} cells expressing specific TCRs with a low affinity for self peptides/HLA class II complex and a high affinity for nonself ones [12, 13]. Unlike self allo-HLApep, the recognition of nonself allo-HLApep provides adequate costimulation for the proliferation and differentiation of B cells into allo-HLA-specific IgG-secreting plasma cells. Therefore, I hypothesize that comparing the highest predicted binding affinity of nonself and self allo-HLApep for a transplant recipient's HLA class II antigens may distinguish immunogenic HLA mismatches (which induce the formation of DSA) from nonimmunogenic ones.

2. Materials and Methods

2.1. Patients with HLA Class II Mismatches. Six renal transplant recipients, with defined HLA class II genotype and IgG DSAs previously characterized at the Terasaki Foundation Laboratory by Dr. El-Awar, were used in this study [38]. Briefly, all patients were transplanted between 2002 and 2010 and had undergone AMR. The HLA-DRB1, HLA-DQA1, and HLA-DQB1 molecular typing were performed using Luminex polymerase chain reaction sequence specific oligo probe hybridization (PCR-SSO) technology (One Lambda, Canoga Park, CA, USA) for all recipient/donor pairs. All patients had IgG DSA detected (MFI > 1000) at the time of AMR using the Luminex platform with single HLA antigen beads (One Lambda, Canoga Park, CA, USA). All the HLA-DQ DSAs (DQA1 and DQB1) had their epitope defined previously [38], and all of the epitopes were located on either the alpha chain or the beta chain, but not to the HLA-DQ heterodimer. The total number of HLA class II mismatches was 25 (including DRB1, DQA1, and DQB1 molecules only).

2.2. Methods to Evaluate Donor HLA-DRB1, HLA-DQB1, and HLA-DQA1 Mismatch Scores

2.2.1. HLAmatchmaker. This program ("HLA-DRDQDP matching for up to 1000 cases (v02)" downloaded from http://www.epitopes.net/downloads.html) was used to evaluate the total eplet mismatch score (EpMS)—antibody verified and others—of each of the HLA class II mismatches, for each individual chain (DRB1, DQB1, and DQA1) separately [39, 40].

2.2.2. PIRCHE-II. This analysis was attempted by manually using the Internet-based NetMHCIIpan 3.1 server

predication tool (found on http://www.cbs.dtu.dk/services/NetMHCIIpan) as described by the group of Otten et al. [34] to evaluate the number of PIRCHE-II for each mismatch. Briefly, the amino acid sequence of each HLA class II mismatch (full-length mature protein comprised of the following: extracellular domain, transmembrane domain, and cytoplasmic tail) was used to predict the binding affinity of all 15-mer nonself peptides to the recipients' HLA-DR antigens' peptide groove. This prediction was made after inter- and intralocus amino acid sequence alignment comparison, and all the nonself 15-mer peptides with high predicted binding affinity (IC_{50} < 1000 nM) were identified. Only the unique nonself nonamer core sequences that aligned to the binding groove of HLA-DR antigens were counted as a PIRCHE-II.

2.2.3. HLA Class II Immunogenicity Algorithm. This program is freely available to download from http://www.hlaimmunogenicity.org/download/Cambridge_HLA_Class_II_Immunogenicity_Algorithm.xls and was used to determine the amino acid mismatch score (AMS), the hydrophobicity mismatch score (HMS), and the electrostatic mismatch score (EMS) for each HLA class II mismatch [25].

2.3. Prediction of the Binding Affinity of Allo-HLA-Derived Peptide to Recipients' HLA Class II Antigens. With the "NetMHCIIpan 3.1" Internet-based predication tool, the amino acid sequence of each HLA mismatch (extracellular domains only, in the FASTA format found on http://www.hla.alleles.org [41]) was used to predict the binding affinity of all allo-HLApep sequences to the recipients' HLA class II antigens' peptide groove. The recipients' HLA class II antigens analyzed were HLA-DR and all possible permutations of DQA1 and DQB1 chains for HLA-DQ. For example, for a hypothetical recipient with DQA1*X1/*X2\DQB1*X3/*X4, all the following HLA-DQ heterodimers were analyzed for their allo-HLApep binding affinity: DQA1*X1\DQB1*X3; DQA1*X1\DQB1*X4; DQA1*X2\DQB1*X3; DQA1*X2\DQB1*X4. Three allo-HLApep peptide lengths were examined: 9 mers, 12 mers, and 15 mers. The prediction output values of the binding affinity of allo-HLA-derived peptide sequences are given as follows: (i) nM IC_{50} values, where IC_{50} < 50 nM are considered high affinity, <500 nM intermediate affinity, and <5000 nM low affinity, and (ii) percentile rank (% rank) of a peptide, which is generated by comparing the peptide's score against the scores of 200,000 random natural peptides (% rank < 2%: strong binder; % rank < 10%: weak binder). After making inter- and intralocus amino acid sequence alignment comparisons, all the allo-HLApep were classified as self when they were identical to the recipients' and as nonself when they were different. As shown in Figure 1, each mismatch was classified based on whether it led to the formation of DSA or not and whether a self or nonself allo-HLApep was predicted to have the highest binding affinity for the recipient's HLA class II antigens. Self presentation was defined as follows: only self allo-HLApep with highest binding affinities for all of the recipient's HLA class II antigens; meanwhile nonself presentation was defined as follows: at least one of the recipient's HLA class II antigen having the highest binding affinity for nonself allo-HLApep.

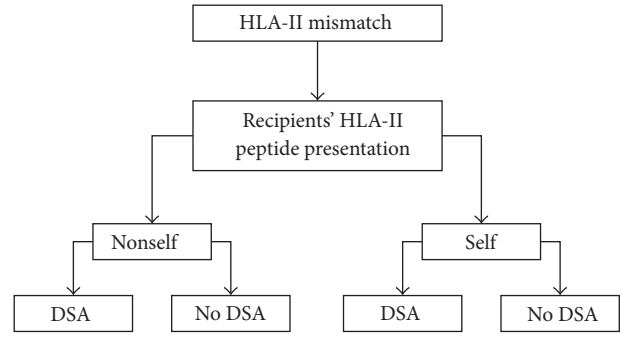

Figure 1: Classification of each HLA mismatch based on the presence of DSA and the nature (self or nonself) of the predicted peptide with highest affinity for the recipient's HLA class II phenotype.

2.4. Statistical Analysis. All statistical analyses were done using STATA 13. All data were tested for normality using the Shapiro-Wilk W test, and the Shapiro-Francia W test. Analysis for significance was performed using the t-test, with equal variance for mean comparison. Fisher's exact test was used to analyze 2 by 2 contingency tables, and the accuracy, specificity, and sensitivity were reported for the same contingency tables. Two-tailed P values less than 0.05 were considered significant.

3. Results

3.1. Patients and HLA Class II Mismatches. The six transplant cases analyzed had 3 to 5 HLA class II mismatches (HLA-DRB1, HLA-DQA1, and HLA-DQB1 molecules) and 2 to 4 DSAs (Table 1). The total number of mismatches was 25 (9 HLA-DRB1, 6 HLA-DQA1, and 10 HLA-DQB1), where 17 led to the formation of DSA (3 anti-HLA-DRB1, 6 anti-HLA-DQA1, and 8 anti-HLA-DQB1) and 8 did not (6 anti-HLA-DRB1, 0 anti-HLA-DQA1, and 2 anti-HLA-DQB1). Only one patient (Tx ID 21) produced DSA against all of his mismatched HLA class II antigens, whereas all other patients did not produce DSA against all their respective mismatches.

3.2. HLA Class II Mismatch Scores. The EpMS (antibody verified and others) and number of PIRCHE-II, HMS, EMS, and AMS are listed in Table 2 for all the mismatches. The EpMS ranged from 3 to 16 (antibody verified: 0–9; others: 1–11), the number of PIRCHE-II from 1 to 18, the HMS from 0.9 to 33.999, the EMS from 0.529 to 31.23, and the AMS from 3 to 21. For all loci combined (Table 3(a)) or separate (Tables 3(b) and 3(c)), there are no significant differences in the EpMS (antibody verified and others), HMS, EMS, and AMS between mismatches that led to the formation of a DSA and those that did not. In contrast, the numbers of PIRCHE-II were significantly higher in mismatches that led to the formation of DSA compared to those that did not for all loci combined (P = 0.0094, Table 3(a)), but not for the separate loci (Tables 3(b) and 3(c)).

Table 1: Transplant cases: HLA-DR and HLA-DQ mismatches and DSA.

Tx ID	Recipient HLA typing	HLA allele mismatches	Mismatch ID	DSA
13 3 mismatches 2 DSAs	DRB1*04:05 DRB1*14:01	DRB1*11:04	1	No
	DQB1*03:02 DQB1*05:03	DQB1*03:01	2	Yes
	DQA1*01:01 DQA1*03:01	DQA1*05:05	3	Yes
18 3 mismatches 2 DSAs	DRB1*11:01 DRB1*16:01	DRB1*07:01	4	No
	DQB1*03:01 DQB1*05:02	DQB1*02:02	5	Yes
	DQA1*01:02 DQA1*05:05	DQA1*02:01	6	Yes
21 4 mismatches 4 DSAs	DRB1*07:01	DRB1*08:01	7	Yes
	DQB1*02:01	DQB1*03:01	8	Yes
	DQB1*02:02	DQB1*03:03	9	Yes
	DQA1*02:01	DQA1*06:01	10	Yes
22 5 mismatches 3 DSAs	DRB1*11:01	DRB1*07:01	11	No
	DRB1*14:07	DRB1*15:01	12	No
	DQB1*05:02	DQB1*02:02	13	Yes
	DQB1*05:03	DQB1*06:02	14	Yes
	DQA1*01:01 DQA1*01:02	DQA1*02:01	15	Yes
33 5 mismatches 3 DSAs	DRB1*11:01	DRB1*07:01	16	Yes
	DRB1*16:01	DRB1*13:01	17	No
	DQB1*03:01	DQB1*02:02	18	Yes
	DQB1*05:02	DQB1*06:04	19	No
	DQA1*01:02 DQA1*05:05	DQA1*02:01	20	Yes
35 5 mismatches 3 DSAs	DRB1*04:03	DRB1*07:01	21	Yes
	DRB1*13:02	DRB1*15:01	22	No
	DQB1*03:02	DQB1*02:02	23	Yes
	DQB1*06:04	DQB1*06:03	24	No
	DQA1*01:02 DQA1*03:01	DQA1*02:01	25	Yes

DSA: donor-specific HLA antibody.

3.3. Predicted Allo-HLA-Derived Peptides Presented by HLA-DRB1 Antigens of the Recipients. Table 4 shows the predicted binding affinity of allo-HLApep to their respective recipient's HLA-DR antigens. Each self or nonself allo-HLApep is characterized by its amino acid (AA) position within the whole allo-HLA molecule and its IC_{50} value and percentile rank (% rank). The same analysis was done for recipient's HLA-DQ antigens, where all possible DQA1 and DQB1 chain permutations were analyzed (data not shown).

Eight HLA class II mismatches did not lead to the formation of DSA (nonbold and nonunderlined mismatches in Table 4), of which five (mismatches ID 1, 11, 12, 19, and 24) had their respective recipient's HLA-DRB1 antigens binding with the highest and higher affinity (lowest IC_{50} and percentile rank values, bolded values in Table 4) for 9-mer self allo-HLApep compared to nonself ones. Of the remaining 3 (mismatches ID 4, 17, and 22), at least one of their respective recipient's HLA-DRB1 antigens had the highest and higher affinity for nonself allo-HLA-derived peptides compared to self ones.

In the HLA class II mismatches that led to the formation of DSA (bold and underlined mismatches in Table 4), fifteen (mismatches ID 2, 3, 5–9, 13–16, 18, 20, 23, and 25) had at least one of their respective recipient's HLA-DRB1 antigens

Table 2: HLA class II mismatch characteristics.

Mismatch ID	DSA	EpMS (ab, ot)	PIRCHE-II	HMS	EMS	AMS
1	No	4 (3, 1)	6	4.399	6.32	4
2	Yes	3 (1, 2)	8	4	4.749	4
3	Yes	8 (6, 2)	18	17.399	16	13
4	No	14 (4, 10)	9	12.499	14.229	16
5	Yes	9 (3, 6)	11	7.199	2.869	9
6	Yes	5 (2, 3)	9	5.499	10.969	9
7	Yes	16 (5, 11)	12	19.4	21.81	16
8	Yes	16 (9, 7)	12	25.499	19.1	19
9	Yes	15 (9, 6)	11	22.399	14.65	16
10	Yes	4 (3, 1)	12	19.899	20.79	10
11	No	13 (4, 9)	8	11.999	15.859	17
12	No	9 (1, 8)	7	14.3	15.43	11
13	Yes	12 (5, 7)	17	14.7	11.259	15
14	Yes	10 (2, 8)	13	3	1.03	7
15	Yes	12 (2, 10)	17	33.999	31.23	21
16	Yes	14 (4, 10)	9	12.499	14.229	16
17	No	6 (0, 6)	3	3.199	4.51	5
18	Yes	9 (3, 6)	11	7.199	2.869	9
19	No	6 (1, 5)	10	5.099	5.79	3
20	Yes	5 (2, 3)	9	5.499	10.969	9
21	Yes	11 (3, 8)	1	11.599	11.589	13
22	No	6 (1, 5)	3	10	10.03	8
23	Yes	8 (4, 4)	7	9	8.05	7
24	No	5 (2, 3)	2	0.9	0.529	3
25	Yes	4 (1, 3)	6	2	4.049	5

AMS: Amino acid mismatch score; DSA: donor-specific HLA antibody; EMS: electrostatic mismatch score; EpMS (ab, ot): eplet mismatch score (antibody verified and others); HMS: hydrophobicity mismatch score; PIRCHE-II: predicted indirectly recognizable HLA epitopes, HLA class II-presented.

binding with the highest and higher affinity for nonself allo-HLA-derived peptides compared to self ones. Of the remaining 2 (mismatches ID 10, 21), their respective recipient's HLA-DRB1 antigens had the highest and higher affinity self allo-HLA-derived peptides (9 mers) compared to nonself ones. The highest binding affinity (IC_{50}) and percentile rank of the predicted self or nonself peptides for the recipients' HLA-DR antigens are below 5000 nM and 5%, respectively, and thus they could all be potential T cell epitopes (Table 4).

3.4. Prediction of DSA Formation: Influence of the Peptide Length and HLA Class II Phenotype. Table 5 shows the classification of each mismatch according to the criteria described in the Materials and Methods and the analysis presented in Table 4. Three peptide lengths (9 mers, 12 mers, and 15 mers) and the predicted HLA-DR and/or HLA-DQ presentation were analyzed for their association with DSA formation. Only the HLA-DR presentation of 9-mer peptides is significantly associated with DSA formation ($P = 0.0169$), in contrast to HLA-DQ or the combined HLA-DR/DQ presentation of 9-mer, 12-mer, and 15-mer peptides (Table 5). If the recipient's HLA-DR antigen has the highest affinity for nonself peptide derived from the mismatch then a DSA will likely be generated against it. The formation of DSA, given the presentation of nonself allo-HLA-derived peptide, can be predicted with an accuracy of 80%, a sensitivity of 88%, and a specificity of 63%.

4. Discussion

This study reveals that comparing the predicted binding affinity of HLA class II mismatched-derived self or nonself peptides helps distinguishing immunogenic HLA class II mismatches that lead to the formation of DSA from nonimmunogenic ones after HLA mismatched kidney transplantation. Indeed, there is a significant association between the predicted presentation of nonself peptide and the presence of DSA in the serum of kidney recipients. This immunologic evaluation of donor-recipient pairs may be useful in optimizing HLA matching and in guiding donor selection by determining potentially nonimmunogenic HLA mismatches, that is, permissible HLA mismatches.

Although the self component of HLA mismatches provides new insight for the understanding of the complex underlying mechanism of HLA antibody development, the computational approach described in this study has some limitations. Indeed, the evaluation of the immunogenicity of allo-HLA cannot rely on the characterization of T cell epitopes alone, as the recognition of B cell epitopes initiates the humoral response to allo-HLA [17]. For example, mismatch

Table 3: Comparison of the different mismatch characteristics between mismatches that led to the formation of DSA and ones that did not.

(a) HLA-DRB1 and DQA1/DQB1 mismatches

	No DSA ($n = 8$)	DSA ($n = 17$)	P value
EpMS	7.9 ± 3.8	9.5 ± 4.3	0.397
Antibody verified	2 ± 1.5	3.8 ± 2.4	0.0714
Others	5.9 ± 3	5.7 ± 3.1	0.8993
PIRCHE-II	6 ± 3	10.8 ± 4.3	**0.0094**
HMS	7.8 ± 5	13 ± 8.2	0.1438
EMS	9.1 ± 5.7	12.1 ± 8.1	0.3492
AMS	8.4 ± 5.7	11.6 ± 4.9	0.1544

(b) HLA-DRB1 mismatches

	No DSA ($n = 6$)	DSA ($n = 3$)	P value
EpMS	8.7 ± 4.1	13.7 ± 2.5	0.0978
Antibody verified	2.2 ± 1.7	4 ± 1	0.1385
Others	6.5 ± 3.3	9.7 ± 1.5	0.1642
PIRCHE-II	6 ± 2.5	7.3 ± 5.7	0.6275
HMS	9.4 ± 4.6	14.5 ± 4.3	0.1516
EMS	11.1 ± 4.9	15.9 ± 5.3	0.2155
AMS	10.2 ± 5.5	15 ± 1.7	0.1919

(c) HLA-DQA1/DQB1 mismatches

	No DSA ($n = 2$)	DSA ($n = 14$)	P value
EpMS	5.5 ± 0.7	8.6 ± 4.1	0.3248
Antibody verified	1.5 ± 0.7	3.7 ± 2.6	0.2707
Others	4 ± 1.4	4.9 ± 2.6	0.6679
PIRCHE-II	6 ± 5.7	11.5 ± 3.7	0.0836
HMS	3 ± 3	12.7 ± 9.8	0.1993
EMS	3.2 ± 3.7	11.3 ± 8.5	0.2105
AMS	3	10.9 ± 5.1	0.0524

Note. Values are expressed as mean ± SD; two-tailed P values are shown. AMS: amino acid mismatch score; DSA: donor-specific HLA antibody; EMS: electrostatic mismatch score; EpMS: eplet mismatch score; HMS: hydrophobicity mismatch score; PIRCHE-II: predicted indirectly recognizable HLA epitopes, HLA class II-presented.

ID 17 did not lead to the formation of DSA, while nonself allo-HLApep had the highest binding affinity for the recipient's HLA-DR antigens. However, mismatch ID 17 carries a total of 6 eplet mismatches, but none are antibody verified; therefore, this mismatch may not be antigenic and may not result in the formation of DSA.

Another limitation relies on the complexity of HLA-DQ heterodimers, where both DQA1 and DQB1 chains are polymorphic and can be mismatched; thus both can contribute to the antigenicity of HLA-DQ antigens. For example, mismatch ID 10 led to the formation of anti-DQA1*06:01 DSA but the DQA1-derived-peptides that presented with highest affinities were only self. Their associated allo-DQB1 chains (DQB1*03:01/03:03) had nonself peptides binding with highest affinity for the recipient's HLA-DR antigens and with higher affinity than the self DQA1-derived-peptides (Table 4). In a similar fashion, mismatch ID 19 (DQB1*06:04), which did not lead to the formation of DSA, had self DQB1-derived peptides binding with highest affinity for the recipient's HLA-DR antigens and with higher affinity than the nonself DQA1-derived-peptides. For mismatch ID 24 (DQB1*06:03), which did not lead to the formation of DSA and self peptides were binding with highest affinity for the recipient's HLA-DR antigens, the associated mismatched DQA1 had nonself peptides binding with highest affinity. In this case, only the expression of the HLA-DQ heterodimer comprised of both mismatched DQA1 and DQB1 (DQA1*02:01\DQB1*06:03) may lead to the formation of DSA against DQB1*06:03, but not if the mismatched DQB1 chain is expressed in association with the matched DQA1 chain. Therefore, to predict the immunogenicity of HLA-DQ mismatches, both alpha and beta chains have to be taken into account, as both can carry peptide sequences recognized as nonself by the recipient (the same is true for antibody epitopes [42, 43]).

The predictive power of this analysis can be improved if the sample size is increased and if the complete HLA class II typing is available. Indeed, the HLA typing of each donor/recipient pair for HLA-DRB3, HLA-DRB4, HLA-DRB5, and DP alleles was not available for this study. This limitation prevented a complete interlocus comparison between donor and recipient, especially for DRB1 antigens. The only mismatches that did not produce DSA but involved nonself peptides that were predicted to be presented were HLA-DRB1 mismatches (ID 17 and 22). Perhaps the nonself peptides predicted to be presented could potentially be self peptides that are shared by the recipient's HLA-DRB3, HLA-DRB4, or HLA-DRB5 antigens.

Furthermore, it can be noted that mismatches ID 4 and 16 were identical. Indeed, TxID 18 and TxID 33 had the same HLA class II typing (DRB1*11:01/*16:01, DQB1*03:01/*05:02, and DQA1*01:02/*05:05), an HLA-DRB1*07:01 mismatch, and at least one of their HLA-DRB1 antigens had the highest affinity for a nonself allo-HLApep. However, only TxID 33 produced an anti-HLA-DRB1*07:01 DSA, but not TxID 18. Therefore, mismatch ID 4 may have the potential to lead to the formation of a DSA, provided the allograft remained longer in the recipient. More direct evidence is needed, however.

This study evaluated the HLA class II (HLA-DR and HLA-DQ) presentation of B cells. Although B cells can be expected to present allo-HLA-derived peptides with all HLA class II antigens (DRB, DQ, and DP) [37, 44], the predicted presentation of recipients' HLA-DR antigens only, and not HLA-DQ or both HLA-DR and HLA-DQ, is associated with the formation of DSA. Therefore, this study suggests that the HLA-DR phenotype is associated with the production of IgG DSA, as previously described [29, 30, 34]. Interestingly, the increased cell surface expression of HLA-DQ and/or HLA-DP (the antigen-presenting phenotype of B cells) was observed in autoimmune diseases (rheumatoid arthritis and psoriatic arthritis) and is possibly associated with the presentation of self antigens [44]. Coincidently, HLA-DQ antigens were often predicted to present self allo-HLA peptides (as shown in Table 5). Evaluating the antigen presentation ability of DRB3, DRB4, or DRB5 and DP antigens remains to be elucidated in the context of transplantation.

Table 4: Recipient's HLA-DR predicted presentation of self and nonself allo-HLA-derived peptide with highest affinity.

Recipient	Donor mismatches																											
	HLA-DRB1												HLA-DQB1												HLA-DQA1					
	DRB1*11:04												**DQB1*03:01**												**DQA1*05:05**					
TxID 13	Self			Nonself									Self			**Nonself**									Self			**Nonself**		
	AA	IC_{50}	% rank	AA	IC_{50}	% rank							AA	IC_{50}	% rank	AA	IC_{50}	% rank							AA	IC_{50}	% rank	AA	IC_{50}	% rank
DRB1*04:05	***158***	***1344***	***0.3***	47	6022	7							***158***	***2053***	***0.7***	11	3057	1.8							146	3369	2.5	***153***	***1032***	***0.15***
DRB1*14:01	***158***	***2007***	***0.9***	47	3918	3.5							87	1426	0.5	***24***	***1203***	***0.4***							66	2605	1.5	***69***	***1813***	***0.7***
TxID 18	DRB1*07:01												**DQB1*02:02**												**DQA1*02:01**					
	Self			Nonself									Self			**Nonself**									Self			**Nonself**		
DRB1*11:01	***158***	***938***	***1.8***	6	1440	3							***87***	***600***	***1.1***	24	715	1.3							63	1465	3	***45***	***349***	***0.5***
DRB1*16:01	158	3272	3.5	***6***	***2455***	***2.5***							122	1910	1.6	***47***	***1819***	***1.5***							148	1063	0.7	***45***	***1060***	***0.7***
TxID 21	**DRB1*08:01**												**DQB1*03:01**						**DQB1*03:03**						**DQA1*06:01**					
	Self			Nonself									Self			Nonself			Self			Nonself			Self			Nonself		
DRB1*07:01	158	2199	2.5	***16***	***1167***	***0.8***							87	1908	1.9	***8***	***1286***	***1***	87	1908	1.9	***23***	***1532***	***1.3***	***148***	***2180***	***2.5***	54	2297	3
TxID 22	DRB1*07:01						DRB1*15:01						**DQB1*02:02**						**DQB1*06:02**						**DQA1*02:01**					
	Self			**Nonself**			Self			**Nonself**			Self			**Nonself**			Self			**Nonself**			Self			**Nonself**		
DRB1*11:01	***158***	***938***	***1.8***	6	1440	3	***47***	***919***	***1.8***	88	1952	4.5	126	1848	4	***87***	***600***	***1.1***	126	1848	4	***87***	***109***	***0.08***	148	1774	4	***45***	***349***	***0.5***
DRB1*14:07	***158***	***3491***	***1.2***	31	7990	9	***158***	***3491***	***1.2***	86	6554	5.5	16	6760	6	***83***	***2957***	***0.8***	16	6760	6	***87***	***1388***	***0.09***	140	5765	4.5	***69***	***2922***	***0.8***
TxID 33	**DRB1*07:01**						DRB1*13:01						**DQB1*02:02**						DQB1*06:04						**DQA1*02:01**					
	Self			Nonself			Self			Nonself			Self			Nonself			Self			Nonself			Self			Nonself		
DRB1*11:01	***158***	***938***	***1.8***	6	1440	3	47	919	1.8	***24***	***526***	***0.9***	***87***	***600***	***1.1***	24	715	1.3	***87***	***166***	***0.17***	24	275	0.4	63	1465	3	***45***	***349***	***0.5***
DRB1*16:01	158	3272	3.5	***6***	***2455***	***2.5***	47	2464	2.5	***24***	***2196***	***1.9***	122	1910	1.6	***47***	***1819***	***1.5***	***87***	***710***	***0.4***	24	1721	1.4	148	1063	0.7	***45***	***1060***	***0.7***
TxID 35	**DRB1*07:01**						DRB1*15:01						**DQB1*02:02**						DQB1*06:03						**DQA1*02:01**					
	Self			Nonself			Self			Nonself			Self			Nonself			Self			Nonself			Self			Nonself		
DRB1*04:03	***157***	***1784***	***0.03***	37	9997	7	***158***	***1784***	***0.03***	67	13081	14	***158***	***4464***	***0.7***	47	7447	3.5	***158***	***4464***	***0.7***	87	7639	3.5	***153***	***5451***	***1.3***	69	6218	1.9
DRB1*13:02	***159***	***3757***	***1.8***	30	5424	4	159	3757	1.8	***67***	***3244***	***1.3***	16	4547	2.5	***24***	***4083***	***2***	***16***	***4547***	***2.5***	87	6480	5	3	1055	0.15	***69***	***431***	***0.01***

Note. Bold and underlined mismatches led to the production of DSA. Bold amino acid position, IC50, and percentile rank are for the peptides with highest affinity for the recipient's HLA-DR antigens.
AA: amino acid position; % rank: percentile rank.

Table 5: Association between the nature (self or nonself) of the peptide predicted to bind with highest affinity to the recipient's HLA-II antigens and the presence of DSA.

Peptide length	HLA class II presentation	Allo-HLApep	DSA	No DSA	*P* value
9 mers	HLA-DR	Nonself	15	3	**0.0169**
		Self	2	5	
	HLA-DQ	Nonself	5	4	0.3942
		Self	12	4	
	Combined (HLA-DR and HLA-DQ)	Nonself	15	6	0.57
		Self	2	2	
12 mers	HLA-DR	Nonself	14	5	0.3442
		Self	3	3	
	HLA-DQ	Nonself	5	6	0.081
		Self	12	2	
	Combined (HLA-DR and HLA-DQ)	Nonself	14	8	0.527
		Self	3	0	
15 mers	HLA-DR	Nonself	14	6	1
		Self	3	2	
	HLA-DQ	Nonself	8	6	0.2337
		Self	9	2	
	Combined (HLA-DR and -DQ)	Nonself	15	8	1
		Self	2	0	

Note. Fisher's exact test two-tailed *P* values are shown.

One interesting finding is that the prediction of DSA formation is significantly associated with the presentation of 9-mer and not with 12-mer or 15-mer peptides. HLA class II antigens have a binding groove that is open at both ends, which allows the binding of peptides that extend beyond the groove and can accommodate peptides of varying lengths (typically 12–18 mers) and possibly whole proteins [45]. However, the core binding motif of HLA class II antigens, which anchors the peptide in the groove, is approximately nine amino acids long [46]. In addition, TCR interaction with the HLA class II/peptide complex requires different contact sites that span the HLA class II alpha and beta chains and across the peptide [47]. Furthermore, changes in the nonamer core sequences that aligned to the binding groove of HLA class II molecules may result in critical effects on peptide contacts and interactions with the TCR [48]. Therefore, when 9-mer allo-HLApep are analyzed, every core binding motif is evaluated separately in contrast to 12- or 15-mer peptides, where different peptides can have the same core binding motif. Similarly, PIRCHE-II relies upon the distinction between identical and nonidentical peptides based upon the exact nonamer sequence that was predicted to occupy the binding groove [34]. Yet the underlying reasons that 9-mer peptides predict the formation of DSA remains to be elucidated.

Surprisingly, the EpMS, HMS, EMS, and AMS were not statistically different between mismatches that led to the formation of DSA and the ones that did not, in contrast to previous reports. Although a trend was seen, where mismatches that led to the formation of DSA had higher EpMS, HMS, EMS, and AMS than mismatches that did not produce DSA, the sample size may have influenced the statistical significance. Nevertheless, HLAmatchmaker, a structurally based matching program, evaluates the possible target of IgG DSAs, and, similarly, Komoliaptsis et al. assessed the number and physiochemical properties of amino acid mismatches. Both approaches focused on the structural basis of antibody-antigen interactions and thus elucidated the antigenicity of HLA molecules. In contrast to the present study, both groups found a correlation between the number and physiochemical characteristics of antigenic determinant mismatches and the incidence of epitope-specific HLA DSA [20–22, 24–28]. Although the immunogenicity of HLA molecules is dependent on the mismatched amino acid residues between the donor and the recipient and on the number and physiochemical properties of these amino acid mismatches that are accessible on the molecular surface of HLA, only the antigenicity of HLA molecules are reported, not the immunogenicity or the humoral response to a specific allo-HLA. In contrast, there was a significant difference between the numbers of PIRCHE-II in mismatches that led to the formation of DSA compared to those that did not for all loci combined; this lends support to the idea that the immunogenicity of HLA class II mismatches is dependent on the recipient's HLA-DR presentation of self or nonself allo-HLA-derived peptides and may be an important factor in the IgM to IgG DSA isotype switch. Lastly, a conclusion drawn by Otten et al. [34] about the antigenicity (B cell epitopes) and immunogenicity (T cell epitopes) of HLA mismatches, being independent parameters in supporting the formation of DSA, is reinforced by this study.

The indirect allorecognition pathway and the thymic selection of CD4+ T cells are the central components underlying the analysis of this study. Figure 2 describes the process

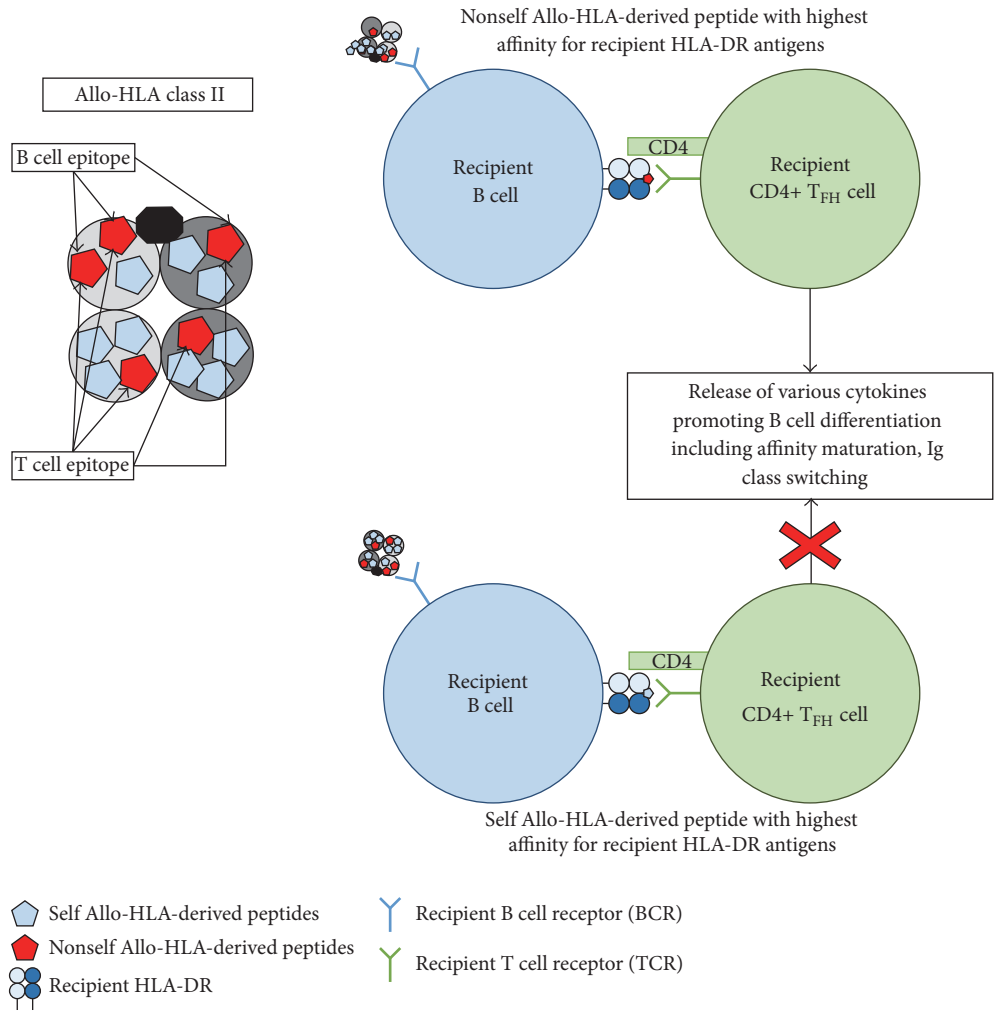

Figure 2: The indirect allorecognition pathway of allo-HLA class II by B cells and the presentation of self or nonself allo-HLA-derived peptide influence CD4+ T_{FH} cells helper function to lead to the formation of DSA. HLA mismatches can become immunogenic when they carry both a B cell epitope and a T cell epitope. B cell epitopes are located on the molecular surface of HLA, whereas T cell epitopes can be located anywhere, exposed or cryptic. B cells expressing a BCR specific for an allo-HLA can capture, internalize, and process the whole allo-HLA class II molecule and then present allo-HLA-derived peptides on the cell surface. Only when CD4+ T_{FH} cells recognize nonself antigenic determinants presented by B cells does the release of various cytokines promote B cell differentiation. This, in turn, may induce affinity maturation and Ig class switching, which eventually leads to the formation of DSA.

by which B cells capture and present allo-HLA molecules to CD4+ T cells. When nonself allo-HLA-derived peptides are presented to CD4+ T cells, the release of various cytokines promotes B cell differentiation into anti-HLA IgG-secreting plasma cells [8]. This process requires allo-HLA molecules to be antigenic (antibody reactive) and immunogenic (ability to elicit an immune response); that is, there has to be a domain recognized by B cells and a domain recognized by T cells, respectively [16]. The B cell domain, referred to as an HLA antibody epitope, should be located on the molecular surface of allo-HLA only, whereas the T cell domain, referred to as a T cell epitope, can be found anywhere, exposed or cryptic. Indeed, for HLA-I antigens, the α3 domain and the N-terminal part of the α1 domain seem to be enriched in T cell epitopes (described as PIRCHE-II), where HLA antibody epitopes (described as eplets) are rare [34]. Moreover, eplets recognized with high frequency by allo-antibodies are generally expressed on the top of the HLA molecule, in contrast to eplets recognized with low frequency that are in less accessible positions [24].

The dynamics of the HLA class II presenting pathways and the processing of allo-HLA molecules through different compartments of the endosomal/lysosomal pathway are a very complex mechanism and remain to be fully elucidated [49]. The cleavage, or proteolysis, of allo-HLA molecules into small antigenic peptides is dependent on the catalysis of different proteases [50–52], although HLA molecules are known to be resistant to protease degradation [53]. The cleavage pattern of HLA molecules by different proteases should help to identify whether certain predicted T cell epitopes (allo-HLApep) can be produced or not. An integrated approach to epitope analysis has been developed [54] and should be used

in the context of transplantation by combining it with the use of databases (such as SYFPEITHI) listing peptide sequences which are known to be presented HLA class II antigens [55]. T cell epitopes can be destroyed if they contain the cleavage site of any protease [56, 57], and it could explain why certain mismatches (ID 4, 17, and 22) that were predicted to present nonself peptides did not produce a DSA, and one mismatch (ID 21) that was predicted to present self peptides led to the formation of a DSA. Lastly, it is possible that relying solely on the peptides with highest affinity for the recipient's HLA class II antigens may carry a risk of missing relevant immunogenic peptides when both self and nonself peptides have similar binding affinities for the same HLA class II antigen. It has been documented that peptide immunogenicity correlates with both its dissociation rate from and its affinity for HLA class I molecules [58, 59], and the same may be true for HLA class II molecules. However, if their binding affinity difference is large enough then this risk may be minimized.

In summary, the approach described in this paper relies on the indirect allorecognition pathway to predict the formation of DSA. The predicted presentation of nonself allo-HLA-derived peptides shows a relationship with the presence of DSA in the serum of kidney recipients. The evaluation of the immunogenicity (self or nonself peptide presentation) and antigenicity (antibody epitopes) of HLA mismatches requires the molecular typing of all HLA-I and HLA class II loci, which is not a standard practice in most organ transplantations. But this approach can benefit mismatched transplantation by optimizing donor-recipient matching and selecting permissible HLA mismatches with low immunogenic potential in order to minimize the appearance of DSA.

Competing Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Computational Approaches to Facilitate Epitope-Based HLA Matching in Solid Organ Transplantation

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Epitope-based HLA matching has been emerged over the last few years as an improved method for HLA matching in solid organ transplantation. The epitope-based matching concept has been incorporated in both the PIRCHE-II and the HLAMatchmaker algorithm to find the most suitable donor for a recipient. For these algorithms, high-resolution HLA genotype data of both donor and recipient is required. Since high-resolution HLA genotype data is often not available, we developed a computational method which allows epitope-based HLA matching from serological split level HLA typing relying on HLA haplotype frequencies. To validate this method, we simulated a donor-recipient population for which PIRCHE-II and eplet values were calculated when using both high-resolution HLA genotype data and serological split level HLA typing. The majority of the serological split level HLA-determined *ln(PIRCHE-II)/ln(eplet)* values did not or only slightly deviate from the reference group of high-resolution HLA-determined *ln(PIRCHE-II)/ln(eplet)* values. This deviation was slightly increased when HLA-C or HLA-DQ was omitted from the input and was substantially decreased when using two-field resolution HLA genotype data of the recipient and serological split level HLA typing of the donor. Thus, our data suggest that our computational approach is a powerful tool to estimate PIRCHE-II/eplet values when high-resolution HLA genotype data is not available.

1. Introduction

Alloimmunity due to Human Leukocyte Antigens (HLA) mismatches between donor and recipient significantly impairs graft survival after solid organ transplantation [1–3]. The risk on graft failure is significantly associated with the number of HLA mismatches [1, 4]. Therefore, some allocation policies prefer deceased donors with zero mismatches at HLA-A, HLA-B, and HLA-DR, whereas others select deceased donors based on the number mismatches at these loci [5].

Although the number of HLA mismatches is a potent predictor of transplant outcome, not every HLA mismatch will have an equal effect on graft failure [6, 7]. Cumulating evidence suggest that some HLA mismatches may induce alloimmunity, whereas others are well-tolerated [6, 7]. This high variability in permissibility might be due to differences in the antigenic load between different donor-recipient couples [8, 9]. Each HLA antigen expresses a unique combination of epitopes, but some of these individual epitopes may be shared between different HLA antigens [8]. These shared epitopes will not induce alloimmunity, whereas those epitopes that are mismatched between donor and recipient may induce alloimmunity. Thus, quantifying the antigenic load (i.e., the number of epitope mismatches) between donor and recipient instead of counting the number of HLA mismatches may be a better approach to predicting transplant outcome [9–12]. This concept of epitope-based HLA matching is an alternative method to define the most suitable HLA mismatch for each patient, thereby reducing the risk on donor-specific HLA antibody formation after transplantation and graft failure.

Two in silico methods, HLAMatchmaker and PIRCHE-II, have incorporated the epitope-based HLA matching concept in their algorithm to find the most suitable donor for a

recipient. HLAMatchmaker determines differences in B-cell epitopes between donor and recipient to estimate the risk of graft failure [13–15]. These B-cell epitopes, designated as eplets, are groups of polymorphic amino acid positions on the three-dimensional molecular surface of HLA to which HLA antibodies can be formed [13–15]. The PIRCHE-II algorithm determines differences between donor and recipient in their HLA-derived T-helper epitopes to estimate the risk of transplant outcome [16]. These T-helper epitopes, designated as PIRCHE-II (Predicted Indirectly ReCognizable HLA Epitopes presented by HLA-DRB1), are involved in the production of HLA-specific IgG antibodies [17–19], as T-helper epitopes are required for B-cell activation and IgM-to-IgG isotype switching [20, 21].

To be able to identify the unique set of donor and recipient HLA epitopes, identification of the exact polymorphisms in donor and recipient HLA is required. Low-resolution HLA typing is, however, not sufficient to identify these polymorphisms, as low-resolution HLA typing can cover numerous HLA alleles at high-resolution HLA level. Thus, low-resolution HLA typing will lead to an ambiguous epitope definition. Therefore, two-field resolution HLA genotype data of both donor and recipient is preferably required to unambiguously determine the HLA compatibility between donor and recipient at epitope level. Indeed, both HLA-Matchmaker and the PIRCHE-II algorithm require high-resolution HLA genotypes of both donor and recipient as input for their algorithm. Although HLA genotyping methodologies have improved over the last few years and high throughput NGS technology became available, the quick availability of reliable high-resolution HLA genotype data remains challenging. High-resolution HLA genotyping is especially for deceased donors hardly feasible, as time is a major limiting factor for deceased organ transplantation.

Instead of high-resolution HLA genotyping of donor and recipients, also alternative approaches can be used to facilitate epitope-based HLA matching. In the present study we describe a computational method to perform epitope-based HLA matching using serological split level HLA typing as input. In this computational method, the most likely high-resolution HLA genotypes that correspond to a serological split level HLA typing are identified using HLA haplotype frequency tables. For all of these high-resolution HLA genotypes, a PIRCHE-II and eplet value can be calculated, which can subsequently be weighed against the normalized frequency of the pair of HLA haplotypes in the general population. To test whether the risk estimation alters when using our computational approach, we calculated the PIRCHE-II/eplet values when using serological split level HLA typing (designated as observed PIRCHE-II/eplet values) and compared these values with the PIRCHE-II and eplet values when using high-resolution HLA genotype data (designated as reference PIRCHE-II/eplet values).

2. Materials and Methods

2.1. Generation of the Representative Recipient Population. To model a representative recipient population, all HLA genotypings ($n = 4{,}579$) that were performed at the University Medical Center Utrecht between January 2009 until July 2016 were extracted from the lab system. HLA genotypings were performed at different resolution levels and for different loci. High-resolution HLA genotypings were performed by SBT (before 2014; SBT kit, genDX, Utrecht, The Netherlands) or NGS (2014 and later; NGSGo, genDX, Utrecht, The Netherlands) whereas PCR-SSO (One Lambda) was used for lower-resolution HLA genotyping. HLA genotypes that did not have a fully unambiguous high-resolution HLA typing for HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 were excluded from the population. A total of 2,373 typings had an unambiguous high-resolution HLA genotype data for HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1. These typings were used to simulate a representative recipient population.

2.2. Generation of the Virtual Donor Population. A virtual Caucasian donor population consisting of 10 million individuals was modeled using HLA haplotype frequency tables from the National Marrow Donor Program of 2007 [22]. The HLA haplotype frequency tables are available via https://bioinformatics.bethematchclinical.org/hla-resources/haplotype-frequencies/. To generate each individual of the 10 million individuals, two HLA haplotypes were randomly assigned to an individual. This assignment was based on the frequency of these HLA haplotypes within the HLA haplotype frequency tables. In this procedure sampling of HLA haplotypes was performed without replacement. Combined with the representative recipient population, this Caucasian population was subsequently used as a virtual donor population to form donor-recipient couples.

2.3. Generation of Donor-Recipient Couples. The virtual donor population and the representative recipient population were combined to form potential donor-recipient couples. Since random allocation will lead to a nonrepresentative distribution of HLA mismatches, donor-recipient couples were formed using the basic guidelines that are currently used for deceased kidney allocation at our local center. For each donor, a recipient was selected that had maximal 3 mismatches at HLA-A and HLA-B and maximal a single mismatch at HLA-DR. To this end, both the HLA genotypes of the representative patient population and the virtual donor population were converted into serological broad level HLA typing, which was used for matching. A donor was randomly selected from the virtual donor population and this donor was subsequently matched to a recipient. When for a certain donor-recipient combination four mismatches at HLA-A and HLA-B and/or two mismatches at HLA-DR were found, another recipient was selected for that donor until the matching criteria were met for a given donor-recipient couple. This procedure was followed until a virtual donor was found for each recipient of the representative recipient population.

This method has resulted in 2,373 donor-recipient couples. A total of 11 donor-recipient couples had zero mismatches, 165 had a single mismatch, 571 had two mismatches,

890 had three mismatches, and 736 had four mismatches at these loci. A total of 2,195 mismatches were at HLA-A, 2,823 at HLA-B, and 1,903 at HLA-DR.

Serological split level HLA typing (observation group) and two-field resolution HLA genotypes (reference group) of these donor-recipient couples were used to calculate the number of PIRCHE-II and eplets, as described below. Although donor-recipient couples were formed based on HLA-A, HLA-B, and HLA-DR only, the complete five loci-haplotypes (HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ) were used to calculate the PIRCHE-II and eplet values. PIRCHE-II and eplet values were also calculated when HLA-C or HLA-DQ was removed from the serological split level HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ typing or when using two-field resolution HLA genotype data of the recipient and serological split level HLA typing of the donor as input for the algorithms.

2.4. The Use of Serological Split Level HLA Typing to Identify Potential High-Resolution HLA Genotypes. For the serological split level HLA typings of the 2,373 donor-recipient couples, a high-resolution extrapolation method was used to identify all possible high-resolution HLA genotypes that correspond to each serological split level HLA typing. To this end, HLA haplotype frequency tables from the National Marrow Donor Program from 2007 and 2011 were both used separately to identify all potential high-resolution HLA genotypes from a serological split level HLA typing [22, 23].

For every given serologic split level HLA typing, the extrapolation algorithm started with setting up all potential two-field resolution HLA haplotype pairs (HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1) that yield the given input serological split level HLA typing. After mapping antigen/allele names for each serologic value, the haplotype frequency table was filtered for matching haplotypes. When no matching high-resolution HLA haplotype was found for a given serological split level HLA typing or subsets thereof, the selection criteria were broadened by removing the link between loci in a step-wise manner. The following order of HLA loci linkage removal was used: [i] A-B-C-DRB1-DQB1, [ii] A-B-C | DRB1-DQB1, [iii] A | B-C | DRB1-DQB1, [iv] A | B-C | DRB1 | DQB1, and [v] A | B | C | DRB1 | DQB1. It has to be noted that mode [v] is equivalent to using allele frequencies for the prediction of each individual locus. Supplementary material 1 in Supplementary Material available online at https://doi.org/10.1155/2017/9130879 shows an example of the linkage breakdown between HLA loci.

Since multiple high-resolution HLA genotypes may correspond to a single serological split level HLA typing, a list of potential high-resolution HLA genotypes was generated for both donor and recipient. For each typing, a frequency was calculated by multiplying both haplotypes' frequencies. The resulting absolute frequencies were normalized within the set of likely high-resolution HLA genotypes for the given input.

This method was applied to both donors and recipients. For all these potential high-resolution HLA genotypes of donor and recipient, the PIRCHE-II and eplets values were calculated as described below. The obtained PIRCHE-II values were subsequently weighted by multiplying the normalized frequency of a certain high-resolution HLA genotype of the recipient with the normalized frequency of a certain high-resolution HLA genotype of the donor. Finally, all weighted PIRCHE-II values were summed up. The same was applied to the eplet values. Thus, the used method takes all potential high-resolution HLA genotypes that are present in the HLA haplotype frequency table into account which corresponds to a certain serological split level HLA typing. By using all these genotypes and by weighing the epitope values, multiple imputation is used to minimize bias towards common HLA genotypes.

In this study, the HLA haplotype frequency tables of the Caucasian population (2007) and the European-Caucasian population (2011) were used in this study to determine all potential high-resolution HLA genotypes.

2.5. Identification of PIRCHE-II. For both the two-field resolution HLA genotypes (designated as reference group) and the serological split level HLA typings (designated as observation group), the number of PIRCHE-II was determined for each donor-recipient couple as described previously [17]. Briefly, the nonameric binding cores of mismatched-HLA derived peptides to recipient HLA-DRB1 (PIRCHE-II) were predicted using the NetMHCIIpan 3.0 algorithm. Peptides that had an $IC_{50} < 1000$ nM were considered as relevant HLA-DRB1 binders. These relevant HLA-DRB1 binders were only classified as a PIRCHE-II when the amino acid residues of the nonameric binding cores were not present in the amino acid sequence of recipient HLA. The PIRCHE algorithm is available via https://www.pirche.org/.

2.6. Identification of HLAMatchmaker Eplets. For both the two-field resolution HLA genotypes and the serological split level HLA typing, the number of mismatched eplets was determined for each donor-recipient couple using eplet definitions as provided in HLAMatchmaker algorithm version 2.1 (available via http://www.epitopes.net/). Mismatched eplets were defined as eplets that were present in donor HLA but absent in recipient HLA. Via interlocus HLA comparisons the number of mismatched eplets was determined.

2.7. Comparison between Serological Split Level HLA Typing and Two-Field Resolution HLA Genotype Data. When using serological split level HLA typing as input for the algorithm, PIRCHE-II and eplet values were obtained for 2,319 donor-recipient couples (97.7%) when using the HLA haplotype frequency tables of 2007 or for 2,369 donor-recipient couples (99.8%) when using the HLA haplotype frequency tables of 2011.

The number of epitopes and alloreactivity are unlikely correlated in a linear fashion; increases from 1 epitope to 10 epitopes are likely to have a higher impact than increases from 200 epitopes to 210 epitopes and the effects are likely to plateau at some point. As such we assume an inverse logarithmic effect of epitope numbers on alloreactivity and we converted the PIRCHE-II and eplet numbers into the natural logarithm thereof. These transformed PIRCHE-II and eplet values were subsequently used to identify differences

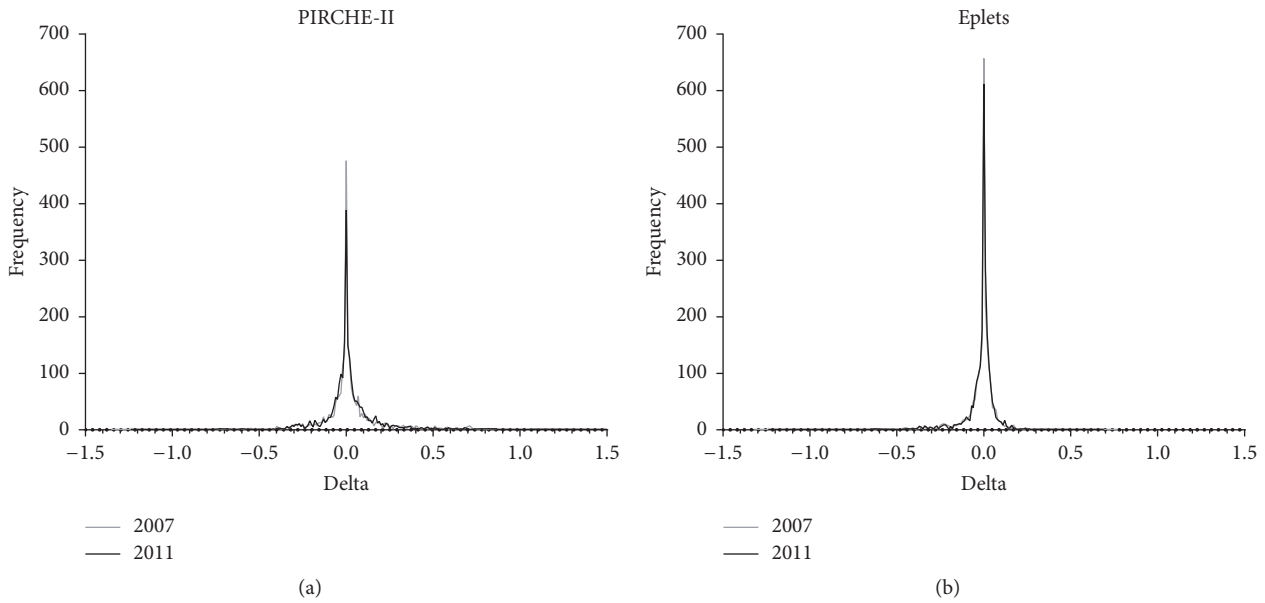

Figure 1: Deviation of the observed *ln(PIRCHE-II)/ln(eplet)* values from reference *ln(PIRCHE-II)/ln(eplet)* values when using HLA haplotype frequency tables from 2007 or from 2011. For both *ln(PIRCHE-II)* (a) and *ln(eplet)* (b), the observed values do not deviate or only slightly deviate from the reference values. The use of haplotype HLA frequency tables from 2007 (gray line) or from 2011 (black line) resulted in similar deviations.

between the two-field HLA genotype-determined PIRCHE-II/eplet values, designated as "reference PIRCHE-II/eplet values," and the serological split level HLA typing-determined PIRCHE-II/eplet values, designated as "observed PIRCHE-II/eplet values." The delta between the observed and reference PIRCHE-II/eplet values was calculated by subtracting the log-transformed reference PIRCHE-II/eplet values from the log-transformed observed PIRCHE-II/eplet values. A delta of >+1 or <−1 was considered a high deviation, as it represents a single unit of *ln(PIRCHE-II)* or *ln(eplet)* difference and thus a significant over- or underestimation of the hazard in graft failure. For all tested settings, we calculated the delta that corresponded to the 50% percentiles, 75% percentiles, 95% percentiles, 99% percentiles, and 99.9% percentiles to test the reliability of our estimations. For example, for the 75% percentile we calculated to which delta 25% of the values above the median stretched and to which delta 25% of the values below the median stretched. A similar approach was used for the other percentiles.

3. Results

3.1. Reliability of Epitope Matching When Using Serological Split Level HLA Typing. For all donor-recipient couples we calculated the PIRCHE-II and eplet values when using two-field HLA genotype data and serological split HLA typing as input for the module. The PIRCHE-II/eplet values determined based on two-field resolution HLA genotypes were designated as "reference PIRCHE-II/eplet values," whereas the PIRCHE-II/eplet values determined based on serological split HLA typing were designated as "observed PIRCHE-II/eplet values."

First we determined whether the use of serological split level HLA typing as input led to different PIRCHE-II and eplet values compared to the use of two-field resolution HLA genotype data as input. To this end, the delta between the observed *ln(PIRCHE-II)* and *ln(eplet)* values and the reference *ln(PIRCHE-II)* and *ln(eplet)* values was calculated (Figure 1). When using the frequency tables of 2007, 20.5% of observed *ln(PIRCHE-II)* values did not show deviation from the reference *ln(PIRCHE-II)* values (Figure 1(a)). Furthermore, 72.8% of the observed *ln(PIRCHE-II)* values deviated maximal 0.1 from the reference *ln(PIRCHE-II)* values. When using the frequency tables of 2007, 28.3% of observed *ln(eplet)* values did not deviate from the reference *ln(eplet)* values and 87.7% deviated maximal 0.1 (Figure 1(b)). These data indicate that majority of the observed *ln(PIRCHE-II)* and *ln(eplet)* values do not deviate or only slightly deviate from reference *ln(PIRCHE-II)* and *ln(eplet)* values.

For both PIRCHE-II and the eplets, similar results were obtained when using the frequency tables of 2011 instead of using the frequency tables of 2007. When using the frequency tables of 2011, 16.4% of observed *ln(PIRCHE-II)* values did not show deviation from the reference *ln(PIRCHE-II)* values and 73.2% deviated maximal 0.1 (Figure 1(a)). When using the frequency tables of 2011, 25.8% of observed *ln(eplet)* values did not deviate from the reference *ln(eplet)* values and 88.4% deviated maximal 0.1 (Figure 1(b)). This observation suggests that the use of these more recent frequency tables in our computational method does not improve or worsen the observed PIRCHE-II/eplet values.

Next, we investigated whether the delta in *ln(PIRCHE-II)* values differed from the delta in *ln(eplet)* values. To this end, we plotted the delta for *ln(PIRCHE-II)* against the delta for *ln(eplet)*. When using serological split level HLA typing of

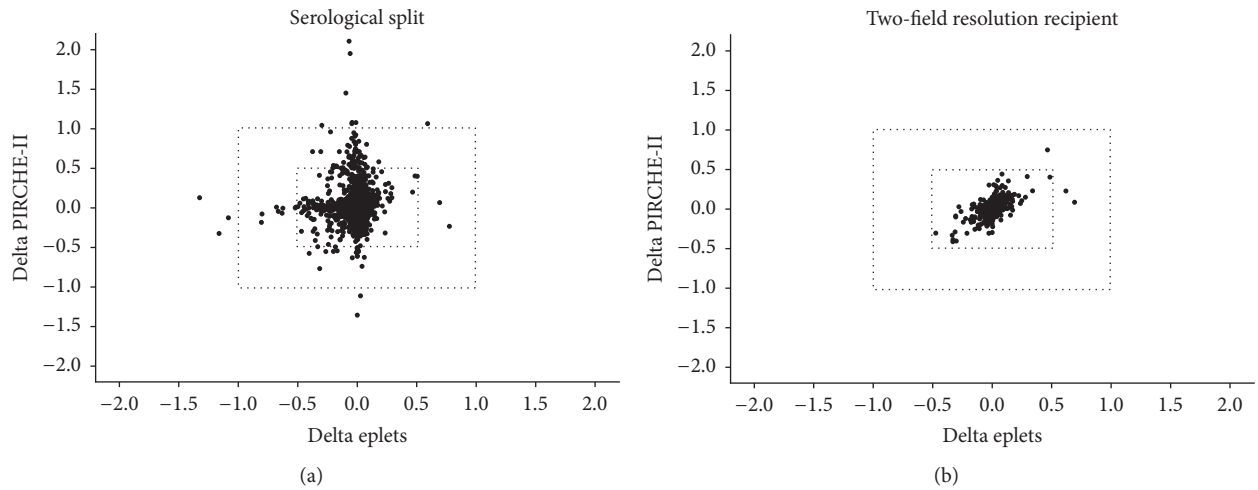

Figure 2: Comparison of the delta in *ln(PIRCHE-II)* and the delta in *ln(eplet)*. (a) When using serological split level HLA typing of both donor and recipient, the majority of the donor-recipient couples had a comparable *ln(PIRCHE-II)* and *ln(eplet)* delta. For 13 donor-recipient couples, the observed *ln(PIRCHE-II)* or *ln(eplet)* values deviated substantially (>+1 or < −1) from the reference *ln(PIRCHE-II)* or *ln(eplet)* values. (b) When using two-field resolution HLA genotype data of the recipient and serological split level HLA typing of donor the deviation between the observed *ln(PIRCHE-II)/ln(eplet)* values and the reference *ln(PIRCHE-II)/ln(eplet)* values substantially diminished. The dashed squares indicate the delta<0.5 and delta<1 borders.

both donor and recipient, the majority of the donor-recipient couples had a comparable *ln(PIRCHE-II)* and *ln(eplet)* delta (Figure 2(a)). However, two donor-recipient couples (0.08% of total) had a *ln(PIRCHE-II)* delta below −1, eight donor-recipient couples (0.34% of total) had a *ln(PIRCHE-II)* delta above +1, and three donor-recipient couples (0.13% of total) had a *ln(eplet)* delta below −1. These data suggests that the PIRCHE-II value is more often overestimated using the extrapolation approach, whereas the eplet value is more often under-estimated using the extrapolation approach. For all the donor-recipient couples who had a high deviation in the *ln(PIRCHE-II)* or *ln(eplet)* values, the corresponding *ln(eplet)* or *ln(PIRCHE-II)* values respectively were within the −1 and +1 range. This observation indicates that an increased delta in *ln(PIRCHE-II)* is not associated with an increased delta in *ln(eplet)* and vice versa.

3.2. Removal of HLA-C or HLA-DQ and Reliability of Estimation. HLA-B is in strong linkage disequilibrium with HLA-C and HLA-DRB1 is in strong linkage disequilibrium with HLA-DQB1. This strong linkage disequilibrium suggests that removal of HLA-C or HLA-DQ from the serological split level HLA typing might only limitedly affect the observed PIRCHE-II/eplet values and thus the delta between the observed and the reference *ln(PIRCHE-II)/ln(eplet)* values. To investigate the effect of HLA-C or HLA-DQ on the PIRCHE-II and eplet estimations, we removed HLA-C or HLA-DQ from the serological split level HLA typing that was used as input for both PIRCHE-II and HLAMatchmaker. Figure 3 shows the percentage of donor-recipient couples with a delta of zero between the observed and the reference *ln(PIRCHE-II)* and *ln(eplet)* values. When omitting HLA-C or HLA-DQ from the input, the percentage of donor-recipient couples who had a *ln(PIRCHE-II)* delta of zero dropped from 20.5% to 9.4% or 12.3% for omitting HLA-C or HLA-DQ, respectively (Figure 3(a)). For the eplets, similar results were obtained. The percentage of donor-recipient couples who had an *ln(eplet)* delta of zero dropped from 28.3% to 12.7% or 16.8% for omitting HLA-C or HLA-DQ, respectively (Figure 3(b)).

To investigate the reliability of the epitope estimation when omitting HLA-C or HLA-DQ, we plotted the *ln(PIRCHE-II)* and *ln(eplet)* delta values for different percentiles (50%, 75%, 95%, and 99.9%). When omitting HLA-C or HLA-DQ from the serological split level HLA typing, the delta values for the higher percentiles slightly increased compared to delta values obtained from serological split level typing with HLA-C and HLA-DQ (Figures 3(c) and 3(d)). These observations indicate that removal of HLA-C or HLA-DQ from the serological split level HLA typing diminishes the reliability of the observed PIRCHE-II/eplet values.

3.3. Effect of Higher Resolution Typing of Recipient. Since high-resolution HLA genotyping is a time-consuming method, high-resolution HLA genotyping of deceased donors is hardly feasible using the currently available typing methodologies. However, in most cases, high-resolution HLA genotyping of the recipient is possible. Therefore, we investigated whether a higher resolution HLA genotyping of the recipient may improve the reliability of the PIRCHE-II and eplet estimations. To this end, we used two-field resolution HLA genotype data of the recipient and serological split level HLA typing of the donor as input for the PIRCHE-II and the HLAMatchmaker algorithm. The percentage of donor-recipient couples who had a *ln(PIRCHE-II)* delta of zero increased from 20.5% to 46.9% when using two-field resolution genotype data of the recipient (Figure 3(a)). For the *ln(eplet)*, this percentage of donor-recipient couples who had a delta of zero increased from 28.3% to 50.9% (Figure 3(b)). The *ln(PIRCHE-II)*

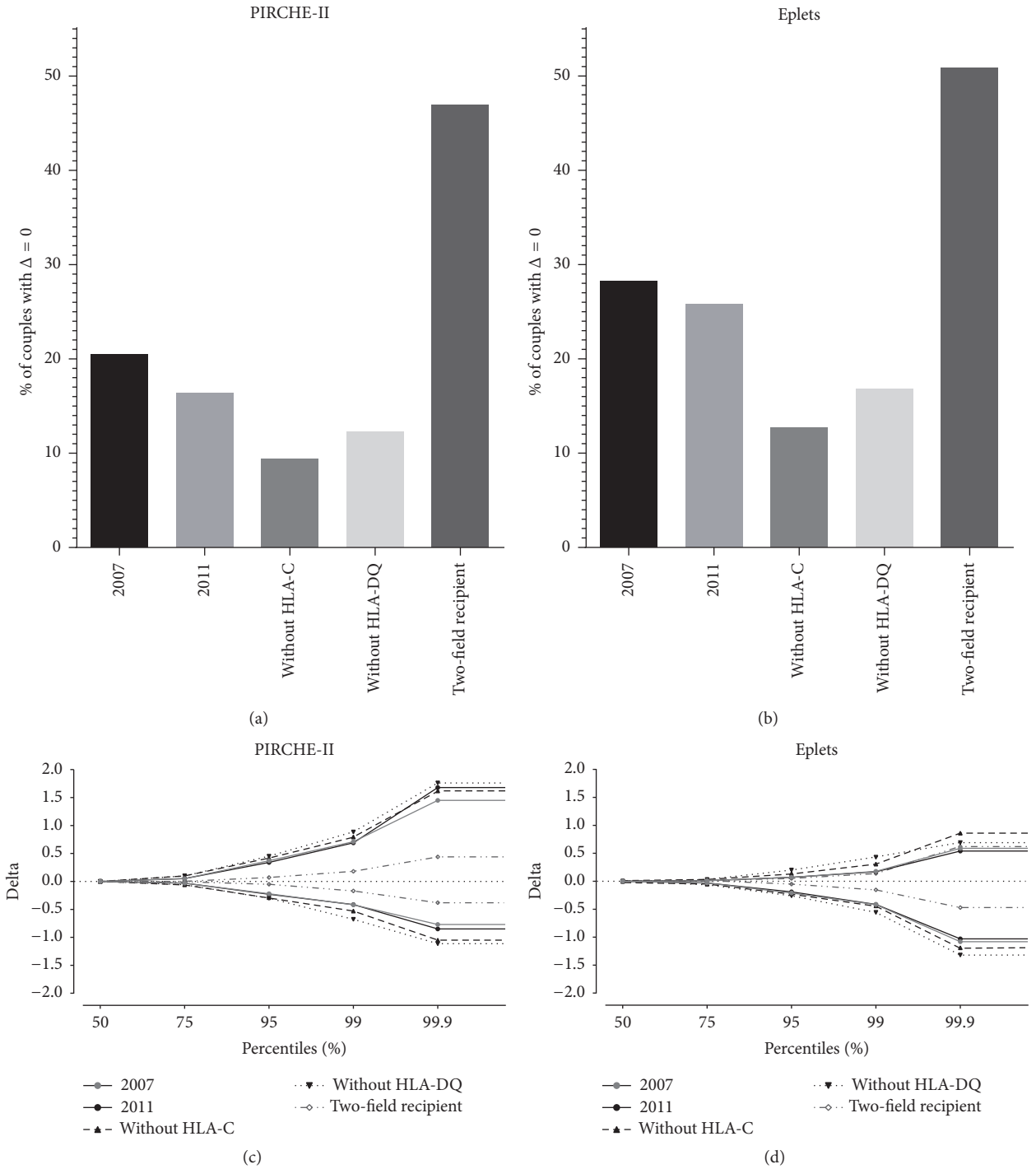

Figure 3: The reliability of the PIRCHE-II and eplet estimations in different settings. The percentage of typing with a delta of zero between the observed and the reference values was plotted for *ln(PIRCHE-II)* (a) and *ln(eplet)* (b). For both *ln(PIRCHE-II)* and *ln(eplet)*, the percentage of typing with a delta of zero was diminished when HLA-C or HLA-DQ was omitted from the typing. The highest percentage was observed when using two-field HLA genotype data of the recipient and serological split level typing of the donor. The different percentiles observed in the different settings were plotted for *ln(PIRCHE-II)* (c) and for *ln(eplet)* (d). The dashed horizontal lines in (c) and (d) indicate a delta of zero.

delta and the *ln(eplet)* delta diminished at all percentile values when using two-field resolution genotype data of the recipient instead of serological split HLA typing (Figures 3(c) and 3(d)). These data indicate that higher resolution genotyping of the recipient decreases the delta between the observed and the reference *ln(PIRCHE-II)* and *ln(eplet)* values and thus increases the reliability of the observed PIRCHE-II and eplet values. The reliability of the estimation was especially improved for PIRCHE-II and to a substantial but lesser extend for eplets. For all different settings tested, the most reliable PIRCHE-II/eplet estimation was achieved when using two-field resolution genotype data of the recipient and serological split level HLA typing of the donor.

When comparing the delta in *ln(PIRCHE-II)* values with the delta in *ln(eplet)* values for the two-field resolution recipient setting, all donor-recipient couples had comparable *ln(PIRCHE-II)* and *ln(eplet)* delta values (Figure 2(b)). For all couples, no outliers for *ln(PIRCHE-II)* and *ln(eplet)* were observed. Thus, our data suggest that two-field resolution HLA genotyping of the recipient further improve the reliability of the PIRCHE-II and eplet estimation.

