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A reverse-engineering strategy utilizing the integration of single antigen beads and NGS HLA genotypes to detect potential antibody inducing epitopes

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ABSTRACT

Central to the idea of antibody recognition is some degree of foreignness of the target antigen compared to the antibody producer. Epitopes are distinct regions on an antigen to which antibody can be elicited and bound. However, for HLA antigens, there is no consensus definition of what represents the minimal functional immunogenic unit of dissimilarity. To assess this in an unbiased way, we developed a reverse engineering software strategy based on donor specific antibodies defined by single antigen beads and full length genomic high resolution HLA typing by NGS of recipients and donors (332 transplant pairs). Starting with the ATG of Exon 1 and moving stepwise one amino acid at a time for each of the following triplets, the algorithm compared every possible amino acid triplet of the recipient and donor for 11 loci (A, B, C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, DPB1). Results were agnostic with respect to HLA class, not restricted to just the mature protein, and not influenced by existing maps (e.g., IMGT, or epitope models). We also developed web-based functions in the 17th IHIWS database to collect the unbiased triplets so that we could group the transplant pairs with the same donor specific antibodies and find shared triplets within the groups as potential core or essential epitopes that trigger the antibody formation. Profiling the pairs where the same DSA was identified led to identification of discrete amino acid triplets shared among the pairs irrespective of HLA match. The potential epitopes were mapped onto the 3D protein structure for reference.

1. Introduction

There is widespread consensus that epitopes, rather than antigens, are key to understanding the induction of *donor specific HLA* antibody. Starting with the understanding of Bw4/Bw6 from polyclonal sera, moving through the era of monoclonal antibodies [1,2] and more recently with the use of state of the art single antigen beads [3] and next generation DNA sequencing (NGS) technology, a variety of approaches and models have been proposed to identify what we refer to as an 'epitope.'

The peptide binding-alpha helical surface of the HLA class I protein is 70 Å in length and 50 Å × 40 Å in cross section [4]. Duquesnoy, studying the amino acid (AA) sequences of the HLA proteins, proposed models wherein the epitope is a linear AA triplet [5] or 2–5 conformationally juxtaposed AA forming an eplet [6], creating a target that is 3–3.5 Å in size. Based on work done in mice with monoclonal antibodies, he proposed further epitope definitions [6]. These include (1) overlapping eplets called 'patches,' (2) non-contiguous AA, triplets, and eplets within a 15 Å radius of the 'central' epitope referred to as 'functional epitopes,' creating a target that is 494 Å in size and, (3) 'structural epitopes' that include AA very distant from the 'central' epitope, covering an area of 750 Å. At the same time, El-Awar and Terasaki [3] described epitopes based on class I monoclonal antibody absorption and elution studies where the eluted antibodies were back tested on single antigen beads (SAB). Analysis of the reactive beads allowed them to map the shared AA. Their results were consistent with the models of linear (triplet), conformational (eplet) and functional epitopes. Immunodominant epitopes showing clear and mutually

Abbreviations: AA, Amino Acid; DSA, Donor Specific Antibody; GL, Genotype List; HLA, Human Leukocyte Antigen; IHIWS, International Histocompatibility and Immunogenetics Workshop; NGS, Next Generation Sequencing; SAB, Single Antigen Beads

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exclusive patterns were later described by Cano and Fernandez-Vina [7] for DPB1 by testing clinical sera on SAB.

