Donor-Specific HLA Alloantibodies: Long-Term Impact on Cardiac Allograft Vasculopathy and Mortality After Heart Transplant

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Abstract

Objectives: **The clinical significance of anti-HLA-alloantibodies remains controversial. Recent studies have linked development of donor-specific HLA-antibodies to chronic allograft rejection and graft loss after heart, kidney, and lung transplants. We investigated the clinical impact of donor-specific humoral alloreactivity during the follow-up of heart transplant recipients.**

Patients and Methods: **The sera of 213 heart transplant recipients were screened by enzyme-linked immunosorbent assay for HLA-antibody production. The antigen specificity of the detected HLA class I and class II antibodies was identified using a Luminex assay. Outcome variables were survival, cardiac allograft vasculopathy, and cellular rejection.**

Results: **The cumulative incidence of alloantibody formation was 23/213 patients (10.8%). The majority of detected alloantibodies were donor-specific for HLA class II. Mean follow-up at antibody measurements was 7 ± 4.9 years. Freedom from vasculopathy at 5 and 10 years was 77.9% and 26% in donor-specific HLA-antibody-positive patients compared with 84.6% and 65.2% in antibody-negative controls (*P* = .025). Freedom from treated, biopsy-proven rejection was 44.4% for donor-specific HLA-antibody-positive patients compared with 70.2% in the controls (*P* = .06). Multivariate analyses identified donor-specific HLA antibody positivity as an independent risk factor for vasculopathy.**

Conclusions: **Our results demonstrate a strong correlation between the development of donor-specific HLA antibodies and adverse outcomes after heart transplant. Detection of donor-specific HLA antibodies might identify high-risk patients and offer an opportunity for early clinical intervention and modification of immunosuppression.**

Key words: *Humoral rejection, Antibody-mediated rejection, Histocompatibility, Donor-specific antibodies, Cardiac allograft vasculopathy*

Advances in immunosuppression have reduced the frequency of cellular rejection, but chronic allograft rejection remains the major obstacle limiting successful long-term outcomes after heart transplant (1). Recent studies have demonstrated that a not-negligible proportion of rejection episodes is not only caused by a T-cell response but also is mediated by preformed and de novo alloantibodies predominantly specific for donor graft HLA class I and class II molecules (2, 3). This type of rejection is an unsolved problem in all fields of organ transplantation because current immunosuppressive regimens are generally intended to interfere in T-cell signaling pathways (2). Manifestation of antibody-mediated (or referred to as biopsy-negative or vascular rejection) occurs in approximately 10% to 20% of cardiac allograft recipients and is associated with hemodynamic compromise, accelerated cardiac allograft vasculopathy, graft loss, and mortality (4).

Cardiac allograft vasculopathy is a rapidly progressive, obliterative form of concentric intimal proliferative arteriosclerosis representing the leading cause of death and retransplant in long-term heart

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transplant survivors. Nonimmunologic risk factors for cardiac allograft vasculopathy encompass a variety of noxious stimuli including ischemia-reperfusion injury, viral infections, and immunosuppressive drugs, as well as hyperlipidemia, insulin resistance, and hypertension (5). Nonetheless, the immunologic response to the allograft is alleged to initiate vascular pathologies culminating in cardiac allograft vasculopathy (6). Previous investigations have shown that donor specific antibodies (DSA) are correlated with poor cardiac allograft survival (7, 8). Circulating antibodies mediate rejection through complement activation and fixation on graft endothelium, thereby predisposing the patient to graft loss, accelerated cardiac allograft vasculopathy, and death (4, 9). With respect to cardiac transplant, clinical and experimental studies have shown that C4d complement component activation and deposition correlate with the presence of DSA subsequently leading to cardiac allograft vasculopathy and thereby contributing to late graft failure (8, 9). The rate of allosensitized patients on waiting lists is increasing as a consequence of growing numbers of retransplants and assist-device implantations (2, 8, 9). Allosensitization is a consequence of exposure to disparate HLA molecules during pregnancy, blood/platelet transfusions, or after cardiac repairs with homograft material.

As a result of the increasing clinical significance and the diagnostic difficulties associated with antibody-mediated rejection, the International Society for Heart Transplantation Task Force for the Standardization of Nomenclature in the Diagnosis of Heart Rejection, and a National Conference for the Assessment of Antibody-Mediated Rejection in Solid Organ Transplantation published modified diagnostic criteria and recommendations for evaluating antibody-mediated rejection (4, 10).

