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ORIGINAL ARTICLE

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Qualitative, rather than quantitative, differences between HLA-DQ alleles affect HLA-DQ immunogenicity in organ transplantation

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Prolonging the lifespan of transplanted organs is critical to combat the shortage of this life-saving resource. Chronic rejection, with irreversible demise of the allograft, is often caused by the development of donor-specific HLA antibodies. Currently, enumerating molecular (amino acid) mismatches between recipient and donor is promoted to identify patients at higher risk of developing HLA antibodies, for use in organ allocation, and immunosuppression-minimization strategies. We have counseled against the incorporation of such approaches into clinical use and hypothesized that not all molecular mismatches equally contribute to generation of donor-specific immune responses. Herein, we document statistical shortcomings in previous study design: for example, use of individuals who lack the ability to generate donor-specific-antibodies

Abbreviations: 2MM1DSA, two mismatches but only one DSA; ABMR, antibody-mediated rejection; CRISPR, clustered regulatory interspaced short palindromic repeats; DSA, donor specific antibody; dnDSA, de novo donor specific antibody; HLA, HLA; HmAb, human monoclonal antibody; IHWS, International Histocompatibility Workshop; mAb, monoclonal antibody; MML, molecular mismatch load; NMDP, National Marrow Donor Program; NPV, negative predictive value; PPV, positive predictive value; SRTR, Scientific Registry of Transplant Recipients.

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(HLA identical) as part of the negative cohort. We provide experimental evidence, using CRISPR-Cas9-edited cells, to rebut the claim that the HLAMatchmaker eplets represent "functional epitopes." We further used unique subcohorts of patients, those receiving an allograft with two HLA-DQ mismatches yet developing antibodies only to one mismatch (2MM1DSA), to interrogate differential immunogenicity. Our results demonstrate that mismatches of DQα05-heterodimers exhibit the highest immunogenicity. Additionally, we demonstrate that the DQα chain critically contributes to the overall qualities of DQ molecules. Lastly, our data proposes that an augmented risk to develop donor-specific HLA-DQ antibodies is dependent on qualitative (evolutionary and functional) divergence between recipient and donor, rather than the mere number of molecular mismatches. Overall, we propose an immunological mechanistic rationale to explain differential HLA-DQ immunogenicity, with potential ramifications for other pathological processes such as autoimmunity and infections.

KEYWORDS
epitope, histocompatibility, immunogenicity, organ-transplantation, HLA-DQ

1 | INTRODUCTION

The development of de-novo HLA-donor-specific-antibody (HLA-dnDSA) posttransplant is associated with increased risk for antibody-mediated rejection (ABMR) and poor allograft outcome.[1–5] While the development of ABMR can be caused by medication noncompliance, it is not clear why some recipients develop dnDSA to their HLA-mismatched donors despite adequate trough-level immunosuppression, while others do not. This suggests that some HLA mismatches are more immunogenic than others.

With the aim to decipher this variability in immunogenicity, several molecular mismatch load (MML) analysis approaches were introduced in recent years.[6–9] As suggested by the name, all MML approaches require knowledge of donor and recipient HLA allele typing at the (molecular) amino acid level. The most utilized software program, HLAMatchmaker,[10–12] postulates that small polymorphic amino acid fragments, termed eplets, have immunogenic significance. HLAMatchmaker combines eplets from both donor alleles into an "eplet universe," compares it to the eplet universe of the recipient, and outputs the number of eplets present only in the donor antigens as the mismatch load. HLAMatchmaker further considers these eplets as "functional epitopes,"[13,14] distinct from the "structural epitope" which refers to the full footprint of the area recognized by an antibody.

Many researchers embraced MML analysis tools, initially proposing the erroneous term "epitope matching"[11,15–17] and later enumerating MML to estimate relative immunogenicity between donor and recipient. Indeed, statistically speaking, many patients that developed dnDSA (and in some studies, developed antibody- and T-cell-mediated rejections) exhibited higher MML compared with patients that did not. However, this was not always the case. Different thresholds for defining high risk were proposed over the past few years (e.g., >10 or >17 eplets for HLA-DR or -DQ, respectively[18]; 11 eplets for both DR and DQ[19]; or 7 eplets for DR and 9 eplets for DQ[20]; etc.[21,22]).

Several fundamental observations suggest that simple enumeration of mismatches (MML analysis) cannot explain immunogenicity. (1) All published studies include some patients that developed dnDSA despite low MML. (2) Lucas et al.[23] demonstrated a specific directionality in the ability to develop DSA, meaning that a recipient typed as X may develop antibodies to a donor typed as Y, but a recipient typed as Y may not develop antibodies to a donor typed as X, although the MML is equivalent. (3) The different MML algorithms claim to use different logic in their calculations, yet all show similar predictive values when applied, indicating no- to minimal-added value of their specific algorithms beyond the mere difference in amino acid sequences.[24] We previously suggested that increased MML may be a surrogate for a yet unexplained immunologic difference.[25–27]

We further hypothesized that qualitative properties of the recipients' HLA alleles influence immunogenicity beyond the number of mismatches.[28] In a recent review,[9] we have summarized multiple limitations of MML approaches, mostly from the theoretical perspective. Here we present the first report of strong, irrefutable evidence, demonstrating that MML should not guide clinical decision making specific for solid organ allocation algorithms nor for drug minimization strategies. Our work focuses on HLA-DQ mismatches, as antibodies against these molecules were shown to have the strongest association with poor allograft outcome.[29,30]

2 | RESULTS

2.1 | Molecular mismatch load analysis

To test the accuracy of MML analysis tools in identifying patients with high risk of developing dnDSA, HLAMatchmaker algorithm was applied for the Northwestern University (NU) cohort. A total of 279 recipient/donor pairs were eligible per inclusion/exclusion criteria (complete exclusion/inclusion strategy is shown in Figure S1, demographic characteristics in Table S1). Patients were assigned as either "dnDSA-positive" ($N = 113$) or "dnDSA-negative" ($N = 166$). Importantly, high-resolution HLA class II typing demonstrated that 39/166 dnDSA-negative pairs were HLA-DQ matched at the allelic level, raising the question whether they should be excluded from analysis as such pairs are not expected to form dnDSA. Analyses were performed with and without these pairs. Analyses were further performed following the HLAMatchmaker algorithm by combining both donor alleles into one "donor-universe", and by analyzing each allele individually ("allele level"). When HLA-DQ-matched pairs were included in the analysis, a strong statistical significance was observed between increased eplet mismatch load and generation of dnDSA ($p < 0.0001$; Figure 1A). However, when HLA-DQ-matched pairs were excluded from the dnDSA-negative group, the statistical significance diminished to $p = 0.0306$ (Figure 1B). Youden's score was calculated to determine risk threshold at 8 eplets, with positive predictive value (PPV) of 56.6%; negative predictive value (NPV) of 74.3%; increasing to 83.2% if HLA identical donors were included. Figure 1C further illustrates the large overlap between the two groups, attributing the strong statistical significance between dnDSA-positive and -negative to the inclusion of DQ-matched pairs.

Analysis at the allele level is presented in Figure 1D, E. The dnDSA-positive allele group ($N = 141$; comprised of the 113 DSA-positive patients developing antibodies to 141 donor-DQ alleles) was compared with either the dnDSA-negative/excluding HLA identical alleles group ($N = 219$; comprised of all mismatches that did not lead to generation of dnDSA), or the dnDSA-negative/all allele group ($N = 399$, including DQ identical alleles). A statistically significant difference was observed between increased eplet mismatch load and generation of dnDSA between all groups including between the two dnDSA-negative groups, $p < 0.0001$, likely driven by the impact of including the 180 HLA-DQ identical alleles. Youden score determined the risk threshold to be 5 eplets, with PPV is 47.1% and NPVs of 81.8% or 93.5% depending on whether donors with HLA identical alleles were included. Notwithstanding, this analysis does not take into consideration the fact that some patients developed dnDSA to both donor-mismatched alleles and therefore are counted twice in this analysis, or develop antibodies to one mismatch but not the other, leading to counting these patients in both the DSA-positive and DSA-negative groups. We therefore adjusted the analysis to account for each patient only once, based on the mismatch contributing to the highest eplet mismatch load. This led to reduction of the statistical significance between increased eplet mismatch load and development of dnDSA to a mere $p = 0.0409$ (Figure 1F,G). This analysis demonstrates the importance of choosing the correct inclusion/exclusion criteria and of performing the correct statistical test.

2.2 | Use of unique cohorts to explore relative immunogenicity—not all mismatches are created equal

We reasoned that analysis of immunogenicity using large cohorts requires correction for multiple confounding variables including the level of immunosuppression, infections, and other patient-specific or external variables that may affect outcome. However, information about these variables is often lacking. To circumvent these limitations, we proposed to study a sub-cohort of dnDSA-positive patients, those receiving a kidney with two HLA-DQ mismatches but forming dnDSA only to one of these mismatches (2MM1DSA cohort[28]). This approach allows us to compare relative immunogenicity of different mismatches in a "fixed," single patient environment. A schematic of how this cohort was analyzed is presented in Figure S2, and examples of how this analysis was applied are shown in Figure 2. To minimize preconceived notions associated with any MML software, we performed our analysis at the amino acid (AA) level. All polymorphic AAs of the recipient's and the donor's HLA-DQ alleles are displayed. Mismatches that are unique to the dnDSA-inducing allele are highlighted yellow, while those that

are unique to the mismatched allele that did not induce dnDSA formation are highlighted green; AA mismatches that are present in both donor alleles are highlighted blue. Using this approach, two sets of lower immunogenicity AA mismatches can be identified. (1) Those that are unique to the non-DSA allele, since they did not lead to dnDSA formation (green), and (2) those that are present in both DSA-allele and non-DSA allele (blue) given that they did not lead to dnDSA formation against the non-DSA allele. Thus, in pair 1, while there are 26 AA mismatches between the recipient and the DSA-inducing allele, 14/26 are shared with the non-DSA allele, leaving