Although there is some commonality to the 'epitopes' defined by these systems, there is, in fact, no common agreement about what constitutes an epitope (e.g., size, spatial relationship, immunogenicity), and there is no uniform nomenclature to describe one [8–15]. In addition, these models incorporate assumptions or biases that may not be true. Some of these assumptions are: (1) only the solvent accessible portions (i.e., top or peptide binding/alpha helical) surface of class I and II are considered relevant, (2) usually only AA encoded by exons 1 and 2 for class I, or exon 2 for class II are included but not AA closer to the cell membrane, (3) only AA sequences within the same class (but not between classes) are compared, and (4) the disparate triplet must be in the same position in the homologous molecule. To avoid these constraints, we undertook an unbiased analysis of fully NGS typed transplant pairs, where immune recognition had occurred as assessed by the presence of donor specific antibody (DSA) from single antigen bead testing. Our question was: why do recipients with diverse HLA genotypes generate antibodies against the same HLA antigens from the donors? Our goal was to define the minimum required region eliciting an immune response. Our only assumptions were that recognition would be to non-self and that any given DSA, regardless of the similarity or difference in donor/recipient match, would require recognition of an essential region of the donor HLA which we would define as the core epitope. We developed a software algorithm that could reverse engineer the location of epitopes without any other assumptions, bias, or external reference data, to try to define what the essential epitope is when a given DSA is formed to particular antigens/alleles.

2. Materials and methods

2.1. Overview

For the 17th IHIWS component "Mapping of Serologic Epitopes", we studied cohorts of primary renal and cardiac transplant pairs in which recipients and donors were typed using full-length genomic DNA NGS technology and patients were assessed for DSA. Based on the typing of each pair, we developed an unbiased in silico tool to reveal and locate specific AA triplets present only in the respective donor to be evaluated for potential epitopes. We then grouped all the transplant pairs sharing the same DSA and enumerated all the donor specific AA triplets within each group. Shared triplets and clusters within a group were considered potential core or essential epitopes and we mapped them onto the 3D protein structures for reference. The study was approved by the Stanford Institutional Review Board (Protocol IRB-38899).

2.2. Data collection and management

In the 17th IHIWS database [16], we created functions to collect and manage the terabytes of data generated in each step for better integration and centralization. The steps and the dataflow are illustrated in Fig. 1 and explained in the subsections below.

2.3. Transplant pairs

We evaluated 241 transplant pairs from Stanford to develop and test the method. We extended the analysis to 91 transplant pairs from the 8 other participating labs that had complete data.

We imported the coded lab specific sample IDs for donors and recipients into the 17th IHIWS database. These IDs were anonymized, such that the contributing lab would not be identified, and then given unique 17th IHIWS IDs to identify recipients, donors, and transplant pairs and eliminate any possibility of release of protected health information. We also created functions in the 17th IHIWS database to collect the transplant information, which includes donor IDs, recipient IDs, organs transplanted, pre/post-transplant status of sera tested, DSA, inclusion criteria, outcomes, if known, etc.

2.4. HLA typing

Stanford donor and recipient samples were sequenced by NGS for eleven loci (HLA-A, B, C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1 and DPB1) using Illumina TruSight HLA v2 Sequencing Panel and analyzed with Assign 2.1. NGS typing of external samples was performed using kits and/or software platforms from Illumina, GenDx, One Lambda, Omixon and Immucor and run on Illumina or IonTorrent sequencers. A total of 406 pairs were submitted.

To make HLA typing results from different platforms comparable, typing results were standardized by converting into the IHIWS XML format and imported into the 17th IHIWS database. The HLA genotype of each sample was output in GL string format [17]. The genotypes were all assigned using the same IMGT/HLA 3.25.0 reference database (as described in [16]) to provide comparability of results while the nomenclature was evolving during the four years of the workshop. The GL strings were linked to the donor-recipient pairs by the IHIWS IDs and could then be exported from the IHIWS database and used for DSA identification and donor-specific triplet detection. In addition to the genotypes, we also collected consensus sequences and aligned them to the corresponding reference sequences to identify novel variants as described in [16]. Variants in exon regions were marked so that we could check if they would alter the AA sequences.

2.5. DSA identification

We used 17th IHIWS standardized protocols, reagents, and cutoffs to determine DSA. We ran IgG SAB I, IgG SAB II, Bio-C1q SAB I, Bio-C1q SAB II using a single IHIWS lot of SAB (One Lambda), on the same set of samples from all recipients. IgG cutoffs were set to 1000 MFI for positive, and 500–999 for possible. C1q cutoffs were set to 1000 MFI above the lower MFI value of the first breakpoint ≥ 300 MFI for assignment of positive and all values in between for possible. Positive reactions on the SAB were compared to the GL strings of the donor and recipient to determine DSA for each pair. Results for IgG and C1q were analyzed separately by the algorithm.

