HLA-DQ eplet mismatch load may identify kidney transplant patients eligible for tacrolimus withdrawal without donor-specific antibody formation after mesenchymal stromal cell therapy

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HLA-DQ eplet mismatch load may identify kidney transplant patients eligible for tacrolimus withdrawal without donor-specific antibody formation after mesenchymal stromal cell therapy

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Recently, the randomized phase-II Triton study demonstrated that mesenchymal stromal cell (MSC) therapy facilitated early tacrolimus withdrawal in living donor kidney transplant recipients. The current sub-study analyzed formation of de novo donor-specific HLA antibodies (dnDSA) in the context of the degree of HLA eplet mismatches. At the time of protocol biopsy at 6 months, 7/29 patients (24%) in the MSC group and 1/27 patient (3.7%) in the control group had developed dnDSA. In the MSC group, all dnDSA were anti-HLA-DQ; two patients had anti-DQ alone and five patients combined with anti-class I, HLA-DR or -DP. Despite excess dnDSA formation in the MSC-arm of the study, the evolution of eGFR (CKD-EPI) and proteinuria were comparable 2 years posttransplant. All dnDSA were complement-binding and three patients had antibody-mediated rejection in the protocol biopsy, but overall rejection episodes were not increased. Everolimus had to be discontinued in nine patients because of toxicity, and tacrolimus was reintroduced in six patients because of dnDSA formation. The HLA-DQ eplet mismatch load independently associated with dnDSA (adjusted hazard ratio = 1.07 per eplet mismatch, $p = 0.008$). A threshold of ≥11 HLA-DQ eplet mismatches predicted subsequent dnDSA in all 11 patients in the MSC group, but specificity was low (44%). Further research is warranted to explore HLA molecular mismatch load as a biomarker to guide personalized maintenance immunosuppression in kidney transplantation.

KEYWORDS

donor-specific antibodies, eplet, HLA, HLA-DQ, mesenchymal stromal cell, tacrolimus, transplantation

1 | INTRODUCTION

Since the introduction of calcineurin inhibitors (CNIs) in transplantation, short-term graft survival has improved remarkably, but long-term survival has not advanced accordingly.[1–4] One of the major factors limiting further improvement in long-term graft survival is the long-term need of immunosuppressants with a narrow therapeutic window. Currently, the majority of kidney transplant recipients are treated with a CNI-based regimen, with tacrolimus recommend as first choice.[5] Apart from the increased risk of opportunistic (viral) infections and malignancies that is inherently associated with the long-term use of clinical immunosuppression, CNIs have adverse cardiovascular effects (hypertension, dyslipidemia, new-onset diabetes) and direct vasoconstrictive and fibrotic nephrotoxic effects.[6,7] Consequently, CNI-sparing regimens using other immunosuppressive agents such as mTOR inhibitors or belatacept have been investigated, as well as CNI minimization or withdrawal.[8–13] Since CNI-withdrawal has been associated with an increased rate of subsequent acute rejection as well as graft loss,[14] there still is an unmet need for immunosuppressive strategies that prevent allograft rejection while preventing the long-term adverse events of current clinical immunosuppression on patient and graft survival.

Mesenchymal stromal cells (MSC) have both regenerative and immunomodulatory properties and are of interest as potential cellular therapy in transplantation because they may promote tolerance through interaction with various immune cell subsets, including regulatory T cells.[15,16] Recently, the Triton study demonstrated that recipients of a first, living-donor kidney allograft who were treated with autologous MSCs, could be safely weaned from tacrolimus early after transplantation.[17] In this sub-study, we analyzed dnDSA formation in kidney-transplant recipients treated with MSC therapy and subsequent tacrolimus withdrawal, and the potential association with the degree of HLA eplet mismatch between recipient and the kidney donor. HLA eplets are polymorphic amino acids that determine HLA antibody-specificity and eplet mismatches have been associated with DSA formation, rejection and graft loss.[18–21] Previous studies have demonstrated that HLA class II eplet mismatch load modulates the tolerance for low tacrolimus trough levels without DSA formation[22] and suggested that HLA eplet mismatches could be informative for selecting patients eligible for minimization of immunosuppression.[8] We hypothesized that the immunomodulatory properties of MSC would limit dnDSA formation in MSC-treated kidney transplant recipients, regardless of HLA eplet mismatches.

