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Epitope load identifies kidney transplant recipients at risk of allosensitization following minimization of immunosuppression

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Abstract

HLA mismatching and minimization of immunosuppression are two major risk factors for the development of *de novo* donor specific antibodies, which is associated with reduced kidney graft survival. Antibodies do not recognize whole HLA antigens but rather individual epitopes, which are short sequences of amino acids in accessible positions. However, compatibility is still assessed by the simple count of mismatched HLA antigens. We hypothesized that the number of mismatched epitopes, or (“epitope load”) would identify patients at the highest risk of developing donor specific antibodies following minimization of immunosuppression. We determined epitope load in 89 clinical trial participants who converted from cyclosporine to everolimus 3 months after kidney transplantation. Twenty-nine participants (32.6%) developed *de novo* donor specific antibodies. Compared to the number of HLA mismatches, epitope load was more strongly associated with the development of donor specific antibodies. Participants with an epitope load greater than 27 had a 12-fold relative risk of developing donor-specific antibodies compared to those with an epitope load below that threshold. Using that threshold, epitope load would have missed only one participant who subsequently developed donor specific antibodies, compared to 8 missed cases based on a 6-antigen mismatch. DQ7 was the most frequent antigenic target of donor specific antibodies in our population, and some DQ7 epitopes appeared to be more frequently involved than others. Assessing epitope load before minimizing immunosuppression may be a more efficient tool to identify patients at the highest risk of allosensitization.

Keywords

Acute rejection; kidney transplantation, antibody-mediated rejection; HLA mismatching; epitopes; immunosuppression minimization

Introduction

Numerous reports have shown a significant influence of the number of HLA mismatches on kidney graft survival¹⁻⁴, although this concept has also been challenged⁵.

The recent advent of solid phase assays detecting anti-HLA antibodies has shed light on how HLA matching influences the occurrence of de novo donor specific antibodies (dnDSA). Indeed, class II HLA matching and inadequate immunosuppression are two independently recognized risk factors for dnDSA development^{6,7}. Moreover, antibody-mediated rejection (ABMR) due to dnDSA is associated with a 40% reduction in 10-years graft survival⁶ and appears as a major cause of late allograft loss⁸⁻¹².

Despite the worldwide use of allocation algorithms taking into account HLA matching, approximately 10-15% of patients still develop dnDSA within an average of six years post-transplant^{6,13,14}, questioning the reliability of current typing for minimizing the risk of allosensitization. Donor/recipient HLA compatibility has always been assessed by a simple count of HLA mismatched antigens, i.e. at the “serologic” level although typing has been more and more performed using molecular techniques for at least a decade. Yet, anti-HLA antibodies do not recognize whole HLA antigens but rather epitopes, i.e. short polymorphic sequences of amino acids in antibody-accessible positions^{15,16}.

Two approaches are used to identify HLA allogenic epitopes. The HLAMatchmaker algorithm developed by Duquesnoy relies on how immunoglobulin paratopes bind to protein epitopes localized on the molecule surface, to identify theoretical HLA epitopes^{17,18}. The empirical approach by Terasaki verifies the existence of epitopes through the ability of antibodies to bind all alleles that share the same amino acid sequences exposed on the HLA molecule^{16,19}. HLAMatchmaker is updated with the epitope registry (www.epregistry.com) and references those verified epitopes, referred to in this manuscript as “antibody-verified”, besides the non-verified epitopes²⁰.

HLAMatchmaker compares donor and recipient HLA molecules at the structural level to infer the number of mismatched epitopes called the epitope load¹⁷. Using HLAMatchmaker, Wiebe et al. showed that class II epitope load was associated with the long-term risk of developing dnDSA²¹.

In our study, we hypothesized that preliminary determination of epitope load, in the daily clinical practice, could be a reliable and easy-to-use tool to identify patients at the highest risk of dnDSA development when a minimization of immunosuppression is

envisioned. We tested this hypothesis in patients who were randomized for switching from cyclosporine (CsA) to everolimus three months post-transplantation²², a modification associated with a high incidence of early dnDSA (up to 27% at one year) and ABMR, and considered as a minimization of immunosuppression²³. We also qualitatively determined whether some mismatched epitopes were more immunogenic than others, i.e., more prone to trigger dnDSA.

Results

Characteristics of the study population and clinical course of transplantations.

The patients who were switched at three months post-transplantation from CsA to everolimus (n=89) all received a first kidney transplant (Table 1). None had DSA either at transplantation or at conversion. After a mean follow-up of 83.8 ± 27.9 months, 29 patients (32.6%: 25 before and 4 after month-12 post-transplantation) developed dnDSA, mainly against class II antigens (Figure 1). Twenty patients (22.5%) experienced a T-cell mediated rejection (TCMR) and five had a clinical episode of antibody-mediated rejection (ABMR) during the first year, all after conversion to everolimus [8 to 12 months post-transplantation]. Four other patients exhibited significant microvascular inflammation ($g+ptc \geq 2$) on the systematic month-12 biopsy. Five patients lost their graft due to TCMR, from 6 to 13 months after transplantation, with three patients presenting concomitant dnDSA and microvascular inflammation. Regarding the impact of dnDSA in the everolimus arm, patient survival was similar in patients with or without dnDSA (Figure 2A), whereas death-censored graft survival was significantly lower in patients with dnDSA: 62.5% versus 93.1% at 8 years, $p=0.0005$ (Figure 2B). Graft survival was similar in the two arms of treatment (Figure 2C). Renal function declined from month-3 in patients who developed dnDSA in the everolimus arm, but was similar in the two arms of treatment (Figure S1).

Evaluation of HLA compatibility with antigenic, allelic and epitopic mismatches

Table S1 shows the different levels of mismatches in the two arms of treatment, class II allelic and antibody-verified epitopic mismatches being higher in the everolimus arm. Table 2 focuses on the distribution of the three levels of mismatches for the patients of the everolimus arm. Figure 3 shows how these values overlap, even if they are clearly correlated. The number of antigenic (Figure 3A/3B) and allelic (Figure 3D/3F) mismatches may under- or overestimate the level of epitopic incompatibility. The correlation of epitopic mismatches with allelic mismatches is not better than with antigenic mismatches. Donor/recipient pairs considered HLA identical at the antigen level may in fact present allelic (Figures 3C/3E) and epitopic (Figures 3A/3B) mismatches. Figure S2 shows that correlation with antibody-verified epitopes is even lower than with the total number of epitopes.

Figure 4A illustrates for two patients how epitope load may be more informative than antigenic mismatches. In both cases, there were one DR and one DQ mismatched antigens and four mismatched alleles (DRB1, DRB3/4/5, DQB1 and DQA1). The epitope load was low in the first case (8 DR – including one antibody-verified– and 8 DQ epitopic mismatches), but higher in the second (20 DR and 28 DQ epitopic mismatches with a high proportion of antibody-verified epitopes). Only the second patient developed DR and DQ dnDSA. Then, a given number of antigenic or allelic mismatches may underlie very different levels of epitopic mismatching.

Moreover, given antigenic mismatches may be associated with distinct epitope loads depending on structural differences between donor and recipient HLA. For example (Figure 4B), a DQ7 antigenic mismatch represents 1 epitopic mismatch for a DQ6/DQ9 recipient (panel-1), 8 mismatches for a DQ2/DQ4 recipient (panel-2), and 16 mismatches for a DQ2/DQ2 recipient (panel-3). The DQ2 homozygous patient can "see" more epitopic mismatches than the heterozygous DQ2/DQ4 recipient because the epitopes shared by DQ4 and DQ7 are not considered as "self". Regarding anti-DQ7 allosensitization, only the last two recipients developed a dnDSA.