4. Discussion

Several studies have shown that the number of PIRCHE-II and eplets are related to the clinical outcome after solid organ transplantation [9–12]. To select donors in an epitope-based manner, high-resolution genotyping of both donors and recipients is currently required. However, high-resolution HLA genotyping is often not feasible, particularly for deceased-donor organs. In this study we describe and validate a computational method to facilitate epitope-based HLA matching using low-resolution HLA typing.

In the present study we provide a methodology for using low-resolution serological split level HLA data to reliably estimate the PIRCHE-II and eplet values for the majority of the tested donor-recipient couples (Figure 1). Most of the observed *ln(PIRCHE-II)* and *ln(eplet)* values do not deviate or only slightly deviate from the reference *ln(PIRCHE-II)* and *ln(eplet)* values (Figure 2(a)). These data suggest that additional high-resolution HLA genotyping may not be necessary for the majority of the donor-recipient couples when using our described computational approach. However, furthers validations in other datasets consisting of different ethnicities are required to identify for which donor-recipient couples additional high-resolution HLA genotyping is required and for which donor-recipient couples low-resolution HLA typing is sufficient.

We also showed that the PIRCHE-II/eplet estimation deteriorated or improved in different settings. The removal of HLA-C and HLA-DQ increased both the *ln(PIRCHE-II)* delta and the *ln(eplet)* delta (Figure 3), indicating that the PIRCHE-II/eplet estimation is deteriorated when HLA-C and HLA-DQ are omitted from the input. Based on these results, we suggest that HLA-C and HLA-DQ typing are a valuable addition when using epitope-based HLA matching algorithms. Two-field resolution HLA genotyping of the recipient in combination with serological split level HLA typing of the donor substantially improved the PIRCHE-II/eplet estimation, as reflected by the decreased delta between the observed and the reference *ln(PIRCHE-II)*/*ln(eplet)* values (Figures 2(b) and 3). Since time allows typing of the recipient at high- or even allelic resolution, the situation of a two-field resolution genotyped recipient and a serological split level HLA typed donor may be a feasible option in many cases and this will substantially improve the reliability of the PIRCHE-II/eplet estimation. This approach is now current practice in our center.

When comparing the PIRCHE-II results with the eplet results for the serological split level data, the delta for *ln(PIRCHE-II)* was more often positive than negative, whereas for *ln(eplet)* the delta was more often negative than positive (Figures 2(a), 3(b), and 3(c)). We also showed that a high deviation in *ln(PIRCHE-II)* values is not related to a high deviation in *ln(eplet)* values; some donor-recipient couples had a high *ln(PIRCHE-II)* delta and a small *ln(eplet)* delta and other donor-recipient couples had a high *ln(eplet)* delta and a small *ln(PIRCHE-II)* delta (Figure 2(a)). These observations suggest that serological epitopes (eplet) can more easily be determined with a serological split level HLA typing than T-helper epitopes (PIRCHE-II). In addition, these observations might also suggest that PIRCHE-II and eplets are indeed two separate entities, as already suggested in previous studies [17, 19].

Our study has several limitations. First, the virtual Caucasian donor population used in our study was constructed upon the HLA haplotype frequency tables from 2007. However, these HLA haplotype frequency tables from 2007 also formed the basis for our PIRCHE-II/eplet estimation approach and therefore may cause bias in our observations or overfitting of the results. When using the updated HLA haplotype frequency tables from 2011, similar observed PIRCHE-II/eplet values were obtained (Figure 1), indicating that the bias is limited. However, usage of the 2007 HLA haplotype frequency tables showed a slightly improved PIRCHE-II/eplet value estimation compared to the 2011 HLA haplotype frequency tables, which might be due to overfitting of the results. Second, by calculating a delta between natural logarithmic transformed data, the difference in the actual PIRCHE-II/eplet count may be masked for several donor-recipient couples. However, since the effect of the PIRCHE-II/eplet count on alloreactivity is likely natural logarithmic, calculating the delta using log-transformed data gives more insight into the alterations of the hazard on alloreactivity rather than differences in actual epitope count. Third, our validation is limited to the Caucasian population and can only limitedly be extrapolated for other ethnicities. Since the Caucasian HLA haplotype frequency tables were based on a large-scale dataset [22, 23], we used these frequency tables to estimate the PIRCHE-II/eplet values. The estimation of the PIRCHE-II/eplet values might less or more deviate when investigating different ethnicities. Considering all these study limitations, further studies, especially with different data sets and with different ethnicities, are required to validate our observations. Ultimately, fine-details on the donor ethnicity and the related frequencies, for instance, as documented by http://allelefrequencies.net [24], may further enhance the reliability of the extrapolations.

Our study showed that the observed PIRCHE-II and eplet values only limitedly deviate from the reference PIRCHE-II and eplet values in a quantitative manner; only differences in PIRCHE-II and eplet numbers were investigated in this study. However, although PIRCHE-II and eplet values do not differ between the observation group and the reference group, both calculations may correspond to different epitopes. Further analyses are required to identify whether the observed and reference calculations will have qualitative differences in epitopes.

To our knowledge this is the first study that uses this computational approach for epitope-based HLA matching algorithms. Other studies have been using the HLA haplotype frequency tables in a similar way but in different settings. For example, a similar approach as our approach has been used by Madbouly et al. for the imputation of high-resolution HLA genotypes from multilocus unphased genotypes with ambiguous or missing typing data [25]. In addition, the HLA Haplotype Validator described by Osoegawa et al. uses HLA haplotype frequency tables to extract all potential HLA haplotype constellations to identify potential errors in HLA genotyping [26]. Furthermore, HaploStats from the National Marrow Donor Program (available via http://www.haplostats.org/) also estimates the most likely high-resolution HLA genotypes, but without HLA loci linkage removal when no matching high-resolution HLA haplotype is found. Thus, imputation of data can only take place when the HLA haplotypes are present in the HLA haplotype frequency tables. For the HLAMatchmaker algorithm, HLA haplotype frequency tables have been used to identify the most frequently present HLA haplotype in the population that corresponds to a given low-resolution HLA typing. This most frequently HLA haplotype is subsequently used as input for the algorithm. By using this approach, other potential less-frequent HLA haplotypes that also fit with the given low-resolution HLA typing are excluded. In our multiple imputation approach, high-resolution HLA genotypes with reduced likelihood were taken along in the PIRCHE-II and eplet calculations. Since our approach does not exclude less likely HLA haplotypes, we believe that our approach is more reliable than selecting the highest frequent high-resolution HLA haplotypes that is present in the general population.

Our algorithm currently only handles serological split level HLA typing, whereas serological broad level HLA typing cannot be used as input for the algorithm. Likely, serological broad level HLA typing will further deteriorate our estimations and, consequently, is not preferred for an epitope-based HLA matching setting. Moreover, since serological split level HLA typing of both donors and recipients is currently mandatory according to the Eurotransplant guidelines [27], serological split level HLA typing is available for all donor-recipient couples and thus can be used in our approach. Extension of the computational method, for example, by providing genotype list string (GL string) [28] or adding NMDP allele codes [29] to the HLA typing input, might eventually further enhance the estimations of the PIRCHE-II/eplet values. Further studies are required to investigate whether addition of these functions will enhance the reliability of the estimations.

Our data show that although the PIRCHE-II/eplet value estimations are quite reliable, the estimations could be further improved. First, for a few donor-recipient couples, the PIRCHE-II/eplet values could not be calculated when using serological split level HLA typing, indicating that our approach cannot be used for a limited number of donor-recipient couples. One of the major improvements will be extension of the Next-Generation Sequencing-based genotype datasets that are used for establishing HLA haplotype frequency tables. As of November 2016, 11,553 HLA class-I alleles and 4,084 HLA class-II alleles are registered in the IMGT/HLA database 3.26 [30]. These high numbers of identified HLA alleles indicate that huge population HLA genotype datasets are required to reliably estimate the HLA haplotype frequencies. Indeed, a previous study has shown that the HLA haplotype frequencies are overestimated when small sample sizes are used [31]. This aspect stretches that more detailed information and reliable typing of different HLA haplotypes is required. Although the Caucasian HLA haplotype frequency table of the NMDP is based on the largest dataset, the sample size is still limited considering all the identified HLA alleles. This limited sample size might bias our results. Indeed, rare alleles are hardly present in the HLA haplotype frequency tables and, consequently, a rare allele among donor and recipients at high-resolution level can often not be identified using the current HLA haplotype frequency tables. Moreover, the NMDP HLA haplotype frequency tables are generally based on exon 2-3 for HLA class-I alleles and on exon 2 for HLA class-II alleles. Therefore, sharing of whole gene NGS sequencing-based high-resolution HLA genotyping may significantly improve the reliability of the HLA haplotype frequency tables and, thus, the reliability of our epitope-based matching estimations.

In conclusion, we have shown that the currently used extrapolation method is a powerful and reliable tool to estimate PIRCHE-II and eplet values. This method provides the opportunity to calculate PIRCHE-II and eplet values when using serological split level HLA typing and, thus, makes high-resolution HLA genotyping presumably redundant for the majority of the donor-recipient couples. When more next-generation sequencing-determined HLA genotype data will become available, HLA haplotype frequency tables will become more reliable and, consequently, the reliability of our epitope-based HLA matching estimations will be further improved.

Abbreviations

HLA: Human Leukocyte Antigens
PIRCHE-II: Predicted Indirectly ReCognizable HLA Epitopes presented by HLA-DRB1.

Competing Interests

The UMC Utrecht has filed a patent application on the prediction of an alloimmune response against mismatched HLA. Eric Spierings is listed as inventor on this patent. Matthias Niemann is employed by PIRCHE AG that publishes the

PIRCHE web-portal. The other authors declare no conflict of interests with regard to this publication.

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Research Article

Impact of Desensitization on Antiviral Immunity in HLA-Sensitized Kidney Transplant Recipients

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Viral infections represent significant morbidity and mortality factors in kidney transplant recipients, with CMV, EBV, and BKV infections being most common. Desensitization (DES) with IVIg and rituximab with/without plasma exchange followed by kidney transplantation with alemtuzumab induction increased successful transplant rates in HLA-sensitized patients but may represent an increased risk for viral infections due to severe lymphocyte depletion. Here, we report on the posttransplant viral infection status in 372 DES versus 538 non-DES patients. CMV and EBV viremia were significantly lower in DES patients, while BKV viremia was similar. This trend was observed primarily in CMV sero(−), EBV sero(+), and sero(−) patients. No patient developed PTLD. The incidence of BKAN, allograft, and patient survival was similar in both groups. These viral infections were not associated with subsequent allograft rejection which occurred within 6 months after the infection. *Conclusions.* The IVIg + rituximab desensitization combined with alemtuzumab induction with triple immunosuppression maintenance does not increase the risk for CMV, EBV, and BKV infections. Possible factors include, in addition to posttransplant antiviral prophylaxis and PCR monitoring, presence of memory T cells and antibodies specific to CMV and likely EBV, NK cell-mediated ADCC despite lymphocyte depletion, elimination of EBV and CMV reservoirs by rituximab and alemtuzumab, and use of IVIg with antiviral properties.

1. Introduction

Viral infections represent significant morbidity and mortality factors for immunocompromised transplant recipients [1, 2]. Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infections are common and have long been associated with significant morbidity in the renal transplant population [1–5]. Polyomavirus BK (BKV) also emerged as an important viral infection associated with risk for allograft loss. [6, 7]. The most common manifestations of CMV infection include flu-like or mononucleosis-like syndromes, leukopenia or thrombocytopenia, infection of native tissues resulting in pneumonia, gastroenteritis, retinitis, and central nerve system disease [4]. Posttransplant lymphoproliferative disorder (PTLD) is one of the most serious complications in transplant recipients and is usually associated with EBV infection [3, 8]. PTLD is a consequence of the failure of the host's immune system to contain EBV-infected B cells, resulting in uncontrolled proliferation. BKV establishes latency in the uroepithelium and persists in the renal tubules without causing disease in immunocompetent individuals [9, 10]. However, BKV reactivation occurring in renal transplant recipients may cause an acute tubulointerstitial nephritis and ureteral stenosis, leading to severe allograft dysfunction and graft loss [6, 7, 11].

We have shown that desensitization (DES) with intravenous immunoglobulin (IVIg) and rituximab with/without plasma exchange (PLEX) followed by a kidney transplantation with alemtuzumab induction increased successful transplant rates in HLA-sensitized (HS) patients [12–15].

Table 1: Patient demographics.

Demographics	Kidney transplant patients		p value
	DES group (n = 372)	Non-DES group (n = 538)	
Transplant date	1/4/07–4/18/15	1/10/07–4/17/15	
Age, mean ± SD	49.6 ± 13.3	51.1 ± 14.2	0.11
Gender (female), n (%)	226 (60.8)	157 (29.2)	**<0.001**
Race, n (%)			0.27
African-American	69/368 (18.8)	84/499 (16.8)	
Hispanic	101/368 (27.4)	167/499 (33.5)	
White	148/368 (40.2)	180/499 (36.1)	
Others	50/368 (13.6)	68/499 (13.6)	
Living donor transplant, n (%)	141 (37.9)	177 (32.9)	0.12
Induction, n (%)			**<0.001**
Lymphocyte depletion	312/365 (85.5)	241/496 (48.6)	
Anti-IL-2 receptor	53/365 (14.5)	255/496 (51.4)	
Maintenance (tacrolimus), n (%)	351/354 (99.2)	452/493 (91.7)	**<0.001**
HLA match*, mean ± SD	1.9 ± 1.6	1.9 ± 1.7	0.78
PRA, n (%)			**<0.001**
>10%	66 (17.7)	470 (87.3)	
10–80%	113 (30.4)	68 (12.7)	
>80%	193 (51.9)	0 (0)	
Reason for DES, n (%)			
HS	314 (84.4)	na	
HS/ABOi	17 (4.6)	na	
ABOi	41 (11.0)	na	
Recipient with CMV sero(−) at Tx, n (%)	57/368 (15.5)	140/524 (26.7)	**<0.001**
Recipient with EBV sero(−) at Tx, n (%)	13/361 (3.6)	33/486 (6.8)	**0.04**
Follow-up (months post-Tx)**, mean ± SD	24.4 ± 20.3	24.6 ± 20.1	0.84
Sample number tested for viral-PCR/patient, mean ± SD	9.7 ± 5.2	9.5 ± 7.4	0.61

*HLA match in 350 DES and 392 non-DES patients with available results. **Follow-up for viral-PCR monitoring.
The number of patients with available results was provided if not available in all the patients. PRA: panel reactive antibody, DES: desensitization, HS: HLA-sensitization, ABOi: ABO incompatible transplantation, and Tx: transplant.

We have also shown acceptable outcomes in patients who received ABO incompatible transplants after the modified DES protocol with IVIg, rituximab, and PLEX [12]. However, profound and prolonged B cell and T cell depletion may result in an increased risk for viral infections [16–22]. To address this, all these patients receive antiviral prophylaxis posttransplant and extensive viral-PCR monitoring to minimize viral infections and their associated complications by early detection and intervention. We have previously shown that DES patients do not exhibit a significant increased risk for viral infection compared to non-DES patients [15, 23–26], except for a significantly higher BKV infection rate in DES patients [27]. In this study, we investigated the status of CMV, EBV, and BKV viral infection and their associated complication in a much larger cohort of patients who received DES and the results were compared with those without DES (non-DES). We also investigated the impact of viral infection on allograft rejection, since an association has been suggested that viral infections may increase this risk through direct effects on allograft-directed immune responses or due to reduced immunosuppression at time of infections. [28–30]. Here, we found significantly lower CMV and EBV infection rates in DES patients and similar BKV infection rates. We then investigated patient and graft survival and immune factors possibly responsible for these findings.

2. Materials and Methods

This study was approved by the Institutional Review Board at Cedars-Sinai Medical Center (IRB numbers Pro00017197, 10969, and 12562). The study was conducted in accordance with the ethical guideline based on federal regulations and the common rule. CSMC also has a Federal Wide Assurance.

2.1. Patient Population and Healthy Volunteers. CMV, EBV, and/or BKV-PCR results in a total of 3614 and 5113 DNA samples obtained from 372 DES and 538 non-DES patients, respectively, were compared. We also examined graft and patient survival, pretransplant viral serological status, virus-associated complication, and allograft rejection. Patients examined were transplanted between January 2007 and April 2015 at Cedars-Sinai Medical Center with patient demographics shown in Table 1. Patients who were <18 years old,

were monitored for viral-PCRs <2.9 months after transplant, or had <3 DNA samples obtained during the viral-PCR monitoring period (median 8.0 DNA samples per patient during median 18.7 months after transplant) were excluded.

Of 372 DES patients, 314 (84.4%) received an ABO compatible and 58 (15.6%) an ABO incompatible kidney transplant after DES. The DES protocols used for ABO compatible transplant in HS and ABO incompatible transplant in non-HS patients have been reported [12]. Briefly, a standard protocol for HLA-DES consisted of 2 doses of IVIg (2 g/kg) one month apart with one dose of rituximab (1 g) in between. The protocol for ABO incompatible transplant consisted of one dose of rituximab (1 g) two weeks prior to initiation of 5–7 sessions of PLEX followed by one dose of IVIg (2 g/kg). The combination of both protocols was used for HS patients who received an ABO incompatible transplant. If a negative or acceptable crossmatch was achieved and/or the antiblood group titer became ≤1 : 8 after DES, patients proceeded to transplantation [14, 15].

Most patients received induction therapy with lymphocyte depleting agent (alemtuzumab or anti-thymocyte globulin) or anti-IL-2 receptor antibody (anti-IL-2R, daclizumab, or basiliximab). Maintenance immunosuppression consisted of calcineurin inhibitor (tacrolimus or cyclosporine A), mycophenolate mofetil (MMF), and steroids. The target levels were dependent on the type of induction as reported elsewhere [27].

All patients received antiviral prophylaxis with ganciclovir (1.25 mg/kg daily) while inpatient and then valganciclovir or acyclovir posttransplant depending on a risk for viral infection. For transplants with CMV R−/D+, 900 mg valganciclovir was given daily for 6 months regardless of induction type. For those with lymphocyte depletion induction, 450 mg valganciclovir was given daily for 6 months for CMV R+/D+, R+/D−, or R−/D−. For those with anti-IL-2R induction, 800 mg acyclovir was given 4x a day for CMV R+/D+ or R+/D− and 800 mg daily for CMV R−/D− for 3–6 months, with dose adjustment for renal function and/or white blood cell count. CMV-, EBV-, and/or BKV-PCR monitoring was performed at 1, 2, 3, 6, 9, 12, 18, and 24 months after transplant or as needed as previously reported [23], and every two weeks in those who developed viremia. CMV and EBV infections were treated with reduction of immunosuppression in conjunction with valganciclovir (900 mg twice daily for 14 to 21 days, regardless of infection during or after antiviral prophylaxis with dose adjustments for renal function and/or white blood cell count). BKV was treated with reduction of immunosuppression, leflunomide, and/or IVIg. Antibody-mediated (ABMR) and cell-mediated (CMR) rejections were diagnosed based on the Banff 2013 [31, 32] and Banff 1997 classification [33], respectively. ABMR was treated with pulse steroids, IVIg and rituximab with or without PLEX, and CMR with pulse steroids. Refractory or Banff 2a rejection was treated with ATG.

Of 372 DES patients, 36 were monitored for lymphocyte subset analysis before and after transplant by flow cytometry, and archived sera obtained from another 38 patients were tested for total IgG and anti-EBV-IgG before and after transplant by ELISA.

Heparinized-peripheral blood samples from 20 normal adult volunteers (7 males) were tested for CMV- and EBV-specific T cell and NK cell activity.

2.2. Viral-PCR Assays. Viral-PCR was performed at the Transplantation and Immunology Laboratory, Cedars-Sinai Medical Center [23, 27, 34]. Briefly, for CMV- and EBV-PCR, total DNA was extracted from blood leukocytes by Qiacube (Qiagen, Valencia, CA) followed by optical density measurement, and 500 ng total DNA was submitted for the real time CMV- and EBV-PCR. CMV and EBV > 5 copies/PCR (500 ng total DNA) were considered viremia. For BKV-PCR, total DNA was extracted from 200 μl of plasma and eluted in 100 μl of Tris-EDTA; 10 μl of the DNA solution was used for the real time BKV-PCR [27]. The result was expressed as BKV DNA copies/ml plasma and >250 copies/ml was considered viremia. Specific primers and probe used were as follows: for the CMV-PCR, specific to the CMV immediate-early antigen region (5′-CAA GCG GCC TCT GAT AAC CA-3′, 5′-ACT AGG AGA GCA GAC TCT CAG AGG AT-3′, 5′-FAM-TGC ATG AAG GTC TTT GCC CAG TAC ATT CT-BHQ-3′) [35], for the EBV-PCR, specific to the BALF5 gene encoding the viral DNA polymerase of human EBV (5′ CGG AAG CCC TCT GGA CTT C 3′, 5′ CCC TGT TTA TCC GAT GGA ATG 3′, 5′ FAM-TGT ACA CGC ACG AGA AAT GCG CC-BHQ 3′) [36], and for the BKV-PCR, specific to the large T antigen of human BKV (5′-AAA GTC TTT AGG GTC TTC TAC CTT TCT TT-3′, 5′-GAG TCC TGG TGG AGT TCC TTT AAT-3′, 5′-FAM-AAT CTG CTG TTG CTT CTT CAT CAC TGG CA-BHQ-3′) and designed by our laboratory.

2.3. Lymphocyte Cell Subset Analysis. The CD4+, CD8+ T cell, CD19+ B cell, and CD56+/CD16+ NK cell numbers were monitored for DES patients before and after transplant by flow cytometry using a standard 6-color direct staining method as previously described with minor modification [37, 38]. Briefly, 5 μl each of the fluorochrome-conjugated antibodies to CD45 (Horizon V500, BD Biosciences, San Jose, CA), CD3 (FITC, Invitrogen), CD8 (Horizon V450, BD Biosciences), CD56 (APC, BD Biosciences), CD16 (PerCP-Cy5.5, eBioscience, San Diego, CA), and CD19 (PE-Cy7, eBioscience) was added to 100 μl of heparinized blood. CD45+ cells were first separated and then plotted against forward/side scatter to separate lymphocytes. Lymphocytes were then plotted against CD3 and CD8, CD3− population was further plotted against CD19 to enumerate CD19+ B cell number, and the remaining cells plotted against CD16 and CD56. CD56+/CD16+, CD56+/CD16−, and CD56−/CD16+ were considered NK cells (CD56+/CD16+ cells). CD3+/CD8− cells were considered as CD4+ cells.

2.4. CMV- or EBV-Specific T Helper (CMV- or EBV-Th) and NK (CMV- or EBV-NK) Cell Analysis. CMV- or EBV-Th and NK cell levels were measured by intracellular cytokine flow cytometry (CFC) developed in our lab and described elsewhere with minor modification [39–41]. Briefly, whole blood was incubated with sucrose density purified CMV or EBV viral lysate (Advanced Biotechnologies, Eldersburg, MD) at the final concentration of 1 μg/ml, together with

brefeldin A and anti-CD28/CD49d overnight. After cells were stained with fluorochrome-conjugated antibodies to CD45 (V500), CD3 (FITC), CD4 (PerCP-Cy5.5), CD8 (V450), and CD56 (APC) and then with PE-anti-IFNγ antibody for intracellular IFNγ staining, followed by cell acquisition, the IFNγ+ cell% in CD4+ T cells and CD56+ NK cells were enumerated and defined as CMV-Th or EBV-Th and CMV-NK or EBV-NK, respectively. CMV-Th $\geq$ 0.20%, EBV-Th $\geq$ 0.10%, and CMV- and EBV-NK $\geq$ 0.5% were considered positive as established based on the levels detected in CMV or EBV sero(+) and sero(−) normal individuals and transplant recipients [37, 39, 40]. Phytohemagglutinin (PHA) at the final concentration of 1 μg/ml was used as positive control for each sample tested. In a separate experiment where degranulation in CMV- or EBV-T and NK cells was assessed, PE-Cy7-conjugated anti-CD107a antibody was also added, and the IFNγ+ cell% and CD107a+ cell% in CD4+ T cells and CD56+ NK cells were enumerated. To assess the involvement of anti-CMV antibody in NK cell activation, whole blood was first incubated with IdeS (Hansa Medical, Sweden), an IgG-degrading enzyme of *S. pyogenes*, that cleaves 4 human IgG subclasses at the hinge region of IgG heavy chains, critical for ADCC [42], at the final concentration of 10 μg/ml at 37°C for 1 hour, and then incubated with CMV lysate to continue the above CFC procedure.

2.5. Total IgG- and Anti-EBV IgG-ELISA. Total IgG (Human IgG-ELISA, Bethyl Laboratories, Inc. Montgomery, TX) and anti-EBV IgG levels (EBV-VCA IgG-ELISA, Calbiotech, El Cajon, CA) were measured by ELISA following the manufacturers' instruction. In the total IgG-ELISA, the results were expressed as mg/ml, and the levels >7, 4–7 and <4 mg/ml were considered normal, mild, and severe hypogammaglobulinemia [43], respectively. In the anti-EBV IgG-ELISA, the results were expressed as anti-EBV IgG index and the index <0.25 was considered EBV sero(−).

2.6. Statistical Analysis. We compared the results in the DES versus non-DES groups, viral sero(+) versus sero(−) groups, or different antiviral prophylaxis groups (Tables 1–6). Continuous variables were analyzed using Student's t-test and categorical variables were analyzed by Chi-square or Fisher's exact test unless otherwise stated. Total IgG and anti-EBV IgG levels before DES versus 12 months after transplant (Figure 4) and IFNγ+ or CD107a+ cell% in CD4+ T or CD56+ NK cells between conditions (Figure 6) were compared by paired t-test. The rates of CMV, EBV, or BKV viremia, allograft rejection, allograft loss, and patient death were estimated by the Kaplan-Meier method and the group differences were assessed by the log-rank test. The p value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Baseline Characteristics. Baseline characteristics in DES and non-DES patients are shown in Table 1. All 372 DES patients showed PRA > 10% and 193 of those (52%) had PRA > 80% before DES. Among DES patients, 314 (84%) received DES for HLA incompatibility, while 41 (11%) and 17 (5%) received DES for ABOi or ABOi + HLA incompatibilities, respectively. There were significantly more females (61% versus 29%, $p < 0.001$), more lymphocyte depletion induction (86% versus 49%, $p < 0.001$), and maintenance with tacrolimus (99% versus 92%, $p < 0.001$) in the DES group. More female patients are HLA-sensitized due to pregnancy, and lymphocyte depletion induction and tacrolimus were used as a standard posttransplant immunosuppressive regimen for DES patients. It should be noted that 96% of DES patients induced with a lymphocyte depleting agent received alemtuzumab, while 81% of non-DES patients received ATG. Significantly more non-DES patients showed CMV (27% versus 16%, $p < 0.001$) and EBV (7% versus 4%, $p = 0.04$) negative serology at transplant. Transplant date, age, race, living donor transplant, HLA match, viral-PCR monitoring follow-up period, and sample number tested for viral-PCR per patient were similar in both groups.

3.2. The Immune Cell Number before and after Transplant in DES and Non-DES Patients. CD8+, CD4+ T cell, CD19+ B cell, and CD56+/CD16+ NK cell number before DES and after transplant in 36 DES patients are shown in Figure 1. CD19+ B cells were nearly undetectable after rituximab treatment during the DES and continued to be low for several months [39]. For patients receiving alemtuzumab induction after rituximab, the levels at 1 month after transplant (after alemtuzumab) were nearly undetectable (Figure 1(c)). Recovery of CD19+ B cells began 2-3 months after transplant. Although most patients still showed <30% of the pre-DES levels of CD19+ B cells at 6 months after transplant, rapid repopulation was also observed in some patients as previously reported by others [44]. The number of T cells significantly decreased after alemtuzumab induction and restoration began 2-3 months after transplant. It should be noted that the CD4+ T cell numbers at 1 month after transplant were nearly undetectable in most patients (Figure 1(b)), while the CD8+ cell numbers were 1–10% of pre-DES levels (Figure 1(a)). The CD8+ cell number continued to be higher than that of CD4+ cells afterward, which was consistent with our previous report [37]. This trend is likely due to CD4+ T cells being more sensitive than CD8+ T cells to alemtuzumab depletion [45] and/or CD8+ T cell restoration being more rapid than CD4+ T cells [46]. In contrast to B cells and T cells, the reduction of NK cell numbers after transplant was minimal; 20–50% of pre-DES levels were already observed at 1 month after transplant and most patients showed >50% of pre-DES levels by 3 months after transplant (Figure 1(d)). This is consistent with previous observations that NK cells were less susceptible to alemtuzumab depletion [47] and NK cell repopulation was faster than T cells in alemtuzumab-treated cynomolgus monkeys [48].

Alemtuzumab is a monoclonal antibody, targeting CD52 positive cells such as mature lymphocytes, including T cell, B cell, NK cells, and monocytes, and then depleting them [49]. On the other hand, another lymphocyte depleting agent, ATG, is a polyclonal antibody prepared from the sera of rabbits or horses immunized with thymocytes. ATG primarily depletes T cells [19], although induction of B cell apoptosis by ATG was reported [50], resulting in slight reduction of B

Table 2: CMV, EBV, and BKV viremia in DES and non-DES patients.

Viral DNA detected	Kidney transplant patients		p value
	DES group (n = 372)	Non-DES group (n = 538)	
CMV-PCR > 5.0 copies/PCR			
Viremia rate (% ± SE)*	30.3 ± 3.6	35.7 ± 2.9	0.19
1st viremia (m post-Tx)	5.8 ± 10.3	7.7 ± 14.1	0.23
Peak levels (copies/PCR)	420 ± 1077	2730 ± 18374	0.12
Duration (m)	0.7 ± 0.7	1.1 ± 2.0	**0.02**
CMV-PCR > 30 copies/PCR			
Vremia rate (% ± SE)*	16.1 ± 2.1	25.2 ± 2.7	**0.04**
1st viremia (m post-Tx)	3.7 ± 4.0	6.7 ± 12.5	**0.02**
Peak levels (copies/PCR)	699 ± 1331	3904 ± 21887	0.13
Duration (m)	0.8 ± 0.5	1.4 ± 2.4	**0.01**
CMV-PCR > 50 copies/PCR			
Viremia rate (% ± SE)*	13.5 ± 1.9	22.5 ± 2.7	**<0.05**
1st viremia (m post-Tx)	3.4 ± 3.1	7.1 ± 13.3	**0.01**
Peak levels (copies/PCR)	811 ± 1410	4462 ± 23360	0.13
Duration (m)	0.8 ± 0.6	1.5 ± 2.5	**0.01**
EBV-PCR > 5.0 copies/PCR			
Viremia rate (% ± SE)*	13.6 ± 3.8	30.0 ± 4.5	**<0.001**
1st viremia (m post-Tx)	18.8 ± 19.5	13.1 ± 19.3	0.20
Peak levels (copies/PCR)	171 ± 512	74 ± 141	0.36
Duration (m)	6.7 ± 17.5	4.5 ± 6.4	0.54
EBV-PCR > 30 copies/PCR			
Viremia rate (% ± SE)*	2.9 ± 1.1	11.3 ± 2.8	**0.001**
1st viremia (m post-Tx)	14.5 ± 20.2	9.4 ± 15.5	0.52
Peak levels (copies/PCR)	474 ± 785	157 ± 187	0.29
Duration (m)	17.1 ± 26.6	7.5 ± 7.7	0.34
EBV-PCR > 50 copies/PCR			
Viremia rate (% ± SE)*	2.3 ± 1.0	6.4 ± 1.5	**0.01**
1st viremia (m post-Tx)	9.4 ± 7.8	6.4 ± 8.4	0.47
Peak levels (copies/PCR)	691 ± 884	216 ± 205	0.29
Duration (m)	23.6 ± 30.6	8.3 ± 8.1	0.32
PTLD, n (%)	0	0	na
BKV-PCR > 250 copies/ml			
Viremia rate (% ± SE)*	20.1 ± 2.5	17.1 ± 1.9	0.21
1st viremia (m post-Tx)	5.2 ± 5.4	6.6 ± 8.7	0.25
Peak levels (copies/PCR)	$7.2 \times 10^5 \pm 4.3 \times 10^6$	$1.6 \times 10^5 \pm 5.8 \times 10^5$	0.31
Duration (m)	5.0 ± 10.4	5.8 ± 11.1	0.70
BKV-PCR >1500 copies/ml			
Viremia rate (% ± SE)*	11.2 ± 1.8	13.0 ± 1.8	0.72
1st viremia (m post-Tx)	4.2 ± 4.0	6.4 ± 9.7	0.14
Peak levels (copies/PCR)	$1.2 \times 10^6 \pm 5.5 \times 10^6$	$2.1 \times 10^5 \pm 6.6 \times 10^5$	0.28
Duration (m)	7.8 ± 12.8	7.3 ± 12.2	0.84
BKV-PCR >2500 copies/ml			
Viremia rate (% ± SE)*	10.9 ± 1.8	10.7 ± 1.7	0.60
1st viremia (m post-Tx)	4.3 ± 4.1	6.9 ± 10.6	0.12
Peak levels (copies/PCR)	$1.3 \times 10^6 \pm 5.6 \times 10^6$	$2.5 \times 10^5 \pm 7.2 \times 10^5$	0.30
Duration (m)	8.0 ± 13.0	8.4 ± 13.2	0.88

Table 2: Continued.

Viral DNA detected	Kidney transplant patients		p value
	DES group (n = 372)	Non-DES group (n = 538)	
BKAN, n (%)	4 (1.1)	10 (1.9)	0.35
Time for BKAN (m post-Tx)	12.3 ± 10.1	11.6 ± 7.1	0.92

*The viremia rates (% ± standard error [SE]) at 5 years after transplant (Tx) were estimated by the Kaplan-Meier method and the group differences were assessed by the log-rank test.
Results for 1st viremia, peak levels, duration, and time for BKAN are mean ± standard deviation.
m post-Tx: months after transplant, PTLD: posttransplant lymphoproliferative disorder, and BKAN: BKV-associated nephropathy.

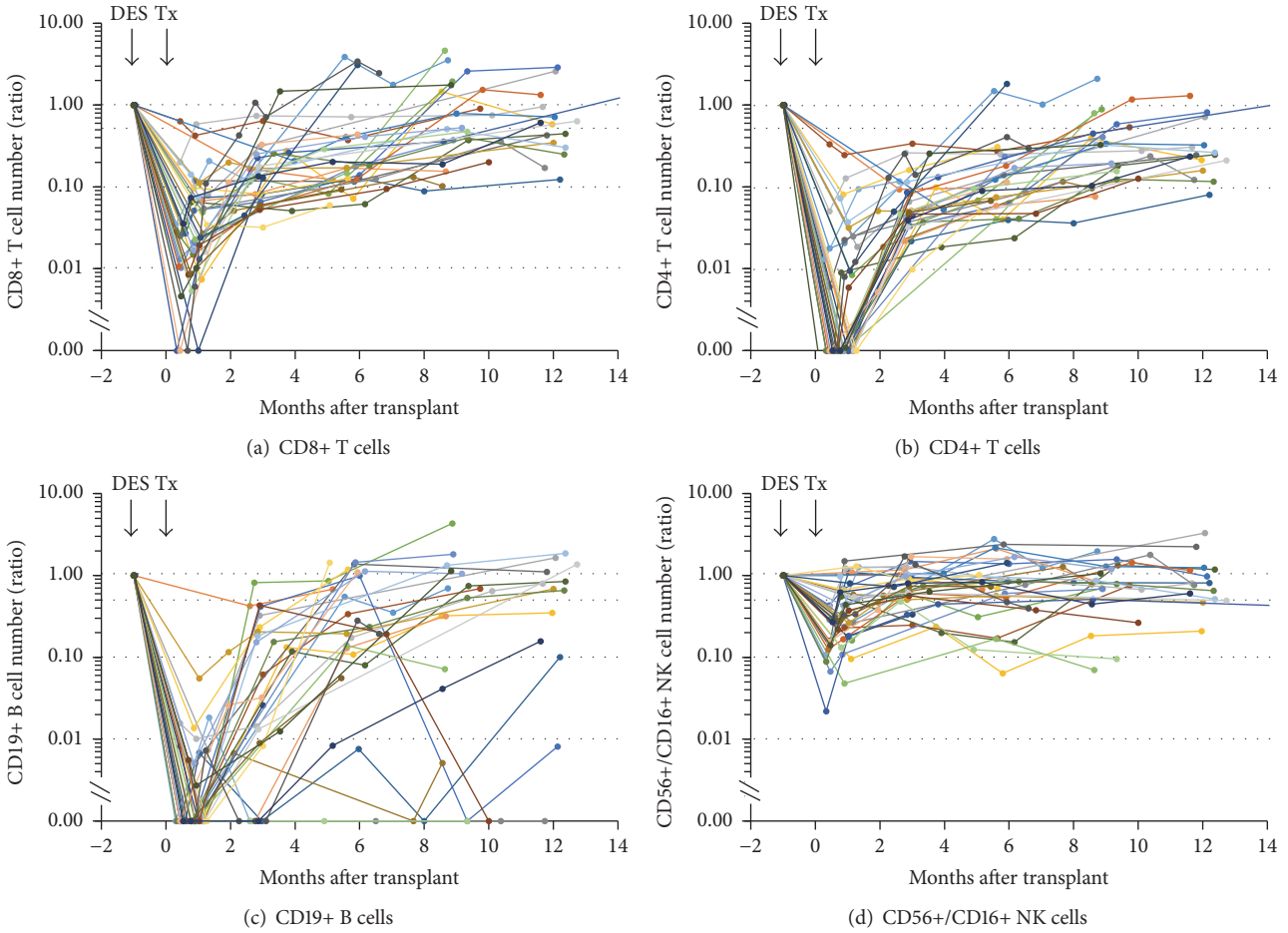


Figure 1: The number of CD8+ (a), CD4+ T cells (b), CD19+ B cells (c), and CD56+/CD16+ NK cells (d) pre-DES and posttransplant in 36 DES patients who received DES with IVIg + rituximab followed by a kidney transplant with alemtuzumab induction. Each line with each symbol describes the result from one patient. The results were expressed as the ratio against the pre-DES level in each patient. DES: desensitization; Tx: transplant.

cells after ATG induction [51]. However, alemtuzumab was reported to be more powerful in reducing T cells than ATG preparations, while reduction of NK cells was similar [51]. In this study, 86% of DES and 49% of non-DES patients received lymphocyte depleting agents, and, of these, 96% of DES patients received alemtuzumab, while 81% of non-DES received ATG. Considering additional B cell depletion by rituximab and alemtuzumab, B cell and T cell depletion in DES was more intense compared to non-DES patients. Theoretically, this should increase the risk for infections in DES patients. Thus, we investigated the viral infection status in DES versus non-DES patients.

3.3. Viral Infection in DES and Non-DES Patients. CMV, EBV, and BKV viremia status after transplant in 372 DES and 538 non-DES patients are summarized in Table 2. CMV or EBV DNA levels > 5 copies/PCR and BKV DNA levels > 250 copies/ml as analyzed by our viral-PCR assays were considered viremia, and the levels > 50 copies/PCR and > 2500 copies/ml, respectively, were usually considered for antiviral

Table 3: CMV viremia in CMV sero(+) versus sero(−) patients in the DES and non-DES groups.

	DES group (n = 368)			Non-DES group (n = 524)			p value (DES versus non-DES)	
	CMV sero(+) (n = 311)	CMV sero(−) (n = 57)	p value (sero+ versus −)	CMV sero(+) (n = 384)	CMV sero(−) (n = 140)	p value (sero+ versus −)	In CMV sero(+)	In CMV sero(−)
CMV-PCR > 5.0 copies/PCR								
Viremia rate (% ± SE)*	32.0 ± 4.0	22.1 ± 5.7	0.37	33.4 ± 3.5	43.5 ± 5.5	**0.01**	0.95	**0.03**
1st viremia (m post-Tx)	6.0 ± 11.0	4.3 ± 2.8	0.25	9.1 ± 16.7	4.9 ± 5.9	**0.02**	0.14	0.60
Peak levels (copies/PCR)	237 ± 578	1652 ± 2236	**0.06**	2951 ± 22399	2407 ± 4370	0.81	0.22	0.42
Duration (m)	0.7 ± 0.6	0.9 ± 0.6	0.22	0.9 ± 1.1	1.6 ± 3.1	0.11	0.15	0.17
CMV-PCR > 30 copies/PCR								
Viremia rate (% ± SE)*	15.5 ± 2.2	20.3 ± 5.5	0.41	22.5 ± 3.3	35.1 ± 5.1	**<0.001**	0.34	**0.09**
1st viremia (m post-Tx)	3.5 ± 4.2	4.4 ± 2.8	0.42	8.1 ± 15.2	4.5 ± 5.9	**0.09**	**0.02**	0.94
Peak levels (copies/PCR)	423 ± 734	1801 ± 2278	**0.09**	4549 ± 27703	2919 ± 4658	0.64	0.23	0.28
Duration (m)	0.7 ± 0.5	1.0 ± 0.6	0.16	1.1 ± 1.3	1.8 ± 3.4	0.20	**0.02**	0.15
CMV-PCR > 50 copies/PCR								
Viremia rate (% ± SE)*	12.7 ± 2.0	18.6 ± 5.4	0.30	18.7 ± 3.3	35.1 ± 5.1	**<0.001**	0.59	**0.05**
1st viremia (m post-Tx)	3.1 ± 3.1	4.4 ± 3.0	0.27	9.2 ± 16.7	4.5 ± 5.9	**0.06**	**0.01**	0.93
Peak levels (copies/PCR)	496 ± 779	1978 ± 2316	**0.09**	5718 ± 30980	2919 ± 4658	0.52	0.23	0.38
Duration (m)	0.8 ± 0.5	1.1 ± 0.6	0.15	1.2 ± 1.4	1.8 ± 3.4	0.29	**0.03**	0.19

*The viremia rates (% ± standard error [SE]) at 5 years after transplant (Tx) were estimated by the Kaplan-Meier method and the group differences were assessed by the log-rank test. Results for 1st viremia, peak levels, and duration are mean ± standard deviation (SD).

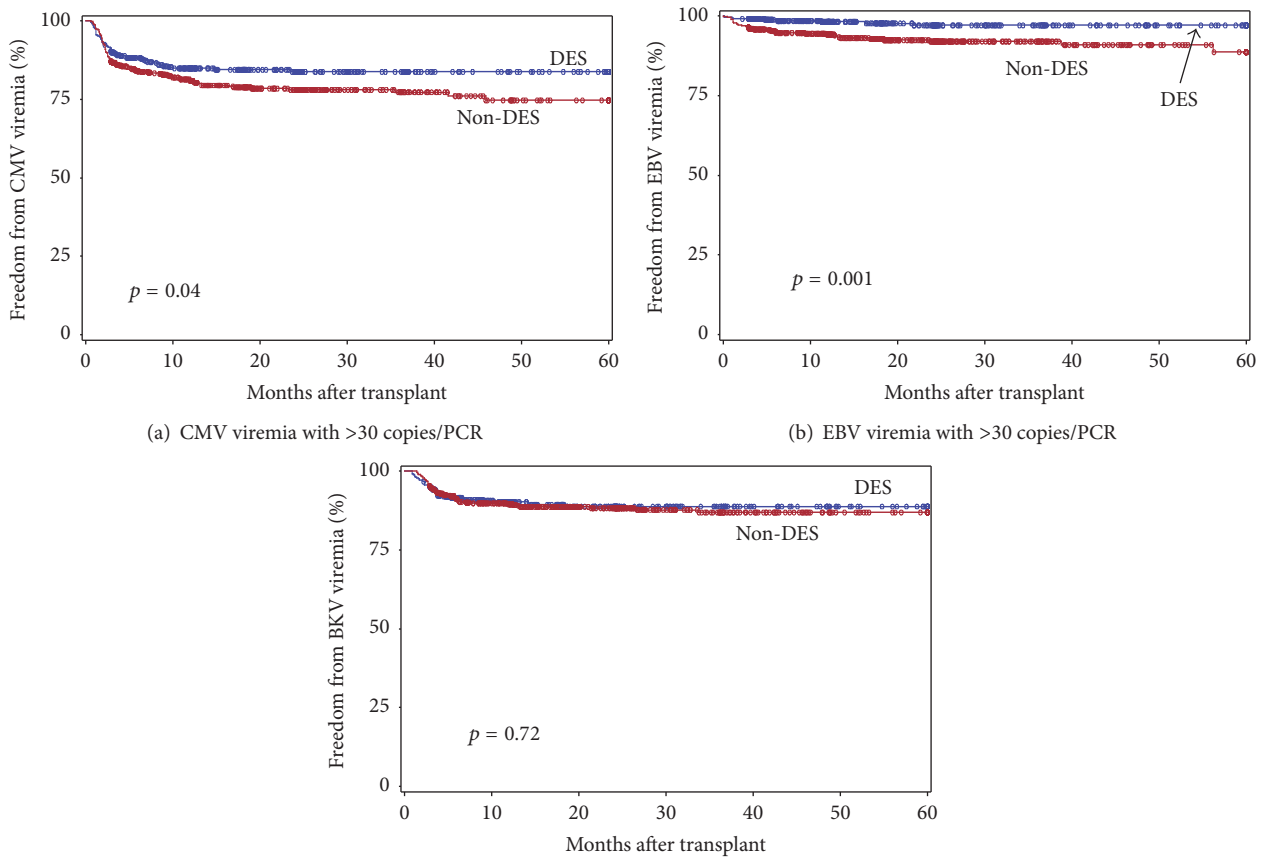
DES: desensitization; post-Tx: posttransplant.

Table 4: EBV viremia in EBV sero(+) versus sero(−) patients in the DES and non-DES groups.

	DES group (n = 368)			Non-DES group (n = 524)			p value (DES versus non-DES)	
	EBV sero(+) (n = 348)	EBV sero(−) (n = 13)	p value (sero+ versus −)	EBV sero(+) (n = 453)	EBV sero(−) (n = 33)	p value (sero+ versus −)	In EBV sero(+)	In EBV sero(−)
EBV-PCR > 5.0 copies/PCR								
Viremia rate (% ± SE)*	14.4 ± 4.1	16.7 ± 10.8	0.29	32.0 ± 5.3	36.7 ± 9.0	**0.02**	**<0.001**	0.22
1st viremia (m post-Tx)	19.8 ± 20.0	7.1 ± 0.9	**0.01**	14.6 ± 20.8	5.5 ± 5.9	**0.00**	0.29	0.45
Peak levels (copies/PCR)	183 ± 531	29 ± 17	0.18	62 ± 130	160 ± 189	0.14	0.29	**0.06**
Duration (m)	7.0 ± 18.1	3.3 ± 2.8	0.45	4.1 ± 6.2	7.1 ± 6.6	0.21	0.46	0.37
EBV-PCR > 30 copies/PCR								
Viremia rate (% ± SE)*	2.8 ± 1.1	8.3 ± 8.0	0.20	11.1 ± 3.3	26.5 ± 8.2	**<0.001**	**<0.01**	0.21
1st viremia (m post-Tx)	15.6 ± 21.2	6.2 ± 0.0	na	11.1 ± 17.3	4.7 ± 6.3	0.12	0.62	na
Peak levels (copies/PCR)	527 ± 817	46 ± 0.0	na	141 ± 185	214 ± 197	0.40	0.25	na
Duration (m)	18.5 ± 27.9	6.0 ± 0.0	na	7.3 ± 7.9	8.0 ± 6.9	0.83	0.33	na
EBV-PCR > 50 copies/PCR								
Viremia rate (% ± SE)*	2.5 ± 1.1	0	0.61	5.4 ± 1.7	23.3 ± 7.9	**<0.001**	**0.09**	**0.08**
1st viremia (m post-Tx)	9.4 ± 7.8	na	na	7.1 ± 9.2	5.2 ± 6.6	0.59	0.60	na
Peak levels (copies/PCR)	691 ± 884	na	na	213 ± 214	239 ± 197	0.79	0.28	na
Duration (m)	23.6 ± 30.6	na	na	8.1 ± 8.5	8.6 ± 7.2	0.89	0.31	na
PTLD, n (%)	0 (0)	0 (0)	na	0 (0)	0 (0)	na	na	na

*The viremia rates (% ± standard error [SE]) at 5 years after transplant (Tx) were estimated by the Kaplan-Meier method and the group differences were assessed by the log-rank test. Results for 1st viremia, peak levels, and duration are mean ± standard deviation (SD).

DES: desensitization, PTLD: posttransplant lymphoproliferative disorder, and post-Tx: posttransplant.



(a) CMV viremia with >30 copies/PCR

(b) EBV viremia with >30 copies/PCR

(c) BKV viremia with >1500 copies/ml

Figure 2: Freedom from CMV (a) or EBV (b) viremia with >30 copies/PCR and BKV (c) viremia with >1500 copies/ml in DES (blue) and non-DES (red) patients during the 1st 5 years after transplant. The group differences were assessed by the log-rank test.

Table 5: CMV and EBV viremia in sero(+) non-DES patients who received valganciclovir (VGCV-LD) versus acyclovir prophylaxis (ACV-anti-IL-2R).

Patients	Number of patients (%)
	w/CMV-PCR > 30 copies/PCR
CMV sero(+) non-DES	
VGCV-LD (n = 176)	31 (17.6)
ACV-anti-IL-2R (n = 175)	26 (14.9)
VGCV-LD-ATZ (n = 25)	7 (28.0)
VGCV-LD-ATG (n = 151)	24 (15.9)
	w/EBV-PCR > 30 copies/PCR
EBV sero(+) non-DES	
VGCV-LD (n = 200)	22 (11.0)
ACV-anti-IL-2R (n = 215)	6 (2.8)*
VGCV-LD-ATZ (n = 39)	2 (5.1)
VGCV-LD-ATG (n = 161)	20 (12.4)

VGCV: valganciclovir; ACV: acyclovir.
LD: lymphocyte depletion; anti-IL-2R: anti-IL-2 receptor.
ATZ: alemtuzumab; ATG: anti-thymocyte globulin.
* $p < 0.001$ versus VGCV-LD by Fisher's exact test.

therapy. Patients with CMV or EBV DNA levels between 30 and 50 copies/PCR and with BKV levels between 1500 and 2500 copies/ml may or may not be treated with antiviral therapy depending on other factors. Thus, the viremia status in the two groups was compared based on 3 viral-PCR cutoff levels. Due to early detection and early intervention, most patients with viremia were asymptomatic.

Freedom from CMV or EBV viremia with >30 copies/PCR and BKV viremia with >1500 copies/ml in the DES versus non-DES groups is shown in Figure 2, and the CMV, EBV, or BKV viremia rates with 3 cutoff levels are shown in Table 2. One of the most striking findings in this analysis was the significantly lower CMV and EBV viremia rates in the DES group except for CMV viremia with >5 copies/PCR, and there was no difference in the BKV viremia rates (Table 2, Figure 2). Estimated viremia rates at 5 years after transplant were 30% versus 36% ($p = 0.19$), 16% versus 25% ($p = 0.04$), and 14% versus 23% ($p < 0.05$) for CMV viremia with >5, >30 and >50 copies/PCR, respectively; 14% versus 30% ($p < 0.001$), 2.9% versus 11% ($p = 0.001$), and 2.3% versus 6.4% ($p = 0.01$) for EBV viremia with >5, >30, and >50 copies/PCR, respectively. Significantly shorter duration of CMV viremia (mean months 0.7 versus 1.1 [$p = 0.02$], 0.8 versus 1.4 [$p = 0.01$], and 0.8 versus 1.5 [$p = 0.01$] for viremia with >5, >30, and >50 copies/PCR, resp.) and the trend of lower CMV DNA peak levels were also observed in the DES compared to non-DES group. The

Table 6: Viral infection and allograft rejection (AR) in DES and non-DES patients.

Allograft rejection (AR)	Kidney transplant patients		p value**
	DES group ($n = 363$)	Non-DES group ($n = 497$)	
AR, n (%)			
Any AR	68 (18.7)	70 (14.1)	**0.07**
ABMR	44 (12.1)	11 (2.2)	**<0.001**
CMR	41 (11.3)	68 (13.7)	0.35
CMV, EBV, or BKV viremia*, n (%)	80 (22.0)	138 (27.8)	**0.06**
AR within 6 months after VI, n (%)			
Any AR after VI	15/80 (18.8)	16/138 (11.6)	0.16
ABMR after VI	8/80 (10.0)	3/138 (2.2)	**0.02**
CMR after VI	11/80 (13.8)	15/138 (10.9)	0.52

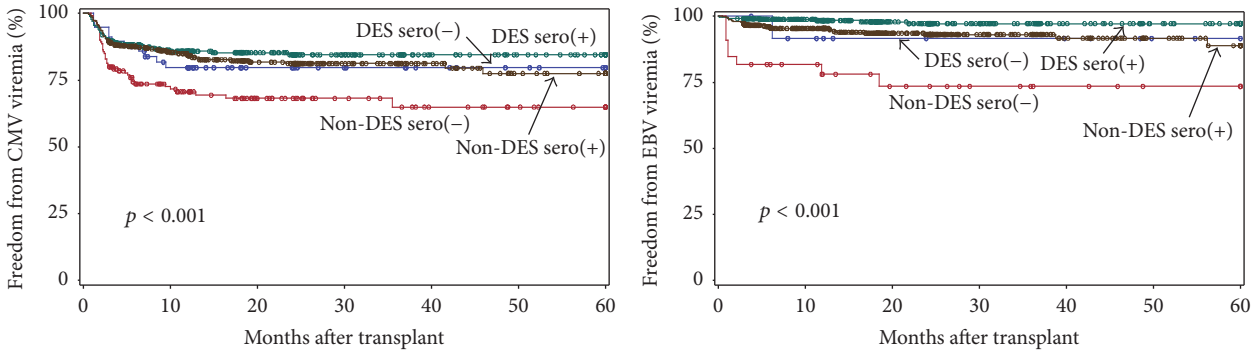
*Viremia (VI) with peak levels > 50 copies/PCR for CMV & EBV and >2500 copies/ml for BKV viremia was analyzed.
**The comparison between the two groups was performed by Fisher's exact test.

1st CMV viremia with >30 and >50 copies/PCR occurred significantly earlier after transplant in the DES group (mean months after transplant 3.7 versus 6.7 [$p = 0.02$] and 3.4 versus 7.1 [$p = 0.01$], resp.). Viral-PCR monitoring was performed every month during the 1st 3 months after transplant and every 3 months afterwards up to 12 months followed by every 6 months during the 2nd transplant year. Shorter duration of CMV viremia and the trend of lower CMV DNA peak levels observed in DES patients could be due to earlier recognition and treatment in the DES group. No PTLD was seen in either group. There was no significant difference in the BKAN rate or the time to BKAN development in the two groups.

We next analyzed viral infection status separately by pretransplant recipient's viral serology status that largely affects posttransplant viral infection rate and its associated complication [52]. Since pretransplant CMV and EBV sero negativity were significantly higher in the non-DES group, this may have contributed to higher CMV and EBV viremia rates in non-DES patients. We divided DES and non-DES patients into 2 subgroups, CMV sero(+) or (−) and EBV sero(+) or (−). The CMV or EBV viremia status was compared among sero(+), sero(−) DES, sero(+), and sero(−) non-DES patients. The analysis for BKV infection was not performed since BKV serology results were not readily available.

Freedom from CMV or EBV viremia with >30 copies/PCR in the 4 groups is shown in Figure 3, and the CMV or EBV viremia rates with 3 cutoff levels are shown in Tables 3 and 4, respectively. Overall, sero(−) non-DES patients showed least freedom from CMV and EBV viremia during the 1st 5 years after transplant (Figure 3, Tables 3 and 4). In the non-DES group, the CMV and EBV viremia rates were significantly higher in sero(−) versus sero(+) patients (44% versus 33% [$p = 0.01$], 35% versus 23% [$p < 0.001$], and 35% versus 19% [$p < 0.001$] for CMV viremia; 37% versus 32% [$p = 0.02$], 27% versus 11% [$p < 0.001$], and 23% versus 5% [$p < 0.001$] for EBV viremia with >5, >30, and >50 copies/PCR, resp.) (Tables 3 and 4, Figure 3). In contrast, significant difference in CMV and EBV viremia rate between sero(−) versus (+) was not observed in the DES group. When the results were compared among CMV sero(−) patients, the CMV viremia rate was lower in the DES group (22% versus 44% [$p = 0.03$], 20% versus 35% [$p = 0.09$], and 19% versus 35% [$p = 0.05$] for CMV viremia with >5, >30, and >50 copies/PCR, resp.), while the viremia rate was similar in sero(+) DES and non-DES patients (Table 3), suggesting that lower CMV viremia rates observed in DES patients resulted in part from lower CMV viremia in sero(−) DES patients. Among the CMV sero(+) patients, significantly shorter duration of CMV viremia with >30 and >50 copies/PCR was again observed in DES than non-DES patients (mean months 0.7 versus 1.1 [$p = 0.02$], 0.8 versus 1.2 [$p = 0.03$], resp.). This might be due in part to earlier onset of CMV viremia in DES than non-DES patients (mean months after transplant 3.5 versus 8.1, [$p = 0.02$], 3.1 versus 9.2 [$p = 0.01$], resp.). Among EBV sero(+) patients, the EBV viremia rate was significantly or near significantly lower in the DES group: 14% versus 32% [$p < 0.001$], 2.8 versus 11% [$p < 0.01$], and 2.5 versus 5.4% [$p = 0.09$] for viremia with >5, >30, and >50 copies/PCR, respectively (Table 4). Among EBV sero(−) patients, only 2 of 13 (15%) DES patients had viremia with >5 copies/PCR and none showed viremia with >50 copies/PCR during the study period. In contrast, 11 of 33 (33%) non-DES had viremia with >5 copies/PCR and 7 (21%) showed viremia with >50 copies/PCR, suggesting EBV viremia also tended to be lower in sero(−) DES patients, although this difference was not statistically significant. These results suggest that lower EBV viremia rates observed in DES patients resulted from lower EBV viremia in sero(+) and to a lesser degree in sero(−) DES patients. Taken together, the standard protocol used for DES patients affects primarily CMV sero(−) patients to reduce CMV viremia as well as EBV sero(+) and to a lesser degree sero(−) patients to reduce EBV viremia rates. The treatment did not increase the BKV viremia and BKAN rate in DES patients.

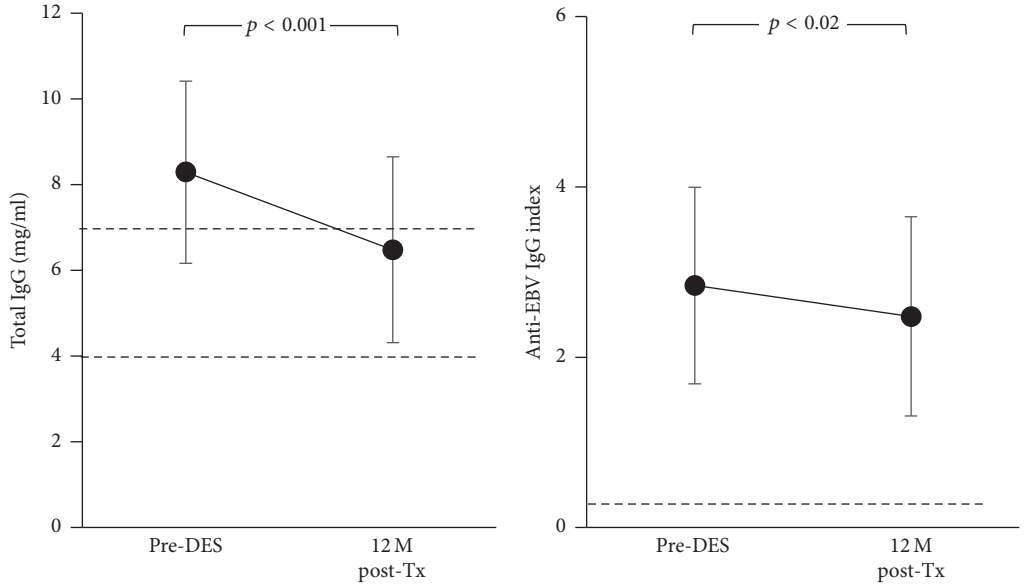
ATG is widely used as an induction and rejection treatment agent in transplant patients, and use of a newer lymphocyte depleting agent, alemtuzumab, is also well established [17, 19]. Use of these agents is essential due to their significant reduction of acute rejection, primarily cell-mediated, especially in high risk HS patients. Although the risk for



(a) CMV viremia with >30 copies/PCR

(b) EBV viremia with >30 copies/PCR

FIGURE 3: Freedom from CMV (a) or EBV (b) viremia with >30 copies/PCR in sero(+) (green) or sero(−) (blue) DES and sero(+) (brown) or sero(−) (red) non-DES patients during the 1st 5 years after transplant. The group differences were assessed by the log-rank test.



(a) Total IgG

(b) Anti-EBV IgG

FIGURE 4: Total IgG (a) and anti-EBV IgG levels (b) before DES and at 12 months (M) after transplant (Tx) in 35 and 33 DES patients, respectively, who received DES with IVIg + rituximab followed by a kidney transplant with alemtuzumab induction. The results are expressed as mean and standard deviation. The dotted lines describe 7 and 4 mg/ml total IgG for the normal and severe hypogammaglobulinemia cutoff, respectively, in (a), and anti-EBV IgG index 0.25 for sero(+) cutoff level in (b).

viral infection is a concern due to severe and prolonged lymphocyte depletion, the viral infection risk reported in studies is inconsistent [17, 19, 22, 53–55]. This must be due to various conditions used in these studies such as type of transplantation, the type and dose of maintenance immunosuppressive drugs, application of rejection treatment drugs, type of viruses, viral serological status of recipient, and donor or viral prophylaxis [19]. Several studies showed similar results to ours. Hanaway et al. [17] reported no difference in CMV, EBV, and BKV infections between alemtuzumab versus ATG or anti-IL-2R induction during the first 3 years after transplant in high and low risk kidney transplant recipients with maintenance using tacrolimus, MMF, and 5-day steroid in a regimen of early steroid withdrawal. No significant difference in CMV and BKV infection in kidney transplant recipients who received alemtuzumab versus ATG or anti-IL-2R induction [22, 56], and no difference in CMV, EBV, and HSV infection in HS patients with alemtuzumab versus ATG induction [57] was shown. In contrast to the above study results in HS patients [17, 57], our study showed significantly lower CMV and EBV infection rates in HS (DES) with alemtuzumab compared to non-DES patients with ATG or anti-IL-2R induction. Our HS patients received DES before transplant, while those included in the previously mentioned

studies did not. This difference might contribute to the observed lower incidence of CMV and EBV infection in our DES patients.

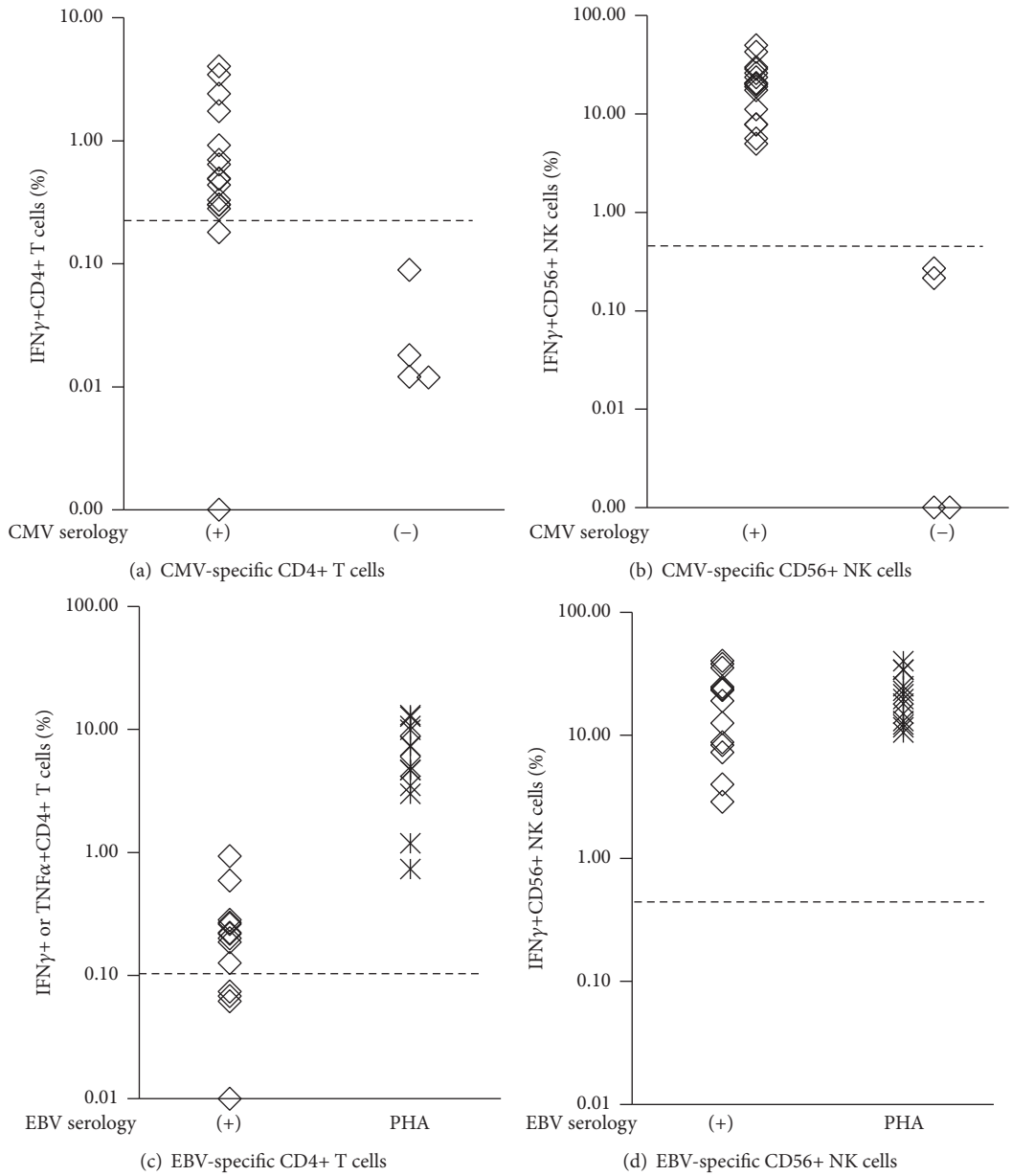
3.4. Possible Factors Contributing to Lower CMV and EBV Viremia Rates in DES Patients. Despite profound and prolonged B cell and T cell depletion from the standard protocol used for DES patients, pretransplant DES with IVIg + rituximab and posttransplant alemtuzumab induction, DES patients showed significantly lower CMV and EBV viremia rates compared to non-DES patients. In the further analysis performed separately by pretransplant CMV and EBV serological status, we found that the standard protocol used for DES patients reduced CMV viremia rate primarily in sero(−) and reduced EBV viremia rate in both sero(+) and sero(−) DES patients to a lesser degree. There are possible factors contributing to these observed beneficial effects of the standard protocol used for DES patients. Possible factors are summarized below.

3.4.1. Viral-Specific T Cells. Viral infections are controlled primarily by antiviral T cells [58]. We have previously shown that CMV-specific CD8+ T cells (CMV-Tc) as analyzed by CFC were detected in most CMV sero(+) healthy individuals as well as kidney transplant recipients, and clearance of CMV DNA was associated with detection of CMV-Tc in those patients [40]. Similar results have also been reported using CFC in solid organ transplant patients [59], ELISPOT in kidney transplant [60], QuantiFeron-CMV® [61], and Tetramer-based assays [62] in allogeneic stem cell transplantation. We also reported that CMV-Tc activity was detected by 2 and 4 months after transplant in 5 of 7 (71%) and 7 of 7 (100%) CMV sero(+) HS patients desensitized with IVIg + rituximab followed by a kidney transplant with alemtuzumab, respectively [39]. Our most recent study in a larger cohort of this patient population (30 patients) also showed similar results [37]: 70% of CMV sero(+) patients showed negativity for CMV-Tc and CMV-specific CD4+ (CMV-Th) cells at 1 month after transplant (after alemtuzumab) due to T cell depletion. However, by 2 months after transplant, 75% showed CMV-Tc and Th cell (+) and 95% did so by 3 months after transplant. These results suggest that a few viral-specific memory T cells that remained after alemtuzumab cell depletion are capable of responding to the virus, resulting in IFNγ production and cytotoxic effector functions against infected cells in CMV sero(+) patients. Preservation of memory T cell function following aggressive depletion by alemtuzumab [63, 64] and low risk of virus infection in transplant recipients treated with alemtuzumab [65, 66] have also been reported by other investigators. In the above study [37], we have also shown that one CMV sero(−) patient who developed CMV viremia with >1000 copies/PCR at 2 months after transplant rapidly developed both CMV-Tc and Th, and the viremia was cleared within a month, demonstrating that even CMV sero(−) patients can develop de novo proliferating CMV-T cells after lymphocyte depletion with alemtuzumab. EBV-specific T cells as assessed by IFNγ or TNFα positivity using the CFC assay were also detected in most EBV sero(+) normal individuals (Figure 5) and transplant recipients (data not shown). Clinical utility of EBV-specific T cell detection in lung, liver, and kidney transplant recipients using tetramer or ELISPOT assays have been reported by other investigators [67, 68]. Taken together, availability of viral-specific T cells in sero(+) patients from early posttransplant and capability of efficiently developing viral-specific T cells in sero(−) patients, despite severe T cell depletion by alemtuzumab, must contribute at least in part to lower CMV and EBV viral infection in this patient population.