We developed a web-based import function in the 17th IHIWS database to import the DSA identification results. The function is in "Sample Profile" - > "Maintain Transplantation Profile" - > "Import calling results" under the tab "Lab Member" in the website.

2.6. Unbiased donor specific AA triplet algorithm

The donor-recipient GL string pairs were also used to identify unbiased donor specific AA triplets. Based on our reverse-engineering strategy, the algorithm enumerates all HLA AA triplets from the donor that have the potential to cause antibody formation. Except for the hypothesis that AA polymorphisms shared between recipient and donor will not be immunogenic and therefore cannot lead to the generation of antibodies, we had no other restrictions. In contrast to prior models, we began the triplet scanning at the start codon of Exon 1 rather than the beginning of the mature protein and we did not restrict comparisons to the same class. We scanned all exonic sequences of all loci without limitation.

We implemented the idea with the following steps.

First, we parse the GL strings to retrieve a list of HLA alleles for the donor and the recipient, respectively. Whenever there is allele-level ambiguity, we pick the lowest numbered allele in the second field. Whenever there is genotype/haplotype-level ambiguity, we pick the genotype/haplotype that has the most alleles with the lowest numbers. Second, we identify the donor specific alleles, which are the alleles in the donor's list but not in the recipient's list. Third, we retrieve the full AA sequences for the donor specific alleles and the recipient list, respectively, from the IPD-IMGT/HLA database [18]; in specific, the

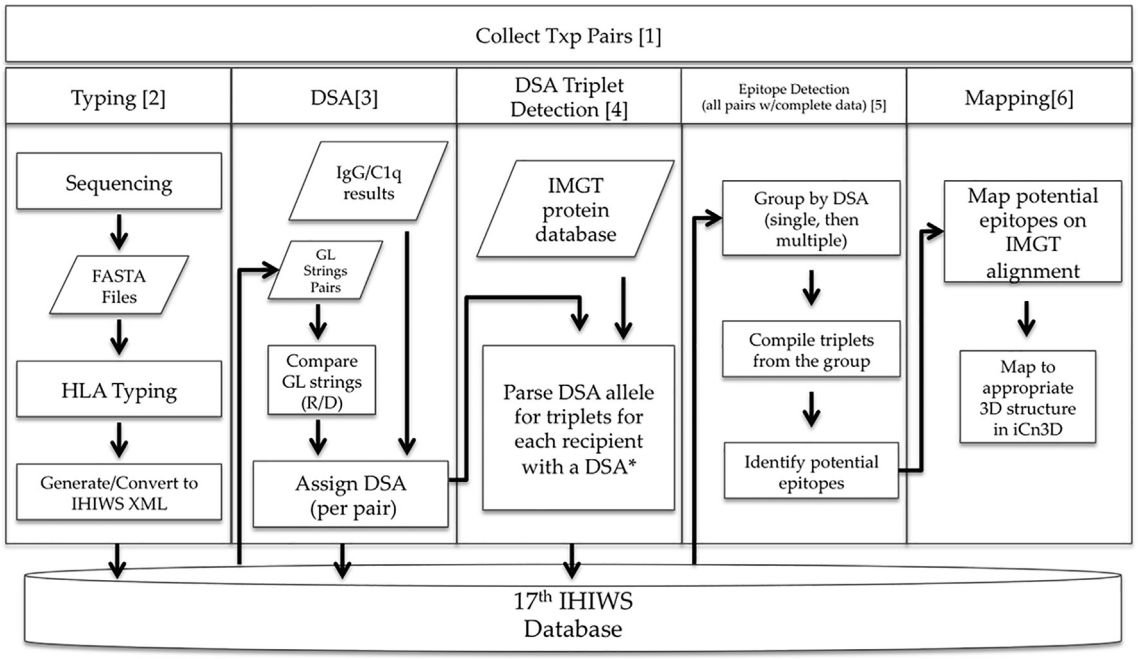


Fig. 1. Data Flow Chart of Epitope Detection The chart illustrates how data of different types are generated and integrated with the centralized IHIWS database. The descriptions of the numbers from (1) to (6) correspond, respectively, to Sections 2.3 to 2.8.