Factors associated with the development of dnDSA

Patients with or without dnDSA had similar baseline characteristics (Table 1) except for age, and similar CsA (until month-3), everolimus (after month-3) and mycophenolic acid exposure (Table 3). Among the 89 patients, 21 experienced a premature discontinuation of everolimus (replaced by a CNI), after 1.9 ± 1.3 month of treatment but the frequency of dnDSA was similar compared with that of patients maintained on everolimus until month-12 (see Figure S3 for details on everolimus and rejections). Patients with dnDSA experienced more TCMR but the difference was not significant (Table 1).

Table 4 summarizes antigenic, allelic and epitopic mismatches in relation with the occurrence of dnDSA. Whatever their class, dnDSA were more strongly associated with the number of antibody-verified epitopic mismatches than with the number of total epitopic mismatches, than with the number of antigenic mismatches. The number of allelic mismatches was only associated with class I or II DSA and with class II DSA, but at a lower level. The occurrence of DR dnDSA was only associated with the number of antibody-verified epitopes.

We performed a ROC curve analysis to determine the mismatch thresholds that best predicted the development of dnDSA for the different loci (Table 5). For class I or II, class I, class II and DQ DSA, AUC were always greater with antibody-verified and then total epitopic mismatches than with antigenic and allelic mismatches. As compared to the AUC of antigenic mismatches, statistical significance was not reached for any mismatches types. To note, the AUC of antibody-verified epitopic mismatches was nearly significant regarding the occurrence of dnDSA (class I or II) ($p=0.06$). The global relative risk for developing any dnDSA, regardless of class, was 2.0 for more than 6 antigenic mismatches, 8.0 for more than 15 verified epitopes and 12.2 for more than 27 total epitopic mismatches. In identifying patients who developed class I or II dnDSA, using the above-mentioned thresholds, antigenic mismatches would have missed 8 patients compared with only one for epitope load (Figure 5). The same results were obtained in the transplantations for which a donor and recipient allelic typing could be performed.

Multivariate logistic regressions (Table 6) showed that epitope load, as a continuous variable, was the only factor independently associated with the risk of developing class I or II dnDSA.

Interestingly, there was no correlation between the occurrence of ABMR or TCMR, or isolated microvascular inflammation on month-12 biopsy, and any level of mismatches (Table S2).

Regarding the influence of immunosuppression, occurrence of dnDSA was delayed and less frequent in the cyclosporine group than in the everolimus arm. Indeed, 16 patients (18.6%), 3 before and 13 after month-12, developed dnDSA whereas 29 patients (32.6%, $p=0.034$) developed DSA in the everolimus group. Five-year graft survival without DSA was 85% in the cyclosporine group versus 69.1% in the everolimus group ($p=0.026$, Figure 6). Under cyclosporine, according to ROC curve analysis, the thresholds associated with the occurrence of class I or II dnDSA were lower: 5 (antigenic mismatches), 6 (allelic mismatches), 22 (total epitopes) and 16 (antibody-verified epitopes). The antibody-verified epitope load was similar in patients in the everolimus arm not developing dnDSA and in the cyclosporine group (14.8 ± 7.0 versus 15.2 ± 6.6 , $p=0.7$). However, the proportion of patients who developed dnDSA with an epitope load above the threshold which is similar (15 for everolimus, 16 for cyclosporine) is higher in the everolimus arm: 48.2% versus 26.1 %, $p=0.02$.

Identification of the most immunogenic epitopes

DQ7 was the most frequent antigenic target of dnDSA. Considering the 23 DQ7^{negative} patients who received a DQ7^{positive} graft, the number of mismatched DQ7 epitopes was significantly higher in the 11 patients (47.8 %) who developed a DQ7 dnDSA (Figure 7A and 7B). Qualitatively, several DQ7 epitopes were significantly more often mismatched when the recipients developed anti-DQ7 dnDSA (Odds-Ratio 11.6 to 23.9), such as 55PP and 77T (antibody-verified) or 37YA, 70RT and 74EL (non-verified). Others were mismatched either with a similar frequency regardless of the development of anti-DQ7 dnDSA (52PL, 140T, 182N, 56PD, 45EV, 14AM, 26Y) (Figure 7C), or in too few cases (0 to 3 cases) for a statistical comparison.

We verified that anti-DQ7 dnDSA often recognized these mismatched epitopes by analyzing serum reactivity using HLAMatchmaker (Table 7). In 6 of the 10 cases we

could analyze, the targeted epitope was 55PP or 77T, two antibody-verified epitopes that were mismatched significantly more often (Figure 7).

Discussion

In this study, we took advantage of the high incidence of dnDSA after switching from CsA to everolimus to show that the epitope load was a more reliable method for assessing HLA compatibility than the mere counting of antigenic mismatches.

In two recent studies, the number of HLA antigenic mismatches was a strong predictor of long-term graft survival^{3,4}. HLA compatibility is involved in the allogenic immune response, and especially in class II dnDSA development^{6,10}, critically associated with worse post-transplant outcomes⁸⁻¹². Given the difficulty of controlling other factors, such as poor adherence, it is important to identify *a priori* the patients at the highest risk of post-transplant sensitization.

HLA matching has been traditionally based on serologically defined HLA antigens, although anti-HLA antibodies react against specific epitopes and not against the antigen as a whole^{15,16}. Epitopes in an HLA molecule can be deduced from allelic typing but not from serologic typing. Therefore, simply counting antigenic mismatches does not allow for the reliable identification of mismatched epitopes.

Molecular techniques for typing and crystallographic studies^{24,25} have provided a better understanding of the central role of epitopes as the targets of alloantibodies. HLA antigens are in fact groups of close alleles with minor sequence differences resulting in the expression of mismatched epitopes that can even stimulate the synthesis of alloantibodies within the same allelic group.

We compared the predictive value of the traditional antigen-based and the new epitope-based approaches. We showed that the two approaches were correlated, but that the epitope load was more informative, as antigenic mismatches may over- or underestimate the real level of incompatibility.

Before the development of single antigen beads, Dankers et al. found a correlation between graft failure, DSA and the number of "triplet" (three amino acid epitope) mismatches²⁶. More recently, using the latest "eplet" version of HLAMatchmaker and single-antigen bead assays, Wiebe et al. showed that class II epitope loads, together with non-adherence and acute rejection, were independent risk factors of long-term dnDSA²¹.

We confirmed that in the context of immunosuppression minimization, epitope load is useful to identify patients at highest risk of developing dnDSA rapidly after the modification, whatever the class of these DSA. Epitope load was more strongly associated with the development of class I or II dnDSA than the number of antigenic or allelic mismatches, with a discriminative threshold of 27 class I+II epitopic mismatches. Furthermore, the number of mismatched antibody-verified epitopes was more closely associated with dnDSA development than the total number of epitopes. It is likely that many (if not all) of the not verified epitopes, have physicochemical properties or localization that do not make them accessible to alloantibodies.

Another advantage of the epitopic approach is the ability to study the immunogenicity of epitopes. Indeed, our study, as the one by Wiebe et al²¹, suggests that some mismatched antibody-verified epitopes are more frequently associated with the development of dnDSA than others and, thus, are more immunogenic, which is possibly based on their exposed position. Pre-transplant identification of mismatched or targeted epitopes (for immunized patients) could be a new tool for assessing immunological risk. One could hypothesize that the greater the epitope load, the higher the probability that the recipient's immune system will respond to at least one immunogenic epitope.

A limitation of our study is the small cohort size and the lack of power that prevented us to show a significantly higher discriminative value of epitope loads in ROC curve analysis, despite the positive result of logistic regression. Thus, the relative role of epitope load should be explored in larger populations. Moreover, for one third of patients, epitope load was calculated with allelic typing deduced from generic typing using the HaploStats application²⁷, because DNA was not available anymore; this may increase the uncertainty of the data. Nevertheless, we confirmed the accuracy of HaploStats for the French Caucasian population²⁸ (Table S3). Moreover, DRB3/4/5 and DQA1 typing were rarely performed because of their strong linkage disequilibrium with DRB1 and DQB1 alleles^{29,30}. We have shown in Table S4 and S5 that the potential errors would have a weak impact on epitope load calculation. Eventually, we did not study DQA1, DPB1 and DPA1 epitope loads in relation with dnDSA because only two patients developed DQA1 and two others DPB1 dnDSA, and always in association with other DSA. Moreover, these DSA are not consensually recognized as pathogenic as those targeting other HLA loci molecules.