3.4.2. Antiviral Antibody. Antiviral antibody functions as one of the early defense mechanisms against viral infection in sero(+) individuals through neutralizing viruses and eliminating virus-infected cells [69–71]. It has been reported that low anti-CMV titer before transplant or at 1 month after transplant was associated with a higher risk of CMV disease in heart transplant recipients [72]. Elevated risk for CMV infection during the 1st year posttransplant was also reported in solid organ transplant recipients with severe hypogammaglobulinemia [73]. In addition, we and others have reported on the benefit of CMV immunoglobulin or IVIg use in the prevention and treatment of viral complications of transplantation including CMV [40, 69, 74], EBV/PTLD [75], parvovirus B19 [76], and BKV infections [21, 77]. These study results demonstrate an important role of antiviral antibody in antiviral immunity in transplant recipients although neutralizing antibodies may not prevent subsequent rounds of infection and the cellular immune response eventually evolves to eradicate the infection [78]. Thus, long-term B cell depletion is always a concern in patients treated with rituximab followed by alemtuzumab induction. Our DES patients often received additional doses of rituximab posttransplant for treatment of ABMR, which may result in more prolonged B cell depletion and possible reduction of antibodies including antiviral antibodies.

We have previously reported [39] that total IgG, IgM, and IgA levels significantly decreased 4–10 months after DES (equivalent to average 9.6 months after transplant) compared to the pre-DES levels in 14 HS kidney transplant recipients with alemtuzumab induction. However, the reduction was only 15–20% and the reduced levels were still within the normal range in most patients. Minimal changes or moderate reductions (15–20% reduction) in total Ig during 6–12 months after rituximab treatment have also been shown in patients with arthritis [79], those with relapsing-remitting multiple sclerosis [80], and those with active rheumatoid arthritis [81]. In addition, anti-CMV IgG levels in CMV sero(+) patients did not change from pre-DES levels up to 10 months after transplant. Based on these results, we suggested that anti-CMV IgG might be produced primarily by CD20− long-lived plasma cells that are not affected by rituximab [82], while 15–20% of total IgG and IgM and IgA producing B cells might be CD20+ peripheral B cells and/or CD20− short-lived plasma cells [83].

In this study, we measured total IgG and anti-EBV IgG levels before DES and 12 months after transplant (15.7 ± 2.9 months after DES) in 35 patients who received DES with IVIg + rituximab, followed by a kidney transplant with alemtuzumab induction. Total IgG levels significantly



(a) CMV-specific CD4+ T cells

(b) CMV-specific CD56+ NK cells

(c) EBV-specific CD4+ T cells

(d) EBV-specific CD56+ NK cells

Figure 5: The levels of CMV-specific CD4+ T cells (CMV-Th) (a) and CD56+ NK cells (CMV-NK) (b) in 16 CMV sero(+) and 4 sero(−) normal individuals and EBV-specific CD4+ T cells (EBV-Th) (c) and CD56+ NK cells (EBV-NK) (d) in 14 EBV sero(+) normal individuals as analyzed by CFC. Each symbol represents the result from one individual. The dotted line describes the positive cutoff level; ≥0.2% for CMV-Th, ≥0.1% for EBV-Th, and ≥0.5% for CMV- and EBV-NK. The PHA (+) control results were also shown in (c) and (d).

decreased at 12 months after transplant compared to pre-DES levels (22% reduction) (Figure 4(a)), which is consistent with our previous results [39]. However, mean total IgG levels at 12 months after transplant was 6.5 mg/ml that is close to normal level, >7 mg/ml, and all patients except for two showed levels >4 mg/ml. Levels <4 mg/ml, considered severe hypogammaglobulinemia, are often associated with increased risk of viral and fungal infections and higher mortality [73, 74]. Pre-DES levels in the two patients with posttransplant total IgG <4 mg/ml were 2.5 and 6.2 mg/ml, already lower than normal levels. Florescu et al. reported based on a meta-analysis [73] that 45% of solid organ transplant recipients had hypogammaglobulinemia (total IgG < 7 mg/ml) within the 1st year after transplant and 15% had severe hypogammaglobulinemia (<4 mg/ml). In the current study, 20/35 patients (57%) showed total IgG <7 mg/ml that was slightly higher than their report, but only 2/35 (6%) showed severe hypogammaglobulinemia requiring transient treatment with IVIg.

In contrast to posttransplant anti-CMV IgG levels observed in the previous study [39], anti-EBV IgG levels significantly decreased in EBV sero(+) patients at 12 months after transplant in this study (Figure 4(b)). However, the reduction was minimal, 11%. Taken together, consistently

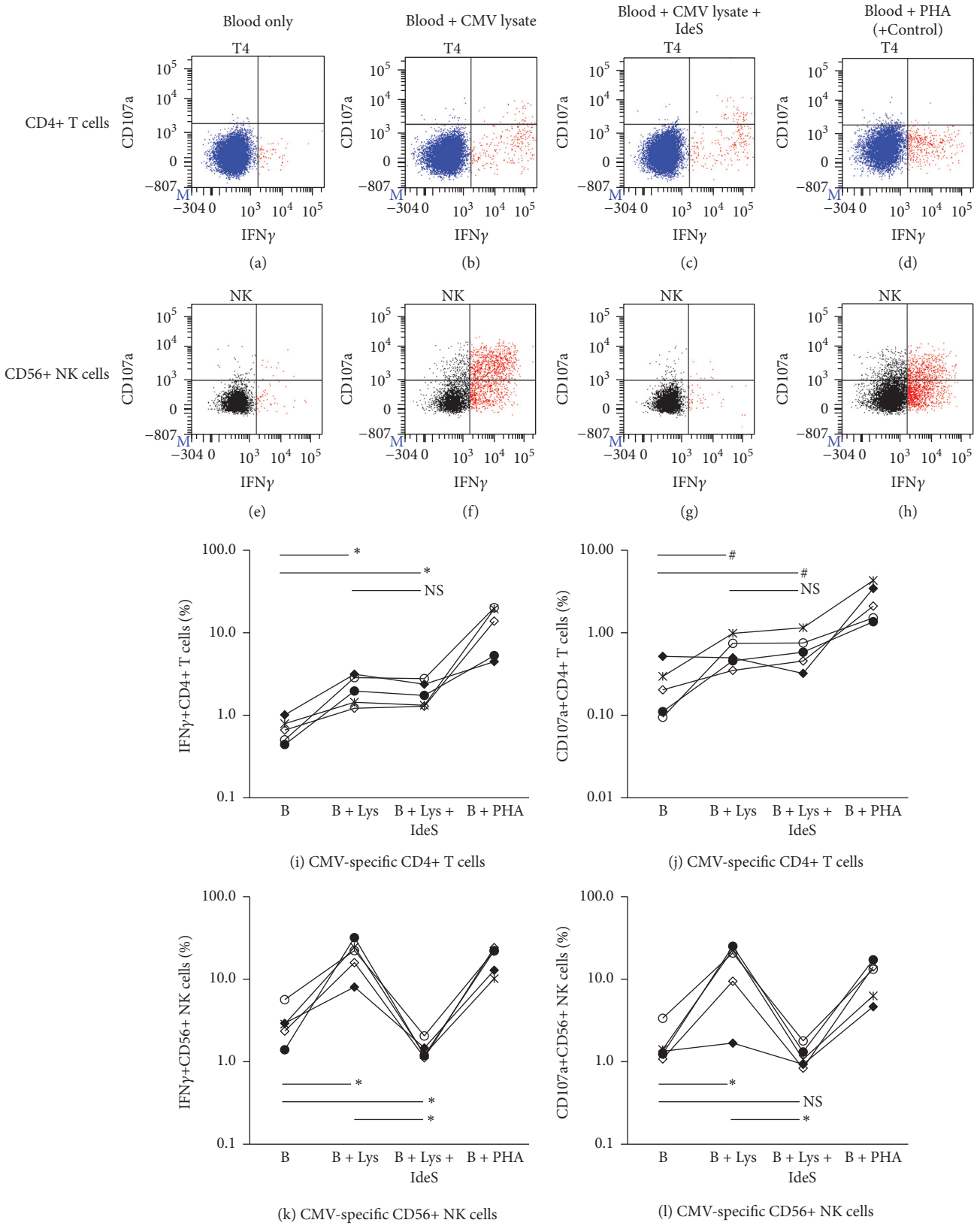


Figure 6: Involvement of anti-CMV IgG in positive reactivity of CMV-Th and CMV-NK cells. Upper (a–h): a typical result of the CMV-Th and CMV-NK assay performed with or without IdeS, IgG-degrading enzyme. Lower (i–l): the summary of 5 sets of experiment results using 5 different normal individuals. Each line with each symbol describes the result from one normal individual. B: blood; Lys: CMV lysate. $^{*}p < 0.05$, $^{\#}p = 0.05$–0.1, and NS: not significant ($p \geq 0.1$) as assessed by paired t-test.

available anti-CMV and anti-EBV IgG that are not affected by prolonged B cell depletion must contribute at least in part to lower CMV and EBV viral infection in sero(+) DES patients.

3.4.3. Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC). ADCC is one of the major antiviral activities and mediated by FcγRIIIa (CD16) bearing cells such as NK cells, monocytes, and a subset of CD8+ T cells through interaction of CD16 with Fc portion of antiviral IgG bound to the viral infected targets [70, 71, 84, 85]. Among the CD16 bearing cells, NK cells are primarily responsible for ADCC. As shown in Figure 1 and in previous studies [37, 39], CD56+/CD16+ NK cell numbers did not decrease as much as T cells after alemtuzumab induction in DES patients. In this study, 20–50% of pre-DES NK cell levels were already detected at 1 month after transplant and >50% of pre-DES levels by 3 months (Figure 1(d)) in most patients. In addition, anti-CMV and anti-EBV IgG levels showed no or minimal changes after transplant (after alemtuzumab) in CMV and EBV sero(+) patients, respectively [39] (Figure 4). These results suggest that NK cells-anti-CMV or anti-EBV IgG-mediated ADCC may be another factor contributing to lower rate of CMV or EBV infection in sero(+) DES patients.

To address this possible ADCC activity in sero(+) patients, we measured NK cell response to CMV or EBV lysate (CMV- or EBV-NK) in vitro by assessing IFNγ production and CD107a expression, degranulation marker [41, 86], using a CFC technique. We first measured the IFNγ+ cell% in CD4+ T and CD56+ NK cells in response to CMV in 20 and EBV lysate in 14 normal individuals; 4 of 20 were CMV sero(−) and all 14 were EBV sero(+). Most CMV sero(+) individuals except for two showed CMV-Th(+) (≥0.2%), while all 4 sero(−) individuals were (−) for CMV-Th (Figure 5(a)). Similar responses were seen in all 16 CMV sero(+) individuals showing (+) for CMV-NK (≥0.5%), while all sero(−) showed CMV-NK(−) (Figure 5(b)). In the EBV-Th and NK assay, 10 of 14 showed EBV-Th(+) (≥0.1%) and the remaining 3 were (−) (Figure 5(c)), while all 14 showed (+) for EBV-NK (Figure 5(d)). All PHA positive controls were (+) for EBV-Th and NK (Figures 5(c) and 5(d)).

It is well accepted that CMV- or EBV-T cells are viral-specific memory T cells and their response to CMV or EBV peptides or lysate in vitro are mediated through T cell receptors [40, 59, 67, 68]. NK cells express various receptors that are critical to their function and have traditionally been classified as important effectors of the innate immune system [87]. As characterization of NK cells has advanced, their crucial role in immunity has been reaffirmed and expanded [88, 89]. Recent studies suggest that NK cells have the capacity for immunological memory [90, 91]. To determine if the NK cell activation in response to CMV lysate observed in this study was mediated by direct interaction of NK cell receptors with CMV antigens on the lysate or antigen presenting cells, or an indirect interaction of NK cells with CMV antigens via CD16 and anti-CMV antibody interaction, similar experiments were performed using IdeS, an IgG-degrading enzyme [92], which enzymatically digest intact IgG into $F(ab')_2$ and Fc fragments, in 5 CMV sero(+) normal individuals, with subsequent analysis of the IFNγ+ cell% and CD107a+ cell% in CD4+ T and CD56+ NK cells. IFNγ+ and CD107a+ cell% in CD4+ T cells and CD56+ NK cells were elevated when blood was stimulated with CMV lysate (Figures 6(b), 6(f), and 6(i)–6(l)). The elevated IFNγ+ and CD107a+ cell% in CD4+ T cells remained when blood pretreated with IdeS was used for the assay (Figures 6(c), 6(i), and 6(j)). In contrast, the elevated IFNγ+ and CD107a+ cell% in CD56+ NK cells was completely abrogated when blood was pretreated with IdeS (Figures 6(g), 6(k), and 6(l)). These results suggest that CD4+ T cell activation in response to CMV lysate is anti-CMV antibody-independent and is memory T cell response specific to CMV antigens via T cell receptor as expected, while CD56+ NK cell activation in response to CMV lysate is anti-CMV antibody-dependent via CD16 and the involvement of other NK cell receptors is unlikely as NK cell activation was similar to background level after IdeS treatment. To determine if the complete inhibition of IFNγ production and CD107a expression by IdeS in NK cells was due to an interaction of IdeS with NK cells, blood stimulated with PHA (positive control) was incubated with or without IdeS in the CMV-T and NK assay. As shown in Figure 7, the elevated IFNγ+ and CD107a+ cell% in NK cells stimulated with PHA remained elevated even when blood was treated with IdeS (Figures 7(e), 7(f), 7(i), and 7(j)), suggesting that IdeS has no direct suppressive effect on NK cells. The same is true for the elevated IFNγ+ cell% in CD4+ T cells (Figures 7(b), 7(c), and 7(g)), while CD107a expression in PHA-stimulated CD4+ cells was minimal (Figures 7(b), 7(c), and 7(h)). Taken together, these results demonstrate that it is likely that ADCC plays an important role in prevention and reduction of CMV and EBV infection in sero(+) DES patients, especially during the first 1-2 months after transplant when less antiviral T cells are available in many patients due to alemtuzumab induction. ADCC-mediated antiviral activity is also likely to take a part of controlling viral infection in sero(−) patients who are treated with CMV-Ig or IVIg as it has been reported that elimination of CMV or EBV-infected cells by CMV-Ig or IVIg was enhanced by addition of NK cells via ADCC [85].

Recent studies showing the important role of antibody-mediated NK cell activity beyond a traditional ADCC mechanism in controlling CMV infection are of interest [93–95]. Elevated number and activity of NK cells during CMV infection in transplant recipients were previously reported [94]. Recently, $NKG2C^{hi}CD57^{hi}$ NK cells have been identified to be expanded exclusively at CMV infection [93, 96, 97] and its effector function is enhanced only in the presence of anti-CMV antibodies [95]. IFNγ+ and CD107a+ NK cells activated via antibody in response to CMV lysate as detected in the CMV-NK assay of this study might be this particular NK cell subset.

3.4.4. Lymphocyte Depletion. Contrary to the widely held concept that lymphocyte depletion increases risk for viral infections, several studies including a meta-analysis showed a similar viral infection rate among patients treated with alemtuzumab, ATG, and anti-IL-2R induction [17, 22, 56]. Our DES patients included in this study received an additional lymphocyte depleting agent, rituximab, for pretransplant

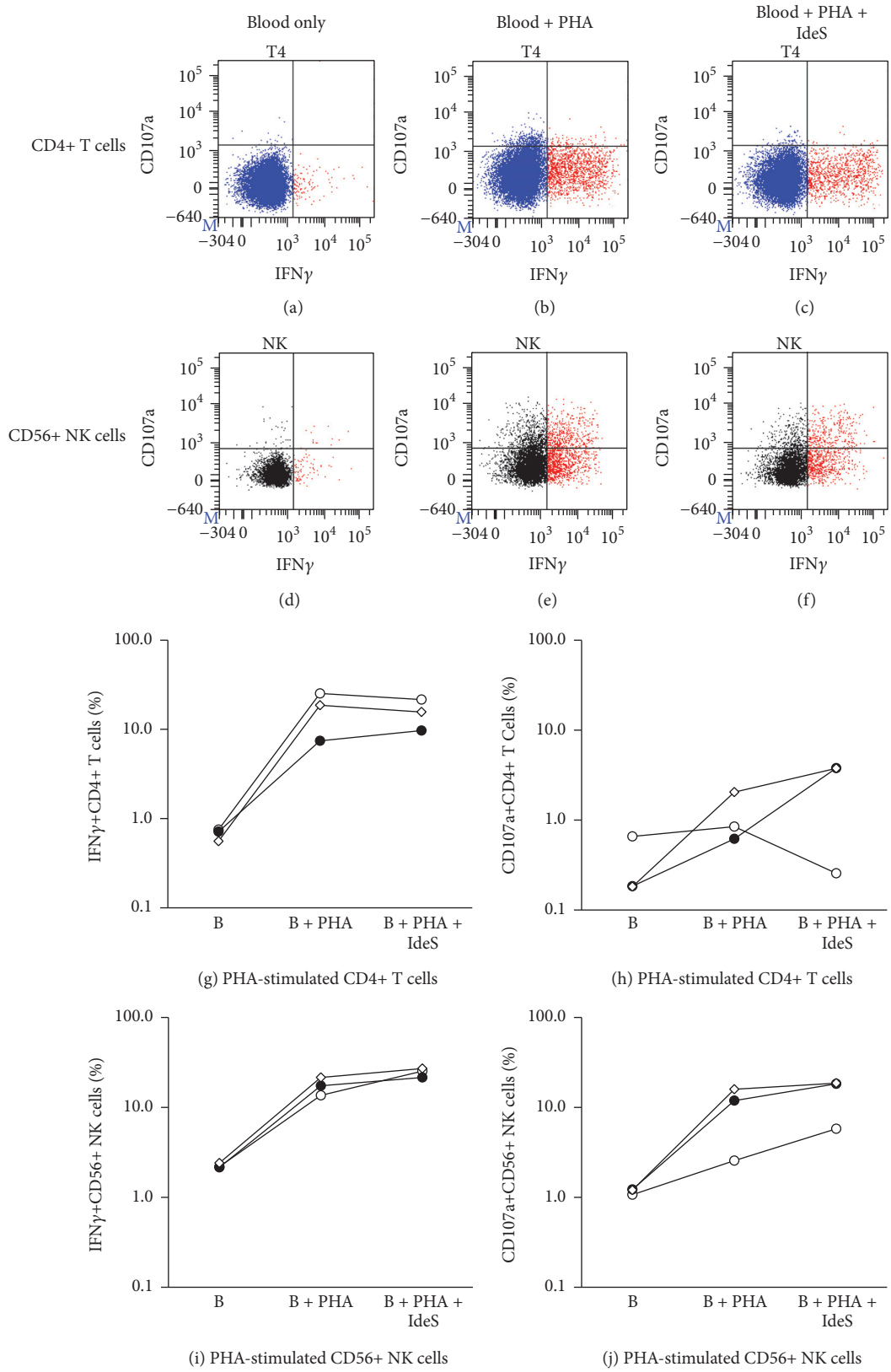


Figure 7: The effect of IdeS on CD4+ T cell and CD56+ NK cell activation in response to PHA. Upper (a–f): a typical result of CD4+ T cell and CD56+ NK cell response to PHA (positive control) with or without IdeS in the CMV-Th and CMV-NK assay. Lower (g–j): the summary of 3 sets of experiment results using 3 different normal individuals. Each line with each symbol describes the result from one normal individual. B: blood.

DES and treatment of ABMR in some patients, and these patients showed even lower CMV and EBV infection rate compared to non-DES patients (Tables 2, 3, and 4 and Figures 2, 3), suggesting that lymphocyte depletion itself might be one of possible factors for the lower viral infection rate in this patient population.

EBV enters B cells via the C3d complement receptor CD21 and establishes its latency on B cells [98]. As EBV is detected in a wide range of B cells, from resting B cells through blast cells to fully differentiated plasma cells, B cells are considered the primary reservoir for EBV [99]. Our study showed that the EBV viremia rates were significantly lower in EBV sero(+) DES and also tended to be lower in EBV sero(−) DES patients (Table 4). Elimination of EBV reservoirs, B cells, by rituximab and alemtuzumab used for DES and induction therapy may reduce the reactivation of latent EBV and/or primary infection in this patient population. In fact, only 2/13 (15%) EBV sero(−) DES patients developed EBV viremia, one with <50 and another with <30 copies/PCR, and none had EBV viremia with >50 copies/PCR, which was in contrast to 7/33 (21%) in EBV sero(−) non-DES patients, although this was not statistically significant ($p = 0.08$, Table 4). Earlier studies showed no EBV reactivation in patients previously treated with rituximab [100, 101] and an association of a lower risk for PTLD by treatment with T cell and B cell depleting agent, alemtuzumab, compared to an agent depleting only T cells, ATG, in hematopoietic cell transplantation [29]. Recently, Schachtner and Reinke [102] reported that a single dose of rituximab 4 weeks prior to transplant significantly reduced posttransplant EBV viremia in EBV sero(−) kidney transplant recipients who received EBV sero(+) kidney, compared to those without rituximab treatment, and no patient developed PTLD. These patients received induction with either anti-IL-2R or ATG, and maintenance with calcineurin inhibitor + MMF + steroid. Thus, we think that the low incidence of EBV viremia observed in DES patients of our study is likely due to rituximab used for DES, although a subsequent use of alemtuzumab induction might further increase this beneficial effect in DES patients.

After primary CMV infection, the virus can persist in a latent form in a variety of tissues, primarily in monocyte-derived macrophages and dendritic cells [29, 103, 104]. The CMV viremia rate was reduced in DES compared to non-DES patients, primarily in CMV sero(−) DES patients. (Table 3). Monocytes and myeloid dendritic cells express CD52 at high levels, but these are less susceptible to alemtuzumab-mediated complement-dependent cell cytotoxicity due to high levels of complement inhibitory proteins expressed on these cells [47]. Thus, unlike B cells for EBV, it is unlikely that the elimination of CMV reservoir is a major reason for lower CMV viremia rate observed in DES patients.

BKV establishes latency in the uroepithelium after a primary infection, [6, 105]. Reactivation and replication occur in immunocompromised patients such as transplant recipients, resulting in viruria. When replication is aggressive, BKV viremia emerges due to injured renal epithelial cells. Currently, it is well accepted that the primary reservoir for BKV is renal epithelial cells, and suggested that the source of BKV in plasma is derived from BKV replication in the allograft of kidney transplant recipients [105]. Thus, alemtuzumab and rituximab have no ability to reduce or prevent BKV infection through elimination of BKV reservoir.

3.4.5. Antiviral Prophylaxis. Antiviral prophylaxis or pre-emptive antiviral therapy is essential for prevention of viral infections and associated complications in transplant recipients [106]. Both strategies are acceptable, but differences are noted [107–110]. Currently, ganciclovir and valganciclovir are commonly used as first-line antiviral prophylaxis and are most effective in preventing CMV infection and disease, although inhibition of other herpes viruses (herpes simplex virus types 1 and 2 [HSV-1, HSV-2], EBV, varicella-zoster virus [VZV], and human herpes virus 6 [HHV-6]) is noted [106, 110]. Acyclovir is also used as antiprophylaxis agent but does not have significant efficacy against CMV compared to HSV-1 and HSV-2, VZV, and EBV [108, 110].

All patients included in this study received antiviral prophylaxis with ganciclovir while inpatient and then valganciclovir or acyclovir posttransplant depending on a risk for CMV infection based on donor and recipient CMV serologies. Briefly, for transplants with lymphocyte depletion induction and those with CMV R−/D+ regardless of induction type, valganciclovir was given. For those with anti-IL-2R induction and CMV R+/D+, R+/D−, or R−/D−, acyclovir was given. In this study, we showed that CMV and EBV viremia rate in DES patients were significantly lower than non-DES patients. Most DES patients (86%) received ganciclovir since they received lymphocyte depletion induction, while 51% of the non-DES patients received acyclovir due to anti-IL-2R induction and CMV sero-status. This difference in antiviral prophylaxis may be a reason for differences in viremia rates in the DES versus non-DES groups. To address this question, we compared the CMV and EBV viremia rates in non-DES patients with valganciclovir versus acyclovir. Since viral sero-status affects the viremia rate, only CMV or EBV sero(+) non-DES patients were included in this analysis. Among CMV sero(+) non-DES patients, 176 received valganciclovir and 175 received acyclovir (Table 5). No significant difference in CMV viremia with >30 copies/PCR was found in the two groups. Among EBV sero(+) non-DES patients, even those with valganciclovir showed higher EBV viremia rates compared to those with acyclovir (22 of 200 [11%] versus 6/215 [2.8%], $p < 0.001$). These results indicate that higher CMV and EBV viremia rates observed in non-DES versus DES patients are unlikely due to acyclovir given to more non-DES patients. Since the most common induction agent used for DES patients was alemtuzumab and most non-DES patients received ATG, we further analyzed the viremia rate in sero(+) non-DES patients with valganciclovir who received alemtuzumab versus ATG. No significant difference in the CMV or EBV viremia rate was found (Table 5), suggesting that the difference in lymphocyte depleting agents is unlikely the reason for higher CMV and EBV viremia rates in non-DES patients in our patient population. It should be noted that non-DES patients who received alemtuzumab showed less EBV viremia rate compared to those with ATG (5% versus 12%) as mentioned in the previous section, but this was not statistically significant.

3.4.6. IVIg. IVIg derived from pooled human plasma from thousands of donors and originally used for the treatment of primary immunodeficiency disorders has also been used for the treatment of autoimmune and inflammatory disorders for nearly 30 years [74, 111, 112] and is currently recognized as a potent immunomodulatory agent. It affects innate and adaptive immune systems, and its effect on most components of immune system including antibodies, complements, cytokines, most immune cells and their receptors, and the interaction of these components have been reported [74, 111, 112]. Precise mechanisms of immune modulation are still not well known although various possible mechanisms have been proposed depending on diseases or its clinical application. We have been using IVIg for the DES therapy as an immunomodulatory agent against allosensitization as previously reported [74, 113–116]. Antiviral properties found in IVIg as discussed at earlier section became an additional benefit for our DES patients. The low CMV and EBV viremia rate observed in DES compared to non-DES patients might be due in part to this IVIg effect.

3.5. Impact of Viral Infection on Allograft Rejection. We next investigated the impact of viral infection on allograft rejection. Of 372 DES and 538 non-DES patients, the rejection information was available in 363 DES and 497 non-DES patients during this study period. Freedom from total allograft rejection, ABMR, and CMR in DES versus non-DES patients is shown in Figure 8. The DES group showed less freedom from overall allograft rejection ($p = 0.08$, Figure 8(a)) during the 1st 5 years after transplant, but this was not statistically significant. When the analysis was performed separately by ABMR and CMR, freedom from ABMR in the DES group was significantly less than non-DES group ($p < 0.001$, Figure 8(b)), which is common and a major obstacle in HS patients [12–15], while rates of CMR were similar in the two groups ($p = 0.27$, Figure 8(c)). This demonstrated that the trend of less freedom from overall rejection observed in the DES was due to significantly less freedom from ABMR in the DES group. The number of patients who had overall allograft rejection (19% versus 14%, resp.), ABMR (12% versus 2.2%), or CMR (11% versus 14%) during this study period in the DES and non-DES groups is also shown in Table 6.

To assess the impact of viral infection on induction of allograft rejection, we next analyzed the rejection rate within 6 months after onset of viral infection. Of 363 DES and 497 non-DES patients, 80 (22%) and 138 (28%) developed at least one CMV with >50, EBV with >50 copies/PCR, or BKV viremia with >2500 copies/ml during this study period (Table 6). Of these, 19% in the DES and 12% in the non-DES groups developed either ABMR or CMR within 6 months after viral infection, which was similar to the overall rejection rate in the whole DES and non-DES groups as shown above (19% versus 14%). In addition, the AMR rate within 6 months after infection in the DES and non-DES groups (10% and 2.2%, resp.) is again similar to that in the whole DES and non-DES groups (12% versus 2.2%), and the same is true for the CMR rate after infection (14% versus 11%). These results suggest that the impact of viral infection on induction of allograft rejection occurring within 6 months after infection is minimal in our patients, both DES and non-DES patients.

The increased risk of viral infections and their complications associated with antirejection therapy is well documented [28, 117]. On the other hand, acute and chronic allograft injuries and rejections caused directly and indirectly by viral infections have also been suggested, but not conclusive [28, 29]. In kidney transplant, CMV infection is known to mediate allograft injury and rejection likely through systemic inflammation, cytokines, and T cell activation induced by CMV [28, 117, 118]. However this trend has been dramatically reduced after application of anti-CMV prophylaxis [28, 107, 119]. All our patients, DES and non-DES, received antiviral prophylaxis for 6 months after transplant, which may minimize the impact of CMV infection on induction of allograft rejection.

3.6. Allograft and Patient Survival in DES and Non-DES Patients. We finally compared allograft and patient survival in DES versus non-DES patients included in this study (Figure 9). There was no significant difference in both allograft and patient survival in the two groups, which was previously reported in our other studies [14, 15, 26, 27]. The estimated graft (death-censored) and patient survival at 2 years after transplant were 93.4% and 95.6% in the DES and 96.1% and 94.9% in the non-DES groups, respectively.

4. Conclusions

Desensitized HS patients are at lower risk for CMV and EBV infections and have a similar risk for BKV infection and BKAN posttransplant. This trend was observed primarily in CMV sero(−) for CMV infection and in EBV sero(+) and sero(−) patients to a lesser degree for EBV infection. No patient developed PTLD in either group. Factors likely responsible for the lower risks for CMV and EBV infections in DES patients include (1) viral-specific memory T cells remaining after lymphocyte depletion with alemtuzumab are capable of efficiently proliferating to clear virus in sero(+) patients. In addition, sero(−) patients are capable of efficiently developing viral-specific T cells even after T cell and B cell depletion; (2) high levels of NK cells remaining after alemtuzumab and consistently available antiviral IgG after T cell and B cell depletion contribute to clearance of CMV and EBV through ADCC in sero(+) patients; (3) elimination of B cells (EBV reservoirs) by rituximab and alemtuzumab, and to a lesser degree, monocytes (CMV reservoir) by alemtuzumab, may reduce or prevent the reactivation of latent infection and/or primary infection; (4) the use of IVIg which contains potent antiviral IgGs that likely have a beneficial effect in preventing or modulating viral infections. We have recently reported that B cell and T cell depletion is unlikely to increase a risk for polyomavirus JC (JCV) and progressive multifocal leukoencephalopathy (PML) in DES patients [24]. Allograft and patient survival were similar in both groups. Taken together, we conclude that the IVIg + rituximab DES combined with alemtuzumab induction with triple immunosuppression maintenance does not increase risk for CMV, EBV, BKV, and JCV infections and their

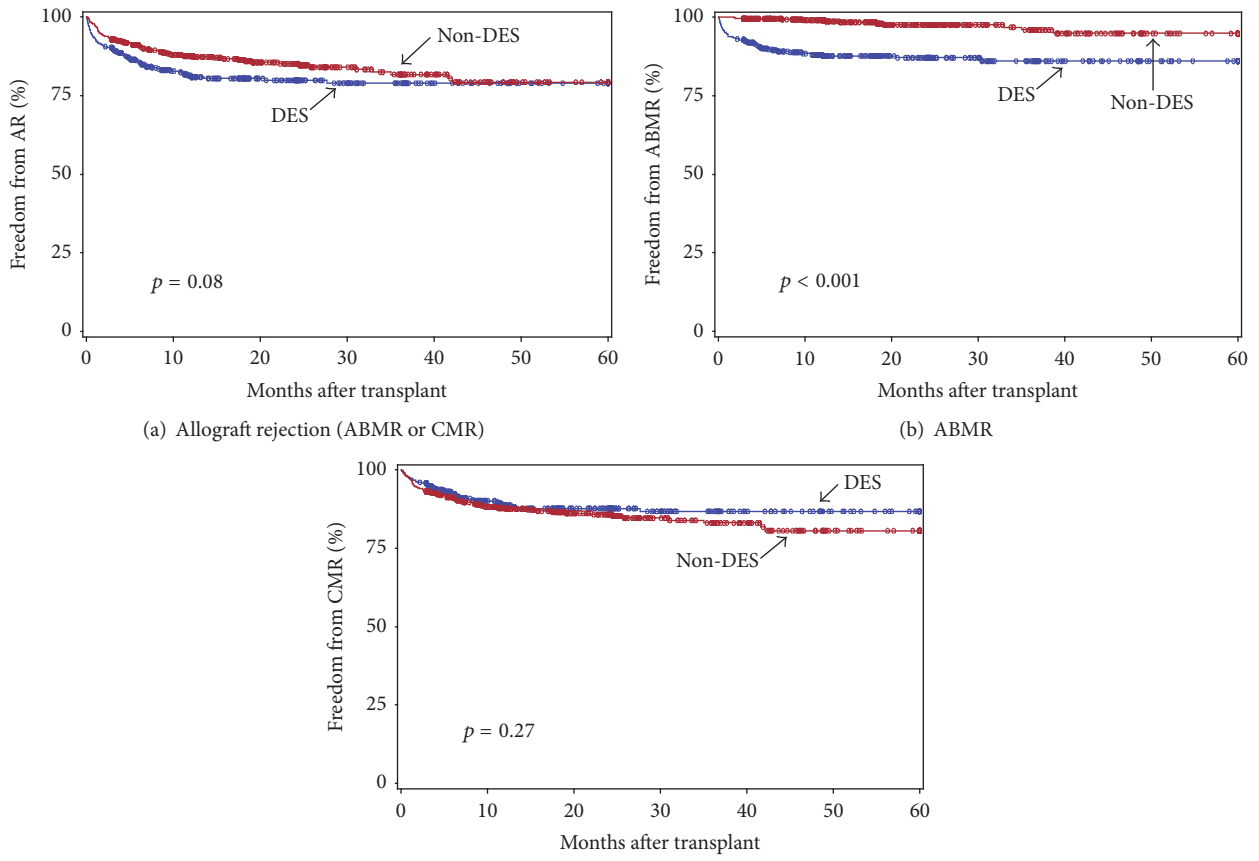


FIGURE 8: Freedom from overall allograft rejection (a), ABMR (b), and CMR (c) in DES (blue) versus non-DES (red) patients during the 1st 5 years after transplant. The group differences were assessed by the log-rank test.

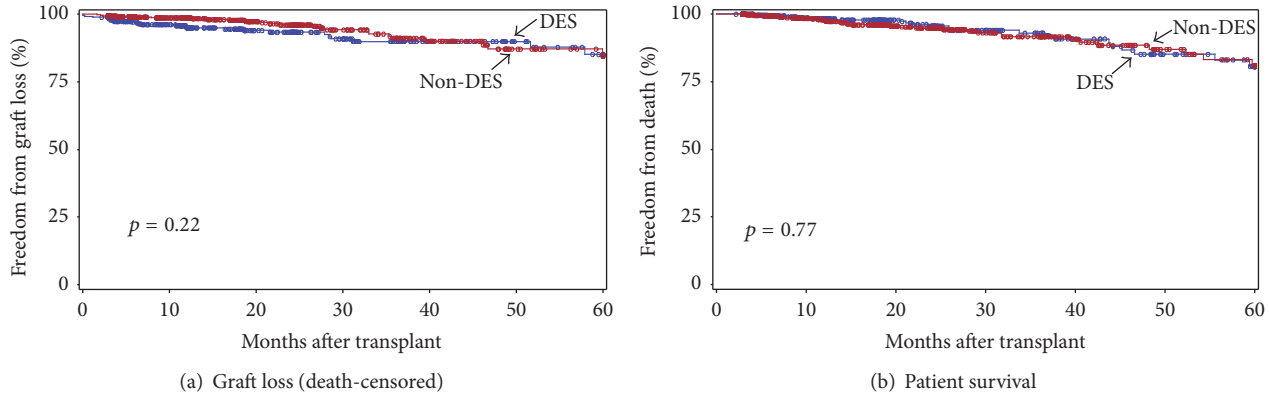


FIGURE 9: Freedom from allograft loss (a) and patient death (b) in DES (blue) versus non-DES (red) patients during the 1st 5 years after transplant. The group differences were assessed by the log-rank test.

associated complications including PTLD, BKAN, and PML in HS kidney transplant patients under antiviral surveillance with antiviral prophylaxis for 6 months after transplant and close monitoring viral infection by PCR for early detection and early intervention.

Competing Interests

Stanley C. Jordan has research grants from Genentech Inc. and owns a patent (US Patent 6,171585B1): "IVIg Immunosuppression for HLA-Sensitized Transplant Recipients," 2001.

The remaining authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Mieko Toyoda participated in the research design, performance of the research, data analysis, data interpretation, and writing the paper. Bong-Ha Shin participated in the research design, performance of the research, and data analysis. Shili Ge participated in the research design, performance of the research, data analysis, data interpretation, and writing the paper. James Mirocha participated in the data analysis. David Thomas participated in the performance of the research, data analysis, and data interpretation. Maggie Chu, Edgar Rodriguez, Christine Chao, Anna Petrosyan, Odette A. Galera, Ashley Vo, Jua Choi, and Alice Peng participated in the performance of the research. Joseph Kahwaji participated in the performance of the research and data analysis. Stanley C. Jordan participated in the research design and writing the paper.

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Review Article

Endothelial Cells in Antibody-Mediated Rejection of Kidney Transplantation: Pathogenesis Mechanisms and Therapeutic Implications

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Antibody-mediated rejection (AMR) has been identified as a main obstacle for stable immune tolerance and long survival of kidney allografts. In spite of new insights into the underlying mechanisms of AMR, accurate diagnosis and efficient treatment are still challenges in clinical practice. Endothelium is the first barrier between recipients' immune systems and grafts in vascularized organ transplants. Considering that endothelial cells express a number of antigens that can be attacked by various allo- and autoantibodies, endothelial cells act as main targets for the recipients' humoral immune responses. Importantly, emerging evidence has shown that endothelial cells in transplants could also initiate protective mechanisms in response to immune injuries. A better understanding of the role of endothelial cells during the pathogenesis of AMR might provide novel therapeutic targets. In the present review, we summarize the antigens expressed by endothelial cells and also discuss the activation and accommodation of endothelial cells as well as their clinical implications. Collectively, the progress discussed in this review indicates endothelial cells as promising targets to improve current diagnosis and therapeutic regimens for AMR.

1. Introduction

Historically, cell-mediated rejection (CMR) was recognized as the predominant form of immune response in organ transplantation. However, progress in the last decade suggested that, besides CMR, antibody-mediated rejection (AMR) also significantly contributes to the rejection and pathogenesis of allografts [1, 2]. Despite the substantial advances in understanding the pathologic process of AMR, accurate diagnosis and efficient treatment are still challenges in clinic. This could be partly ascribed to our limited knowledge of the underlying mechanisms of AMR.

Vascular endothelium is the first barrier between recipients' immune system and allograft in solid organ transplantation. As endothelial cells express a number of antigens that can be targeted by various allo- and autoantibodies (Abs), endothelial cells play an important role in the pathogenesis of AMR [3–5]. Furthermore, increasing evidence has demonstrated that endothelial cells in allograft are not only passive participants, but also active regulators of pathophysiology in recipients [6]. Exploring the role of endothelial cells in AMR, therefore, will facilitate the improvement of current diagnosis and therapeutic regimens for AMR.

This review will summarize the cross talk between endothelial cells and antibodies in allograft rejection and its clinical relevance. We will also discuss the mechanism of activation and accommodation of endothelial cells and their clinical implications. Finally, we will put forward perspectives that could be a valuable subject of research in the future.

Table 1: Endothelial antigens in antibody-mediated immune responses.

Types of Abs	Endothelial antigens	Time course of Ab formation	Hyperacute rejection	Acute rejection	Long-term graft injury	Reference
Alloantibodies	ABO	Preformed & de novo	Yes	Yes	Yes	[10, 11]
	HLA	Preformed & de novo	Yes	Yes	Yes	[14–16]
	MICA	Preformed & de novo	Yes	Yes	Yes	[20–33]
Autoantibodies	AT_1R	Preformed or de novo	No	Yes	Yes	[34–47]
	ET_AR	Preformed or de novo	No	No	Yes	[41, 48]
	Vimentin	De novo	No	No	Yes	[49, 50]
	Perlecan	Preformed or de novo	No	Yes	Yes	[51, 52]
	Endoglin	Preformed	No	Yes	N/A	[53]
	FLT3 ligand	Preformed	No	Yes	N/A	[53]
	EDIL3	Preformed	No	Yes	N/A	[53]
	ICAM4	Preformed	No	Yes	N/A	[53]

2. Endothelial Cells as Targets in Antibody-Mediated Rejection

2.1. Endothelial Antigens Targeted by Alloantibodies

2.1.1. ABO Blood Group Antigens. As early as the 1900s, the ABO blood group system was discovered by Karl Landsteiner, who later won the Nobel Prize in Physiology or Medicine for this extraordinary contribution [7]. The ABO system is composed of genetically determined blood group antigens and corresponding antibodies (namely, isohaemagglutinins) in circulation [8]. Interestingly, these blood group antigens, including A, B, and H, are expressed not only on red blood cells, but also on other tissue cells, such as endothelial cells [9]. Anti-A/B antibodies are preformed natural antibodies, which are the main barriers for ABO-incompatible (ABOi) blood transfusions and organ transplantation. Early practice revealed that ABOi kidney transplantation without special treatment could result in unavoidable disastrous AMR [10, 11] (Table 1). In this respect, kidney transplantation that breaches the ABO system was considered an absolute contraindication for a long period of time. However, the organ-specific pattern of ABO antigens allows an exception for ABOi kidney transplantation. Individuals who are A2 subtypes express low levels of A antigens within kidneys [12]. Therefore, it is acceptable to perform incompatible transplant using kidneys from A2 donors even without adequate preconditioning [7]. With the improved understanding of the ABO-related AMR, ABO blood group compatibility has no longer been a prerequisite for kidney transplantation. Feasible desensitization regimens including anti-A/B antibody deletion and preemptive modulation of B-cell immunity have been developed and thus expand the donor pool significantly. More importantly, such transient treatment is able to induce long-term stable function of allografts even after the reappearance of anti-A/B antigens. This phenomenon is termed accommodation, which will be discussed later.

2.1.2. Human Leukocyte Antigens (HLA). HLA, also known as major histocompatibility complex, are genetically determined and highly heterogeneous proteins in human beings [13]. HLA is able to present antigens to T-cells and thereby regulate immune responses. There are 2 distinct classes of HLA that possess different functions. Class I HLA is expressed on all kinds of cells and exposes antigens within cells to CD8+ cytotoxic T-cells; class II HLA is expressed selectively on antigen presenting cells (APCs) as well as some special cell types, and they can present antigens to helper T-cells. Endothelial cells express both kinds of HLA.

HLA molecules themselves can also be recognized as antigens and induce allogeneic specific antibodies in the process of organ transplantation. The deleterious role of HLA antibodies has been studied extensively in the past decade. Preformed anti-HLA donor-specific antibodies (DSAs) due to pregnancies, blood transfusions, and organ transplantation contribute to higher risk for AMR and allograft failure [14]. Besides, the generation of de novo anti-HLA DSA is considered as a major risk factor for acute and chronic antibody-mediated rejection and graft loss, especially the complement fixing antibodies [15, 16].

2.1.3. Major Histocompatibility Complex Class I Related Chain A Antigens (MICA). MICA are highly polymorphic glycoproteins that are expressed on different types of cells including endothelial cells, and the expression of MICA can be induced upon stresses, which make them ideal targets in organ transplantation [17–19]. In 2002, Sumitran-Holgersson et al. identified preformed MICA antibodies as risk factor for graft loss [20]. Subsequent studies obtained similar results and showed that both preformed and de novo MICA antibodies could result in acute as well as chronic rejections [21–26].

It has been suggested that MICA-associated rejection is highly associated with C4d deposition [27, 28]. In addition, MICA antibodies were reported to cause cell death through complement-dependent cytotoxicity [29]. A very recent research showed that 23% of anti-MICA-positive sera from pretransplant patients could fix C1q and further confirmed that the deleterious effect of MICA antibodies relied on fixation and activation of the complement cascade [30]. All

these data indicate an indispensable role of complement system in the pathogenesis of MICA antibodies.

Considering that MICA is not expressed on resting T- or B-cells, standard lymphocyte cross-matching fails to detect MICA antibodies [20, 31]. Mismatched MICA episodes in allografts are the main targets of MICA antibodies generated by recipients [32]. In this regard, it is of importance to perform MICA genotyping that is not available in the present clinical practice. Tonnerre et al. found that polyreactive anti-MICA sera bound preferentially to MICA∗008 (the most common allele of MICA) donor endothelial cells, indicating that MICA∗008(A5.1) molecules are the predominant determinants of MICA antibodies-related physiopathology [33]. A better understanding of MICA episodes in the background of kidney transplantation may provide feasible strategies for clinical monitoring and treatment.

2.2. Endothelial Targets of Autogenous Anti-Endothelial Cell Antibodies (AECAs)

2.2.1. Angiotensin Type 1 Receptor (AT_1R). AT_1R is a transmembrane G-protein coupled receptor that is expressed at the vascular endothelium [60]. Angiotensin II is the endogenous ligand for AT_1R and exerts most of its effects through AT_1R. Angiotensin II-AT_1R signaling plays an important role in vasoconstriction, cell migration, protein synthesis, inflammation, and fibrosis in various physiologic and pathophysiologic context [61]. Recently, AT_1R autoantibodies have drawn much attention due to their direct involvement in the pathogenesis of autoimmune diseases and solid organ allograft rejections [62, 63]. AT_1R autoantibodies belong to IgG1 and IgG3 subclass and serve as AT_1R agonists. Accordingly, malignant hypertension is recognized as one of the most prominent clinical symptoms in AT_1R-associated disorders.

Dragun et al. first revealed the presence and pathogenic role of AT_1R-Abs in a cohort of renal transplantation recipients with steroid-refractory vascular rejection and malignant hypertension in 2005 [34]. In this study, it is demonstrated that AT_1R-Abs-positive and HLA-Abs-negative patients with vascular refection were at higher risk for allograft loss, in contrast to those with HLA-Abs and without AT_1R-Abs.

Subsequently, other researches provided more evidence for the initial findings [35–47]. The largest retrospective study by now was conducted by Giral et al. in a cohort of 599 kidney transplant recipients [38]. In this study, the authors found that preformed AT_1R-Abs were associated with a higher risk of acute rejection within the first 4 months after transplantation and graft failure after 3 years from transplantation. On the other hand, another research consisting of 351 patients demonstrated that, except for preexisting antibodies, de novo AT_1R-Abs could also cause allograft failure [39].

2.2.2. Endothelin-1 Type A Receptor (ET_AR). ET_AR is a receptor for endothelin-1 and plays an important role in the regulation of blood pressure [64]. It is reported that anti-ET_AR antibodies (ET_AR-Abs) are strongly correlated with anti-AT_1R antibodies in heart transplantation [65]. In renal transplantation, however, researches on ET_AR-Abs are limited. Banasik et al. evaluated ET_AR-Abs in a cohort of 116 kidney transplant recipients and found that ET_AR-Abs existed in almost half of the recipients before transplantation and were related to reduced renal function and increased intimal arteritis after transplantation [48]. But there was no evidence that ET_AR-Abs could deteriorate rejection rates. Another research from the same group demonstrated that ET_AR-Abs were associated with higher risk for graft loss [41].

2.2.3. Vimentin. Vimentin is an intermediate filament protein existing within cells of mesenchymal origin, including endothelial cells. Upon the settings of endothelial injuries, vimentin is exposed to the immune system and thereby results in the production of autoantibodies against vimentin [49, 66]. There have been studies demonstrating the presence of anti-vimentin antibodies (AVA) in kidney transplantation [49, 50]. Besarani et al. further correlated AVA with interstitial fibrosis and tubular atrophy of kidney allografts in a retrospective study including 70 recipients [50]. An experimental research of murine cardiac transplant demonstrated the colocalization of AVA and C3d on endothelium of allografts, indicating a vital role of complement in the AVA-mediated injuries [67]. The destructive effect of AVA, however, seemed to be dependent on alloimmune responses, for vascular lesions were not observed in syngeneic hearts.

2.2.4. Perlecan. Perlecan is an important component of vascular basement membrane that contains 3 laminin-like globular (LG) domains in its C-terminal [68]. LG3 can be cleaved from perlecan and elicits the production of autoantibodies during endothelial injuries [69, 70]. It has been shown that increased serum level of LG3 itself was highly associated with acute vascular rejection [51]. Moreover, another report from the same group revealed elevated anti-LG3 IgG titers before and after transplantation in kidney recipients with acute vascular rejection rather than those with tubule-interstitial rejection or normal graft function [52]. It should be noted that patients who were concomitantly positive for both pretransplantation DSAs and posttransplantation LG3 antibodies had inferior graft survival, indicating the synergy effect between DSAs and LG3 antibodies.

2.2.5. Endoglin, FLT3 Ligand, EDIL3, and ICAM4. Recently, four novel targets on endothelial cells for AECAs were identified using high-density protein arrays: endoglin, Fms-like tyrosine kinase 3 ligand (FLT3 ligand), EGF-like repeats and discoidin I-like domain 3 (EDIL3), and intercellular adhesion molecule 4 (ICAM4) [53]. Enzyme linked immunosorbent assay was performed to detect these AECAs in a validation cohort consisting of 151 renal recipients. Result showed that these four AECAs were obviously related to HLA-DSAs and AMR.

3. Endothelial Cells Act as Participants in AMR: Activation versus Accommodation

Vascular endothelium is the main interface for the direct contact between recipients' immune system and allografts in kidney transplants as well as other solid organ transplantation

procedures. Considering abundance of antigens expressed by endothelial cells as discussed previously, vascular endothelial cells serve as a preferential target for host immune response. However, endothelial cells are not only "passive victims" in the settings of transplantation, but also "active participants" in this pathophysiologic process. Notably, accumulating evidence indicated endothelial cells as a potential promoter for immune tolerance. Therefore, it is of vital importance to understand the role and mechanism of endothelial cells in modulating immune responses and allograft pathogenesis.

3.1. Activation of Endothelial System: Cross Talk between Endothelial and Immune Cells. Activation of endothelial cells refers to the proinflammatory transition under certain microenvironment based on resting condition [71]. Classically, endothelial activation can be divided into 2 distinguished types. Type I activation is induced by histamine or thrombin and type II activation is initiated in response to cytokines such as TNF-α or IL-1. Type I activation acts as a quick fashion independently of de novo gene transcription. In contrast, type II activation relies on gene expression and thereby exhibits a slower process. Activation of endothelial cells could result in various pathophysiologic effects, of which the most important one in the context of allograft rejection is the recruitment and priming of circulating leukocytes. Expression of adhesion molecules and chemokines contributes to this process.

It should be noted that endothelial cells are semiprofessional APCs and are able to activate T-cells, including CD8+ and CD4+ T-cells [72]. In this context, it is of interest to consider whether endothelial cells could exert a direct effect on B-cells and humoral immunity. Given the indispensable role of helper T-cells in the generation of antibodies, endothelial cells are proposed to influence antibody production indirectly via presenting self-antigens to helper T-cells.

Interestingly, a recent research found that endothelial cells could also recruit regulatory T-cells (Tregs) [73]. Recognition of self-antigens of endothelial cells plays a key role in the trafficking of Tregs into target organs. And this recruitment effect of endothelial cells is dependent on IFN-γ-associated microenvironment. Such accumulated Tregs contribute to peripheral immune tolerance. However, how this process could be related to organ transplantation remains unclear and requires further investigation.

Moreover, endothelial cells may also participate in graft nephropathy through regulating thrombosis. Normally, resting endothelial cells could maintain blood fluidity and regulate blood flow. Upon activation, however, they upregulate procoagulant molecules expression and subsequently promote thrombi formation [6].

3.2. Accommodation: Resistance to Allograft Rejection. Nowadays, ABOi transplantation has become a routine option for kidney recipient candidates. This breakthrough, to a great extent, is ascribed to the long-term acceptance of allografts after transient treatment of desensitization. Even though the titers of anti-A/B antibodies increased again after a period of time, ABOi transplant recipient could avoid the assumed higher risk for AMR and keep allograft function stable, which is termed "accommodation" [74]. Current perspectives recognize accommodation as the self-protection and resistance of endothelial cells against AMR. The scientific community has paid much attention to the exploration of the underlying mechanisms of accommodation and determining whether this protective effect could be augmented in clinic practice.

Various mechanisms of endothelial cell-mediated graft protection have been reported by a number of studies (Table 2). In 1997, Bach et al. reported that heart xenografts could acquire accommodation by upregulation of a number of antiapoptotic and anti-inflammatory genes including A20, Bcl-2, Bcl-xl, and hemeoxygenase-1 in endothelial cells [54]. Accordingly, similar mechanisms have been confirmed in renal grafts in the subsequent studies. Salama et al. examined endothelial behavior during accommodation in renal recipients [55]. Immunohistochemistry of the graft biopsies demonstrated increased expression of antiapoptotic protein Bcl-xl in glomerular and peritubular capillary endothelial cells. The authors further performed in vitro experiments to confirm that endothelial cells with upregulated Bcl-xl were rendered resistant to complement-dependent cytotoxicity. Chen et al. reported that antiapoptotic proteins and complement regulatory proteins such as Bcl-2, CD59, CD46, and clusterin might contribute to allografts' accommodation [56]. Interestingly, Iwasaki et al. compared molecular signaling of accommodation under different conditions in vitro [57]. They found that accommodation for anti-A/B antibodies relied on unregulated complement regulatory proteins CD55 and CD59 induced by suppressed ERK1/2 pathway, whereas in the background of anti-HLA antibodies activated PI3K/AKT pathway of endothelial cells led to expression of cytoprotective molecules such as hemeoxygenase-1 and ferritin H. These results indicated that, specifically, induction of anticomplement or antiapoptosis molecules on endothelial cells might be a promising strategy to improve antirejection regimens in clinic. However, the mechanism in depth and feasible treatment modality needs further investigation.

Another explanation for accommodation is the ABO blood group changes on endothelial cells. A study by Tanabe et al. showed time-dependent downregulation of donor's blood-type antigen on the graft endothelium, which might contribute to the long-term accommodation after ABOi kidney transplantation [58]. Besides, the same group confirmed detectable antigenic chimerism on the graft endothelium in another research [59]. The establishment of antigenic chimerism is still not fully understood and warrants further exploration.

Taken together, although substantial breakthroughs have been made in researches of endothelial accommodation, it is still not feasible to develop endothelial cell-targeted therapeutic strategies currently. Investigations therefore are urgently needed in the future.

4. Endothelial Cell-Related Diagnostic Biomarkers in AMR

AMR is recognized as the major obstacle for long survival of kidney grafts. Efficient treatment for AMR relies on accurate diagnosis. The diagnosis of AMR, however, is sophisticated

Table 2: The proposed mechanisms of endothelial cell-mediated accommodation in ABOi transplantation.

Study	Design	Key findings	Reference
Alleviation of apoptosis and complement			
Bach et al.	Hamster to rat heart xenografts	Heart xenografts could acquire accommodation by upregulation of a number of antiapoptotic and anti-inflammatory genes including A20, Bcl-2, Bcl-xl, and hemeoxygenase-1 in endothelial cells.	[54]
Salama et al.	Human renal transplantation with HLA antibodies	Immunohistochemistry of the graft biopsies demonstrated increased expression of antiapoptotic protein Bcl-xl in glomerular and peritubular capillary endothelial cells. In vitro experiments confirmed that endothelial cells with upregulated Bcl-xl were rendered resistant to complement-dependent cytotoxicity.	[55]
Chen et al.	Renal transplantation in skin-presensitized nonhuman primates	Antiapoptotic proteins and complement regulatory proteins such as Bcl-2, CD59, CD46, and clusterin might contribute to allografts' accommodation.	[56]
Iwasaki et al.	In vitro study of the effects of anti-HLA and anti-A/B antibody binding on complement-mediated cytotoxicity and signal transduction	Accommodation for anti-A/B antibodies relied on unregulated complement regulatory proteins CD55 and CD59 induced by suppressed ERK1/2 pathway, whereas in the background of anti-HLA antibodies activated PI3K/AKT pathway of endothelial cells led to expression of cytoprotective molecules such as hemeoxygenase-1 and ferritin H.	[57]
Blood group alteration or chimerism			
Tanabe et al.	ABOi renal transplant recipients	Time-dependent downregulation of donor's blood-type antigen on the graft endothelium was observed, which might contribute to the long-term accommodation after ABOi kidney transplantation.	[58]
Tanabe et al.	ABOi renal transplant recipients	Detectable antigenic chimerism on the graft endothelium was confirmed.	[59]

due to the paucity of characteristic hallmarks under heterogeneous conditions. Recently, several literatures exhibited that some molecular markers of endothelial activation were highly connected to AMR and were able to serve as diagnostic indicators.

In 2009, Sis et al. screened 119 endothelial-associated transcripts in 173 renal grafts to determine their possible role in diagnosis of AMR [75]. They found that increased expression of kidney endothelial transcript successfully predicts active antibody-mediated allograft damage and poor graft outcome. The result was confirmed in an independent validation cohort containing 82 kidneys. Predictive endothelial markers were further explored subsequently. Most recently, a study from Xu-Dubois et al. discovered that endothelial-to-mesenchymal transition (EMT) is of vital importance in the process of AMR, and 3 EMT markers, that is, fascin1, vimentin, and heat shock protein 47, are sensitive and reliable markers for diagnosis for AMR [76]. Taken together, exploration for predictive markers in endothelial cells might provide alternatives for accurate diagnosis for AMR.

5. Final Remarks

Thanks to the progress in organ preservation and immunosuppressive regimens, 1-year survival of kidney allografts has reached 95%. However, the improvements in long-term graft survival are limited and remain unsatisfactory. AMR is recognized as one of the leading causes of graft loss. In this regard, understanding the underlying mechanisms of AMR will facilitate better therapeutic strategies.

Due to the abundance of surface and inside antigens, vascular endothelial cells act as preferential targets for both allo- and autoantibodies. More importantly, endothelial cells in allograft are not only passive participants, but also active regulators in the process of AMR. Upon injuries or inflammation, endothelial cells can increase the expression of allo- and autoantigens, as well as adhesion molecules and chemokines, and thereby recruit and activate circulating leukocytes. On the other hand, endothelial cells are able to initiate self-protection pathways under similar conditions. The balance between their proinflammatory capacities and accommodation statement might decide the final fate of the allograft. Therefore, it is of great value to explore how to modulate this balance favorably towards reducing immunogenicity and increasing graft acceptance.

Taken together, endothelial cells are indispensable participants in the pathophysiology of AMR, and therapeutics targeted at endothelial cells hold great promise to improve the current immunosuppressive regimens, which warrant urgent researches in the near future.

Abbreviations

Ab: Antibody
ABOi: ABO-incompatible
AECA: Autogenous anti-endothelial cell antibody
AMR: Antibody-mediated rejection
APC: Antigen presenting cell
AT_1R: Angiotensin type 1 receptor
AVA: Anti-vimentin antibodies
CMR: Cell-mediated rejection
DSA: Donor-specific antibody
EDIL3: EGF-like repeats and discoidin I-like domain 3
EMT: Endothelial-to-mesenchymal transition
ET_AR: Endothelin-1 type A receptor
HLA: Human leukocyte antigen
FLT3: Fms-like tyrosine kinase 3
ICAM4: Intercellular adhesion molecule 4
LG: Laminin-like globular domain
MICA: Major histocompatibility complex class I related chain A antigen
Treg: Regulatory T-cell.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

Shuo Wang collected and analyzed the literatures and wrote the draft. Chao Zhang, Jina Wang, and Cheng Yang collected the literatures. Ming Xu and Ruiming Rong revised the manuscript. Tongyu Zhu conceived and designed the review. Dong Zhu conceived and designed the review and revised the manuscript.

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Clinical Study

Treatment of Antibody-Mediated Renal Allograft Rejection: Improving Step by Step

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Throughout the past years we stepwise modified our immunosuppressive treatment regimen for patients with antibody-mediated rejection (ABMR). Here, we describe three consecutive groups treated with different regimens. From 2005 until 2008, we treated all patients with biopsy-proven ABMR with rituximab (500 mg), low-dose (30 g) intravenous immunoglobulins (IVIG), and plasmapheresis (PPH, 6x) (group RLP, $n = 12$). Between 2009 and June 2010, patients received bortezomib (1.3 mg/m^2, 4x) together with low-dose IVIG and PPH (group BLP, $n = 11$). In July 2010, we increased the IVIG dose and treated all subsequent patients with bortezomib, high-dose IVIG (1.5 g/kg), and PPH (group BHP, $n = 11$). Graft survival at three years after treatment was 73% in group BHP as compared to 45% in group BLP and 25% in group RLP. At six months after treatment median serum creatinine was 2.1 mg/dL, 2.9 mg/dL, and 4.2 mg/dL in groups BHP, BLP, and RLP, respectively ($p = 0.02$). Following treatment, a significant decrease of donor-specific HLA antibody (DSA) mean fluorescence intensity from 8467 ± 6876 to 5221 ± 4711 ($p = 0.01$) was observed in group BHP, but not in the other groups. Our results indicate that graft survival, graft function, and DSA levels could be improved along with stepwise modifications to our treatment regimen, that is, the introduction of bortezomib and high-dose IVIG treatment.

1. Introduction

Antibody-mediated rejection (ABMR) is one of the most challenging complications following renal transplantation. Paul Terasaki proposed in his humoral theory of transplantation that the majority of transplants are rejected by the action of antibodies, not cells [1]. In a cross-sectional study, we were able to show that about 30% of patients may have HLA antibodies (HLAab) after transplantation [2]. In more than 30% of HLAab positive patients donor-specific HLAab (DSA) were present. Renal allograft survival at 5.5 years after HLAab testing was significantly lower in patients with detectable DSA as compared to HLAab negative patients (49% versus 83%). In a series of 60 patients Sellarés et al. observed that graft failure was caused by ABMR in the majority of cases [3]. To date, plasmapheresis (PPH) together with the application of intravenous immunoglobulins (IVIG) has been the mainstay of ABMR treatment [4, 5]. Over the last years monoclonal antibodies directed against B cells (rituximab) as well as inhibitors of the proteasome (bortezomib) have expanded our therapeutic repertoire [6]. In a previous retrospective analysis, we observed a trend towards an improved graft survival in patients treated with a combination of bortezomib (1.3 mg/m^2, 4x), low-dose IVIG (30 g), and PPH (6x) as compared to patients treated with the same regimen but a fixed dose of rituximab (500 mg) instead of bortezomib [7]. However, even the bortezomib-based regimen was not sufficient to treat all episodes of ABMR effectively.

IVIG preparations containing the pooled serum IgG fractions from thousands of donors have been used for treatment of various autoimmune diseases for more than 30 years. Usually, low doses of IVIG (0.1–0.2 g/kg) are used to substitute immunoglobulins ("replacement") in patients with inherited hypogammaglobulinaemia or following removal

of immunoglobulins by PPH. Pursuing immunomodulation higher doses ("therapeutic") are necessary (1-2 g/kg). Mechanistically, the effects of IVIG on the immune system can be differentiated into effects mediated by the dimeric antigen-binding [$F(ab')_2$] fragment and the Fc fragment [8]. $F(ab')_2$-dependent mechanisms include neutralization of pathologic antibodies (anti-idiotypic) and cytokines, depletion of neutrophils and eosinophils, scavenging of anaphylatoxins such as C3a and C5a, and blockade of cellular receptors. More recent research has shown that much of the immunosuppressive effect of IVIG is mediated via the Fc fragment [8]. These effects include upregulation of the inhibitory Fcγ receptor FcγRIIB, downregulation of activating Fcγ receptors, reduction of antibody half-life by competition of IVIGs with pathological antibodies for binding to the neonatal Fc receptor which recycles IgG, and expansion of regulatory T-cells. Interestingly, Fc fragment glycosylation including terminal sialic acid residues seems to be crucial for the effectiveness of IVIG [9]. The fact that this important structure is present only in a minority of the total serum IgG pool [9] explains why high doses of IVIG are necessary to achieve therapeutic efficacy.

Based on the abovementioned evidence on the dosage and the underlying mechanism of action of IVIG, we increased the applied IVIG dose from a low-dose (30 g fixed dose) to a high-dose regimen (1.5 g/kg) in July 2010, in order to further improve the efficacy of our bortezomib-based treatment protocol. Here, we report on the long-term efficacy and safety of treatment with bortezomib, high-dose IVIG, and PPH (group BHP). The obtained results are compared with two preceding groups of patients treated either with rituximab, low-dose IVIG, and PPH (group RLP) or with bortezomib, low-dose IVIG, and PPH (group BLP).

2. Patients and Methods

Between January 2005 and November 2008 nine consecutive patients with biopsy-proven ABMR were treated with a fixed dose of rituximab (500 mg i.v.), six sessions of PPH (2.5 L/session, 4% albumin), and low-dose (30 g) polyvalent human IVIG (KIOVIG®) after the last PPH (group RLP). Since 2009, all of our patients with a diagnosis of ABMR have received bortezomib-based treatment. However, three patients received rituximab between 2010 and 2013 because of a diagnosis of preexisting polyneuropathy, which is a well-known side effect and consequently a contraindication for bortezomib treatment. Therefore, group RLP finally comprised 12 patients. Between January 2009 and June 2010 eleven consecutive patients received bortezomib (1.3 mg/m^2 i.v., days 1, 4, 8, and 11), PPH (6x), and low-dose (30 g) IVIG (group BLP). In July 2010 we increased the IVIG dose and treated 11 consecutive patients with bortezomib, PPH (6x), and high-dose (1.5 g/kg) IVIG (group BHP). In order to avoid removal of rituximab and/or IVIG by means of PPH, rituximab was given one day after the last PPH. IVIG treatment was started two days after rituximab, in order to reduce potential adverse interactions between IVIG and rituximab. In addition, we monitored $CD19^+$ peripheral B cells following the administration of rituximab. $CD19^+$ B cells were either not measurable or slightly above the detection limit following rituximab treatment [0.01/nL (0.00–0.01); normal range 0.1–0.4/nL]. Patients of all groups additionally received a three-day pulse of methylprednisolone (500 mg/day i.v.). After discharge all patients were regularly monitored in our outpatient clinic.

Renal transplantation was performed at the Charité Hospital based on a negative complement-dependent cytotoxicity crossmatch (CDC-XM) with and without dithiothreitol using T- and B-lymphocytes with current and historical serum. In addition, graft allocation was based on a negative virtual crossmatch by considering current and historical unacceptable antigens as defined by Luminex® based single antigen bead assays. Based on this procedure, none of the patients underwent any kind of desensitization before transplantation. Consequently, only patients with de novo DSA were included.

Altogether, 76% (26/34) of all patients received induction therapy with either basiliximab ($n = 22$) or daclizumab ($n = 4$). The remaining eight patients were transplanted before the introduction of basiliximab or daclizumab and received no induction therapy. The distribution of patients, who received no induction therapy, was not significantly different between groups (group RLP $n = 4/12$, group BLP $n = 3/11$, and group BHP $n = 1/11$, $p = 0.37$).

Renal biopsies were taken on indication only. All patients presented with clinically relevant allograft dysfunction after transplant manifesting as an otherwise unexplained increase of serum creatinine (≥0.3 mg/dL), proteinuria (≥1 g/d), or primary nonfunction in the early phase after transplantation. The diagnosis of ABMR was based on the presence of circulating DSA and significant allograft pathology according to the definitions of the Banff classification [10]. C4d staining was done by indirect immunofluorescence on paraffin sections using a polyclonal rabbit anti-human C4d IgG antibody (Biomedica, Vienna, Austria). Only patients who gave their written informed consent were considered eligible for enrollment. All patients were treated in accordance with the Declaration of Helsinki.

Serum samples before and after treatment were screened for HLA antibodies (HLAab) by the Luminex bead-based assay LABScreen® Mixed (One Lambda, Canoga Park, CA, USA). In addition, HLAab specificities were determined by LABScreen Single Antigen beads assay (One Lambda). As an indicator for the antibody level, the normalized MFI was used. HLAab were considered positive when exceeding an MFI value of 500. The DSA showing the highest MFI at the time of ABMR diagnosis (DSAmax) and the MFI sum of all DSA (DSAsum) were tracked to indicate the effectiveness of treatment.

End of follow-up was June 30th, 2016. Renal allograft survival was defined as the interval between diagnosis of ABMR and return to maintenance dialysis treatment or end of follow-up. The estimated glomerular filtration rate (eGFR) was calculated according to the chronic kidney disease epidemiology collaboration (CKD-EPI) formula [11]. All adverse events, abnormal laboratory values, and hospitalizations were tracked from our web-based electronic patient record system "TBase" [12]. Adverse events occurring during

Table 1: Patient characteristics.