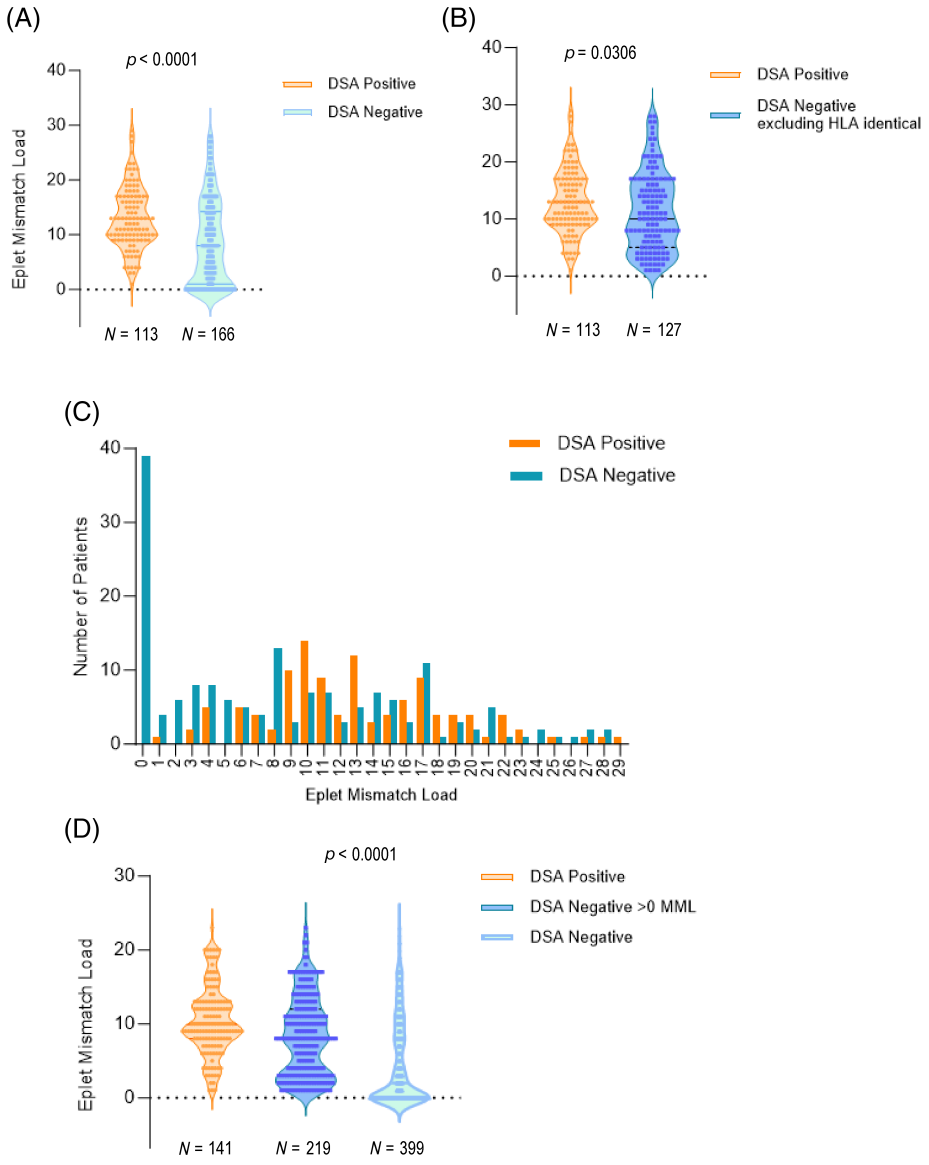


FIGURE 1 Statistical "bounded rationality" in determining association between eplet mismatch load and generation of de novo donor specific antibody (dnDSA) is skewing the association of molecular mismatch load (MML) analysis with generation of dnDSA. We tested the impact of including transplant pairs with no mismatches at the investigated locus on the perceived statistically significant correlation between eplet mismatch load and generation of dnDSA. Data were first analyzed following the HLAMatchmaker "eplet universe" approach (A) considering all transplant pairs, including those that are matched for HLA-DQ, or (B) excluding pairs that are matched for HLA-DQ. (C) A large overlap between the two groups, apart from those pairs that have 0 eplets mismatches, further demonstrates how inclusion of patients that have no mismatches cause inflation of the DSA-negative population and skew the calculation. Data were further analyzed at the individual donor allele level (D) with and without including the 0 eplet mismatched alleles, (E) raw data showing the large proportion of 0 eplet mismatches. (F) Once calculations were corrected to account for each pair only once, the statistical difference is lowered to $p = 0.409$; (G) raw data demonstrating the high overlap between the number of eplet mismatches in the DSA-positive and DSA-negative groups once immunological irrationality is corrected.

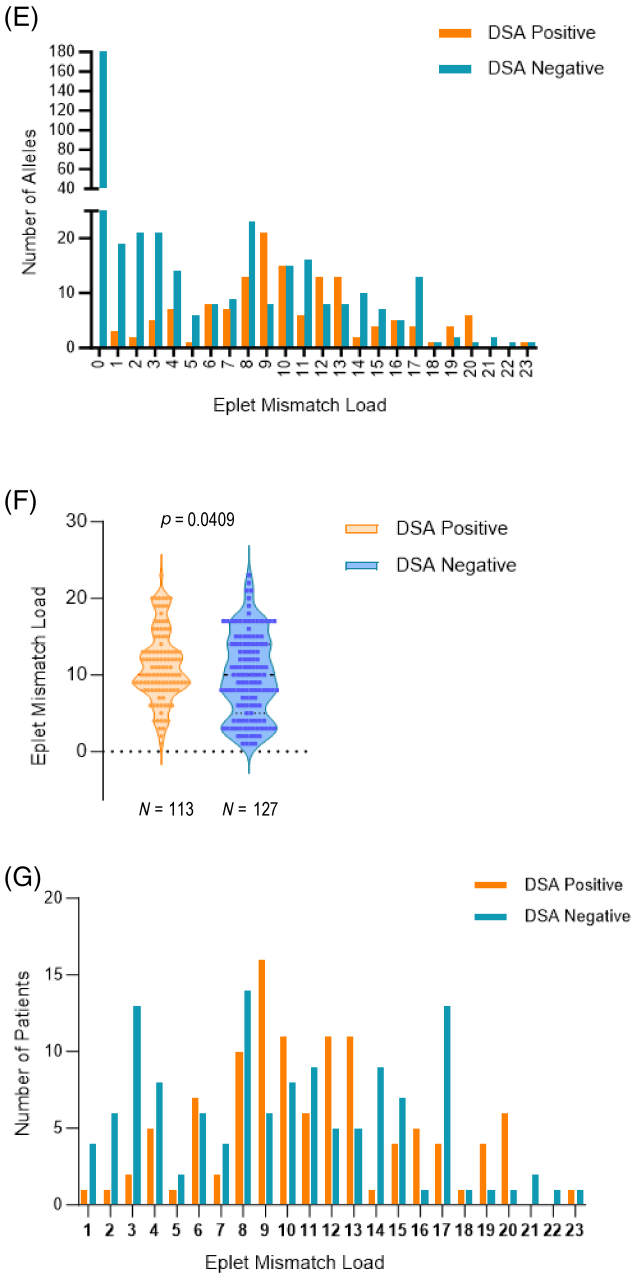
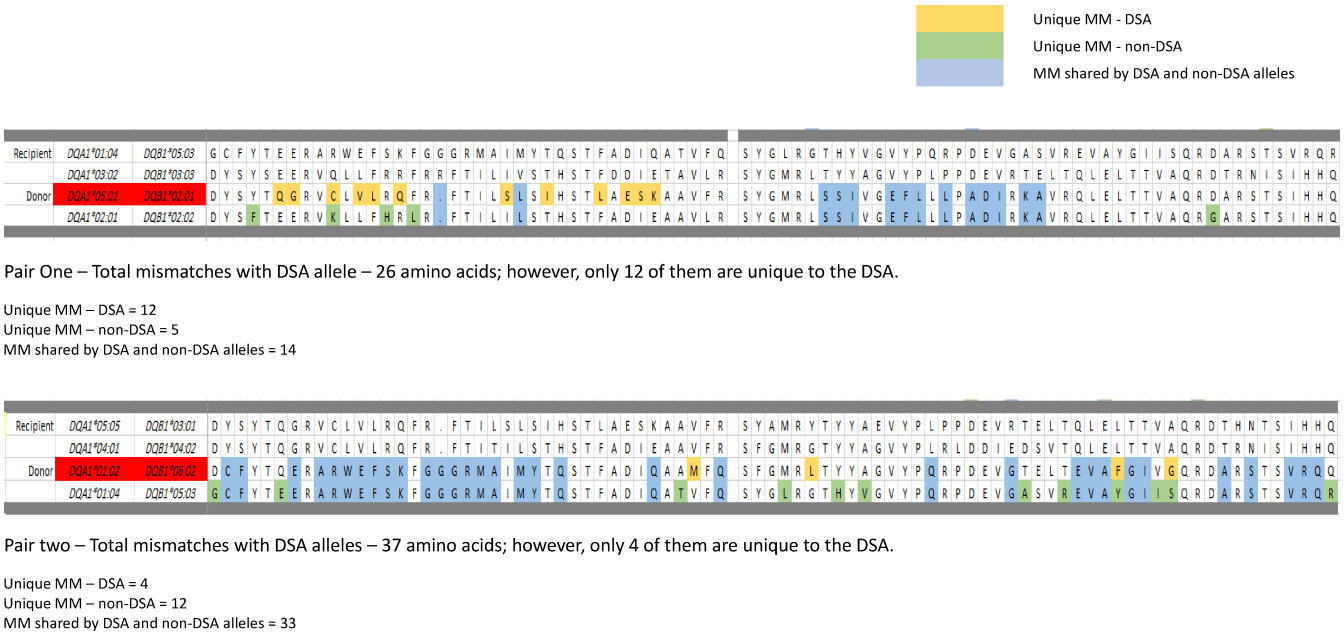


FIGURE 1 (Continued)

only 12 mismatches that are unique to this DSA-inducing allele. In pair 2, there are 37 amino acid mismatches between the recipient and the DSA-inducing allele. 33/37 of them are shared with the non-DSA allele and thus cannot be given a high immunogenicity value, leaving only 4 mismatches that are unique to the DSA-inducing allele. Similar observations apply to many subjects in the 2MM1DSA sub-cohort. This analysis demonstrates the even within the dnDSA-inducing allele, not all mismatches contribute equally to the immunogenicity of this mismatch.