We did not identify any association between epitope load and TCMR or ABMR. TCMR involves T-cell epitopes that are different from B-cell epitopes, although they may be involved in dnDSA generation³¹ Sapir-Pichhadze et al. described an association between transplant glomerulopathy and the DR+DQ eplet load in kidney transplant recipients³². Using an electronic system of adherence monitoring, Wiebe et al. found that the combination of poor adherence and higher epitope load acted synergistically, but not separately, to determine the risk of ABMR (DQ) or graft loss (DR and DQ)³³. In our study, the adherence was not specifically studied, but the close follow-up during the first post-transplantation year and the context of a clinical trial²² make the probability of poor adherence low. Moreover, the similar exposure to everolimus in patients who developed or not DSA, suggests that it did not play a major role in DSA occurrence. Interestingly, we found a lower epitope load threshold for the development of DQ dnDSA than Wiebe et al²¹ (8 versus 17), possibly because we did not take into account DQA epitopes. In our study, conversion to everolimus may have played a critical role in the development of dnDSA and therefore cannot be advocated, even if patients at risk may be identified. Given that antibody-verified epitope load was higher in the everolimus arm, we cannot rule out that patients under everolimus would have eventually developed dnDSA while remaining on cyclosporine. However, dnDSA appeared more frequently and earlier after conversion to everolimus. Moreover, even if TCMR was observed in 34.5% of patients who developed dnDSA, most of the patients (19/29) who developed dnDSA did not experience TCMR. This was confirmed in the multivariate analysis that found that epitope load was the only independent factor associated with dnDSA. Our study was similar to the one by Hricik et al.³⁴ but we benefited of a larger cohort. They showed retrospectively in 14 kidney transplant recipients randomized to wean off tacrolimus that the 5 patients who developed anti-DQ dnDSA had an epitope load above the threshold of 16 epitopic mismatches³⁴.

In conclusion, our study revealed that epitope load is a more informative and predictive tool than the number of antigenic or allelic mismatches for determining the risk of post-transplant allosensitization, particularly the short-term risk following minimization of immunosuppression. We think that in a daily practice, when reduction of immunosuppression is discussed, clinicians could right now implement epitope load for the identification of patients for whom such a minimization may be hazardous. If epitope

load is above the threshold, minimization should only be performed in case of clinical indication and with a strict follow up of dnDSA. We think that epitope load, and not solely antigenic mismatches, should also be calculated and provided in published clinical studies to compare the level of incompatibility of two groups of patients, particularly in clinical trials assessing a modification of immunosuppression. This study provides a basis for large-scale studies and for the use of epitope load in allocation policies.

Methods

Patient population

The patients included in our study had been previously randomized in a controlled trial for conversion from CsA to everolimus three months post-transplantation²². In brief, kidney transplant recipients were randomized into two treatment groups corresponding to maintenance on CsA or conversion to everolimus, both in conjunction with mycophenolic acid and steroid treatment. Patients were excluded if they had experienced TCMR prior to randomization, or were positive for DSA. Patients randomized to CsA withdrawal received everolimus adjusted to target a C0 concentration of 6–10 ng/mL. Mycophenolic acid was started at 720 mg b.i.d and decreased at 360 mg b.i.d. after month-3 only in the everolimus arm. The diagnosis of ABMR was made according to the 09' update of the Banff 97 classification³⁵.

Determination of HLA allelic typing

DNA was available for retrospective complete allelic typing (A,B,C,DRB1,DQB1) for 68 recipients and 62 donors in the everolimus arm. This retrospective typing was performed at the laboratory of Immunology of Saint-Louis Hospital, with next generation sequencing (Reagents : GenDX company, analyzed on a Illumina MiSeq sequencer with the NGSEngine software) (Bedia, Chavenay, France) or high resolution PCR-specific sequence oligonucleotides (HD-SSO LabType, One Lambda, Ingen, Chatenay-Malabry, France), the latter when only two loci or less were to be analyzed. For the remaining cases, only the initial typing locally performed by lymphocytotoxicity, sequence-specific PCR primers (SSP) or low-resolution SSO, was available. DQA typing was only available for 27 donors and 14 recipients. For the remaining patients, DQA1 typing was inferred from the well-described tight linkage disequilibrium with DRB1 and

DQB1 alleles²⁹. DRB3/4/5 typing is not performed in France but is also inferred from the well-known associations with DRB1 genes³⁰. DPB and DPA typing were not performed. The missing alleles were inferred from their haplotype associations with HaploStats, a modified expectation-maximization algorithm provided by the National Marrow Donor Program²⁷. The frequency of haplotypes was questioned for Caucasian populations (including Middle East and Africa's North Coast). HaploStats outputs the list of all possible unphased allelic typings for HLA A, B, C, DRB1, DRB3/4/5, and DQB1 loci and the likelihood for each pair of haplotypes. These typings were confirmed when possible with the list of common French HLA haplotypes²⁸, which may lead to some infrequent errors (Table S3).

Determination of epitope load with HLAMatchmaker software

Once computed, allelic donor and recipient HLA typings were entered into HLAMatchmaker¹⁸, a Microsoft Excel-based software (ABCepletmatching Vs1 for class I and DRDQDPepletmatching Vs1 for class II). This algorithm applies two principles: (1) each HLA antigen is represented as a distinct string of structurally defined epitopes that correspond to many potential immunogens that can induce specific antibodies, and (2) patients cannot make antibodies against epitopes expressed by their own HLA molecules. It assesses donor-recipient compatibility through intra-locus and inter-locus comparisons. Class I and II epitope loads are the number of epitopes expressed by the donor but not by the recipient.

Identification of DSA and of targeted epitopes

To rule out the presence of DSA before transplantation, all centers conducted pre-transplant systematic Luminex single antigen (SA) or Luminex screenings (every 3 to 6 months). A Luminex SA test was performed if the screening became positive.

During the post-transplantation period, all patients underwent Luminex screening at month-3 (randomization), month-12 (end of the study) and then annually. A Luminex SA test was performed if the screening became positive. Luminex assays were performed using One Lambda kits (Canoga Park, CA) except for three patients tested using Lifecodes kits (Immucor, Norcross, GA).

In DQ7^{negative} patients who received a DQ7^{positive} graft and developed anti-DQ7 dnDSA, we used the HLAMatchmaker DRDQDP software to determine which DQ7 epitopes were

targeted by serum antibodies. Briefly, we entered the MFI of the class II Luminex single antigen beads into HLAMatchmaker (“DRDQDP antibody analysis V01”). The DQ epitopes of the recipient and of the negative beads were excluded as potential candidates for targeted epitopes. We examined the positive beads and determined which epitopes shared across these positive alleles could explain the reactivity pattern.