	Group RLP ($n = 12$)	Group BLP ($n = 11$)	Group BHP ($n = 11$)	p
First/repeat transplantation	8/4	7/4	11/0	n.s.
Donor age	52.4 ± 13.8	42.3 ± 14.9	52.1 ± 15.2	n.s.
Living/deceased donor	5/7	5/6	7/4	n.s.
Interval between transplantation and diagnosis (months)	34.6 ± 56.9	58.1 ± 51.4	46.1 ± 44.7	n.s.
Early/late antibody-mediated rejection	6/6	2/9	2/9	n.s.
Pathology scoring				
Glomerulitis (g)	0.8 ± 1.1	0.9 ± 1.0	1.6 ± 1.2	n.s.
Peritubular capillaritis (ptc)	1.1 ± 1.1	0.8 ± 1.1	1.5 ± 1.0	n.s.
Intimal arteritis (v)	0.7 ± 0.9	0.3 ± 0.6	0.5 ± 0.7	n.s.
Transplant glomerulopathy (cg)	1.1 ± 1.2	2.2 ± 1.3	0.8 ± 1.3	0.049 (BLP versus BHP)
C4d (immunohistochemistry)	1.4 ± 1.3	2.0 ± 1.1	1.3 ± 1.3	n.s.
IVIG dose (g)	30	30	120 (80–150)	<0.001 (BHP versus RLP and BHP versus BLP)
Maintenance immunosuppression after diagnosis				
Steroids	12	11	11	n.s.
Cyclosporine A	0	3	1	n.s.
Tacrolimus	12	6	10	0.01 (RLP versus BLP)
Everolimus	0	2	0	n.s.
Mycophenolic acid	12	11	11	n.s.
Median observation time after treatment (months)	101 (39–137)	88 (73–90)	64 (55–71)	<0.001 (BHP versus RLP); 0.015 (BHP versus BLP)

Group RLP, rituximab + low-dose IVIG + plasmapheresis; group BLP, bortezomib + low-dose IVIG + plasmapheresis; group BHP, bortezomib + high-dose IVIG + plasmapheresis. IVIG, intravenous immunoglobulin. Comparison between groups was carried out using Fisher's exact test for categorical variables and Kruskal-Wallis test with Dunn-Bonferroni post hoc test for continuous variables.

the first year after treatment were documented and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 [13]. Normally distributed variables are summarized as mean ± standard deviation. Not normally distributed variables are summarized as median and interquartile ranges. Comparison between groups was carried out using Fisher's exact test for categorical variables and Kruskal-Wallis test with Dunn-Bonferroni post hoc test for continuous variables. Wilcoxon signed-rank test was used for pairwise comparison between different time points. Graft survival was analyzed according to Kaplan-Meier with a log-rank test. A multivariate Cox proportional hazard model was used to identify covariates which independently contribute to allograft loss following ABMR treatment. A probability of less than 0.05 was considered as statistically significant. Statistical analysis was carried out using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA), and STATA 11 IC software (StataCorp., College Station, TX, USA).

3. Results

Relevant patient characteristics are shown in Table 1. The interval between transplantation and diagnosis was not different between groups. In all groups there were patients with "early" ABMR occurring during the first year after transplantation and patients with "late" ABMR. Scoring of renal allograft tissue according to the Banff classification revealed no differences between groups except for the transplant glomerulopathy (cg) score, which was higher in group BLP as compared to group BHP (2.2 ± 1.3 versus 0.8 ± 1.3, $p = 0.049$). The applied IVIG dose for group BHP on average was four times as high as that of both other groups [120 g (range: 80–150 g) versus 30 g, $p < 0.001$]. Following diagnosis, all patients received triple drug maintenance immunosuppression comprising a calcineurin inhibitor or mammalian target of rapamycin inhibitor in combination with steroids and mycophenolic acid. In group RLP more patients received tacrolimus as compared to group BLP (100% versus 55%, $p = 0.01$). Median observation time following diagnosis was 101, 88, and 64 months for groups RLP, BLP, and BHP, respectively. Due to the sequential nature of our treatment protocol modifications follow-up was shortest for the BHP group.

None of the patients died with a functioning graft. Graft survival following diagnosis improved stepwise in the three groups along with the sequential modifications of our treatment protocol, that is, the substitution of rituximab by

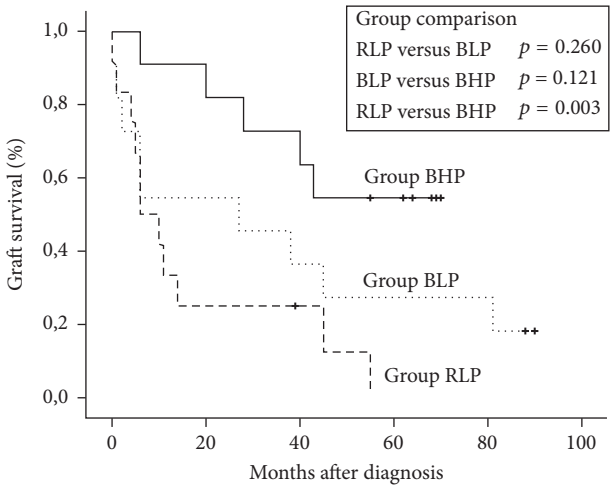

Figure 1: Graft survival according to Kaplan-Meier. Differences between groups were calculated by log-rank test. Note: none of the patients died with a functioning allograft. Group RLP, rituximab + low-dose IVIG + plasmapheresis; group BLP, bortezomib + low-dose IVIG + plasmapheresis; group BHP, bortezomib + high-dose IVIG + plasmapheresis. "+", end of follow-up.

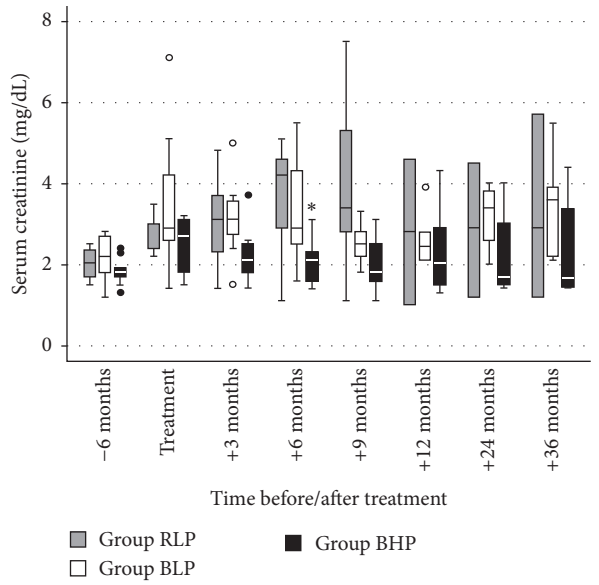

Figure 2: Serum creatinine before, during, and after treatment of antibody-mediated rejection of all patients with a functioning graft at each time point. Differences between groups were calculated by Kruskal-Wallis test with Dunn-Bonferroni post hoc test. Group RLP, rituximab + low-dose IVIG + plasmapheresis; group BLP, bortezomib + low-dose IVIG + plasmapheresis; group BHP, bortezomib + high-dose IVIG + plasmapheresis. $^{*}p = 0.02$ versus group RLP and group BLP.

bortezomib (group RLP → BLP) and the increase of the IVIG dose from a low-dose (30 g fixed dose) to a high-dose (1.5 g/kg) IVIG regimen (group BLP → BHP) (Figure 1). A significant difference in graft survival was observed between group BHP and group RLP ($p = 0.003$). Graft survival at one and three years after treatment was 91% and 73% in group BHP, as compared to 55% and 45% in group BLP and 33% and 25% in group RLP, respectively. At the end of follow-up, graft survival was 55% (6/11) in group BHP, 18% (2/11) in group BLP, and 8% (1/12) in group RLP. At diagnosis, serum creatinine [2.7 mg/dL (1.9–2.9) versus 2.9 mg/dL (2.6–4.1) versus 3.0 mg/dL (2.6–3.0), $p = 0.23$] (Figure 2) and proteinuria [1.3 g/d (0.5–2.1) versus 1.1 g/d (0.45–3.9) versus 0.9 g/d (0.5–1.8), $p = 0.79$] (Figure 3) were not significantly different between groups BHP, BLP, and RLP, respectively. Following treatment, serum creatinine was lower in group BHP as compared to both other groups (Figure 2). A significant difference of group BHP as compared to group BLP and group RLP was observed at six months after treatment [2.1 mg/dL (1.6–2.3) versus 2.9 mg/dL (2.7–3.8) versus 4.2 mg/dL (2.9–4.6), $p = 0.02$] (Figure 2). In accordance, eGFR at six months after diagnosis was higher in group BHP as compared to both other groups [33.0 mL/min/1.73 m^2 (26.9–41.4) versus 26.0 mL/min/1.73 m^2 (15.5–32.0) versus 16.3 mL/min/1.73 m^2 (15.4–19.0), $p = 0.03$]. In addition, proteinuria was significantly lower in group BHP as compared to both other groups at six months after diagnosis [0.15 g/d (0.1–0.4) versus 1.2 g/d (1.0–2.1) versus 1.2 g/d (0.7–2.4), $p = 0.049$] (Figure 3).

The immunological characteristics with respect to HLA mismatches and HLA antibodies are summarized in Table 2. The count of HLA class I and II mismatches was equally distributed between groups. Similarly, HLA antibody panel reactivity (% PRA) at the time of diagnosis did not differ significantly although the HLA class I% PRA among patients

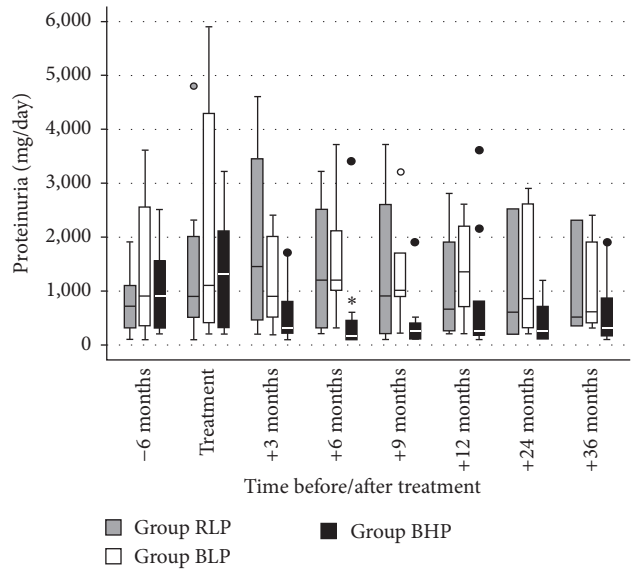

Figure 3: Proteinuria before, during, and after treatment of antibody-mediated rejection of all patients with a functioning graft at each time point. Differences between groups were calculated by Kruskal-Wallis test with Dunn-Bonferroni post hoc test. Group RLP, rituximab + low-dose IVIG + plasmapheresis; group BLP, bortezomib + low-dose IVIG + plasmapheresis; group BHP, bortezomib + high-dose IVIG + plasmapheresis. $^{*}p = 0.049$ versus group RLP and group BLP.

Table 2: HLA mismatches and HLA antibodies.

	Group RLP ($n = 12$)	Group BLP ($n = 11$)	Group BHP ($n = 11$)	p
Number of HLA mismatches per patient (mean ± SD)				
Class I (A, B)	2.2 ± 1.2	2.0 ± 1.3	2.7 ± 0.9	n.s.
Class II (DR, DQ)	1.7 ± 0.9	2.5 ± 1.1	2.0 ± 1.2	n.s.
HLA antibody panel reactivity (% PRA) at diagnosis (mean ± SD)				
Class I	85 ± 14	50 ± 30	41 ± 20	n.s.
Class II	66 ± 20	63 ± 19	63 ± 28	n.s.
DSA_{max} MFI (mean ± SD)				
At diagnosis	8539 ± 5478	9747 ± 8363	8467 ± 6876	n.s.
After treatment	8196 ± 5425	7891 ± 6475	5221 ± 4711[a]	n.s.
DSA_{sum} MFI (mean ± SD)				
At diagnosis	11,235 ± 10,116	12,937 ± 11,196	10,657 ± 8973	n.s.
After treatment	12,900 ± 11,018	12,653 ± 12,744	5587 ± 4509[b]	n.s.
Patients with a decrease of [% (count)]				
DSAmax	33% (4)	72% (8)	82% (9)	0.036 (group BHP versus RLP)
DSAmax < 500 MFI	8% (1)	9% (1)	18% (2)	n.s.
DSAsum	33% (4)	72% (8)	72% (8)	n.s.

Group RLP, rituximab + low-dose IVIG + plasmapheresis; group BLP, bortezomib + low-dose IVIG + plasmapheresis; group BHP, bortezomib + high-dose IVIG + plasmapheresis. DSA_{max}, donor-specific HLA antibody showing the highest mean fluorescence intensity (MFI) at time of diagnosis. n.s., not significant. [a] $p = 0.01$ in comparison to before treatment. [b] $p = 0.04$ in comparison to before treatment.

of groups BLP and BHP was lower as compared to group RLP (50% and 41% versus 85%, $p = 0.07$). Concerning the level of DSAmax and DSAsum before treatment as indicated by MFI, there was no significant difference between groups. Interestingly, following treatment, DSAmax and DSAsum could be decreased significantly as compared to the pretreatment status in group BHP, but not in the other two groups. The BHP treatment scheme was statistically more efficient in the decrease of DSAmax MFI levels than the RLP scheme [9/11 (82%) versus 4/12 (33%), $p = 0.04$]. In all three groups, there were a few patients with a posttreatment DSAmax level below 500 MFI.

The observed side effects during the first year after treatment are shown in Table 3. The most frequent side effects were haemoglobin reduction (94%), thrombocytopenia (76%), and leukopenia (59%). We observed two cases of grade IV leukopenia in the RLP group and four cases of grade III leukopenia, two of which in the RLP group and two in the BLP group. In the BLP group there were two cases of grade III thrombocytopenia in patients with preexisting thrombocytopenia. All episodes of leukopenia and thrombocytopenia were spontaneously reversible. During episodes of thrombocytopenia no bleeding events were evident. A mild to moderate (grade I-II) increase of transaminase levels occurred in 41% of all patients. Altogether 35 episodes of infection were observed in 16/34 (47%) patients. No difference between groups was found regarding the frequency of infections, neither in general nor with respect to specific infections. Gastrointestinal side effects were observed more frequently in patients, who received bortezomib. In the BHP group significantly more patients suffered from diarrhea as compared to the RLP group (64% versus 8%, $p = 0.009$). Reversible peripheral sensory neuropathy (grade I-II) occurred in two patients of the BHP group. One patient in each group experienced a mild allergic reaction during the IVIG infusion. These events were successfully treated with antihistamines and prednisolone. The number of hospitalizations as well as the number of hospitalized patients was not significantly different between groups. In two patients of group BHP a nonmelanoma skin cancer was diagnosed and successfully treated by local excision.

To correct for any covariate with a potential impact on allograft outcome following ABMR treatment, we performed a Cox proportional hazard analysis with clinical, immunological, and therapeutic covariates as summarized in Table 4. Based on the univariate analysis we identified an impaired allograft function (i.e., eGFR < 30 mL/min/1.73 m^2) at the time of ABMR diagnosis as well as treatment with rituximab, bortezomib, and high-dose IVIG as the only statistically significant predictors for allograft survival following ABMR treatment. Impaired graft function at diagnosis and ABMR treatment by rituximab were identified as risk factors for subsequent allograft survival. On the contrary, bortezomib and high-dose IVIG revealed a beneficial effect. Subsequent multivariate analysis was performed using impaired graft function at diagnosis and the treatment options rituximab, bortezomib, and IVIG dose as combined covariates. Taken together, impaired allograft function contributed significantly as a risk factor to the regression model (HR = 3.26, 95% CI: 1.29–8.24, $p = 0.01$) and bortezomib plus high-dose IVIG treatment revealed the strongest beneficial effect on allograft survival following ABMR treatment (HR = 0.21,

Table 3: Main adverse events during the first year after treatment.

	Group RLP ($n = 12$)	Group BLP ($n = 11$)	Group BHP ($n = 11$)	p
Haemoglobin reduction: baseline – nadir (mg/dL)	2.1 ± 1.5	2.3 ± 1.8	2.8 ± 1.3	n.s.
Thrombocytopenia (patients)	9	10	7	n.s.
Leukopenia (patients)	8	6	6	n.s.
Increase of serum transaminase levels (patients)	6	5	3	n.s.
Infections (events/patients)	14/7	12/7	9/2	n.s./n.s.
Urinary tract infection	7/4	2/1	7/1	n.s./n.s.
Otitis media	2/2	0/0	0/0	n.s./n.s.
Tonsillitis	0/0	1/1	1/1	n.s./n.s.
Pneumonia	1/1	0/0	0/0	n.s./n.s.
Enterocolitis	4/4	4/4	0/0	n.s./n.s.
Central venous catheter infection	0/0	2/2	0/0	n.s./n.s.
CMV reactivation	0/0	2/2	1/1	n.s./n.s.
Fever of unknown origin	0/0	1/1	0/0	n.s./n.s.
Nausea (patients)	0	0	2	n.s.
Vomiting (patients)	0	0	2	n.s.
Diarrhea (patients)	1	2	7	0.009 (RLP versus BHP)
Peripheral sensory neuropathy (patients)	0	0	2	n.s.
Allergic reaction to IVIG (patients)	1	1	1	n.s.
Hospitalizations (events/patients)	16/8	9/7	10/5	n.s./n.s.
Nonmelanoma skin cancer (patients)	0	0	2	n.s.

Adverse events during the first year after treatment are shown except for malignancies, where the whole follow-up period was considered. *Note*. Some patients suffered from more than one gastrointestinal adverse event (nausea, vomiting, or diarrhea) simultaneously. Group RLP, rituximab + low-dose IVIG + plasmapheresis; group BLP, bortezomib + low-dose IVIG + plasmapheresis; group BHP, bortezomib + high-dose IVIG + plasmapheresis.

Table 4: Univariate and multivariate cox regression analysis of clinical, immunological, and therapeutic covariates to predict allograft loss following ABMR treatment. Covariates were only considered for the multivariate cox regression model if statistically significant in the univariate analysis.

	HR	95% CI	p
Univariate			
Retransplantation	1.70	0.66–4.37	0.27
Induction therapy by IL-2R antibody	0.82	0.31–2.13	0.68
Early ABMR	0.97	0.38–2.47	0.95
eGFR < 30 mL/min/1.73 m^2 at ABMR diagnosis	2.67	1.07–6.63	**0.03**
Chronic glomerulopathy (cg) score ≥ 1 at ABMR diagnosis	1.69	0.71–4.01	0.23
DSA class I	0.65	0.28–1.50	0.31
DSA class II	0.82	0.30–2.24	0.70
DSA class I + II	0.77	0.32–1.88	0.57
DSAmax > 10,000 MFI	1.57	0.69–3.57	0.28
Everolimus-based maintenance immunosuppression	0.52	0.07–3.94	0.52
ABMR treatment by rituximab	2.74	1.16–6.47	**0.02**
ABMR treatment by bortezomib	0.37	0.15–0.86	**0.02**
ABMR treatment by high-dose IVIG	0.34	0.13–0.93	**0.04**
Multivariate			
ABMR treatment by rituximab + low-dose IVIG (RLP)	1.00	n.a.	n.a
ABMR treatment by bortezomib + low-dose IVIG (BLP)	0.58	0.22–1.52	0.27
ABMR treatment by bortezomib + high-dose IVIG (BHP)	0.21	0.07–0.62	**0.005**
eGFR < 30 mL/min/1.73 m^2 at ABMR diagnosis	3.26	1.29–8.24	**0.01**

DSA, donor-specific HLA antibody(ies), HR, hazard ratio, MFI, mean fluorescence intensity, n.a., not applicable, 95%CI, confidence interval 95%.

95% CI: 0.07–0.62, $p = 0.005$) as compared to rituximab plus low-dose IVIG.

4. Discussion

The existing literature on the treatment of ABMR is characterized by a marked heterogeneity concerning the definition of ABMR as well as the applied treatment protocols [5]. Notably, a considerable number of the available studies was performed before the introduction of a pathology-based definition for ABMR in 2003 [14]. At that time, various and nowadays outdated criteria to diagnose ABMR were oftentimes used. In addition, new therapeutics more specifically targeting B cell-mediated immune response have become available. The efficacy of these substances as well as their combination with established treatment protocols has not yet been studied sufficiently. A recent randomized study could not demonstrate a benefit of rituximab compared to placebo [15]. Therefore, current evidence for the treatment of ABMR is anything else but satisfactory.

To date, IVIG preparations are widely used for treatment of ABMR [4]. Yet, it is not known, whether high-dose IVIG treatment is advisable in combination with modern antihumoral therapeutics such as bortezomib. Here, we addressed this important question by comparing the efficacy and safety of high-dose IVIG treatment in combination with bortezomib and PPH in 11 consecutive patients with biopsy-proven ABMR (group BHP) with a group of 12 patients treated with low-dose IVIG together with a fixed dose of rituximab and PPH (group RLP) and with a group of 11 patients treated with low-dose IVIG together bortezomib and PPH (group BLP). Group RLP and group BLP have already been partially described in our previous study [7]. Extending the results of this study group RLP now comprises three additional patients, that is, altogether 12 patients, and the median observation time of the present study has been markedly prolonged as compared to our previous study (group RLP: 101 versus 18 months, group BLP: 88 versus 18 months). Notably, the median observation time exceeded five years in all groups. The three groups presented here were comparable regarding the underlying patient characteristics, renal function, and renal pathology at diagnosis except for the transplant glomerulopathy (cg) score, which was significantly higher in group BLP as compared to group BHP.

Our results indicate that treatment with bortezomib in combination with high-dose IVIG and PPH (group BHP) is statistically superior as compared to treatment with rituximab, low-dose IVIG, and PPH (group RLP). In addition, high-dose IVIG with bortezomib and PPH seemed to be advantageous as compared to low-dose IVIG with an identical regimen comprising bortezomib and PPH. Both the change from rituximab-based treatment to bortezomib-based treatment and the increase of the IVIG dose from a low-dose to a high-dose regimen resulted in a stepwise improvement of graft survival and graft function. The observed reduction of DSA following treatment in the BHP group but to a lower extent in both other groups reflects the clinical course and is in concordance with the literature. Terasaki and colleagues demonstrated that a decrease in HLA antibody levels can be used as a surrogate marker for the efficacy of an antirejection treatment and directly correlates with superior allograft survival [16]. Therefore, our results confirm and extend our previous observations [7] inasmuch as bortezomib-based treatment proved to be superior to rituximab-based treatment and that the addition of high-dose IVIG further increased graft survival. Our findings are in line with the results from the randomized RITUX-ERAH trial [15], which demonstrated no statistically significant effects of rituximab for the treatment of ABMR. Our results also suggest that high-dose IVIG seems to be advisable in the presence of modern antihumoral therapeutics such as bortezomib. This conclusion is supported by experimental data showing that only a small proportion of IgG in the available IVIG preparations is responsible for the immunosuppressive effect [8, 9]. Therefore, the amount of applied IVIG seems to be crucial for the success of treatment.

Theoretically, it would have been interesting to investigate a group of patients treated with rituximab, PPH, and high-dose IVIG, in order to further elucidate the efficacy of rituximab and high-dose IVIG treatment. However, in our previous study [7] we observed a clear trend towards an improved graft survival in patients, who received a bortezomib-based treatment over a rituximab-based treatment, so that we deliberately decided to treat all upcoming patients with a bortezomib-based protocol and refrained from further trials on rituximab-based treatment in order not to expose patients to a (in our opinion) potentially higher risk of graft loss.

First reports on the effects of IVIG in the context of renal transplantation have been published 20–30 years ago. In 1984, Steiner et al. investigated the impact of prophylactic IVIG administration on the rate of infection in a prospective randomized trial [17]. Because the proportion of patients discharged with a functioning graft was lower in the treatment group, the authors concluded that prophylactic administration of IVIG is "not only useless but probably dangerous." About one decade later, Peraldi et al. showed that early high-dose IVIG treatment (2 g/kg) following renal retransplantation improved 5-year graft survival [18]. One of the first reports demonstrating that high-dose IVIG (2 g/kg) may be effective in the treatment of ABMR following kidney transplantation was published by Jordan et al. in 1998 [19]. In 2001, Casadei and colleagues observed that high-dose IVIG treatment (3.5 g/kg) was equally effective in the treatment of steroid-resistant rejection as OKT3 [20]. At the same time, Luke and colleagues also found that high-dose IVIG (2 g/kg) was effective to reverse steroid- or antilymphocyte antibody-resistant rejection [21]. Importantly, a consistent definition of ABMR based on pathological findings and the presence of circulating DSA was not introduced into the Banff classification until 2003 [14]. Consequently, studies performed before 2003 must be regarded with caution. In the more recent era, Lefaucheur et al. reported that treatment of ABMR with high-dose IVIG alone (2 g/kg ×4) was inferior to a combination of high-dose IVIG, PPH, and rituximab, indicating that IVIG alone, even if applied in very high doses, is not sufficient to treat ABMR [22]. In 2014, Cooper et al. showed that high-dose IVIG alone (5 g/kg) caused a modest

DSA reduction, especially concerning class I DSA in patients with previous acute ABMR [23].

The effectiveness of IVIG therapy on circulating HLAab has also been investigated in various studies on pretransplant desensitization. In 1993, Glotz et al. described that pretransplant administration of high-dose IVIG (1.6 g/kg) suppresses HLAab formation [24]. More than ten years later, Jordan et al. showed that high-dose IVIG treatment (2 g/kg ×4) reduced PRA levels [25]. In 2006, Stegall et al. observed that high-dose IVIG treatment (2 g/kg) decreased DSA titers [26]. Vo and colleagues reported in 2008 that treatment with high-dose IVIG (2 g/kg ×2) and rituximab (375 mg/m^2×2) reduced PRA levels from 77% to 44% [27]. Recently, the same group compared desensitization with high-dose IVIG (2 g/kg ×2) plus rituximab (1 g) to high-dose IVIG (2 g/kg ×2) plus placebo in a randomized clinical study [28]. Patients received an additional dose of IVIG at transplantation plus an additional dose of rituximab or placebo at six months after transplant. Following transplantation of 13 patients, the study was halted and unblinded because of three episodes of ABMR. All episodes had occurred in the placebo group (3/7) indicating that high-dose IVIG alone did not sufficiently prevent ABMR. Notably, 1/6 patients in the rituximab group died at 12 months after transplantation from a *Nocardia* brain abscess.

In group BLP, two patients received maintenance immunosuppression including everolimus following diagnosis. One of these patients returned to maintenance hemodialysis at 6 months after diagnosis; in the other patient serum creatinine at 88 months after diagnosis is stable at 2.4 mg/dL. In this patient we did not reinitiate calcineurin-inhibitor treatment because of a history of cyclosporine A-induced hemolytic uremic syndrome soon after transplantation. Notably, we were not yet aware of the fact that everolimus-based immunosuppression is associated with an increased risk for the development of DSA and ABMR in 2009 [29]. In addition, a higher cg score was observed in patients of group BLP as compared to group BHP. This might be important, as Billing et al. showed that the degree of transplant glomerulopathy is associated with the response to treatment [30]. Therefore, we included maintenance immunosuppression and cg scores at the time of ABMR diagnosis as covariates in our Cox proportional hazard analysis but could not find a statistically significant impact on allograft outcome.

Taken together, our results demonstrate that graft survival of patients with a diagnosis of ABMR following renal transplantation improved during the past years along with the introduction of bortezomib and high-dose IVIG treatment resulting in a 5-year graft survival of about 50% following diagnosis. In conclusion, the use of high-dose IVIG in combination with a bortezomib-based treatment regimen seems to be useful, especially when compared to a historical group of patients treated with low-dose IVIG, PPH, and rituximab. The degree of DSA reduction in group BHP supports this conclusion and may be used as biomarker for the efficacy of treatment. In our view, stepwise controlled modifications of the established treatment protocols are helpful to gradually improve the prognosis following a diagnosis of ABMR. The main limitation of our study is the fact that it is a retrospective study with a limited number of patients comprising episodes of early and late ABMR and comparing cohorts treated at different time periods that differ in some important variables. Therefore, the results must be interpreted with caution, and further studies are necessary to confirm our findings. In future, IVIG preparations containing a higher proportion of Fc fragments with terminal sialic acid residues may be even more effective. Until such preparations will be available, the amount of applied IVIG seems to be crucial for the success of treatment.

Abbreviations

ABMR: Antibody-mediated rejection
DSA: Donor-specific HLA antibodies
DSAmax: DSA showing the highest MFI at the time of ABMR diagnosis
DSAsum: MFI sum of all DSA exceeding 500 MFI
HLAab: HLA antibody(ies)
IVIG: Intravenous immunoglobulins
MFI: Mean fluorescence intensity
PPH: Plasmapheresis.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

Nils Lachmann, Michael Dürr, and Johannes Waiser were responsible for conception, data acquisition and analysis, and preparation and writing of the manuscript. Constanze Schönemann, Axel Pruß, and Klemens Budde reviewed and proofread the manuscript. Nils Lachmann and Michael Duerr contributed equally.

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Review Article

The Potential of MicroRNAs as Novel Biomarkers for Transplant Rejection

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The control of gene expression by microRNAs (miRNAs, miR) influences many cellular functions, including cellular differentiation, cell proliferation, cell development, and functional regulation of the immune system. Recently, miRNAs have been detected in serum, plasma, and urine and circulating miR profiles have been associated with a variety of diseases. Rejection is one of the major causes of allograft failure and preventing and treating acute rejection are the central task for clinicians working with transplant patients. Invasive biopsies used in monitoring rejection are burdensome and risky to transplant patients. Novel and easily accessible biomarkers of acute rejection could make it possible to detect rejection earlier and make more fine-tuned calibration of immunosuppressive or new target treatment possible. In this review, we discuss whether circulating miRNA can serve as an early noninvasive diagnostic biomarker and an expression fingerprint of allograft rejection and transplant failure. Understanding the regulatory interplay of relevant miRNAs and the rejecting allograft will result in a better understanding of the molecular pathophysiology of alloimmune injury.

1. Introduction

MicroRNAMicroRNAs (miRNAs, miRs) are a class of small (~22 nt) noncoding molecules that inhibit translational initiation and stimulate decay of mRNA targets [1, 2]. MiRs are transcribed by RNA polymerase II/III and processed by the RNAse III enzymes Drosha and its binding partner DGCR8 in the nucleus and Dicer in the cytoplasm, to produce short double-stranded RNAs. One strand of the double-stranded RNA is loaded into the Argonaute (Ago) protein and forms the miR-mediated silencing complex (miRISC). MiRs guide miRISC to pair with imperfect complementarity to sequences in target mRNAs, resulting in their subsequent destabilization and translational repression [3]. The target mRNA recognition by the miRISC is mediated by the "seed sequence" nucleotide 2 to 8 [4, 5]. Recent data show that 35–40% of miR binding sites are found in the 3′UTRs, 40–50% in coding regions, and <5% in the 5′UTR mRNA regions [6, 7]. Greater than 60% of the human transcriptome is predicted to be under miR regulation, making this posttranscriptional control pathway as important as proteins in the regulation of cell functions [2]. It is clear that miRs play vital roles in regulating diverse functions in normal and diseased cells [8, 9]. Recent studies have shown that in addition to intracellular regulatory functions, miRs can be secreted and detected in bodily fluids such as blood and urine. The secreted miRs are associated with proteins (Ago2), lipoprotein complexes, or packaged into microvesicles like exosomes. Circulating miRNAs are very stable and resistant to treatment with ribonucleases, freezing/thawing cycles, and other drastic experimental conditions [10]. Several studies have shown that secreted miRNAs can function as a second messenger. MiRNAs packed into exosomes or HDL can be taken up as an active component by neighboring cells and induce cell modification/regulation [10, 11]. The biogenesis, function, and export of miRNAs are shown in Figure 1. Recent reports have gone even further by reporting a much more complex picture of the strong regulatory functions of a diversity of other small ncRNA species such as piwi-interacting RNAs (piRNA) or long noncoding RNAs (lncRNA). PiRNA were

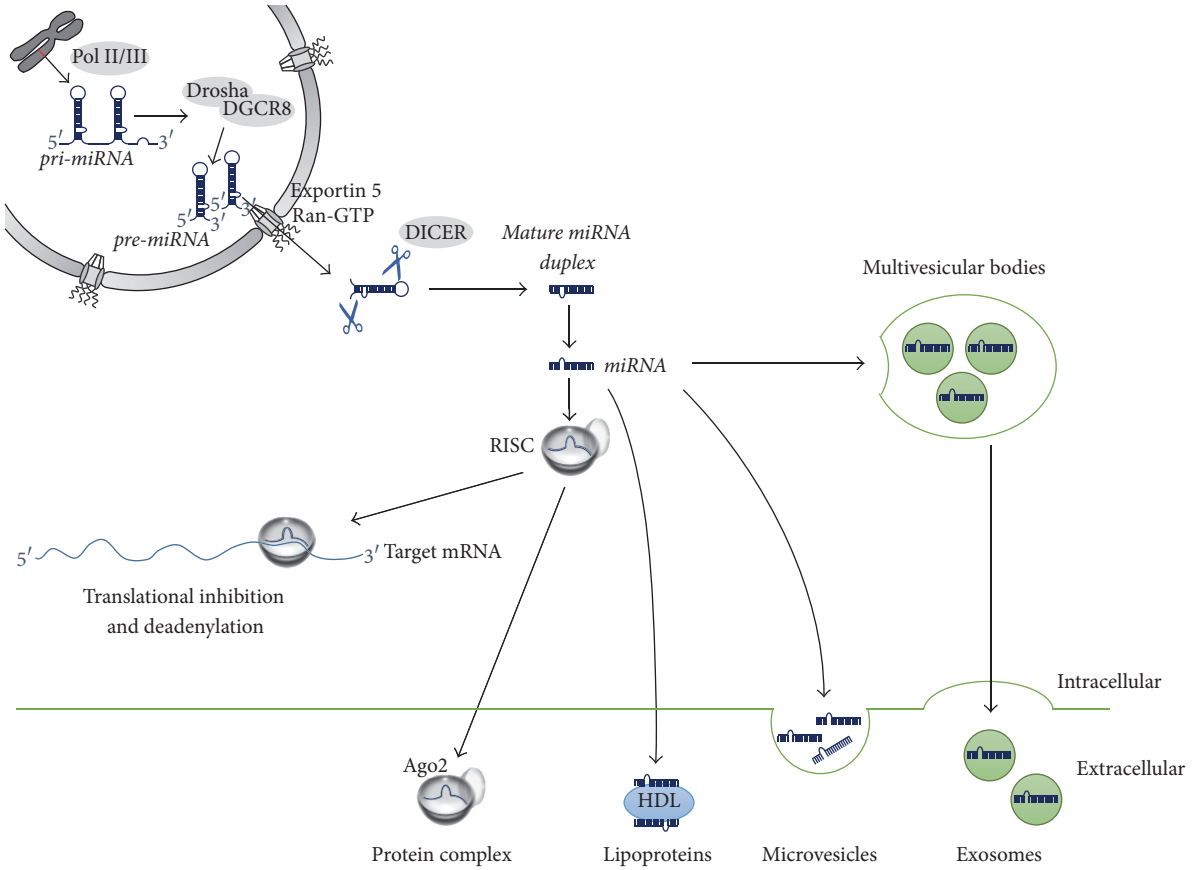

Figure 1: Biogenesis and release of miRNAs. Pri-miRNAs are transcribed in the nucleus by RNA polymerase II/III and processed by the ribonuclease Drosha into hairpin RNAs (pre-miRNA). The stem loops are exported into the cytoplasm using Exportin 5 and Ran-GTP and further cleaved by Dicer to yield 21–23 nucleotide duplexes. The duplexes are unwound and can be loaded directly into the RISC complex and guide translational repression of target mRNAs or they can be released from the cells in protein complexes, bound to lipoproteins, packed in microvesicles, or secreted in exosomes.

identified in germline cells as regulators of transposons. They depend on a specific PIWI clade protein and their biogenesis is independent of Dicer [12, 13]. LncRNAs are a large and diverse class of transcribed RNA molecules with a length of more than 200 nucleotides that do not encode proteins. To date, very few lncRNAs have been characterized in detail. However, it is clear that lncRNAs are important regulators of gene expression and are thought to have a wide range of functions in cellular and developmental processes [14, 15]. A short overview of selected RNA species and their functions is shown in Table 1. In summary, the identification of miRNAs and small RNA species seems to represent only the tip of the iceberg and the prediction of an individual miRNA; its target and function in health and disease are one of the big challenges in research.

Table 1: Categories, characteristics, and functions of selected ncRNAs.

	Length	Function
Short ncRNAs		
miRNA	~22 nt	Posttranscriptional regulation of gene expression
siRNA	~21 nt	Gene silencing
piRNA	~30 nt	Transposon regulation, development
Long ncRNAs		
rRNA	+1.9 kbases	Protein biosynthesis
lncRNA	~200 nt	Epigenetics and gene regulation

2. Use of miRNAs in Transplantation

The few publications that exist on the topic of miRNAs and transplants focus on miRNAs isolated from biopsies. An overview of the different studies is given in Table 2. This review focuses on a short overview of the transplanted organ, the identified miRNA pattern, and shared common pathways.

2.1. miRNAs and Pathways Involved in Transplant Rejection

2.1.1. TGF-Beta Signaling Pathway. An inflammatory reaction takes place during the acute or chronic rejection of

Table 2: Human miRNA expression in different types of transplantation.

	MicroRNA	lncRNA and piRNA	Target	Source	Method	Groups	Ref.
Liver	miR-122, miR-148a, miR-194	N/A	N/A	Biopsy Serum	TaqMan miRNA assay	AR (n = 13) Control (n = 12) Total LivertTx (n = 43)	[16]
	miR-122, miR-155	N/A	BAAT, STAT-1	Biopsy	qPCR	RHC (n = 17) AR (n = 12)	[17]
	miR-146a, miR-19a, miR-20a, miR-200a, miR-141, miR-203, miR-20b, miR-205, miR-33a, let-7e, miR-150, miR-34c-5p, miR-342-5p, miR-181c-5p, miR-29a, miR-19a, miR-204, miR-20a, miR-328, miR-1336, miR-223, miR-210, miR-503	N/A	TGFβR2, SMAD4, EGFR, VGFA, IL-8, IL-6, CCL8, CD40L, IRS2, TLR4, c-Myb, STAT-1, IGF1, VGFA	Biopsy	GeneChip 2.0 Affymetrix and qPCR	Slow fibrosis progressors (n = 11) Fast progressors (n = 9) AR (n = 5) Control (n = 4)	[18]
	let-7c, miR-23b, miR-122, miR-150	N/A	N/A	Serum	TaqMan miRNA Assay	OBI (n = 11) HBV (n = 29) Control (n = 30)	[19]
Lung	miR-144	N/A	TGIF1 (TGF beta signaling)	Biopsy/BAL cells	TaqMan miRNA assay	BOS+ (n = 20) BOS− (n = 19)	[20]
	let-7c, **miR-10a**, **miR-98**, miR-99a, miR-101, miR-125a-5p, miR-125b, miR-135b, miR-137, miR-148b, miR-184, miR-190, miR-199b, miR-203, miR-219, miR-299-5p, miR-302b, miR-335, miR-338-3p, **miR-369-5p**, miR-381, miR-450a, miR-499, **miR-518f**, miR-548c-5p, miR-551b, miR-627, miR-708, miR-874, miR-208, miR-875-5p, miR-302c*, miR-18b, **miR-133b**, miR-134, miR-145, miR-187, miR-214, miR-433, miR-489, miR-494, miR-503, miR-542-5p, **miR-548d**, miR-628-5p, miR-144*	N/A	TGF beta and B- cell receptor signaling	BAL cells/PBMCs	TaqMan low-density array, TaqMan miRNA assay	DSA+BOS− (n = 10) DSA+BOS+ (n = 10) DSA−BOS− (n = 10)	[21]
	364 differentially expressed, **miR-299-3p, miR-29b-1*, miR-34a, miR-451, miR-519e, miR-629, miR-590-5p, miR-381, miR-374a, miR-28-5p, miR-126, miR-27b**	N/A	TCF4, LRRC8B, C14orf2, FUT8, C14orf135, ATR, PYHINI, TCF4, CA1, NFKBIA, NFIL3, DOCK4, PLK2	PBMCs	Exiqon miRCURY LNA array	LTx (n = 18) Control (n = 35)	[22]
	miR-16, miR-195	N/A	Rfx5 MHC CII	BAL cells	TaqMan miRNA assay, qPCR	DSA+ (n = 15) DSA−BOS− (n = 15)	[23]

Table 2: Continued.

	MicroRNA	lncRNA and piRNA	Target	Source	Method	Groups	Ref.
	miR-658, miR-629, miR-628, miR-602, miR-381, miR-125a, miR-663, miR-654, miR-611, miR-524, miR-483, miR-346, miR-326, miR-324, miR-125b-2, miR-125b-1	NR_001562, NR_002791, NR_002909, NR_002941, NR_003024, NR_003130, NR_003573, NR_023318, NR_024080, NR_024332, NR_024400, NR_024418, NR_024611, NR_026550, NR_026576, NR_026695, NR_027303, uc001pyd, uc002nyb, uc002zic, uc002zpx, uc003akf, uc003bgk, uc003dwf, uc003syy, uc003tsq, uc003wcs, uc003zfx, uc010akv, uc010gqe, uc010kwo, uc010lqx	AP-1, AP-4, STATx, c-Myc, p53	Biopsy	Exiqon MiRNA microarray, LncRNA expression microarray	AR ($n = 3$) Control ($n = 3$)	[24]
	miR-99b, miR-23b, let-7b-5p, miR-30a, miR-145	N/A	N/A	Biopsy	NanoString assay, TaqMan miRNA assay	APN ($n = 11$), AR ($n = 5$) Control ($n = 4$)	[25]
	miR-146a C>G (rs2910164), miR-149 T>C (rs2292832), miR-196a2 (rs11614913), miR-499a A>G (rs3746444)	N/A	N/A but twofold increased risk for overall survival	Biopsy	Genomic DNA sequencing	ESRD: AR ($n = 52$) Non-AR ($n = 218$) Control ($n = 350$)	[26]
	miR-142-5p, miR-155, miR-223, miR-30a-3p, miR-10b, let-7c	N/A	CD3, NKCC-2	Biopsy (PBMCs/HRECs)	TaqMan low-density array, qPCR analysis	AR ($n = 12$) Normal ($n = 21$)	[27]
Renal	miR-10b	N/A	BCL2L11	Biopsy	Next-generation sequencing, qRT-PCR	AR ($n = 15$) Normal ($n = 15$)	[28]

Table 2: Continued.

	MicroRNA	lncRNA and piRNA	Target	Source	Method	Groups	Ref.
	miR-99a, miR-100, miR-151a, let-7a, let-7c, let-7f	N/A	N/A	Serum	TaqMan miRNA Assay	AR (n = 12) Control (n = 11) DGF (n = 15)	[29]
	miR-324-3p, miR-611, miR-654, miR-330_MM1, miR-524*, miR-17-3p_MM1, miR-483, miR-663, miR-516-5p, miR-326, miR-197_MM2, miR-346, miR-658, miR-125a_MM1, **miR-320**, miR-381, miR-628, miR-602, miR-629, miR-125a	N/A	RIMBP2, GTDC1, NTRK2, CCDC21, SAMD4B, SYS1, SYNGR1, PTPN9, ETF1, BCL6, ACBR2B, NFIB, RAB11FIP2, ARID4B, PTCH1, HD, HABP4, FOXG1B, H1F0, EDEM1, ZNF673	Biopsy	Exiqon MiRNA microarray	AR (n = 3), Control (n = 3)	[30]
	miR-182-5p, miR-21-3p	N/A	FOXO1, BCL2	Biopsy	GeneChip miRNA 3.0, qPCR	DSA+ AR (n = 16) AKI (n = 8) Control (n = 10)	[31]
	miR-450b-5p, **miR-142-3p**, miR-876-3p, miR-106b, miR-508-3p, miR-148b, miR-324-5p, miR-98	N/A	TGFβ-1	PBMCs, B-cells	TLDA microRNA cards pool A, TaqMan qPCR	Operationally tolerant (n = 15) STA (n = 20)	[32]
	miR-10a, miR-10b, miR-210	N/A	N/A	Urine	TaqMan qPCR	Acute cellular rejection (n = 62) Control (n = 19)	[33]
	miR-25, miR-181a, miR-204, miR-192, miR-10b, miR-142-3p, miR-215, miR-342-3p, miR-615-3p	N/A	PRMT5, TP53, CDX2, ATM, HIPK2, TGFβR1, TGFβR2, SNAI1, SPDEF, MAD2L1, HRH1, LMNB2, DTL, NCOR2, RAC1, ACVR2B	Biopsy	Whole genome microarrays, microfluidic qPCR	AR (n = 18) Control (n = 52)	[34]
Cardiac	**miR-10a, miR-21, miR-31, miR-92a, miR-142-3p, miR-155, miR-451**	N/A	Inhibition of NFkB signaling pathway, inflammatory pathways E-selectin, ICAM-1, α5, S1P1, MKK4, eNOS	Serum	qPCR analysis	AR (n = 30) Control (n = 30)	[35]
	miR-326, miR-142-3p, miR-101, miR-144, miR-27a, miR-424, miR-339-3p	N/A	N/A	Serum	qPCR analysis	Before AR (n = 10) During AR (n = 10) After AR (n = 10)	[36]

Table 2: Continued.

	MicroRNA	lncRNA and piRNA	Target	Source	Method	Groups	Ref.
BM	**miR-27b-3p, miR-10a-5p, miR-21-5p, miR-181a-5p, miR-92a-3p,** miR-3168, miR-22-3p, miR-378a-3p, miR-205-5p, miR-423-5p, miR-146b-5p, miR-26a-5p, miR-148a-3p, miR-486-5p, miR-28-3p, miR-4792, let-7a-5p, miR-3182, miR-423-3p	piR_004307_DQ575881, piR_017723_DQ594464, piR_020814_DQ598650, piR_016745_DQ593052, piR_002732_DQ573682	EGR2, CEBPA, ANXA1, MPL, ZFP36, CCL3, CSF2, IL1b, CXCR4, GATA1, MPO, SLC2A5, SLAMF8, CYP1B1	MSC and cord blood CD34+	Next-generation sequencing and qPCR	CD34+ with EVs ($n = 3$) CD34+ Control ($n = 3$) EVs ($n = 3$)	[37]

Occult HBV infection (OBI), hepatitis B virus (HBV), acute rejection (AR), sustained responders (SR), acute pyelonephritis (APN), bronchiolitis obliterans syndrome (BOS), development of antibodies to HLA (DSA), end-stage renal disease (ESRD), delayed graft function (DGF), acute tubular necrosis without rejection (AKI), stable patients treated with conventional immunosuppression (STA), extracellular vesicle (EV), recurrent hepatitis C (RHC), peripheral blood mononuclear cell (PBMC), bronchoalveolar lavage (BAL), human renal epithelial cell (HRECs), and bone marrow (BM) bold = second validation by quantitative polymerase chain reaction. ∗ = star strand of the miRNA duplex (less predominant loaded to RISC).

an organ. Among different cytokines such as IL-6 [38] and inflammatory mediators elicited during inflammation, TGF-β1 is considered the main mediator and inducer of fibrosis [39]. TGF-β1 belongs to a family of growth factors that includes TGF-βs, activins, and bone morphogenic proteins (BMPs). TGF-β1 and BMP-7 are key determinant factors in peritoneal cell plasticity and in particular, the predominance of one or the other may determine the epithelial or mesenchymal phenotype [40, 41]. Among the different organs and studies, the TGF-beta signaling pathway is the predominant pathway for organ rejection. Wilfingseder et al. (kidney) [42], Xu et al. (lung) [21], and Joshi et al. (liver) [18] described in their studies that miR-548d, miRNA-203, and miRNA-146a are regulators of SMAD4. After activation, SMAD4 forms homomeric and heteromeric complexes and translocates to the nucleus to initiate TGF-beta induced transcription. The downregulation of the miRNAs during rejection leads to an overexpression of SMAD4 and enhanced signaling. The receptor of TGF-beta (TGFβR2) is regulated by miR-548d and miRNA-200a and miRNA-141 and downregulation leads to a higher density of the receptor on the cell surface. The miRNA-200 family is described in the literature as involved in inflammation and IL-6 regulation. In an additional publication, Xu et al. defined the role of miRNA-144 in fibroproliferation leading to bronchiolitis obliterans syndrome (BOS) [20]. MiRNA-144 is strongly upregulated in BOS patients and downregulates TGIF1, a suppressor of TGF-beta induced transcription. The absence of the suppressor leads to an elevated production level of FGF, TGF-beta, and VEGF and promotes the onset of fibroses and organ rejection [20]. Discrimination of cases with fibrosis by miRNA profiles is certainly of interest in acute rejection as well as chronic rejection. The microRNA let-7c targets the TGF-beta and the WNT1 signaling pathway and can enhance the function of the other miRs [43]. In mouse transplantation models, it has been shown that miR-21 is one of the driving agents of fibrosis in acute cardiac allograft rejection [44]. In general, all miRs that are able to promote TGF-beta signaling and the development of fibrosis are drivers of rejection. The aforementioned publications lay out the mechanisms of miRNA and their function in the TGF-beta signaling pathways as a model for the interplay and modulation coursed by miRNAs.

2.1.2. T-Cell, B-Cell, and Macrophage Signaling and Development. Wilflingseder et al. showed that miR-182-5p expression is profoundly correlated with kidney tissue injury. MiR-182-5p can be activated by IL-2 and STAT5 and act as an inhibitor of FOXO1 expression. FOXO1 acts as a master cellular regulator of a variety of cellular processes including cell survival, apoptosis, proliferation, and metabolism. FOXO1 also plays a critical role in the homeostasis of cells of the immune system including T-cells, B-cells, and neutrophils [31]. Further, the absence of FOXO1 was shown to severely reduce the development of FOXP3+ regulatory T-cells (T_{regs}). Those T_{regs} that developed were found to be nonfunctional in vivo and downregulation of FOXO1 in T-cells resulted in lymphocyte infiltration and promotion of inflammation and immune response induced rejection of the transplant [45]. In addition, Wei et al. observed in their study that mice with rejecting cardiac allografts had significantly higher levels of miR-182 in the grafts, infiltrated mononuclear cells, and plasma compared to syngeneic transplants. These findings further support the potential of miRNAs as a biomarker for organ rejection [46]. One miRNA that has an overlap in three of the analyzed organs (see Figure 2) is let-7c. In the literature, let-7c is discussed as an important regulator of HSCs by controlling signaling of TGF-beta and WNT1 and plays a role in the development of T_H1 cells [43]. Other publications show that the polarization and activation of macrophages are dependent on let-7c [47, 48]. The development of T_H1 cells in addition to the regulation of macrophages may play an important role in the recognition of the transplant and result in its rejection. Additionally, let-7c plays an important role in cardiomyogenesis [49] and may interfere with the replacement of tissue that is damaged by the immune system in heart transplants. Surprisingly, none of the cardiac tissue relevant miRs (miR-1, miR-133a, miR-208a/b, and miR-499) [50] are found to be affected by acute rejection. In particular for cardioprotection, a recent review discusses the importance of exosomes during normal health and injury and the interaction with the immune system [51].

Xu et al. found four differentially expressed miRNAs (miR-133b, miR-134, miR-433, and miR-628-5p) in the lungs that modulate the B-cell signaling pathway in different stages and regulate autoimmunity, immunoglobulin (Ig) production, and immune response. The study showed a significant downregulation of these miRNAs in donor specific antibody (DSA) positive BOS positive patients. The study argues that the downregulation leads to overactivation of the B-cell mediated immune response and to the DSA-induced rejection of the transplant [21].

2.2. miRNAs as Potential Noninvasive Biomarkers for Acute and Chronic Rejection

Liver. In many studies, miRNA-122 was discovered in mice and humans as a very liver-specific miRNA. In liver patients, it was demonstrated that hepatocyte-derived miRNAs miR-122, miR-148a, and miR-194 correlated with hepatic injury and acute rejection after liver transplantation [16]. The serum level of these miRNAs was significantly increased in patients with liver injury induced by rejection and there was a strongly positive correlation with the clinically used aminotransferase diagnostic marker. Liu et al. discussed the findings in their study on the potential roles of the miR-148 family in immune homeostasis and regulation. When they examined dendritic cells (DCs) stimulated by TLR agonists, they discovered that the miRNA was overexpressed upon activation, which inhibited cytokine production, upregulation of MHC class II expression, and DC-mediated T-cell proliferation. They also observed that miR-148/152 targets calcium/calmodulin-dependent protein kinase II, which could increase production of proinflammatory cytokines in response to DC activation by TLR in mice [52]. Farid et al. were able to show that the miR-122 in the serum of patients with acute rejection reaches a high level even at the beginning of rejection in comparison with the classical aminotransferase marker and

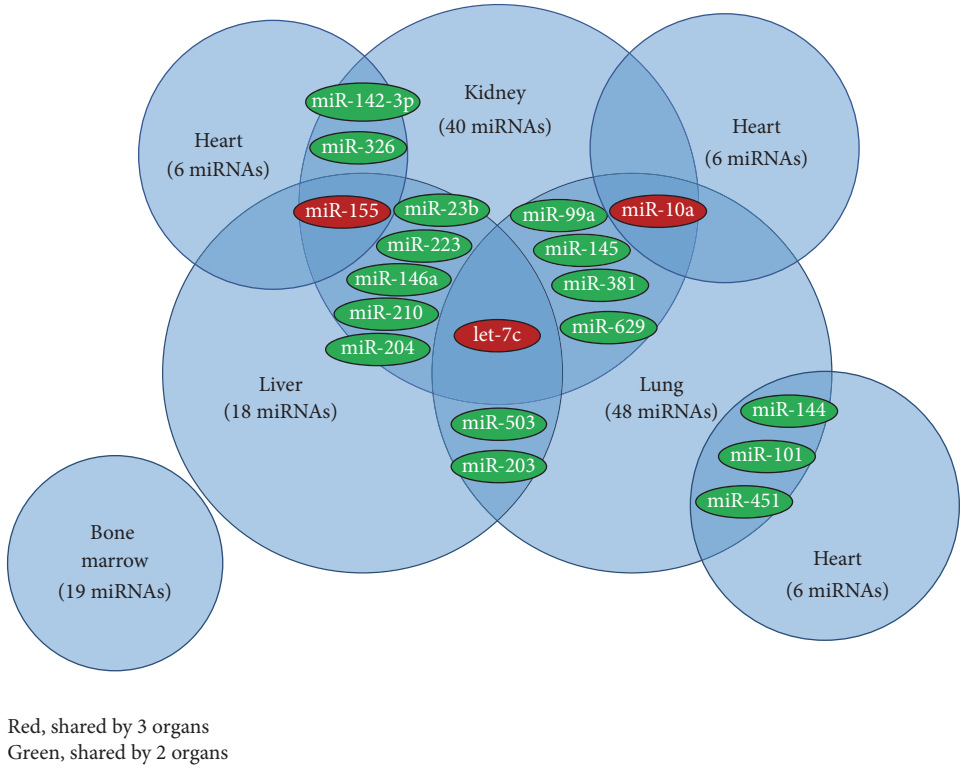

FIGURE 2: Comparison of overlapping miRs in the different studies. Color-labeled miRs indicate a miRNA shared by 2 (green) and 3 (red) organs, respectively. The number in parentheses represents nonoverlapping miRNAs detected for the corresponding organ.

may perhaps provide a new marker for early diagnosis and faster intervention during an acute liver rejection [16]. The significance of miRNA in liver transplant rejection has also been supported by animal studies conducted by Morita et al. They investigated the miRNAs involved in acute rejection of liver allografts in mice and found that miR-146a, 15b, 223, 23a, 27a, 34a, and 451 were significantly increased in the grafts while miR-101a, 101b, and 148a decreased in their expression levels [53]. Several studies have quantified circulating miRNAs as potential biomarkers for hepatitis B (HBV) and hepatitis C (HCV) infections and identified four that are differentially expressed between HBV and control groups (let-7c, miR-23b, miR-122, and miR-150) [19, 54]. HCV infection studies found a significant increase of miR-21 [55–57], and this finding may be translated into transplant studies as well. In summary, miR-122 in combination with other miRNAs seems to be a good diagnostic marker for rejection.

As a novel diagnostic marker miRNAs in exosomes can be a sensor for injury and inflammation. Bala et al. studied several miRNAs packed in exosomes of liver disease patients and they were able to identify a specific pattern of miRNA reflecting liver injury [58].

Lung. Guo et al. found tissue-specific miR-126 to be a lung marker [59]. Zhang et al. identified miR-126 after lung transplantation when they analyzed peripheral blood mononuclear cells [22]. In mice, it has been reported that miR-126 is highly expressed by plasmacytoid DCs and it regulates the survival and function of these cells. Moreover, this miRNA was shown to regulate production of type 1 Interferon by controlling expression of KDR that encodes VEGF-receptor 2 [60]. It is unclear if this is a contamination of lung cells during the preparation or if this is due to the fact that lung tissue is damaged after lung transplantation. There is a huge variety among the detected miRNA profiles during lung rejection and even studies using the same parameters such as DSA+ and/or BOS+ end up with no significant overlap in the miRNA profiles. Further investigation is needed to define a specific marker for lung injury.

Renal. The greatest number of miRNA and transplant studies is done in renal transplantations. The kidney specific miRNA-146a seems to be a risk factor in development of rejection because as Misra et al. were able to demonstrate, the mutation in miR-146 (SNP) is associated with double the risk for rejection [26]. MiRNA-146a expression is highly elevated in response to inflammatory stimuli such as cytokines. Specifically, a recent study on miRNA expression in human activated CD8+ T-cells showed that when these cells were treated with IL-2 or IL-15, miR-146a was significantly upregulated. Also, they were able to demonstrate that subsets of CD8+ T-cells, including naïve and memory cells, differentially expressed certain miRNAs, with 146a being strongly upregulated in the memory T-cell subset [61, 62]. MiR-10b is another kidney specific miRNA that regulates the expression of BCL2L11. Downregulation of miR-10b directly depressed the expression of BCL2L11. Transfecting miR-10b into human renal

glomerular endothelial cells recapitulated key features of acute allograft rejection, including endothelial cell apoptosis, release of pro/inflammatory cytokines (IL-6, TNF-alpha, IFN-gamma, and CCL2) and chemotaxis of macrophages, whereas transfection of miR-10b mimics had the opposite effects [28]. Members of the miR-10 cluster were also found in other organ transplantation studies. Despite the numerous studies in kidney transplantation, there is no common marker for acute rejection and further investigation to find a marker for clinical use is necessary. Sui et al. investigated mechanisms that are related to rejection by integrating protein, mRNA, miRNA, and lncRNA in biopsies of patients with acute rejection versus controls. They were able to predict five transcription factors that are active and responsible for rejection and these transcription factors correlate with 12 miRNAs and 32 lncRNAs. In a previous study, the same author was able to confirm and validate two additional miRNAs (miR-320 and miR-324) by quantitative polymerase chain reaction [24].

Cardiac. Many studies describe miRNA-142-3p as an expression marker of organ grafts. It is associated with lymphocyte alloimmunity during rejection and organ damage. This miRNA is found as an overlap in the three presented studies of heart transplantation [32, 34, 35]. Van Huyen et al. identified and validated a set of four miRNAs (miR-10a, miR-31, miR-92a, and miR-155) as a specific signature for cardiac rejection. The correlation in the receiver operating curves showed a very strong and significant relationship between these miRNAs and rejection [35]. In different studies, miR-10b has been shown as an inhibitor of NFkB signaling and as a regulator of the proinflammatory markers MCP-1, IL-6, IL-8, IL-1, and VCAM [63]. For miRNA-155, Liod et al. described several inflammatory functions that include its enhanced expression after activation of the T-cell receptor, the repression of the IFN receptor, and the contribution to Ig class switch in B cells. Increased expression of this miRNA molecule is also associated with activation of DCs. MiRNA-155 is believed to possess the ability to modulate the antigen presentation activity of DCs to activate T-cells according to animal studies using a mouse model [64]. In DCs derived from human monocytes, following activation by lipopolysaccharides, the expression of miRNA-155 was upregulated as high as 50-fold. In the same study, the knockout of miR-155 in the activated DCs led to an increase in some cytokine gene expression, suggesting its potential role as a negative regulator of cytokine production [65]. Moreover, miR-155 has been found to be upregulated in graft infiltrating lymphocytes, T-cells in spleen, and circulating lymphocytes during acute cardiac rejection in mice. GSK3β was identified to be a direct target of miR-155, which decreased GSK3β expression and thereby increased proliferation of T-cells [66]. MiRNA-31 mainly regulates the expression of E-selectin and ICAM-1 when it is induced by the TNF pathway. It also regulates the infiltration of immune cells into the tissue. Finally, miR-92 targets integrin α5, S1P1, MKK4, and eNOS and plays a potentially important role in the vascular inflammatory response. Dawi et al. discovered three miRNAs, miR-326, miR-142-3p, and miR-101, which have crucial functions in the regulation and maintenance of self-tolerance. Further investigation of miR-142-3p in combination with an miRNA that shows organ damage could prove to be a good candidate biomarker for ongoing rejection.

More systematical research is needed to determine whether miRNAs can be applied as biomarkers, therapeutic targets, or therapeutic agents for specific organs. For other inflammatory diseases like atherosclerosis, a full network between miRNA, organs, and immune cells are described and can help to understand the function in an ongoing inflammation that can lead to chronic rejection [67]. As additional information, a great overview about organ-specific miRNAs as a potential profile for allo- and xenotransplantation is given in the review from Zhou et al. [68].

2.3. miRNAs as a Tool to Enhance Transplantation. The last study we would like to discuss in this review is a study that was done in hematopoietic stem cell (HSC) transplantation and that shows the positive effect of miRNAs packaged in extracellular vesicles on HSC transplantation. De Luca et al. identified miRNAs and piRNA derived from extracellular vesicles (EVs) secreted by mesenchymal stem cells. The group found 87 miRNAs and five piRNAs differentially expressed and predicted to regulate cell differentiation and apoptosis. Validation of four miRNAs and further experiments showed that the EV treatment of HSCs enhances host engraftment and HSC plasticity and function. These findings create a new perspective on how miRNA and piRNA in EVs can positively influence the outcome of transplantations [37].

In addition to this study several publications are showing a specific regulatory function of exosomes and the proteins and miRNAs transmitted by them. As an example Song et al. were able to show that donor-derived peripheral exosomes have the potential to inhibit immune inflammation in allograft heart transplantation by the specific induction of Treg cells [69]. For microvesicle-delivered miRNAs derived from endothelial progenitor cells, it was demonstrated that they are able to reprogram residential renal cells and protect the kidney from ischemia-reperfusion injury [70]. The review from Monguió-Tortajada et al. gives a great overview of the variety of these regulatory functions, from classical immunosuppression to novel extracellular vesicles [71].

3. Conclusion

MiRNAs are emerging as important regulatory molecules of gene expression. They play a significant role in many physiological and pathological processes and have revolutionized cellular biology in the past decade. Research has focused on different expression profiles in health and disease. In the field of transplantation, several miRNAs have been described and it has been shown that miRs have the potential to be a novel diagnostic marker. Therefore, they represent a group of promising candidates for early detection of organ rejection with the potential to affect clinical decision making. However, further investigation and standardization in the profiling of miRNAs in serum, plasma, and urine samples are needed to find a robust diagnostic marker and to develop insights into pathways responsible for the rejection process as well as novel targets for therapy. A few miRNA mimics (miR-34 in phase I)

and miRNA inhibitors (anti-miR-122 in phase II) are already in their first clinical trials and show promising results in HCV and primary liver cancer treatment [72, 73]. Today we are on the verge of implementing many of these new technologies into the clinical routine to improve diagnosis and treatment of transplant patients and to enhance their quality of life.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Renal Transplant Patients Biopsied for Cause and Tested for C4d, DSA, and IgG Subclasses and C1q: Which Humoral Markers Improve Diagnosis and Outcomes?

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The association between donor specific antibodies (DSA) and renal transplant rejection has been generally established, but there are cases when a DSA is present without rejection. We examined 73 renal transplant recipients biopsied for transplant dysfunction with DSA test results available: 23 patients diffusely positive for C4d (C4d+), 25 patients focally positive for C4d, and 25 patients negative for C4d (C4d−). We performed C1q and IgG subclass testing in our DSA+ and C4d+ patient group. Graft outcomes were determined for the C4d+ group. All 23 C4d+ patients had IgG DSA with an average of 12,500 MFI (cumulative DSA MFI). The C4d− patients had average DSA less than 500 MFI. Among the patients with C4d+ biopsies, 100% had IgG DSA, 70% had C1q+ DSA, and 83% had complement fixing IgG subclass antibodies. Interestingly, IgG4 was seen in 10 of the 23 recipients' sera, but always along with complement fixing IgG1, and we have previously seen excellent function in patients when IgG4 DSA exists alone. Cumulative DSA above 10,000 MFI were associated with C4d deposition and complement fixation. There was no significant correlation between graft loss and C1q positivity, and IgG subclass analysis seemed to be a better correlate for complement fixing antibodies in the C4d+ patient group.

1. Introduction

In *Humoral Theory of Transplantation* [1] Terasaki argued against Sir Peter Medawar's evidence for cellular rejection through thymus directed T-cell immunity that had for decades biased the transplantation community against antibodies as a cause of transplant rejection and loss. Terasaki first proposed a compelling hypothesis that linked antibodies (particularly to human leukocyte antigens (HLA)) with occurrence of transplant rejection. Antibody rejection was particularly associated with complement activation and shown specifically by the deposition of C4d on the kidney peritubular capillaries [2–4].

Interestingly, Terasaki showed in his studies a significant correlation of non-donor specific antibodies, HLA antibodies with poor outcomes [5–7], and later revealed the specific correlation of HLA donor specific antibodies (DSA) resulting in poor outcomes, that is, a more rigorous proof of the antibodies' role in rejection.

During the early days circa 2000, the elution of antibodies from rejected kidneys, biopsies, and C4d deposition results showed that both Sir Peter Medawar and Terasaki were correct. In several publications until the 1990s ([8] histological review) allograft dysfunction was accounted for by acute cellular rejection (ACR), and antibodies had a minor role with the exception of hyperacute rejection [9, 10]. Antibody mediated rejection (AMR) assumed a prominent role in allograft dysfunction and loss with the discovery of the complement protein C4d on the peritubular capillaries [2–4] and the principles described in *Humoral Theory of Transplantation* [1]. In fact, the association of antibodies was clearly shown by histologic and antibody examination of 232 transplant recipients, 67 undergoing acute dysfunction. In this study, 30% of the patients showed AMR only, 45% exhibited AMR plus cell mediated rejection (CMR), 15% CMR only, and only 10% acute tubular necrosis [11]. Clearly this data shows 75% of the patients had AMR. It is notable that antibody class switch from IgM to IgG is under the modulation of T-helper cells. Therefore, one can conclude that the T-cells are indirectly identified with AMR, and, of course, 60% of the group studied also had diagnosed CMR. Since AMR has been shown to be the prevalent component in graft rejection and loss, immunosuppressant drugs for AMR have become one of the most unmet needs for treatment. Graft rejection is currently controlled primarily by increasing T-cell immunosuppression, which one could argue is a good AMR immunosuppressant because of T-helper cell function in antibody formation. Albeit Rituximab, IVIg, Atgam, and Bortezomib seem to have an effect on B-cells and/or antibodies, there is no good plasma cell-targeting immunosuppressant agent.

With the discussion above as background, we have chosen to study antibody mediated rejection in a patient population that had allograft dysfunction with primary focus on C4d positive/DSA positive (C4d+ DSA+) patients. Our patient groups were long term graft survivors and had an average of >7 years after transplant at the time of dysfunction, biopsy, and DSA analysis. We examined 73 transplant recipients biopsied for transplant dysfunction, whereof 23 of these patients were diffusely positive for C4d (C4d+), 25 patients were focally positive for C4d, and 25 patients tested negative for C4d (C4d−). DSA test results for these patients were available within 1–10 days of the biopsy. In order to compare DSA and C4d results, we performed C1q and IgG subclass testing in our DSA+ and C4d+ patient group. Graft outcomes were determined for the C4d+ group. The antibody strength was ascertained by measurement of the mean fluorescence intensity (MFI) in the various tests.

Although there are commercially available kits for identifying C1q-binding HLA antibodies, IgG subclasses of HLA antibodies were measured by using several murine antibody clones recognizing human IgG subclasses. These clones have been tested by several other investigators with variable outcomes and correlations to the different subclasses [12–15]. In our hands, these clones behave differentially depending on the dilution tested and whether they were deployed in a direct or sandwich assay. Cross-reactivity was a major issue for us, whereas in other articles either cross-reactivity of clones was not determined [12, 13] or clones were described to be specific with minimal cross-reactivity [14, 15]. We primarily assessed the IgG subclasses using direct labeling. However, data is presented that suggests indirect "sandwich" assays could give a more specific result.

2. Material and Methods

2.1. Study Patients. Seventy-three renal transplant recipients (Table 1) were biopsied for cause and analyzed by immunofluorescence for C4d deposition in the peritubular capillaries (PTC) from frozen material. Twenty-three patients were diffusely positive (>50% PTC staining), 25 focally positive (20–50% PTC staining), and 25 negative (<20% PTC staining). The patients were chosen sequentially and reviewed retrospectively, with the requirement that a DSA test had been performed within ten days of the biopsy. All data review and study testing were performed under IRB approval.