2 | METHODS

2.1 | Study population

Patients that were enrolled in the Triton Study, a 24-week, randomized, prospective, open-label, single-center clinical study, were included. The description of the population and the main results of the trial have been published previously.[17] In brief, 70 recipients of a first living-donor kidney transplant were enrolled between March 2015 and January 2020 and randomized to receive either MSC therapy with concomitant early withdrawal of tacrolimus or standard therapy including tacrolimus. Patients in the MSC group received two infusions of autologous MSC ($1\text{-}2 \times 10^6$ per kilogram body weight) intravenously at week 6 and 7 after transplantation. All patients received alemtuzumab induction therapy (2×15 mg subcutaneously at day 0 and 1) and triple maintenance therapy with tacrolimus, everolimus and prednisolone.[17] In the MSC group, tacrolimus dose was reduced to 50% at the time of the second MSC infusion and was completely withdrawn 1 week later, while in the control group tacrolimus therapy was continued. Patients that were unable to receive the allocated treatment, patients that had a contra-indication for the baseline kidney biopsy and patients that withdrew informed consent were excluded, resulting in a total of 56 subjects included in this analysis (Figure S1).

2.2 | HLA antibody detection

The Triton study protocol predefined analysis of anti-HLA dnDSA in the different treatment groups. According to the protocol, patient serum samples were screened for dnDSA at 0, 4, 12 and 24 weeks post-transplantation. After the 24-week study period, screening was performed at the time of graft dysfunction, or annually depending on serum availability. All sera were screened for anti-HLA antibodies using the LABScreen Kit (One Lambda, Inc., Canoga Park, CA, USA) or the LIFECODES LifeScreen Deluxe kit (Immucor Transplant Diagnostics, Stamford, CT, USA). In case of positive screening, HLA antibody specificities were assessed using the Lifecodes Single Antigen Bead (SAB) kit (Immucor). Data were analyzed with Match It! Antibody software version 1.3.0 (Immucor). Background corrected mean fluorescence intensity (MFI) $\geq$ 1000 was considered positive. In case the donor allele was not present in the SAB panel, the allele with the least amino acid mismatches that was present in the SAB panel was used for DSA assignment, taking the negative beads and complete reactivity pattern into consideration.

2.3 | Characterization of donor-specific antibodies

For determining IgG subclasses of HLA antibodies, sera of patients that developed dnDSA were tested with a modified Lifecodes HLA class I or II SAB assay by using anti-IgG1 (10 μg/mL; HP6001), anti- IgG2 (2.5 μg/mL; HP6002), anti-IgG3 (10 μg/mL; HP6050), and anti-IgG4 (2.5 μg/mL; HP6025) PE-conjugated detection antibodies (Southern Biotech, Birmingham, AL, USA).[23] A positive result was defined by an MFI value greater than five times the mean of four negative control sera from healthy, non-sensitized and HLA antibody-negative individuals. The cut-off value was calculated for each individual bead for every IgG subclass detection assay. The ability of dnDSA to bind complement component 3d (C3d) was tested with Lifecodes C3d Detection kit (Immucor) according to the manufacturer's instructions.

2.4 | HLA eplet analysis

High resolution HLA typing at the second field level for 11 loci was performed using next-generation sequencing on an Illumina platform (Illumina, San Diego, CA, USA) using NGSgo kits (GenDx, Utrecht, The Netherlands) as previously described[24] and using AlloSeq Tx17 (CareDx, South San Francisco, CA, USA). HLA antigen mismatches are presented at the split level. HLAMatchmaker ABC Eplet Matching Program 2.0 and DRDQDP Eplet Matching Program 2.0 were used to define total HLA class I and class II eplet mismatches between donor and recipients. Eplet calculations were performed interlocus for HLA class I, and intralocus for HLA class II. HLA-DQ antibody-verified eplets as defined in the DRDQDP Eplet Matching Program 2.0 were used in further analyses.

2.5 | Kidney allograft biopsies

Subjects in the Triton study underwent protocol kidney biopsies at week 4 and 24 after transplantation. For-cause biopsies were performed at the time of graft dysfunction. All protocol biopsies were clinically screened for acute rejection by a nephropathologist. A detailed structural evaluation according to the Banff 2019 classification[25] was performed retrospectively by one nephropathologist (JK).

2.6 | Statistical analyses

Data analysis was performed using IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA) and graphs were created using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). Categorical variables were summarized using counts and percentages, and continuous variables were described as mean ± standard deviations. A p value <0.05 was considered statistically significant. Analysis of time to dnDSA formation was performed using Kaplan- Meier curves and the log-rank test with subjects censored at the time of death or graft loss. The slopes of eGFR data were calculated and analyzed using linear regression analysis. Patients that experienced graft loss, defined as return to dialysis or re-transplantation, were included with a eGFR of 10 mL/min/1.73 m^2 from graft loss onwards. Univariate comparisons were performed using Fisher's exact test for categorical variables and t tests for continuous variables. Uni- and multivariate Cox regression models were performed with HLA eplets as continuous variables.

TABLE 1 Baseline characteristics.