Statistical analysis

The results are expressed as the mean \pm SD for continuous variables or percentages for categorical data. Comparisons were based on Student’s t tests for normally distributed continuous data or Wilcoxon Rank Sums tests for nonparametric univariate analysis, and on χ^2 tests or exact Fisher’s tests for categorical data. Receiver operating characteristic (ROC) curve analysis was performed with the pROC package³⁶ to determine the optimal epitopic mismatch threshold that best predicted dnDSA development. This value was associated with the highest true positive rate and the lowest false positive rate. A two-sided p-value of 0.05 was considered statistically significant. AUC corresponding to the different types of mismatches were compared using the Delong method. Crude and adjusted odds ratio of graft loss according to number of epitopic mismatches were estimated using logistic regression. Potential adjustment covariates were antigenic mismatches, mean everolimus level, recipient and donor ages, and TCMR. Only covariates associated with a p-value $<$ 0.1 were included in the final model using stepwise forward selection. Statistical analyses were performed using R software. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Disclosures

None

Supplementary Materials

'Supplementary information is available at Kidney International's website'

Supplementary Table 1: HLA matching of the two arms of the randomized trial

Supplementary Table 2 HLA mismatches according to the occurrence of TCMR or ABMR and the occurrence of dnDSA in patients with rejection

Supplementary Table 3: Comparison of allelic typing assumed with HaploStats application based on generic typing and true allelic typing for frequent French haplotypes.

Supplementary Table 4: Modification of total and antibody-verified DR epitope loads with the different DRB3/4/5 alleles

Supplementary Table 5. Difference in DQA epitope expression in case of error in DQA allele inference

Supplementary Figure 1. Evolution of renal function during the follow-up.

Supplementary Figure 2. Comparison of class I and class II antigenic and antibody-verified epitope mismatches.

Supplementary Figure 3. Distribution of patients with everolimus discontinuation, T-cell mediated rejection and de novo DSA

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Figure Legends

Figure 1. Number of patients with occurrence of de novo DSA in the study population. Patients were included in the trial before transplantation, received a treatment based on cyclosporine and were randomized at three months post-transplantation to be maintained on cyclosporine or switched to everolimus. Abbreviations: MPA mycophenolic acid

Figure 2. Graft and patient post-transplantation survival.

A) Patient survival according to the development of dnDSA (red line) or not (black line). **B)** Death-censored graft survival according to the development of dnDSA (red line) or not (black line). **C)** Death-censored graft survival according to the treatment arm: cyclosporine (red line) or everolimus (black line). p value corresponds to log-rank test.

Figure 3. Comparison of the numbers of antigenic, allelic and epitopic mismatches in the study population. A and B) Comparison of class I (A) and class II (B) antigenic and epitope mismatches. For example, patients with a maximal value of 6 class I antigenic mismatches actually displayed an epitope load ranging from 13 to 36. Conversely, patients with the median value (n=15) of class I epitopic mismatches displayed 2 to 6 antigenic mismatches. The maximal number of antigenic mismatches is 6 for class I (2 for each locus HLA-A, B and Cw) and 4 for class II (2 for DR and DQB locus). **C and D)** Comparison of class II antigenic and allelic mismatches including DQA alleles (C), and of class II allelic and epitopic mismatches including DQA alleles (D). Patients with 3, 4, 5, 6 and, to a lesser degree, 7/8 class II allelic mismatches had very similar distributions of epitope load, although all these patients would be considered as having a growing level of incompatibility. Conversely, patients with a given number of allelic mismatches, for example 6, may have many epitopic mismatches ranging from 10 to 51. **E and F)** Comparison of class II antigenic and allelic mismatches excluding DQA alleles (E) and class II allelic and epitope mismatches excluding DQA alleles (F). Each floating bar represents the minimum and maximum values, and the line corresponds to the median value. The maximal number of allelic mismatches is 8 (Panels C/D: 2 for each locus HLA-DRB1, DRBW, DQB, DQA) or 6 without DQA (Panels E/F).

Figure 4. Evaluation of HLA compatibility with antigenic, allelic and epitope mismatches. A) An example of two patients with the same number of class II antigenic (n=2) and allelic (n=4) mismatches but with different epitope loads (11 and 48), including DQA epitopes. The second patient developed de novo class II DSA. **B)** An example of three DQ7-negative recipients who received a DQ7-positive transplant. The same DQ7 antigenic mismatch was associated with different DQ7 specific epitope loads according to the recipient's DQB typing. Alpha chains are shown in pink, beta chains are in blue, and peptides are in brown. Mismatched epitopes are in yellow (beta chain epitopes are shown in normal font, and alpha chain epitopes are shown in italic font).

Figure 5. Distribution of patients who developed or not dnDSA, according to antigenic and epitopic mismatches. Patients with (black dots) or without (empty dots) class I or II dnDSA, according to the total number of antigenic (A+B+C+DR+DQB1) and epitopic (A+B+C+DR+DQB1) mismatches. Horizontal dashed/dotted line corresponds to the threshold of 27 epitopic mismatches and vertical line to the threshold of 6 antigenic mismatches (see Table 5).

Figure 6. Survival without de novo DSA.

In patients from the cyclosporine arm (red line) or everolimus (black line). p-value corresponds to log-rank test

Figure 7. Comparison of specific DQ7 epitopic mismatches in patients who developed or did not develop DQ7 DSA. DQ7 specific epitope load including all DQ7 epitopes (A) or only antibody-verified epitopes (B) in DQ7-negative patients who received a DQ7-positive transplant and did or did not develop a DQ7 dnDSA after transplantation. C) Frequency of mismatched DQ7 epitopes in DQ7-negative patients who received a DQ7-positive transplant and did or did not develop a DQ7 dnDSA. *p<0.05. RR: relative risk of DQ7 dnDSA in cases of mismatched epitopes.

Table 1: Characteristics of the study population

Recipients	Whole group	dnDSA	no dnDSA	p
N	89	29	60	
Age, yrs (mean ± SD)	48.2 ± 12.5	42.9 ± 14.0	50.8 ± 10.9	0.005
Sex, N (%) of males	59 (66.3)	22 (75.9)	37 (61.7)	0.18
Nephropathy, N (%)				0.80
Diabetes	9 (10.1)	4 (13.8)	5 (8.3)	
Glomerular disease	28 (31.5)	9 (31.0)	19 (31.7)	
Nephroangiosclerosis	5 (5.6)	2 (6.9)	3 (5.0)	
Interstitial nephritis	12 (13.5)	5 (17.2)	7 (11.7)	
Polycystic disease	16 (18.0)	4 (13.8)	12 (20.0)	
Other	11 (12.4)	4 (13.8)	7 (11.7)	
Unknown	8 (9.0)	1 (3.4)	7 (11.7)	
No dialysis before transplantation, N (%)	6 (6.7)	1 (3.4)	5 (8.3)	0.66
Positive PRA, N^a (%)	5 (6.5)	0 (0)	5 (10)	0.16
Mean PRA (%)	0.8 ± 3.8	0	1.2 ± 4.7	0.09
Donors				
Age, yrs (mean ± SD)	47.5 ± 14.9	43.4 ± 14.8	49.4 ± 14.6	0.07
Deceased donor / living donor (%)	92.1 / 7.9	93.1 / 6.9	91.7 / 8.3	1.0
Cerebrovascular death, N^b (%)	46 (56.8)	13 (50.0)	33 (60.0)	0.47
Delayed graft function, N (%)	16 (18.0)	7 (24.1)	9 (15.0)	0.38
T-cell Mediated Rejection, N(%)	20 (22.5)	10 (34.5)	10 (16.7)	0.10

a: information available for 77 patients

b: information available for 81 patients

Abbreviations. dnDSA: de novo donor-specific antibody; SD: standard deviation;

PRA: panel reactive antibody

Table 2: Antigenic, allelic and epitopic mismatches

	Min	Max	Median	IQR	Mean ± SD
Class I					
Antigenic MM	1	6	4	3-5	3.8 ± 1.4
Allelic MM	1	6	4	3-5	4.1 ± 1.3
All Epitopic MM	2	36	15	10-19	15.2 ± 6.4
Abv Epitopic MM	1	19	8	5-11	8.5 ± 4.0
Class II					
Antigenic MM (DR+DQ)	0	4	2	1-2	2.0 ± 1.1
Allelic MM (w/o DQA)	1	6	3	3-4	3.3 ± 1.4
Allelic MM (with DQA)	1	8	4	3-5	4.5 ± 1.8
All Epitopic MM (w/o DQA)	0	50	20	11-31	20.9 ± 12.5
Abv Epitopic MM	0	26	8	4-12	8.5 ± 6.3
All Epitopic MM (with DQA)	0	65	24	15-38	26.3 ± 15.8
Abv Epitopic MM	0	26	9	4-14	9.4 ± 6.8
Class I + II					
Antigenic MM	1	10	6	4-7	5.7 ± 1.9
Allelic MM	2	12	7	6-9	7.4 ± 2.1
All Epitopic MM (w/o DQA)	2	66	36	25-49	36.1 ± 14.6
Abv Epitopic MM	1	33	16	12-22	17.0 ± 7.5