The objective of this study is to review the impact of DSA on survival, cardiac allograft vasculopathy, and cellular rejections after heart transplant.

Patients and Methods

Blood samples were obtained from 213 patients during routine transplant follow-up at our outpatient department. Mean follow-up at antibody measurements was 7 ± 4.9 years. The sera were screened by enzyme-linked immunosorbent assay (ELISA) for HLA-antibody production. Donor specificity of the detected HLA class I and class II antibodies was identified using a Luminex bead assay, while the antibody specificity was differentiated by various single-antigen–coated beads. The presence of DSA was correlated with outcome variables including survival, cardiac allograft vasculopathy, and cellular rejection episodes. A rejection episode was defined as biopsy-proven cellular rejection greater than 1B/1R or any treated rejection episode of 1B/1R or more according to the old and new International Society for Heart Transplantation classification. Patient demographics and major outcome-influencing variables are listed in Table 1.

Table 1. Demographic data of screened patients including incidence of anti-HLA-alloantibodies.

Characteristic	Value	Percentage
Number of patients screened	213	100
Mean recipient age, years	48.2 ± 13	
Sex		
Male	180	84.5
Female	33	15.5
Indication		
DCM	145	68.1
ICM	54	25.4
Other	14	6.5
Mean ischemic time, min	212 ± 62.8 min	
Mean follow-up time at Ab-screening, y	7 ± 4.9 y	
pos. for anti-HLA-alloantibodies	23	10.8

Values are expressed as number or means ± SD

Abbreviations: DCM, dilative cardiomyopathy; ICM, ischemic cardiomyopathy

Pretransplant HLA antibody detection

Pretransplant HLA antibody detection was performed using the complement-dependent lymphocytotoxicity assay when patients were listed for transplant. Antibody reactivity was measured as the percentage of the panel reactive to the patient's serum panel reactive antibody. Patients with a pretransplant panel reactive antibody level higher than 10% were excluded from this analysis.

Immunosuppressive regimen

All patients received triple immunosuppressive therapy consisting of cyclosporine or tacrolimus in combination with mycophenolate mofetil, azathioprine or sirolimus, and corticosteroids. Since 1995, we have routinely withdrawn steroids after 6 months. Induction therapy is reserved for special indications and is not given routinely at our center.

HLA typing

Donors and recipients were typed for major histocompatibility class I (HLA-A and -B) by conventional microlymphocytotoxicity. Major

histocompatibility class II (HLA-DR and -DQ) typing was performed by molecular techniques using polymerase chain reaction and amplification with sequence-specific oligonucleotide primers.

Anti-HLA alloantibody screening

Anti-HLA class I and II IgG antibody detection was performed automatically by a solid-phase ELISA (AbScreen HLA class I [111405-ASI] and II [013006-ASII], Biotest Quickstep, Dreieich, Germany). Microtiter plates are coated with highly purified HLA class I antigens (HLA-A,-B,-C) from a pool of human platelets. HLA class II molecules (HLA-DR, -DQ) are purified from selected Epstein-Barr virus-transformed cell lines. If the sample contained specific antibodies against HLA class I or II, they bound to the antigens on the microtiter plate. Unbound antibodies were removed in a washing step. The resulting antibody-antigen complex was detected using a specific enzyme-labeled antibody directed against human IgG. Results greater than twice the mean of the negative controls were defined as positive.

Anti-HLA alloantibody panel-reactivity and specification

Anti-HLA alloantibody specification was performed with a set of 54 (HLA class I) and 34 (HLA class II) color-coded microsphere beads bound with affinity-purified class I (HLA-A,B,C) and II (HLA-DR,DQ) antigens (Tepnel, Lifecodes 0621106LM1, 010906LM2, CT, USA) for the qualitative detection of IgG panel reactive antibodies. An aliquot of the beads was incubated with a small volume of test serum sample. The sensitized beads were then washed to remove unbound antibody. An anti-human IgG antibody conjugated to phycoerythrin was added. After a second incubation, the test sample was diluted and analyzed on the Luminex instrument. The signal intensity from each bead was compared to the signal intensity of a negative control. In all patients, the same cut-off level was used. The interpretation was performed manually and by using the Tepnel Quicktype for Lifematch software version 2.2.