	MSC group ($n = 29$)	Control group ($n = 27$)
Recipient age (years) - mean ± SD	50 ± 14	50 ± 15
Donor age, mean (years) - mean ± SD	53 ± 14	52 ± 10
Male sex - no. (%)	26 (90)	20 (74)
Donor male sex - no. (%)	13 (45)	10 (37)
Related donor - no. (%)	16 (55)	12 (44)
Cold-ischemia time (h) - mean ± SD	3.1 ± 0.6	3.0 ± 0.5
HLA class I antigen mismatch - mean ± SD	3.3 ± 1.9	3.7 ± 1.5
HLA class II antigen mismatch - mean ± SD	2.8 ± 1.3	3.1 ± 1.4

Abbreviations: MSC, mesenchymal stromal cell; SD, standard deviation.

3 | RESULTS

A total of 56 randomized recipients were evaluated: 29 in the MSC group and 27 in the control group (Figure S1). Median time of follow-up after transplantation was 4.8 years (95% CI 3.4–6.2) and 3.7 years (95% CI 3.0–4.5) for the control group and MSC group, respectively. Baseline characteristics of the patients are summarized in Table 1.

3.1 | De novo donor-specific antibody formation

The majority of dnDSA formation was within 6 months after transplantation, with 7/29 patients (24%) in the

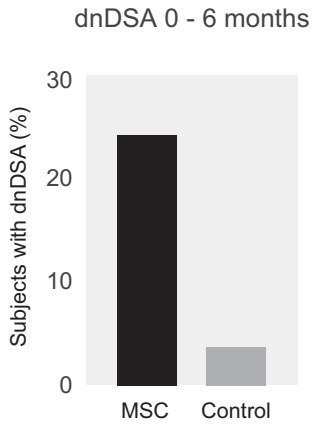

FIGURE 1 Prevalence of dnDSA in the MSC and control group at 6 months after transplantation. dnDSA, de novo donor-specific antibodies; MSC, mesenchymal stromal cell.

MSC group, and 1/27 patient (3.7%) in the control group (Figure 1). Beyond this period, dnDSA formation was similar between the two groups; dnDSA were detected in four patients in the MSC group at 1 ($n = 1$), 2 ($n = 2$) and 4 ($n = 1$) years and in two control patients at 2 ($n = 1$) and 3 ($n = 1$) years after transplantation. Overall, 11 patients (38%) in the MSC group and three patients (11%) in the control group had developed dnDSA after a median follow-up of 4.2 years post-transplantation (Figure S2). In the MSC group, all dnDSA were directed against HLA-DQ. Four patients (36%) had dnDSA against HLA-DQ alone, three patients (27%) had dnDSA against both HLA-DQ and HLA-DR, one patient (9%) had dnDSA against HLA-DQ, HLA-DR and HLA-DP, and three patients (27%) had dnDSA against HLA-DQ, HLA-DR and HLA class I. In the control group, all dnDSA were directed against HLA class II; one patient had dnDSA against HLA-DR alone, one patient against HLA-DP alone and one patient against both HLA-DQ and HLA-DP.

3.2 | Kidney function

3.2.1 | MSC patients versus controls

Previously published results of the Triton study demonstrated that kidney function parameters (eGFR and proteinuria) in the MSC patients were comparable to those in the control group at 6 months after transplantation.[17] The extended follow-up, with all patients having a follow-up of least 2 years post-transplantation, demonstrated that the evolution of kidney function and proteinuria in the MSC and control patients remained comparable, with an eGFR-slope of −0.89 and −2.26 between year 1 and 5 in the MSC and control group respectively ($p = 0.288$) (Figure S3).

3.2.2 | MSC patients with dnDSA formation within 6 months

Mean eGFR of dnDSA positive MSC patients was not significantly different from MSC patients without dnDSA formation at 2 years after transplantation (57.3 versus 53.4 mL/min/1.73 m^2) ($p = 0.57$) (Figure 2A) and proteinuria was similar (Figure 2B).

3.3 | IgG DSA subclass distribution and C3d-binding

Since dnDSA development did not lead to inferior kidney function in the MSC group, we further characterized the DSA to determine their possible inability of causing complement-mediated damage. IgG subclasses could be determined in 12/14 patients. No dnDSA IgG subclass could be determined in two patients, likely because of the lower sensitivity of the subclass assay. Regular SAB assay MFI values for these dnDSA specificities were relatively low with background-corrected MFI of 1325 and 2109. The prevalence of the different combinations of anti-HLA antibody IgG subclasses for the MSC group is presented in Figure 3. In the MSC group, IgG subclass distribution revealed that IgG1 was positive in 10 patients, IgG2 in three patients, IgG3 in six patients and IgG4 in one patient. In the control group, IgG1 was detected in two patients, one in combination with IgG2, and one in combination with IgG2 and IgG4. The majority of patients had a mixture of IgG subclasses. C3d-binding was positive in 10 out of 11 MSC patients and in two out of three control patients. The two C3d-negative samples were from the two patients in whom also no IgG subclasses could be determined.