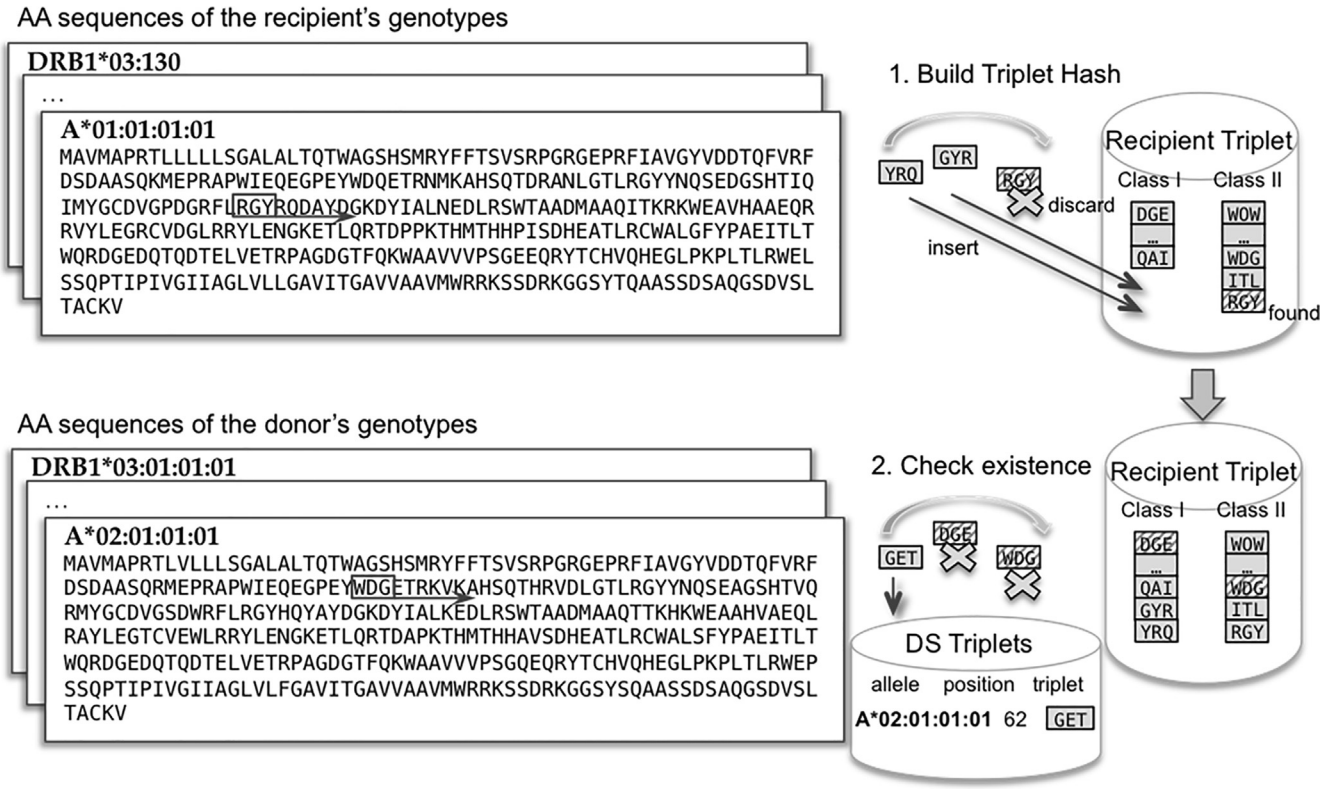