2.3 | Use of CRISPR-Cas9-modified cells and human monoclonal antibodies to gain insight into "functional epitopes"

We further explored the claim that an eplet can be considered a "functional epitope" (loosely used to describe the critical area for antibody recognition). Under this paradigm, the rest of the antibody footprint, the "structural epitope," is ignored. The homozygous cell line SWEIG007 (wild-type *DQA1*05:05/DQB1*03:01*; SWEIG-WT) was mutated in a nonpolymorphic region of the DQB1 gene,



Pair One – Total mismatches with DSA allele – 26 amino acids; however, only 12 of them are unique to the DSA.

Unique MM – DSA = 12
Unique MM – non-DSA = 5
MM shared by DSA and non-DSA alleles = 14

Pair two – Total mismatches with DSA alleles – 37 amino acids; however, only 4 of them are unique to the DSA.

Unique MM – DSA = 4
Unique MM – non-DSA = 12
MM shared by DSA and non-DSA alleles = 33

FIGURE 2 Not all mismatches are created equal. Polymorphic amino acid sequences of two recipient/donor pairs from the 2MM1DSA cohort are presented. Color code for mismatches is shown on the top right corner and helps demonstrate a hierarchy between potentially more immunogenic and less immunogenic mismatches. The non-donor specific antibody (DSA) allele has multiple mismatches that, since they did not lead to generation of de novo DSA (dnDSA), qualify as less immunogenic. These mismatches are highlighted green if unique to the non-DSA allele and blue if present in both the non-DSA allele and the DSA allele. When evaluating the DSA allele, the mismatches that have lower immunogenicity are highlighted blue, as above, and only those mismatches highlighted yellow, those that are unique to the DSA allele, can be qualified as potentially having a higher immunogenicity. Following this rationale, only 12/26 mismatches in pair 1, and only 4 mismatches in pair 2, should be considered as potentially having higher immunogenicity.

position 61–64 (converting amino acid sequence from WNSQ to W–K; location and 3D-change are presented in Figure 3A; SWEIG-Mut). The impact of this structural change was assessed by three commercial pan-HLA-DQ monoclonal antibodies (mAbs). As seen in Figure 3B, the binding of two mAbs (REA303 and 1a3) was minimally affected, whereas the third mAb, clone HLADQ1, completely lost its ability to bind its target (Figure 3C). Consequently, we deduced that the mutated area is required to maintain the epitope recognized by HLADQ1 mAb. However, since the binding of REA303 and 1a3 is only marginally affected by the mutation, the epitopes recognized by these antibodies are likely located at a different area of the HLA-DQ molecule (Figure 3D,E). The SWEIG-WT and the SWEIG-Mut cells can therefore serve to investigate the relative areas recognized by an antibody.

We then tested the binding of a recombinant human mAb (HmAb), generated from an individual who was immunized due to pregnancy, directed at an *HLA-DQA1*05:05/DQB1*03:01* heterodimer (HmAb; LB_DQB0301_A,[31]; amino acid sequences of antibody producer and immunizer and MML are shown in Figure 4A). HLAMatchmaker analysis suggested that glutamic acid "E" at position 45 of the DQβ chain contributes to the 45EV eplet and equates it to the "functional epitope," marked with a blue arrow in the figure. The "functional epitope" as well as the putative footprint of the antibody (the "structural epitope" as depicted by a radius of 15 Å) are illustrated in Figure 4B. The location of the CRISPR/Cas9 mutation on the backbone of the "structural epitope" is depicted in Figure 4C. As seen, the mutation is located at the periphery of the "structural epitope" and is quite remote from the "functional epitope." Adsorption elution experiments using the L_DQB0301 HmAb and either the SWEIG-WT or the SWEIG-Mut demonstrated full adsorption of the HmAb by the SWEIG-WT, but complete abrogation of binding by the mutation (Figure 4D). Thus, while the mutation did not directly affect the "functional epitope," changes to the "structural epitope" significantly impact the ability of this antibody to bind its cognate target. These results demonstrate that the assignment of eplets as "functional epitopes" is not supported by experimental data.

2.4 | Comparing mismatches within 2MM1DSA sub-cohorts

Reasoning that immunogenicity can best be explored using the 2MM1DSA cohort, we turned to survey characteristics that are present only in the DSA, but not in the

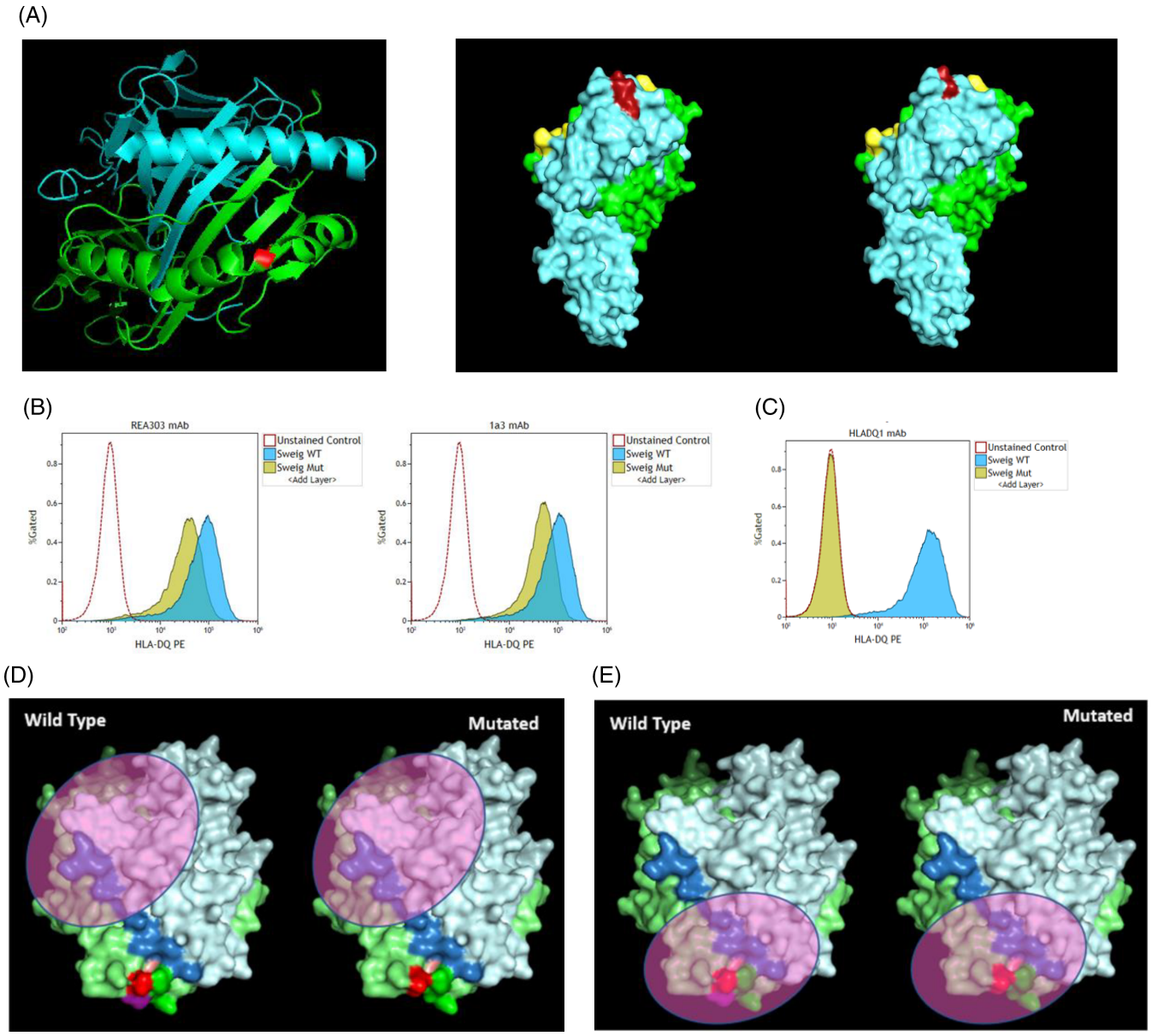


FIGURE 3 Generation of CRISPR-Cas9 modified cells as a tool to "visualize" antibody binding area. (A) Location of CRISPR-Cas9 edits are highlighted red on the background of a HLA-DQ ribbon diagram and on the 3D sphere structure of wild type and mutated cells; generated with PyMol V2.1.4 (B) flow cytometry histograms of antibody binding show minimal impact of mutation (olive color) on the binding of two monoclonal antibodies, REA303 and 1a3, compared with wild type binding (blue color), while (C) a complete abrogation of binding by mAb clone DQ1 is observed. These results demonstrate that the epitope recognized by REA303 and by 1a3 mAbs is different from the epitope recognized by mAb DQ. While the exact footprint of the different epitopes cannot be defined by these experiments, it is likely to deduce that (D) the binding area of REA303 and 1a3 is likely remote from the mutation site, while (E) the biding area of DQ1 requires the intact molecule (depicted by pink ellipsoids).

non-DSA, mismatched alleles. The 2MM1DSA cohort comprised about 30% of the full dnDSA-positive group. We first analyzed differences in amino acid size, charge, location, or polarity of amino acids that may be associated with peptide binding or T Cell Receptor recognition site. None of these approaches yield a marker of increased immunogenicity. Applying lessons learnt from our previous work, we compared the frequency of all mismatches that led to DSA formation (DSA-positive alleles) with all mismatches, from the same donors, that did not lead to DSA formation (DSA-negative alleles; Figure 5A). Comparison was performed using the complete α/β heterodimer. As control population, we used frequencies of DQα/β heterodimers representing effective donors used over the past 3 years in the United States/ Organ Procurement & Transplantation Network (OPTN)