3.4 | Protocol biopsies and clinical immunosuppression

3.4.1 | Histological assessment

Activity and chronicity indices (integrated RejectionClass algorithms[26]) did not differ between MSC patients versus controls (Table S1). Banff scores of the protocol biopsies at month 6 showed that MSC patients with dnDSA more often had glomerulitis ($n = 5$), peritubular capillaritis ($n = 2$) and C4d deposition ($n = 3$) as compared with the MSC patients without dnDSA at 6 months (Figure 4;

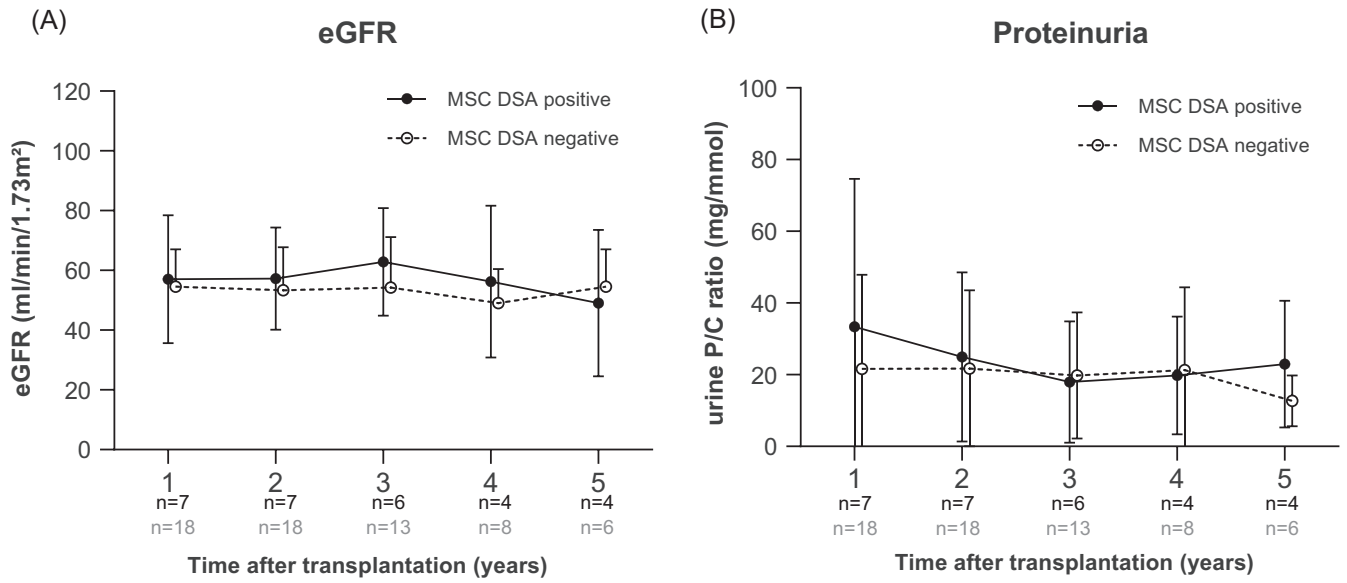

FIGURE 2 Kidney function is comparable between MSC patients with and without dnDSA. (A) eGFR as calculated by the CKD-EPI formula and (B) urine protein/creatinine ratio for MSC patients that developed dnDSA within the first 6 months after transplantation versus MSC patients that did not develop dnDSA during a follow-up up to 5 years. Data points with error bars represent the mean ± standard deviation. dnDSA, de novo donor-specific antibodies; MSC, mesenchymal stromal cell.