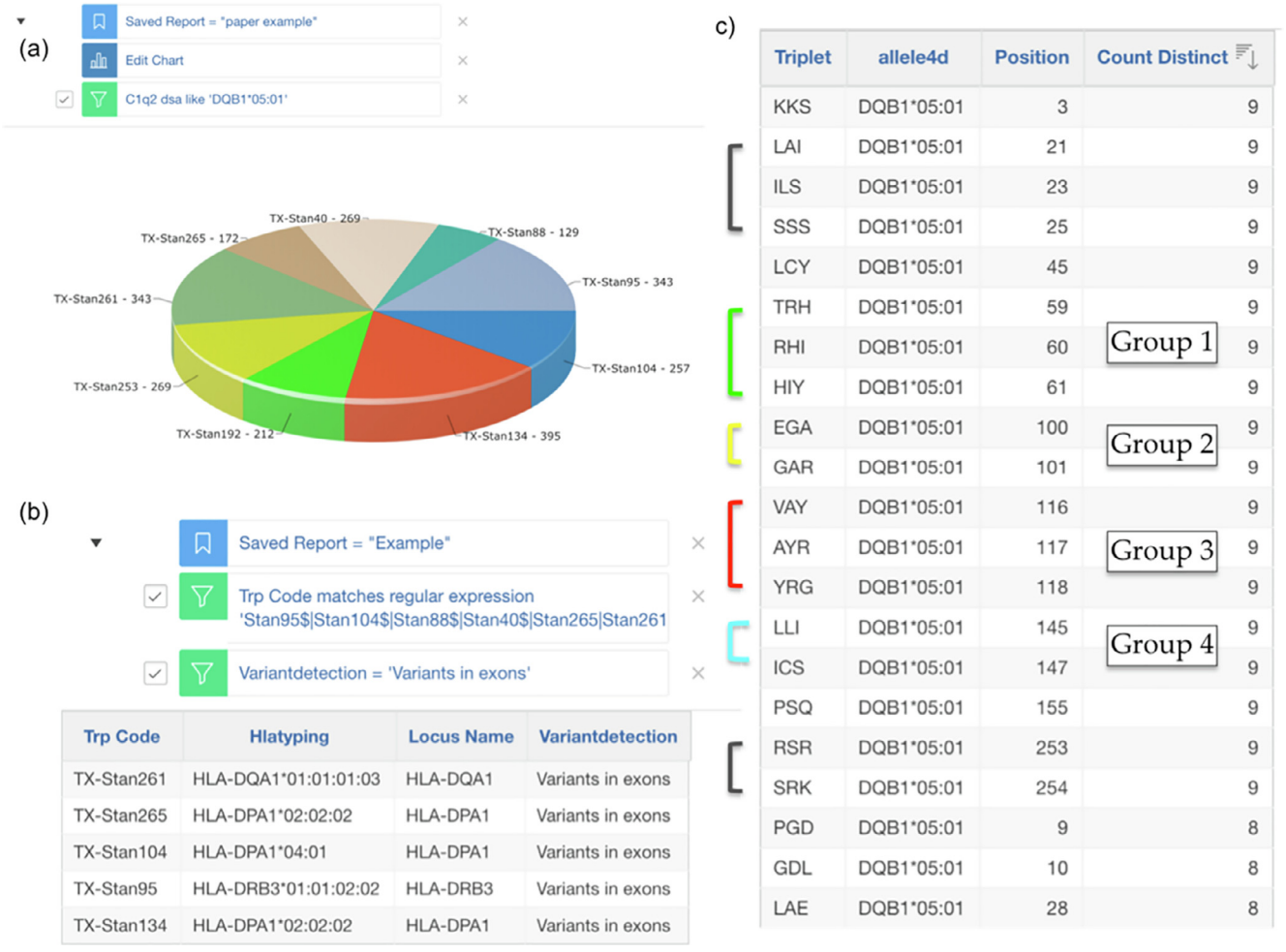