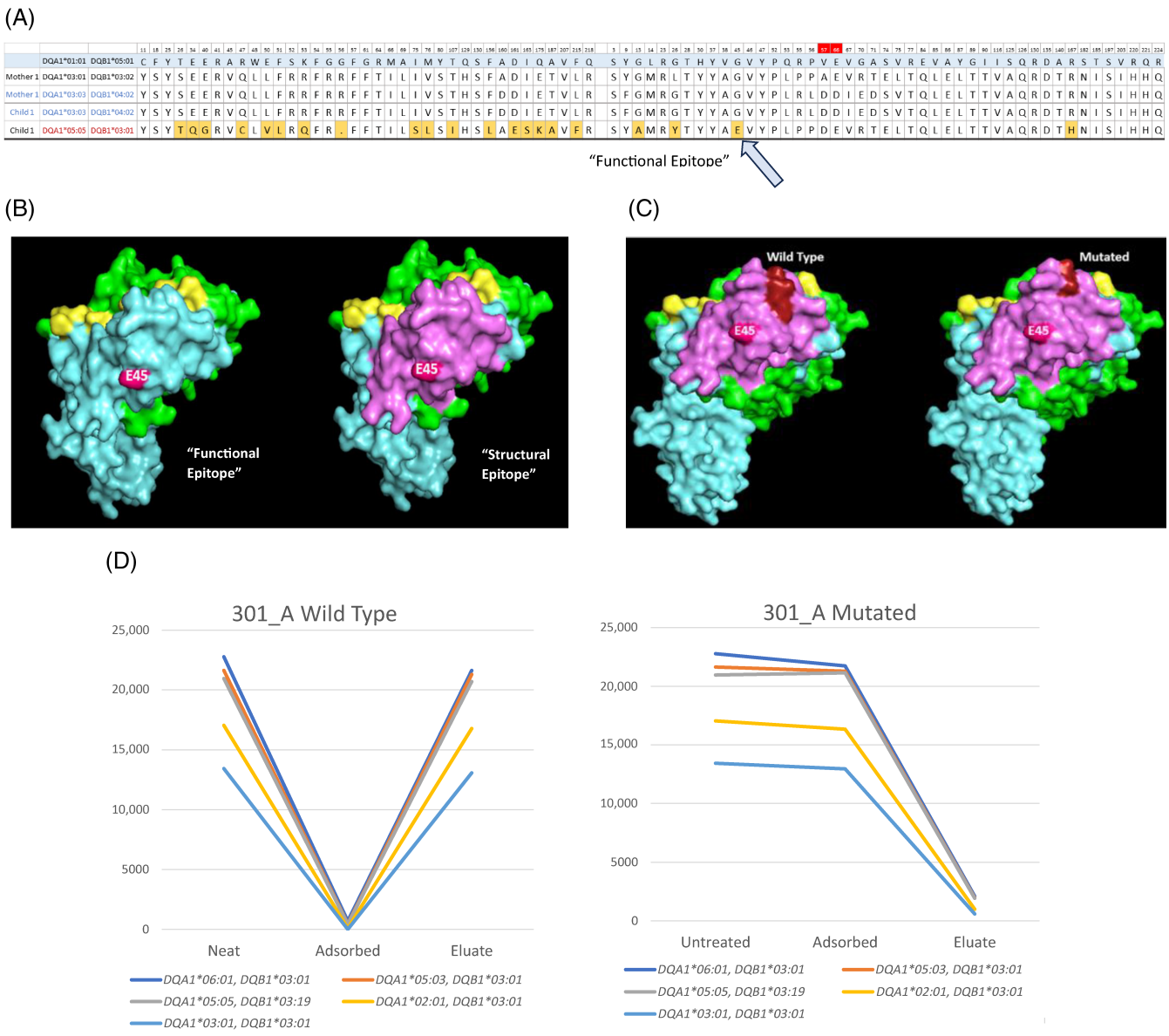


FIGURE 4 An eplet is not a "function epitope" and is not the only critical structure of the molecule that affects antibody binding. The tools demonstrated in Figure 3 were used to interrogate binding of a human monoclonal HLA-DQ antibody. (A) Polymorphic amino acid sequences of antibody producer and immunizer are shown. Yellow highlights represent mismatched amino acids ($n = 21$), red highlight indicates area affected by the mutation; HLAMatchmaker analysis identified 45EV as the eplet "leading" to the immune response or as the "functional epitope." The arrow points at position 45 in the DQβ chain where the mismatched amino acid is "E" (glutamic acid). (B) The identified "functional epitope" (eplet—designated as E45) is depicted in red on the background of the wild type SWIEG cells. The projected footprint of the complete epitope, referred to as the "structural epitope", is depicted in pink, generated by PyMol V2.4.1 (C) overlay of the area affected by the CRISPR-Cas9 editing on the backdrop of the "structural epitope" on wild type and mutated SWIEG cells. (D) Human monoclonal antibody LB_DQB0301_A was testing by adsorption/elution. The presence of HLA antibodies was measured by Mean Fluorescence Intensity (MFI) in neat sample as well as after adsorption, and in the eluate, using either the wild type or mutated cells as target for antibody binding. The mutation abrogated the ability of the antibody to bind its target.

(using heterodimer information associated with DR/DQ linkages from a recent study performed and shared under Data Use Agreement with the NMDP and the CIBMTR, adjusted to effective kidney donors' ethnic frequencies obtained from the OPTN). The most prominent observation, as seen in Figure 5A, is that in about half of the 2MM1DSA pairs (18/34), the mismatch that led to DSA formation carried DQα05 chain as part of the HLA-DQ heterodimer, henceforth referred to as DQα05-heterodimers (orange and red circles). Specifically, the frequencies of *DQA1*05/DQB1*02* and *DQA1*05/DQB1*03* among DSA-positive alleles compared with the DSA-negative alleles and

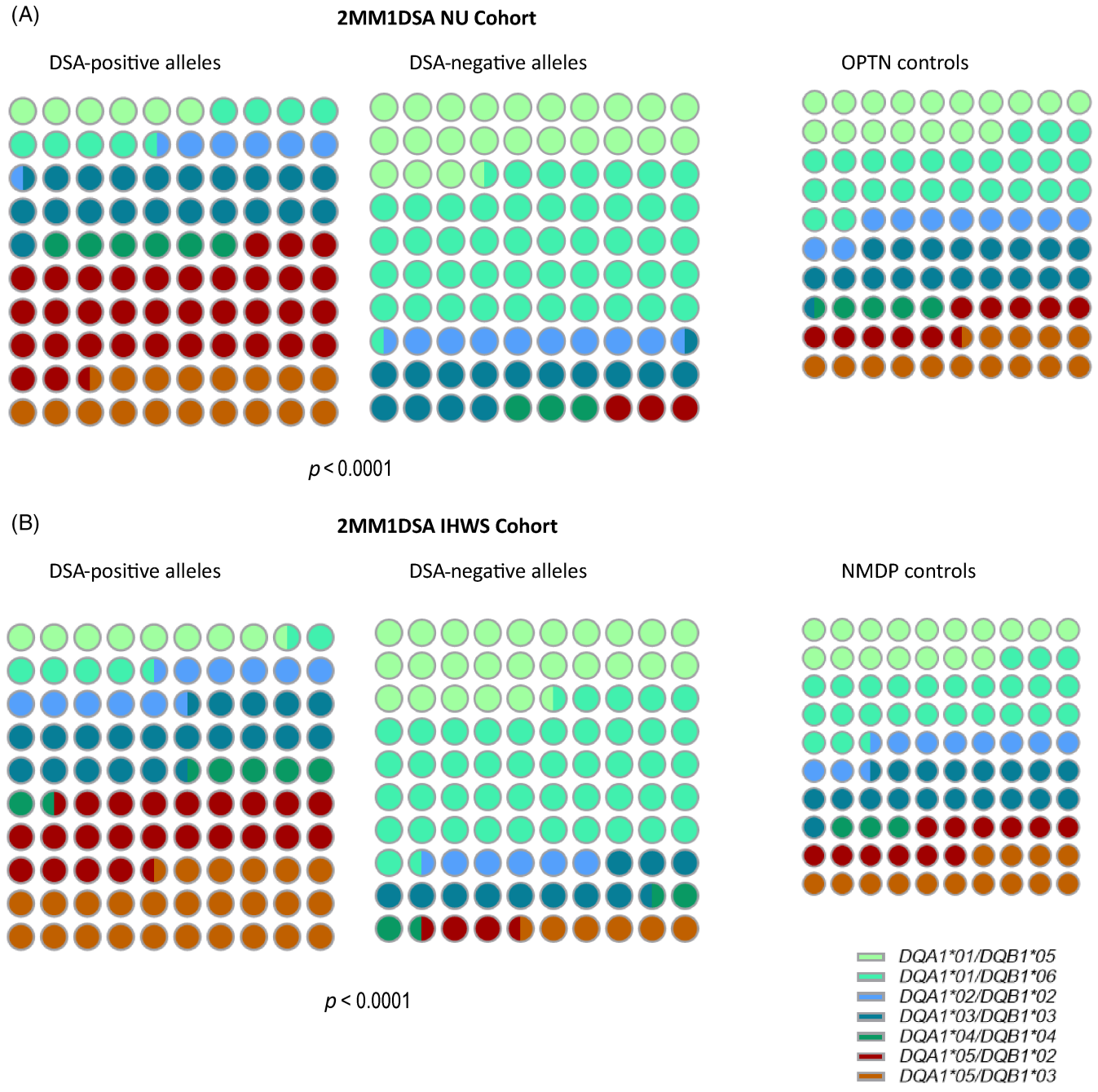


FIGURE 5 Frequency of de novo donor specific antibody (dnDSA)-positive versus dnDSA-negative donor alleles within 2MM1DSA cohorts. Donor HLA-DQ alleles were grouped based on their paired DQα and DQβ chain heterodimers, as shown by the legend, on the bottom right corner. The frequency (%) of each DQαβ heterodimer group within the DSA-positive alleles was compared with that of their DSA-negative alleles counterparts, and with the frequency of these alleles within a relevant, ethnically similar, control group for (A) the Northwestern University (NU) cohort and (B) the International Histocompatibility Workshop (IHWS) cohort. These results demonstrate a significantly increased frequency of DQα05-heterodimers among the DSA inducing mismatched alleles.

the control population were 35.3% and 17.6% versus 2.9% and 0% versus 10.3% and 14.3%, respectively, $p < 0.001$. Correspondingly, the frequency of all non-DQα05-heterodimers including DQ5, DQ6, and a subset of DQ2 = *DQA1*02:01/DQB1*02:02* alleles was reduced (5.9%, 8.8%, and 5.9% vs. 23.5%, 47%, and 8.8% vs. 17%, 24.7%, and 10.2%, respectively, $p < 0.0001$). Within the NU 2MM1DSA cohort there was only one pair in which a mismatched DQα05-heterodimer was not the DSA allele. This one outlier pair involved a recipient that could, by trans-dimerization, form the same DQ heterodimer expressed by the donor (i.e., paring an α chain from one of the self-DQ

molecule with a β chain from the other self-DQ molecule). In this case, the patient was typed as *DQA1*05:05/DQB1*03:01* and *DQA1*02:01/DQB1*02:02*, thus can potentially form a DQA/B trans-dimer that is identical to the mismatched, non-DSA, donor allele (*DQA1*05P/DQB1*02*).