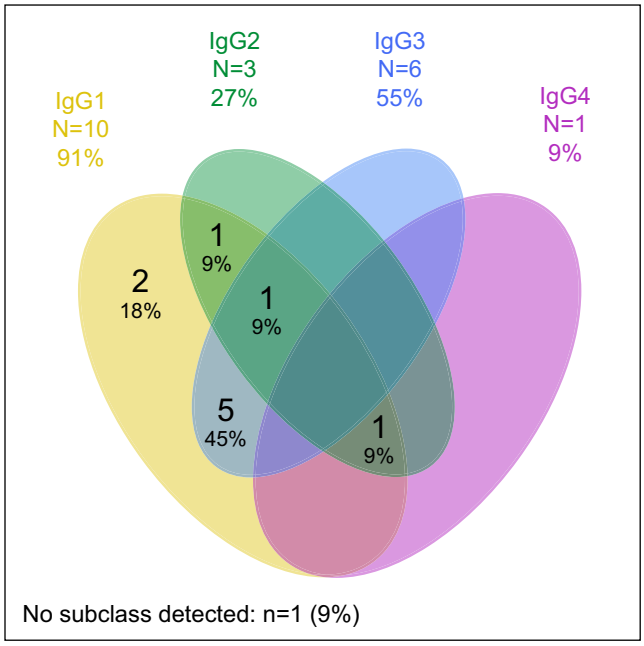

FIGURE 3 Venn diagram showing the IgG1-4 subclass distribution of dnDSA positive patients in the MSC group. dnDSA, de novo donor-specific antibodies; MSC, mesenchymal stromal cell.

chronic lesion scores in Figure S4). Additionally, total inflammation (ti) and IFTA were 15.7% and 4.3% in MSC patients with dnDSA and 12.4% and 9.0% in MSC patients without dnDSA (ti-score $p = 0.68$; IFTA $p = 0.24$). Based on these protocol biopsies, three DSA positive patients were classified as having active antibody-mediated rejection (ABMR), one as suspicious for acute T cell-mediated rejection (TCMR) and three patients had no evidence for rejection. Notably, one protocol biopsy at week 4, before the MSC infusions and tacrolimus withdrawal, already showed ABMR. This biopsy was initially assessed as being normal, but structural classification according to the Banff classification at a later timepoint classified this biopsy as ABMR. The other two patients that were retrospectively diagnosed with ABMR were restarted on tacrolimus and remained clinically stable for 5 years of follow-up, without need for a for-cause biopsy or anti-rejection treatment. No graft loss was observed and eGFR did not decrease below 30 mL/min/1.73 m^2 in any patient. No differences regarding HLA specificity, MFI, C3d-binding or IgG subclasses of dnDSA between the patients with and without rejection was observed (Table S2).

3.4.2 | Immunosuppression

Because of the detection of dnDSA ($n = 6$) and a vascular rejection episode without detectable DSA ($n = 1$), tacrolimus was resumed in seven MSC patients (24%). Additionally, nine patients (31%) were converted to a CNI or mycophenolate mofetil because everolimus had to be discontinued because of adverse effects (3 wound healing complications, 2 anemia, 1 thromboembolism, 1 edema, 1 pulmonary toxicity, 1 drug interaction). In total, 13/29 (45%) patients from the MSC group remained of a

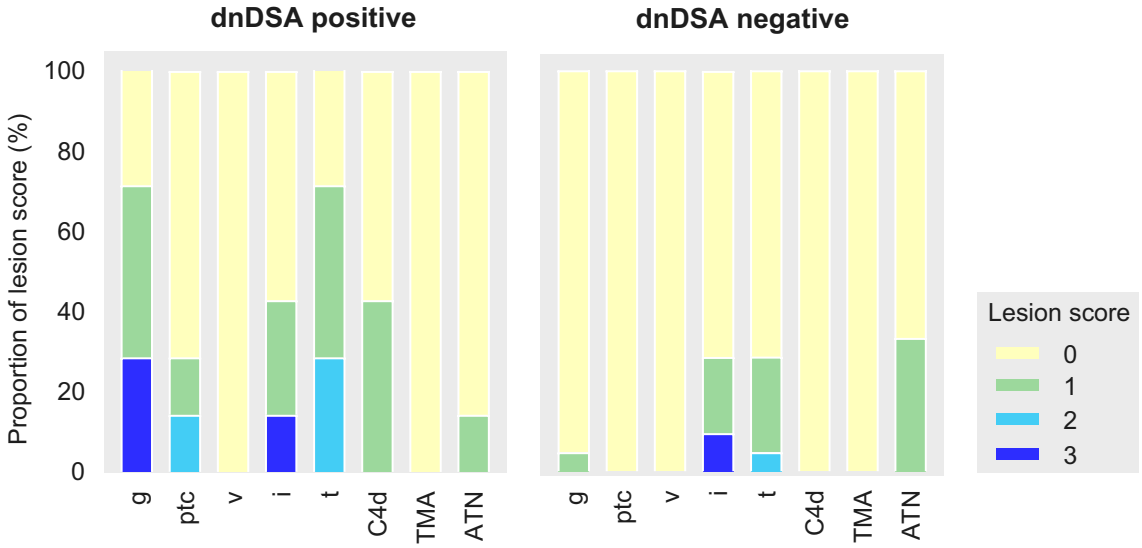

FIGURE 4 Histopathology of protocol biopsies at 6 months after transplantation in the MSC group. Distribution of acute histopathologic lesions according to the Banff classification between DSA positive ($n = 7$) and DSA negative ($n = 21$) patients in the MSC group. ATN, acute tubular necrosis; C4d, complement split product 4d; DSA, donor-specific antibodies; g, glomerulitis; i, interstitial inflammation; MSC, mesenchymal stromal cell; ptc, peritubular capillaritis; t, tubulitis; TMA, thrombotic microangiopathy; v, endarteritis.

tacrolimus-free treatment based on everolimus and prednisone.