Fig. 2. Illustration of detecting unbiased donor specific AA triplets (1). The script generates recipient triplets by sliding a window of size three and a step of size one. If a triplet is found in more than one recipient allele, only one instance of that triplet is inserted into the recipient hash irrespective of allele of origin or position. (2). Afterward, the script generates donor triplets in the same manner, except that the allele of origin and position are retained. If a triplet is found in more than one donor allele, every instance of that triplet is inserted into the donor hash with its allele of origin and position in that allele. Any instance of a triplet is discarded if it is found in the recipient triplet hash; otherwise, it is inserted into the donor specific triplet hash.

'hla_prot.fa' file. Fourth, we retrieve all the AA triplets of the recipient by sliding a window of size 3 with a step of size 1 on the full AA sequences of all alleles in the recipient's list. Last, we retrieve all the AA triplets for the donor specific alleles as in the fourth step and only keep those not in the recipient's AA triplet set as the output. The last two steps are illustrated in Fig. 2. The output is a table with four columns (Fig. 3b): the transplantation ID, the donor-specific allele where the triplet is found, the position of the triplet on the allele and the AA



Trp Code	Hlatyping	Locus Name	Variantdetection
TX-Stan261	HLA-DQA1*01:01:01:03	HLA-DQA1	Variants in exons
TX-Stan265	HLA-DPA1*02:02:02	HLA-DPA1	Variants in exons
TX-Stan104	HLA-DPA1*04:01	HLA-DPA1	Variants in exons
TX-Stan95	HLA-DRB3*01:01:02:02	HLA-DRB3	Variants in exons
TX-Stan134	HLA-DPA1*02:02:02	HLA-DPA1	Variants in exons

Triplet	allele4d	Position	Count Distinct
KKS	DQB1*05:01	3	9
LAI	DQB1*05:01	21	9
ILS	DQB1*05:01	23	9
SSS	DQB1*05:01	25	9
LCY	DQB1*05:01	45	9
TRH	DQB1*05:01	59	9
RHI	DQB1*05:01	60	9
HIY	DQB1*05:01	61	9
EGA	DQB1*05:01	100	9
GAR	DQB1*05:01	101	9
VAY	DQB1*05:01	116	9
AYR	DQB1*05:01	117	9
YRG	DQB1*05:01	118	9
LLI	DQB1*05:01	145	9
ICS	DQB1*05:01	147	9
PSQ	DQB1*05:01	155	9
RSR	DQB1*05:01	253	9
SRK	DQB1*05:01	254	9
PGD	DQB1*05:01	9	8
GDL	DQB1*05:01	10	8
LAE	DQB1*05:01	28	8

Fig. 3. Illustration of AA triplets within DSA groups (a) An example of the pie chart showing the transplant pairs passing the filter "C1q2 DSA like 'DQB1*05:01'" (n = 9) and the numbers of donor specific triplets detected for each. (b) An example of the variant detection results for the consensus sequences of a DSA group. Since there is no shared exonic variant matching the putative epitope groups, the exonic variants don't affect or alter these epitope groups. (c) An example of grouping and counting of the triplets within the entire DSA group. The "Count Distinct" column indicates which triplets are shared among all transplant pairs in the DSA group and the "Position" column shows that some continuous triplets could form a longer donor specific AA sequence. In this example, we marked four donor specific AA sequences as Group 1 to Group 4.

triplet. Fig. 3c is an example of the table after grouping the 4-digit allele and the position.

We implemented the steps with a perl script and created an import function in the 17th IHIWS database. The script is maintained in GitHub https://github.com/IHIW/bioinformatics/ and the import function could be accessed in the IHIWS website through "Epitope data" - > "Epitope Triplet" - > "Import Triplets" under the "Project Leader" tab.