To validate our data, we used a second cohort, collected as part of the HLA-DQ epitope component of the 18th International Histocompatibility Workshop (IHWS). The full IHWS DQ-antibody cohort included 460 recipient/donor pairs of which 154 developed HLA-DQ-specific dnDSA. Of note, this cohort includes many recipient/donor pairs that are part of the Eurotransplant collaboration, where HLA matching receives a higher value compared with the UNOS system, and thus, there is a lower proportion of patients who received organs from donors with 2 HLA-DQ mismatches (67/154 = 43.5% for IHWS cohort vs. 66/113 = 58.4% for NU cohort). Among the dnDSA cohort, 35/154 (22.7%) exhibit 2MM1DSA. Of those, 20 donors carried a DQα05-heterodimer, and in 17/20 pairs, (85%) this was the target for dnDSA. Figure 5B illustrates the frequencies observed for the DSA-positive alleles compared with the DSA-negative alleles of the IHWC 2MM1DSA cohort, as well as comparing them to ethnically matched controls obtained from the NMDP. As above, the frequency of DQα05-heterodimer/DSA-inducing alleles is statistically higher compared with the frequency of these alleles among the control population, $p < 0.0001$ (specifically skewed toward both *DQA1*05/DQB1*02* and *DQA1*05/DQB1*03*; 22.9% and 25.7% vs. 2.8% and 5.7% vs.11.7% and 14.1%, respectively). Of the 3/20 pairs who had a mismatched DQα05-heterodimer that was not the target of DSA formation, the recipient in two pairs had the ability to form a trans-dimer equivalent to the donor mismatched, non-DSA, allele (recipients typed as *DQA1*03:01/DQB1*03:01* and *DQA1*05:01/DQB1*02:01*, and donors typed as *DQA1*05P/DQB1*03:01*). In the last pair, the patient was typed as *DQA1*05:05/DQB1*03:01* homozygote, whereas the donor typed as *DQA1*05:01/DQB1*02:01*.

Evaluating the complete dnDSA cohorts, the overall frequency of donors carrying a DQα05-heterodimer was quite comparable among the two cohorts, with 41.5% for the full NU cohort and 45.2% for the full IHWS cohort. The frequency of donors carrying a mismatched DQα05 allele among the dnDSA-positive cohorts was higher than in the respective dnDSA-negative cohorts. Specifically, a ratio of 3:1 was observed for the NU cohort (48.7% vs. 16.5%; $p < 0.0001$, two-sided Fisher's exact test) and about 2:1 for the IHWS cohort (42.3% vs. 22.9%; $p < 0.0001$), Table 1. Importantly, in both cohorts, almost all recipients mismatched for DQα05-developed dnDSA to this mismatch (98.2% in NU cohort and 95.4% in IHWS cohort).

Lastly, to explore whether our observations can be explained by random events, we tested a model where each mismatch adds a probability q to develop dnDSA.[32] As shown in Figure S3A, the probability of random events is ruled out (q is 0.26 [26%]). We further compared the expected and observed number of patients that developed dnDSA as a function of the donor's-specific HLA-DQ mismatch typing (broad families as presented in Figure 5). Our results, shown in Figure S3B, demonstrate a low fit probability, with significant difference compared with the control population ($\chi^2 = 36.5349$ and 79.1330 for NU and IHWS groups, respectively, $p < 0.0001$) thus again ruling out a possibility of a random effect.

2.5 | Functional and evolutionary divergence of HLA-DQ heterodimers

The intriguing increased frequency of DQα05-heterodimers among mismatches that led to DSA formation resonated with our clinical experience in which these DQ alleles are the most frequently affected by

TABLE 1 Frequency of DQα05 heterodimers among dnDSA-positive alleles.

Cohort	Frequency of donors carrying MISMATCHED DQα05 heterodimer, N (%)	Frequency DQα05 heterodimers among dnDSA-positive alleles, N (%)	Frequency of DQα05 heterodimers in control population, N (%)
			UNOS
NU—dnDSA pos	55/113 (48.7%)	**54/55 (98.2%)***	24.8%
NU—dnDSA neg	21/127 (16.5%)	N/A	24.8%
			NMDP-Caucasians
IHWS—dnDSA pos	65/154 (42.3%)	**62/65 (95.4%)***	25.8%
IHWS—dnDSA neg	40/182 (22%)	N/A	25.8%

Abbreviations: dnDSA, de novo donor specific antibody; IHWS, International Histocompatibility Workshop; NMDP, National Marrow Donor Program; NU, Northwestern University.

*Yellow shding and bold indicate $p < 0.0001$.

prozone/inhibition and are exhibiting very high titers. There are two common DQα05-heterodimers worldwide, each belonging to a different serologic antigen family—DQ2 and DQ7. Each of these serologic families has an additional common allele, with a different DQα chain. Specifically, the two alleles of DQ2 are *DQA1*02:01/DQB1*02:02* and *DQA1*05:01/DQB1*02:01*, and the two alleles of DQ7 are *DQA1*03:01/DQB1*03:01* and *DQA1*05:01/DQB1*03:01*. We measured the impact of the different DQα chains on the overall physicochemical properties of these molecules by calculating the EMS-3D scores. Figure 6 demonstrates a rather high score between the two DQ2 alleles and the two DQ7 alleles, 0.341 and 0.346 respectively (EMS-3D scale ranges between 0 and 0.65), while the eplet mismatch load is only 2. Conversely, the EMS-3D between the two DQα05-heterodimers (shown in boxed data), despite being part of different serologic families, is lower: 0.257, although the eplet mismatch load is higher (12 mismatched eplets). This information suggests that the α chain is quite prominent in determining the electrostatic value of an HLA-DQ molecule.

We further evaluated the distances between all common* HLA-DQ heterodimers based on their amino acid sequences (genetic evolutionary distances, calculated using MEGA software and the PAM250 distance metric) and compared it with distances based on these molecule's EMS3D scores, as shown in Figure 7A,B, respectively. For the genetic distance (Figure 7A), one clear cluster of relatively low genetic distance is shown on the bottom left, including all heterodimers using the DQα01 chain with either DQβ05 or DQβ06 chains (distance values 0–0.05; serologic terminology of DQ5 or DQ6, previously known as DQ1). We will refer to this group as the DQα01-heterodimers alleles. The rest of the DQ alleles, the non-DQα01-heterodimes group, also demonstrate relatively low distance values between 0 and 0.05 and are clustered in the top right portion of the heatmap. The distance between the main two groups, DQα01-heterodimers and the non-DQα01-heterodimers, is much larger, correlating with the main branching of this phylogenetic tree (based on a ML-tree algorithm).

Evaluating divergence between different HLA-DQ heterodimers based on their physicochemical/electrostatic qualities (EMS3D scores; Figure 7B) confirms the clustering of all DQα01-heterodimers as one group, lower left corner of the heatmap, purple branch, as well as the higher

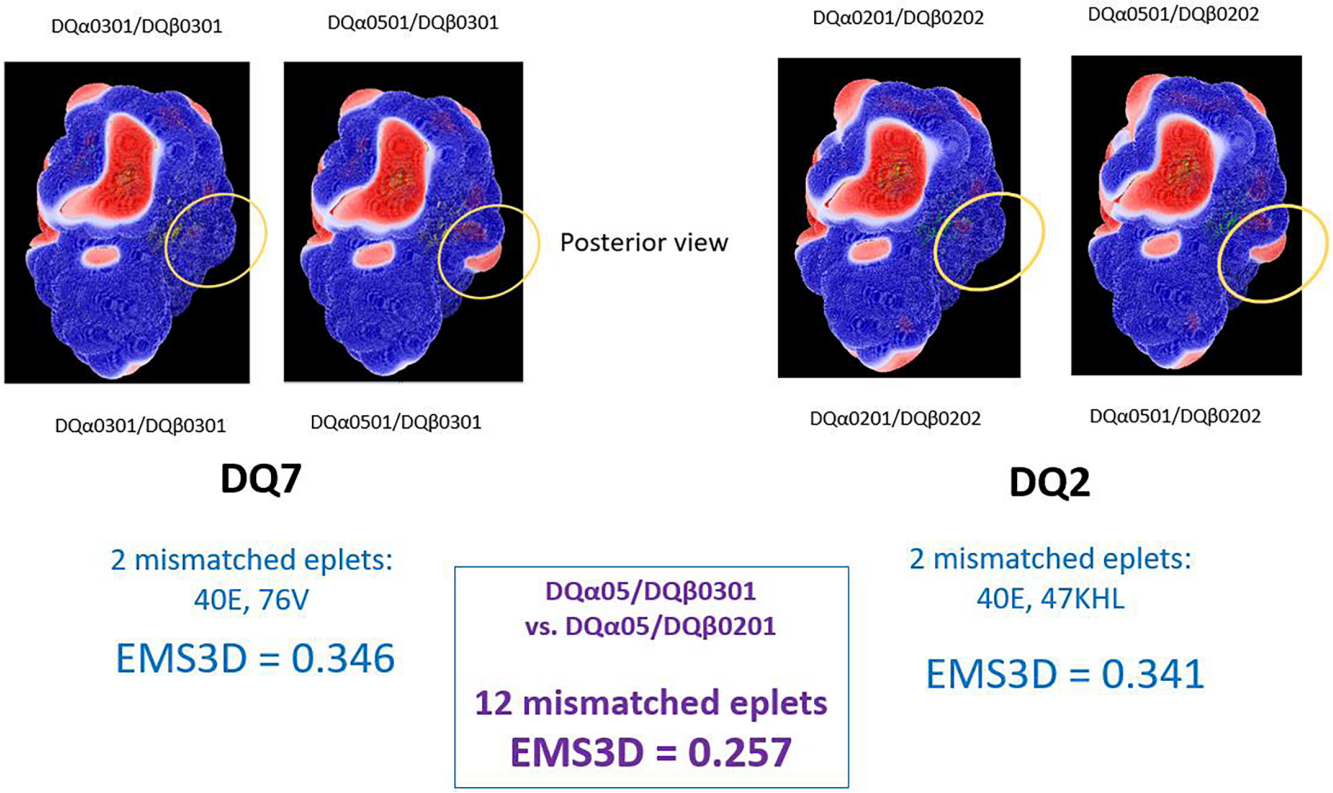


FIGURE 6 Contribution of the alpha chain to divergence of HLA-DQ heterodimers. Physicochemical properties of the two main alleles of alleles of DQ7 and DQ2 are presented as posterior and peptide views. While the eplet mismatch load between the alleles within the same serologic group is low,[2] the EMS3D score is relatively high (0.346 and 0.341, respectively). Conversely, while the EMS3D score between the two DQα05 heterodimer is lower (0.257), the number of eplet mismatch load is much higher (12 eplets). Structures generated using the program MODELLER v9.17 (https://salilab.org/modeller/).