Abbreviations. IQR: Interquartile range. MM: mismatch. Abv : antibody-verified

Table 3: Exposure to immunosuppressive drugs and de novo DSA

	De novo DSA	No de novo DSA	p
T° cyclosporine* at randomization	169.0 ± 54.0 (N=14)	166.0 ± 33.5 (N=34)	0.93
T2 cyclosporine* at randomization	851.2 ± 290.8 (N=7)	925.7 ± 381.8 (N=21)	0.84
T° everolimus M6, mean ± SD	5.7 ± 1.8	6.9 ± 2.9	0.08
T° everolimus M9, mean ± SD	7.0 ± 2.1	7.2 ± 2.8	0.78
T° everolimus M12, mean ± SD	7.1 ± 2.1	8.3 ± 4.5	0.62
Average T° everolimus, mean ± SD	6.4 ± 1.8	7.4 ± 2.6	0.08
T° everolimus M6 < 6 ng/mL, % of patients	52.2%	37.3%	0.23
T° everolimus M9 < 6 ng/mL, % of patients	31.3%	31.0%	1.0
T° everolimus M12 < 6 ng/mL, % of patients	34.8%	30.2%	0.69
Dose Mycophenolate M3 (mg/day)	1386 ± 356	1316 ± 388	0.40
Patients below 1440 mg M3, n(%)	8 (13.3)	5 (17.2)	0.75
Dose Mycophenolate M12 (mg/day)	711 ± 328	734 ± 238	0.75
Patients below 720 mg M12, n(%)	12 (20.3)	3 (11.5)	0.54

*Some patients in the study were monitored with cyclosporine trough level (T°) or with cyclosporine level two hours after drug intake (T2). N: number of patients corresponding to the presence of DSA and to the type of monitoring. M6/M9/M12 correspond to the protocol visit (at 6, 9 or 12 months after transplantation) at which the level was measured.

Table 4: Relationship between the occurrence of de novo DSA and antigenic, allelic and epitopic mismatches

N. patients (DSA+ / DSA-)	N. of Antigenic mismatches		N. of Allelic mismatches		N. of Epitope mismatches		N. of abv Epitope mismatches	
	(DSA+ vs DSA-)	p	(DSA+ vs DSA-)	p	(DSA+ vs DSA-)	p	(DSA+ vs DSA-)	p
Class I or II DSA								
29 / 60	6.5 ± 1.8 vs 5.4 ± 1.8	0.015	8.2 ± 2.1 vs 7.1 ± 2.1	0.026	44.5 ± 11.6 vs 32.0 ± 14.2	<0.0005	21.6 ± 6.3 vs 14.8 ± 7.0	5.7 e-5
Class I DSA								
5 / 84	4.8 ± 0.8 vs 3.7 ± 1.4	0.07	5.0 ± 0.7 vs 4.1 ± 1.3	0.11	23.2 ± 7.0 vs 14.7 ± 6.1	0.014	13.2 ± 3.1 vs 8.2 ± 3.9	0.009
Class II DSA *								
28 / 61	2.5 ± 0.9 vs 1.7 ± 1.2	0.003	3.8 ± 1.4 vs 3.0 ± 1.3	0.024	27.6 ± 9.9 vs 17.9 ± 12.4	0.0003	12.1 ± 5.4 vs 6.8 ± 6.1	4.8 e-5
DR DSA								
6 / 83	1.2 ± 0.4 vs 1.0 ± 0.6	0.45	1.2 ± 0.4 vs 1.3 ± 0.6	0.41	16.8 ± 8.2 vs 10.4 ± 7.8	0.07	7.0 ± 4.5 vs 3.5 ± 3.5	0.04
DQ DSA *								
25 / 64	1.3 ± 0.5 vs 0.9 ± 0.7	0.006	1.4 ± 0.5 vs 1.2 ± 0.6	0.18	13.5 ± 5.2 vs 8.7 ± 8.2	0.0005	6.8 ± 3.3 vs 3.9 ± 4.8	<0.0005

Abbreviations: N number, abv: antibody-verified, * DQ and Class II mismatches do not include DQA

Table 5. Thresholds of antigenic, allelic and epitope mismatches predictive of the occurrence of dnDSA

	Antigenic MM	Allelic MM	Epitopic MM	Abv Epitopic MM
DSA I or II				
AUC	0.658	0.645	0.749	0.764
p ^a (comparison with antigenic MM)	--	0.71	0.10	0.06
Threshold	6	7	27	15
N patients(%) above the threshold	50(56.2%)	60(67.4)	62 (69.7)	56(62.9)
Freq of DSA (above vs below,%), p ^b	42.0 vs 20.5, p=0.03	41.7 vs 13.8, p=0.009	45.2 vs 3.7 p=0.0001	48.2 vs 6.1 p=4.2 e-5
RR 95CI	2.05 [1.02-4.12]	3.02 [1.2-7.9]	12.2 [1.7 -85.1]	8.0 [2.0-31.3]
DSA CLASS I				
AUC	0.738	0.710	0.830	0.849
p ^a (comparison with antigenic MM)	--	0.59	0.36	0.16
Threshold	4	5	19	10
N patients(%) above the threshold	55 (61.8)	37 (41.6)	26(29.2)	35(39.3)
Freq of DSA (above vs below,%), p ^b	9.1 vs 0.0, p=0.07	10.8 vs 1.9 p=0.16	15.4 vs 1.6, p=0.025	14.3 vs 0.0, p=0.008
RR 95CI	NA	5.6 [0.7-48.3]	9.7 [1.1-82.6]	NA
Class II DSA^c				
AUC	0.684	0.644	0.738	0.769
p ^a (comparison with antigenic MM)	--	0.40	0.34	0.15
Threshold	2	3	18	10
N patients(%) above the threshold	64(71.9)	67(75.3)	50(56.2)	61(68.5)
Freq of DSA (above vs below,%), p ^b	39.1 vs 0.0, p=0.002	32.8 vs 13.6, p=0.08	44.0 vs 7.7, p=1.10-4	39.3 vs 3.6, p<5.10-4
RR 95CI	NA	2.4 [0.8-7.3]	5.7 [1.8-17.7]	11.0 [1.6-77.4]
DQ DSA^c				
AUC	0.667	0.577	0.737	0.741
p ^a (comparison with antigenic MM)	--	0.02	0.23	0.22
Threshold	1	2	8	2
N patients(%) above the threshold	69(77.5)	26(29.2)	56(62.9)	57(64.0)
Freq of DSA (above vs below,%), p ^b	34.8 vs 0.0, p=0.002	34.6 vs 23.8, p=0.30	41.1 vs 3.0, p<1.10-4	40.4 vs 3.1, p=1.10-4
RR 95CI	NA	1.5 [0.7-2.9]	13.6 [1.9-95.8]	12.9 [1.8-91.2]

Abbreviations. MM: mismatch, AUC: Area Under the Curve, exc: excluding, N: Number, RR: Relative Risk, CI: Confidence Interval, NA: not applicable, Freq: Frequency, a: the first p-value corresponds to the comparison with the AUC of antigenic mismatches with the De Long methods. The second p value corresponds to the Fisher test performed to compare the proportion of patients with dnDSA in patients with a number of mismatches above the threshold and in patients with a number of mismatches below the threshold, c: DQ and Class II mismatches do not include DQA