2.7. Grouping the transplant pairs with identical DSA

After DSA identification, we reviewed the 406 transplant pairs for complete NGS, SAB, and QC acceptable data. We were able to include 332/406 pairs from the combined Stanford and non-Stanford samples for analysis and maximize the number of pairs where the same DSA was made. We generated the donor specific AA triplets for these pairs and imported them into the 17th IHIWS database. The triplets were integrated with the DSA and the HLA genotypes in a web-based interactive report implemented with Oracle APEX so that users could filter the triplets with regular expressions on the DSA or the GL string and count the shared triplets (see Fig. 3a). For example, to select transplant pairs with the specific DSA: DQB1*05:01, we would apply the regular expression "(DQB1*05:01|DQ5)" on the DSA columns and further select those with donor GL string containing DQB1*02:01. We could also filter the triplets using sets of transplant IDs, which are unique identifiers for the pairs, selected according to DSA profiles. After selection of the target DSA group and transplant pairs, using the grouping function in the interactive report, we could further group the triplets by their AA sequence, allele of origin, position within the allele, and sort the triplets according to the count of the number of pairs with the same triplets (see Fig. 3c). As in the example, we found some continuous triplets that could be concatenated to form a longer potential epitope. The flexibility of applying combinations of user-defined filters, groupings, counts and sorting allows users to gain more insight about the shared triplets. For instance, sometimes we found a discrepancy between the count of pairs in a DSA group and the count of pairs sharing a triplet, where there were more pairs with the DSA but not included in the triplet counts. We found that other recipient alleles contained the triplet or cluster attributed to the DSA epitope and therefore would have been excluded by our single hypothesis that recipients cannot make DSA to a shared epitope.

This would imply that even though the shared triplet is contained in the recipient's HLA AA sequences, the triplet might still cause an immune response in some recipients. We could further investigate the locus and position of the recipients containing the same triplet to provide an explanation.

Table 1
Surveyed DSA groups and their PDB IDs.

DSA groups	PDB ID
A2	1HHI
B8	4QRP
C7	5VGE
DQ2	5KSV
DQ5, DQ6	1UVQ
DQ8	5UJT
DR4	5JLZ
DR53	3C5J

2.8. Verification with 3D structures

We mapped the regions of potential epitopes onto the protein 3D structures using iCn3D for reference. Only a small number of HLA alleles have experimentally determined structures; therefore, we picked the proteins with experimentally determined structures with the same first two digits or those with the closest AA sequences to the structure of a targeted DSA group. The DSA groups we surveyed and their corresponding PDB IDs that we used for 3D structures are listed in Table 1. Since the AA sequences of the 3D protein structures only contain mature proteins and there are many HLA alleles, not all the potential epitopes can be directly mapped on the 3D structure by their positions and AA sequences. We marked the potential epitopes manually with the help of the HLA sequence alignment results in IMGT/HLA. We built a website to mark all the potential epitopes we found on the corresponding 3D structures with iCn3D Web APIs.

3. Results

We identified DSAs and detected unbiased donor specific AA triplets for 332 transplant pairs. The triplets were joined with the DSAs and the GL strings in interactive web reports in the 17th IHIWS database (https://ihiws17.stanford.edu/). Users are able to group the transplant pairs according to the DSAs and/or genotypes. Fig. 2 gives an illustration. First, we defined the DSA group by selecting transplant pairs with only a single DSA, DQB1*05:01, detected by the C1q panel. With the “Chart” function in the interactive report, we could easily see the number of transplant pairs passing the filter, which is nine in this example, and the numbers of donor specific AA triplets for each pair (Fig. 3a). With the tool to identify novel polymorphisms in the 17th IHIWS database, we checked to see if there were shared exonic variants that might alter the results within the donors in the DSA group as in Fig. 3b. To be noted, we did not find any shared exonic variants in all the DSA groups. Next, in the triplet interactive report, the eight-digit (four-field) alleles were trimmed to four digits (two fields) so that alleles with the same proteins could be grouped together. With the “Group By” and “Sort” function, we grouped and counted the distinct transplant code with the same triplets, four-digit allele and position and sorted them by counts, in descending order, and by position, in ascending order. In Fig. 3c, we listed only the beginning part of the table. There are six discrete regions (shown in brackets) shared by all the donors. Only four of them are contained in the AA sequence in the reference 3D structure. The colors of the brackets correspond to the mapped epitope colors in the 3D structure in Fig. 4.