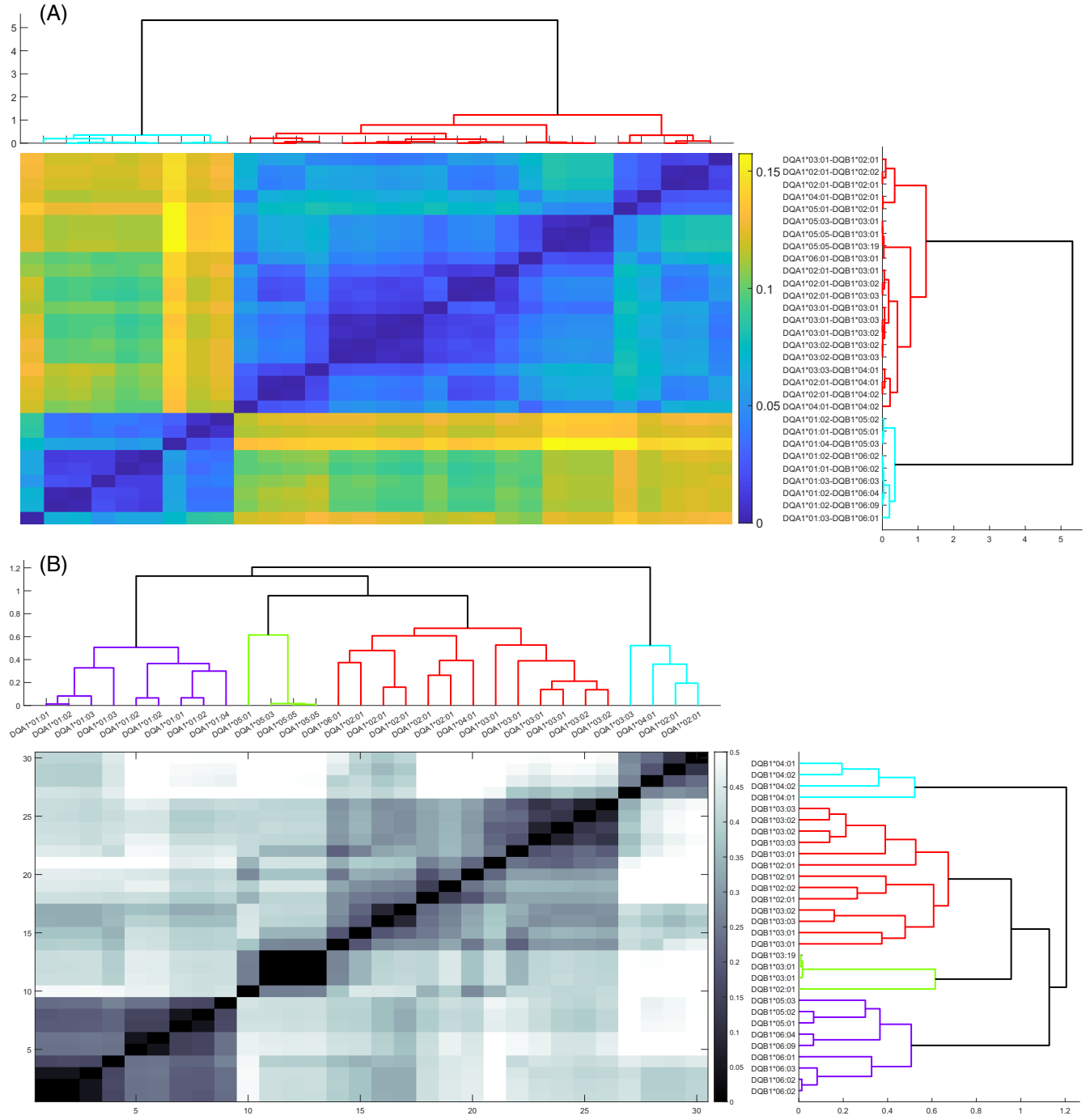


FIGURE 7 Quantitative (genetic/evolutionary) and qualitative (functional?) divergence of HLA-DQ heterodimers. Heatmap analysis of (A) genetic/evolutionary divergence between the more common HLA-DQαβ heterodimers demonstrates a prominent brunch separating all heterodimers into two main groups of lower genetic distance (scale shown to the right of the heatmap with blue colors showing lower distance). The light-blue branch includes all the DQα01-positive alleles, lower left portion, and the red branch includes all other DQ heterodimers. Note that DQα05-heterodimers are not separated as a unique branch. (B) Physicochemical properties of alleles (based on EMD3D values). Four separate branches are noted. A unique branch separates all alleles of the DQ4 family (top group, light blue). The lowest branch (purple) includes all DQα01, as above. The last two branches separate all heterodimers including DQα05 (green branch) from the rest of the other alleles (red branch).

EMS3D scores between this group and the rest of the HLA-DQ heterodimers. In difference from the genetic/evolutionary tree, this heatmap analysis indicates a first branching of all alleles containing the *DQβ*04* alleles (light-blue branch). Importantly, additional grouping demonstrates distinct branching between all DQα05-heterodimers

(green branch) and an additional group that includes the rest of the DQ heterodimers (red branch). Of note, the highest EMS3D values are observed between members of the non-DQα01-heterodimers group: *DQA1*02:01/DQB1*04:01* and *DQA1*05:01/DQB1*02:01* (EMS3D = 0.665) and *DQA1*02:01/DQB1*04:02* and *DQA1*05:01/DQB1*02:01* (EMS3D = 0.645), indicating high functional distance between DQα05-heterodimers and other alleles of the non-DQα01-heterodimers "evolutionary" group.

Hypothesizing that HLA-DQ immunogenicity correlates with the degree of genetic and functional distances as shown above, we reevaluated our HLA-DQ dnDSA cohorts. Patients' and donors' alleles were assigned into the two main Evolutionary Groups based on the presence of DQα01-heterodimers (i.e., serologic family of DQ5 and DQ6), or its absence (serologic families of DQ2, DQ3, DQ4; non-DQα01-heterodimers). We then evaluated the pairs for the presence of a DQ-α05-heterodimer mismatch and for transplantation across Evolutionary Group mismatched. Specific for the NU-2MM1DSA cohort, 79% (27/34) of pairs fulfill one of the two criteria: (i) in 18/34 pairs, the mismatched allele that induced generation of dnDSA was a DQα05-heterodimer, while the non-DSA allele was not; (ii) in 9/34 additional pairs, the recipient was homozygous for one of the Evolutionary Groups, and the donor was heterozygous (carry alleles belonging to both Evolutionary Groups); the allele that induced dnDSA formation belong to the opposite Evolutionary Group while the non-DSA inducing allele was part of the recipient's Evolutionary Group. For the remaining 7/34 pairs, since the recipients themselves had alleles belonging to both Evolutionary Groups, this type of analysis could not be performed (Figure 8A). Similar data were observed for the IHWS-2MM1DSA cohort with overall 77% (27/35) following one of the two rules as above (Figure 8B). Analysis of the full NU cohort further

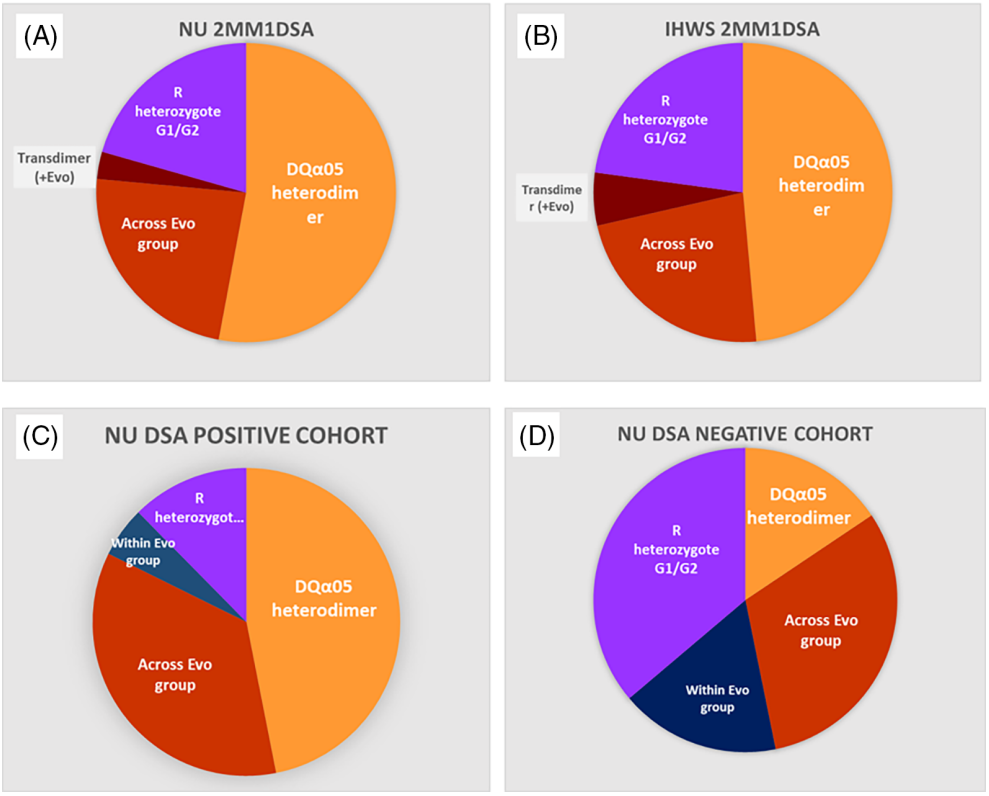


FIGURE 8 Functional and evolutionary divergence impact generation of de-novo HLA-DQ donor specific antibodies (DSAs). Immunogenic mismatches were identified as being either DQα05-heterodimers or across an evolutionary group barrier. Pie charts represent frequency of HLA-DQ mismatches belonging to either of these group within 2MM1DSA cohorts (A) specific for the Northwestern University (NU) cohort and (B) specific for the International Histocompatibility Workshop (IHWS) cohort. Frequency of HLA-DQ mismatches within the complete DSA-positive group of the NU cohort (C) and (D) specific for the DSA-negative group of the NU cohort. Recipient/donor pairs that exhibit mismatches across a DQα05-heterodimers mismatches and colored yellow; recipient/donor pairs that exhibit mismatches across an "evolutionary divergence barrier" are colored red; recipients that carry one DQ allele, that is, part of one "evolutionary group" and the other allele is part of the other group—called here heterozygote for the evolutionary groups are colored purple. Recipients that have the potential ability to generate trans-dimers that then be identical to the otherwise mismatched donor DQ molecules are colored burgundy (relevant only for insets A and B); pairs in whom both the recipient and the donor have their DQ alleles within the same evolutionary group are colored dark blue.

demonstrated enrichment of the dnDSA-positive group with patients receiving a mismatched organ positive for a DQα05-heterodimer, or patients that were homozygotes for one Evolutionary Group receiving an organ carrying at least one allele from the opposite Evolutionary Group. Conversely, among DSA-negative patients, more patients were heterozygous for the Evolutionary Groups or received an organ within their Evolutionary Group (Figure 8C,D).