Definition and grading of cardiac allograft vasculopathy

Angiographic controls were done after 1 month and on a yearly basis thereafter. Alternatively, after 4 years, patients underwent a computer-assisted tomography scan or dobutamine-induced stress echocardiography. Catheterization was only performed in patients revealing irregular findings in these examinations. Nevertheless, at least every 2 years, a coronary angiogram was mandatory. The incidence of cardiac allograft vasculopathy was defined as any new-onset coronary stenosis of more than 30% or severe rarefacation of the small coronary branches. The angiograms were evaluated by an independent cardiologist without knowledge of HLA compatibility. Coherent intravascular ultrasound data were only available in a minority of the patients and therefore, were not considered in this study.

Statistical analyses

All analyses were done using SPSS software (Statistical Product and Services Solutions, version 15.0, SPSS Inc, Chicago, IL, USA). The Kaplan-Meier analysis, log-rank test, and *t* test were used for parametric, and the chi-square test was used for categorical, variables. A multivariate Cox regression model was used for statistical data analysis. Survival and freedom from cardiac allograft vasculopathy and rejection were calculated using a Kaplan-Meier analysis. Differences in actuarial survival as stratified by the presence of HLA antibodies were determined by the log-rank test. For multivariate analyses, a Cox regression analysis for metric and ordinal variables was calculated. A value for *P* less than .05 was considered statistically significant. All variables are expressed as a number or as a mean ± standard deviation unless otherwise indicated.

Results

The demographic characteristics of screened patients are summarized in Table 1. Of the 213 heart transplant recipients, 23 showed positivity for the presence of DSA (10.8%). The majority of DSA were directed against HLA-DR and –DQ (major histocompatibility class II). Four patients developed only HLA class I (n=4; 1.9%), and 12 patients developed only class II (n=12; 5.6%) antibodies. Seven patients developed both anti-HLA class I and class II antibodies (n=7; 3.3%). Patients with detectable amounts of antibodies were younger than DSA-negative controls (mean recipient age, 39.5 ± 16 years vs 49.2 ± 13 years) (*P* = .001). With respect to donor age, recipient sex, assist-device implantation before cardiac transplant and ischemic time, no

differences between the groups were found. The number of patients having a retransplant and rare indications for heart transplant (eg, congenital) were more frequent in the DSA-positive group (Table 2).

Table 2. Demographic data as classified by presence or absence of anti-HLA alloantibodies.

Characteristic	HLA-Ab. negative (n=190)	HLA-Ab.-positive (n=23)	*P* value
Mean recipient age, years	49.2 ± 13	39.5 ± 16	.001
Mean donor age, years	36.1 ± 13.4	34.6 ± 15.3	NS
Sex (male/female)	163/27	17/6	NS
Mean follow-up, years	6.9 ± 4.7	8.2 ± 6.4	.007
Indication (DCM/ICM/other)	132/50/8	12/5/6	.001
Mean ischemic time, min	215 ± 62	193 ± 70	NS
Retransplant	3	2	.09
Assist device	17	4	NS

Values are expressed as numbers or means ± SD.

Abbreviations: DCM, dilative cardiomyopathy; ICM, ischemic cardiomyopathy; NS, not significant

Survival of all screened patients after a mean follow-up of 7 ± 4.9 years at the time of antibody screening was 98.1%, 97.5%, 96.5%, and 84.6% after 1, 5, 10, and 15 years. This is a positive selection because only long-term survivors were screened during routine follow-up visits. However, Kaplan-Meier survival analyses revealed significant differences when comparing survival rates of DSA-positive and DSA-negative patients. Cumulative survival of DSA-positive patients after 5, 10, and 15 years was 89.3%, 80.3%, and 53.6% compared with 98.4% after 5 and 97.3% after 10 and 15 years for DSA-negative controls ($P = .001$) (Figure 1A).

Freedom from cardiac allograft vasculopathy in DSA-positive patients was 94.4%, 81.5%, 41.2%, and 10.3% at 1, 5, 10, and 15 years compared with 96.2%, 83.4%, 67.3%, and 34.7% in DSA-negative controls ($P = .025$) (Figure 1B).

Freedom from cellular rejection was 60.0% at 1 year and 46.7% at 5 years in DSA-positive recipients compared with 92.1% at 1 and 70.2% at 5 years in negative controls. A marked trend toward more cellular rejection episodes in DSA-positive patients was detected ($P = .06$) (Figure 1C).