2.2. C4d Immunostaining. Biopsied renal tissue was placed in Zeus fixative (Zeus Scientific, Branchburg, NJ), sectioned using a cryostat, and then stained as follows in a multistep procedure. Initially, the Zeus-fixed tissue was placed in Zeus fixative wash solution (Zeus Scientific) and sectioned in a cryostat at 4 μm thickness, and the resultant sections were placed on poly-L-lysine-coated slides at room temperature, allowed to dry, and then fixed in cold acetone for 10 minutes. The tissue sections were then washed in PBS times 3 for 10 minutes each. The sections were then stained using a 3-step procedure as follows: (1) mouse anti-human C4d (Quidel Corp., San Diego, CA) was diluted 1 : 200, for 30 minutes and then washed in PBS; (2) FITC-conjugated rabbit anti-mouse immunoglobulin (Dako Corp, Carpinteria, CA) diluted 1 : 30, for 20 minutes, with interval wash in PBS; and (3) finally FITC-conjugated swine anti-rabbit immunoglobulin (Dako Corp), diluted 1 : 30, for 20 minutes, with final wash in PBS. Slides were then mounted with a cover slip in glycerol/Optimax solution and reviewed in an Olympus BX Fluorescence Microscope.

2.3. HLA Typing. HLA Class I & II antigens were detected using the standard monoclonal trays using the microcytotoxicity method (One Lambda, Inc., Canoga Park, CA). In the case of ambiguous results or uncertainty, additional testing using PCR-SSP was performed (One Lambda, Inc.) and (QIAGEN Inc., Valencia, CA).

2.4. HLA Single Antigen Bead (SAB) Specificity Analysis. All patients were tested for the presence of antibodies of IgG isotype using SAB Luminex technology (LABScreen Single Antigen, One Lambda, Inc.). The tests were performed according to the vendor's instructions using DTT-treated

Table 1: Patient demographics.

	C4d diffusely positive ($N = 23$)	C4d focally positive ($N = 25$)	C4d negative ($N = 25$)	P value
Age				
At biopsy [yr.]	44.5 ± 12.7	45.7 ± 9.9	49.5 ± 13.7	0.343
At transplantation [yr.]	39.0 ± 10.9	37.1 ± 12.1	42.1 ± 13.3	0.356
Female sex [%]	7 (30.4)	8 (32.0)	8 (32.0)	0.938
Race or ethnic group [%]				0.104
White Hispanic	15 (65.2)	11 (44.0)	16 (64.0)	
Asian	2 (8.7)	4 (16.0)	2 (8.0)	
Black non-Hispanic	4 (17.4)	1 (4.0)	1 (4.0)	
Mixed	0 (0.0)	4 (16.0)	0 (0.0)	
White non-Hispanic	3 (13.0)	2 (8.0)	5 (20.0)	
White unknown	0 (0.0)	1 (4.0)	0 (0.0)	
Native American	0 (0.0)	1 (4.0)	0 (0.0)	
Previous Transplantation [%]	7 (30.4)	2 (8.0)	4 (16.0)	0.168
Simultaneous kidney pancreas [%]	1 (4.3)	3 (12.0)	1 (4.0)	0.423
Deceased donor [%]	15 (65.2)	20 (80.0)	19 (76.0)	0.211
Cause of ESRD [%]				0.868
Diabetes	4 (17.4)	4 (16.0)	7 (28.0)	
Hypertension	8 (34.8)	7 (28.0)	7 (28.0)	
Miscellaneous	9 (39.1)	10 (40.0)	9 (36.0)	
Unknown	3 (13.0)	3 (12.0)	1 (4.0)	
Allograft year at Bx [yr.]	5.4 ± 5.0	8.7 ± 6.2	7.4 ± 5.6	0.138
Allograft loss [%]	9 (39.1)	16 (64.0)	7 (28.0)	0.023
Graft year at loss [yr.]	7.8 ± 3.1	11.1 ± 7.0	10.9 ± 6.0	0.61

sera. Antibody specificity was analyzed manually using baseline mean fluorescent intensity (MFI) values. Positive MFI thresholds were defined on the basis of >500 MFI when a DSA was noted for a donor mismatched HLA antigen.

2.5. IgG Subclass Determination. SAB Luminex technology was used to determine the specificity of HLA Class I & II IgG antibody subclasses. The "direct" subclass assay was performed essentially in the manner of the standard SAB assay, with the replacement of the PE-labeled polyclonal goat anti-human IgG antibody with a PE-labeled monoclonal murine anti-human IgG subclass-specific antibody. DTT-treated graft recipient serum was reacted with SAB for 30 min, washed four times with 1x wash buffer, incubated with PE-labeled subclass-specific monoclonal antibody for 30 min, and washed twice before acquisition. The "sandwich assay" was performed with an unlabeled subclass-specific antibody and a PE-labeled secondary antibody. DTT-treated graft recipient serum was reacted with SAB for 30 min, washed four times with 1x wash buffer, incubated with murine antihuman IgG subclass-specific monoclonal antibodies for 30 min, washed four times, incubated with a polyclonal anti-murine IgG-PE conjugate for 30 min, and washed twice before acquisition. The antibodies used for the direct assay were purchased from Southern Biotech (Birmingham, AL) and are detailed in Table 2. The antibodies used for the sandwich assay were unlabeled murine monoclonal antibodies specific for human G1 (Clone HP6001, Millipore, Billerica, MA), G2 (Clone HP6002, Millipore), G3 (Clone HP6047, Alpha Diagnostic Intl. Inc., San Antonio, TX), and G4 (Clone HP6023, Millipore) subclasses and a goat polyclonal F(ab)2 anti-murine IgG-PE conjugate (R & D Systems, Minneapolis, MN). Positive control beads were produced by Acuimmune (Chatsworth, CA) using IgG1–IgG4 and IgM purified from myeloma plasma (Sigma-Aldrich, Saint Louis, MO) and coupled to Microplex Microspheres (Luminex, Austin, TX). Antibody specificity was analyzed manually using baseline mean fluorescent intensity (MFI) values. Positive MFI thresholds were defined on the basis of >500 MFI when a DSA was noted for a donor mismatched HLA antigen.

2.6. C1q SAB Specificity Analysis. C1q testing was performed using the commercially available kit (C1qScreen, One Lambda, Inc.) according to the manufacturer's instructions. Antibody specificity was analyzed manually using baseline mean fluorescent intensity (MFI) values. Positive MFI thresholds were defined on the basis of >500 MFI when a DSA was noted for a donor mismatched HLA antigen.

2.7. Statistical Analysis. Statistical analysis was performed using Fishers T-test and/or ANOVA. Allograft survival was analyzed using Kaplan Meier curves and the log-rank test.

3. Results and Discussion

IgG subclass analysis and how it is determined is a critical issue with regard to the interpretation of complement fixing

Table 2: PE conjugated secondary antibodies for IgG subclass detection.

Secondary Ab	MFI [relative%]				
	IgG1 bead	IgG2 bead	IgG3 bead	IgG4 bead	IgM bead
IgG1-PE 1 μg/mL HP6001	9989 (100)	341 (3)	27 (0)	3733 (37)	1814 (18)
IgG2-PE 5 μg/mL HP6014	31 (0)	11621 (100)	576 (5)	117 (1)	556 (5)
IgG3-PE 5 μg/mL HP6050	19 (0)	21 (0)	10666 (100)	307 (3)	215 (2)
IgG4-PE 1 μg/mL HP6023	5 (0)	113 (0)	12 (0)	28872 (100)	50 (0)

and noncomplement fixing antibodies. We will endeavor herein to explicate the best way to determine the IgG subclasses of HLA antibodies. In the literature [12–15] comparative results for the IgG1 and IgG2 subclasses as correlate to outcomes should be viewed in the context of cross-reactivity. Therefore, in our results we present methodology for IgG subclass analysis.

Our data represent a unique group of patients with an average of greater than seven years of renal allograft function at the time of biopsy and DSA determination. Therefore it should be interpreted in this context.

The demographics of the studied patients are depicted in Table 1. There is a significant correlation to graft loss of the C4d+ and focally positive transplant recipients compared to the C4d− patients when analyzed by ANOVA (Table 1), but comparison of allograft survival among the groups did not reach significance using Kaplan Meier curves and the log-rank test (Figure 1). As noted, the average time of biopsy was greater than seven years after transplant, and some of these patients had incidences of rejection prior to inclusion in this study.

The murine antibody clones that were utilized in the subclass experiments at concentrations selected to minimize cross-reactivity are shown in Figure 2 and Table 2. IgG1 had significant binding to the IgG4 control bead at the concentration at 1 μg. These murine antibody clones were chosen from the available clones binding to the subclasses and represented the least cross-reactivity seen. The murine monoclonal subclass clones were the same as used in most of the subclass papers (Table 3) with the notable exception of IgG4 HP6023, which showed a strong MFI and was monospecific for IgG4. The concentration of IgG1 used by most of the investigators was different than used by us, and it is worth observing that individual lots of HP6001 can be quite different in cross-reactivity. Hence, it is problematic when switching lots, and IgG1 could give false reading for IgG4. In fact, Figure 3 shows the hypervariability of the murine monoclonal antibodies to IgG subclasses with three different sera on the control beads. As examples, Serum 1 shows results similar to that found in the PBS. Sera 2 and 3 show the extreme variation one finds with background in different sera. IgG3 and IgG4 showed the least serum-dependent differences. We point to these results as a caution in the interpretation of not only our results but those given by others, particularly for IgG1 and IgG2 and their propensity for

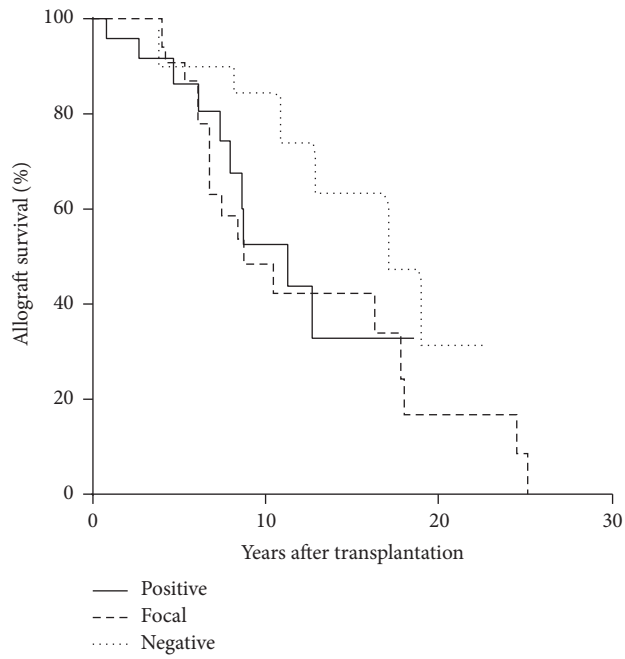


Figure 1: Comparison of renal allograft survival among study patients. The three cohorts of patients are separated according to the results of their with renal biopsies: diffusely positive C4d immunostaining (Positive), focally positive C4d immunostaining (Focal), and negative C4d immunostaining (Negative). The log-rank test result for the Kaplan Meier curves is 0.163.

cross-reactive results. We interpreted different serum binding results (Table 2) as nonspecific binding causing spurious results in the direct binding assay. However, in preliminary results when we used the indirect or "sandwich" assay at least some of the cross-reactivity could be eliminated (Table 4). We attribute the better results with the sandwich assay to the increased washing and secondary antibody binding, which we postulate caused elimination of lower affinity nonspecific adsorbed serum proteins causing cross-reactivity. Although our results below are based on the direct assay, we propose that the indirect assay provides better discrimination of IgG subclasses.

Table 5 shows a comparison of the cumulative DSA MFI in the patients, stratified by C4d status. In the C4d+ patients, all had DSA, and the average cumulative MFI was 12,353,

Table 3: Reported IgG subclass antibodies used for DSA detection.

		IgG1	IgG2	IgG3	IgG4	Control
Cicciarelli et al. (2013) [16]	Clones	HP6001	HP6014	HP6050	HP6023	Control bead
	Concentration	1 μg/mL	5 μg/mL	5 μg/mL	1 μg/mL	
	MFI					
Lefaucheur et al. (2016) [17]	Clones	HP6001	31-7-4	HP6050	HP6025	Negative sera
	Concentration	5 μg/mL	5 μg/mL	20 μg/mL	20 μg/mL	
	MFI	Mean Pan-IgG 1784				
Hönger et al. (2011) [14]	Clones	HP6001	31-7-4	HP6050	HP6025	Negative sera
	Concentration	1.3 μg/mL	1.3 μg/mL	10.6 μg/mL	0.68 μg/mL	
	MFI	Median, 1524; range, 1083–3584, ratio above the cutoff (i.e., ratio = MFI IgG subclass divided by MFI cutoff)				
Kaneku et al. (2012) [15]	Clones	4E3	HP6002	HP6050	HP6025	Control bead
	Concentration	?	?	?	?	
	MFI	Median IgG subclass MFI in chronic rejection patients with post-OLT DSA (71%) was 5596				
Khovanova et al. (2015) [13] Lowe et al. (2013) [18]	Clones	4E3	31-7-4	HP6050	HP6025	Control bead
	Concentration	250 μg/mL	250 μg/mL	250 μg/mL	250 μg/mL	
	MFI	Median MFI of IgG ~1000				
Arnold et al. (2014) [12]	Clones	HP6001	31-7-4	HP6050	HP6025	Isotype control
	Concentration	?	?	?	?	
	MFI	?	?	?	?	

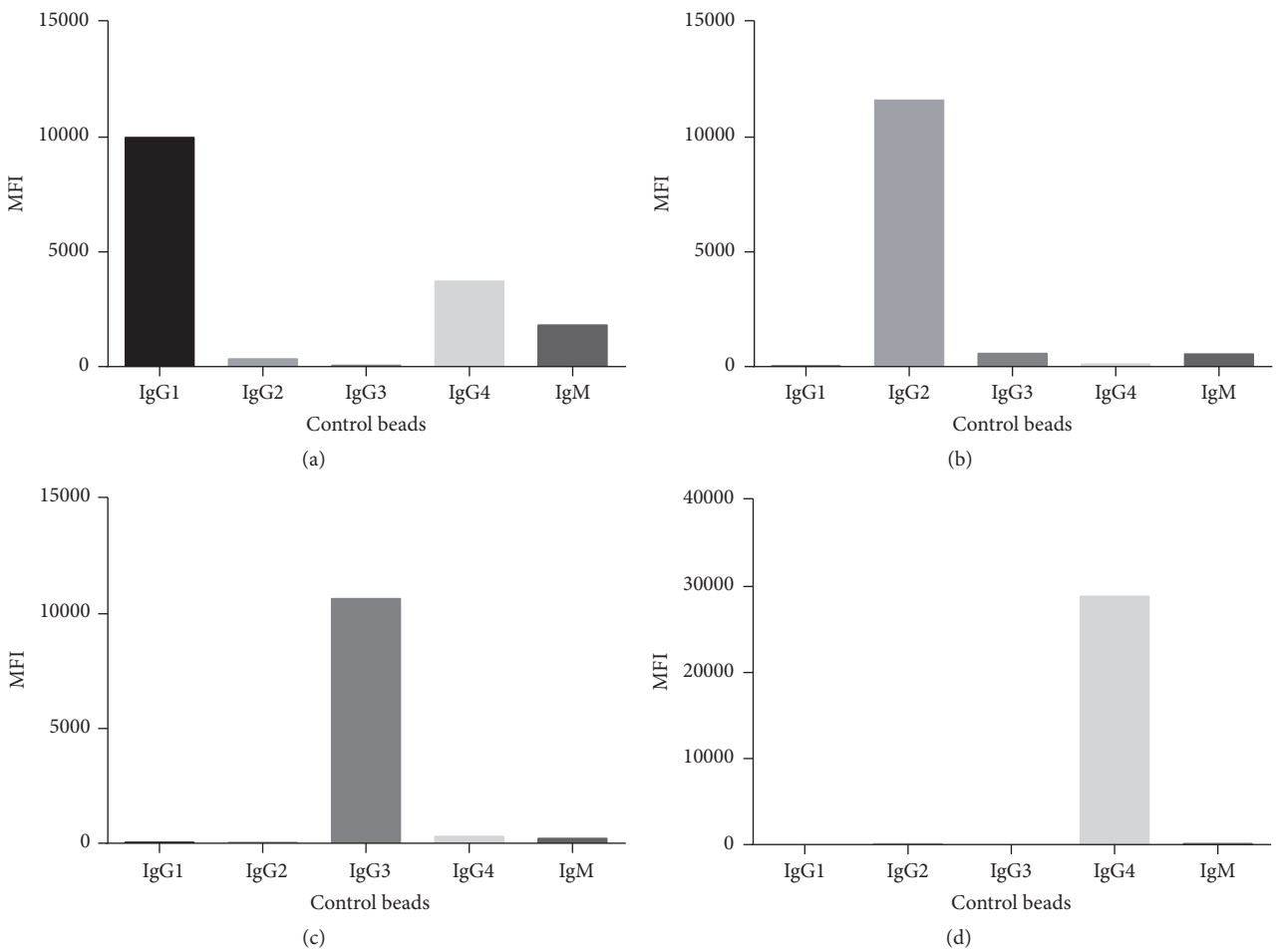
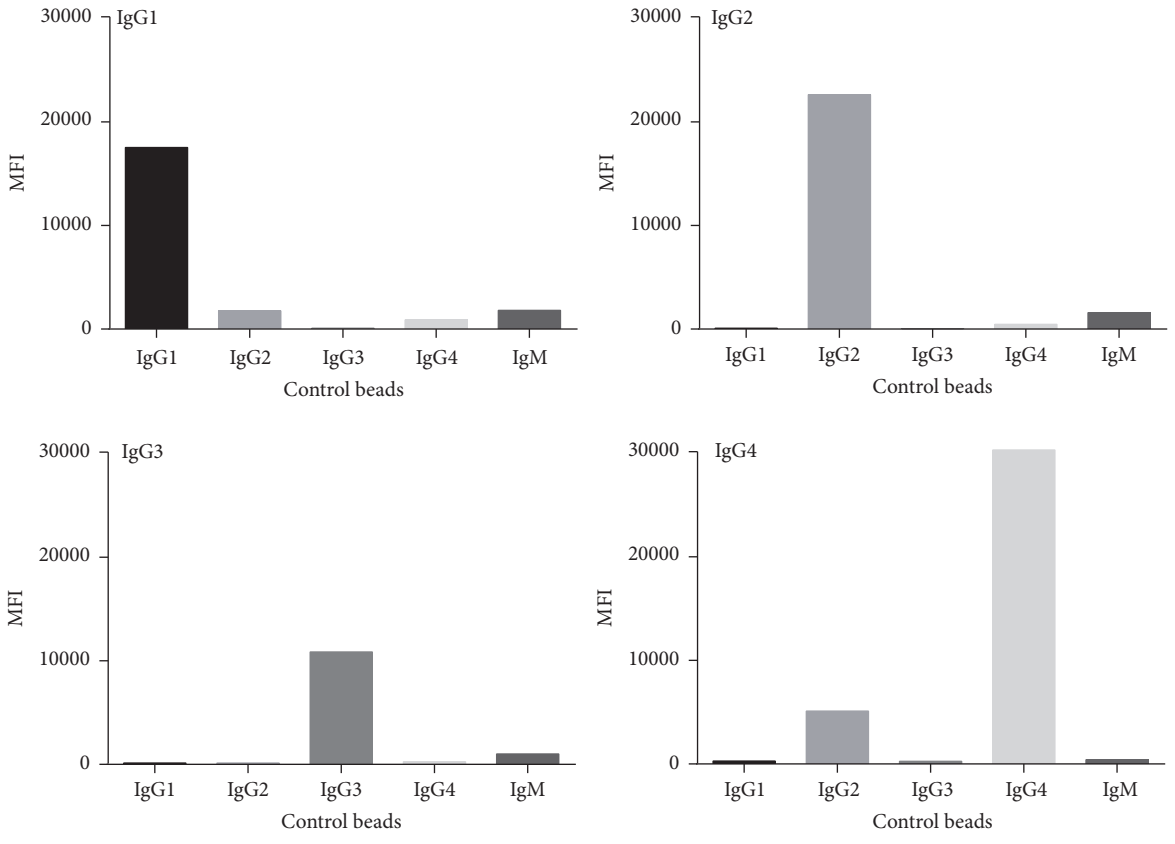
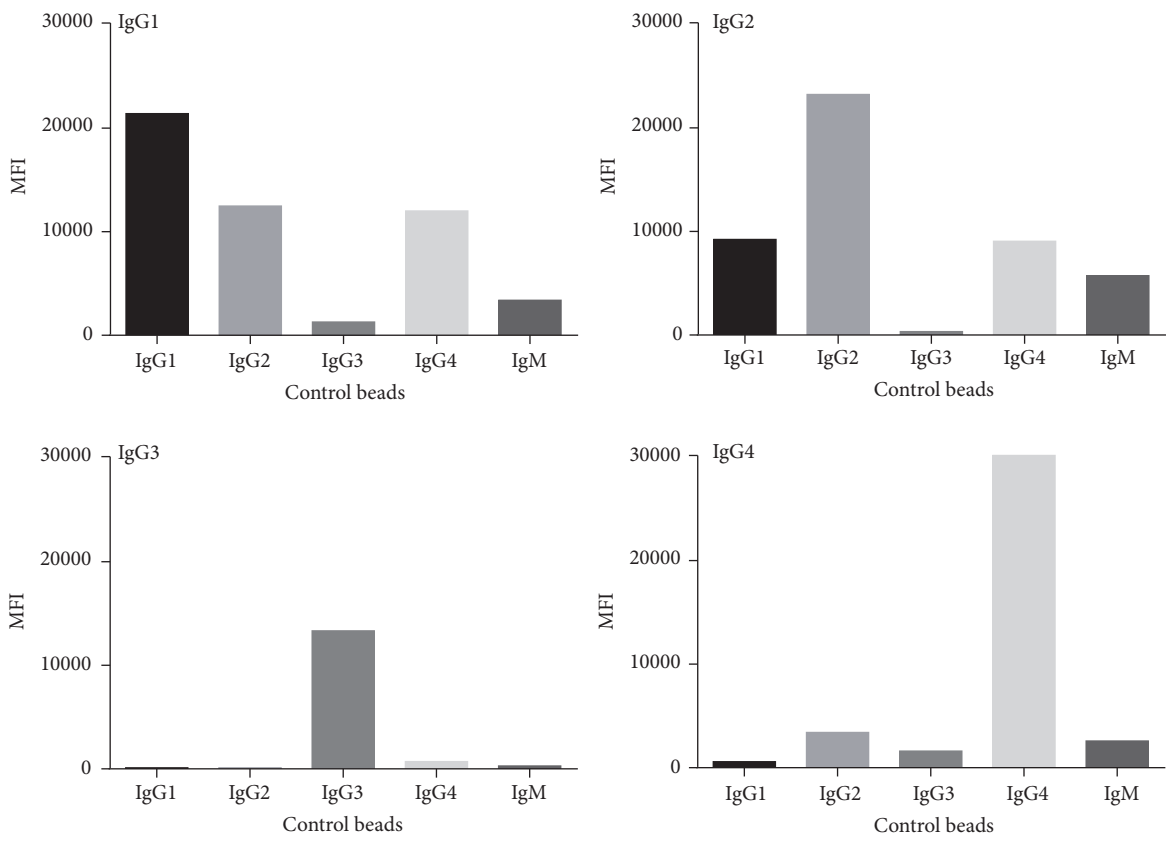


Figure 2: PE-conjugated secondary antibodies for IgG subclass detection. The control beads were incubated with negative control serum, washed, and then detected with subclass-specific antibodies ((a): IgG1-PE; (b): IgG2-PE; (c): IgG3-PE; (d): IgG4-PE).

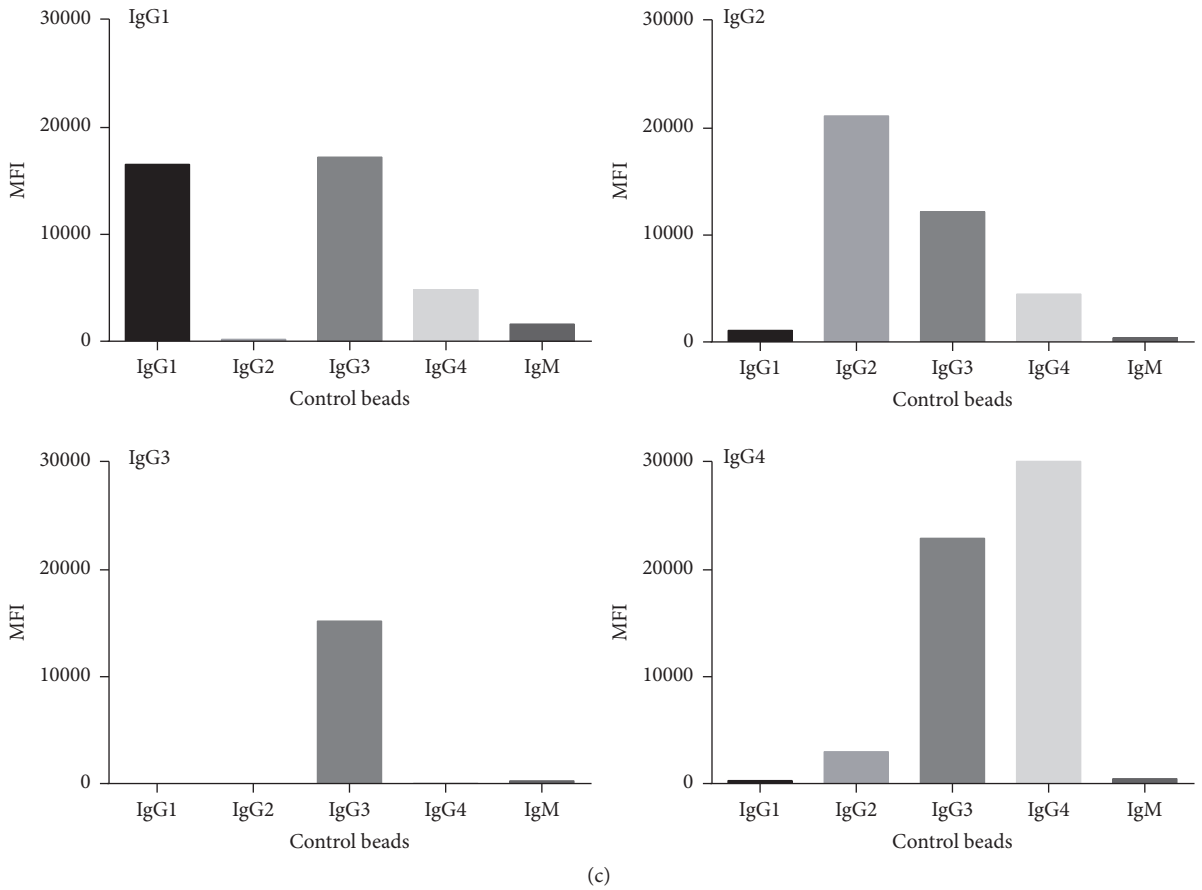


(a)



(b)

Figure 3: Continued.



(c)

Figure 3: Examples of serum-dependent cross-reactivity of IgG subclass detection on control beads. The control beads were incubated with different patient sera, washed, and then detected with subclass-specific secondary antibodies ((a): patient serum 1; (b): patient serum 2; (c): patient serum 3).

Table 4: Comparison of direct and sandwich IgG subclass assays.

		IgG1 beads	IgG2 beads	IgG3 beads	IgG4 beads	IgM beads
Sandwich assay	IgG1 HP6001	100%	14%	41%	15%	2%
	IgG2 HP6002	8%	100%	27%	25%	2%
	IgG3 HP6047	1%	0%	100%	4%	0%
	IgG4 HP6023	1%	1%	5%	100%	0%
Direct assay	IgG1 4E3	100%	228%	260%	5%	1%
	IgG1 HP6001	100%	2%	88%	3%	1%
	IgG2 HP6002	5%	100%	105%	126%	6%
	IgG2 31-7-4	14%	100%	245%	185%	2%
	IgG3 HP6050	0%	0%	100%	1%	0%
	IgG4 HP6023	0%	0%	2%	100%	0%

Table 5: Reactivity of monoclonal IgG subclass detection antibodies against control microbeads.

	C4d+ mean MFI	C4d focally positive mean MFI	C4d− mean MFI
Total	12,353	4,771	963
Class I	5,487	443	442
Class II	7,373	4,317	520

MFI cutoff for DSA positivity set at 500.

as compared to the focally positive group average, which was 4,771 MFI. The predominant HLA for C4d+ patients was HLA Class II and also for the focally positive patients. However, in the focally positive group, nearly all of the DSA specificities were against HLA Class II and almost none against HLA Class I. The C4d− group had very low average DSA. One could argue that C4d− groups were potentially subclinical rejection, but it was clear from our results that in order to see C4d deposition on the PTC an average DSA of greater than 10,000 MFI was needed.

A more in depth analysis of DSA and C4d groups is presented in Table 6. All of the C4d positive groups were significantly positive when compared to the C4d−, and C4d+ DSA was significant when compared to the DSA in the focally positive group. This indicated that C4d reactivity was a function of the DSA at a certain threshold level. DSA used alone would predict a C4d+ result with a 95% confidence limit range between 6,436 and 16,343 MFI. C4d+ compared to focally positive C4d was significantly different, indicating that the DSA associated with C4d+ biopsies had significantly higher MFI. In addition, when we compared HLA Class I to HLA Class II in the focally positive group there was a significant difference, since the average HLA Class I DSA associated with the focally positive recipients were below 500 MFI. However, when HLA Class II DSA were compared between C4d+ and focally positive groups, there was no significant difference. Therefore, we saw that focally positive C4d and C4d+ were both associated with HLA Class II DSA but only C4d+ had a significant correlation to DSA in HLA Class I. Even though the focally positive C4d group tended had positive HLA Class II, it was only just significant compared to the C4d− with a broad 95% confidence limit range (351–7,716 MFI).

Table 7 shows standard DSA specificities, IgG subclass, and C1q all listed with MFI for the C4d+ patients. These results reiterate the specific antibodies that we saw in the analysis and the specific correlation of the results where there appears to be a threshold DSA for the C1q positive reaction. Also, one should note that the IgG subclass analysis seems to give more complete information regarding the complement fixing antibodies and IgG class switching. All of the patients had standard DSA, whereas seven patients had no C1q DSA, and this observation carries over into the total numbers of DSA for both HLA Class I and II. That is, fifteen patients had standard DSA to both Class I and Class II and only one class of C1q DSA. The threshold of DSA before seeing the C1q+ reactivity was ~4 DSA at 12,485 MFI and the C1q+ averaged 16,729 MFI with an average of only a single DSA (Table 8).

When we looked at the C4d+ patients outcomes there was no significant correlation with C1q or IgG subclass results (Table 9). There seemed to be a trend toward increased graft loss and thus possibly severity of rejection in the patients with C1q+ DSA. The profile of rejection and loss was in general a mixed, nonsignificant correlation. Looking at the pattern of C1q and IgG subclass DSA among the C4d+ patients (Table 10), we saw that all patients with complement fixing IgG1 subclass had DSA and eleven had IgG4 subclass. Also, 19 of 23 patients who were C4d+/DSA+ had complement fixing subclass DSA, as compared to 16 of 23 with C1q+ DSA. In this analysis, IgG1 subclass provided the best indication of the C4d+ reactivity, but was not a significant predictor of graft loss (Table 9). IgG4 subclass seemed to have little to do with the outcome and the complement fixation; that is, there was no blocking of the C4d+ complement fixation. An interesting result was the absence of IgG1 at the time of biopsy in four patients with significant dysfunction (average sCr of 4.4 mg/dL). Although only one of the four lost the transplant, it would be expected that there would be complement fixing antibodies. However, as noted above, the IgG1 assay could give quite variable results.

The absence of IgG3 subclass for the most part was likely a function of our longer term transplant patients that were analyzed. Also, patients had a large variation with regard to the cross-reactive of the IgG subclasses owing to the unknown variations intrinsically associated with the sera (Figure 2).

4. Conclusions

Many articles have been written regarding de novo DSA early after transplant [19–24]. The incidence of these de novo DSA ranged from 10 to 30% and the studies included 1–5 year follow-ups. In our study, a group of 73 patients were biopsied for cause with an average of greater than seven years after transplant, and the incidence of DSA was 41%. It is reasonable to suspect that patients biopsied for cause would have a higher incidence of DSA than patients without cause for biopsy. One should note that this is much less than has been reported by investigators using histology C4d criteria [11], but these patients likely exhibited accelerated acute rejection. However, 41% of patients with HLA DSA biopsied for cause can stand alone as a unique observation for our groups of patients biopsied after an average of seven years after transplant. There is a paucity in the literature of correlates between C4d+ and DSA MFI ranges but a few references show that immediately after transplant if DSA increase sharply or are greater than 9,500 MFI outcomes are significantly worse [20, 22]. A recent article reported on the diagnostic contribution of various assays to the diagnosis of silent AMR in renal transplant recipients [25]. This prospective study included patients who were determined to be DSA positive by protocol screening and underwent renal allograft biopsy, somewhat distinct from our patients who exhibited allograft dysfunction and were biopsied and tested for cause. The assays analyzed included the standard SAB assay, the C1q SAB assay, and a C3d SAB assay. The authors concluded that higher MFI DSA were associated with higher risk for rejection or allograft loss, but

Table 6: In depth analysis of DSA and C4d+, C4d focally positive, and C4d− patient groups.

	P value	95% confidence interval MFI
C4d+ DSA MFI versus C4d− DSA MFI	<0.0001	6,436–16,343
Class I C4d+ DSA MFI versus Class I C4d− DSA MFI	<0.0005	2,473–7,616
Class II C4d+ DSA MFI versus Class II C4d− DSA MFI	<0.0011	3,053–10,652
C4d+ DSA MFI versus C4d focally positive DSA MFI	<0.012	1,718–13,443
Class I C4d+ DSA MFI versus Class I C4d focally positive DSA MFI	<0.0005	2,455–7,634
Class II C4d+ DSA MFI versus Class II C4d focally positive DSA MFI	<0.2	NS
Class I C4d focally positive DSA MFI versus Class I C4d− DSA MFI	<0.4	NS
Class II C4d focally positive DSA MFI versus Class II C4d− DSA MFI	<0.0431	351–7,716

that the addition of complement fixing assays did not provide an enhanced diagnostic benefit. These results are concordant with our own findings, despite the differences in patient populations and utilized assays.

Our data show that the DSA in C4d+ patients was greater than 12,000 MFI, composed of both HLA Class I and II, and was significantly associated with C4d+ AMR. Also, focally positive patients had predominantly HLA Class II with an average of HLA Class I less than the 500 MFI negative cutoff (a consequence of the number of patients with no Class I DSA at all). Our observations showed that when C4d was focally positive there was dysfunction significantly associated only with HLA Class II. Others have shown that mismatching HLA Class II resulted in HLA-DQ DSA antibodies [26]. Certainly there may be occurrences where the DSA is lower but significant ACR glomerulopathy is present. However, for our C4d− group, the MFI range averaged just slightly above our negative cutoff of 500 MFI. Part and parcel of the C4d+ deposition on the PTC is the DSA detected in the serum but when one looks for antibodies on the PTC they are not present [27]. This may be a capping and stripping occurrence [28]. However, the important point may be that serum DSA is more stable and indicative of glomerulopathy and therefore suggests more attention be paid to DSA occurrence versus C4d. The question remains as to the quantity and the quality of the DSA present. In our study we have endeavored to quantify the DSA and also measure the complement activity of the antibodies. The measure of the complement inhibitory factors (CD55, CD45, and CD35) has yet to quantified but CD59 has been found on the PTC, thus inhibiting complement activity [29]. Again, this suggests that not just C4d but the quantity and quality of DSA are in general overarching factors in glomerulopathy.

Complement has a multitude of functions which include death of target cell or organism, proinflammatory effects, histamine release, phagocytosis, and chemotaxis [30, 31]. Therefore, looking for the activation of the complement pathway, assays have been developed that show activation of C1q as the initiation target which occurs when an antibody of the complement fixing class (IgM, IgG 1, 3) binds to the target HLA epitopes. The C1q assay should give a good concordance for with presence of damaging antibodies. Several authors have shown the effect C1q binding assay as a positive predictor of graft loss in de novo antibody patients, in both renal and heart transplants [32, 33]. However, there is a DSA threshold below which the C1q assay is negative, suggesting the quantity of the DSA antibody gives rise to the C1q+ result and the DSA MFI has the best predictive value for the C1q test. In our experience, C1q was negative in 30% of the DSA and C4d positive patients. Conversely, when complement fixing antibody subclasses were measured directly only 18% were negative. Furthermore, when C1q was positive, DSA was greater than 12,000 MFI and always appeared in the presence of C4d+ DSA+ plus complement fixing subclass antibodies. Among our patients who tested negative for C1q DSA, there were three patients who had complement fixing IgG subclass antibodies in the presence of DSA and C4d+ results.

IgG subclasses as mentioned above were a better correlate for complement fixation compared to C1q in the C4d+ DSA patients. We feel that IgG subclasses give a more complete picture of the complement fixing antibodies present. Indeed, strong complement fixing IgG3 along with DSA had the highest risk for graft loss in liver transplant recipients [34]. C1q+ correlated with high risk of graft loss but seemed to add little to the risk associated with IgG3+, DSA+. In our current study, there was a paucity of IgG3+ recipients, possibly owing to the average posttransplant biopsy times. That is, IGg3 is the first complement fixing antibody in class switching from IgM and may not be present except in acute, initial AMR response which is in contrast with our greater than seven years posttransplant, biopsied patient population. Interestingly, in the 82% of patients IgG1+ C4d+, eleven patients had antibodies of the IgG4 subclass, which is one of the last antibodies to class switch. IgG4 has been often described as a blocking antibody, because it does not fix complement. IgG4 has been found in some patients to be present solely and with salutary effect for transplantation [16]. However, in this study we could not see any association between the presence of IgG4 DSA and lower sCr or better graft survival. One study suggests that IgG4 has a detrimental effect on graft outcomes [35]. We suggest because class switching is governed by time and T regulatory cells [36], when IgG4 occurs in these long term biopsied patients it may be associated with accommodation and T regulatory cell but concomitant presence of IgG1 negates these beneficial effects. In a recent case [37], we found a patient C4d− DSA+ C1q+ IgG1+ and IgG4+ (22,000 MFI) and a sCr < 0.9 mg/dL. The patient was treated for rejection with IVIg, but this therapy was discontinued because of infusion reactions. In this case, it is possible that the IgG4 antibody was blocking

Table 7: Pan-DSA specificities, IgG subclass, and C1q in C4d+ patients.

Patient no.	PAN-IgG				IgG1				IgG2				IgG3				IgG4				C1q	
	Class I		Class II		Class I		Class II		Class I		Class II		Class I		Class II		Class I		Class II		Class I	Class II
1	B7	1608																			0	0
2	Cw7 B44 A68	16888 2233 1040			Cw7	14869			Cw7	5271							Cw7	752			20236	0
3	Cw7 A30 B8	9642 6939 5643	DQ6	4396	Cw7 A30	4509 1254	DQ6	1190													11331	0
4	B7 Cw15 Cw10 A31	16700 6627 5268 1988			B7 Cw10	4733 1420															1604	0
5	Cw1 Cw10 B55	1349 1342 1035	DP2 DR4 DQ9 DP5 DQ4	11354 2718 2155 1603 1366			DP2	3714											DP2 DR4	2462 5517	0	0
6	A11	1371	DQ5 DR51 DR16	4682 2765 1351			DQ5	3376													0	0
7	A2 Cw4	999 1000	DQ2 DR52	3350 926	Cw4	1071	DQ2	2884							DQ2	714					0	5892
8	B35 B61	4059 843	DQ4 DR53	6038 673			DQ4	2747			DQ4	6612							DQ4	5397	0	16072
9	A2	2647	DR52	1099	A2	1099															0	0
10	Cw4 A33 A68 B53	3039 1028 924 879			Cw4	4541															0	0
11	A31 B65 B64 B51	5220 4383 2680 2458	DR7 DQ2	11314 5223	A31 B65 B64	2195 2129 867	DQ2	701			DQ2	1012									20551	0
12	A68 B49 A1	13440 6052 2023	DQ8 DQ9 DQ7 DR4	14106 13986 11822 1800	A68 B49	6021 1238	DQ8 DQ9 DQ7	4727 5506 4283			DQ9 DQ7	982 1978					A68	779	DQ8 DQ9 DQ7	17214 18600 17779	509	50289
13	A31 A30	13834 6813	DR8 DQ4	3593 3289	A31 A30	4506 2171	DQ4 DR8	2386 1270													24668	0
14			DQ7	12435			DQ7	4488			DQ7	4352							DQ7	7343	0	28347
15	A24 Cw4	4141 1881	DQ7 DR52	3611 1271	A24 Cw4	9132 8717	DQ7	3503									A24 Cw4	7291 4366	DQ7	4555	0	7411
16	A31	11549			A31	3902							A31	2242			A31	7998			25084	0
17	A32	1193	DR1 DR51	1732 1127																	0	0

Table 7: Continued.

Patient no.	PAN-IgG				IgG1				IgG2				IgG3				IgG4				C1q	
	Class I		Class II		Class I		Class II		Class I		Class II		Class I		Class II		Class I		Class II		Class I	Class II
18																					0	28441
19	A2 A29 Cw7	4105 1442 1078	DQ7	8907	A2 A29 Cw7	2112 1585 717	DQ7	4052	A2	4099	DQ7	1702	A2	4181	DQ7	843	A2	19117	DQ7	1782	0	26224
20			DQ5 DQ4 DR16	8804 5672 925			DQ5 DQ4	4457 2743											DQ5	2988	0	28688
21			DQ5 DR1 DR4	9454 1883 1712																	0	1836
23	A31	3505																			0	0
24	B62	894	DR53 DR7 DR4	8265 2371 1771			DR53	954											DR4	1214	0	16627

Table 8: DSA, IgG subclasses, and C1q detection in C4d+ patients.

Assay	Type of DSA detected [# of patients]			
	No DSA	Class I only	Class II only	Class I+II
Standard	0	5	3	15
C1q	7	6	9	1
Assay	Mean total DSA MFI			Average # of DSA
		Class II only	Class I+II	
Standard	7,046	8,320	12,485	4
C1q	14,460	17,864	16,729	1

Table 9: Analysis of C1q, IgG subclass, and Cd4d+ DSA.

	C1q− ($N = 7$)	C1q+ ($N = 16$)	IgG1− ($N = 4$)	IgG1+ ($N = 19$)	IgG1+ IgG4− ($N = 8$)	IgG1+ IgG4+ ($N = 11$)
Mean sCr (at biopsy)	3.5 mg/dL	2.9 mg/dL	4.4 mg/dL	2.8 mg/dL	2.7 mg/dL	2.9 mg/dL
Mean sCr (most recent)	2.2 mg/dL	1.9 mg/dL	3.4 mg/dL	1.9 mg/dL	1.8 mg/dL	1.9 mg/dL
Graft loss ($N = 5$)	1 (14%)	4 (25%)	1 (25%)	4 (21%)	1 (13%)	3 (27%)

Values did not reach statistical significance.

Table 10: DSA detected by C1q and IgG subclass.

Patient	C1q	IgG1	IgG2	IgG3	IgG4
1	+	+	+		+
2		+			
3		+			
4		+			+
5	+	+			+
6	+	+			
7	+	+		+	
8	+	+	+		+
9	+	+			
10		+			
11	+	+	+		
12	+	+			
13	+	+	+		+
14	+	+			+
15	+	+		+	+
16					
17	+	+	+		+
18	+	+	+	+	+
19	+	+			+
20	+				
21	+	+	+		+
22					
23	+	+			+

the complement fixation in vivo. Indeed we could show CDC blocking by this patient's serum in vitro in preliminary experiments using human serum as a complement source (unpublished data). Thus, if an IgG4 antibody is present in high enough concentration we expect that the cross-linking of two immunoglobulin molecules is blocked and complement is not activated. Such a mechanism may not occur in the C1q bead assay because of the quantity and proximity of the HLA bound to the beads.

The presence of IgG4 in transplant recipients may be most analogous to the phenomenon observed in subcutaneous allergic desensitization. Allergic desensitization occurs after weekly subcutaneous allergen injection and successful results show IgG4 specific allergen antibodies. The class switching is governed by T regulatory cells secreting IL-10 and TGF-β predominantly [36]. Thus the allograft may in many cases, when DSA is present, eventually cause the switch to IgG4 and a T regulatory phase. However, it may be too late for the allograft since the ongoing presence of complement fixing antibodies typically precedes IgG4. We have proposed using the allergic desensitization model in pretransplant patients with high cPRA using HLA proteins specific to the detected antibodies [16].

Lastly, IgG1 and IgG2 subclasses have a very high variability regarding their cross-reactivity when detected by the available murine antibodies (Table 4). Although in this study we used the direct assay for subclass identification, we have seen less cross-reactivity using an indirect sandwich assay. We postulate that the extra washing and additional antibody strip off the lower affinity cross reacting antibodies. We feel utilization of a standard indirect assay technique can bring about a better correlation to the complement fixing antibodies and aid in our understanding of the presence or absence of subclass antibodies using standardized kits to ascertain IgG subclass values.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Immune Responses to Tissue-Restricted Nonmajor Histocompatibility Complex Antigens in Allograft Rejection

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Chronic diseases that result in end-stage organ damage cause inflammation, which can reveal sequestered self-antigens (SAgs) in that organ and trigger autoimmunity. The thymus gland deletes self-reactive T-cells against ubiquitously expressed SAgs, while regulatory mechanisms in the periphery control immune responses to tissue-restricted SAgs. It is now established that T-cells reactive to SAgs present in certain organs (e.g., lungs, pancreas, and intestine) are incompletely eliminated, and the dysregulation of peripheral immuneregulation can generate immune responses to SAgs. Therefore, chronic diseases can activate self-reactive lymphocytes, inducing tissue-restricted autoimmunity. During organ transplantation, donor lymphocytes are tested against recipient serum (i.e., cross-matching) to detect antibodies (Abs) against donor human leukocyte antigens, which has been shown to reduce Ab-mediated hyperacute rejection. However, primary allograft dysfunction and rejection still occur frequently. Because donor lymphocytes do not express tissue-restricted SAgs, preexisting Abs against SAgs are undetectable during conventional cross-matching. Preexisting and de novo immune responses to tissue-restricted SAgs (i.e., autoimmunity) play a major role in rejection. In this review, we discuss the evidence that supports autoimmunity as a contributor to rejection. Testing for preexisting and de novo immune responses to tissue-restricted SAgs and treatment based on immune responses after organ transplantation may improve short- and long-term outcomes after transplantation.

1. Introduction

Human leukocyte antigens (HLA) have traditionally been thought to play a dominant role in the development of alloimmunity and allograft rejection [1–4]. Although the effect of HLA matching on improving posttransplant survival is controversial in racially mixed populations, most agree that preexisting donor-specific HLA antibodies (Abs) significantly predispose to hyperacute or acute antibody-mediated allograft rejection (AMR), particularly if the cross-match is positive. This has led to the universal practice of cross-matching prior to solid organ transplantation and, by extension, a reduction in the rate of hyperacute AMR [5]. Nevertheless, acute allograft dysfunction and acute and chronic allograft rejection have remained unaffected [6]. In the context of lung transplantation (LTx), preexisting donor-specific antibodies (DSA) are known to significantly worsen both short- and long-term outcomes [7–13]. Murine and human studies have established the notion that lung-restricted autoimmunity also plays a central role in lung allograft failure at many levels, suggesting that it may represent the common pathogenic pathway of many injury mechanisms which lead to chronic lung rejection [7, 14]. Importantly, the current system of cross-matching cannot detect preexisting immune responses to lung-restricted antigens [15]. In this review, we will discuss recent advancements in defining immune responses to tissue-restricted self-antigens (SAgs)—that is, autoimmunity—and the role of these immune responses in posttransplant allograft survival, focusing specifically on LTx.

2. Pathogenesis of Lung-Restricted Autoimmunity (LRA)

It has traditionally been postulated that self-reactive lymphocytes are deleted in the thymus. However, recent data indicate that nonubiquitous antigens present in organs such as the lung, pancreas, and small intestine are not expressed on the thymocytes, and lymphocytes specific to these SAgs do not undergo thymic deletion [16]. $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs) dynamically suppress these self-reactive lymphocytes against tissue-restricted SAgs [16]. Because the SAgs are normally sequestered, activation of self-reactive lymphocytes is further prevented. LTx recipients and patients with end-stage lung disease undergo many injury-repair cycles that create an inflammatory milieu, which can lead to the expansion of autoreactive lymphocytes. Some mechanisms that have been proposed for this phenomenon include release of the sequestered SAgs, lowering of activation thresholds of self-reactive lymphocytes [17], and epitope spreading [18, 19]. Recruitment of innate immune cells such as monocytes and neutrophils after ischemia-reperfusion can further contribute to this phenomenon. Innate immune cells recognize pathogens using pathogen-recognition receptors (PRRs), which include Toll-like signaling receptors, nucleotide-binding oligomerization domain- (NOD-) like receptors, and retinoic acid-inducible gene- (RIG-) like helicases. However, the PRRs may also cross-react with SAgs released after cellular damage during transplantation (e.g., hyaluronan and heat shock proteins) triggering an immune response [20]. Therefore, the inflammatory cascade that results after transplantation may play an important role in the development of de novo LRA.

Antibodies against donor human leukocyte antigens predispose to LRA. Hachem et al. demonstrated that 70% of all LTx recipients develop LRA within the first three years of transplantation. However, over 96% of recipients with preexisting donor-specific HLA Abs develop LRA [21]. It has been postulated that the donor-specific HLA Abs can cause lung injury and inflammation [22], which can expose otherwise-sequestered SAgs [23–25]. Similarly, acid aspiration from gastroesophageal reflux disease can lead to lung injury [26] and is a known risk factor for chronic rejection [27–29]. Hence, lung allografts are susceptible to several injury mechanisms that can cause local inflammation [14, 30, 31] and increase the risk of lung-restricted autoimmunity [12, 32]. Tregs are known to suppress both inflammation and immune responses by effector lymphocytes [33–35] by inhibiting cytokine production and proliferation of effector cells [36–39]. Loss of Tregs is associated with both acute and chronic lung allograft rejection [36–41]. Therefore, it stands to reason that an injury mechanism that reveals the sequestered SAgs to the host immune system, combined with loss of Tregs, may lead to further activation of self-reactive lymphocytes and development of tissue-restricted autoimmunity.

We have previously shown that respiratory viruses can induce loss of Tregs in murine models. Specifically, draining lymph nodes of murine recipients of orthotopic tracheal transplantation prompted apoptosis in Tregs after infection of the airway with Sendai virus [42, 43]. This is a transient effect, and Tregs levels return to baseline upon clearance of the viruses. Infection of tracheal epithelial cells with Sendai virus in vitro was found to mediate Treg apoptosis through Fas-FasL interactions. It is of interest that viruses are often associated with a variety of autoimmune diseases [24, 44]. In this context, we demonstrated that LTx recipients who develop respiratory viral infections demonstrate a transient loss of Tregs [42, 45]. Interestingly, if these recipients had a preexisting lung injury mechanism (e.g., donor-specific HLA Abs or gastroesophageal reflux), they are at increased risk of developing de novo lung-restricted autoimmunity.

Both murine and human LTx recipients infected with respiratory viruses show increases in FasL in the bronchoalveolar lavage fluid. We therefore tested whether lung injury and concomitant loss of Tregs in wild-type hosts would lead to LRA. Immunocompetent mice were injected with either hydrochloric acid or Abs to MHC class I, and Tregs were depleted by either murine parainfluenza Sendai virus (in wild-type mice) or diphtheria toxin (in Foxp3-DTR mice) [45]. This resulted in the development of both cellular and humoral immunity against lung-restricted SAgs. Lung injury with the MHC Abs, hydrochloric acid without depletion of Tregs, or depletion of Tregs without lung injury did not trigger autoimmunity. In human subjects, patients with cystic fibrosis and idiopathic pulmonary fibrosis are predisposed to ongoing lung injury and respiratory infections, and individuals with these diseases have the highest prevalence of LRA before LTx compared to patients with other diseases (e.g., emphysema and alpha-1-antitrypsin deficiency) [46–50]. The evidence from murine and human models suggests a "two-hit" mechanism for the development of lung-restricted autoimmunity wherein both lung injury and loss of Tregs are essential.

Recent data show that LTx recipients develop exosomes containing lung-restricted antigens, and this might be a biomarker for allograft rejection [51]. However, detection of exosomes, both in serum and in bronchoalveolar lavage fluid, raises the possibility that these exosomes might be involved in the development of lung-restricted autoimmunity. It is possible that the underlying lung injury mechanism leads to the formation of exosomes that incorporate lung-restricted SAgs as well as several immunoregulatory microRNAs, which are released into the circulation and facilitate the generation of autoimmunity due to their immunogenic potential [52–55]. The mechanisms that lead to exosome formation and how these mechanisms may trigger autoimmunity remain to be elucidated.

3. Role of Tissue-Restricted Abs in Organ Transplantation

3.1. Lung Transplantation. Compared with other types of solid organ transplantation, LTx has the lowest survival rate. Development of primary graft dysfunction (PGD) within the first 72 hours and chronic allograft rejection within six months are the two predominant causes of this poor outcome [6, 56, 57]. Intriguingly, PGD has emerged as one of the strongest risk factors for chronic lung allograft rejection. We

have previously demonstrated that PGD is associated with a robust inflammatory response that promotes development of alloimmunity, autoimmunity, and chronic rejection [3, 11]. PGD has been thought to be the result of ischemia-reperfusion injury [58, 59], but this hypothesis conflicts with the recent observation that ischemic time may not correlate with PGD development [60, 61]. In other words, it is not uncommon to observe high-grade PGD development in lung allografts with very short ischemic times, or grafts with more than 8 hours of ischemia that do not develop PGD. It is also noteworthy that several histological hallmarks of PGD, such as alveolar edema, capillaritis, hyaline membrane formation, and neutrophil infiltration [62, 63], are similar to features observed in antibody-mediated rejection (AMR) after LTx, which raises the possibility of PGD being caused by some form of preexisting Abs [64–68].

As previously discussed, LTx recipients, like other solid organ transplant recipients, are rigorously screened for donor-specific HLA Abs. Nevertheless, we detected complement deposition and increases in soluble complement in the allograft biopsies and bronchoalveolar lavage fluid, respectively, in patients with PGD who did not have Abs against HLA [11]. Similar findings have been noted by Westall et al., who found complement deposition in human transbronchial allograft biopsies obtained from patients who developed PGD after LTx [69]. Further longitudinal analysis revealed that about 30% of patients undergoing LTx have preexisting Abs against the lung-restricted antigens collagen type V (Col V), collagen type I, and k-alpha 1 tubulin (Kα1T). These lung-restricted SAgs strongly predispose patients to PGD, development of de novo alloimmunity, and chronic lung rejection [11, 12, 70]. In fact, the presence of all three Abs before transplantation was associated with PGD by over 7-fold magnitude [8–11].

Because a number of clinical factors can confound the association between preexisting Abs to lung SAgs and PGD, we tested whether these two variables are mechanistically linked using the murine model of unilateral LTx [71]. In this recent study, recipients were passively given one or more Abs to lung-restricted antigens before transplantation of syngeneic lung grafts. Each of the Abs demonstrated a dose-dependent graft dysfunction of the syngeneic grafts. Interestingly, preexisting LRA led to epitope spreading wherein administration of Col V Abs induced de novo Kα1T Abs after LTx and vice versa. We further used allogeneic LTx to investigate whether preexisting LRA could prevent development of tolerance. Using MR1 and CTLA4-Ig, tolerance can reliably be achieved toward MHC-mismatched lung allografts. However, preexisting LRA prevented tolerance development and led to dose-dependent development of donor-specific alloimmunity and chronic lung allograft dysfunction. The same held true for de novo LRA [72]. Similar to preexisting LRA, de novo development of LRA after LTx can lead to rejection of a syngeneic lung allograft and can prevent allotolerance toward MHC-mismatched lungs. Development of PGD associated with preexisting LRA has also been demonstrated in the rat LTx model. In that study, the authors administered Col V Abs in rats prior to syngeneic graft transplantation. Rats who received the Col V Abs developed a syndrome of PGD [12]. The authors demonstrated that lung allografts with PGD associated with Col V Abs demonstrated both Ab and complement deposition.

Other reports have confirmed the presence of Col V-specific T-cells after allogeneic rat LTx [9]. When adoptively transferred into recipients of syngeneic lung grafts, these Col V-specific T-cells induced rejection [73]. We previously found that expansion of IFN-γ-producing, Col V-specific Th-1 cells together with reduction in IL-10 secreting T-cells is associated with development of chronic lung allograft rejection [8, 74, 75]. In an experimental model of chronic lung allograft rejection, adoptive transfer of lymphocytes with high levels of IL-17 and IL-23 gene transcripts from Col V-sensitized mice induced histological lesions of obliterative airway disease observed in chronic lung allograft rejection after syngeneic LTx [9].

Another non-HLA antigen associated with lung allograft rejection is MHC class I-related chain A (MICA). MICA is a glycoprotein expressed on cellular membrane which, when expressed, indicates cellular stress and triggers a variety of immune effector mechanisms [76, 77]. MICA can bind to the immune-receptor NKG2D and provides costimulatory signal for the activation of natural killer (NK), $CD8^+$ T, and $\gamma\delta$ T-cells after LTx [78]. Abs to MICA after solid organ transplantation have been associated with chronic rejection [79]. It appears that anti-HLA often precedes the development of anti-MICA, and peak titers of anti-MICA are present at the time of clinical diagnosis of chronic lung allograft rejection [80].

The importance of de novo LRA after transplantation has been established in clinical settings. In an important study by Hachem et al., more than 96% of LTx recipients with preexisting donor-specific HLA Abs developed de novo LRA within three years of LTx and were strongly predisposed to development of chronic rejection [21]. However, Ab-directed therapy was only effective in reversing the increased risk of chronic rejection if it cleared LRA. Patients who cleared HLA Abs but had persistent LRA demonstrated the same risk of developing chronic rejection as those with both HLA Abs and LRA, suggesting that LRA is an important contributor to chronic rejection.

3.2. Heart Transplantation. Over 40% of patients with cardiac allografts develop chronic rejection—which is manifested by vasculopathy—within 5 years [81]. Th17 cells have been shown to mediate a proinflammatory response leading to chronic allograft vasculopathy (CAV) in the absence of Th1 response [82]. T-cell autoreactivity against cardiac myosin, a SAg present in the heart, can develop in the absence of alloimmune responses and is associated with the development of chronic CAV [83, 84]. Humoral immunity has also been implicated in the pathogenesis of CAV [85, 86]. Abs against vimentin, a cytoskeleton protein, independently increase the risk of CAV and accelerate its course [83, 87, 88]. Additionally, humoral immunity to mismatched MICA has been reported to contribute to the immunopathogenesis of both AMR and CAV after heart transplantation [89, 90].

3.3. Kidney Transplantation. Chronic allograft nephropathy is the predominant cause of kidney graft failure [91]. Although alloimmune responses are important in renal allograft rejection, clinically refractory rejection may be associated with Abs against non-HLA antigens. Abs against angiotensin II type 1 (AT1) receptor [92] have been shown to increase the risk for refractory allograft rejection. Other non-HLA antigens that have been shown to play a role in kidney allograft rejection include perlecan, Col IV, Col VI, and the glomerular basement membrane protein, agrin [93, 94]. Recent studies have also suggested a role for antivimentin in the development of chronic renal rejection [95]. Transplant glomerulopathy (TG) is another form of renal allograft dysfunction that can affect over 20% of patients within 5 years of transplantation [93]. TG usually results from humoral injury to the endothelial cells [93], and both HLA and non-HLA Abs have been shown to play roles in its development. Non-HLA Abs include those against AT1, Col IV, fibronectin, MICA, and agrin. Antiglomerular basement membrane Abs against heparan sulphate proteoglycan agrin can also predispose to TG [96].

3.4. Liver Transplantation. Chronic rejection after liver transplantation manifests itself as allograft fibrosis. Recurrence of hepatitis C virus (HCV) is universal after orthotopic liver transplantation (OLT) in HCV-infected recipients [97]; this recurrence is associated with the remodeling of extracellular matrix and its components, including collagen (which promotes fibrogenesis). This process can generate Abs against liver collagen and further augment this process [98–101]. Increased levels of Abs against Col II, Col IV, and vimentin are found in patients with liver fibrosis before transplantation and in patients who develop allograft fibrosis. Patients with native liver and allograft fibrosis also demonstrate significantly higher T helper 2 (Th2) and T helper 17 (Th17) cytokine levels and lower T helper 1 cytokine levels than recipients without fibrosis. Our previous results have also demonstrated that, in HCV-infected patients, levels of Abs to extracellular matrix protein positively correlate with liver fibrosis, which is associated with a predominant Th2 and Th17 cytokine profile [98]. Taken together, these results suggest that development of liver-restricted autoimmunity might play a role in liver allograft fibrosis following OLT.

3.5. Mechanism of Action of Lung-Restricted Abs. The precise mechanisms of action of Abs to lung-restricted SAgs remain unknown—a hurdle which has resulted in inconsistent diagnosis of humoral allograft rejection mediated by such Abs. For example, complement deposition is used as a marker of humoral rejection even though it has not been conclusively shown that LRA can activate complement. We recently reported a case series of human LTx recipients in which a form of hyperacute and acute humoral rejection was caused by preexisting LRA [62]. This was associated with deposition of LRA and complement on the allograft. Furthermore, both types of rejection were successfully treated using Ab-directed therapy, including intravenous immunoglobulin and plasmapheresis. This suggests that the acute effects of LRA may indeed involve complement activation. Nevertheless, patients with LRA-mediated lung injury demonstrate neutrophil recruitment, but whether the newly arriving neutrophils play a mechanistic role remains unclear. Additionally, several immune cells including monocytes, macrophages, dendritic cells, neutrophils, and NK cells have Fc receptors. LRA ligation with the cognate antigens can potentially activate these immune cells and mediate their pathogenic effects. These mechanisms must be investigated in future studies.

In lung allografts, respiratory epithelium may be the primary target of a recipient's immune system. Abs to Kα1T, an epithelial cell surface gap junction protein, cause upregulation of profibrotic growth factors [10]. Lipid rafts may also play a critical role in the surface ligation of Kα1T Abs to their antigens on the surfaces of airway epithelial cells [102, 103]. Normal human bronchial epithelial cells demonstrated upregulation of profibrotic growth factors (e.g., VEGF, HGF, and TGF-β), after ligation with Kα1T Abs. Additionally, hypoxia-inducible factor (HIF-1α) was increased as a consequence of Kα1T Ab ligation on airway epithelial cells. Suppression of HIF-1α reversed the production of profibrotic growth factors upon ligation with Kα1T Abs. These studies suggest that LRA promotes fibrosis and can contribute to chronic rejection.

4. Current Limitations and Future Directions

A wealth of recent literature convincingly supports the role of tissue-restricted Abs in allograft dysfunction and rejection, particularly in the context of LTx. Both human and murine studies have mechanistically linked LRA with lung allograft rejection; however, several questions remain unanswered. First, the treatment thresholds for LRA in LTx recipients are unknown. If a recipient is positive for donor-specific HLA Abs before transplantation, the donor can be excluded or the recipient can be desensitized. However, because these SAgs are nonpolymorphic and are present in all humans, the presence of pretransplant LRA poses a clinical dilemma pertaining to management. LRA likely become pathogenic above a certain titer, but perhaps their pathogenicity is determined by the expression of cognate SAgs in the donor lungs. Because donor lungs are exposed to multiple injury mechanisms (e.g., brain death, mechanical ventilation, variable levels of warm and cold ischemia, and pneumonia), the levels of expression of SAgs may vary. Hence, equivalent titers of LRA could cause varying effects in different donor lungs. Second, although the testing of these Abs is based on ELISA, there are no commercial tests presently available for clinical application and it is unlikely that they will be made available until Ab testing is widely adopted. Third, LRA were detected based on lung epithelial and endothelial cells lines, and it may be possible that patients with end-stage lung disease might have Abs against additional undetected SAgs. Therefore, testing of the LRA present at a given moment may be insufficient to prevent PGD and chronic lung allograft rejection. Fourth, the mechanisms of action of LRA remain unclear: although complement deposition is linked to LRA-associated lung dysfunction, it is unknown whether complement activation is indeed necessary for the pathogenic effects of LRA. Because lung myeloid cells such

as macrophages, monocytes, neutrophils, and dendritic cells express Fc receptors, LRA might directly activate the immune cells and mediate lung rejection. It is important to elucidate these mechanisms, as the mechanism of action will inform the treatment selected. Lastly, it remains unclear whether expansion of self-reactive T-cells plays a role in lung allograft rejection. Further investigation is imminently required to answer these questions, as targeting lung-restricted autoimmunity represents a clinically applicable therapeutic avenue with the potential to significantly improve outcomes in LTx and transplantation of other solid organs.

Glossary

Abs: Antibodies
AMR: Antibody-mediated rejection
AT1: Angiotensin II type 1
CAV: Chronic allograft vasculopathy
Col V: Collagen type V
HLA: Human leukocyte antigens
Kα1T: k-alpha 1 tubulin
LRA: Lung-restricted autoimmunity
LTx: Lung transplantation
MHC: Major histocompatibility complex
MICA: MHC class I-related chain A
NOD: Nucleotide-binding oligomerization domain
OLT: Orthotopic liver transplantation
PGD: Primary graft dysfunction
PRR: Pathogen-recognition receptors
RIG: Retinoic acid-inducible gene
SAgs: Self-antigens
TG: Transplant glomerulopathy
Tregs: $CD4^+CD25^+Foxp3^+$ regulatory T-cells.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

The Role of Humoral Alloreactivity in Liver Transplantation: Lessons Learned and New Perspectives

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More than ten years after the initial description of the humoral theory of transplantation by Dr. Paul I. Terasaki, the significance of humoral alloimmunity in liver transplantation has yet to be clearly defined. The liver allograft has an inherent tolerogenic capacity which confers its resistance to cell-mediated as well as antibody-mediated rejection. Nevertheless, the protection against alloimmunity is not complete, and antibody-mediated tissue injury can occur in the liver graft under specific circumstances. In this article the evidence on the clinicopathologic effects of donor-specific alloantibodies in liver transplantation will be examined and interpreted in parallel with lessons learned from renal transplantation. The unique anatomic and immunologic features of the liver will be reviewed to gain new insights into the complex interactions between humoral immune system and the liver allograft.

1. Introduction

An increasing body of evidence has been published over the past two decades in support of Dr. Paul I. Terasaki's humoral theory of transplantation [1]. In kidney transplantation, the presence of donor-specific anti-human leukocyte antigen (HLA) antibodies has been associated with acute and chronic rejection, as well as impaired graft function and accelerated graft failure [2, 3]. Donor-specific HLA antibodies (DSA) cause vasculitis and rejection in cardiac allografts which contribute to graft dysfunction and poor clinical outcomes [4]. In lung transplantation, the emergence of de novo DSA has been linked with the bronchiolitis obliterans syndrome and inferior patient survival [5]. The presence of de novo DSA has also been identified as a strong independent predictor of allograft failure among pancreas transplant recipients [6].

The role of humoral alloreactivity in liver transplantation, on the other hand, remains unclear. Since the earliest days of experimental liver transplantation (LT), the liver has been recognized as an immunologically privileged organ with relative resistance to rejection [7]. The tolerogenic capacity of the liver graft is not limited to cell-mediated alloimmunity but also appears to extend to antibody-mediated inflammation as well. Several mechanisms have been proposed for the tolerogenic properties of the liver, and it is likely that multiple pathways act in concert to circumvent immunologic rejection [8]. One such theory revolves around the liver allograft's ability to secrete soluble HLA class I antigens [9]. Together with the plethora of cell-bound HLA class I antigens expressed within the liver, the organ has a tremendous ability to absorb or neutralize alloantibodies directed against HLA antigens [10]. Indeed, an estimated 85% of LT recipients with preformed alloantibodies will eliminate circulating DSA within the first few months after transplantation [11]. These mechanisms, however, do not confer complete protection against allospecific HLA antibodies; LT recipients who develop de novo DSA demonstrate inferior survival, particularly when DSA against HLA class II antigens [12–14] and IgG3 subclass DSA [15] are present at high titers. Other reports have associated DSA with late acute rejection [16] and chronic ductopenic rejection [17]. A summary of the recent studies investigating the effects of de novo DSA on LT outcomes is presented in Table 1.

Many questions remain with regard to the effects of alloantibodies on liver allografts. Are HLA antibodies a cause or consequence of liver injury? What are the histopathologic characteristics of antibody-mediated rejection in the liver

Table 1: Summary of recent studies of de novo DSA on clinical outcomes ABO-compatible liver transplantation.