Finally, we ventured to study an additional large multicenter cohort. The data stored by the US Scientific Registry of Transplant Recipients (SRTR) has an obvious appeal given the sheer magnitude of data collected. However, there are known limitations regarding the granularity of the data from the SRTR. Specifically, HLA typing is available only at a low resolution. Information on dnDSA or granular ABMR data is lacking, and one must resort to analyzing graft loss, which can be due to multiple factors. Despite these limitations, we reasoned that a "mate kidney" sub-cohort should be able to provide insight at least to the genetic/evolutionary aspect of DQ divergence. "Mate kidney" cohorts include cases where kidneys from one donor were transplanted into two different recipients, effectively controlling for multiple donor-specific variables. We hypothesized that at least when donor and recipient both belong to the DQα01-heterodimer Evolutionary Group (due to the high homology between its members) we should be able to detect the effect of compatibility. Given the low-resolution HLA typing, this cohort is not amenable to testing the immunogenicity of the mismatched DQα05-heterodimers, and thus using the non-DQα01-heterodimer Evolutionary Group would not provide useful information. Data from 51,406 recipients were analyzed, corresponding to 25,703 kidney transplant pairs. Donors and recipients were characterized based on their HLA-DQ Evolutionary Group assignment. Indeed, when the donor was homozygous for DQα01-heterodimers, recipients who had both DQ alleles within the same Evolutionary Group showed less risk for (death censored) graft loss, independent of the level of HLA-DR mismatch and other clinical-demographic variables, compared with mate recipients that were either heterozygous for their DQ Evolutionary Group or were homozygous for the non-DQα01-heterodimers ($n = 2922$ univariate Cox regression HR 0.737, p-value <0.05; multivariate HR 0.791, $p < 0.1$; Figure S4).

3 | DISCUSSION

The ever-increasing need for organ transplantation as a lifesaving therapy, the shortage of available donors, and, the significant side effects associated with the use of immunosuppressive medications require continuous search for approaches to prolong the lifespan of transplanted organs.[33] While multiple factors can increase the risk for transplant rejection, a major driver of immune responses is the degree of histoincompatibility between the patient and their donor. In recent years, MML approaches were proposed to improve recipient-donor matching,[11,15,18] leading to discussions of changing allocation algorithms to incorporate these tools. Further, HLAMatchmaker specifically claims to define the epitope recognized by HLA antibodies, reducing it to a much smaller structure—an eplet (and referring to it as a "functional epitope"). We have previously highlighted multiple theoretical flaws in current approaches.[9] Herein we investigated the use of HLAMatchmaker in our own cohort, demonstrated debatable decisions made in previous study design/determining appropriate control population, experimentally disputing the belief that epitope definition can be reduced to considering eplets, and pointing to overall flaws in using simple mismatch enumeration approaches (MML) to determine immunogenicity. We then built on unique observations from the 2MM1DSA cohort, proposing a mechanistic hypothesis to explain immunogenicity.

In designing studies, consecutive enrollment is emphasized to minimize sampling bias. However, the control population should be determined based on the question asked. Specifically, when studying the effect of HLA mismatch on the likelihood to develop dnDSA, recipient-donor pairs who are HLA identical should be excluded as, immunologically speaking, they are unable to form such antibodies. Most published studies, though, include transplant pairs that are HLA identical for the analyzed locus, suggesting suboptimal decision-making as part of bounded rationality.[34] As we demonstrate here, excluding recipient-donor pairs who do not have at least one mismatched HLA-DQ allele diminished the statistical difference (from $p < 0.0001$ to $p = 0.03$; Figure 1A,B) highlighting the large overlap between DSA-positive and-negative patients' groups, when observing the raw eplet mismatch data (Figure 1C). Among patients with eplet mismatch load below the calculated threshold, 17/113 (15%) developed dnDSA, and using a previously reported threshold (11 eplets,[20]), that number would have increased to 38% (43/113).

Addressing the limitation of the "eplet universe"[9,20] approach, we have calculated eplet mismatch load per each of the mismatched donor alleles, separately. In 180/540 cases, we saw a donor allele that matches the recipient (zero eplet mismatch load; Figure 1D,E), but in many others the donor contributed two different metrics associated with their two mismatches. This means that some pairs were counted twice and thus inflating their

role in the cohort. Once correcting for this statistical problem by using only the highest mismatch load allele, the significance diminished again (Figure 1F,G; $p = 0.04$). The threshold calculated using the "single donor allele" approach was five eplets, yet, even within this low threshold group 21.4% (9/42) of patients developed dnDSA. This number would have been higher using previously reported thresholds. Overall, our results reinforce the notion that it is not the number of mismatched eplets that determines whether a recipient will form dnDSA. Moreover, it highlights the risk for patients' miscategorization as exhibiting low risk for the purpose of immunosuppression minimization or worse, for the purpose of organ allocation. Statistical limitations notwithstanding, other investigators discussed the limitation of previously reported rigid thresholds.[22,35]

A different approach to scrutinize the validity of enumeration approaches as predictors of immunogenicity is the use of the 2MM1DSA cohort. We reasoned that when analyzing immunogenicity in a population, multiple patient-specific factors (e.g., trough levels of immunosuppression, competing immune events such as infection, etc.) may influence the observed transplant outcome, which may hinder analysis of HLA compatibility. We have previously reported on a unique cohort in which, within a single patient, differences in immunogenicity can be demonstrated.[28] We further chose to conduct our studies analyzing amino acid sequences directly, rather than using either of the MML software algorithms, to minimize potential bias. Briefly, and as demonstrated in Figure 2, the 2MM1DSA cohort can classify AA mismatches as associated with higher or lower immunogenicity for each patient and each individual donor allele. Using this approach, we clearly demonstrated that not all AA mismatches, even within a DSA-inducing allele, carry the same immunological significance. These results further refute the claim that increased MML informs on increased immunogenicity, and contest the rationale behind MML enumeration approaches, in which every mismatch is assigned the same immunogenicity value. To the best of our knowledge, this is the first time where irrefutable evidence demonstrated that some AA mismatches, even within the DSA allele, have lower immunogenetic value (those that are shared with the non-DSA allele).

Beyond MML analysis, HLAMatchmaker was purported to identify the antibody's "functional epitope" by evaluating antibody recognition patterns from patients' sera and identifying eplets shared by reactive alleles. This terminology was borrowed from the field of vaccine development (and others), but in this case, in a leap of faith, was applied to simply describe an eplet. The semifamiliarity with the "epitope" terminology led to embracing this phrase without experimental evidence to support its euphemistic use. To investigate the validity of the "functional epitopes" statement in the context of HLA antibodies, we used a human mAb, developed by Heidt and colleagues,[36] recognizing an allele of HLA-DQ7 (*DQA1*05:05/DQB1*03:01*). Per HLAMatchmaker, the "functional epitope," or the critical area relevant for antibody binding, was determined to be glutamic acid ("E") at position 45 of DQβ chain (Figure 4A). However, using CRISPR-Cas9-mutated cells, we demonstrated that a small mutation in the "structural epitope," remote from the "functional epitope," was sufficient to hinder binding of this antibody. The STAR workgroup, in all its iterations,[7,8,37] cautioned against the misuse of the term "epitope" instead of "eplet." Our results provide experimental evidence refuting the notion that only the "functional epitope" (or the "molecular mismatch") is critical for antibody recognition. It further confirms that an eplet should not be considered, in isolation, an EPITOPE. Further evidence was recently published by Killian et al. in a highly innovative study, providing a framework for understanding the structural and molecular basis of B-cell epitope/antibodies.[38]

The advantage of the 2MM1DSA cohort is in its ability to compare mismatches that are associated with DSA formation and those that did not lead to DSA formation, within a specific patient. It thus represents a cohort in whom the immune system was sufficiently activated to form dnDSA to one of the mismatches, yet the other mismatched allele evaded this response. In both cohorts studied herein, the frequency of DQα05-heterodimers was significantly higher compared with frequencies in control population or within the non-DSA alleles of the 2MM1DSA cohorts. This observation was further supported by the significantly higher frequency of DQα05-heterodimer mismatches leading to dnDSA in the full DSA-positive groups (98.2% and 95.4% for the NU and IHWS cohorts, respectively). These observations resonated with our clinical experience in which DQα05-heterodimers are not only a frequent occurrence when monitoring for DSAs posttransplantation but are also often the ones exhibiting the highest inhibition (prozone phenomenon) and demonstrating highest titers (up to 1:65,536[39]). Increased frequency of DQα05-heterodimers was observed also in studies of heart and lung transplant recipients[40,41] as well as in pediatric kidney transplant recipients.[42]

Importantly, our observation emphasizes the contribution of the DQα to the full heterodimer, as molecules that have the same DQβ chain (and are considered serologically equivalent) but different DQα chain have quite different EMS-3D scores (Figure 6). The significant difference between two alleles of a serologic family (DQ2 and DQ7), on its own, has important implications when

discussing organ allocation algorithms and when attempting to dissect the role of HLA-DQ mismatching from SRTR, or from other larger data repositories that include only serologic DQ typing. Given the information provided here, the data hosted in these repositories are clearly insufficient to accurately analyze the role of HLA-DQ incompatibility in organ transplantation.