Furthermore, Kaplan-Meier analysis for freedom from cardiac allograft vasculopathy at 1, 5, 10, and 15 years after transplant was 94.4%, 81.5%, 41.2%, and 10.3% for recipients with anti-HLA class II antibodies and 96.3%, 83.1%, 67.3%, and 32.9% for recipients without these antibodies ($P = .02$).

A multivariate Cox regression analysis was performed to identify independent risk factors for the development of cardiac allograft vasculopathy. Univariate risk factors such as recipient age, diagnosis, sex, ischemic time, and several acute

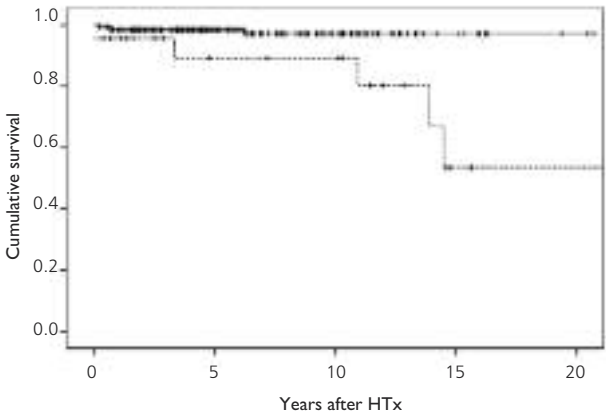

Figure 1A. Kaplan-Meier survival estimate. Comparison between antibody-positive recipients (dashed line) and the antibody-negative controls (bold line). Higher mortality risk in alloantibody-positive recipients ($P = .001$).

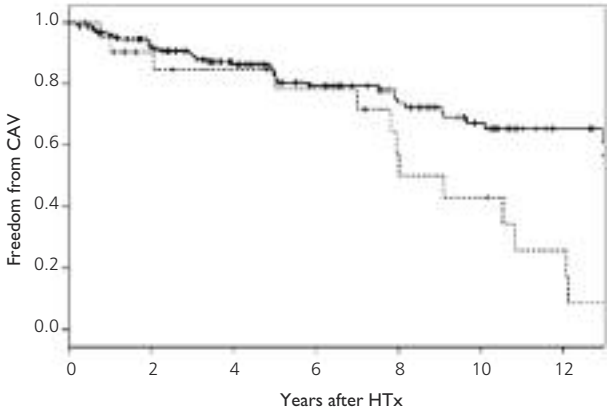

Figure 1B. Freedom from CAV. Comparison between antibody-positive recipients (dashed line) and the antibody-negative controls (bold line). Faster CAV development in alloantibody-positive recipients ($P = .025$).

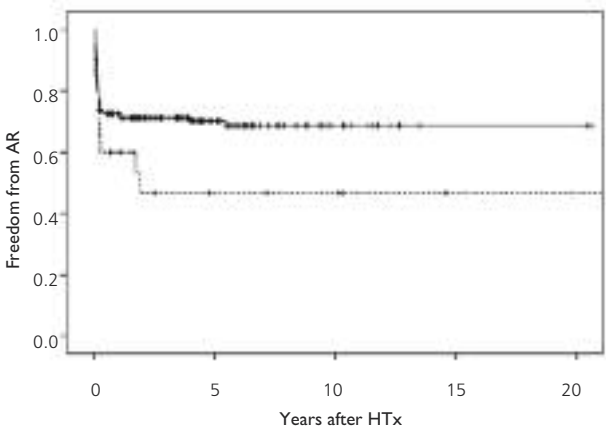

Figure 1C. Freedom from acute rejection episodes. Comparison between antibody-positive recipients (dashed line) and antibody-negative controls (bold line). A marked trend toward more cellular rejection episodes in alloantibody-positive recipients ($P = .06$).

cellular rejection episodes were included. Only presence of anti-HLA alloantibodies remained as an independent risk factor for cardiac allograft vasculopathy in the multivariate analysis ($P = .009$; odds ratio 2.96).