3.5 | HLA eplet mismatch and risk of developing DSA

Since previous studies reported a correlation between HLA molecular mismatch loads and the risk of dnDSA development, we determined eplet mismatch levels. Overall, the HLA antigen mismatch numbers and eplet mismatch numbers were similar in the MSC and control group (Table S3). Within the MSC group, mean HLA class II eplet mismatches and HLA-DQ eplet mismatches were higher in dnDSA positive patients (46.5 versus 30.4 HLA class II eplet mismatches, $P = 0.008$ and 23.5 versus 11.5 HLA-DQ eplet mismatches, $P = 0.002$) (Table S4).

Univariable Cox regression demonstrated that HLA class II and HLA-DQ eplet mismatches as a continuous variable significantly increased the hazard ratio for dnDSA formation. Recipient and donor age, recipient and donor sex, and donor type (living related or unrelated) were not associated with dnDSA formation (Table S5). Multivariable Cox models demonstrated that both total HLA class II eplet mismatches (model 1) and HLA-DQ eplet mismatches (model 2) were independently associated with dnDSA formation (adjusted hazard ratio 1.04 per single HLA class II eplet mismatch, 95% CI 1.01–1.07, $p = 0.004$) (adjusted hazard ratio 1.07 per single HLA-DQ eplet mismatch, 95% CI 1.02–1.13, $p = 0.008$) (Table 2). In the ROC curve analysis, the HLA-DQ eplet mismatch threshold that best correlated with dnDSA development in the entire cohort was ≥11 mismatches (Figure S5). Using this threshold to define high risk demonstrated that a high HLA-DQ epitope mismatch load was associated with a higher dnDSA formation among all patients (13 of 38 versus 1 of 18 patients; $P = 0.023$; sensitivity = 93%; specificity = 40%; positive predicting value = 34%; negative predicting value = 94%) and among MSC patients (11 of 21 versus 0 of 8 patients; $P = 0.012$; sensitivity = 100%; specificity = 44%; positive predicting value = 52%; negative predicting value = 100%) (Figure 5). Since specificity in this analysis was low, we also analyzed antibody-verified HLA-DQ eplets, which

TABLE 2 Multivariable HRs for dnDSA occurrence.

	HR (95% CI)	*P* Value
Model 1		
MSC treatment/Tac withdrawal	4.46 (1.21–16.37)	0.024
HLA Class II eplets (range 8–84)	1.04 (1.01–1.07)	0.004
Model 2		
MSC treatment/Tac withdrawal	4.38 (1.17–16.40)	0.028
HLA-DR eplets (range 0–45)	1.04 (0.97–1.10)	0.282
HLA-DQ eplets (range 0–38)	1.07 (1.02–1.13)	0.008

Abbreviations: dnDSA, de novo donor-specific antibodies; HR, hazard ratio; MSC, mesenchymal stromal cell; Tac, tacrolimus.

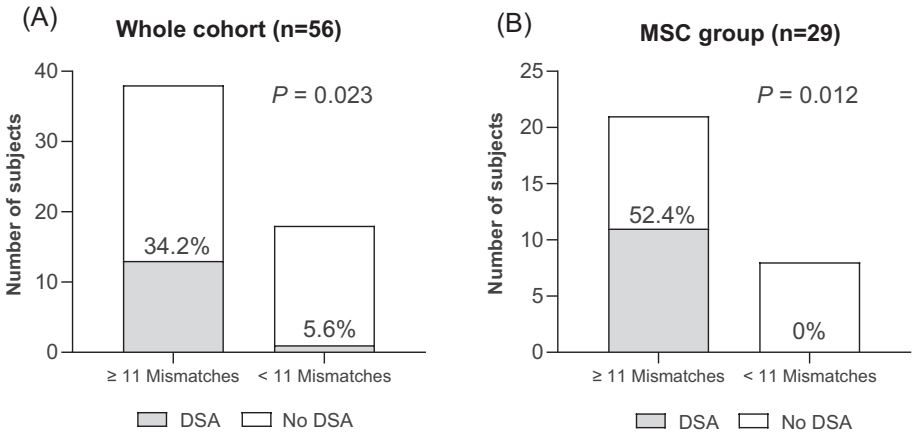

FIGURE 5 HLA-DQ eplet mismatch levels correlate with dnDSA development. The bars depict the total number of patients with ≥11 HLA-DQ eplet mismatches and <11 HLA-DQ eplet mismatches. The shaded portions of each bar depict those patients in each group that developed dnDSA in the (A) total study population and (B) the MSC group. dnDSA, de novo donor-specific antibodies; MSC, mesenchymal stromal cell.