An interesting insight gained from the 2MM1DSA cohort addresses the concept of forming HLA-DQ heterodimers not only by cis-dimerization (where both the A and B genes are encoded on the same chromosome) but also by trans-dimerization (where the α-chain, encoded by an A gene on one chromosome, pairs with on a β-chain encoded by a B gene on the other chromosome). The model of DQα/β trans-dimers has been previously explored,[43] and in fact is reported to confer risk in some autoimmune diseases.[44] Our study demonstrates that patients with the ability to form a trans-dimer that resembles the donor mismatched DQα05-heterodimer avoided the risk conferred by that mismatch and did not develop dnDSA.

Kwok et al demonstrated several decades ago that only particular DQα/β pairings can form physiologically stable molecules.[45–47] Thus, HLA-DQ heterodimers can be divided into two major groups: the DQα01-positive heterodimers, that include the serologic HLA-DQ1 alleles (split into DQ5, 6), and the non-DQα01-heterodimers, that include the serologic HLA-DQ2, DQ3 (split into 7, 8, 9), and DQ4.[45] The dichotomy between the two groups based on the presence of absence of the DQα01 chain is clearly demarcated in a heatmap comparing genetic/AA sequences for multiple DQα/β heterodimer combinations (Figure 7). Raymond et al.[48] recently calculated an exceptionally high divergence between the DQB1-DQA1-DRB1 extended haplotypes of the two groups, probably due to a long history of independent haplotype evolution. This dramatic pattern of haplotype divergence likely extends back ∼40 million years, effectively separating the DQα01-heterodimers from the other DQα heterodimers. The functional implications of this large evolutionary divergence likely have immune consequences in the context of transplantation. In fact, Petersdorf et al[49] recently reported that the presence of the DQα01-hetreodimer haplotype was associated with higher rates of relapse and decreased disease-free survival in patients with malignant diseases requiring hematopoietic stem cell transplantation. This data suggests that genetic divergence has a significant role in immune responses.

Analysis of divergence based on the physicochemical properties of HLA-DQ molecules confirmed the branching of DQα01-hetreodimers, but importantly demonstrated a unique branching of the DQα05-hetreodimers, despite being members of two serologically distinct families (DQ2 and DQ7). These DQα05-hetreodimers further showed the highest EMS-3D values when compared with members of their own Evolutionary Group, supporting our conclusion from the 2MM1DSA cohort regarding their increased immunogenicity.

For kidney transplant recipients, the ability to explore a role for evolutionarily and functionally divergent populations is complicated by the fact that many donors and recipients are mismatched at multiple loci. Nonetheless, the unique insight gained by using the 2MM1DSA cohorts provides compelling evidence for hierarchy in immunogenicity, as illustrated in Figure 8. While the data from the SRTR mate kidney transplant cohort showed only relatively weak correlation, we were encouraged to see these relationships even with the significant limitations in granularity of the data (low resolution HLA typing, no data on generation of dnDSA or ABMR, relatively low numbers of transplant pairs). It is yet to be proven, but we anticipate that studying well annotated cohorts, with all relevant information, will provide the necessary support to consider implementation of immunology-based algorithms to risk stratify solid organ transplant recipients. Of note, a role for functional immunological distance between recipient and donor HLA-DPB1 alleles, at the high resolution, is now being used in determining nonpermissive mismatches in hematopoietic stem cell transplantation,[50] providing strategy for risk stratification for these patients.

We acknowledge that the number of amino acid (or eplet) differences between DQ alleles across the two major Evolutionary Groups is rather large, as shown in Figure S5. This explains the reported association between increased mismatch load and poor transplant outcome (despite all the limitations that were identified herein). However, this correlation applies only to a small portion of the transplant population. Specifically, if the donor and recipient are within the same Evolutionary Group, or if they are heterozygous for the Evolutionary Group, MML approaches do not correlate with the outcome, as seen in the cohorts studied herein (detailed information in Figure S5).

We caution the readership to view this report only as a first step toward deciphering HLA-DQ immunogenicity. Our observations must be validated in additional cohorts, with broad coverage of HLA-DQ alleles across multiple ethnic groups, prior to consideration in any clinical decision-making process. We must further develop a better understanding of immune divergence within the non-DQα01-heterodimer group and understanding how to apply evolutionary and functional distance approaches in the context of patients that are DQα-evolutionary group heterozygous is required.

4 | MATERIALS AND METHODS

4.1 | Study approval

Serum and DNA samples were originally obtained for the purpose of clinical testing. Leftover samples were stored according to local center regulations. Permission to use leftover samples was obtained through Northwestern Institutional Review Board, with waiver of informed consent for deidentified samples prior to initiation of the study.

4.2 | Study design

The overall goal of this study was to critically analyze the correlation between increased MML and generation of dnDSA in renal transplantation and assess their utility as a biomarker to prognosticate this outcome. We further aimed at gaining new mechanistic insights into immunogenicity stratification. In this study, we examined a cohort of transplant recipients from Northwestern University, Chicago, IL, USA, as well as a cohort stemming from an international collaboration under the auspices of the 18th IHWS. We further performed empirical experiments on CRISPR-Cas9-modified cells to assess antibody binding and epitope characteristics, and interrogated qualities of HLA-DQ alleles using computations approaches (such as EMS-3D, generation of phylogenetic trees, etc).

4.3 | Inclusion criteria

All recipients (age >18 years old) of kidney alone allografts, transplanted and followed during 2008–2018 at the Northwestern Memorial Hospital, were evaluated ($n = 2316$), NU = Northwestern University cohort. To fit inclusion criteria, recipients must have had (1) at least two serum samples tested in our laboratory prior to transplant, confirming absence of preformed HLA antibodies; (2) availability of DNA samples for high-resolution HLA class II allele typing (at least 2-field resolution for DRB1, DQA1, and DQB1) of both donor and recipient. To be considered dnDSA-positive, the first detection of DSA had to be at least 3 months posttransplant, with at least moderate levels (to mitigate assay variability or nonspecific reactivity: mean fluorescence intensity > 3000 or titer >1:8; One Lambda, Thermo Fisher Scientific), persistent over time and with a concordant biopsy-proven diagnosis of ABMR. To be considered DSA-negative, patients must have had at least 3 years of clinical follow-up posttransplant, with corresponding solid phase-based antibody testing and no HLA-DSA detected during the follow-up period. Patients who did not fulfill these criteria were excluded. The 3 years of follow-up requirement was used due to temporal US insurance coverage of immunosuppression medication rules. Indeed, many patients were lost to follow-up after 2 years and 10–11 months. The IHWS cohort, served as validation cohort, was submitted by multiple laboratories according to the workshop criteria. Information was obtained from the HLA-epitope component and cleaned to achieve similar inclusion/exclusion criteria when possible.

4.4 | Eplet/molecular mismatch load analysis

Eplet mismatch load analysis was performed using HLA-Matchmaker (v3.1; AbVer), applying two approaches[1]: as recommended by the software developer, meaning combining both donor HLA-DQ alleles (DQA1/DQB1 heterodimer) into one "universe" and comparing this with both recipient alleles' universe—analysis at the patient's level; and[2] analyzing the mismatch between the donor individual alleles compared with both alleles of the recipient—analysis at the allele level. Receiver operating characteristic curve (ROC) analysis was used to assess model discrimination and the Youden score to determine specific thresholds for eplet mismatch load analysis as previously described.[24]

4.5 | CRISPR work

CRISPR/Cas9 target region was identified as previously described and performed on the cell line SWEIG007 (SWEIG-WT) as described.[51] Genetic modification of HLA-DQB1 was confirmed by sequencing. Staining for HLA-DQ cell surface expression was performed using the following monoclonal antibodies: REA303 IgG1 PE (Miltenyi, Bergisch Gladbach, Germany), HLADQ1 IgG1 PE, and 1a3 IgG2a PE (BioLegend, San Diego, CA, USA). Recombinant HmAbs were received from Heidt, as described in.[31]

4.6 | EMS-3D

Quantitative comparison of 3D electrostatic potential between HLA molecules was performed as previously described.[52]

Phylogenetiv trees of molecular and functional evolutionary analyses were conducted using MEGA software[53] (v11.0.13) using ML-tree algorithm was used. Distances were measured using the PMA250 distance metric.

4.7 | SRTR Registry study

All adult patients receiving a deceased donor first kidney transplant between 2010 and 2021 were included if both kidneys from each donor were transplanted into two different recipients (mate kidney approach). Further exclusion criteria were lack of HLA molecular typing for HLA A, B, C, DR, and DQ loci, follow-up shorter than 31 days and recipients of another solid-organ transplant.

4.8 | Statistics

Statistical analysis was performed using GraphPad Prism software (V9.5.1). To compare the number of donors in different groups, we used a chi-square test, unpaired *T*-test with Welch correction, maximum likelihood estimation.[54] All in vitro experiments were repeated at least three times. For SRTR Registry study, death censored graft loss was analyzed by univariate and multivariate Cox regression with clustered standard errors.[55] Other variables included in the model were: number of DR mismatches, recipient sex, recipient age, recipient race, insurance (Medicaid, Medicare), pretransplant cPRA, cold ischemic time, time on dialysis (no dialysis), cause of end-stage renal disease.

AUTHOR CONTRIBUTIONS

Conceptualization: A.R.T. *Methodology*: C.M., P.C., K.F., J.S., and Y.L. *Investigation*: C.M., M.M., M.G., and H.C.C. *Clinical data acquisition*: D.I. and A.C. *IHWS data organization*: C.M., S.H., and S.C.M.K. *SRTR data*: M.M., M.G., and J.S. *Supervision*: A.R.T., J.S., S.H., and V.K. *Writing—original draft*: A.R.T. *Writing—review & editing*: C.M., P.C., K.F., D.I., C.S.M.K., H.C.C., S.H., M.M., J.S., and Y.L.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no competing interest.

DATA AVAILABILITY STATEMENT

All data associated with this study are available upon request from the corresponding author once requirements from the Northwestern Institutional Review Board have been satisfied, and approval for data transfer agreement has been obtained.

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ENDNOTE

* Common HLA-DQ heterodimers refers to those DQ heterodimers available for single antigen bead testing by both vendors of these reagents.

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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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