Table 6. Factors associated with the development of dnDSA in univariate and multivariate analysis

	Univariate Analysis			Multivariate Analysis		
	OR	95%CI	p	OR	95%CI	p
Epitope load (per 1 MM increment)	1.07	[1.03-1.12]	0.0004	1.05	[1.01-1.11]	0.029
Antigenic MM (per 1 MM increment)	1.41	[1.09-1.88]	0.011	1.09	[0.76-1.57]	0.62
Mean everolimus level (per 1 ng/mL)	0.82	[0.65-1.01]	0.08	0.92	[0.69-1.18]	0.52
Recipient Age (per 1 year increment)	0.95	[0.91-0.98]	0.007	0.95	[0.89-1.01]	0.11
Donor Age (per 1 year increment)	0.97	[0.94-1.0]	0.08	1.00	[0.95-1.06]	0.95
TCMR (Yes versus No)	2.63	[0.94-7.43]	0.064	1.56	[0.48-5.36]	0.48

Abbreviations. MM: mismatch, TCMR: T-cell mediated rejection

Table 7. Patients with a DQ7 mismatch

#	DSA DQ7	Recipient DQB1 Allelic typing		Donor DQB1 Allelic typing		AL MM	DQ7 Epitope Load	Mismatched DQ7 epitopes (Abv)	Mismatched DQ7 epitopes (non-Abv)	Targeted epitope	DQ antigens sharing this epitope
1	P	*04:02	*02:02	*03:01	*02:02	1	8	45EV 55PP	14AM 26Y 56PD 66EV 70RT 74EL	55PP/ 74EL*	DQ6,DQ7,DQ8,DQ9
15	P	*02:02	*05:01	*03:01	*05:01	1	12	45EV 52PL 55PP 77T 140T 182N	14AM 26Y 37YA 56PD 70RT 74EL	55PP	DQ7,DQ8,DQ9
25	P	*06:09	*02:01	*03:01	*02:01	1	8	45EV 52PL 55PP 140T 182N	14AM 26Y 56PD	52PL3	DQ4,DQ7,DQ8,DQ9
27	P	*05:03	*02:01	*03:01	*02:01	1	11	45EV 52PL 55PP 77T 140T 182N	14AM 26Y 37YA 70RT 74EL	77T	DQ4,DQ6,DQ7,DQ8,DQ9
41	P	*05:01	*04:02	*03:01	*06:04	2	7	45EV 55PP	14AM 26Y 56PD 70RT 74EL	45EV	DQ7
45	P	*02:02	*05:01	*03:01	*05:01	1	12	45EV 52PL 55PP 77T 140T 182N	14AM 26Y 37YA 56PD 70RT 74EL	55PP	DQ7,DQ8,DQ9
65	P	*02:02	*02:02	*03:01	*02:02	1	16	45EV 46VY 28T 52PL 55PP 52P 77T 140T 182N	14AM 26Y 37YA 56PD 70RT 74EL 135D	DQαβ epitope?	DQB1*03:01- DQA1*05:03/05:05/06:01
73	P	*06:03	*03:02	*03:01	*03:01	1	3	45EV	14AM 26Y	45EV	DQ7
107	P	*05:02	*02:02	*03:01	*02:01	1	12	45EV 52PL 55PP 77T 140T 182N	14AM 26Y 37YA 56PD 70RT 74EL	NA	-
173	P	*05:02	*05:01	*03:01	*05:01	1	16	45EV 52PL 55PP 77T 84QL 125A 53L 140T 182N	14AM 26Y 37YA 56PD 70RT 74EL 116V	55PP	DQ7,DQ8,DQ9
187	P	*06:02	*02:02	*03:01	*02:02	1	8	45EV 52PL 55PP 140T 182N	14AM 26Y 70RT	55PP	DQ7,DQ8,DQ9
43	N	*06:02	*03:02	*03:01	*02:02	2	3	45EV	14AM 26Y		
49	N	*03:03	*06:01	*03:01	*06:02	1	1	45EV			
63	N	*03:03	*05:02	*03:01	*06:03	1	3	45EV	14AM 26Y		
71	N	*03:02	*03:02	*03:01	*03:01	1	5	45EV	14AM 26Y 56PD 185T		
83	N	*02:02	*02:01	*03:01	*02:01	1	16	45EV 46VY 28T 52PL 55PP 52P 77T 140T 182N	14AM 26Y 37YA 56PD 66EV 70RT 74EL		
101	N	*06:04	*02:01	*03:01	*05:03	2	8	45EV 52PL 55PP 140T 182N	14AM 26Y 56PD		
127	N	*05:01	*03:02	*03:01	*02:01	2	4	45EV	14AM 26Y 56PD		

133	N	*03:02	*06:02	*03:01	*05:01	2	3	45EV	14AM 26Y	
135	N	*06:02	*06:02	*03:01	*05:02	2	12	45EV 52PL 55PP 84QL 125A 53L 140T 182N	9Y 14AM 26Y 70RT	
163	N	*05:01	*03:02	*03:01	*03:02	1	4	45EV	14AM 26Y 56PD	
189	N	*03:02	*06:03	*03:01	*06:03	2	3	45EV	14AM 26Y	
195	N	*02:01	*06:03	*03:01	*06:03	1	8	45EV 52PL 55PP 140T 182N	14AM 26Y 70RT	

Abbreviations P: positive, N: negative, AL MM: number of DQB1 allelic mismatches, Abv: antibody-verified epitope. * 74EL was the sole non-exposed epitope.

175 Kidney transplant recipients
Treatment from day 0:
Basiliximab, steroids,
MPA, Ciclosporine

RANDOMIZATION
at 3 months

EVEROLIMUS
ARM
N=89

N=60
(67.4%)
no DSA
de novo

N=29
(32.6%)
DSA de
novo

N=24
Class II
DSA

N=4
Class
I+II
DSA

N=1
Class I
DSA

N=3
Anti-
DR
DSA

N=22
Anti-
DQ
DSA

N=3
Anti-
DR+DQ
DSA

CYCLOSPORINE
ARM
N=86

N=70
(81.4%)
no DSA
de novo

N=16
(18.6%)
DSA de
novo

N=12
Class II
DSA

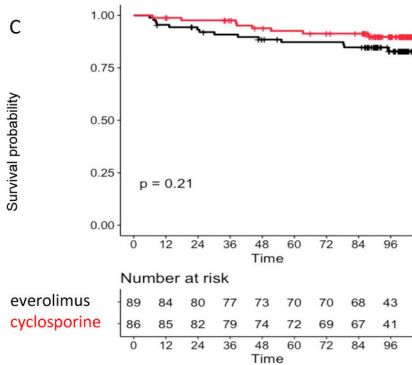
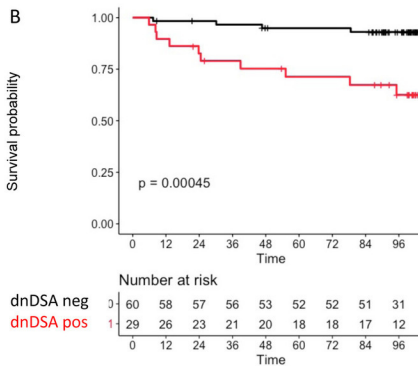
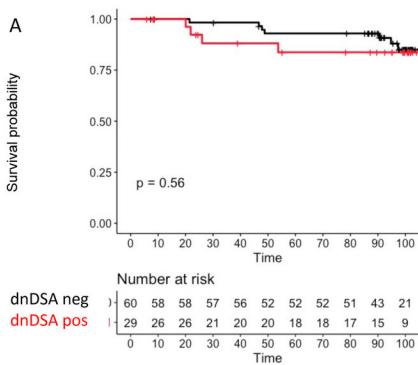
N=3
Class
I+II
DSA