Reference	Study design	Sample size	Prevalence of de novo DSA	Study findings
Kaneku et al. (2013) [13]	Retrospective	749 adult	8.1% at 1 year	(i) Presence of DSA associated with inferior patient and graft survival (ii) Almost all de novo DSA were against HLA class II antigens (majority DQ) (iii) Risk of de novo DSA formation increased by low calcineurin inhibitor levels and the use of cyclosporine (versus tacrolimus)
Grabhorn et al. (2015) [17]	Retrospective	43 pediatric	33% in stable recipients; 68% in chronic rejectors	(i) Higher rate of de novo DSA among pediatric LT recipients with chronic rejection (ii) Antibodies predominantly against HLA class II antigens
O'Leary et al. (2015) [15]	Retrospective	749 adult	8% at 1 year	(i) IgG3 subclass DSA-positive patients at highest risk for death (ii) IgG3-negative, DSA-positive patients still had inferior outcomes compared to DSA-negative patients
Wozniak et al. (2015) [16]	Cross-sectional	50 pediatric	54%	(i) Younger age associated with presence of DSA (ii) Nontolerant patients more likely to have DQ DSA (61%) compared with stable (20%) and tolerant (29%) patients (iii) DQ DSA associated with de novo autoimmune hepatitis and late acute rejection
Del Bello et al. (2015) [18]	Prospective	152 adult	14%	(i) Younger age, low exposure to calcineurin inhibitors, and noncompliance were risk factors for de novo DSA emergence (ii) Nine of 21 (43%) DSA-positive recipients developed acute rejection (iii) No differences in patient or graft survival with DSA presence
Levitsky et al. (2016) [19]	Retrospective analysis of an observational cohort study	195 adult (129 LDLT, 66 DDLT)	5.4% in LDLT; 6.1% in DDLT	(i) No differences in the prevalence of de novo DSA between LDLT and DDLT recipients (ii) Presence of DSA was an independent risk factor for graft failure in LDLT and DDLT

LDLT, living donor liver transplantation; DDLT, deceased donor liver transplantation.

graft? Why does the liver appear to resistant to antibody-mediated injury? Are all HLA antibodies pathogenic, and how do we predict which recipients with alloantibodies will progress to graft failure? In this article we will examine the available data pertaining to DSA in LT, and draw parallels to lessons learned from renal transplantation. We will also introduce novel perspectives and potential explanations for which the liver is less susceptible to injury mediated by HLA antibodies.

2. Known Effects of Alloantibody on the Liver Allograft

2.1. Acute Antibody-Mediated Injury. Demetris et al. have described two distinct histopathologic phenotypes associated with antibody-mediated rejection (AMR) in the liver graft, acute and chronic AMR [20]. Acute AMR is extraordinarily rare, occurring in less than 1% of all LT cases, and is almost exclusively limited to the first few weeks after transplantation in highly sensitized recipients [9, 21]. The few cases of acute AMR [22–29] reported among recipients of ABO-compatible LT in the era of solid-phase antibody testing are summarized in Table 2. Ischemia-reperfusion (IR) injury in the immediate posttransplant period activates the innate immune system and other nonimmune mechanisms, generating an inflammatory milieu that predisposes to allograft rejection. The clinical features of acute AMR resemble those seen with ABO-incompatible transplants and include allograft dysfunction, DSA persistence, refractory thrombocytopenia, and hypocomplementemia. Histopathologically, acute AMR is characterized by portal edema, endothelial cell hypertrophy, and eosinophilia within the portal microvasculature, hepatocyte swelling, ductular reaction, and cholestasis [20, 30]. These patterns of injury are analogous to findings indicative of capillaritis as seen with AMR of other solid organ allografts.

2.2. Mixed Cell-Mediated and Antibody-Mediated Rejection. In contrast to acute AMR, chronic AMR often encompasses features of both cellular and humoral immune reactivity. It is conceivable that, in moderate to severe cases of T-cell-mediated rejection (TCMR), helper T cells can activate humoral immunity and stimulate the production of alloreactive antibodies. In turn, donor-specific HLA antibodies can further potentiate the cellular alloimmune response, thereby increasing the severity of rejection. Compared with acute AMR which occurs in a small minority of sensitized recipients, this clinical picture of mixed TCMR/AMR is much more commonly encountered in actual practice.

In a study of 65 LT recipients presenting with acute allograft dysfunction, paired serum and tissue samples were obtained at the time of hospital admission. Of the 48 recipients with biopsy-proven rejection, donor-specific HLA class I and/or class II antibodies were identified by Luminex-based single antigen bead testing in 25 (52%) of recipients. The presence of strong class II DSA (mean fluorescence intensity $\geq$ 10,000) was associated with steroid-resistant rejection, as well as increased rejection severity. Furthermore, in the majority of cases DSA quickly diminished following resolution of the acute rejection episode, while the few remaining patients with persistent DSA were likely to progress to chronic rejection (unpublished data).

Similar findings were reported by Musat et al. [31], who retrospectively reviewed tissue and serologic data on 43 LT recipients presenting with graft rejection. The authors reported that diffuse portal C4d deposition, together with DSA positivity in the serum, predicted the frequency of acute rejection episodes, as well as the likelihood of steroid-resistant rejection and ductopenia. Taken together, these observations suggest that humoral alloreactivity is closely intertwined with cellular mechanisms during acute rejection; alloantibodies may be a direct consequence of cell-mediated immunity, but antibodies may in turn intensify the degree of tissue injury.

3. Humoral Alloimmunity and Chronic Liver Graft Rejection

A more interesting question pertains to whether HLA antibodies elicit insidious inflammation, fibrosis, and chronic rejection of the liver allograft. Addressing this question requires the prospective collection of serial biopsies and serum samples to document the association between circulating DSA and the histopathologic progression from inflammation to fibrosis. Until recently, this data has not been available as protocol biopsies and alloantibody testing are not routinely performed for LT recipients, and most studies in this subject area have been retrospective in nature. In a prospective study of 89 stable pediatric LT recipients, Varma et al. [32] obtained serial protocol biopsies and analyzed the tissue for evolution of inflammation and fibrosis. The authors found an association between class II DSA and portal inflammation, which over time predisposes to fibrosis progression in the portal areas.

The story with alloantibodies in LT is much more complicated, however, as not all recipients with donor-specific HLA antibodies will develop clinicopathologic evidence of graft injury. Preformed DSA is present in an estimated 13–17% of LT recipients [19], and an additional 8% will develop de novo DSA within the first year after transplant [13, 15]. Yet chronic rejection is an infrequent reason for allograft loss following LT, affecting only 3-4% of liver allografts among adult recipients maintained on tacrolimus-based immunotherapy [33]. Furthermore, results from immunosuppression withdrawal trials in pediatric LT recipients indicate that even operationally tolerant patients may harbor DSA, and the mere presence of DSA does not necessarily correlate with progressive increase in histologic inflammation or fibrosis [34].

4. Perspectives on the Liver Allograft's Resistance to Antibody-Mediated Injury

4.1. Incidence of Donor-Specific HLA Antibodies after Liver Transplantation. One of the potential explanations for the relative infrequency of AMR in LT is the lower incidence of

Table 2: Summary of reported cases of acute antibody-mediated rejection following ABO-compatible liver transplantation.

Reference	Age/gender	Onset of graft dysfunction	DSA detection method	Type of DSA	DSA specificity	AMR treatment	Clinical outcome
Rostron et al. (2005) [22]	23/F	POD 6	Luminex SAB	Preformed	Bw6	Steroids, MMF, PP, IVIG	Alive with functioning graft
Wilson et al. (2006) [23]	36/F	4 years	Luminex SAB	De novo	DR52	Steroids, MMF, PP, IVIG, Rituximab, ATG	Alive with functioning graft
Watson et al. (2006) [24]	50/F	POD 5	Flow cytometry SAB	Preformed	B7	Steroids, MMF, PP, IVIG, Rituximab	Death
Kamar et al. (2009) [25]	49/F	POD 10	Luminex SAB	Preformed	A2, DR7	Steroids, MMF, PP, Rituximab, OKT3	Death
	39/F	POD 6	Luminex SAB	Preformed	A2, A24, B27, DR4	Steroids, PP, Rituximab	Alive with functioning graft
Kozlowski et al. (2011) [26]	N/A	POD 5	Flow cytometry or Luminex SAB	Preformed	3 DSA (specificity not provided)	Steroids, PP, IVIG, Rituximab	Death
	N/A	POD 7		Preformed	A30, A74, B7, B45, DR15, DR51, DQ7	Steroids, PP, IVIG, Rituximab, ATG	Death
	N/A	POD 7		Preformed	4 DSA (specificity not provided)	Steroids, PP, IVIG, Rituximab, OKT3	Retransplant, alive
Paterno et al. (2012) [27]	62/F	POD 8	Luminex SAB	De novo	DR13, DR15, DR51, DR52	Steroids, OKT3, ATG, Bortezomib	Alive with functioning graft
	28/F	POD 452	Luminex SAB	De novo	DQ2, DQ6	Steroids, PP, Rituximab, ATG, Bortezomib	Alive with functioning graft
	53/F	POD 6	Luminex SAB	Preformed	B51, Cw2, DQ7	Steroids, ATG, Bortezomib	Alive with functioning graft
Kheradmand et al. (2014) [28]	43/F	POD 1	Luminex SAB	Preformed	B35, B51, DR4, DR53, DQ8	Steroids, PP, IVIG, Rituximab, ATG	Alive with functioning graft
Wozniak et al. (2016) [29]	22 mo/M	POD 45	Luminex SAB	De novo	B44, DQ2	Steroids, MMF, IVIG, Rituximab	Alive with functioning graft
	3/F	POD 13	Luminex SAB	N/A	A1, DQ5	Steroids, MMF, IVIG	Alive with functioning graft
	19 mo/F	POD 8	Luminex SAB	Preformed & de novo	Cw7, Cw17, DR4, DR53, DQ8	Steroids, MMF, IVIG, Rituximab	Alive with functioning graft
	11/M	POD 7	Luminex SAB	De novo	DR53, DQ8	Steroids, MMF, PP, IVIG, ATG, Bortezomib	Alive with functioning graft
	6 mo/M	POD 38	Luminex SAB	De novo	DQ7, DQ9	Steroids, MMF, PP, IVIG, Rituximab	Retransplant, death
	3/M	POD 7	Luminex SAB	Preformed	A1, B8, Cw7, DR17, DQ2, DP1	Steroids, MMF, PP, IVIG, Rituximab, Bortezomib, Eculizumab	Alive with functioning graft

N/A, not available/information not provided; SAB, single antigen bead-based testing; PP, plasmapheresis; IVIG, intravenous immunoglobulin; ATG, antithymocyte globulin.

DSA observed among LT recipients. Although up to 25% of LT candidates may be sensitized heading into transplantation, the vast majority of LT recipients clear all preformed DSA by four months after transplant [11]. The incidence of de novo DSA is approximately 5–14% after LT [13, 15, 18, 19], whereas de novo DSA has been reported in up to 28% of recipients following kidney transplantation [35]. The reasons for the apparently lower incidence of de novo DSA in LT have yet to be completely elucidated but may be attributable to the same mechanisms which confer tolerogenic properties to the liver, including the ability to absorb or neutralize alloantibodies [10, 36], as well as the immunomodulatory milieu imparted by nonparenchymal liver cells, including sinusoidal endothelial cells, Kupffer cells, resident dendritic cells, and hepatic stellate cells [8]. The unique structural and functional features which contribute to tolerance in the liver graft will be further explored below.

4.2. Unique Cellular Architecture of the Liver. The liver has several unique anatomic and immunologic features which renders its ability to avert antibody-mediated injury. First, the liver receives a dual blood supply from two distinct circulatory routes, from the systemic circulation via the hepatic artery and from the mesenteric system via the portal vein. On a microscopic level, hepatocytes are arranged in sheets surrounded by vascular sinusoids, which are enlarged capillaries lined by a fenestrated endothelium without an underlying basement membrane. Blood collected in these sinusoids drain into the central venule which then returns the blood to the systemic circulation.

The pathologic lesions indicative of AMR have been clearly defined in renal transplantation and are characterized by microvascular endothelial cell injury, as manifested by peritubular capillaritis, glomerulitis, and basement membrane duplication [3, 37, 38]. The distinct morphology of the liver sinusoidal endothelium, with a larger luminal diameter, fenestrated endothelium, and lack of a basement membrane may confer its resistance to microvascular damage. Accordingly, most of the histopathologic evidence of AMR in the liver has been observed in the small vessels with continuous endothelia such as the portal microvasculature, hepatic arterial capillaries, and the peribiliary plexus [39, 40], not within the hepatic sinusoids.

The peribiliary plexus is thought to be derived from hepatic arterial branches and appears exceptionally susceptible to ischemia and immunologic insults [41, 42]. Disruption of the peribiliary plexus by DSA causes arterial insufficiency which, in turn, incurs damage to the biliary epithelium and predisposes to the formation of bile duct strictures [43]. These mechanisms likely account for bile duct atrophy and ductopenia which are typically seen in late acute or chronic rejection [31, 44]. The temporal relationships between rejection, endothelial cell injury, and bile duct loss have been described by Matsumoto et al. [45] in comparing biopsies from rejecting and normal allografts. Both acute and chronic rejection are associated with a reduction in the number of portal microvascular structures, a finding most pronounced in severe cases of rejection. They further demonstrated that components of the microvasculature were destroyed prior to disappearance of the bile ducts. Taken together, the unique cellular architecture of the liver likely plays an important role in its resistance to antibody-mediated inflammation and fibrosis.

4.3. The Immunomodulatory Liver Parenchyma. The hepatic portal circulation is constantly exposed to microbial products and foreign antigens from the gut, and the liver has acquired a number of molecular adaptations to avoid unnecessary immune responses to innocuous antigens. Liver sinusoidal endothelial cells (LSECs), together with Kupffer cells and resident dendritic cells found within the hepatic sinusoids, play essential roles in the maintenance of immunologic tolerance. At steady state, the liver demonstrates strong, diffuse expression of class I major histocompatibility complex (MHC) antigens, but weak class II MHC expression. The normal liver also secretes soluble class I HLA molecules, which can complex with alloantibody and then become vigorously cleared by Kupffer cells in the hepatic sinusoids [46]. Additionally, antigen presentation by LSECs often leads to the release of anti-inflammatory cytokines and the preferential expansion of tolerogenic T-cell subsets, creating an immunomodulatory microenvironment within the liver.

This pattern of tolerance can be broken by pathogenic stimuli such as infectious microorganisms and endotoxin, as well as by endogenous damage-associated molecular patterns generated during preservation injury. Following inflammatory insults, class II MHC expression is upregulated on biliary epithelial cells, portal and hepatic artery endothelia, resulting in increased DSA targeting and further immune stimulation. An exhaustive review of the tolerogenic mechanisms and immune reactions within the liver is beyond the scope of this article, and the interested reader is encouraged to refer to an excellent review article written by Demetris et al. [41].

Because of the high tolerogenic threshold within the liver, humoral alloimmunity does not typically elicit tissue injury without coexisting insults. This is best illustrated by normal biopsies without histologic signs of tissue injury which can be obtained from clinically stable LT recipients with circulating DSA, even among patients who have undergone complete withdrawal of immunosuppression therapy [11, 47]. Based on this evidence, Kim et al. have proposed the "two-hit hypothesis," in which a concurrent insult causing allograft inflammation is needed for alloantibodies to incur observable dysfunction [48].

Further support for the "two-hit hypothesis" originates from epidemiologic studies of chronic rejection among primary LT recipients [49]. Among patients receiving tacrolimus-based immunotherapy, the risk of chronic rejection is increased by the occurrence of acute cellular rejection, advanced donor age, hepatitis B or hepatitis C viral infections, and diagnoses of primary biliary cirrhosis, primary sclerosing cholangitis, or autoimmune hepatitis. Each of these etiologic factors introduces an alternate source of allograft injury, such as T-cell-mediated alloimmunity, augmented IR injury in a suboptimal donor organ, chronic active viral hepatitis, or autoimmunity. These insults tip the delicate immune balance

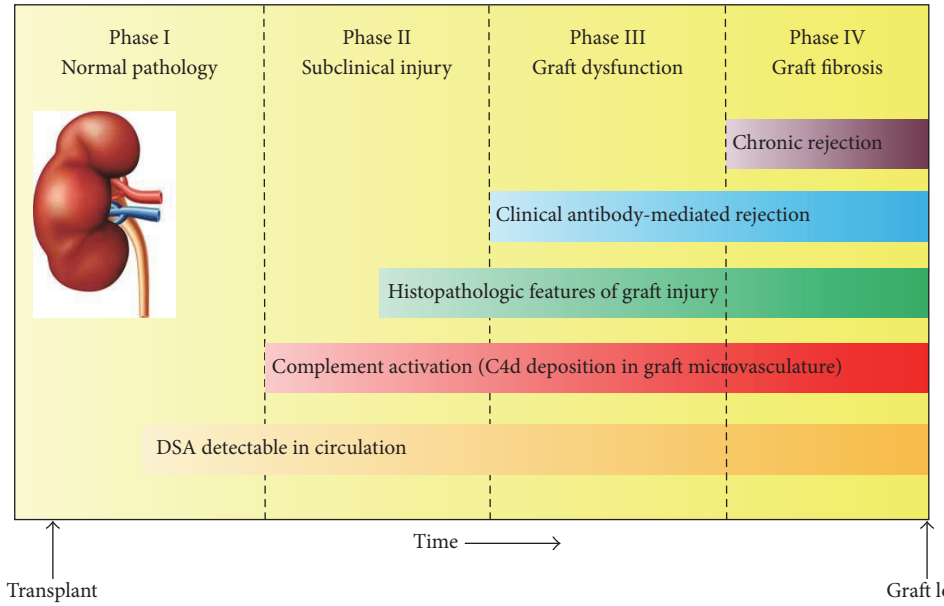

Figure 1: Natural progression of antibody-mediated rejection in renal transplantation. DSA, donor-specific antibody.

within the liver towards a proinflammatory phenotype and may act in concert with alloantibodies to promote fibrosis and graft loss.

5. Implications and Conclusions

In comparison to other solid organ transplants, the liver allograft appears exceptionally tolerant to cellular and humoral alloimmune activity. While the unique anatomic and functional properties of the liver contribute at least in part to its tolerogenic capabilities, the protection conferred by these attributes is not complete. Alloantibody-mediated inflammation in the liver graft can occur, particularly in the presence of class II DSA and in the face of coexistent insults, which if left untreated will likely culminate in tissue injury and irreversible graft damage.

Because humoral alloimmunity is closely intertwined with other mechanisms of inflammation (such as cell-mediated alloimmunity and IR injury), the identification of unique histopathologic features indicative of antibody-mediated injury has been a particularly challenging task. C4d deposition, which is a specific marker for humoral activity in kidney transplantation, lacks the same degree of accuracy in liver allografts and can often be detected among recipients with nonrejection causes of dysfunction. In the 2016 update of the Banff Working Group on Liver Allograft Pathology, histopathologic criteria for the diagnosis of AMR was introduced for the first time [40]. Clearly more research efforts are needed to establish the link between humoral immune pathways and histopathologic features of antibody-mediated injury in liver grafts.

Kidney transplant experts have proposed a hypothetical chain of events (Figure 1) which describe the temporal progression of AMR, starting from the serologic presence of HLA alloantibodies (Phase I), to histopathologic evidence of tissue injury via C4d deposition (Phase II), to clinically evident graft dysfunction (Phase III) and irreversible graft fibrosis (Phase IV). Great efforts have been made to identify patients in the earlier phases of this sequence by routine serologic testing and protocol biopsies, in an attempt to hinder the humoral immune response before irreversible damage to the graft is incurred. Despite advances in DSA testing with the sensitive and specific Luminex-based single antigen testing, currently available antibody-reduction protocols have failed to demonstrate reliable and sustained eradication of DSA after transplant. Consequently, AMR and chronic rejection still remain as frequent causes for renal allograft loss.

I herein propose a modified sequence of events for liver transplant recipients who harbor persistent DSA based on the evidence presented in this review (Figure 2). In many LT recipients, humoral alloimmunity requires a concomitant insult to overcome the immunoregulatory tendency of the liver allograft to induce tissue injury. Occasionally, the emergence of DSA may even be a direct result of these alternative sources of inflammation, such as stimulation from innate immune pathways and from T helper cells. Acting together, the coexisting insult and humoral immunity will quickly initiate allograft inflammation and clinically evident dysfunction. This model introduces significant implications for the monitoring and management of LT recipients.

(1) Antibody-mediated injury can be ameliorated or prevented altogether by addressing the coexistent causes of allograft injury. Our treatment strategies for these alternative insults to the liver allograft have generally been much more effective than the protocols designed for antibody reduction. For instance, the activation of cell-mediated immunity can be avoided by adequate

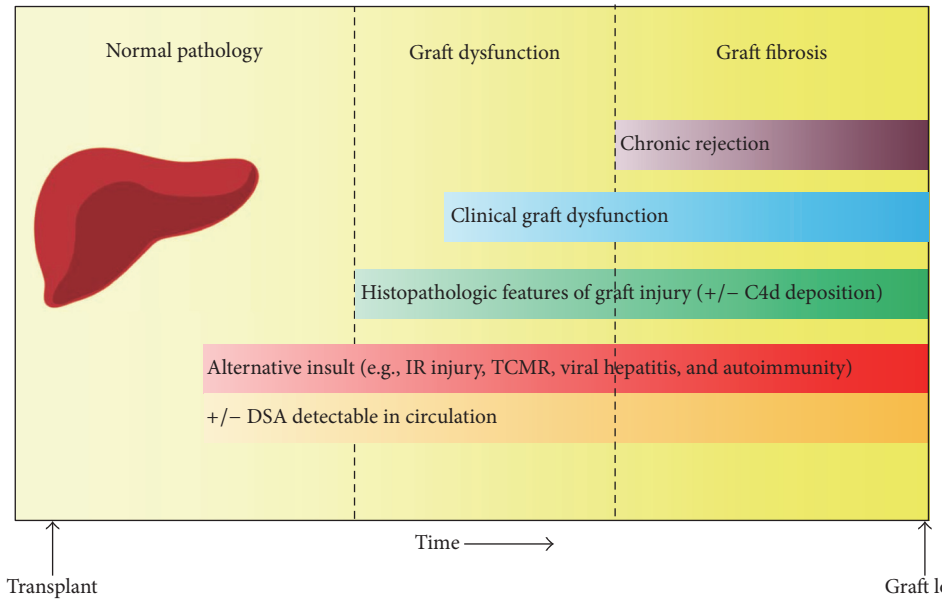

Figure 2: Proposed sequence of events leading to the development of chronic rejection in liver transplantation. DSA, donor-specific antibody; IR, ischemia-reperfusion; TCMR, T-cell-mediated rejection.

immunosuppression and by promoting medication adherence. IR injury can be minimized by appropriate donor selection and minimization of ischemia times, and a great deal of research efforts have been dedicated to identifying strategies that attenuate IR injury following transplantation. Impressive advances have been made in the treatment of hepatitis B and hepatitis C virus infections over the past few decades, and sustained viral responses can be consistently achieved to prevent viral reinfection after LT [50].

(2) Routine posttransplant monitoring for HLA antibodies is not necessary for all LT recipients but may be beneficial for selected patients with coexisting reasons for graft inflammation, such as those with diagnoses of autoimmune liver diseases or acute TCMR. This recommendation is based on the premise that alloantibodies require an alternative insult or a "second hit" to incur tissue injury. Testing for DSA in these subgroups of patients may help identify those at highest risk for severe graft damage and accelerated graft loss.

Knowledge gaps remain pertaining to the immunologic mechanisms leading to liver allograft failure. Given the relative immunologic ease of managing LT recipients and the small percentage of grafts lost to chronic rejection, transplant professionals have historically focused on more urgent issues such as the critical donor organ shortage, recurrent diseases after transplantation, and complications from the long-term use of immunosuppressive drugs. However, given recent trends in LT, including the rising use of marginal donor organs, advances in the treatment of recurrent diseases, and attempts to withdraw or minimize immunosuppression, we are likely to witness an increase in the immunologic consequences of humoral reactivity. More work is needed to decipher the complex interactions between the humoral immune system and the liver allograft and to identify the contexts in which alloantibodies incur tissue injury following transplantation.

Abbreviations

AMR: Antibody-mediated rejection
DSA: Donor-specific antibody
HLA: Human leukocyte antigen
IR: Ischemia-reperfusion
LSEC: Liver sinusoidal endothelial cell
LT: Liver transplantation
MHC: Major histocompatibility complex
TCMR: T-cell-mediated rejection.

Competing Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Desensitization: Overcoming the Immunologic Barriers to Transplantation

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HLA (Human Leucocyte Antigen) sensitization is a significant barrier to successful kidney transplantation. It often translates into difficult crossmatch before transplant and increased risk of acute and chronic antibody mediated rejection after transplant. Over the last decade, several immunomodulatory therapies have emerged allowing for increased access to kidney transplantation for the immunologically disadvantaged group of HLA sensitized end stage kidney disease patients. These include IgG inactivating agents, anti-cytokine antibodies, costimulatory molecule blockers, complement inhibitors, and agents targeting plasma cells. In this review, we discuss currently available agents for desensitization and provide a brief analysis of data on novel biologics, which will likely improve desensitization outcomes, and have potential implications in treatment of antibody mediated rejection.

1. Introduction

Kidney transplantation is the treatment of choice for patients with end stage kidney disease as it is associated with improved patient survival, and better quality of life [1, 2]. HLA (Human Leucocyte Antigen) sensitization, resulting from previous pregnancies, blood product transfusions, or previous transplant, and ABO incompatibility pose significant immunologic barriers to kidney transplantation. HLA sensitized patients present vexing problems as they express multiple alloantibodies that often result in crossmatch positivity and hence longer wait times due to the presence of donor-specific antibodies (DSAs). Patients transplanted across these barriers without sufficient desensitization are at high risk for early graft loss from antibody mediated rejection (ABMR). However, those that survive still are at a much higher risk of chronic antibody mediated rejection (CABMR) posttransplantation with decreased overall allograft survival [3, 4]. Approximately 30% of patients on the kidney transplant waitlist in the US are sensitized against HLA antigens, which reduces the opportunities for successful transplantation. With the new kidney allocation system (KAS) giving priority to patients with a calculated panel reactive antibody (cPRA) of 99-100%, there has been an increase in rate of transplants in this group (from 2.3% pre-KAS to around 10% at year one after KAS); however transplants have declined for patients with cPRA 80–94% (10% pre-KAS to 4.9% post-KAS) [5]. Thus, other approaches are needed to improve the access and success of kidney transplants in this disadvantaged group.

To this end, desensitization protocols (probably best termed immunomodulation) emerged in the late 1990s to overcome this humoral incompatibility and optimize the availability of compatible or acceptable donors. The development of novel immunomodulatory therapies (see Table 1) in the last decade has allowed for refinement of desensitization protocols. This emerged in conjunction with better immunological risk stratification with sensitive DSA screening assays and avoidance techniques and has led to improved transplantation rates and favorable short- and long-term outcomes in these high immunological risk patients. This is an important advancement since ESRD patients who remain on dialysis die at high rate while waiting for an allograft [6, 7]. The benefits of desensitization in improving the life expectancy of ESRD patients were shown in a multicenter study of 1025 kidney transplant recipients by Orandi et al. [8]. Patients who received kidney transplants from HLA incompatible live donors had a substantial survival benefit compared to those who waited for HLA compatible transplants from deceased

Table 1: Agents of desensitization.

Immunotherapy	Mechanism of action	Dosing
IVIg*	Exact mechanism unclear; however some mechanisms include regulation of B-cell antibody production, induction of B-cell apoptosis through FcyR mediated signals, inhibition of dendritic and macrophage cell maturation and function, inhibition of various proinflammatory cytokines, inhibition of complement mediated inflammation	1 g/kg max 70 g daily × 2 doses OR 2 g/kg max 140 g (given over HD) [88]
Rituximab*	Anti-CD20	375 mg/m^2× Body Surface Area IV over 5–7 hours [89]
Obintuzumab*	Anti-CD20	1000 mg IV titrated per package insert
Bortezomib* Carfilzomib*	Inhibiting proteasomes	Bortezomib: 1.3 mg/m^2/dose × 6–8 doses [89] Carfilzomib: 20, 27, 36 mg/m^2 [90]
Tocilizumab*	Anti-IL6 receptor blocker	8 mg/kg (max 800 mg) monthly × 5–7 doses [53]
IgG endopeptidase*	Cleaving Igg leaving behind Fc and F(ab′)$_2$	0.24 mg/kg IV over 15 minutes [NCT02426684]
Belimumab	Inhibiting binding of B lymphocyte stimulator protein to the B-cell receptors	10 mg/kg IV over 1 hour every 2 weeks for the first 3 doses [88]
Eculizumab*	Blocking complement protein C5 and preventing generation of the terminal complement complex C5b-9	1200 mg IV over 1hour then 900 mg IV over 1 hour weekly × 3 doses or more per clinical response [88]
C1 esterase inhibitor*	Inactivating complement pathway players C1r and C1s	20 units/kg IV twice weekly × 4 wks [65]
Belatacept	CTLA4-Ig may have potent effects on de novo donor specific antibody generation and plasma cell inhibition	Not used [70]

*Immunotherapy agents require premedication with acetaminophen, antihistamine, and glucocorticoid thirty minutes before infusion.

donors or those who did not undergo transplantation at 1, 3, and 8 years (1 yr, 95.0% versus 94.0% versus 89.6, 3 yrs, 91.7% versus 83.6% versus 72.7%, and 8 yrs, 76.5% versus 62.9% versus 43.9% resp., $P < 0.001$ for all comparisons). Our group has also shown that desensitization is cost effective and leads to better patient survival when compared to remaining on dialysis [9].

2. Therapeutic Approaches for Immunomodulation of HLA Sensitized Patients

2.1. Intravenous Immunoglobulin (IVIg). The immunomodulatory effects of IVIg were first recognized in the early 1980s when this agent, developed primarily for replacement of humoral immunity, was found to have beneficial effects in autoimmunity and vasculitis [10]. IVIg affects innate and adaptive immune systems, regulating most components of the immune system including antibodies, complements, cytokines, most immune cells, and their receptors [11–13]. Precise mechanism(s) of immune modulation are still not well known although several have been proposed depending on the specific disease. Plasma-derived IgG has since evolved as a critical biologic for replacement therapy in primary and secondary immunodeficiency. Newer manufacturing methodologies based on gentle chromatographic purification have resulted in IgG products expressing higher concentrations and avidities. In addition these formulations are suitable for i.v. (intravenous immunoglobulin, IVIg) or s.c. (subcutaneous immunoglobulin, SCIG) administration [14]. Proof-of-concept studies in the early 1980s in idiopathic thrombocytopenia (ITP) patients [15] were the cornerstone for the use of IVIg/SCIg in autoimmune inflammatory diseases, particularly those mediated by autoantibodies. Labeled autoimmune indications for IVIG include ITP, Kawasaki's disease, Guillain–Barré syndrome (GBS), Chronic Inflammatory Demyelinating Polyneuropathy (CIDP), and Multifocal Motor Neuropathy (MMN); in addition, IVIg has multiple off-label use in autoimmune indications and prevention and treatment of antibody mediated rejection (ABMR) of kidney allografts [10]. Recent data suggest that IVIg can be modified in vitro using tetra-Fc sialylation to produce a candidate drug with 10-fold greater immune modulatory capacity than seen with IVIg [16].

The advantages of IVIg as a desensitizing agent were clearly demonstrated by the only randomized placebo-controlled trial of IVIg conducted by our team through the NIH (1997–2002) [12]. This multicenter study showed improved transplantation rates for highly sensitized patients, 35% in IVIg (2 g/kg monthly × 4 doses before transplant and 4 doses monthly after transplant) versus 17% in placebo; $P < 0.05$. The deceased donor transplantation rates were 31% versus 12% in placebo; $P = 0.0137$, with a graft survival of 80% for IVIg group and 75% for placebo group at 30 months (P = NS). Subsequently, Montgomery et al. [6] used a protocol of low dose IVIg (100 mg/kg) and plasma exchange (PLEX) and demonstrated a significant survival benefit for

patients with HLA sensitization who underwent living donor transplantation in comparison to patients who remained on dialysis or underwent HLA compatible transplantation. This survival advantage more than doubled by 8 years. Several desensitization protocols using high dose IVIg (2 g/kg) have been described [13, 17].

Today, IVIg remains the cornerstone of all desensitization protocols. Most protocols use either high dose IVIg or low dose IVIg in combination with PLEX. Various mechanisms by which IVIg has been proposed to be beneficial include its inhibitory effect on B cells and T-cell proliferation, upregulation of anti-inflammatory Th2 cytokines and anti-idiotypic blockade of alloantibodies, and enhanced clearance of pathogenic IgG through blockade of FcR receptors [10, 11, 18].

Since these initial studies of IVIg as a desensitizing agent, our group [17, 19] has shown that use of IVIg alone is not sufficient to sustain low levels of anti-HLA antibody and is associated with antibody rebound posttransplantation with antibody mediated rejection (ABMR). In addition, better understanding of the role of cryptic B-cell responses in antibody mediated rejection and decreased allograft survival has led to the acceptance of addition of anti-CD20 monoclonal antibody rituximab to the IVIg protocols for desensitization [20].

2.2. Rituximab (Rituxan®). It is a monoclonal antibody specific for CD20, a member of the membrane-spanning-4-domain A family of proteins. Our group has reported extensively on the efficacy of combination of high dose IVIg and rituximab in lowering anti-HLA antibodies and improving rates of transplantation in the HLA sensitized group [17, 19]. In 79 highly sensitized patients, a combination of IVIg (2 doses of 2 g/kg on day 0 and 30) and rituximab (1 g on day 15) led to significant reductions in T-cell flow cytometry crossmatch from pretreatment (T cell 183.5 ± 98.4 mean channel shifts (MCS) for LD and 162.8 ± 41 MCS for DD) to time of transplant (T cell 68.2 ± 58 MCS for LD [$P < 0.00006$] and 125 ± 49 for DD [$P = 0.05$]), respectively. Time on wait list for DD recipients was reduced from 95 ± 46 months to 4.2 ± 4.5 months after treatment. Twenty-eight patients (37%) experienced acute rejection. Patient and graft survival up to 24 months were 95% and 84%, respectively, with good allograft function at 1 year. The infusions were well tolerated with minimal side effects [21].

Despite good early results from our group, there were questions that arose regarding the efficacy of rituximab in desensitization. To address this directly, we conducted a blinded, placebo-controlled trial of IVIg + placebo versus IVIg + rituximab for desensitization [19]. Shortly after initiation of this study which aimed to have 75 patients entered, we noted 3 SAEs in our first 15 patients entered. When the study code was broken, we noted all SAEs (severe ABMR) were in the IVIg + placebo group. Due to the severe nature of the SAEs and graft losses, we terminated the study with results reported. Although the evaluation of results was limited in this small cohort, clinically important trends were observed where IVIg + rituximab appeared more effective in preventing DSA rebound, ABMR, and development of transplant glomerulopathy determined by protocol biopsies after transplant. Since publication, other reports have demonstrated an important impact of rituximab on anamnestic responses to HLA antigens after transplant [20]. Kohei et al. reported on the benefits of rituximab in preventing early HLA sensitization after transplant in ABOi patients [22]. This intriguing paper showed significant long-term benefits in reducing de novo DSA generation and preventing chronic ABMR compared to a group of nonsensitized living donors that did not receive rituximab. These findings codify the importance of rituximab in controlling allosensitization and prevention of anamnestic responses. These likely represent the most important impact of anti-CD20 therapy in transplantation since there is little evidence that rituximab alone can reduce antibody levels sufficiently to allow for incompatible transplantation [23].

An important question to address is the risk of infectious complications with this protocol. We evaluated infectious outcomes in a retrospective study including 361 patients, 170 of whom underwent desensitization with IVIg and rituximab [24]. No differences were observed in the desensitized and nondesensitized groups. Bacterial infections were similar in both groups, with urinary tract infections accounting for 50%. The rate of viral and fungal infections was similar. However, a trend toward a higher rate of BK viremia was noted in the desensitized group. In another study comparing BKV viremia in desensitized ($n = 187$) and nondesensitized group ($n = 284$), BK viremia was observed in 20% of the desensitized and 10% of the nondesensitized ($P < 0.001$) groups by 2 years after transplant. More patients in the desensitized group had a peak viral load greater than 10,000 copies per milliliter ($P < 0.001$). However, there was no significant difference in BKV-associated nephropathy or graft loss in the two groups. There was an association of BKV viremia with desensitization and lymphocyte induction [25].

To date, other important observations include the absence of cases of progressive multifocal leukoencephalopathy and PTLD in the desensitized patients [26]. A more comprehensive analysis of the impact of desensitization on risk for viral infections after transplant is presented by Dr. Toyoda in this issue.

Loupy et al. [27] described a posttransplant approach to desensitization in deceased donor kidney transplant recipients using high dose IVIg, rituximab and PLEX. This was successful in decreasing DSA, chronic ABMR, and transplant glomerulopathy at 1 year. Renal function was significantly better in this group when compared to the group that received only IVIg after transplantation. Our group has also reported on the significant benefits of IVIg + rituximab versus IVIg alone in improving long-term graft survival [19].

Jackson et al. [28] retrospectively examined posttransplant DSAs in 50 HLA incompatible living donor kidney transplant recipients. All patients received plasmapheresis and IVIg, and those deemed to be at higher immunological risk (multiple transplants, repeated HLA mismatches, and higher CPRA) received rituximab. At 1 month after transplantation, patients who received rituximab had significantly lower numbers of DSAs ($P = 0.03$) and non-DSAs

($P = 0.003$) than those in the control group. The magnitude of the increase in all HLA antibodies at 1 month was also lower in the rituximab group. However, rituximab induction did not significantly impact the persistence of DSA at 1-year posttransplantation (detected in 52% of the patients treated with rituximab versus 40% in the nonrituximab treated group). Importantly, no significant difference was observed in rates of ABMR and in allograft survival at 5 years after transplantation.

Van Den Hoogen et al. [29] conducted a placebo-controlled trial of rituximab as an induction agent for kidney transplant recipients. 280 patients were examined (138 randomized to rituximab and 142 placebo). Overall, no difference in the graft rejection rates was noted. However, when data for high-risk patients (repeat transplants and HLA sensitized) were analyzed, there was a significant reduction in rejection episodes for those who received rituximab (17.9% versus 41.1%, $P = 0.039$). The authors concluded that a single dose of rituximab given as an induction agent at transplant significantly reduces rejection rates in sensitized patients.

A recent prospective study by Shaffer et al. [30] reported 3-year outcomes in 29 highly sensitized patients who were desensitized with high dose IVIg and one dose of Rituximab after transplantation. The study showed a 46% reduction ($P < 0.001$) in the strength of DSA at 1 month after transplant that was sustained throughout the 3-year follow-up period and was observed for both class I and class II DSAs regardless of pretreatment MFI (mean fluorescence intensity) levels. 3-year patient and graft survival were 95% and 90%, respectively, and acute rejection was diagnosed in 4 patients (14%) during the follow-up period. In a Korean study by Hwang et al. [31], patients with high panel reactive antibody (PRA scores) but negative crossmatch who received pretransplant rituximab ($n = 32$) had significantly lower risk of ABMR and higher 3-year graft and patient survival rates ($P = 0.007$ and $P = 0.037$, resp.).

In summary, the use of IVIg + rituximab ± plasma exchange offers acceptable outcomes and improves long-term patient survival compared to remaining on dialysis [9], especially for patients with DSAs of low to moderate intensity. However, Orandi et al. [32] have shown that outcomes of patients transplanted across a complement-dependent cytotoxicity (CDC) crossmatch are unacceptably low and should not be pursued. Thus, the most successful approaches include desensitization and a strategy to avoid offers from donors where the recipient has strong C1q+ DSAs that will likely result in early ABMR and graft loss. In our experience C1q+DSAs are the most difficult to immunomodulate and are the most pathogenic. Thus, limitations do exist. To respond to these deficiencies investigators have developed other approaches [33, 34].

2.3. Bortezomib (Velcade®). It was FDA approved in 2008 for treatment of refractory multiple myeloma and has been successfully used in ABMR treatment in transplantation [35, 36]. Bortezomib inhibits the 26s proteasome which ultimately leads to plasma cell apoptosis. Woodle et al. [37] showed a significant decrease in immunodominant (iDSAs) and successful transplantation in 19 of 44 highly sensitized patients with low acute rejection rates (18.8%) at 6 months, with a protocol incorporating bortezomib, plasmapheresis, and rituximab. Jeong et al. [38] ($n = 19$) used a desensitization protocol of high dose IVIg, bortezomib, and rituximab and demonstrated an increased rate of deceased donor transplantation. These studies were small, open labeled and nonrandomized. Thus interpretation of efficacy is limited. Moreno Gonzales et al. [39] evaluated the efficacy of 32 doses of monotherapy with bortezomib in 10 highly sensitized patients and found only modest reductions in both class I and class II antibodies with no change in cPRA or flow cytometric crossmatch. In addition, the therapy was not well tolerated. Treatment was interrupted or discontinued in half the patients due to symptoms of fatigue, anorexia, insomnia, anemia, thrombocytopenia, peripheral neuropathy, and disseminated varicella zoster in one patient. Currently, the limited data on efficacy and significant AE/SAEs limits enthusiasm for incorporation of this drug into most desensitization protocols. Recent data was presented on the use of a second generation proteasome inhibitor carfilzomib. Currently, only limited data on efficacy are available. However, the posttransplant use of this drug will likely be limited due to nephrotoxicity and risk of thrombotic microangiopathy [40].

2.4. Interleukin-6 (IL-6) Receptor Antagonist (Tocilizumab, Actemra®). IL-6 is a cytokine critical to numerous inflammatory and immunomodulatory pathways and is essential for the maintenance of host defenses [41, 42]. However, excessive and unregulated production of IL-6 result in a number of chronic immune disorders, including a role in the chronic inflammation seen in transplant rejection, patients on dialysis, in crescentic glomerulonephritis, and graft versus host disease (GVHD) [43–45]. IL-6 is one of the major cytokines involved in differentiation of B cells to IgG-secreting plasmablasts and finally to plasma cells [46, 47]. In addition, IL-6 stimulates Th17 cells that increase inflammation and allograft rejection and inhibits the generation of Treg cells [48].

The IL-6R is expressed constitutively only on hepatocytes and some immune cells [49], while soluble IL-6R can bind IL-6 and then transsignal through gp-130 expressed on virtually any cell type. Membrane bound IL-6R signaling is responsible for host defenses while transsignaling likely mediates the pathologic functions of IL-6. Tocilizumab antagonizes both soluble and membrane bound forms of the IL-6 receptor, resulting in inhibition of the classic and transsignaling pathways. Tocilizumab is a humanized monoclonal antibody that is FDA approved for moderate to severe rheumatoid arthritis, systemic juvenile idiopathic arthritis (SJIA), and polyarticular juvenile idiopathic arthritis (PJIA) [50]. Animal models reveal that anti-IL-6 receptor therapy weakens alloantibody responses by increasing Treg and suppressing B-effector and plasma cells in bone marrow [51]. By targeting the IL-6/IL-6R pathway, reduction in antibody production and increases in Treg cells (CD4+, CD25+, FoxP3+) are likely [52]. We recently completed a phase I/II trial of anti-IL-6R therapy for HS patients who failed standard desensitization (IVIg + Rituximab ± PLEX). Patients received IVIg on days

0 and 30 at 2 g/kg and TCZ 8 mg/kg on day 15 and then monthly for 6 months. If transplanted, patients received IVIg once and TCZ monthly for 6 months. With this protocol, 7 of the 10 patients were transplanted and 6-month protocol biopsies showed no evidence of antibody mediated rejection or transplant glomerulopathy. eGFR (MDRD) at 1 year was 60 ± 25 mL/min. DSA strength and numbers were reduced by TCZ at transplant ($P = 0.024$) and 12 months ($P = 0.0003$) after transplantation. The infusions were well tolerated with the most common side effects of elevated blood pressures, thrombocytopenia, and anemia. The results of this small trial are very encouraging and suggest a need for larger randomized controlled trials to determine the overall efficacy of anti-IL6R drug therapy in desensitization [53].

Over the past 5 years, we have studied tocilizumab as an agent to treat chronic ABMR and TG. To date, we compared a group of 37 patients with TG and CABMR treated with tocilizumab, monthly for 6 to 12 months to a historical cohort of 39 CABMR and TG patients treated with IVIg + rituximab. We have noted a significant benefit in graft survival and reductions of immunodominant DSAs as well as stabilization of GFRs over a 5-year observation period. Pretocilizumab and posttocilizumab treatment biopsies performed on selected patients revealed significant improvements in features of ABMR, including decreased C4d + scores and reduced glomerulitis and peritubular capillaritis scores. Although preliminary, these data are encouraging and possibly suggest a role for disruption of IL-6/IL-6R signaling for treatment of CABMR and TG [54].

2.5. IgG Endopeptidase (Ides®). IgG endopeptidase is a bacterial enzyme produced by *Streptococcus pyogenes* that cleaves all four human IgG subclasses at the lower hinge region of IgG heavy chains yielding $F(ab')_2$ and Fc fragments [55]. The rapid inactivation of IgG molecules inhibits both complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADCC). This likely explains the high pathogenic potential of *S. pyogenes* infection which account for more than 500,000 deaths world-wide each year [55]. However, the isolated enzyme could have a large impact on many antibody mediated autoimmune diseases and transplantation. To this end, phase 2 trials are currently taking place in Europe and US, focusing on safety, tolerability, pharmacokinetics, and efficacy of use in chronic kidney disease patients (NCT02224820 and NCT02426684). Initial data are promising, showing complete removal of DSAs prior to incompatible transplantation with prevention of early ABMR. This drug will likely become part of desensitization protocols and may give hope to highly HLA sensitized patients who have received multiple rounds of desensitization without success.

2.6. Obinutuzumab (Gazyva®). It is a humanized anti-CD20 monoclonal antibody that received FDA breakthrough status in November 2013 for the treatment of chronic lymphocytic leukemia (CLL) [56]. It differs from rituximab in that it recognizes the type II epitope of the CD-20 antigen present on the pre-B and mature B cells versus type I epitope recognized by rituximab. In a clinical trial, obinutuzumab was superior to rituximab in B-cell depletion and yielding significantly better outcomes for patients with CLL and non-Hodgkin's lymphoma [57, 58]. Currently, two multicenter clinical trials are taking place in Europe and US to assess safety, efficacy, tolerability, and pharmacokinetics in the highly HLA sensitized chronic kidney disease patients awaiting kidney transplantation (NCT02224820, NCT02475551, and NCT02426684). Obinutuzumab will also likely represent a significant advancement in desensitization and treatment of antibody rejection as it allows for more complete and durable B-cell depletion. It is also the first drug to be brought forward in more than 10 years for potential indication in kidney transplantation and also the first agent being investigated for an FDA indication as a desensitization agent.

2.7. Anti-B-Cell Activating Factor (Belimumab, Benlysta®). Belimumab inhibits growth and differentiation of B cells by blocking B lymphocyte stimulator (also known as BlyS) and is FDA approved for treatment of adults with active systemic lupus erythematous [59]. Belimumab monotherapy was studied as a desensitization agent in kidney transplantation (NCT01025193). However, the study was terminated early for reported lack of efficacy. Currently, a phase 2 double-blinded, randomized, placebo-controlled trial of Belimumab plus standard of care is being examined for prevention of allograft rejection in renal allograft recipients (NCT01536379).

Several other B-cell depleting immunomodulatory agents are currently in pipeline but have not yet been evaluated for use in desensitization.

2.8. C5 Inhibitor (Eculizumab®, Soliris) for Prevention of ABMR. The complement system plays an important role in tissue damage, graft dysfunction and loss induced by alloantibodies, and ischemia reperfusion injury [60, 61]. Eculizumab is a monoclonal antibody that binds protein C5 of the complement cascade, inhibiting its cleavage to C5a and C5b and formation of membrane attack complex C5b-9. A recent study by Bentall et al. [62] at the Mayo Clinic reported significantly decreased incidence of early ABMR in 26 highly sensitized recipients with a positive crossmatch against their living donor after treatment with eculizumab. The incidence of ABMR at 3 months was 7.7% (2/26) in the eculizumab group compared to 41.2% (21/51) in the historical control group who received similar plasma exchange based protocol without eculizumab to achieve acceptable crossmatch. The percentage of patients who developed high levels of DSA (MFI > 10,000) in the first three months after transplant was similar in both groups. However, a follow-up on the eculizumab group beyond 1 year reported by Cornell et al. [63] showed similar graft survival rates in the 2 groups at 3 years. A striking finding of the study was the incidence of TG in the anti C5-treated patients who had persistence of DSAs with BFXM > 200 (50% versus 35.7% in control group $P = 0.75$), suggesting other mechanisms for antibody mediated graft injury besides complement activation, that is, ADCC and direct endothelial cell activation. Thus, eculizumab may be helpful in improving short term outcomes in patients who

develop low levels of DSA, but in patients with persistent high levels of DSA, the benefits are lost. In addition, a recent multicenter phase 2 clinical trial of eculizumab for prevention of ABMR in HLA sensitized patients (NCT02113891) failed to achieve the primary composite endpoint (defined as the occurrence of biopsy-proven ABMR, graft loss, patient death, or loss to follow-up at week 9 after transplant).

2.9. C1 Esterase Inhibitor (Berinert®, C1-INH). C1-INH is the only plasma protease that regulates the classic complement pathway [64]. C1-INH can also inhibit the mannose-binding serine protease lectin pathway of complement activation. During C1qrs activation by immune complexes, C1-INH can dissociate C1r and C1s from the activated C1 macromolecule, thus preventing proteolytic activation of C4 and C2, blocking the formation of C3 convertase. We recently completed a blinded, placebo-controlled trial of C1-INH for prevention of ABMR in highly HLA sensitized patients. Twenty patients were enrolled in this phase I/II trial and results showed that no patient in the C1-INH group developed ABMR during the 1-month study period [65]. Analysis of complement levels during the study suggested an important inhibitory effect on systemic complement activation and complement activating antibodies by C1-INH. Montogomery et al. evaluated the use of C1 INH as an add-on therapy to standard of care (IVIg/plasmapheresis) for the treatment of ABMR in a multicenter double-blind randomized placebo-controlled pilot study. While there was no statistical difference between groups in the primary end point of posttreatment day 20 histopathology or graft survival, the C1 INH group demonstrated a trend toward sustained improvement in renal function through day 90. Six-month biopsies showed no transplant glomerulopathy (TG) in the C1 INH group (n = 7), whereas 3 of 7 placebo subjects had TG [66]. Similar results were seen in a single-arm pilot study from France investigating the potential effects and safety of C1-INH added to high dose intravenous immunoglobulin for the treatment of acute ABMR nonresponsive to conventional therapy (IVIg, rituximab, and plasmapheresis). This small study showed significant improvement in allograft function at 6 months and a decrease in complement C1q-binding capacity of DSA together with reduced C4d deposition in allograft capillaries [67]. These results are encouraging and support the need for larger studies of C1-INH in the prevention and treatment of ABMR.

Both anti-C5 and C1-INH are also being investigated in larger clinical trials for prevention of ischemia reperfusion induced delayed graft function (NCT01756508 and NCT02134314).

2.10. Belatacept (Nulojix®). It is an anti-CD80/CD86 humanized IgG1 conjugate with CTLA4 (CTLA4-Ig) that blocks T-cell costimulation [68]. Belatacept was FDA approved in June 2011 for prevention of rejection in renal transplant patients. Recent data regarding the seven-year follow-up of patients in the belatacept clinical trials revealed a significant benefit of belatacept in reducing de novo DSA generation compared to patients maintained on cyclosporine based therapy (4.6% versus 17.8%) at 7 years [69]. Belatacept has not been examined as a desensitization agent, but data from our animal models suggests CTLA4-Ig is a potent inhibitor de novo DSA generation and also modifies DSA rebound responses [70]. Our study suggests that there may be inhibitory effects of CTLA4Ig on plasma cell IgG production in mice. This could prompt further studies of CLTA4Ig as a desensitization agent.

3. Defining Acceptable Crossmatch and DSA Parameters after Desensitization

Several studies have shown that a positive CDC-CXM at time of transplantation is associated with poor outcomes [71, 72]. However, desensitization can reduce alloantibody titers to a level sufficiently low to create an acceptable CXM that allow for transplantation with low-risk for ABMR [73]. In this regard, it is important to recognize that not all DSAs are susceptible to reduction with desensitization. Here, those that are C1q+ and/or have MFI strength ≥ 10,000 are difficult to reduce to an acceptable level. To deal with this, we have adapted a protocol to identify unacceptable antigens as those expected to produce a positive CDC-CXM and when reacted with the sensitized patient's sera [74]. At our center, we define negative CXM by a flow cytometric crossmatch (FCXM) less than 130 mean channel shift (MCS) for B cell and less than 70 MCS for T-cell FCMX after pronase digestion. Pronase treatment is used to remove CD20 from B cells and non-HLA antigens from T and B cells, allowing more precise determinations of HLA specific antibodies and eliminating rituximab effect. DSA binding is determined by the multi-analyte bead assay performed on the Luminex platform. The strength of the reactions is graded as weak (<5000 MFI), moderate (5,000–10,000 MFI), and strong (>10,000 MFI). Antibody specificities and strengths are compared to those obtained before desensitization. To simplify analysis, we have created a scoring system to represent MFI intensity of DSAs. The DSA-RIS (relative intensity scale) gives 0 points for no DSA, 2 points for each weak DSA (MFI < 5,000), 5 points for each moderate DSA (MFI 5,000–10,000), and 10 points for each strong DSA (MFI > 10,000) [4, 74]. Desensitization is considered successful if posttherapy donor-specific CXM is acceptable, as determined by negative CDC in 1 : 2 or higher dilution or FCXM with a shift of less than 225 MCSs and DSA-RIS scores ≤17. Using this approach, we have reduced our ABMR rate to ~16% in the first year after transplant.

A critical aspect which deserves to be mentioned here is the absolute need to develop standardized solid phase DSA assays which will allow reproducibility of results over time and from site to site. Establishing standards for reporting and interpreting data is crucial for comparisons of desensitization therapies [75].

4. Novel Therapeutics: From Use in Desensitization to Potential Application in Treatment of ABMR

Chronic immunologic injury to the allograft is now recognized as the leading cause of allograft dysfunction and

long-term loss [76, 77]. DSAs, which are the target of desensitization strategies, have been implicated in ABMR leading to decreased allograft survival [3, 78, 79]. Hence, advancements in desensitization will potentially have significant implications on ABMR treatment. DSAs can be present before transplant or emerge after transplant in 20–30% patients. These de novo DSAs are mainly class II and associated with a poorer prognosis compared to DSAs to HLA class I [80]. DSAs can damage allografts in several ways including antibody-dependent cellular cytotoxicity, acceleration of graft atherosclerosis, direct endothelial activation, and complement-dependent cytotoxicity, which if untreated can result in rapid loss of the graft [81].

The development of assays to detect complement-fixing DSAs (C1q-DSA Luminex) has provided new insights into the clinical significance of complement-fixing DSAs. Loupy et al. [82] demonstrated a dramatic reduction in long-term allograft survival in patients who developed C1q-DSAs after transplant (HR, 4.78; 95% confidence interval [CI], 2.69 to 8.49). C1q-DSAs in this study also had a higher mean fluorescent intensity score when compared with non-C1q-DSAs indicating a considerable overlap between DSA strength and complement activating capacity. Better understanding of the pathophysiology of ABMR has stimulated interest in development of therapies aimed at depletion of B cells, antibody, and complement inhibition, much like the drugs being studied for desensitization. To date, there are no FDA approved drugs for desensitization or antibody mediated rejection [74].

5. Kidney Paired Donation (KPD)

Paired kidney donation offers another opportunity for transplantation of patients who have a living donor but are HLA incompatible. In many circumstances, it provides a good alternative to desensitization. However, patients who are very broadly sensitized with strong binding HLA antibody will still be difficult to match without the use of desensitization therapies. A combination of desensitization therapies with kidney paired donation may allow for a better immunologic match in such cases. Montgomery et al. [83] reported excellent patient and graft survival with no ABMR at a median follow-up of 13 months in 5 highly sensitized patients who received living donor transplants at Johns Hopkins via paired kidney exchange. One of these patients required desensitization prior to kidney transplantation. Yabu et al. [84] reported successful transplantation of five patients with a cPRA of 100% utilizing this hybrid approach. Utilizing high dose IVIg after transplant, Blumberg et al. [85] reported 100% patient and graft survival at a median follow-up of 22 months in 12 highly sensitized patients with DSA (median cPRA 98%) who underwent transplantation via kidney paired program at UCLA. 3 of these 12 patients had ABMR. Several multicenter consortia have been created in an endeavor to increase the donor pool and facilitate multicenter KPD transplants. A key to success of such multicenter programs is a careful assignment of unacceptable antigens in virtual crossmatch [86]. Unfortunately, the very highly sensitized patients are difficult to find matches for even with desensitization.

6. Financial Implications of Desensitization

Kidney transplant is well established as the most cost-effective strategy for ESRD patients when compared to long-term dialysis. According to the recent US Renal Data System annual report, the cost of maintaining a patient on dialysis is $84,550/yr, and the cost of uncomplicated transplantation is $29,920 but could increase to $106,000/yr with complicating events [87]. However, with a functioning graft, the annual cost per transplant patient is much lower at $18,000.

When discussing desensitization strategies, it is important to study the impact of these interventions on the overall healthcare expenditure. Our group performed a study assessing the cost/benefit analysis of desensitization with IVIg + rituximab compared with dialysis over a 3-year study period. In the study, 71% of patients were successfully desensitized with IVIg + rituximab at a cost of $28,090 followed by renal transplant (80% LD and 67% DD) at a cost of $92,799 for each patient. The cost of continuing dialysis ($84,639 annually per patient) in 29% percent of sensitized patients who were unresponsive to the desensitization regimen was included in the desensitization arm of the model according to an intention-to-treat analysis. After accounting for the cost of treatment of antibody mediated rejection that occurred in 22% of transplanted patients and cost of return to regular dialysis after graft loss that occurred in 19% patients, the analysis showed $18,753 cost saving in the desensitization arm ($219,914 per patient compared with $238,667 per patient treated in the dialysis arm). This amounts to saving 7.9% of 3-year dialysis patient costs. Most importantly, transplantation was associated with a 14.7–17.6% increased survival compared to conventional dialysis [9].

7. Conclusions

The development of desensitization protocols has been a significant advancement in the field of kidney transplantation offering hope for the immunologically disadvantaged group of highly sensitized patients. Despite the recently highlighted successes in desensitization therapies, there is no consensus regarding the need for desensitization and development of new drugs in this area. Clinical trials of novel therapeutic agents are critical to our persistent efforts to increase the longevity of kidney allografts. The collaboration of transplant physicians, immunologists, and pharmaceutical industries is crucial to delineate a path forward to improve access to kidney transplantation.

Competing Interests

Drs. Sethi, Choi, Vo, Peng, and Toyoda declare no conflict of interests. Dr. Jordan has research grants from CSL-Behring and Genentech and has served as a consultant for CSL-Behring, Genentech, and Bristol Myers Squib, Inc.

Authors' Contributions

Supreet Sethi and Jua Choi participated in literature review and preparation of manuscript. Mieko Toyoda participated in data extraction and drafting of the manuscript. Ashley Vo, Alice Peng, and Stanley C. Jordan participated in drafting, critical revisions, and final approval of manuscript.

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Review Article

From Humoral Theory to Performant Risk Stratification in Kidney Transplantation

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The purpose of the present review is to describe how we improve the model for risk stratification of transplant outcomes in kidney transplantation by incorporating the novel insights of donor-specific anti-HLA antibody (DSA) characteristics. The detection of anti-HLA DSA is widely used for the assessment of pre- and posttransplant risks of rejection and allograft loss; however, not all anti-HLA DSA carry the same risk for transplant outcomes. These antibodies have been shown to cause a wide spectrum of effects on allografts, ranging from the absence of injury to indolent or full-blown acute antibody-mediated rejection. Consequently, the presence of circulating anti-HLA DSA does not provide a sufficient level of accuracy for the risk stratification of allograft outcomes. Enhancing the predictive performance of anti-HLA DSA is currently one of the most pressing unmet needs for facilitating individualized treatment choices that may improve outcomes. Recent advancements in the assessment of anti-HLA DSA properties, including their strength, complement-binding capacity, and IgG subclass composition, significantly improved the risk stratification model to predict allograft injury and failure. Although risk stratification based on anti-HLA DSA properties appears promising, further specific studies that address immunological risk stratification in large and unselected populations are required to define the benefits and cost-effectiveness of such comprehensive assessment prior to clinical implementation.

1. Introduction

Circulating anti-donor-specific HLA antibodies (anti-HLA DSA) were recognized in hyperacute rejection in 1969 [1]; however, it took more than 40 years for the transplant community to consider the presence of anti-HLA DSA as the main reason for allograft rejection and long-term failure [2, 3]. There is mounting evidence both experimental and clinical in support of Dr. Terasaki's prediction as outlined in "the humoral theory of transplantation" [4, 5]. Furthermore, the transplant community has recognized circulating anti-HLA DSA detected prior to or after transplantation as one of the most informative biomarkers for predicting worse allograft outcome [6].

Although the detection of anti-HLA DSA is widely used in clinical practice for the assessment of pre- and posttransplant risks of rejection and allograft loss, it has become indisputable that not all anti-HLA DSA carry the same risk for transplant outcomes [7]. These antibodies have been shown to cause a wide spectrum of effects on allografts, ranging from the absence of injury to indolent or full-blown acute antibody-mediated rejection (ABMR) [8, 9]. Consequently, the presence of circulating anti-HLA DSA does not provide a sufficient level of accuracy for the risk stratification of allograft outcome. Enhancing the predictive performance of anti-HLA DSA is currently one of the most pressing unmet needs for facilitating individualized treatment choices that may improve outcomes [7].

Over the last decade, studies have been focused on defining how the level of circulating anti-HLA DSA may explain the substantial phenotypic variability in allograft injury. First, anti-HLA DSA strength (mean fluorescent intensity [MFI] as defined by Luminex single antigen bead testing [SAB]) has been associated with antibody-mediated allograft injury

Table 1: Patterns of Class I and Class II HLA specific antibodies in sensitized renal transplant recipients as determined by various modifications of SAB assay (MFI): total IgG, C1q-screen, and IgG1–4 subtypes.

Specificity	Total IgG (MFI)	C1q (MFI)	IgG1 (MFI)	IgG2 (MFI)	IgG3 (MFI)	IgG4 (MFI)
B53	14522	1247	5280	2023	1022	19999
B35	10128	44	2473	178	1516	20667
A23	11440	89	4733	1413	40	0
A2	10605	0	4265	985	475	4
A68	10062	6	29	3463	3	4
B13	8056	1	2763	88	0	0
DR12	11741	30	3864	89	0	5
DR10	19469	6737	8863	1472	0	1551
DQ6	16639	22113	14577	6045	20	9009
DQ7/DQA1∗05	16592	7431	14151	5467	21	2811
DQ7/DQA1∗03	15287	21936	3901	479	3828	0
DQB1∗05:01	16026	20787	14030	5668	0	8066
DR1	10008	3	2388	12	0	0

and risk of allograft loss. Currently, the strength of anti-HLA DSA defined by MFI is used in allocation policies and immunological monitoring after transplantation. However, recent data have demonstrated that the level of HLA antibodies cannot be determined by SAB testing of undiluted sera and serial dilutions are required to assess the titer of the antibody [10]. In addition, a more comprehensive assessment of circulating anti-HLA DSA that includes their capacity to bind complement and their IgG subclass composition would also provide clinically relevant information with respect to the prediction of allograft injury and loss.

The purpose of the present review is to describe how we improve the model for risk stratification of transplant outcomes in kidney transplantation by incorporating the novel insights of anti-HLA DSA characteristics.

2. Contemporary Multidimensional Assessment of Circulating Donor-Specific Anti-HLA Antibodies

Introduction of multiplex-bead array assays has significantly improved the sensitivity and precision of circulating anti-HLA DSA detection. The benefits and limitations of the solid-phase assays using SAB have been captured in many reviews identifying potential problems that may impact test interpretation of antibody strength and patient management [7, 12]. For example, false positive results may be reported due to antibodies to denatured HLA molecules, or false weak or negative results may occur in the presence of intrinsic and extrinsic factors inhibiting the SAB assay [13]. It was elegantly demonstrated in two studies that the false low MFI in SAB assays, "prozone," was caused by C1 complex formation that initiates classical complement activation culminating in dense C3b/d deposition, thus preventing secondary antibody binding [14, 15]. Furthermore, biologic confounding factors related to epitope-sharing may also impact the MFI values. Currently SABs may provide a semiquantitative measurement of antibody strength but are not approved for quantitative assessment of antibody level. Removing potential inhibitors in the sera with various treatment modalities has improved HLA antibody detection, but it did not address the potential oversaturation of the beads in the presence of high titer antibody. Tambur et al. demonstrated that serial dilution of sera pre-SAB testing provided a reliable measure of antibody strength over time and was informative for monitoring antibody levels pre- and postdesensitization protocols [10, 16].

Although the standard SAB assay has improved the sensitivity of HLA antibody testing, it does not discriminate between complement-binding IgG and noncomplement-binding subclasses [7]. Flow cytometry based detection of HLA antibody using FlowPRA beads was the first cell-independent assay to demonstrate complement activation in vitro [17]. Recently, two SAB assays have been developed to detect C1q- or C3d-binding antibodies [18–27]. The ability of HLA antibody to bind complement has been shown to depend on the composition of IgG subtypes: complement-binding IgG1 or IgG3 versus noncomplement-binding IgG2 and IgG4 subtypes [28]. However, we have shown in sensitized renal transplant recipients that merely the presence of complement-binding IgG subtype in the mixture was not enough to detect C1q-binding antibody [28]. Many studies attempted to show a strong correlation between strength of antibody (>8000 MFI) and C1q-binding reactivity [29]. The best correlation, however, was found between HLA antibody titer >1 : 16 or 1 : 32 and complement-binding ability [10, 30]. We have also compared the neat MFI, C1q reactivity, and IgG subtype level (MFI) in a group of sensitized renal transplant recipients [28]. For example, despite the strong total IgG SAB MFI (8000–11000), C1q reactivity was negative for anti A2, A68, A23, B13, DR12, and DR1; IgG subtypes for these specificities consisted of only low level IgG1 and/or IgG2 (Table 1). In contrast, HLA antibodies that consisted of a combination of multiple IgG subtypes were more often C1q-reactive, as long as one of the subtypes was IgG1 or IgG3 (anti-B53, DR10, DQ6, DQ7/DQA1∗05, DQ7/DQA1∗03, and

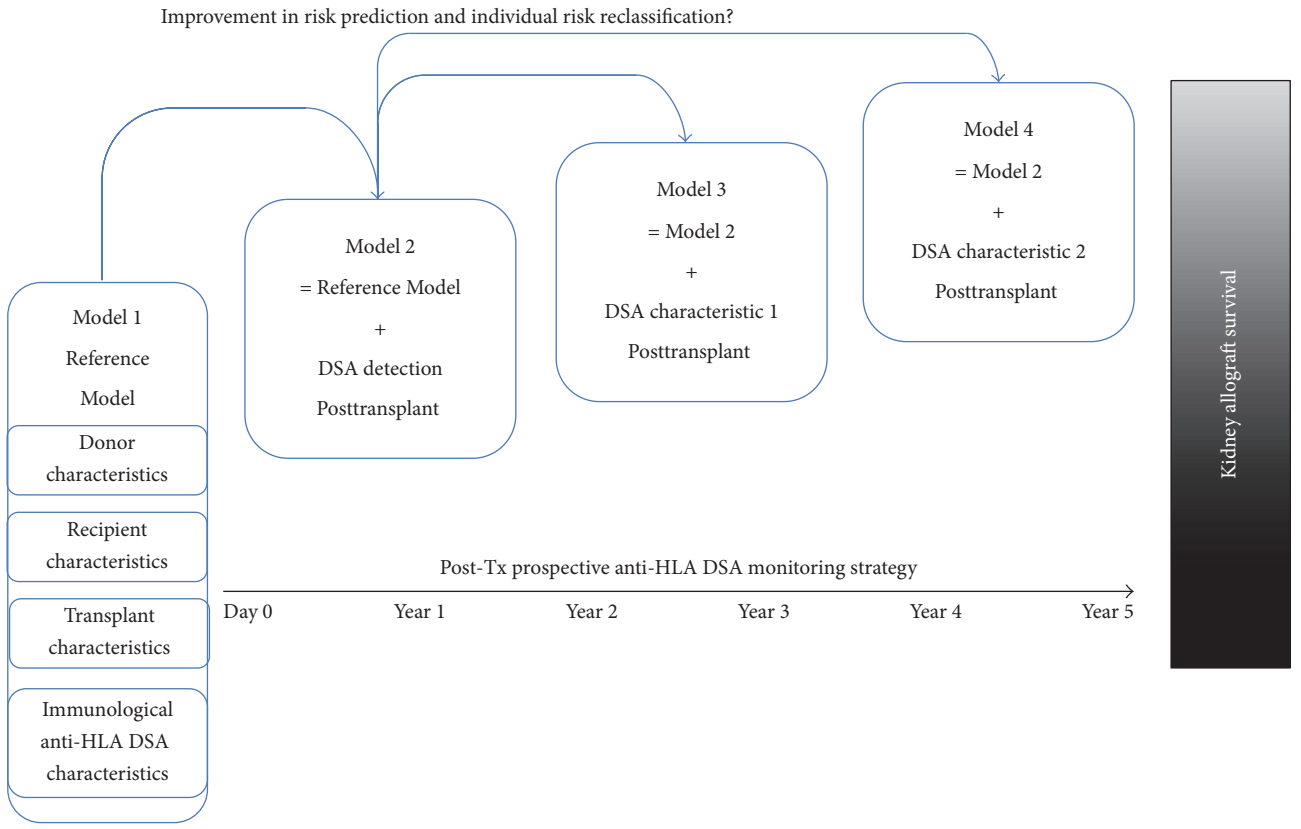

Figure 1: Prospective strategy of dynamic, incremental modeling to assess improvement in risk prediction of allograft loss based on circulating anti-HLA DSA monitoring and characterization. DSA, donor-specific antibody; HLA, human leucocyte antigen; Tx, transplant.