Discussion

Our results demonstrate that presence of circulating HLA-directed donor-specific alloantibodies is correlated with increased mortality, cardiac allograft vasculopathy, and a trend toward more cellular rejection episodes. An increasing number of studies has demonstrated the clinical impact of preformed and de novo anti-HLA alloantibodies on outcome after solid-organ transplant (7, 8). Histopathologically, humoral rejection of the heart is defined by capillary endothelial changes, infiltration of macrophages and neutrophils, interstitial edema, and linear accumulations of immunoglobulins and complement, especially C4d. Graft rejection is triggered by alloantibody-mediated activation of endothelial and smooth muscle cells, activation of complement-dependent mechanisms, interaction of antibodies with macrophages and other leucocytes via their Fc receptors, and finally, enhanced presentation of alloantigens to T cells stimulating concomitant cellular immune responses (2). Further evidence for the pathomechanistic role of HLA-antibodies emerges from experimental studies whereby ligation of class I and class II molecules on the surface of endothelial cells triggered by anti-HLA alloantibodies promotes diverse biological functions including platelet and macrophage activation, thrombosis, and pathological endothelial growth and smooth muscle cell proliferation (9).

Approximately 10% to 20% of patients will present with episodes of hemodynamic compromise with no evidence of cellular rejection in endomyocardial biopsies (11). Up to 68% of early antibody-mediated rejecting patients have graft dysfunction; only 13% of those presenting late exhibit graft dysfunction (10). Additionally, all of the mentioned processes predispose for the development of cardiac allograft vasculopathy. Michaels and associates reported that humoral rejection without concurrent cellular rejection in cardiac transplant recipients correlates with an increased risk of mortality, and patients who developed antibody-mediated rejection progressed to cardiac allograft vasculopathy earlier and at an increased frequency (12).

Our results confirm the results observed in previous investigations. In a retrospective study, Stastny and associates (13) analyzed the impact of HLA-antibodies in the sera of 113 adult heart transplant recipients that were collected before the transplants, as well as 1, 6, and 12 months after the transplants and annually thereafter. In all, 27.4% of all included patients screened positive for the presence of antibodies; antibody specificity was determined by single-antigen–coated beads. The authors found a highly significant association between the presence of donor-specific HLA alloantibodies and the incidence of transplant coronary artery disease, the frequency of acute rejection episodes, and graft failure. Furthermore, they revealed that obviously only the presence of antibodies that can bind to the graft—thus, donor-specific antibodies—is associated with an adverse outcome. Inherent to the cross-sectional design of our study, which must be considered a limitation, we cannot conclude at which time antibodies occurred or even disappeared during the posttransplant follow-up. Nevertheless, we found similar results

The prevalence of DSA in our patient population was 10.8%, which is less than the rate reported by other groups (7, 8). Most of our screened patients belong to the group of long-term survivors. Antibody screenings were conducted after a mean follow-up of 6.5 years at different times after transplant. Many antibody-mediated rejection episodes, especially those with hemodynamic compromise, occur within the first years after heart transplant. Some patients with early graft loss due to rejection and cardiac allograft vasculopathy are not available for screenings, which contributes to a positive selection bias. This can be considered a limitation of this analysis; however, in our opinion, a prospective study including patients with early graft loss would most probably strengthen our results.

Another possible explanation for the low prevalence of DSA might be related to the use of mycophenolate mofetil, which became a new standard between 1995 and 1997. Furthermore, sirolimus was introduced to our maintenance protocols in 2000. These immunosuppressants have beneficial effects on cardiac allograft vasculopathy prevention and might also reduce antibody production (14, 15).

Somewhere between 15% and 82% of patients will

develop detectable levels of circulating antibodies by 6 months after transplant (10). The majority of patients will remain asymptomatic but probably will progress to cardiac allograft vasculopathy earlier and at an increased frequency. It is important to monitor these antibodies on a routine basis.

Not only in cardiac but also in other fields of transplant, mismatches on the HLA class II locus seem to have great effect on the development of an alloimmune response against transplanted organs. For the presence of only HLA class I antibodies, no significant correlation with adverse outcome was found. We have previously reported that the HLA-DR mismatch, which is known to be a major risk factor for the development of HLA-antibodies (1), has a significant effect on long-term survival and cardiac allograft vasculopathy in heart transplant patients, whereas HLA-A and HLA-B show no such correlation (16).