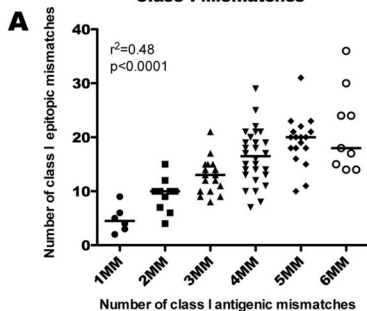
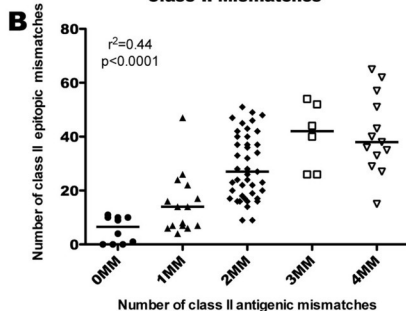
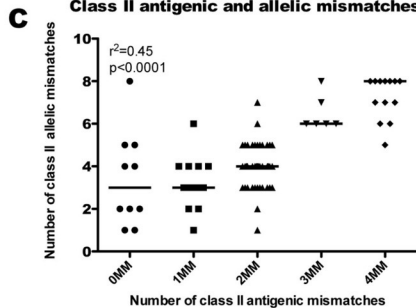
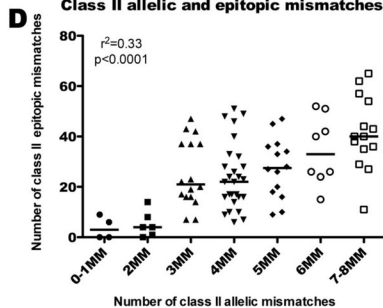
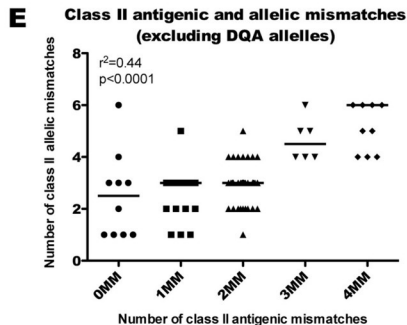
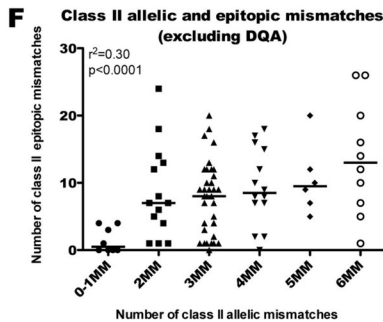
N=1
Class I
DSA

N=2
Anti-
DR
DSA

N=12
Anti-
DQ
DSA

N=1
Anti-
DR+DQ
DSA



Class I Mismatches**Class II Mismatches****Class II antigenic and allelic mismatches****Class II allelic and epitopic mismatches****Class II antigenic and allelic mismatches (excluding DQA alleles)****Class II allelic and epitopic mismatches (excluding DQA)**

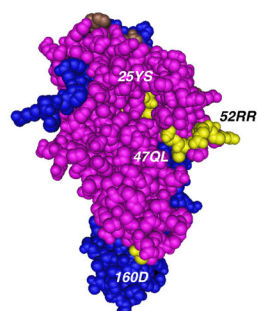
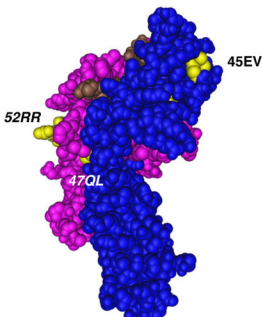
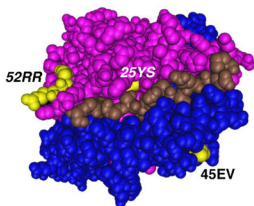
	DRB LOCUS			DQB LOCUS			DQA LOCUS		
Recipient #77 generic (antigenic) typing	DR11		DR14		DQ7		DQ5		
Donor generic (antigenic) typing	DR11		DR13		DQ7		DQ6		
Antigenic mismatches	1DR				1DQ				
Recipient #77 deduced allelic typing	DRB1*11:01	DRB1*14:01		DRB3*02:02	DRB3*02:02		DQB1*03:01	DQB1*05:03	
Donor deduced allelic typing	DRB1*11:01	DRB1*13:01		DRB3*02:02	DRB3*01:01		DQB1*03:01	DQB1*06:03	
Allelic mismatches	1 DRB1			1 DRBX			1 DQB1		1 DQA1
	DRB1 + DRBW			DQB1			DQA1		
	TOTAL	ABV	NON-ABV	TOTAL	ABV	NON-ABV	TOTAL	ABV	NON-ABV
Epitopic mismatches		74R			-			-	
				37NV 37FL 257V 60S			13GM 26L 70GT		25FT 41KA 130A
				67I 71E 183A			87F 125GQ		
Locus specific epitope load including DQA	8	1	7	5	0	5	3	0	3
Class II abv epitope load including DQA	1								
Class II total epitope load including DQA	16								
dnDSA	NO								
Recipient #173 generic (antigenic) typing	DR1		DR16		DQ5				
Donor generic (antigenic) typing	DR1		DR4		DQ5		DQ7		
Antigenic mismatches	1DR				1DQ				
Recipient #173 deduced allelic typing	DRB1*01:01	DRB1*16:01		DRB5*02:02			DQB1*05:01	DQB1*05:02	
Donor deduced allelic typing	DRB1*01:01	DRB1*04:01		DRB4*01:01			DQB1*05:01	DQB1*03:01	
Allelic mismatches	1DRB1			1DRBX			1DQB1		1DQA1
	DRB1 + DRBW			DQB1			DQA1		
	TOTAL	ABV	NON-ABV	TOTAL	ABV	NON-ABV	TOTAL	ABV	NON-ABV
Epitopic mismatches		4Q 18L 26WN 37YV		37YA 70R 71K 74E				-	
							45EV 52PL 55PP		18S 25YS 45VL 47QL
							14AM 26Y 37YA		
		48Q 40YNL 96Y 81Y		85VV 98E 120N 133RS			77T 84QL 125A		61FT 66L 52RR 80S
							56PD 70RT 74EL		
		104A 180LT 181M		140TV 189S			53L 140T 182N		129H 175E 187T
							116V		
Locus specific epitope load including DQA	21	11	10	16	9	7	11	0	11
Class II abv epitope load including DQA	20								
Class II total epitope load including DQA	48								
dnDSA	DR4 DQ7								

1. DQ7 mismatch seen by recipient #49 HLA DQ6/DQ9

Top view

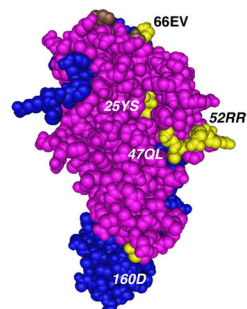
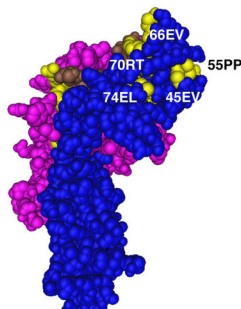
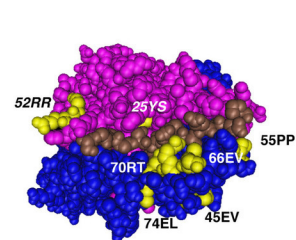
β -chain side view