have been proven to interact with HLA-specific antibodies and are therefore clinically relevant. A threshold of ≥8 antibody-verified HLA-DQ eplets was associated with dnDSA formation in the MSC group (9 of 14 versus 2 of 15 patients; $P = 0.008$; sensitivity = 82%; specificity = 72%; positive predicting value = 64%; negative predicting value = 87%) and the whole cohort (11 of 24 versus 3 of 32 patients; $P = 0.002$; sensitivity = 79%; specificity = 69%; positive predicting value = 46%; negative predicting value = 91%).

4 | DISCUSSION

Since 2011, several trials have investigated the safety of both autologous and allogeneic MSC therapy in kidney transplantation.[27,28] While two clinical trials demonstrated safety of MSC therapy in combination with a low-dose CNI regimen,[29,30] the Triton Study is the first randomized study that investigated autologous MSC therapy in combination with early tacrolimus withdrawal.[17] Earlier clinical trials investigating tacrolimus withdrawal in kidney transplant recipients without MSC therapy reported increased rates of DSA formation and rejection, which led to early termination of these trials.[8,9] We hypothesized that dnDSA formation in MSC-treated patients would be limited, because of the immunomodulatory effects of the MSC. However, our data show that MSC patients actually developed more dnDSA than patients in the control group. In all MSC patients that developed dnDSA, and in two out of three control patients, dnDSA were directed against HLA-DQ; in several cases in combination with HLA-DR or HLA-DP antibodies or (less frequent) HLA class I. These data are concordant with previous studies demonstrating the formation of predominantly HLA-DQ-specific antibodies after kidney transplantation.[31–36] The reason for the abundance of anti-HLA-DQ antibodies might be explained by the fact that most matching algorithms do not consider HLA-DQ. Despite the linkage disequilibrium between HLA-DR and HLA-DQ, a zero HLA-DR antigen mismatch does not automatically correspond to a zero HLA-DQ antigen mismatch. Furthermore, unlike HLA-DR, HLA-DQ consists of a polymorphic alpha and beta chain, which increases the number of potentially immunogenic epitopes on HLA-DQ molecules that can be targeted by HLA antibodies.[37]

Although MSC patients had more dnDSA formation in the first 6 months after transplantation, there was no difference in dnDSA occurrence at longer follow-up. Importantly, despite a higher rate of early dnDSA formation, evolution of eGFR of MSC patients was not inferior to the controls up to 5 years of follow-up.

To determine whether the dnDSA in the MSC group were qualitatively different because of possible immunoregulation, we further characterized the DSA. The majority of MSC patients had a combination of different IgG subclasses, in which the complement-binding subclass IgG1 was always present. The distribution of the IgG subclass pattern was comparable to previously published patterns, in which the complement-binding subclasses were most prevalent.[38] The complement-binding capacity of the majority of DSA was confirmed using the C3d Luminex assay. Hence, the dnDSA that are formed after MSC therapy do not appear to be of a less damaging phenotype, considering the complement-binding capacity of IgG1 and IgG3 subclasses. Banff scores of the protocol biopsies indeed showed that several MSC patients with dnDSA had lesions associated with antibody-mediated damage and three patients were diagnosed with ABMR. Importantly, MSC therapy was not associated with a higher overall rate of rejection episodes at 6 months after transplantation.[17] Also at longer follow-up, MSC patients did not experience increased rejection rates compared with the control group. In addition to the previously described for-cause biopsies,[17] a total of six patients

received a for-cause biopsy; three patients in the MSC group and three in the control group, which all showed ABMR. In total, four out of 29 patients in the MSC group and 11 out of 27 patients in the control group received a for-cause biopsy, with a total of three rejection diagnoses in the former and six rejection diagnoses in the latter (Table S6). These results are in contrast to the two tacrolimus withdrawal trials without MSC therapy that were stopped because of the high rates of DSA formation and acute rejection.[8,9]

The limited rate of acute rejection episodes in MSC patients despite withdrawal of tacrolimus might be correlated to the use of the leukocyte depleting agent alemtuzumab. It has been hypothesized that alemtuzumab has different effects on the two types of rejection, with the humoral component being dominant over cellular rejection.[39,40] The 3C study demonstrated that alemtuzumab induction followed by CNI and MMF minimization and steroid avoidance compared with basiliximab induction was associated with a decrease in acute rejection in the first 6 months after transplantation, primarily because of a significant decreased incidence of cellular rejection, but not of humoral rejection.[40] Use of alemtuzumab as induction therapy has also been associated with a higher incidence of dnDSA formation, although this was not corroborated in a more recent study.[41,42] In our study, dnDSA rate was increased in MSC patients, but not in controls, suggesting that the increased dnDSA occurrence must be primarily seen in the context of tacrolimus withdrawal and is not primarily correlated to alemtuzumab induction.