DQB1∗05:01). Interestingly, anti-HLA-B53 was complement-binding even though it consisted of strong level IgG4 but in combination with IgG1, IgG2, and IgG3 whereas anti-HLA-B35 was not complement-binding; it consisted of similar strong level IgG4 in combination with low level IgG1 and IgG3 (Table 1). These few examples illustrate the complexity of complement-binding capacity of HLA antibody and considering the composition of the IgG subtypes and their level may be more informative to predict C1q reactivity rather than the neat MFI of SAB assay. Of note, none of the examples depicted in Table 1 were considered "prozone" since the total IgG MFI was >8000 with or without C1q binding. In contrast, in prozone the SAB MFI value for the HLA antibody is low while the C1q-SAB MFI is high [7, 10, 13, 30]. Removing the complement interference by DTT, heat, or EDTA treatment has improved the interpretation of the SAB assay; however, it did not address the limitations of SAB assay for determining the titer of DSA nor the composition of the IgG subtypes.

In summary, based on the current knowledge of SAB testing, to use a single MFI value to predict clinical outcomes is not sufficient. Comprehensive monitoring to facilitate risk assessment and patient-tailored management should incorporate an algorithm that addresses HLA antibody characteristics.

3. Circulating Donor-Specific Anti-HLA Antibodies for Risk Stratification in Organ Transplantation

The present review was focused on prospective cohort studies that used hard endpoint (allograft loss) among observational studies that assessed the clinical value of anti-HLA DSA in order to provide the best level of evidence. To date, most studies in kidney transplantation have been limited to association analyses between the anti-HLA DSA and ABMR occurrence, allograft histological lesions, or allograft failure. Furthermore, the detection of anti-HLA DSA in an individual patient has not been shown to improve the accuracy of existing prediction model based on conventional risk factors [31]. In contrast, in other fields such as cancer or cardiovascular diseases, emerging biomarkers have made an important impact on risk prediction [32, 33]. A novel strategy using a dynamic integration of anti-HLA DSA and their characteristics should be addressed using dedicated metrics for discrimination and risk reclassification [34–36]. An illustration of such a strategy is provided in Figure 1.

3.1. The Value of Donor-Specific Anti-HLA Antibody Detection for Predicting Outcomes of Kidney Transplantation: Role of Systematic Monitoring. Short-term and long-term kidney

allograft survival have been shown to be substantially worse among patients with pretransplant anti-HLA DSA detected by cell-based assays using complement-dependent cytotoxicity testing [1] or flow cytometry crossmatching [37], compared with both sensitized patients without anti-HLA DSA and nonsensitized patients. This observation remains valid even in patients with preexisting anti-HLA DSA detected only by solid-phase assays such as the SAB Luminex technique with a 1.98-fold increase in the risk of ABMR and a 1.76-fold increase in the risk of allograft failure [38]. Because of the detrimental effect of preexisting anti-HLA DSA on kidney allograft outcome it became important to include this factor in national, regional, and local allocation policies worldwide. These policies have implemented rules to prevent transplantation in the presence of preexisting anti-HLA DSA by defining acceptable and unacceptable mismatches and performing virtual crossmatching [39–41].

In the posttransplant setting, the development of de novo anti-HLA DSA has also been reported to dramatically increase the risk of ABMR and allograft loss. Wiebe et al. [42] found a 10-year allograft survival rate of 57% in patients with de novo anti-HLA HLA DSA compared to 96% in patients without de novo anti-HLA DSA. Recently, the relevance of a prospective strategy of systematic posttransplant anti-HLA DSA monitoring using SAB Luminex for the prediction of the risk of allograft loss was demonstrated at the population level [11]. In this study, the detection of posttransplant anti-HLA DSA improved the performance of a conventional model defined at the time of transplantation (which included donor age, donor serum creatinine, cold ischemia time, and anti-HLA DSA status at day 0) for predicting allograft loss (increase in c-statistic from 0.67 to 0.72) [11].

Importantly, the detrimental effects of posttransplant anti-HLA DSA can occur in the absence of initial allograft dysfunction, and 12 to 58% of sensitized recipients with preexisting or de novo anti-HLA DSA might develop subclinical forms of ABMR and have an increased risk of allograft loss [42–45]. This further emphasizes the need for anti-HLA DSA monitoring to identify patients who might be at risk for developing ABMR. However, the low positive predictive value of anti-HLA DSA for identifying subclinical ABMR [11, 42, 46] has required allograft biopsies to be performed when posttransplant anti-HLA-DSA are detected to accurately determine if subclinical ABMR is present. Recent advances for characterizing anti-HLA DSA have been implemented to improve their predictive performance by identifying harmful anti-HLA DSA that are responsible for allograft injury and failure.

3.2. The Strength of Donor-Specific Anti-HLA Antibodies for Predicting Outcomes of Kidney Transplantation. Currently, the assessment of circulating anti-HLA DSA strength is widely used by transplant centers worldwide to stratify the pre- and posttransplant risks for ABMR and allograft loss [7]. Anti-HLA DSA strength is commonly assessed by the MFI value provided by SAB tests or the mean channel shift provided by cell-based flow cytometry crossmatches [47]. Although determining anti-HLA DSA level by solid-phase assay was not approved by the US Food and Drug Administration as a quantitative measurement [48], studies have defined clinically relevant anti-HLA antibodies detected only by this assay. Several groups have demonstrated correlations between increased MFI/mean channel shift levels and increased incidences of ABMR and allograft loss [49, 50]. These studies may imply that additional clinically relevant information beyond the presence or absence of anti-HLA DSA may be derived by considering the numeric values reported by these assays. Higher strength defined by MFI of circulating anti-HLA DSA have also been correlated with increased microvascular inflammation and increased C4d deposition in the peritubular capillaries of the allograft [47, 51]; thus, a biological relationship exists between anti-HLA DSA strength and the allograft lesion intensity. However, the correlation between the MFI and the antibody level is far from perfect. Despite recent efforts toward the standardization and normalization of solid-phase multiplex-bead arrays [52], there are significant limitations that compromise the use of MFI as a surrogate marker of the antibody level as previously summarized [7, 10, 53, 54]. As a consequence, no consensual threshold for risk categories based on anti-HLA DSA MFI have been defined, a limitation that was pointed out by the Transplantation Society Antibody Consensus Group in 2013 [7]. Recently, Tambur et al. addressed the importance of how best to determine antibody strength and have suggested that the quantification of the antibody level is best achieved by titration [10]. However, the use of anti-HLA DSA titration to predict ABMR and allograft loss has not been incorporated in the routine assessment of anti-HLA DSA and for patient management.

3.3. Additional Value of the Complement-Activating Capacity of Donor-Specific Anti-HLA Antibodies for Predicting Outcomes of Kidney Transplantation. Since the pioneering discovery in 1969 that anti-HLA antibodies are lymphocytotoxic [1], activation of the complement cascade has been considered to be a key component of antibody-mediated allograft rejection. However, complement-dependent cytotoxicity assays lack sensitivity and specificity and cannot be used in large scale in transplantation follow-up. The recent development of sensitive solid-phase assays for detecting complement-binding anti-HLA antibodies has revealed novel insights into the associations between anti-HLA DSA and transplant outcomes. Growing evidence supports the notion that the capacity of anti-HLA DSA to bind complement significantly improves our ability to predict ABMR and allograft loss. The clinical relevance of posttransplant complement-binding anti-HLA DSA detected using C1q or C3d assays has been recently shown by several groups in kidney transplantation in the United States and in Europe [18, 20–27] and has also been extended to other solid transplant organs, including heart [19, 30], liver [55], and lung [56]. In the study by Loupy et al. [24], posttransplant C1q-binding anti-HLA DSA detected within the first year after transplantation were found to be an independent determinant of allograft loss with a 4.8-fold increased risk.

Patients with posttransplant C1q-binding anti-HLA DSA exhibited a higher incidence of ABMR and an increased rate of allograft injuries, including microvascular inflammation,

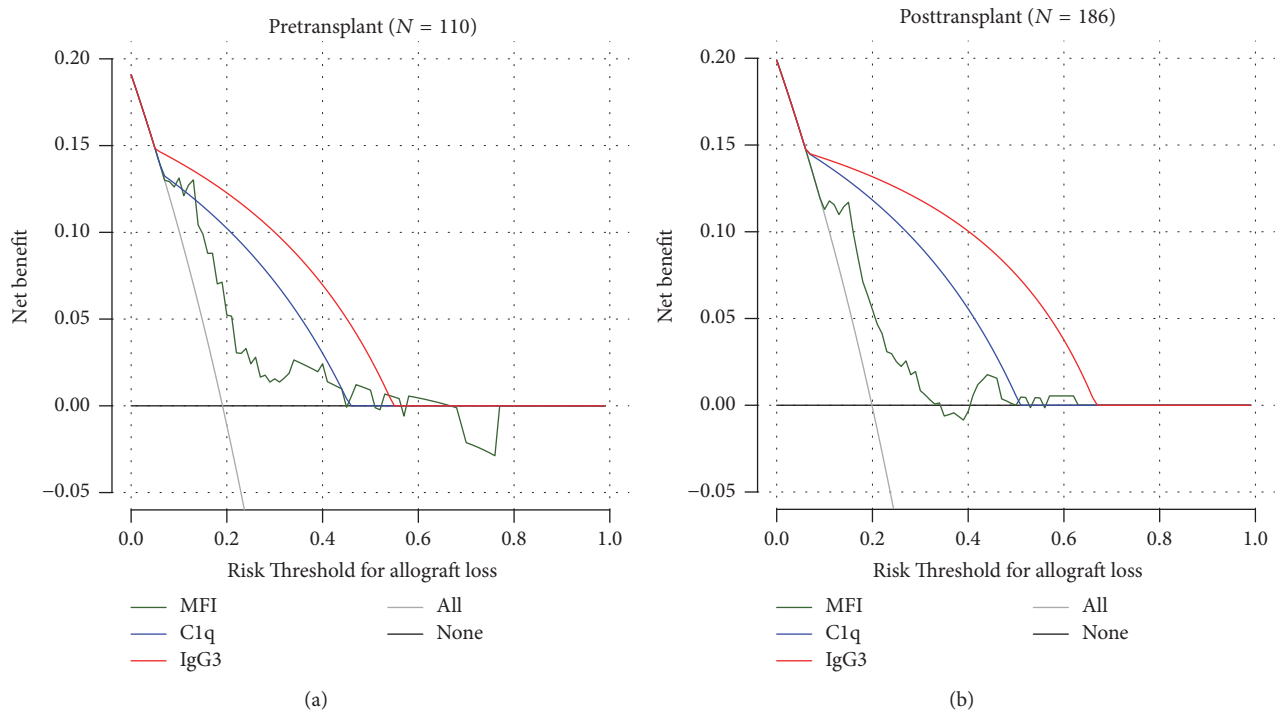

Figure 2: Improvement in clinical decision-making provided by circulating anti-HLA DSA characterization beyond antibody detection: decision curve analysis. Data are based on a prospective study performed in 851 kidney transplant recipients who were screened for the presence of circulating anti-HLA DSA at the time of transplantation, systematically at 1 and 2 years after transplantation, and at the time of any clinical event occurring within the first 2 years after transplantation [11]. Net benefit is shown in the 110 patients identified with pretransplant anti-HLA DSA (a) and in the 186 patients identified with posttransplant anti-HLA DSA (b). Net benefit of a clinical intervention is provided assuming that all patients will lose their graft at 5 years after transplantation (grey) and none of patients will lose their graft at 5 years after transplantation (black), based on anti-HLA DSA MFI level (green), C1q-binding status (blue), and IgG3 subclass status (red). The net benefit is determined by calculating the difference between the expected benefit and the expected harm associated with each decisional strategy. The expected benefit is represented by the number of patients who will lose their allograft and who will undergo clinical intervention (true positives) using the proposed decision rule. The expected harm is represented by the number of patients without allograft loss who would undergo clinical intervention in error (false positives) multiplied by a weighting factor based on the risk threshold. The highest curve at any given risk threshold is the optimal strategy for decision-making in order to maximize net benefit.

tubular and interstitial inflammation, endarteritis, transplant glomerulopathy, and C4d deposition in the peritubular capillaries compared with patients with nonC1q-binding anti-HLA DSA and patients without anti-HLA DSA [24].

Many centers feel that MFI strength is the best predictor of anti-HLA DSA pathogenicity and complement-activating capacity. Recently, anti-HLA DSA complement-binding status following transplantation has been shown to be associated with ABMR occurrence and allograft loss independently of the anti-HLA DSA MFI [24, 57], suggesting an additional value beyond MFI level for outcome prediction. Our team confirmed in a recent prospective study [11] that the detection of complement-binding anti-HLA DSA improved the prediction accuracy for allograft loss at the population level. In this study, the information provided by anti-HLA DSA complement-binding capacity adequately reclassified the individual risk of allograft loss in more than 62% of patients compared with anti-HLA DSA MFI level alone.

3.4. The IgG Subclass Composition of Donor-Specific Anti-HLA Antibodies for Predicting Outcomes of Kidney Transplantation. The determinants of anti-HLA DSA complement-binding capacity are complex as discussed previously, including the presence of complement-fixing IgG subclasses (IgG1 and IgG3) and the levels of IgG subclasses [29, 30] (Table 1). Experimental data suggest that antibodies exhibit different abilities to bind complement, to recruit immune effector cells through the Fc receptor, and to display different kinetics of appearance during the immune response according to their IgG1-4 subclass status. [58–60]. Emerging data support the clinical relevance of the IgG subclass composition of anti-HLA DSA and their relationships with allograft injury phenotype and survival in kidney [11, 28, 61–63] and liver [55, 64] transplantation. In particular, several teams have showed a significant association between the IgG3 subclass status of circulating anti-HLA DSA and worse transplant outcome [11, 28, 55, 61, 63, 64].

In a study [28] that included 125 kidney transplant recipients the majority of patients with IgG3 anti-HLA DSA that were detected within the first year after transplantation had acute clinical ABMR that was characterized by intense microvascular inflammation and increased complement deposition in the allografts. In contrast, the majority of

patients with IgG4-containing anti-HLA DSA had features of subclinical ABMR with a predominance of chronic features represented by transplant glomerulopathy and interstitial fibrosis. In this study, the IgG3 and IgG4 positivity showed good predictive performance to identify patients with clinical and subclinical ABMR, respectively. Furthermore, it was also shown that circulating anti-HLA DSA IgG3 status improved the performance of MFI level in predicting the individual risk for allograft loss in more than 76% of patients [42].

Overall, in future studies we should evaluate how IgG subtype information may add value to the assessment of sensitized patients and to our current available tools for anti-HLA DSA analysis.

4. Risk Stratification Based on Donor-Specific Anti-HLA Antibody Characterization for Transplant Outcome Management

The ultimate goal of accurate risk stratification for allograft injury and failure is to improve clinical transplantation outcomes. The risk-stratified approach is greatly needed to tailor therapeutic strategies in the pre- and posttransplant periods, incorporating predicted risks for adverse outcomes to maximize benefits and minimize harms and costs from medical care (Figure 2) [65]. Moreover, risk stratification is also needed to improve our ability to design and interpret therapeutic trials [66]. Averaged results of clinical trials may obscure treatment effect on specific populations, because their aggregated results including patients at various risk levels can be misleading when applied to individual patients [67]. Finally, the risk-stratified approach using anti-HLA DSA properties has direct consequences for patient care. In the pretransplant setting, this approach has the potential to increase allocation policy efficiency by providing more reliable discrimination of the antibodies that are more or less harmful, thereby potentially expanding the donor pool for sensitized patients. In hypersensitized patients with an insufficient stream of potential donors, immunological risk stratification will help to more accurately select the patients in whom specific intensive pretransplant conditioning should be considered to eliminate deleterious antibodies. In the posttransplant setting, systematic monitoring and characterization of circulating anti-HLA antibodies provide a non-invasive tool for clinical decision-making regarding further tests and treatment. In terms of therapeutic strategies, risk assessment based on anti-HLA DSA properties could provide a basis for more targeted pathogenesis-driven therapies. The identification of specific injury phenotypes based on anti-HLA DSA characteristics could provide a rationale for the development of more specific therapeutic approaches, such as B-cell depletion with rituximab in patients with IgG4-associated allograft injury [68] and complement blockade using the C5 inhibitor [69, 70] Eculizumab or C1 inhibitors [71, 72] in patients with complement-binding and/or IgG3-positive anti-HLA DSA. Thus, collaborative prospective analysis of anti-HLA DSA using multiple assays will be critical to reconcile these issues and to create recommendations for best practices.

Competing Interests

The authors declare that they had no financial relationships with any organizations that might have an interest in the submitted work and no other relationships or activities that could appear to have influenced the submitted work.

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Review Article

The Humoral Theory of Transplantation: Epitope Analysis and the Pathogenicity of HLA Antibodies

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Central to the humoral theory of transplantation is production of antibodies by the recipient against mismatched HLA antigens in the donor organ. Not all mismatches result in antibody production, however, and not all antibodies are pathogenic. Serologic HLA matching has been the standard for solid organ allocation algorithms in current use. Antibodies do not recognize whole HLA molecules but rather polymorphic residues on the surface, called epitopes, which may be shared by multiple serologic HLA antigens. Data are accumulating that epitope analysis may be a better way to determine organ compatibility as well as the potential immunogenicity of given HLA mismatches. Determination of the pathogenicity of alloantibodies is evolving. Potential features include antibody strength (as assessed by antibody titer or, more commonly and inappropriately, mean fluorescence intensity) and ability to fix complement (*in vitro* by C1q or C3d assay or by IgG subclass analysis). Technical issues with the use of solid phase assays are also of prime importance, such as denaturation of HLA antigens and manufacturing and laboratory variability. Questions and controversies remain, and here we review new relevant data.

1. Introduction

Central to the humoral theory of transplantation so closely identified with the pioneering work of Terasaki [1, 2] is the ability of the recipient's immune system to produce antibodies against donor mismatched HLA antigens, as well as other polymorphic systems. HLA matching determines that a given transplant can proceed without fear of hyperacute rejection initially, while at the same time minimizing the chances of acute and/or chronic alloimmune mediated rejections in the longer term. In addition, a prominent concern is that sensitization induced by HLA mismatches may impair the ability to receive future transplants should the initial one fail. However, not all mismatches (MMs) result in sensitization, and not all antibodies preclude transplantation. Furthermore, not all antibodies detectable after transplantation injure a graft, whether persisting from pretransplantation or developing *de novo* [3]. In this review, we address recent data relative to 2 important issues regarding antibodies in solid organ transplantation: the role of epitope analysis in optimizing HLA matching and the assessment of the pathogenicity of HLA antibodies.

2. Epitopes in HLA Matching

The determination of the three-dimensional structure of an HLA molecule by Cn3D modelling together with amino acid (AA) sequencing led to the definition of polymorphic AA residues on the surface of the molecule accessible to antibody binding. An antibody does not recognize an entire HLA molecule but rather a 15 to 25 AA segment termed an epitope [4]. Epitopes have an area of 700–900 A^2 within a radius of about 15 Å that represents the *structural* epitope. The corresponding antibody binding surface (paratope) contains 6 complementarity determining regions (CDR), 3 in the hypervariable region of the light chains and 3 in the hypervariable region of the heavy chains.

At the center of an epitope is a polymorphic region of 1 to several AAs within a 3 Å radius, termed an eplet or alternatively the *functional* epitope. These eplets need not be continuous AAs, but they must lie upon protein folding within the 3 Å radius. The third and most variable CDR of the heavy chain lies in the center of the paratope and recognizes the foreign nature of the mismatched eplet that defines the functional epitope. The other 5 CDRs allow for stabilization of the synapse. Eplets are named by their amino acid sequence number followed by one or more AAs. Many epitopes are defined simply by the functional epitope (eplet) alone, whereas others require pairing of that eplet with one or more additional residues within the structural epitope. These secondary configurations may be superficial on the surface of the molecule, where they interact with another CDR. Other times they are hidden, often in the peptide grove, but in this case they have their effect by altering the configuration of the functional eplet.

Duquesnoy developed the HLAMatchmaker program (http://www.HLAMatchmaker.net/) that predicts epitopes based on surface expression of polymorphic amino acid(s) located within a 3 Å radius. This program has the ability to determine epitope specificities of highly sensitized individuals and, by intra- and interlocus subtraction, to compare eplet mismatches between 2 individuals (donor and recipient) [5]. While initially aimed at identifying polymorphisms in three consecutive amino acids (triplets) of class I alleles, newer versions consider 1 to several polymorphic amino acids within a 3 Å radius, including both continuous and discontinuous residues. Class II epitopes have been described as well [6].

The ability of an epitope to be antigenic has been verified by monoclonal antibody binding to single antigen beads by Professor Terasaki's group. Mouse monoclonal antibodies or human alloantibodies absorbed and then eluted from single antigen cell lines, or other sources of single antigens, were tested with single antigen beads (SAB). AAs common to all reactive beads were determined by comparing AA sequences as listed in the Anthony Nolan website. From 1 to 4 AAs common to all reactive alleles that were exposed on the surface and within area of 750 A^2 were used to define an epitope [7]. By this method, 110 class I, 83 class II, 7 MHC class I chain related gene A (MICA), and 96 cross-reacting epitopes (found in nontransfused healthy males or cord blood) have been defined [8] and named TerEps in Professor Terasaki's honor.

Duquesnoy and Marrari correlated HLA class I TerEps with HLAMatchmaker defined epitopes and found 90% concordance, although about 10% could not be reconciled [9]. Similarly for class II, 49 of 53 HLA-DR TerEps corresponded to HLAMatchmaker eplets, as did 17 of 18 DQ TerEps [10]. Many more eplets are predicted by HLAMatchmaker that have not yet been verified by antibody binding. The international Registry of Antibody Defined HLA Epitopes developed a website (http://www.epregistry.com.br) to record all possible HLA epitopes, denoting those that have been antibody verified [11, 12]. This registry contains 5 databases, HLA-ABC, HLA-DRB, HLA-DQ, HLA-DP, and MICA. As of July, 2016, 289 HLA-ABC epitopes are listed on the website, with 81 noted to be antibody verified. One hundred forty-three HLA-DRB epitopes have been identified, of which 25 are antibody verified.

In the case of HLA-DQ, the α and β chains are considered separately in the registry with 60 β-chain epitopes (16 antibody verified) and 25 α-chain epitopes (3 antibody verified) listed. The registry does not consider epitopes defined by specific α/β parings but determines epitope specificity solely by the β-chain [12]. However, Tambur et al. provide evidence that specific α/β-chain parings can determine a DQ epitope, not either chain in isolation. For example, using Cn3D software and HLAMatchmaker analysis, 39 of 40 recipients with failed allografts had *de novo* DQ antibodies whose paratope covered both α and β chains [13].

A given alloantibody may be specific for a particular eplet, such that *every* allele carrying that eplet will react with that antibody. Another alloantibody may also react with that same eplet, but *only* if paired with one or more particular additional residue(s). Alleles containing the eplet without the additional residue(s) will not react. These additional eplets are often identical with those of the recipient. This has led to the "non-self-self" theory of epitope recognition, but these additional eplets can also be nonself, or even locus specific monomorphic residues [9]. The additional epitope is usually on the surface, but it can also be hidden with an effect via conformational alteration of the functional eplet [9]. The annotation of such multiply defined epitopes utilizes a "+" sign between the required eplets.

Over half of defined HLA class I epitopes are restricted to a single antigen (private epitopes), whereas the others are shared by 2 or more antigens (public epitopes) [14]. Such public epitopes result in both intra- and interlocus cross-reactions, often referred to as cross-reactive groups (CREGs) [13]. This explains the development of apparent nondonor specific HLA sensitization following solid organ transplantation, although the epitopes are indeed donor specific (discussed below). Also, individual alleles usually contain multiple epitopes. For example, El-Awar et al. found between 6 and 19 epitopes for each HLA-A, HLA-B, and HLA-C antigen [8]. Similarly, HLA-DR antigens had between 8 and 21 epitopes per antigen and DQB chains between 4 and 8.

To better predict and understand the immunogenicity of HLA molecules, the group from Cambridge assessed the physiochemical properties of polymorphic AAs that define eplets, including hydrophobicity and electrostatic charge disparity [15–18]. The binding of antibody to antigen is initially determined by electrostatic interactions [19] and is stabilized by hydrogen bonding, salt bridges, and van der Waals forces [15, 20, 21]. Kosmoliaptsis et al. assessed immunogenicity by determining the AA sequences of HLA molecules (http://www.ebi.ac.uk./imgt/hla/) and then the number of AA mismatches (AAMs) after interlocus subtraction between 32 highly sensitized patients and alleles of a panel of class I SABs exposed to their sera [15]. Both the presence and magnitude of positive responses were highly correlated with the number of AAMs, confirming their prime importance. Independently, however, hydrophobicity mismatch scores (HMS) and electrostatic mismatch scores (EMS, determined as the sum of the difference in isoelectric

points of each mismatched AA) also correlated with reaction frequency. The immunogenicity of class II antigens was also shown to be related to the number of AAMs, although it was not independently related to HMS or EMS by multivariable analysis [22].

Kosmoliaptsis et al. generated atomic resolution 3D structural models of HLA class I molecules and calculated the surface electrostatic potential [17]. Using the public Bw4 and Bw6 epitopes as examples, a remarkable consistency of the surface electrostatic potential was shown among all alleles expressing either epitope that reacted with a given alloantibody. AA substitutions that did not affect surface potential did not affect binding, whereas those that did affect surface potential abrogated binding [17, 23].

Finally, immunogenicity of class I HLA mismatched epitopes may depend on the DR phenotype of the recipient. The ability of recipient CD4 T-cells to respond indirectly to donor HLA class I epitopes depends on the ability of such epitope to be presented in the groove of the recipients HLA class II DR molecules on antigen presenting B-cells. Certain anchor amino acids in the peptide are preferred, and this can be predicted [24]. These epitopes have been designated "predicted indirectly recognizable HLA epitopes, HLA class II presented" (PIRCHE-II) [25]. Hence, not all mismatched eplets can be presented to CD4 cells which would inhibit appropriate class switching (IgM to IgG) of B-cells potentially reactive to those eplets. Otten et al. found 49 HLA class I mismatches containing HLAMatchmaker predicted immunogenic eplets among 21 recipients following allograft nephrectomy [25]. DSA were detected for 38 of these mismatches, and these immunogenic epitopes contained a larger number of PIRCHE-II compared to nonimmunogenic ones. Interestingly, 68% of PIRCHE-II epitopes were not part of HLAMatchmaker designated eplets.

3. HLA Matching and Epitope Analysis

Current solid organ allocation in the US and elsewhere relies on low resolution serologic HLA matching to determine calculated PRA values. A major question is whether it is worth the time and expense to incorporate epitope analysis in order to better define the immunogenicity of an HLA mismatch, whether by simply determining the AAM load, the EMS, documented antibody binding (TerEps), PRICHE-II epitopes, or the number of epitope mismatches (MMs) using HLAMatchmaker. Data are accumulating in support of these methods as a means of predicting subsequent sensitization, as well as transplant outcomes.

Using the original HLAMatchmaker algorithm that considered defined epitopes based on polymorphic amino acid triplets [26], studies on the utility of epitope matching of class I antigens (HLA-A and HLA-B) in predicting graft survival compared to standard HLA matching have given conflicting results. Some studies show a benefit on graft survival [27, 28], whereas one large study did not [29]. In an earlier study, Laux et al. compared epitope mismatches (EpMMs) at the DPB1 locus (defined as amino acid differences in 4 of the 6 hypervariable regions) with conventional allelic matching in 1,478 kidney retransplant patients [30]. Patients with 2 allelic mismatches but with only 0–2 EpMMs had better 2-year graft survival than those with 1 allelic mismatch but more than 3 EpMMs.

More recently, Wiebe et al. evaluated the development of *de novo* class II (DR and DQ) donor specific antibodies (DSA) in 286 recipients using the current HLAMatchmaker program integrated with antibody verification (TerEps) [31]. Locus specific EpMMs were significantly more frequent in those who developed *de novo* DSA (dnDSA), whereas high resolution typing alone was not helpful. The optimal thresholds below where dnDSA were highly unlikely were 10 epitope mismatches for HLA-DR and 17 for HLA-DQ. Overall, 6 epitopes (3 DFR and 3 DQ) were immunodominant, 5 of which correlated with TerEps. Epitope mismatches at the DP locus were not associated with development of anti-DP DSA.

In a follow-up study of 195 recipients with prospective evaluation of medication adherence, Wiebe et al. evaluated the interaction of noncompliance with these thresholds in predicting late acute rejections and graft loss [32]. The combination of ≥17 DQ EpMMs and medication noncompliance was associated with 3 times the rate of rejection and graft loss as compared to all other groups and ≥10 DR EpMMs plus noncompliance nearly double so. This study suggests that patients under consideration for immunosuppression minimization/withdrawal should have epitope analysis performed.

The Clinical Trials in Transplantation-09 was a multicenter trial aimed at determining if tacrolimus could be safely withdrawn at 6 months from immunologically quiescent patients [33]. It was terminated very early after only 21 patients were enrolled (14 in tacrolimus withdrawal arm) due to safety concerns, as 8 of the 14 withdrawal patients developed acute rejection and/or dnDSA. Using the DQ EpMM cut-off of 17 noted above, 7 of 13 patients with ≥17 DQ EpMMs as defined by HLAMatchmaker developed dnDSA as compared to 0 of 8 with <17 EpMMs ($p = 0.028$). Obviously, these numbers are too small to draw any firm conclusions, but together with the data of Wiebe et al. cited above they do indicate further research is warranted.

Sapir-Pichhadze et al. compared HLA-DRB1/3/4/5, DQA1, and DQB1 EpMM loads using HLAMatchmaker in 52 patients with transplant glomerulopathy (TG) and 104 case-controls [34]. A significantly increased odds ratio (OR) for having TG was found for the highest and middle tertiles of HLA-DR + DQ MMs as compared to the lowest tertile. This held whether modelled as a binary variable or a continuous variable. Surprisingly, DR eplet load appeared to confer a greater risk than DQ eplet load, similar to the findings of Kosmoliaptsis et al. for the immunogenicity of class II HLA [16].

The significance of epitope matching on sensitization following allograft failure has been studied. Singh et al. from our institution studied 66 previously nonsensitized patients that received a kidney transplant that subsequently failed and found that 34 became highly sensitized (cPRA ≥ 80%) [35]. Epitopes were assessed by HLAMatchmaker. Multivariable analysis revealed that DQB1 EpMMs, immunosuppression withdrawal, and the graft intolerance syndrome were significantly associated with high sensitization, whereas HLA-A,

HLA-B, DRB1/3/4/5, and DQA1 EpMMs were not. These data highlight the potential importance of epitope matching in those who may require retransplantation, such as pediatric or young adult cases.

Lachmann et al. studied 54 patients with failed kidney allografts: 28 with concurrent nephrectomy/immunosuppression withdrawal (IWD), 14 with just nephrectomy (prior IWD), and 12 with just IWD [36]. HLA antibodies were detectable in 100%, 100%, and 92%, respectively. An increase in breath and intensity of antibodies against class I antigens followed nephrectomy, whereas an increase in class II followed IWD. In a subgroup of 9 patients with concurrent nephrectomy/IWD, epitope specificities were determined by monoclonal antibody binding (TerEps). Overall, they identified 26 mismatched donor epitopes in these 9 patients. Although only 18 of 243 class I antibodies in these patients' serum could be called DSA by traditional matching, 145 of the remaining 225 non-DSA HLA were actually donor epitope specific antibodies (DESA). These data again suggest that epitope matching may reduce sensitization after allograft failure, an issue of prime importance for those requiring retransplantation.

Kosmoliaptsis et al. studied 131 patients with a failed first kidney transplant. Initially, they determined that standard matching (0, 1, or 2 MMs) at HLA-A, HLA-B, HLA-C, DRB1, 3, 4, 5, and DQB1 loci contributed independently to HLA sensitization with an incremental effect [37]. Subsequently, they determined in this same cohort that the AAM score (AMMS), the epitope mismatch score (EpMMS), and the electrostatic mismatch score (EMS) contributed independently to sensitization [18]. All 3 scores independently correlated to HLA-DRB1/3/4/5 and DQ DSA, but only EMS did so with HLA-A and HLA-B DSA. For these analyses they used the algorithms freely available online (http://www.hlaimmunogenicity.org/download/Cambridge_HLA_Class_I_Immunogenicity_Algorithm.xls for class I and http://www.hlaimmunogenicity.org/download/Cambridge_HLA_Class_II_Immunogenicity_Algorithm.xls for class II).

In summary, it is still unclear whether epitope matching should be incorporated into the HLA matching algorithm of all potential kidney transplants. Optimally, it requires high resolution (4 digit) typing, as compared to the low resolution (2 digit) typing currently required by UNOS, which may add significantly to cost and labor [38]. Technology is improving, however, with turnaround time down to several hours. Furthermore, the most likely 4-digit allele can be statistically predicted from serologic typing based on demographic features and haplotype frequencies [39], as is used by the Be The Match Registry of the National Marrow Donor Program (https://bioinformatics.bethematchclinical.org/). Some feel this approach is warranted [38], whereas others feel sufficient information can be obtained from serologic matching with consideration of known CREGs in place of epitopes [40]. Directionality was noted in the protective effect of CREG matching, a finding suggesting that more than just AMMs are involved [40]. The optimal method of epitope analysis is also unclear, be it a simple AMMS, an HLAMatchmaker defined EpMMS, an antibody defined EpMMS, PIRCHE-II, or an EMS.

In our opinion, all highly sensitized patients on the waiting list should have epitope analysis by some method to better define acceptable and unacceptable mismatches. The Eurotransplant Acceptable Mismatch program now incorporates the HLAMatchmaker program for such analysis [41]. In patients likely to need another transplant, epitope analysis for optimal matching of the first transplant is also warranted. At least one pediatric transplant center now incorporates class II epitope matching using HLAMatchmaker when confronted with 2 potential donors (e.g., parents) [42], and we feel this should be the norm. Finally, the degree of EpMM should potentially be assessed in any patient considered for immunosuppression minimization or withdrawal, although more data are clearly required.

4. Pathogenicity of HLA Antibodies

Not all antibodies capable of reacting with a kidney allograft are necessarily pathogenic, and significant variability exists in those that are. Initially, the detection of HLA antibodies was dependent on their ability to lyse lymphocytes in the presence of complement, the complement dependent cytotoxicity crossmatch (CDCXM) assay, which is still in use as the final determinant of the safety of a particular transplant [43]. Subsequently, flow cytometry crossmatch (FCXM) technology was developed with higher sensitivity, and it remains in use for the majority of current kidney transplants. In general, outside of desensitization protocols, the presence of DSA detected by either type of crossmatch would abrogate the transplantation, but various exceptions exist [44].

The current standard, however, for evaluating HLA antibodies in most laboratories involves HLA molecules attached to a solid phase matrix, typically a bead, which may contain multiple HLA antigens from multiple cell lines per bead (mixed antigen beads), multiple HLA antigens from a single cell line per bead (phenotypic beads), or a single HLA antigen (single antigen beads or SAB) [45]. The breath of sensitization ("panel reactive antibody" or PRA) is determined along with donor specificity (DSA). These bead-based assays are typically used in conjunction with the cell-based assays (CDCXM, FCXM) to determine if a given transplant should proceed, a decision that also depends on the immunologic risk of a recipient as determined by sensitizing events (prior transplantation, pregnancy, and transfusions), PRA, quality of life on dialysis, life expectancy, and so forth. Various algorithms have been in use [46]. In our laboratory at Thomas Jefferson University Hospital, SABs are used for screening, whereas other centers prefer a screening assay with beads derived from cell lines [47]. In our laboratory all patients undergo autocrossmatch, T and B-cell CDCXM, and FCXM in all live donors and any deceased donor when recipient has any past or current sensitization. If SABs used for screening are completely negative in a patient with no sensitizing events, crossmatching may be superfluous [44, 48].

Features that are possibly significant in determining the pathogenicity of any given DSA include antibody isotype (IgM versus IgG), class specificity (HLA class I versus class II), mean fluorescence intensity (MFI), antigenic specificity (i.e.,

native HLA class I molecules (nHLA) complexed with β2-microglobulin and peptide versus denatured HLA (dHLA) containing α-chain only), titer, and ability to fix complement. The latter may be demonstrable by detection of C4d in allograft tissue histologically, or by *in vitro* detection of C1q, C3d, or C4d on synthetic surfaces containing alloantigen epitopes after exposure to patient serum. Also, immunoglobulin subclass determination may be relevant given the differential ability of IgG subclasses to fix complement and recruit inflammatory cells.

Before transplantation, the first order is to predict which DSA will result in a positive crossmatch to obviate unnecessary incompatible offers, as well as to enhance the chances of highly sensitized patients receiving a kidney. In addition, the ability to predict which pretransplant DSA will result in acute antibody-mediated rejection (AAMR) and/or affect long term graft survival (GS) is critical to guide post-transplantation surveillance and immunosuppression. Any DSA detected after transplantation, whether persisting from pretransplantation or developing *de novo*, requires the same scrutiny owing to their documented association with both acute and chronic AMR, as well as graft and patient survival.

5. Antibody Isotype

IgM autoantibodies are not pathogenic, although they may result in a positive CDCXM that would be determined by a concurrent positive auto-CDCXM. IgM alloantibodies have been considered to be of low or no potential pathogenicity [49–51], and their presence has even been linked to a more favorable outcome [52]. By contrast, reduced allograft survival has been reported [53]. More recently, a potentially pathogenic role for IgM alloantibodies was detected in eculizumab-treated patients receiving a positive crossmatch kidney transplant, as posttransplantation IgM DSA were found in 3 of 3 such patients with early AAMR, as compared to 1 of 23 without rejection ($p = 0.006$) [54, 55]. In a study of 179 nonsensitized kidney transplant patients, 100 developed DSA, including 53 with IgM alone and 42 with concurrent IgG DSA [56]. A complete IgG subclass profile was not performed, although IgG3 specifically was determined. Only, 5 had IgG DSA without any detectable IgM. IgM alone was not associated with reduced allograft survival, but the 19 IgG3 positive patients with persisting IgM DSA did have reduced allograft survival ($p = 0.02$). IgM alloantibodies may be pathogenic, but more research is obviously needed to clarify this issue. In any case of AAMR not explainable by IgG alloantibodies, we would search for IgM antibodies.

6. Class Specificity

Preformed class I antibodies are the major mediators of hyperacute rejection as well as early AAMR. Class II antibodies can also be involved, alone or in combination with class I antibodies [57, 58]. Class specificity of pretransplant DSA does not seem to be an independent variable for determining adverse outcomes by multivariable analysis [59–61]. Class II DSA after transplantation, however, are more deleterious than those against class I for predicting risk of TG and GS [62–64]. This is especially true for DQ antibodies, which are also the most common to develop *de novo* after transplantation [62, 64, 65]. Several factors may explain the prominence of DQ antibodies after transplantation, including linkage disequilibrium with DR [66], *cis/trans* combinations, greater potential peptide variability, and a high degree of self-chain paired with nonself epitope [67]. Further support of the importance of class II loci, especially DQ, comes from a recent registry study from Australia and New Zealand, where mismatches at the DQ locus were significantly associated by multivariable analysis with any rejection or specifically late rejections. In those with 1 or 2 mismatches at the DR locus, any mismatch at the DQ locus was significantly associated with AMR as well [68].

7. Mean Florescence Intensity

The single antigen bead assays currently in use are approved as semiquantitative analyses only. The way to assess the potential concentration of antibodies is usually by mean fluorescence intensity (MFI). Only an imperfect correlation exists, however, between MFI and true antibody concentration. Variability in MFI may arise from manufacturing issues regarding density and quality of HLA antigens attached to SAB [69], as well as operator implementation of protocols [45]. Even under conditions of optimal standardization and normalization, the coefficient of variation (CV) between laboratories is 20% to 25% at best [45]. This is above the standard FDA requirement of 15–20% maximal CV to be considered a quantitative test. Numerous factors additionally may alter the relationship between observed MFI and actual antibody concentration and pathogenicity. In states of marked antibody excess where beads are completely saturated, MFI would underestimate antibody concentration. This can be shown by simple dilution, which would not result in a corresponding reduction of MFI. A given antigen may have multiple alleles, each represented on individual beads, and this would tend to lower the MFI relative to an antigen with fewer alleles represented [70]. An antibody directed against a public epitope (e.g., Bw4) would be spread out over multiple beads, resulting in a falsely low MFI, as would relatively low antigen expression with use of phenotype or mixed antigen screening beads [71].

A well-known phenomenon resulting in a marked underestimation of true antibody concentration is often termed the "prozone effect." Initially, this was ascribed to the inability of the detection antibody to bind the HLA antibody on the bead as a result of interference from bound C1. More recently, the downstream complement activation products C4d and especially C3d have been shown to be responsible [72]. Again, a prozone effect can be uncovered by simple dilution, where the MFI would in this case increase. Also, it can be reversed by disruption of the C1 component of complement by ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), or heating [73]. Heat and DTT denature C1q, and EDTA chelates calcium to inactivate C1q binding [74]. Interference from IgM antibodies has also been reported, [75] as well as from

undefined serum factors, and these effects can be mitigated by hypotonic dialysis or DTT [76]. At a minimum, we feel that some methods, such as EDTA or a simple one-time dilution, should be routinely used to eliminate concerns over the prozone effect.

Alloantibodies reacting only with denatured class I antigens would inappropriately inflate the MFI relative to pathogenicity and are discussed below. Also, alloantibodies that do not activate complement *in vitro* may be less pathogenic. This too is discussed below.

Evolution of MFI over time may be more meaningful than an isolated reading. Burns et al. studied the early posttransplant course of 41 crossmatch-positive patients using B-cell flow crossmatch intensity and its correlate, total DSA MFI [58]. All patients had a decrease in intensity by day 4, presumably by adsorption to the graft. These levels remained low in those who did not develop an AAMR but increased significantly in those who did, a result suggesting that serial MFI monitoring may better identify the potential pathogenicity of preformed DSA. In studying patients receiving a FCXM-positive kidney transplant, Kimball et al. showed by SAB analysis that persistence of preformed DSA as compared to their elimination during the course of the first posttransplantation year was significantly associated with AAMR (43% versus 3%), chronic rejection (43% versus 0%), and graft loss (33% versus 5%) [77]. Dieplinger et al. studied 24 patients with dnDSA in the first 2 years after transplantation and found that, over an additional 24 months, 16 lost significant estimated GFR (>25%) [78]. Initial MFI was not different as compared to the 8 with preserved GFR, but the subsequent peak MFI and delta MFI (>20% or >50%) were significantly higher. Altogether, these 3 studies suggest that MFI evolution over time is much more meaningful than any single isolated reading. Nevertheless, more data are clearly needed before immunosuppression can be routinely altered by such information.

Most patients with DSA have antibodies reacting with more than one antigen. In those with multiple DSA, the best way to determine the actual risk for a particular patient remains uncertain. For example, risk may be determined by the single highest MFI. By contrast, the algebraic sum of the MFIs of all DSA may be more relevant. When multiple DSA are present, it would seem reasonable to consider the sum of their MFIs when they share an epitope or epitopes, as opposed to the single immunodominant MFI [47]. Some studies suggest that summing DSA is superior to using the immunodominant MFI when multiple DSA exist [69, 79]. Most recently, Visentin et al. found a greater predictability of positive crossmatches using immunodominant MFI of class I DSA as compared to the sum of class I DSA MFI [80]. The specific use of epitope analysis to answer this question has not been addressed.

8. *In Vitro* Complement Activation Assays

The C1q *in vitro* assay detects C1q on SAB after adding human C1q, and many studies have evaluated its role in predicting outcomes. This assay would appear to be an excellent way to assess antibody pathogenicity given the known role of complement in mediating antibody injury [81]. Antibodies may induce injury by mechanisms other than activating complement, however, including antibody-dependent cellular cytotoxicity [82] and direct antibody activation of endothelial cells [83]. Furthermore, C1q-positivity may merely be a surrogate for antibody titer [84, 85]. In order to activate C1q, IgG molecules must be in close proximity to typically form a hexamer [86], which requires a high concentration of antibody. Yell et al. demonstrated that concentration of relatively lower MFI C1q-negative DSA to higher concentration uniformly converted them to C1q-positive, and dilution of C1q-positive DSA converted them to C1q-negative [87]. Low MFI sera may contain C1q-positive DSA, and this is explainable by the prozone effect, at least in some instances [88]. Likewise, high MFI DSA may be C1q-negative [89], possibly if restricted to noncomplement fixing IgG2/IgG4 subclasses (rare), IgM antibodies, abnormalities of IgG Fc glycosylation [90], or due to variable percentages of denatured HLA molecules on individual SAB [91].

In an elegant technical study, Taylor et al. studied 25 highly sensitized, wait-listed patients and looked at the relationship between pan-IgG SAB MFI and C1q-positive MFI in order to assess factors affecting this relationship [91]. Sera were compared neat and following EDTA to abrogate complement interference and simple 1 : 20 dilution to detect very high titers. Also, the percent of denatured HLA class I chains (without β-2 microglobulin) was assessed as a contributing factor. There was a poor correlation between neat pan-IgG MFI and C1q-positive MFI ($r^2 = 0.42$). The correlation increased following EDTA ($r^2 = 0.57$) and dilution ($r^2 = 0.77$). A consistent expression of intact HLA (with β-2 microglobulin) molecules per SAB was found for all HLA-A and B specificities and most C specificities. Marked variability, however, existed in the amount of denatured HLA molecules between bead populations, ranging from 19% to 91% (mean 69%). The greater the percentage of denatured HLA the lesser the correlation between pan-IgG MFI and C1q-positive MFI, irrespective of which assay was used. Restricting diluted serum to bead populations with ≤30% denatured HLA increased the r^2 to 0.86. These data suggest that C1q positivity merely reflects MFI if appropriately assessed and does not add significant additional information to justify the excess cost.

Nevertheless, many studies have assessed the ability of C1q-positive DSA to predict adverse outcomes. As we [92] and others [93] have reviewed, at best only conflicting evidence exists to support the pretransplantation use of this assay for such prediction. After transplantation, however, there appears to be a significant relationship [94], and new studies have appeared in the past 2 years to further this notion.

Calp-Inal et al. studied 284 patients with no pretransplant DSA (group 1) and compared them to 405 patients from an earlier era (group 2) with unknown pretransplant status [95]. Over 2.5 years of prospective follow-up, 11% of group 1 patients developed *de novo* DSA, of which 4% were C1q-positive and 7% C1q-negative. AAMR was significantly higher (45%) with C1q-positive DSA as compared to

C1q-negative DSA (5%) and no DSA (1%) with $p < 0.001$. The incidence of chronic AMR/TG was significantly higher as well (36% versus 5% versus 2%, respectively, $p < 0.001$). Overall, GS was nonsignificantly reduced with C1q-positive DSA. Similar results were obtained with group 2 patients, with 19% having detectable DSA after transplantation (8% C1q-positive and 11% C1q-negative).

Guidicelli et al. studied 346 nonsensitized patients with DSA evaluation at 2 and 5 years with 10-year follow-up [88]. At 2 years, 25 had *de novo* DSA, 12 C1q-positive and 13 C1q-negative. At 5 years, 30 were *de novo* DSA positive, 8 C1q-positive and 22 C1q-negative. Patients with C1q-negative DSA at 2 years had the same death censored GS (DCGS) at 5 years as DSA-negative patients, but C1q-positive patients had significantly worse DCGS, a result indicating a rather rapid effect on GS if C1q-positive. Those with C1q-positive results at either 2 or 5 years had worse DCGS at 10 years than those without DSA. Interestingly, those with C1q-negative DSA at *both* 2 and 5 years also had worse DCGS than those without DSA, a result suggesting a slower but still pathogenic effect of these C1q-negative DSA.

Lefaucheur et al. studied 125 patients (of 635 consecutive kidney transplants) with DSA detected in the first posttransplant year, with 42% of immunodominant DSA being C1q-positive [96]. By multivariable analysis (MVA) C1q-positivity was independently associated with allograft loss (HR3.6, $p = 0.03$). In a larger follow-up study of 851 consecutive kidney transplants that included the same patients, Viglietti et al. found that 13% were DSA positive at transplantation and 23% were positive after transplantation [97]. C1q-positivity at either time improved significantly the *c* statistic for allograft loss independent of MFI and significantly improved the net reclassification index (NRI) at both time points as well.

In 69 patients with AAMR, Sicard et al. compared the ability of the DSA to activate complement *in vitro* by detecting C1q or C3d with flow bead assays [98]. By MVA, C3d-positivity was significantly associated with graft loss, whereas C1q-positivity was not. This result was validated in an independent cohort. Even C3d+ patients with a low MFI had reduced GS. Similarly, Comoli et al. studied 114 nonsensitized, pediatric, first kidney transplant recipients and found that 39 developed dnDSA at a median of 25 months [99]. Of these, 25 were C1q-positive, and 9 were additionally C3d-positive. Some that were C1q-negative on initial detection progressed to C1q-positive over time, and some of these further progressed to C3d-positive, all with the same antigenic specificity. Any such progression was associated with significant increase in MFI. C3d-positivity significantly enhanced the predictability of dnDSA for 10-year graft survival.

To summarize, while these new data are promising, they must be validated in other populations and tested prospectively. It remains unclear whether determination of *in vitro* complement activating capability by C1q or C3d binding on SABs justifies the additional cost for routine use, although there certainly may be a role in higher risk candidates. Prospective outcome studies with therapy based on such testing could determine the optimal approach.

9. IgG Subclass Determination

Given the pathogenic potential of complement activation by IgG HLA antibodies, interest has arisen regarding IgG subclass determination as a means of assessing the pathogenic potential of DSA. The germline order of IgG subclasses begins with IgG3 and proceeds through IgG1, IgG2, and IgG4, sequentially. The strength of complement activation parallels this order with IgG3 and IgG1 considered strong activators and IgG2 and IgG4 weak or not at all. The vast majority of HLA antibodies as detected by SABs are composed of strong complement activators (IgG1 and/or IgG3) alone or in combination with weak/nonactivators (IgG2 and/or IgG4). Isolated weak and/or nonactivators in the absence of strong activators are distinctly unusual, ranging from about 1% [100, 101] to 5% [102, 103] of SAB reactions. Hence, strong activators are nearly always detectable, and the absence of C1q positivity does not rule out their presence [103]. As we reviewed previously [92], the majority of evidence at that time did not support routine subclass determination.

The recent study by Lefaucheur et al. (noted above for C1q) evaluated the characteristics of DSA detected in the first year after transplantation in 125 patients with biopsy, either for indication or as a 1-year protocol, and with 5-year follow-up [96]. These DSA were evaluated for MFI, C1q binding, and subclass distribution. Altogether, 40% of patients had a clinical AAMR, 29% had subclinical AAMR, and 30% were free of AAMR. As in prior studies, IgG1 was most often found (75%), followed by IgG2 (44%), IgG3 (28%), and IgG4 (26%), and 17% had no subclass detectable with a relatively low MFI by pan-IgG. Only 4% had just noncomplement fixing IgG2 and/or IgG4. Overall, 32 of 35 IgG3-positive patients had clinical AAMR, and IgG3 was the only class significantly associated with shortened GS including by MVA. IgG4-positivity was associated with subclinical AAMR and more chronic lesions on biopsy. Viglietti et al. expanded this database to 851 patients and found by MVA that detection of IgG3-positive DSA, either at the time of transplantation or after transplantation, significantly increased the *c* statistic for allograft survival at both time points and improved the NRI as well [97]. While very provocative, these data require independent validation.

10. Antibody Titration

Antibody strength is a measure of antibody affinity and avidity and is reflected in the kinetics of antigen-antibody dissociation [104]. Affinity measures the strength of interaction between an antibody paratope and the corresponding epitope and denotes the relative amount of antigen-antibody complex at equilibrium. Avidity is a more global measure of strength and includes affinity, but also valency and structural orientation (e.g., antigen bound to a solid phase matrix). Strength can be determined by serial dilution studies, with the titer being the dilution at which the test becomes negative. Relative strengths of different antibodies can then be defined as the relative MFIs at the highest dilution.

Tambur et al. accumulated over 7000 individual data points from 27 class I assays and 49 class II assays of 55

sensitized patients [104]. A prozone effect, defined here as at least a 100% increase from neat to peak MFI, was detectable for at least one antibody specificity in 71% of patients and for at least one specificity in 40% of class I assays and 65% of class II assays, although overall less than 1% of beads were affected. The effect was often but not always abrogated with EDTA treatment. Looking at the correlation with the ability to fix complement (via the C1q assay), a much higher correlation coefficient was found for the peak MFI on serial dilutions as compared to the standard neat MFI. An even higher correlation was obtained with titers. This study also showed the relative insensitivity of C1q assay, as titers of 1 : 16 to 1 : 32 represented a threshold for C1q positivity for class I antibodies and 1 : 32 to 1 : 64 for class II.

In a follow-up study, the neat IgG MFI, C1q-positive MFI, and antibody titers were compared for their ability to best monitor the effect of desensitization procedures on 40 patients [105]. Titration studies provided a better estimation of strength and more uniformly demonstrated reduction of antibody in response to treatment. Serial titration does add cost, and it remains to be determined by prospective studies the actual role in clinical practice. In our opinion, a one-time dilution may be sufficient to rule out the prozone phenomenon, but serial dilutions would be in order for desensitization protocols or to monitor treatment.

11. HLA Antigen Conformation

Class I HLA molecules are present *in vivo* on the cell surface of activated lymphocytes both as intact trimolecular complexes (α-chain, β2-microglobulin, and peptide) and open conformers (α-chain only) [106]. These isolated ("denatured") α-chains (dHLA) may homodimerize and/or bind to intact class I molecules to enhance antigen presentation [107]. They can also heterodimerize with other cell surface receptors, such as the insulin receptor, and alter cell signaling [107, 108]. Such dHLA exist in varying proportions on SAB as a result of different manufacturing techniques as compared to multiantigen screening beads [109], but they may exist on mixed antigen beads as well [110]. Antibodies specific for intact HLA class I can be determined by SABs that lack dHLA, so-called iBeads [111]. Unfortunately, iBeads are no longer commercially available [80]. The relative proportion of intact HLA molecules and dHLA molecules on SABs can be assessed by relative binding of monoclonal antibodies specific for intact HLA (W6/32) or dHLA (HC-10) [112].

Alternatively, antibodies against dHLA can be detected by acid treating regular mixed antigen or SABs, as acid treatment will denature all HLA antigens on the beads. Such treatment would significantly reduce MFI of antibodies reactive with epitopes determined by the trimolecular structure of intact (nondenatured) HLA (nHLA). During the process of denaturing, hidden epitopes may be exposed that are not accessible to binding on intact antigens [14]. Hence, the MFI of a particular serum against a particular bead may markedly increase after acid exposure, thereby indicating the antibodies are directed against dHLA [14, 113]. If the MFI markedly decreases, the antibodies are directed against nHLA. If it stays roughly the same, they are also against nHLA, but anti-dHLA may coexist [113]. Interestingly, antibodies against dHLA may be found in nontransfused healthy male patients as well as in cord blood, a finding suggesting they arise by cross-reactivity with environmental antigens, vaccines, or microorganisms [14, 114].

Other studies indicate anti-dHLA antibodies do not shorten GS. In a study of 156 sensitized patients with 241 class I DSA by regular SAB analysis before transplant, Otten et al. found that 152 DSA were also positive by iBeads and 28 were found only by dHLA beads [112]. Allograft survival was significantly reduced in those with either regular SAB positivity or iBead positivity as compared to nonsensitized patients, whereas in the 20 patients with 28 isolated antibodies to only dHLA, GS was not different from controls. Furthermore, iBead-positive DSA were associated with sensitizing events, whereas dHLA only DSA were not. Likewise, Cai et al. found that 379 (38%) of 994 KT recipients had class I HLA antibodies by mixed antigen bead testing, including 200 with antibodies also reacting with acid-eluted mixed antigen beads (dHLA) and 179 lacking these antibodies (i.e., only containing anti-native HLA antibodies) [110]. Overall GS was equal between sensitized and unsensitized recipients, but it was significantly lower in the 179 that had only anti-native HLA and not in those who also had anti-dHLA.

C1q positivity may signify pathogenicity of antibodies against denatured HLA antigens. Cai et al. studied 975 kidney transplant patients and found that 30% had antibodies against denatured HLA class I, class II, or MICA, using heat-treatment of mixed antigen beads as a means of denaturation [115]. Overall, 8.5% were C1q-positive and 21.5% C1q-negative [115]. Again there was no difference in graft survival comparing those with to those without these antibodies against denatured antigens. However, the 8.5% with C1q-positive dHLA antibodies had lower graft survival compared to recipients with only C1q-negative anti-dHLA or no such antibodies. C1q-positivity was significantly associated with graft failure due to AMR and mixed AMR/CMR.

In summary, MFI on neat serum is clearly an imperfect measure of antibody concentration, strength, and potential pathogenicity. At a minimum, treatment with EDTA or a simple one-time dilution should be routine. Formal titration is indicated in desensitization procedures and possibly in the course of treating acute AMR. The role of *in vitro* complement activating capability as a routine or in the management of the individual patient remains uncertain at this time, as does IgG subclass determination. Consideration of dHLA to explain a high MFI would be most applicable in those without sensitization history. Most importantly, bead-based data must not be used in isolation, but only in conjunction with cell-based assays and with appropriate consideration of sensitization history and other clinical features of any particular patient.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

The Effect of Histological CD20-Positive B Cell Infiltration in Acute Cellular Rejection on Kidney Transplant Allograft Survival

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Background. It is controversial whether lymphocyte infiltration exhibited in biopsy specimens is associated with transplant outcomes. This study focused on the effect of CD20-positive B cell infiltration in biopsy specimens from allografts with acute cellular rejection (ACR) in a Chinese population. *Methods*. Altogether, 216 patients transplanted from Sep. 2001 to Dec. 2014 with biopsy-proved ACR (Banff I or Banff II) were included in the analysis. Biopsies were immunostained for CD20 and C4d. Baseline information, serum creatinine and GFR before and after treatment, steroid resistance, response to treatment, graft loss, and survival were analyzed. *Results*. Eighty-three patients were classified into CD20-negative group, and 133 patients were classified into CD20-positive group. Significantly more CD20-negative patients (49/83, 59.0%) received steroid plus antibody therapy compared with the CD20-positive group (52/133, 39.1%) ($P = 0.004$). The response to treatment for ACR did not differ between these two groups. The CD20-positive group had less graft loss (18.8% versus 32.5%, $P = 0.022$) and a better graft survival rate. Further exploration of the infiltration degree suggested that it tended to be positively related to graft survival, but this did not reach statistical significance. *Conclusion*. CD20-positive B cell infiltration in renal allograft biopsies with ACR is associated with less steroid resistance and better graft survival. The presence of CD20-positive B cells is protective for renal allografts.

1. Introduction

Acute rejection (AR) is a major risk factor for chronic allograft nephropathy and renal allograft failure after kidney transplantation [1]. The Banff classification criteria divided AR into acute cellular rejection (ACR) and acute humoral rejection [2]. ACR is also known as "T cell mediated rejection," as it is associated with cytotoxic T cell infiltration. However, long-term allograft survival has not been completely improved by controlling factors that affect the T cell pathway [3–5]. In addition to T cells, other inflammatory cells, including CD20-positive B cells [6–8], plasma cells [9–11], macrophages [12–15], eosinophils [16–18], and NK cells [19], can also infiltrate grafts in ACR and may affect the severity of rejection and the therapeutic response.

The role of CD20-positive B lymphocytes in renal allograft biopsy specimens is still controversial. Using DNA microarrays and immunohistochemical staining, Sarwal et al. reported the presence of CD20-positive B lymphocytes in the graft interstitium of pediatric recipients with ACR for the first time, and they concluded that it was strongly associated with clinical glucocorticoid resistance and graft loss [7]. Subsequently, Hippen et al. classified ACR biopsies into the CD20-positive group if they demonstrated strong and diffuse staining characteristics, while trace or rare CD20-positivity was recognized as the CD20-negative group [6]. Their research suggested that CD20-positive infiltrates correlated with worse clinical outcomes. Other studies also suggested a correlation between CD20 graft infiltration and steroid-resistant rejection [20, 21]. However, relevant studies

Table 1: Distribution of Banff diagnosis stratified by CD20 staining.

Type (grade)	CD20-negative ($n = 83$)	CD20-positive ($n = 126$)	$P = 0.002$
IA	30	65	
IB	3	13	
IIA	45	34	
IA + IIA	3	13	
IB + IIA	1	5	
IA + IIB	0	2	
IIB	1	1	

have argued that CD20-positive B cells exhibited in biopsies have no effect on clinical outcome [22, 23]. Clatworthy et al. conducted a clinical trial comparing rituximab (an anti-CD20 monoclonal antibody) with daclizumab (an anti-CD25 monoclonal antibody) as induction therapy in nonsensitive kidney transplant recipients, but this trial was suspended because of an excess incidence of ACR in the rituximab group [24]. The authors surmised that this anti-CD20 monoclonal antibody might have cleared immunoregulatory B cells, including B cells in the allograft tissue, which led to a marked increase in ACR. Disagreements that exist in published studies may be because of a relatively small sample size and lack of a unified standard for the definitions of what is CD20-positive and CD20-negative.

The aim of this study was to determine the effects of CD20-positive B cell graft infiltration during ACR on allograft outcome in a Chinese population.

2. Patients and Methods

2.1. Patients. This is a retrospective study of patients who underwent kidney transplantation between September 2001 and December 2014 at the Kidney Disease Center of the First Affiliated Hospital of Zhejiang University (Hangzhou, China). This study was approved by the Committee of Ethics in Biomedical Research of Zhejiang University. Pathological records, clinical data, test results, and follow-up information of all patients were collected from the electronic medical record system and the kidney transplantation database of our center.

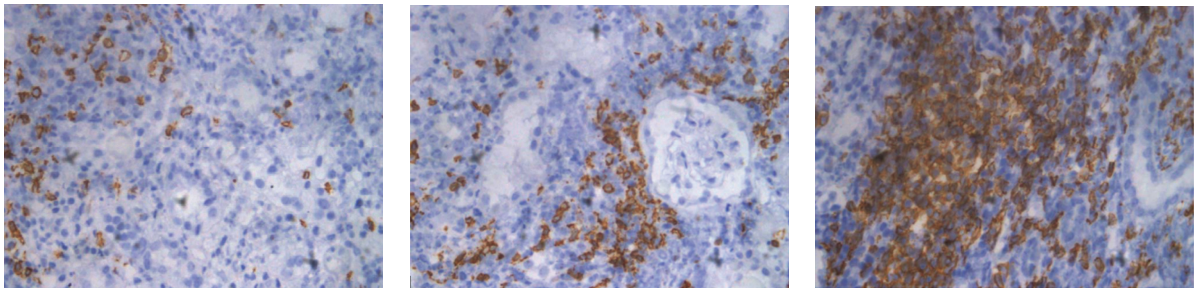
Altogether, 217 patients were identified with biopsy-proven ACR (grade I or II) according to the Banff 2005 criteria and were negative for C4d staining. Excluding one patient who was lost of follow-up after antirejection treatment, 216 patients were included in this analysis. All patients were followed up until June 30, 2015. According to the presence of CD20-positive B cell infiltration, 83 recipients were classified into the CD20-negative group, and 133 were classified into the CD20-positive group. The pathologic types of ACR in these two groups were listed in Table 1.

Most of the recipients received calcineurin inhibitor (CNI) (cyclosporine or tacrolimus) in combination with mycophenolate mofetil (MMF) and steroids as maintenance immunosuppressive regimen, while some received rapamycin in place of CNI. Cyclosporine (CsA) was initiated at 5 mg/kg/d and tacrolimus (FK506) at 0.10–0.15 mg/kg/d. The drug dosage was adjusted according to the plasma concentration. The target plasma concentration for CsA and FK506 was 250–300 μg/L and 8–12 μg/L, respectively, during the first month after transplantation; 200–250 μg/L and 6–10 μg/L, respectively, from the second to the third month; 150–200 μg/L and 4–8 μg/L, respectively, from the fourth to the sixth month; and around 150 μg/L and 3–6 μg/L, respectively, after the sixth month. MMF was started at 1.5–2 g/d for half a month and maintained at 1 g/d thereafter. Methylprednisolone was given at 6 mg/kg on the third postoperative day. Since then, starting from 80 mg/d, with a daily reduction of 10 mg, prednisone was maintained at 10–15 mg/d.

Once acute rejection was proven by allograft biopsy, intravenous methylprednisolone was administrated at 6–10 mg/kg daily for 3 days as pulse therapy. If the serum creatinine (Cr) level decreased more than 50% or went back to the baseline level within 1-2 weeks, the treatment was considered effective. If not, ACR was further treated with OKT3 at 5–10 mg/d or ATG at 100–200 mg/d for 5–7 days. Response to therapy was determined by comparing the serum Cr level measured two weeks after completion of the antirejection treatment to the baseline serum Cr level measured before rejection [25]. Response was considered complete if a decrease in serum Cr level was maximally 125% of baseline and partial if the Cr was 125%–175% of baseline, and it is no-response if Cr was still more than 175% of the baseline or graft loss (back to dialysis or nephrectomy).

2.2. Allograft Biopsy and Histopathology. Percutaneous ultrasound-guided renal biopsy was performed in recipients experiencing allograft dysfunction. Kidney allograft pathology diagnosis was made by an experienced renal pathologist (W. H.) according to the Banff 2005 criteria. We only included ACR grade I and grade II. ACR grade III and antibody-mediated rejection were excluded. Vascular rejection refers to the existence of intimal arteritis in the ACR sample, that is, grades IIA, IIB, IIA + IA, IIA + IB, IIB + IA, and IIB + IB, according to the Banff 2005 criteria.

Immunohistochemical staining for CD20 and C4d was routinely performed on paraffin sections using the CD20 monoclonal antibody from Zhongshan (Cat number ZA0549, Beijing, China) and the C4d polyclonal antibody from Abcam (Cat number ab36075, Cambridge, UK). Using the same definition as those of Hippen et al., CD20-positive was defined as strong and diffuse staining characteristics, while trace or rare CD20-positivity was assigned to CD20-negative [6]. The CD20-positive specimens were further assessed independently by two authors (W. R. and W. H.) and defined as mild-positive if CD20-positive cells accounted for less than 25% of the inflammatory cells, moderate-positive if CD20-positive cells were 26%–49% of the inflammatory cells, and severe-positive if CD20-positive cells were more than 50% of the inflammatory cells. Representative images are shown in Figure 1. All the patients included in this study were negative for C4d staining.



(a) (b) (c)

Figure 1: Representative pictures of CD20 immunostaining in ACR (×400): (a) CD20 mild-positive; (b) CD20 moderate-positive; (c) CD20 severe-positive.

2.3. Statistical Analysis. Continuous variables are expressed as mean ± standard deviation (SD) or median (range). Categorical variables are presented as numbers (frequencies). Normally distributed continuous variables were analyzed using student's t-test or one-way ANOVA, and nonnormally distributed continuous variables were analyzed using Mann–Whitney test. Chi-square test was used for categorical variables. Graft/patient survival was analyzed with Kaplan-Meier method and differences between survival curves were calculated by the log-rank test. Factors associated with graft loss on the univariate analysis with $P < 0.1$ were included into a Cox multivariate analysis. All statistical calculations used SPSS 19.0. Two sided $P < 0.05$ was considered as significant difference.

3. Results

3.1. Baseline Characteristics. The baseline characteristics of the patients were listed in Table 2. Eighty-three patients were assigned to the CD20-negative group, and 133 patients were classified as CD20-positive. No significant differences in age, gender, cold/warm ischemia time, donor type, primary disease, induction regimen, and prerejection immunosuppressive drugs were observed between these two groups. ACR was diagnosed earlier after kidney transplantation in the CD20-negative group compared with CD20-positive group (median time to ACR, 29 days versus 142 days, $P = 0.016$). As presented in Table 1, the pathologic types differed in two groups ($P = 0.002$). There was significantly more vascular rejection (IIA, IIB, IIA + IA, IIA + IB, IIB + IA, and IIB + IB) in the CD20-negative group (50/83 patients, 60.2%), compared with the CD20-positive group (55/133 patients, 41.4%) ($P = 0.005$).