α -chain side view



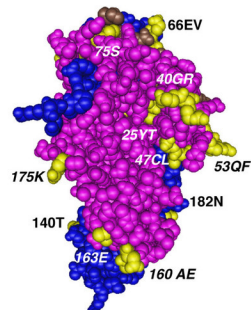
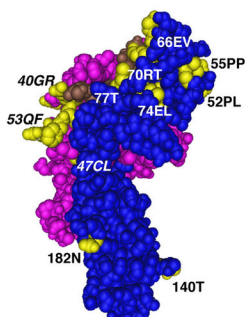
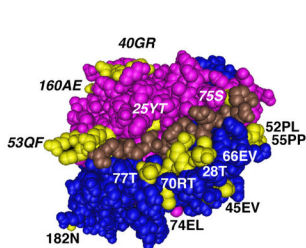
β epitope load : 1
 α epitope load : 5

2. DQ7 mismatch seen by recipient #1 HLA DQ2/DQ4

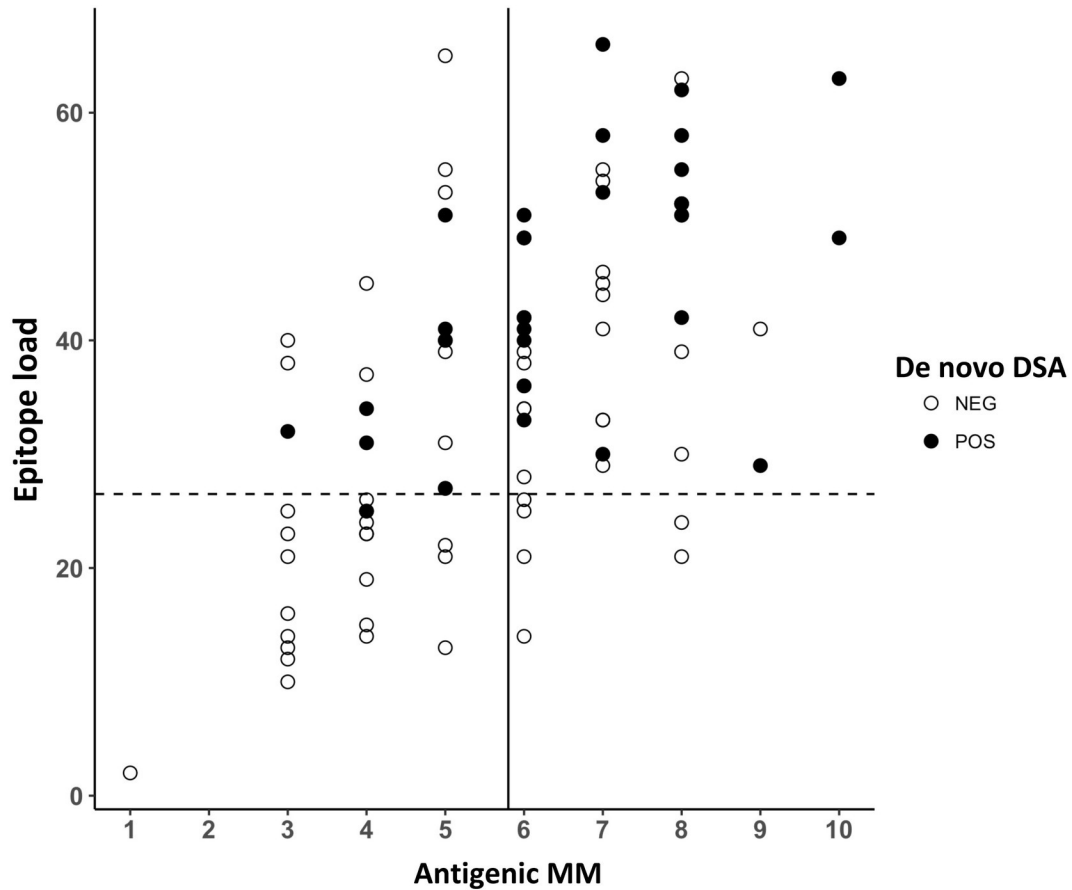


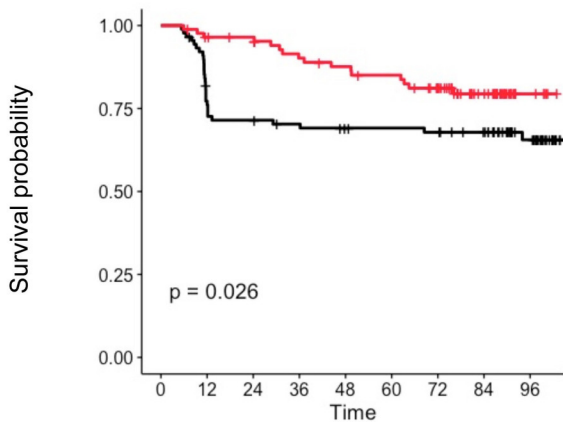
β epitope load : 8
 α epitope load : 5

3. DQ7 mismatch seen by recipient #65 HLA DQ2/DQ2



β epitope load : 16
 α epitope load : 8

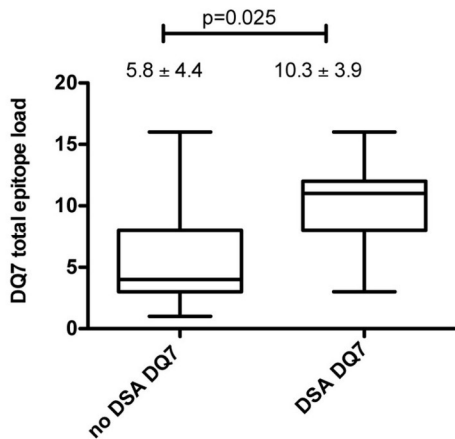




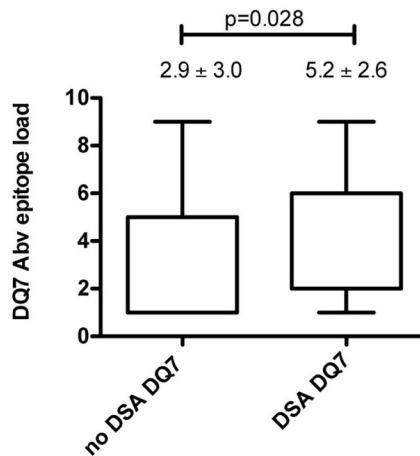
Number at risk

everolimus	89	67	62	59	55	54	53	48	29	
cyclosporine	86	81	79	71	68	65	57	34	6	
	0	12	24	36	48	60	72	84	96	
	Time									

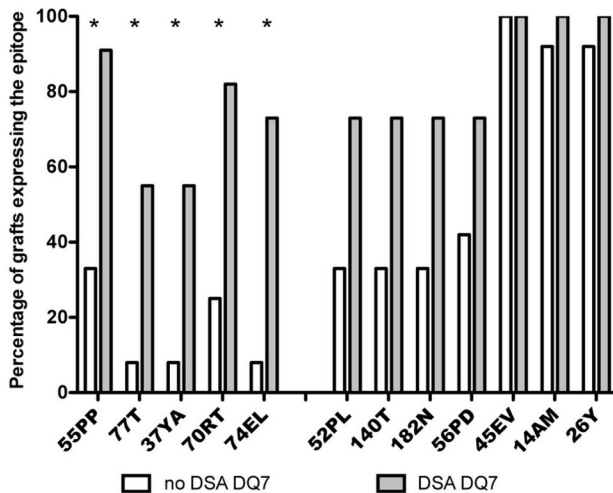
A DQ7^{neg} patients receiving a DQ7^{pos} transplant



B DQ7^{neg} patients receiving a DQ7^{pos} transplant



C Mismatched DQ7 epitopes



Epitopes	Odds Ratio	95% CI	p
55PP	17,1	1.5 : 966.4	0.009
77T	11.6	1.0 : 657.7	0.27
37YA	11.6	1.0 : 657.7	0.27
70RT	11.7	1.4 : 174.4	0.012
74EL	23.9	2.0 : 1399.4	0.003