Tambur and associates (17) investigated the influence of de novo DSA on survival, rejection episodes, and cardiac allograft vasculopathy. Only the presence of donor-specific HLA class II antibodies was correlated with cardiac allograft vasculopathy and graft failure. Vasilescu and associates (18) analyzed the relation between cardiac allograft vasculopathy and HLA mismatches, presence of anti-HLA-antibodies, growth of lymphocyte-infiltrations, and biopsy-proven acute rejection. The study revealed a significant correlation between cardiac allograft vasculopathy and generation of anti-HLA class II antibodies. Monitoring of these antibodies facilitates the prevention and diagnosis of rejection episodes before clinical manifestations of irreversible organ damage. It must be kept in mind that the application of the above-mentioned, more-sensitive methods for detection of circulating alloantibodies fails to detect antibodies that are already bound to target antigens in the allograft. Therefore, the search for reliable alternative diagnostic markers is of obvious importance (19, 20).

Recognition of anti-HLA class II antibodies is problematic only when using standard panel reactive antibody assays. The standard for panel reactive antibody testing is complement-dependent lymphocytotoxicity, which is conducted with T lymphocytes and not B cells and therefore, is unable to detect HLA class II alloreactivity. Additionally, complement-dependent lymphocytotoxicity carries the risk of detecting clinically less-relevant non-HLA antibodies. Patients without antibodies detectable by a complement-dependent lymphocytotoxicity assay still experienced early and accelerated antibody-mediated graft rejection and loss, underscoring the need for more-sensitive assessments. Solid-phase–based assays such as Luminex, ELISA, and flow cytometry provide higher sensitivity even at low antibody titers and enable the determination of single epitope specificity of detected alloantibodies in multispecific sera (21, 22). The information provided by these reliable tests would be useful in predicting crossmatch results in sensitized patients and identifying donor HLA antigens prognosticated to be nonreactive with the recipient's alloantibodies. Vaidya and associates (23) demonstrated that crossmatch outcome could be predicted with greater-than-90% accuracy by using a labeled-streptavidin-biotin-method assay and suggest that this approach to donor selection might allow wider geographic sharing of organs without performing time-consuming crossmatches. Duquesnov and associates (24) showed identical long-term graft survival in patients with HLA-A and -B mismatched kidneys compared with HLA-matched kidneys when they were defined as "compatible" by HLA-Matchmaker—a computer program that simultaneously considers the HLA type of transplant recipients and their HLA antibody status. Virtual crossmatching realized by a single antigen bead-based analysis can facilitate transplant or retransplant, even in highly sensitized patients (25). Although the majority of patients with detectable levels of HLA alloantibodies remain asymptomatic, some will develop acute antibody-mediated rejection. Currently, therapeutic options for neutralizing circulating alloantibodies and preventing alloantibody formation by B-cell depletion include plasmapheresis, immunoadsorption, intravenous immunoglobulin, cyclophosphamide, and high-dose immunosuppression (26). We previously reported the successful removal of DSA associated with antibody-mediated rejection by a combination of immunoadsorption and anti-CD20 monoclonal antibody treatment. Hemodynamic compromise and clinical symptoms were completely reversible, and the patient recovered (26). However, treatment of antibody-mediated rejection is still challenging, and standardized schemes must be defined. It remains to be elucidated whether asymptomatic patients at risk of developing chronic allograft rejection would benefit from removing

circulating alloantibodies. Therefore, preventing antibody formation, desensitizing sensitized patients, careful monitoring of alloantibodies to identify recipients at risk, and future development of effective approaches to treat antibody-mediated rejection are needed to achieve better long-term results.

Conclusions

Our results demonstrate a correlation between presence of DSA and adverse outcome after heart transplant. Detecting DSA identifies patients who are at high risk for acute rejection episodes and cardiac allograft vasculopathy. Because the development of anti-HLA antibodies often precedes deterioration of graft function, routine screening for DSA might open the opportunity for modification of immunosuppressive strategies and early clinical intervention. We presume that DSA detection with ELISA and Luminex assay is highly specific and sensitive, even at low antibody titers. Characterization of single-antigen reactivity is a useful tool for prospective virtual crossmatching in sensitized patients. Further prospective studies and investigations on more patients are needed to formulate recommendations for immunological screening algorithms. Finally, the role of antibodies directed against non-HLA-molecules such as major histocompatibility class I chain-related antigen or vimentin must be defined to further elucidate the mechanisms of antibody-mediated cardiac allograft rejection (27, 28).

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