The current immunosuppression protocol resulted in a substantial proportion of patients in whom everolimus had to be discontinued because of adverse effects. Additionally, tacrolimus was reinstated in six patients at the time of detection of dnDSA and in one patient during a vascular rejection episode. Although dnDSA formation may not lead to clinical ABMR in every kidney transplant recipient, dnDSA in general carry a risk for developing ABMR, a major cause of allograft dysfunction and graft loss.[43] Furthermore, sensitization will decrease the chances to find a suitable organ donor if a future re-transplantation would be necessary. Therefore, tools are required to identify patients that would be eligible for minimization of immunosuppression, without an increased risk of dnDSA formation. Data from the CTOT-09 Trial, which was prematurely ended because of high rates of DSA and rejection, suggested that HLA-DQ eplet mismatch levels can be informative to select patients eligible for calcineurin inhibitor withdrawal without additional risk for dnDSA development.[8] In our cohort, a threshold of ≥11 HLA-DQ eplet mismatches was significantly associated with dnDSA formation. However, our study was underpowered to define a general threshold. Notably, in accordance with the CTOT-09 trial,[8] the previously established threshold of ≥12 HLA-DQ eplet mismatches[18] also demonstrated an association between high HLA-DQ eplet mismatch load and dnDSA formation in the MSC group, albeit not significant ($p = 0.064$) (Figure S6). Although the threshold of ≥11 HLA-DQ eplet mismatches had a high sensitivity, specificity was low. This might be related to the fact that total HLA-DQ eplet mismatches were analyzed in this study and that not every HLA eplet mismatch will lead to antibody formation.[44] Indeed, antibody-verified eplet mismatches might be a more suitable predictor, since specificity and positive predicting value were increased when antibody-verified HLA-DQ eplets were analyzed. However, sensitivity of this marker was decreased. This emphasizes the need for defining the most immunogenic eplet mismatches in transplantation, in order to develop more specific HLA eplet mismatch tools that can guide personalized immunosuppression strategies in transplant patients.[45]

There are limitations to the present study. Firstly, median times of follow-up were 4.8 and 3.7 years in controls and MSC patients, respectively. Although all patients were followed up at least 2 years at the time of this study, the follow-up time is still relatively short. The long-term consequences of increased dnDSA formation after MSC therapy remains to be investigated. Furthermore, no protocol kidney biopsies were performed after the initial 6-month study period. Lastly, the SAB assay was only performed when Luminex screening was positive. However, when patient serum was tested because of a suspicion of ABMR and antibody screening was negative, the sample was still tested in SAB. This approach did not lead to detection of HLA antibodies in the SAB assay when the Luminex screening was negative. A strong aspect of the current study as compared with the post-hoc analysis[8] of the CTOT-9 study is that HLA typing was performed at the second field level for 11 loci for all patient and donors, without the need for imputation, which allowed for accurate eplet and DSA assignment.[46]

In conclusion, kidney function in MSC-treated patients with subsequent early tacrolimus withdrawal was not inferior to the control group at 2 years post-transplantation and rejection episodes were not increased, despite a higher incidence of dnDSA. This can possibly be explained by the use of alemtuzumab induction therapy. However, MSC patients developed more often dnDSA, which were directed against at least HLA-DQ in all patients and had the capacity to bind complement. We showed that HLA-DQ eplet mismatch loads could be instrumental to identify patients eligible for

minimization of immunosuppression without increased risk of dnDSA development. Further research is warranted to investigate eplet mismatch analysis as a tool to identify patients eligible for weaning of immunosuppression in immunosuppression minimization trials.

AUTHOR CONTRIBUTIONS

Suzanne Bezstarosti performed the research and analyzed the data. Kim Voogt-Bakker performed the experiments. Soufian Meziyerh, Koen E. Groeneweg and Dave L. Roelen contributed to clinical data. Jesper Kers evaluated the biopsies. Marlies E.J. Reinders, Jesper Kers and Sebastiaan Heidt designed the study. Suzanne Bezstarosti wrote the paper. All authors contributed to the manuscript and approved the final version.

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CONFLICT OF INTEREST STATEMENT

All the authors declared no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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