3.2. Antirejection Therapy. In general, CD20-negative patients presented with higher prerejection serum Cr levels, compared with the CD20-positive group (180.1 ± 128.4 versus 130.6 ± 68.1 μmol/L, $P = 0.002$). The same results were obtained with peak Cr at rejection (352.7 ± 242.3 versus 274.1 ± 265.6 μmol/L, $P = 0.027$). No significant differences were observed at any other time point during follow-up (Table 3). Corresponding to this, worse GFR was observed in the CD20-negative group before rejection (47.2 ± 21.3 versus 60.4 ± 21.6 mL/min, $P < 0.001$), and the same results were obtained at the time of rejection (25.0 ± 15.0 versus 30.6 ± 13.3 mL/min, $P = 0.005$). No significant differences between CD20-positive and CD20-negative groups were observed at any other time point during follow-up (Table 4).

Patients in the CD20-positive and CD20-negative groups received similar maintenance immunosuppressive regimen after rejection (Table 5). After ACR, significantly more CD20-negative patients (49/83, 59.0%) received steroid plus antibody therapy (defined as steroid-resistant rejection) compared with the CD20-positive group (52/133, 39.1%) ($P = 0.004$). The response to treatment for ACR did not differ between these two groups.

3.3. CD20 Staining and Patient/Graft Survival Rates. More patients in the CD20-negative group (27/83, 32.5%) experienced graft loss compared with the CD20-positive group (25/133, 18.8%), which reached a significant difference ($P = 0.022$). Figure 2(a) displayed the graft survival over time analyzed by the Kaplan-Meier death-censored method for CD20-positive and CD20-negative groups. CD20-positive infiltration was associated with significantly better allograft survival ($P = 0.049$). There was no significant difference in the patient survival rate between these two groups.

3.4. Association of the Degree of CD20 Infiltration and Patient/Graft Survival. We further divided the CD20-positive group into CD20 mild-positive subgroup ($n = 76$), CD20 moderate-positive subgroup ($n = 36$), and CD20 severe-positive subgroup ($n = 31$) according to the percentage of CD20-positive B cells found in the inflammatory cell population. Figure 3(a) showed that the CD20 severe-positive subgroup tended to have better graft survival compared to the other three groups, but this difference was not significant. Patient survival was similar among these four groups (Figure 3(b)).

3.5. Predictor of Graft Loss in a Cox Proportional-Hazards Model. Univariate analysis showed that the CD20-positive infiltration, prerejection immunosuppressive regimen,

Table 2: Patient baseline characteristics stratified by CD20 staining.

Characteristics	CD20-negative ($n = 83$)	CD20-positive ($n = 133$)	P value
Sex (male/female)	54/29	94/39	0.387
Age at transplantation (years)	39.1 ± 12.0	37.5 ± 11.5	0.332
Warm ischemia time (min)	5.5 ± 1.9	5.5 ± 2.4	0.657
Cold ischemia time (min)	442 ± 204	448 ± 209	0.792
Donor type			0.863
Living	17 (20.5%)	26 (19.5%)	
Deceased	66 (79.5%)	107 (80.1%)	
Primary disease			0.445
Chronic nephritis	75 (90.4%)	119 (89.5%)	
AKPD	6 (7.2%)	5 (3.8%)	
Diabetic nephropathy	0 (0)	2 (1.5%)	
Gouty nephropathy	0 (0)	2 (1.5%)	
Hereditary nephropathy	0 (0)	2 (1.5%)	
Others	2 (2.4%)	3 (2.2%)	
Number of HLA mismatches	3.11 ± 1.22	2.98 ± 1.26	0.468
Induction regimen			0.498
CD25 monoclonal antibody	35 (42.2%)	46 (34.6%)	
ATG/OKT3	4 (4.8%)	9 (6.8%)	
None	44 (53.0%)	78 (58.6%)	
PRA > 10% – number (%)			
After transplant	4 (4.8%)	2 (1.5%)	0.149
At rejection	3 (3.6%)	5 (3.8%)	0.956
Prerejection immunosuppressant arms			0.230
FK506 + MMF +Pred	53 (63.9%)	76 (57.1%)	
CSA + MMF + Pred	27 (32.5%)	53 (39.8%)	
Rapamycin + MMF + Pred	1 (1.2%)	2 (1.5%)	
CSA + rapamycin + Pred	2 (2.4%)	0	
CSA + AZA + Pred	0 (0)	2 (1.5%)	
Median days to ACR (range) (days)	29 (3–3878)	142 (3–3398)	0.016

PRA, panel reactive antibody; AZA, azathioprine; CSA, cyclosporine; FK506, tacrolimus; MMF, mycophenolate mofetil; PRED, prednisone.

Table 3: Serum creatinine values during follow-up.

	CD20-negative	CD20-positive	P value
Before rejection	180.1 ± 128.4 (83)	130.6 ± 68.1 (133)	0.002
Peak	352.7 ± 242.3 (83)	274.1 ± 265.6 (133)	0.027
After biopsy			
1 month	183.2 ± 143.2 (80)	160.1 ± 171.8 (132)	0.313
3 months	150.6 ± 102.5 (62)	135.8 ± 55.6 (113)	0.293
6 months	127.4 ± 50.9 (63)	142.6 ± 68.7 (125)	0.121
12 months	126.9 ± 43.7 (64)	144.7 ± 97.9 (110)	0.101
24 months	127.1 ± 49.5 (58)	131.0 ± 80.0 (91)	0.742
36 months	114.1 ± 49.5 (38)	129.4 ± 58.2 (69)	0.173
48 months	116.8 ± 77.1 (26)	122.0 ± 55.6 (52)	0.734
60 months	113.0 ± 72.0 (23)	116.4 ± 36.5 (47)	0.788

Table 4: GFR values during follow-up.

	CD20-negative	CD20-positive	P value
Rerejection	47.2 ± 21.3 (83)	60.4 ± 21.6 (133)	<0.001
Peak	25.0 ± 15.0 (83)	30.6 ± 13.3 (133)	0.005
After Biopsy			
1 month	47.2 ± 21.8 (80)	52.5 ± 19.8 (132)	0.070
3 months	54.0 ± 20.2 (62)	55.6 ± 19.4 (113)	0.641
6 months	58.5 ± 18.1 (63)	54.8 ± 20.6 (125)	0.224
12 months	58.1 ± 19.8 (64)	57.3 ± 22.7 (110)	0.804
24 months	58.2 ± 17.4 (58)	60.6 ± 23.7 (91)	0.502
36 months	66.7 ± 25.0 (38)	59.8 ± 20.5 (69)	0.123
48 months	69.3 ± 24.1 (26)	62.6 ± 21.1 (52)	0.212
60 months	68.3 ± 20.3 (23)	64.1 ± 21.4 (47)	0.428

Table 5: Antirejection therapy and response to treatment.

	CD20-negative (n = 83)	CD20-positive (n = 133)	P value
Postrejection immunosuppressant arms			0.898
FK506 + MMF + Pred	69 (83.1%)	113 (85.0%)	
CSA + MMF + Pred	13 (15.7%)	18 (13.5%)	
Rapamycin + MMF + Pred	1 (1.2%)	2 (1.5%)	
Antirejection therapy			0.004
Steroids	34 (41.0%)	81 (60.9%)	
Steroids + antibody	49 (59.0%)	52 (39.1%)	
Response to treatment			0.232
Complete	55 (66.2%)	86 (64.7%)	
partial	11 (13.3%)	28 (21.0%)	
No response	17 (20.5%)	19 (14.3%)	

Table 6: Cox regression hazard ratios for renal allograft failure.

	Univariate			Multivariate		
	RR	95% CI	P	RR	95% CI	P
CD20-positive infiltrates	0.506	0.293–0.872	0.014	0.570	0.327–0.995	0.048
Prerejection immunosuppressive regimen	0.392	0.221–0.696	0.001	0.621	0.356–1.083	0.093
Antirejection therapy (combination versus MMP)	3.142	1.724–5.728	<0.001	3.316	1.677–5.958	<0.001
Response to treatment			<0.001			<0.001
Partial versus complete response	2.613	1.129–6.048	0.025	2.538	1.078–5.974	0.033
No-response versus complete response	13.410	7.032–25.570	<0.001	13.847	7.018–27.321	<0.001

RR, relative risk; CI, confidence interval.

antirejection therapy, and antirejection response were the factors influencing renal allograft loss. Further multivariate Cox regression analysis revealed that CD20 infiltration was a protective factor for graft loss. Antirejection therapy is another independent risk factor. The adjusted risk ratio of graft loss for steroid plus antibody treatment was 2.316 compared with steroid alone. Compared with the complete response, the adjusted risk ratio of graft loss was 2.538 for partial-response and 13.847 for no-response, as exhibited in Table 6. The prerejection immunosuppressive regimen, which was significant in the univariate analysis, did not reach significance in the multivariate analysis.

4. Discussion

Our study demonstrated that CD20-positive infiltration in the biopsy specimens from the allografts with ACR was associated with less steroid-resistant rejection and better allograft survival. Further exploration of the infiltration degree suggested that it tended to be positively related with graft

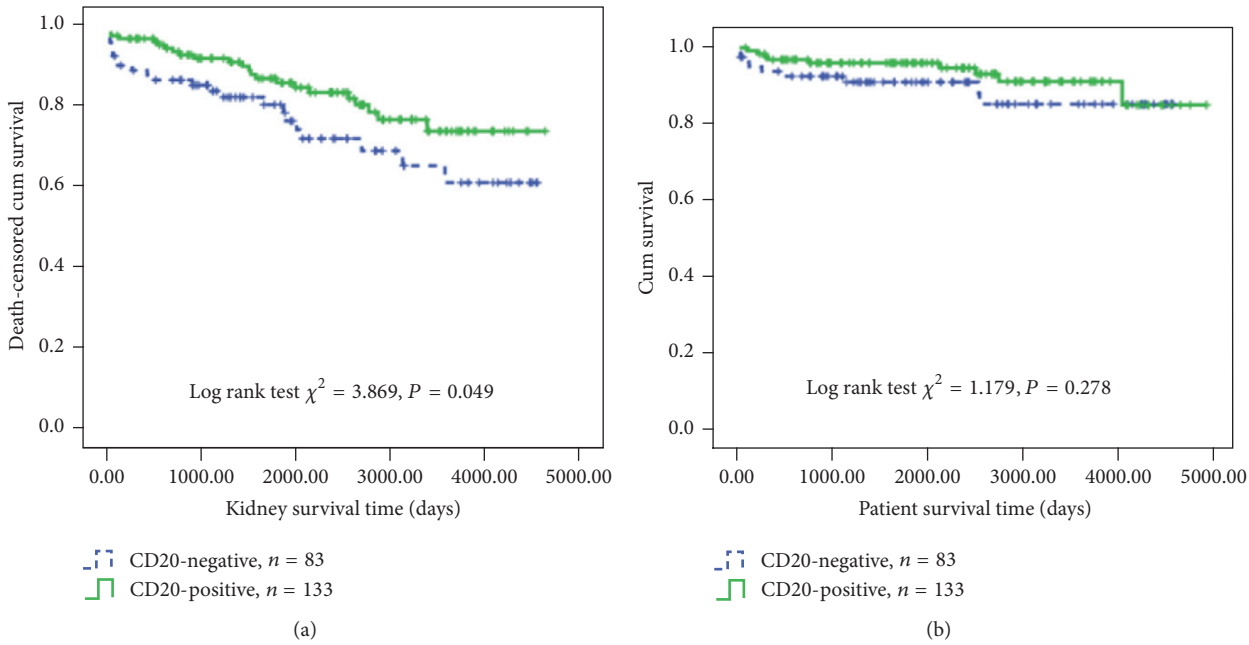


Figure 2: Effects of CD20 staining on (a) death-censored renal allograft survival; (b) patient survival.

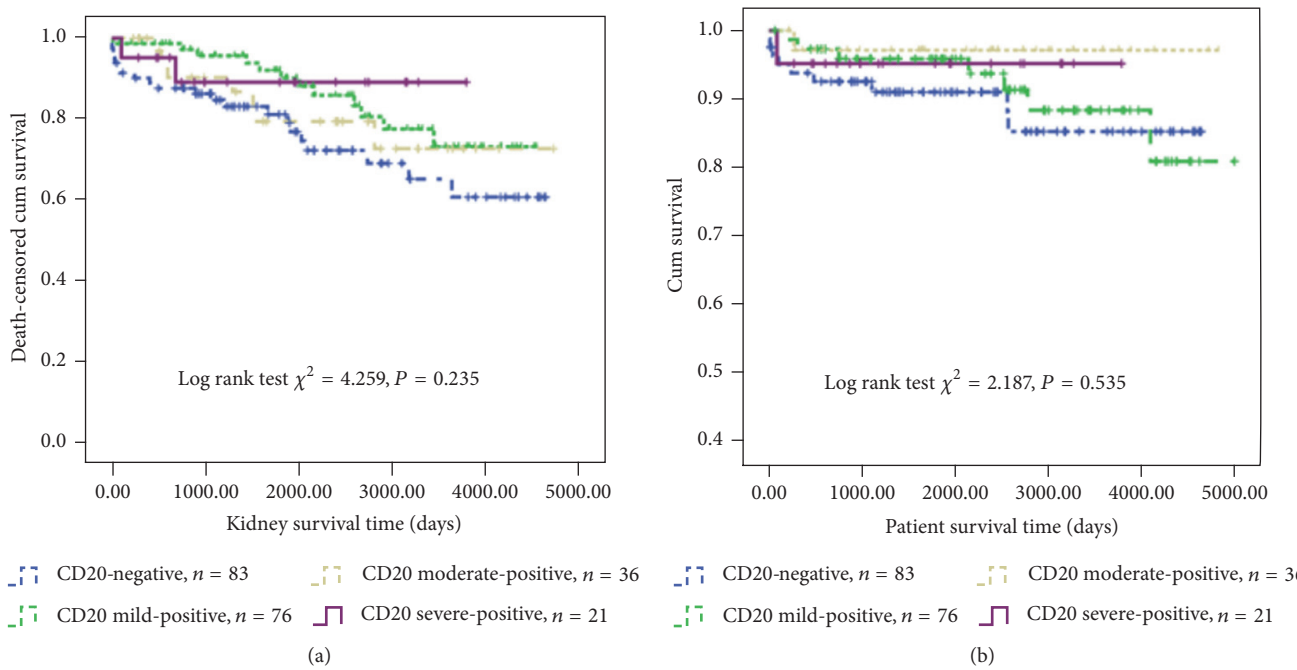


Figure 3: Effects of CD 20 infiltration degrees on (a) death-censored renal allograft survival; (b) patient survival.

survival, but without statistical significance. Multivariate Cox regression revealed that CD20-positive infiltration, antirejection therapy, and antirejection response were independent predictors of graft loss. The presence of CD20-positive B cells was a protective factor for graft loss.

B cells are very common in solid organ transplantation. B cells and plasma cells in pathological tissues have been considered as nonspecific effector cells in the past [26, 27]. In 2003, Sarwal et al. first demonstrated the presence of CD20-positive B cells in the graft interstitium of pediatric transplant recipients experiencing ACR [7]. Their study suggested that CD20-positive infiltration was associated with steroid-resistant rejection and worse graft survival. Since then, the role of CD20-positive B cells in ACR has attracted more attention. In adult kidney transplantation, 22% of acute rejection (IA, IB) specimens had CD20-positive infiltration, and it was associated with worse outcome [6]. A mouse model of acute cardiac allograft

rejection demonstrated that recipients' B cells participate in indirect alloantigen presentation and play an important role in the progression of acute vascular rejection [28]. Studies analyzing the CD20-positive B cells' infiltration pattern in adult kidney transplantation reported higher serum Cr levels in recipients with clusters of B cells [29]. Some studies have suggested that once recruited into injured grafts, B cells can act as antigen-presenting cells to promote T cell mediated rejection, which is resistant to conventional steroid therapy [30].

Different result was observed, suggesting that the early infiltration of B cells can be beneficial [23, 31]. Scheepstra et al. performed immunostaining for CD20 on 54 biopsy-proven ACR samples, and no correlation was found between the number of CD20 cells, in clusters or in a scattered pattern, and clinical outcome [23]. Increased peripheral blood B cells were detected in the steady recipients [32], instead of those experiencing rejection. CD20 transcription was found to be increased in tolerant kidney transplant recipients [33–35]. Kayler et al. suggested that B cells were not indicators of graft loss or steroid resistance in their analysis of 120 ACR biopsies [36]. This result was proved by other studies [37].

Thus, the role of CD20-positive B lymphocytes in acute cellular rejection is controversial. One of the possible explanations for this is the lack of a unified standard on the definition of CD20-positive and CD20-negative. Sarwal and her colleagues defined CD20-positive as more than 275 CD20-positive cells in a single high power field (HPF) and came to the conclusion that CD20-positive cells were strongly associated with severe graft rejection [7]. Hippen et al. used a qualitative method in their research and defined biopsies with strong and diffuse staining characteristics of CD20-positive cells as the CD20-positive group, while trace or rare CD20-positive cells were recognized as the CD20-negative group [6]. Their research indicated that CD20-positive infiltration was more likely to have steroid-resistant rejection and worse graft survival. In Bagnasco's research, CD20-positive patients (at least one cluster containing more than 100/HPF CD20-positive cells) and CD20-negative patients (the count was below 50/HPF) were compared [37]. No association was found between CD20-positive infiltrates and worse graft outcome using this method. CD20-positive lymphoid clusters, which were defined as any dense cluster of lymphoid cells containing more than 15 CD20-positive B cells by Kayler et al., did not predict steroid resistance or worse graft survival [36].

The definition of CD20-positive and CD20-negative in our study is in line with Hippen et al.'s research. However, our study's findings that CD20-positive infiltration is associated with better clinical outcomes are not consistent with Hippen et al.'s results [6]. Differences exist between our two studies. First, our study included a larger number of ACR biopsies than any published studies so far. A total of 216 cases of biopsy-proven ACR were included in our analysis; 83 samples were assigned to the CD20-negative group, and 133 were classified as CD20-positive. Hippen et al.'s study cohort contained 27 patients and only 6 biopsies were classified as CD20-positive. As mentioned in the discussion by the authors, the relative small sample sizes in their analysis may limit the generalization of their conclusions. Secondly, Hippen et al.'s research was conducted in biopsies with proven Banff IA or IB rejection within the first year after transplantation. Our study included patients with ACR grade I and grade II at any time point after kidney transplantation. The differences in the inclusion criteria of study population may contribute to the different results. Similarly, Scheepstra et al. [23] adopted the same method to assess CD20-positive as mentioned in Sarwal et al.'s work [7], but they reported different results. Thus, besides the lack of a uniformed definition of CD20-positive and CD20-negative, a relatively small sample size, different study populations (pediatrics versus adults; different subgroups of ACR), differences in the induction and maintenance immunosuppression therapy, different follow-up durations, and other factors can contribute to the inconsistency in the studies concerning the role of CD20-positive infiltration in ACR.

The prognostic study of CD20-positive cells in ACR helps to select future treatment, in particular, whether or not to apply B-cell-depleting agents in steroid-resistant rejection. Rituximab, an anti-CD20 monoclonal antibody, was first applied in the treatment of lymphoma. It has been proven effective in treatment of a number of hematological diseases and autoimmune diseases [38–40]. In the aspect of transplantation, rituximab has been used for desensitization of panel reactive antibody, anti-HLA antibody, and anti-ABO antibody before transplantation as induction regimen and treatment of humoral rejection after transplantation [41–47]. In 2002, a cardiac transplant patient with vascular rejection refractory to plasmapheresis was successfully treated with rituximab, which was the first documented application of an anti-CD20 monoclonal antibody in the aspect of rejection [48]. Later, other case reports confirmed that CD20 monoclonal antibody was effective in the treatment of vascular rejection in heart and pancreas transplantation [49–51]. At present, rituximab is applied to treat steroid-resistant rejection after renal transplantation. It can clear DSA and B cells to improve outcomes after renal allograft rejection [52]. After treatment, the reconstruction of peripheral B cells requires 6 to 9 months. The CD20-positive B cells in graft are significantly reduced after treatment, but the studies of the reconstruction of B cells in grafts are rather rare [53]. Our results showed that CD20-positive infiltration in patients with ACR appeared to have a protective effect on graft outcome. Additionally, a clinical trial which planned to use rituximab in nonsensitive kidney transplant recipients was forced to stop because of increases in rejection [24]. Thus, the administration of anti-CD20 monoclonal antibody in patients with ACR requires careful consideration.

5. Conclusion

CD20-positive B cell infiltration in renal allograft biopsies with ACR is associated with less steroid-resistant rejection and better graft survival. The presence of CD20-positive B cells is protective for renal allografts.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Yan Jiang and Rending Wang contribute equally to this paper.

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Clinical Study

Patterns of Early Rejection in Renal Retransplantation: A Single-Center Experience

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It has been reported that kidney retransplant patients had high rates of early acute rejection due to previous sensitization. In addition to the acute antibody-mediated rejection (ABMR) that has received widespread attention, the early acute T-cell-mediated rejection (TCMR) may be another important issue in renal retransplantation. In the current single-center retrospective study, we included 33 retransplant patients and 90 first transplant patients with similar protocols of induction and maintenance therapy. Analysis focused particularly on the incidence and patterns of early acute rejection episodes, as well as one-year graft and patient survival. Excellent short-term clinical outcomes were obtained in both groups, with one-year graft and patient survival rates of 93.9%/100% in the retransplant group and 92.2%/95.6% in the first transplant group. Impressively, with our strict immunological selection and desensitization criteria, the retransplant patients had a very low incidence of early acute ABMR (6.1%), which was similar to that in the first transplant patients (4.4%). However, a much higher rate of early acute TCMR was observed in the retransplant group than in the first transplant group (30.3% versus 5.6%, $P < 0.001$). Acute TCMR that develops early after retransplantation should be monitored in order to obtain better transplant outcomes.

1. Introduction

Renal transplantation is regarded as the optimal treatment for patients with end-stage renal disease. However, as long-term graft survival is still limited, most transplant patients will face graft loss after 9-10 years [1]. These patients are generally more fragile and in considerable need of new grafts, in comparison to naïve patients waiting for their first renal transplantation.

It has been reported that the best approach to treat most patients suffering from chronic renal allograft failure is to perform a kidney retransplant, in hopes of avoiding the high risk of morbidity and mortality with a return to dialysis [2]. These patients, however, are commonly human leukocyte antigens- (HLA-) sensitized because of exposure to previous allograft(s); thus there is a lower chance of their receiving a retransplant. Retransplantation accounts for 13–15% of the annual transplants performed in USA and only approximately 5% of those performed in Europe [3]. Therefore, every retransplant case needs to be evaluated and managed very carefully.

Renal retransplant patients had high rates of acute rejection, from 33% to 69%, as reported in previous studies [4–6]. About two-thirds of these rejections were verified as antibody-mediated rejection (ABMR), comprising the primary cause of early graft loss. Thus, it is well recognized that the risk of ABMR in retransplantation increases markedly and needs to be prevented as much as possible. In contrast, the risk of T-cell mediated rejection (TCMR) in retransplantation is less of a concern. Compared to first transplant patients, it is unclear whether the incidence of acute TCMR would significantly increase in retransplant patients without early ABMR. In other words, if de novo donor-specific

antibody (DSA) and its mediated ABMR could be prevented successfully in retransplantation, would TCMR be brought to the forefront as an important issue?

Here, we report on the early transplant outcomes of 33 second, third, and fourth kidney transplants performed at our hospital within the last 3 years. Analysis focused particularly on the incidence and patterns of the early acute rejection episodes, as well as one-year graft and patient survival.

2. Patients and Methods

2.1. Study Population. Between January 2013 and December 2015, a total of 703 kidney transplants were performed at Tongji Hospital, including 521 transplants from deceased donors (donation after brain death or cardiac death) and 182 from living-related donors. Of these, 662 (94%) were first transplantations and 41 (6%) were retransplantations.

In the current retrospective study, for the retransplant group, we included 33 adult patients, who received a second, third, or fourth renal allograft with Thymoglobulin induction therapy and Tacrolimus-based maintenance therapy. The exclusion criteria were as the following: (1) pediatric recipients; (2) renal allografts from pediatric donors; (3) patients who received no induction therapy or received induction therapy other than Thymoglobulin; (4) patients who received a multiorgan transplant. For the control group, we selected 90 patients who received a first renal allograft during the same period and fulfilled the same inclusion and exclusion criteria. This study was performed after approval by the ethics committee at Tongji Hospital, Tongji Medical School, Huazhong University of Science and Technology.

2.2. Data Collection. Data on transplantations and hospital stays, as well as follow-up data, were collected from hospital records. Baseline characteristics, such as recipient age and gender, donor type (deceased or living), number of previous transplants, cold ischemia time, number of HLA mismatches, pretransplant panel reactive antibody (PRA) percentages divided into groups (0–10%, >10%–50%, and ≥50%), and preformed DSA, were collected and analyzed. In addition, early clinical outcomes, including the generation of de novo DSA, rate of delayed graft function (DGF), the frequency and type of acute allograft rejection (cellular or antibody-mediated rejection), and one-year graft and patient survival, were analyzed. DGF was defined as the need for more than 1 dialysis during the first week after transplant. HLA class I and II antibody screenings were performed using FlowPRA® (One Lambda, Inc., Canoga Park, CA), and the specificity determination was measured by Luminex using LABScreen® single antigen beads (One Lambda, Inc., Canoga Park, CA).

2.3. Immunosuppression. All patients received induction therapy with Thymoglobulin and maintenance immunosuppression with Tacrolimus, mycophenolate mofetil, and steroids. Because of differences in immunological risk, the dosage and duration of the Thymoglobulin administration were slightly different for the retransplant group, compared to the control group. The initial administration of Thymoglobulin was finished intraoperatively, before the graft reperfusion, at a dose of 50 mg in the retransplant group and a dose of 25 mg in the control group. Then, it was used daily by the retransplant group from day 1 to day 4, reaching a total dosage of 125–150 mg. In the control group, Thymoglobulin was given from day 0 to day 2 at a daily dose of 25 mg. The peripheral blood lymphocyte counts were frequently monitored within the first 2 weeks after renal transplantation. Methylprednisolone was given intravenously from day 0 to day 2 (500 mg/d), followed by oral doses of prednisone at 50 mg/d, which was then tapered every other day to a maintenance dose of 10 mg/d. Mycophenolate mofetil was administered at a dose of 1.5 g/d and was subsequently reduced to 1 g/d depending on the individual's white blood cell count. Tacrolimus was started at day 3, with a targeted trough level of 8–10 ng/ml initially and 6–8 ng/ml one month after transplantation.

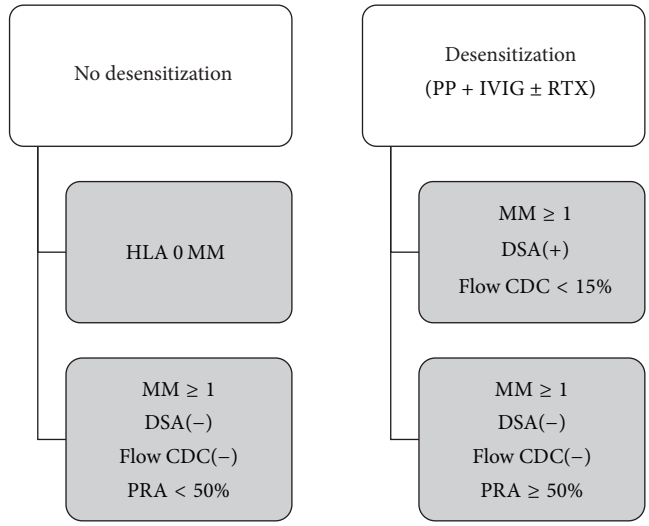

Figure 1: Indications of desensitization therapy for our retransplant patients. No desensitization was initiated if patients had HLA 0 MM, or HLA MM ≥ 1, but PRA < 50% with negative DSA and CDC. Desensitization was suggested if patients had (1) HLA MM ≥ 1, DSA positive, and negative or weakly positive flow CDC (<15%) or (2) HLA MM ≥ 1 and PRA ≥ 50% with negative DSA and CDC.

2.4. Desensitization. In our center, the decision of desensitization was mainly based on the laboratory testing results for pretransplant PRA, preformed DSA, HLA mismatch (MM), and flow complement-dependent cytotoxicity (CDC). As shown in Figure 1, sensitized patients were not required to be desensitized in the following two situations: (1) HLA 0 MM between donor and recipient and (2) HLA MM ≥ 1, PRA < 50% with negative DSA and flow CDC. In contrast, desensitization was suggested for sensitized patients in any of the following conditions: (1) HLA MM ≥ 1, DSA positive, and negative or weakly positive flow CDC (10–15%); (2) HLA MM ≥ 1 and PRA ≥ 50% with negative DSA and flow CDC. For a few highly sensitized patients with flow CDC ≥ 15%, the upcoming transplantation was then avoided due to the

Table 1: Demographics and early transplant outcomes.

	Second and subsequent transplant (n = 33), n (%)	First transplant (n = 90), n (%)	P value
Recipient age, year	46 ± 11	43 ± 11	0.170
Recipient gender			0.334
Male	25 (75.8)	60 (66.7)	
Female	8 (24.2)	30 (33.3)	
Type of donor			
Deceased	24 (72.7)	90 (100)	NA
Living-related	9 (27.3)	0	
Times of previous transplant			NA
1	28 (84.9)	0	
2	4 (12.1)	0	
3	1 (3.0)	0	
Cold ischemia time (h)	7.7 ± 5.8	8.6 ± 4.6	0.122
HLA-A, B, DR MM	3.3 ± 1.2	3.5 ± 0.8	0.261
Pretransplant PRA			<0.001
Class I and class II < 10%	14 (42.4)	87 (96.7)	
Class I or class II 10~50%	8 (24.3)	2 (2.2)	
Class I or class II ≥ 50%	11 (33.3)	1 (1.1)	
Preformed DSA+	3 (9.1)	0	NA
Delayed graft function	4 (12.1)	16 (17.8)	0.451
De novo DSA+	4 (12.1)	5 (5.6)	0.215
TCMR	10* (30.3)	5 (5.6)	<0.001
ABMR	2 (6.1)	4 (4.4)	0.712
1 yr graft survival	93.9%	92.2%	0.746
1 yr patient survival	100%	95.6%	0.218

*4 of 10 patients had 2 episodes of acute TCMR.

limitations of current desensitization therapy. The protocol we used to desensitize patients involved a combination of plasmapheresis (PP), intravenous immunoglobulin (IVIG), and/or rituximab (RTX). IVIG was used at a dose of 300–400 mg/kg each time and RTX was given once at a dose of 200 mg.

2.5. Diagnosis of Acute Rejection. In general, acute rejection was diagnosed using kidney biopsies upon Banff 2007 or 2013 classification. When a tissue analysis was not available, the clinical diagnosis was based on an otherwise unexplained elevation of serum creatinine levels, coupled with appropriate physical signs (including edema, oliguria, fever, or weight gain). All allograft biopsies were routinely stained for hematoxylin and eosin (HE) and immunohistochemistry for C4d, CD3, CD4, and CD8. The diagnosis of acute TCMR was based on the following criteria: IA, cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3), and foci of moderate tubulitis (t2); IB, cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3), and foci of severe tubulitis (t3); IIA, cases with mild-to-moderate intimal arteritis (v1); IIB, cases with severe intimal arteritis comprising >25% of the luminal area (v2); III, cases with "transmural" arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3) [7]. Acute ABMR was defined as a biopsy with or without C4d, evidence of acute renal injury and microvascular inflammation, in the presence of circulating DSA [8]. If an episode of ABMR occurred together with acute TCMR, it was also defined as ABMR. Early acute rejection referred to a rejection that developed < 90 days after transplantation.

2.6. Statistical Analysis. In our descriptive statistical analysis, results are expressed as numerical values and percentages for categorical variables and as mean values with standard deviation (SD) for continuous variables. The frequencies procedure was used to compare baseline characteristics between the two groups. Graft and patient survival was evaluated according to Kaplan-Meier survival statics. Statistical analysis was performed using SigmaStat software version 3.5.

3. Results

3.1. Patient Population. The patient cohort included 33 kidney retransplants and 90 first transplants. The retransplant group comprised 28 (84.9%) second, 4 (12.1%) third, and 1 (3%) fourth kidney transplants. Baseline and demographic characteristics are shown in Table 1. The mean recipient age and sex ratios were similar in both groups. The majority of

patients received kidneys from deceased donors (72.7% in retransplant group and 100% in control group). No statistical difference was observed between the two groups in terms of cold ischemia time and the global HLA mismatch (3.2 ± 1.2 in the retransplant group versus 3.5 ± 1.2 in the control group). However, in relation to the pretransplant PRA levels, a much higher incidence of HLA presensitization was present in the retransplant recipients than in the first transplant recipients (57.6% versus 3.3%, $P < 0.001$). In particular, 11 (33.3%) retransplanted patients were highly presensitized, as evidenced by the high pretransplant PRA levels that were detected (class I or class II ≥ 50%). Based on our criteria, 7 sensitized patients, including 3 patients with positive preformed DSA, received desensitization therapy to decrease anti-HLA antibody levels and ensure a negative flow CDC before their retransplants. Early transplant outcomes were then evaluated on the basis of the frequency of DGF and acute rejection, as well as one-year graft and patient survival.

3.2. DGF. The incidence of DGF in both groups was not high, with 12.1% in the retransplant group and 17.8% in the control group ($P > 0.05$). All patients with DGF were able to achieve normal renal function within 2–4 weeks.

3.3. Acute ABMR. The rates of acute ABMR in both groups were similarly low, with 6.1% (2/33) in the retransplant group and 4.4% (4/90) in the control group ($P > 0.05$). In the retransplant group, two patients unexpectedly generated large amounts of de novo DSA in the first week after transplantation, resulting in severe acute ABMR of renal allografts and eventual graft loss. In the control group, 3 grafts developed mild or moderate acute ABMR, which was successfully reversed by treatment with PP + IVIG. Unfortunately, severe acute ABMR was also seen in the control group, leading to graft loss in 1 patient.

3.4. Acute TCMR. In the control group, acute TCMR was only seen in 5.6% (5/90) of the patients with an induction therapy of low Thymoglobulin doses. However, even when a higher total dosage of Thymoglobulin was administered, resulting in a satisfactory decline in peripheral blood lymphocyte count (Figure 2), a much higher rate of TCMR was still observed in the retransplant group (30.3% versus 5.6% in the control group, $P < 0.001$). A total of 14 acute TCMR episodes were observed in 10 of 33 retransplant patients. Among them, 4 patients had 2 sequential episodes of early acute TCMR. The acute TCMR usually occurred around 2 weeks after the retransplantation and could be successfully reversed by either the high-dose steroid pulse therapy alone or its combination with Thymoglobulin.

A typical example of TCMR in the retransplant group is shown in Figure 3. The recipient was a 47-year-old man. His first renal allograft was from his sister (half HLA match), and the graft developed chronic failure after 10 years. He returned to dialysis without discontinuation of his immunosuppressive drugs. His preretransplant PRA levels were not significantly elevated (class I: 18% and class II: 2%). For his second transplant with HLA 3 MM, the flow CDC was negative. To prevent ABMR, IVIG was given at a daily dose of 10–20 g from day 0 to day 11. The second renal graft achieved immediate function with serum creatinine (sCr) levels decreasing rapidly and reaching normal levels at day 7. However, increased sCr levels were subsequently observed at around day 14 without detected DSA. Acute TCMR was the suspected clinical diagnosis, and thus 3 doses of Methylprednisolone (MP, 500 mg/d) were administered. After the treatment, the patient's sCr levels decreased to 117 μmol/L at day 23. Surprisingly, at day 31 after transplantation, the patient once again had elevated sCr levels (221 μmol/L), and a biopsy was performed (Figure 3(a)). Pathological results indicated acute TCMR, Banff 2007 grade IIA (i3, t2, v1, and g0), with extensive T-cell infiltration and negative C4d staining (Figure 3(b)). The patient fully recovered after treatment with Thymoglobulin (25 mg/d) for 4 days and subsequent MP doses (500 mg/d) for 3 days (Figure 3(a)).

Interestingly, almost all the episodes of TCMR were observed in lower presensitized retransplant patients with pretransplant PRA < 50% (Table 2). In contrast, only 1 of 10 highly sensitized patients (PRA ≥ 50%) developed acute TCMR. This patient did not receive desensitization due to 0 MM of HLA to his donor. Furthermore, none of the highly

Table 2: Impact of desensitization on incidence of TCMR in retransplant patients[#].

	TCMR (+) n (%)	TCMR (−) n (%)	P value
PRA 10% < 50% ($n = 21$)	9 (42.9)	12 (57.1)	
PRA ≥ 50% ($n = 10$)	1[*] (10)	9 (90)	0.067
Nondesensitization ($n = 24$)	10 (41.7)	14 (58.3)	
Desensitization ($n = 7$)	0	7 (100)	<0.05

[#]Two patients with ABMR were censored from the analysis.
[*]No desensitization due to HLA 0 MM.

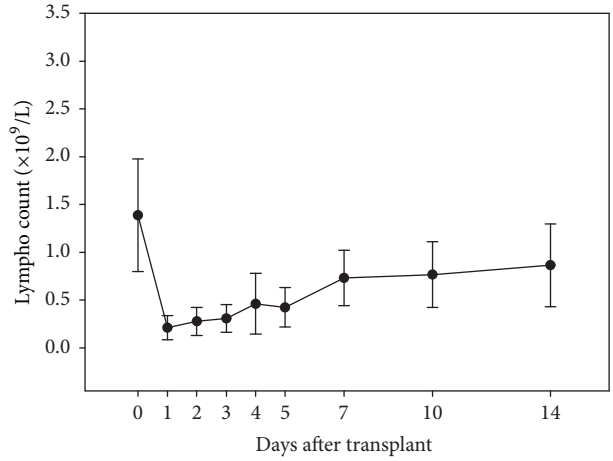

Figure 2: The average blood lymphocyte counts in retransplant patients before and after Thymoglobulin induction therapy. A satisfactory decline of peripheral blood lymphocyte count in the retransplant group was achieved with a 5-day period of Thymoglobulin induction therapy (total dosage: 125–150 mg).

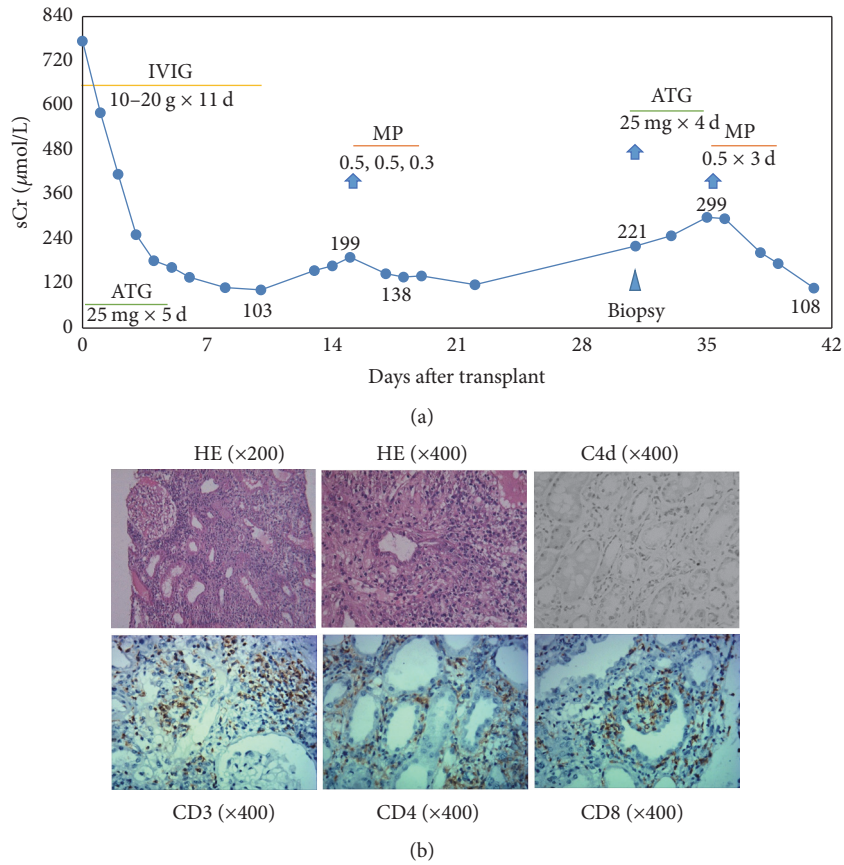

Figure 3: The clinical course of a second graft recipient who had early acute TCMR twice after transplantation. A 47-year-old male patient received his second kidney transplantation. (a) Early clinical course after retransplantation: renal graft gained immediate function with serum creatinine (sCr) levels decreasing rapidly and reaching normal levels at day 7. Increased sCr was observed at around day 14 and 3 doses of Methylprednisolone (MP, 500 mg/d) were then administered. After treatment, sCr levels decreased to 117 μmol/L at day 23 and then were elevated again (221 μmol/L) at day 31, and a biopsy was performed. sCr levels returned to normal after treatment with Thymoglobulin (25 mg/d) for 4 days and subsequent MP (500 mg/d) for 3 days. (b) Pathological results indicated acute TCMR, Banff 2007 grade IIA (i3, t2, v1, and g0), with extensive T-cell infiltration (positive staining for CD3, CD4, and CD8) and negative C4d staining.

sensitized patients with desensitization therapy ($n = 7$) developed acute TCMR, indicating that desensitization may have some role in the prevention of early TCMR (Table 2).

3.5. One-Year Graft and Patient Survival. Both groups had high rates of graft and patient survival at the one-year mark (Table 1). In the retransplant group, graft and patient survival at 1 year were 93.9% and 100%, respectively. None of the retransplant patients lost their grafts due to early acute TCMR. Renal allograft failure was only seen in 2 retransplant patients, as a result of early severe acute ABMR. In the control group, graft and patient survival at 1 year were 92.2% and 95.6%, respectively. One female patient lost her graft in the early period after transplantation because of severe acute ABMR. Two patients lost their grafts due to renal artery rupture caused by donor-derived infections of *Candida albicans*. Additionally, the other 4 patients in the control group died with a functioning graft: 3 patients died from severe interstitial pneumonia which might be cytomegalovirus- (CMV-) relevant, while 1 patient died of cardiac arrest during surgery for renal graft rupture.

4. Discussion

Since the recipients of retransplantation are usually sensitized to certain mismatched HLA antigens because of exposure to previous allograft(s), they are at high risk for the development of acute ABMR. This process is mediated by either preformed or induced DSA that is produced as a result of an anamnestic response by memory B cells [9]. Consequently, considerable efforts have been made to prevent ABMR in kidney retransplantation, including a stringent immunological selection of donors, pretransplant desensitization therapy, and more potent induction therapy. As a result, second kidney transplants have been reported to have similar outcomes to first transplants [10]. Since the HLA presensitization may also result in the generation of alloantigen-specific memory T cells, which could mediate the so-called second-set rejection that is rather difficult to block or inhibit [11, 12], it is important to investigate the incidence and patterns of acute rejection in kidney retransplantation. In the current retrospective study, we included 33 retransplant patients and 90 first transplant patients with similar protocols of induction therapy and

maintenance therapy. Excellent short-term clinical outcomes were obtained in both groups, as evidenced by high rates of one-year graft and patient survival and low incidences of early acute ABMR. Even so, a much higher rate of early acute TCMR was observed in the retransplant group than in the first transplant group, which has been rarely reported or emphasized in the literature.

It is well known that despite attempts devoted to improving outcomes in highly sensitized patients by using desensitization protocols, acute ABMR rates remain high, afflicting about 28%–40% of all cases [13, 14]. In our cohort, however, a much lower incidence of acute ABMR (6.1%) was observed in retransplant patients, leading to significantly improved short-term graft survival. This result is different from previous studies, which could be explained by our cautious selection of patients and donors (by avoiding flow CDC positive transplantation), the expanded indications for desensitization (patients who had negative DSA and flow CDC but with PRA $\geq$ 50% were given desensitization), and the lack of routine primary allograft nephrectomies (implying no massive reduction in immunosuppressive drugs after the return to dialysis) [15]. In addition, compared to other studies which mainly focused on third and fourth renal transplant patients, the majority of our retransplant population were second graft recipients, who may have fewer anti-HLA immunization and memory responses. Finally, the ethnic background of our patients differs from Caucasian and African American patients, which could influence transplant outcomes as well [16].

Notably, even with more potent Thymoglobulin induction therapy, the retransplant patients in our study had a much higher incidence of early acute TCMR compared to the first transplant group. This phenomenon has been rarely reported and emphasized in the literature. One study showed a relatively higher rate of both ABMR (22%) and TCMR (40%) in 37 presensitized kidney transplant patients without any desensitization treatment [17]. However, they mainly focused on ABMR in their study and did not discuss anything about the problem of acute TCMR. In the present study, we found that the type of acute TCMR had the following characteristics: (1) usually occurring around 2 weeks after the retransplantation; (2) repeated occurrence in some of the patients; and (3) the fact that it could be successfully reversed by high-dose steroids and/or Thymoglobulin. Since almost all these episodes of TCMR were effectively reversed, the short-term clinical outcome was not affected in any way. However, long-term graft survival still warrants further observation. The mechanisms for the elevated occurrence of early acute TCMR in retransplant patients also remain unclear. Memory T cells may play a major role in mediating the acute cellular rejection. In retransplant patients, there could be two potential approaches for the generation of alloreactive memory T cells: (1) direct alloantigenic stimulation of naïve T cells during the course of the previous transplantation and (2) homeostatic proliferation in response to transient lymphopenia resulting from T-cell depletion therapy, which induces the proliferation and differentiation of naïve T cells into memory cells [18]. In contrast to naïve T cells, memory T cells have lower activation thresholds and are less dependent on costimulation signals [19], are more resistant to killing by T-cell depletion therapy and regulation by regulatory T cells (Treg) [20], and are less susceptible to conventional immunosuppressive agents [21]. These features lead us to speculate that memory T cells may be responsible for the early acute TCMR observed in our retransplant patients. In the future, we will focus on memory T cells and try to provide direct evidence for our hypothesis. Additionally, we found that none of the highly presensitized retransplant patients with desensitization therapy developed early acute TCMR, indicating that desensitization may have some role in the prevention of early TCMR. However, we cannot draw a clear conclusion due to the small sample size in our current study.

In conclusion, our data on kidney retransplantation show excellent clinical outcomes with low incidence of early acute ABMR and satisfactory one-year patient and graft survival. However, the retransplant patients are at higher risk for the development of early acute TCMR, which requires accurate diagnosis and prompt treatment.

Competing Interests

The authors declared that they have no conflict of interests.

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Research Article

The Polymorphism −308G/A of *Tumor Necrosis Factor-α* Gene Modulates the Effect of Immunosuppressive Treatment in First Kidney Transplant Subjects Who Suffer an Acute Rejection

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The −308G/A SNP of *tumor necrosis factor-alpha (TNF-α) gene* affects TNF-α production. As its impact on transplant outcome remains open to debate, we decided to genotype it in a cohort of transplant subjects. A retrospective analysis of 439 first kidney recipients randomly divided into two subgroups (discovery and validation cohorts) was performed to identify the best predictors of acute rejection (AR). The effect on transplant outcome was analyzed by an adjusted logistic regression model. Carriers of the A allele, associated with elevated TNF-α production, presented a higher risk of AR (OR = 2.78; 95% CI = 1.40–5.51). Logistic regression analyses for AR showed an interaction between the polymorphism and treatment with thymoglobulin (p-interaction = 0.03). In recipients who did not receive thymoglobulin, carriers of A allele had higher risk of AR (OR = 4.05; 95% CI = 1.76–9.28). Moreover, carriers of A allele not treated with thymoglobulin presented higher risk of AR than those who received thymoglobulin (OR = 13.74; 95% CI = 1.59–118.7). The AUC of the model in the discovery cohort was 0.70 and in the validation cohort was 0.69. Our findings indicate that the −308G/A *TNF-α* polymorphism is associated with AR risk and it modulates the effectiveness of thymoglobulin treatment. This pharmacogenetic effect lets us propose this SNP as a useful predictor biomarker to tailor immunosuppressive regimens.

1. Introduction

Acute rejection after kidney transplantation is a major cause of allograft dysfunction and can lead to rapid loss of graft function despite antirejection therapy. Even after initial recovery of kidney function, acute rejection is associated with an increased risk of long-term graft failure [1]. The identification of variables that can trigger rejection or modulate its severity could enable us to improve long-term allograft survival. The variables identified to date include younger age and African American ethnicity in the recipient, older donor age, the degree of donor-recipient human leukocyte antigen (HLA) mismatch, pretransplant anti-HLA alloantibodies, panel-reactive antibodies, ischemia-reperfusion injury (e.g., manifested by delayed graft function), and the adequacy of baseline immunosuppression [2]. It remains unknown why variations in the incidence of acute rejection are observed in patients with similar matching status who have received identical immunosuppressive protocols [3]. Much evidence exists to support the role of cytokines in the inflammatory and immune responses that mediate allograft rejection [4–6]. Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine produced by monocytes/macrophages and, to a lesser extent, by T cells and B cells [7, 8]. TNF-α is released

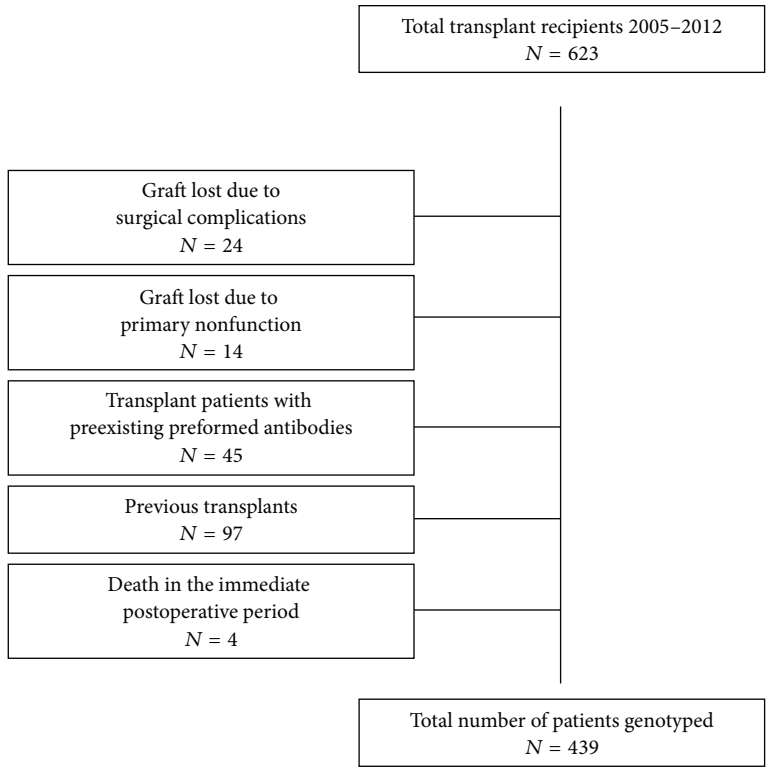

Figure 1: Flow-chart: kidney recipients.

at the site of inflammation, where it causes endothelial cell activation, upregulation of cell adhesion molecules and MHC expression, and increased vasodilatation and vascular permeability [9]. Therefore, TNF-α helps to maintain the inflammatory response to the allograft by facilitating recruitment and activation of leukocytes.

Many researchers have reported increased serum concentrations of TNF-α during acute rejection of liver [10], heart [11], and kidney [12, 13] allografts. The impact of TNF-α has been also reported in human renal allograft biopsies and in rat models of acute and chronic rejection [14, 15].

On the other hand, polymorphism in cytokine genes could explain differences in cytokine production and, therefore, in severity of rejection between individuals [16–18]. Polymorphism associated with cytokine production has been described in the gene encoding TNF-α [19]. The TNF-α gene is located in HLA class III region of the major histocompatibility complex (MHC) on chromosome 6p21.3 [15]. G-to-A single-nucleotide polymorphism (SNP) at position −308 in the *TNF-α* promoter region results in two forms related to their production, carriers of allele A, and GG genotype [20]. The presence of allele A is associated with increased transcriptional activity [19, 21] and elevated TNF-α production [22].

As local TNF-α release promotes endothelial cell activation and intragraft leukocyte migration, increased production of TNF-α could trigger rejection [10, 23, 24]. Consequently, the role of TNF-α polymorphism in acute graft rejection has been studied. Nonetheless, findings for the association between TNF-α and rejection are inconsistent. Some authors report that kidney recipients with the high-producing TNF-α−308A allele are at greater risk of rejection [16, 18, 22, 24–31], whereas other authors found no association [32–38]. Given the apparently controversial results of the studies performed to date, we investigated the impact of this polymorphism in a large cohort of well-characterized kidney recipients and validated our findings in a second cohort.

2. Subjects and Methods

2.1. Patients and Data Collection. Between January 2005 and December 2012, a total of 623 Caucasian adult patients (≥18 years) received a deceased donor organ and were followed up for at least 24 months in our center. We excluded 184 patients (Figure 1). The data recorded were as follows: demographic characteristics (recipient and donor), number of mismatches, immunosuppressive treatment, immediate or delayed graft function (need for dialysis in the first week after transplant), and type of donor (brain death or circulatory death). All diagnoses of rejection were confirmed by biopsy, and acute rejection was categorized according to the Banff classification [39]. Graft loss was defined as returning to chronic dialysis or death with a functioning graft. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul, as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism. The protocol was approved by the Local Ethics Committee, and written informed consent was obtained from all patients.

2.2. Histopathology and C4d Staining. An ultrasound-guided graft biopsy was performed when clinically indicated, that is, in patients with elevated serum creatinine levels. All patients with delayed graft function underwent protocol biopsy every 7 days until kidney function began to improve. A representative biopsy involved at least 1 artery and more than 7 glomeruli. All Banff-scored lesions were assessed [39]. Deposition of C4d was studied by immunohistochemistry. Each patient with an acute rejection episode was tested for serum alloantibody. We classified acute rejection as follows: acute T cell rejection without vascular lesions, acute T cell rejection with vascular lesions, and antibody-mediated rejection according to the Banff classification [39, 40].

2.3. Immunosuppression. Patients who received a kidney from a brain dead donor were treated mainly with tacrolimus, mycophenolate mofetil, and methylprednisolone; when the donors had expanded criteria or when ischemia time was long, they also received basiliximab or thymoglobulin. When the organ was donated after circulatory death, most patients received treatment with tacrolimus, mycophenolate mofetil, and methylprednisolone combined with basiliximab or thymoglobulin. In patients who received thymoglobulin, tacrolimus was introduced between days 4 and 6 after transplant.

2.4. Cytokine Polymorphism Genotyping. Genomic DNA was extracted from EDTA-anticoagulated peripheral whole blood. The −308G/A *TNF-α* polymorphism (rs1800629) was genotyped in a 7900HT Fast Real-Time PCR System using a TaqMan assay (C_7514879_10, Applied Biosystems, Foster City, California, USA), as recommended by the manufacturer.

2.5. Statistical Analysis. Sample size was calculated based on an alpha risk of 0.05 and a beta risk of 0.2 in a 2-tailed contrast to detect a minimum relative risk of 2, assuming that 20% of patients not exposed to treatment would experience vascular rejection. It was calculated that 57 patients would be necessary in the high-producing group and 285 in the non-high-producing group (STATA, version 12.0). Kidney transplant recipients were randomly divided into two groups (2/3 and 1/3). The study of predictive factors was performed in 286 patients (discovery cohort) and subsequently validated in 153 patients (validation cohort). The influence of cytokine genotypes on acute rejection was expressed as a dichotomous variable, namely, low producers (GG) or high and intermediate producers (AA and GA). Qualitative variables were compared using the chi-square test or Fisher exact test and expressed as frequency distributions. Qualitative variables are expressed as mean (SD) or median (IQR) in the case of nonnormally distributed variables. They were compared using the t-test or nonparametric tests where necessary. An adjusted logistic regression model was constructed and included variables with $p < 0.15$ in the univariate analysis or variables that were biologically relevant in the population analysis. Interactions with TNF-α polymorphism were evaluated. The p value for the interaction was obtained from the models constructed. The adjusted odds ratios (Adj. OR) are presented with their 95% confidence intervals. Discriminatory power was evaluated using area under the receiver operating characteristic (ROC) curves (AUC) of the predicted probabilities obtained in the model. Calibration was assessed using the Hosmer-Lemeshow goodness-of-fit test in both the discovery cohort and the validation cohort. Survival of the kidney transplants as functioning organs was analyzed using the Kaplan-Meier method with a log-rank test. Null hypotheses with an alpha error < 0.05 were rejected. The statistical package used was SPSS version 15.0.

3. Results

Of the total 439 patients in the cohort, 119 (27.1%) developed acute rejection (AR); of these, 83 experienced vascular involvement (18.9%). Median follow-up was 62.5 (39.9–87.2) months. The median time to rejection was 9 (7–15) days, and 96.6% of all rejections were during the first year after transplantation. The genotype distribution of −308A/G *TNF-α* was 82.9% ($N = 364$) for GG, 15.3% ($N = 67$) for GA, and 1.8% ($N = 8$) for AA.

The study of predictive factors was performed in 286 patients and subsequently validated in 153 patients. The distribution of risk factors was similar between the two cohorts (Table 1). The results of the univariate analysis for AR in the discovery cohort are shown in Table 2. The variables significantly associated with a greater risk were age (donor and recipient), the −308A/G polymorphism, and the immunosuppressive treatment. Carriers of the A allele had a greater risk of AR than patients with the GG genotype (Table 2).

The model was adjusted to evaluate the development of acute cellular rejection and only the statistically significant variables were shown (Table 3). In this multivariate analysis, a significant interaction was recorded between induction treatment with thymoglobulin and the polymorphism in *TNF-α* ($p = 0.03$) (Table 3). Carriers of the A allele who were not treated with thymoglobulin had a 4.05 times greater risk of vascular rejection than those harboring GG (Table 3) (Figure 2). Furthermore, in the subgroup of carriers of A allele, the probability of rejection was considerably higher (13-fold) in patients not receiving thymoglobulin than in those receiving thymoglobulin (Table 3). The area under the ROC curve of the model in the discovery cohort was 0.70 (95% CI = 0.62–0.78); the p value of the Hosmer-Lemeshow test was 0.916. The model was subsequently applied in the validation cohort, and the area under the ROC curve was 0.69 (95% CI = 0.54–0.76).

4. Discussion

Alloimmune responses and differences in susceptibility to rejection may be influenced by individual variations in cytokine genes. Indeed, cytokine gene polymorphism types have been extensively explored in transplantation because they are thought to explain the heterogeneous outcomes of the allograft and can thus help clinicians to tailor immunosuppression [5, 31, 33, 41–43].

Several studies have assessed the association between the −308A/G TNF-α polymorphism and acute rejection

Table 1: Characteristics of the two randomly divided cohorts of kidney recipients.

	Discovery cohort (n = 286)	Validation cohort (n = 153)	p value
Recipient age, years, mean ± SD	52.2 ± 13.5	51.8 ± 13.0	0.78
Male recipient, *N* (%)	191 (66.8%)	100 (65.4%)	0.76
Time on dialysis, months	17.8 (7.1–31.2)	16.8 (6.1–26.9)	0.58
Cause of chronic renal failure, *N* (%)			0.08
Glomerulonephritis	86 (30.1%)	53 (34.6%)	
Chronic tubulointerstitial nephropathy	31 (10.8%)	23 (15.0%)	
Nephroangiosclerosis	22 (7.7%)	11 (7.2%)	
Polycystic kidney disease	45 (15.3%)	21 (13.7%)	
Diabetic nephropathy	42 (14.7%)	8 (5.2%)	
Unknown cause	48 (16.8%)	32 (20.9%)	
Others	12 (4.2%)	5 (3.3%)	
Donor age, years, mean ± SD	43.0 ± 14.3	42.6 ± 14.1	0.73
Male donor, *N* (%)	209 (73.6%)	112 (74.2%)	0.90
Donor type, *N* (%)			0.65
Brain death	111 (38.8%)	56 (36.6%)	
Circulatory death	175 (61.2%)	97 (61.2%)	
Immunosuppressive treatment, *N* (%)			0.87
Thymoglobulin + FK + MMF + P	98 (34.3%)	54 (35.3%)	
IL2R + FK + MMF + P	127 (44.4%)	69 (45.1%)	
FK + MMF + P	54 (18.9%)	28 (18.3%)	
CsA + MMF + P	2 (0.7%)	0 (0)	
FK + SRL + P	1 (0.3%)	1 (0.7%)	
Belatacept + MMF + P	4 (1.4%)	1 (0.7%)	
Follow-up time, months (median [IQR])	74.9 (53.8–99.5)	73.0 (50.2–97.5)	0.54
Delayed graft function, *N* (%)	143 (50.0%)	80 (52.3%)	0.65
HLA-A mismatch, *N* (%)			0.56
0	24 (8.4%)	17 (11.3%)	
1	118 (41.4%)	64 (42.4%)	
2	143 (50.2%)	70 (46.4 %)	
HLA-B mismatch, *N* (%)			0.55
0	12 (4.2%)	4 (2.6%)	
1	112 (39.3%)	66 (43.4%)	
2	161 (56.5%)	82 (53.9%)	
HLA-DR mismatch, *N* (%)			0.13
0	34 (11.9%)	18 (11.8%)	
1	123 (43.2%)	80 (52.6%)	
2	128 (44.9%)	54 (35.5%)	
Acute total rejection, *N* (%)	80 (28.0%)	39 (25.5%)	0.58
Acute rejection Banff ≥ 2, *N* (%)	55 (19.2%)	28 (18.3%)	0.81
Acute humoral rejection, *N* (%)	20 (7.0%)	10 (6.5%)	0.86
Genotype frequency GA/AA TNF-α −308, *N* (%)	49 (17.1%)	26 (17.0%)	0.97
Graft loss, *N* (%)	49 (17.1%)	21 (13.7%)	0.35

FK: tacrolimus; MMF: mycophenolate; P: prednisone; IL2R: interleukin- (IL-) 2 receptor antagonist; CsA: cyclosporin A; SRL: sirolimus.

in kidney recipients of different populations; however, the results are apparently inconsistent and inconclusive. Our data support reports that found association of this SNP with a higher incidence of acute rejection [16, 18, 22, 24–31]. In their meta-analysis, Hu et al. [44] concluded that the TNF-α high producers genotypes in the recipient were associated with an increased risk of acute allograft rejection. In this meta-analysis, authors recommended performing additional studies with large sample size and better study designs.

Discrepancies in previously reported findings may be due to a small size that compromised the statistical power and heterogeneity of the studies. Only few reports included cohorts of consecutive transplants performed over a specific time period [16, 24, 33, 34, 37] and some even recruited

Table 2: Univariate analysis for acute rejection in the discovery cohort ($n = 286$).

	Acute rejection N (%)	OR (95% CI)	p value
Recipient age			0.04
<60 years	43 (22.6%)	2.04 (1.02 to 4.17)	
≥60	12 (12.5%)	1	
Recipient sex			0.17
Male	41 (21.5%)	1.59 (0.81 to 3.03)	
Female	14 (14.7%)	1	
Donor age			0.05
<60 years	54 (20.6%)	5.88 (0.81 to 50.0)	
≥60	1 (4.2%)	1	
Donor sex			0.22
Male	44 (21.1%)	1.72 (0.83 to 3.70)	
Female	11 (14.5%)	1	
Time on dialysis			0.64
<15 months	24 (18.0%)	1	
≥15 months	31 (20.3%)	1.15 (0.64 to 2.09)	
Donor type			0.47
Brain death	19 (17.1%)	1	
Circulatory death	36 (20.6%)	1.25 (0.68 to 2.32)	
Delayed graft function			0.45
Yes	30 (21.0%)	1.25 (0.70 to 2.26)	
No	25 (17.5%)	1	
TNF-α −308 polymorphism			0.003
GG	38 (16.0%)	1	
GA/AA	17 (34.6%)	2.78 (1.40 to 5.51)	
Immunosuppressive treatment			0.003
Thymoglobulin + FK + MMF + P	8 (8.2%)	1	
IL2R + FK + MMF + P	32 (25.2%)	3.74 (1.63 to 8.57)	
Other (belatacept, SRL)	15 (24.2%)	3.55 (1.40 to 8.98)	
HLA mismatch			0.75
<3	4 (22.2%)	1.20 (1.38 to 3.85)	
≥3	51 (19.1%)	1	
HLA-DR mismatch			0.22
≤1	34 (21.9%)	1	
2	21 (16.2%)	0.69 (0.38 to 1.25)	
HLA-A mismatch			0.86
≤1	28 (19.7%)	1	
2	27 (18.9%)	0.95 (0.52 to 1.71)	
HLA-B mismatch			0.56
≤1	22 (17.7%)	1	
2	33 (20.5%)	1.20 (0.66 to 2.18)	

FK: tacrolimus; MMF: mycophenolate; P: prednisone; IL2R: interleukin- (IL-) 2 receptor antagonist; SRL: sirolimus.

hyperimmunized and retransplanted patients [16, 26, 30, 31, 33, 34, 37]. Moreover, rejection was not histologically confirmed in most studies and incidence of rejection also varies considerably, ranging from 17% [16] to 63% [24]. In addition, discrepancies in terms of the impact of the TNF-α polymorphism in kidney rejection could also arise because of differences in the immunosuppressive therapy used, as the type of medication administered is not usually included. The most common immunosuppressive agents are cyclosporine, prednisone, and azathioprine or mycophenolate [16, 18, 24–27, 29–34, 36–38]. Our results suggest that use of triple therapy based on tacrolimus, mycophenolate, and corticosteroids may not be sufficient to block release of TNF-α in patients with the high producer genotype. TNF-α is mainly generated by monocytes and macrophages [45] and it has been reported that the aforementioned triple therapy does not have a clear effect in these cells [46–48]. However, antithymocyte globulin promotes expansion of regulatory T cells [49], the

Table 3: Multivariate analysis for acute rejection in the discovery cohort ($n = 286$).

Variable	OR (95% CI)*	p value
No thymoglobulin treatment		
TNF-α −308 GG	1	
TNF-α −308 GA/AA	4.05 (1.76 to 9.28)	0.001
Thymoglobulin treatment		
TNF-α −308 GG	1	
TNF-α −308 GA/AA	0.65 (0.12 to 3.69)	0.65
TNF-α −308 GG		
Thymoglobulin treatment	1	
No thymoglobulin treatment	2.72 (1.05 to 7.05)	0.04
TNF-α −308 GA/AA		
Thymoglobulin treatment	1	
No thymoglobulin treatment	13.74 (1.59 to 118.7)	0.02
Recipient age		
≥60 years	1	
<60 years	2.29 (1.10 to 4.78)	0.03

*Adjusted for recipient sex, donor age and sex, HLA-DR mismatches, and delayed graft function.
p-interaction (thymoglobulin treatment and TNF-α −308 polymorphism) = 0.03.

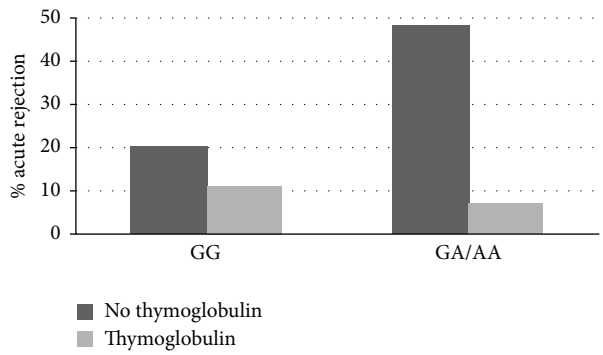

Figure 2: Percentage of AR in renal transplant patients stratified both −308G/A *TNF-α* gene polymorphism and treatment with thymoglobulin.

main producers of interleukin-10, which in turn inhibits production of TNF-α by macrophages [50]. Therefore, antithymocyte globulin could help to control the immune response in patients who produce high levels of TNF-α.

Our study is limited by the fact that TNF-α genotyping was not performed in the donor. Nevertheless, bearing in mind that most TNF-α is produced by macrophages, we think that more emphasis should be placed on the receptor genotype.

Before a model can be relied upon to draw conclusions or predict future outcomes, it is important to ensure that it is correctly specified; that is, the data do not conflict with assumptions made by the model. Logistic regression is the most popular modeling approach for binary outcomes. The Hosmer-Lemeshow test makes it possible to compare goodness of fit by comparing observed and predicted risks across subgroups in a population. Prediction models allow clinicians to estimate prognosis [51, 52] and are increasingly used in clinical practice to guide decision-making [52]. Our study is the first one that retrospectively analyzed a prospective cohort of first transplants in patients with no preformed antibodies who were randomly assigned to two cohorts, a discovery and a validation cohort. We attempted to predict the risk of acute rejection according to variables that can modify the effect simultaneously (age, delayed graft failure, immunosuppression protocol, and the TNF-α polymorphism).

5. Conclusions

The *TNF-α gene* polymorphism that was previously associated with differential production of this cytokine is associated with AR risk and modulates the effectiveness of thymoglobulin treatment. Screening of this polymorphism will enable us to predict those patients (carriers of A allele) more likely to experience rejection and, therefore, require more intense immunosuppressive therapy. Similarly, it will enable us to identify patients with a potentially optimal response, who can be treated with less potent immunosuppression.

Competing Interests

The authors declare that they have no conflict of interests.

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