4. Discussion

Current epitope models are based on known mature protein sequences with the exception of the work done by absorption/elution and biochemical purification. Only the eluted monoclonal antibodies actually pinpoint the AA or determinants with which the antibodies react. Other epitope descriptions, such as those with Matchmaker or EpVix partially coincide with the antibody proven epitopes, but our data suggest that by ‘forward’ analysis from the protein sequences, the epitope load is overestimated. Because further absorption/elution studies are unlikely or cannot be performed for the array of all known HLA directed antibodies, it is not possible to sort out the ‘true’ epitopes from the potentially irrelevant ones. The current models are both biased and restricted by some of the following assumptions. One: only the mature protein can be immunogenic. This overlooks the fact that the leader sequence (coming from Exon 1 and cleaved during maturation) can be presented as a peptide in the groove of HLA molecules [19]. Two: only the solvent accessible portion of the molecule and usually only sequences from Exons 2 and 3 for class I or Exon 2 for class II are evaluated for donor disparity. Results from absorption/elution studies of sera clearly show reactivity close to the membrane in the Exon 4 region [20]. Three: the transmembrane and cytoplasmic sequences have never been considered in the belief that they are buried and therefore cryptic. This presumes that every HLA molecule is intact, never degraded, and the cryptic regions never exposed during cellular injury or ischemia. It also assumes that changes in the transmembrane region are irrelevant for the intact molecule, even though it is known that the substitution of certain AA (e.g., proline) can bend the molecule such that it could lead to different regions of the external domains being more exposed. Four: it is assumed that disparities in donor sequences must be limited to the sequences derived from the same class of, and sometimes the same position in, the HLA molecule, despite cross-comparisons between classes not having been performed. The underlying (and untested) assumption would be that the recipient can only see disparities in a particular locus or class compared to their own, as opposed to recognizing any disparity among the donor HLA mismatched alleles.

We chose to avoid all assumptions/restrictions with regard to recognition, except two: (1) we restricted the analysis to overlapping triplets, and (2) the assumption that a recipient would not make antibody to their own HLA. (Some of our data suggest that even this might not be true, Tyan, unpublished observations). Using the methodology described, stepwise scanning the entire ‘immature’ (full length) proteins for 11 HLA loci for donor differences, the algorithm found and enumerated the exact mismatched triplets, their gene of origin, and how often they were found in the total cohort having that particular DSA (O/E). The unbiased results obtained were then mapped to the IMGT database and coincided with some of the polymorphic regions in the IMGT database, lending support to the validity of the approach. The algorithm defined some of the same epitopes as have been described in Matchmaker and EpVix (see paper by Yamamoto in this issue), while showing that other putative epitopes are not, or only sporadically, found for the same DSA. The identification by the algorithm of a uniform set of target DSA triplets among the multiple HLA disparate pairs with a given DSA suggests that the tool is revealing the essential or core epitope(s) necessary for that DSA to arise. The fact that these results can be obtained with polyclonal patient sera suggest that the algorithm is robust.

With the reverse-engineering strategy and the integrated tools, we plan to collect more transplant pairs so that less common DSA can also be studied. The tool can also be used to interrogate pairs where the same mismatched donor antigen was present but no DSA was formed to determine whether the mismatched triplets identified in those producing DSA were absent or different in those who did not. To accelerate the process, the next step is to automate the mapping of the potential epitopes on the 3D structure. With more comprehensive data, our goal is to find more general traits that are shared within the transplant pairs forming DSA and develop methods to predict donor compatibility given the genotypes of the donor-recipient pairs.

5. Conclusion

Using this unbiased tool, we now have the opportunity to compare results among epitope models to zero in on the critical epitope(s) and regions and to eliminate other putative, but unconfirmed, epitopes or

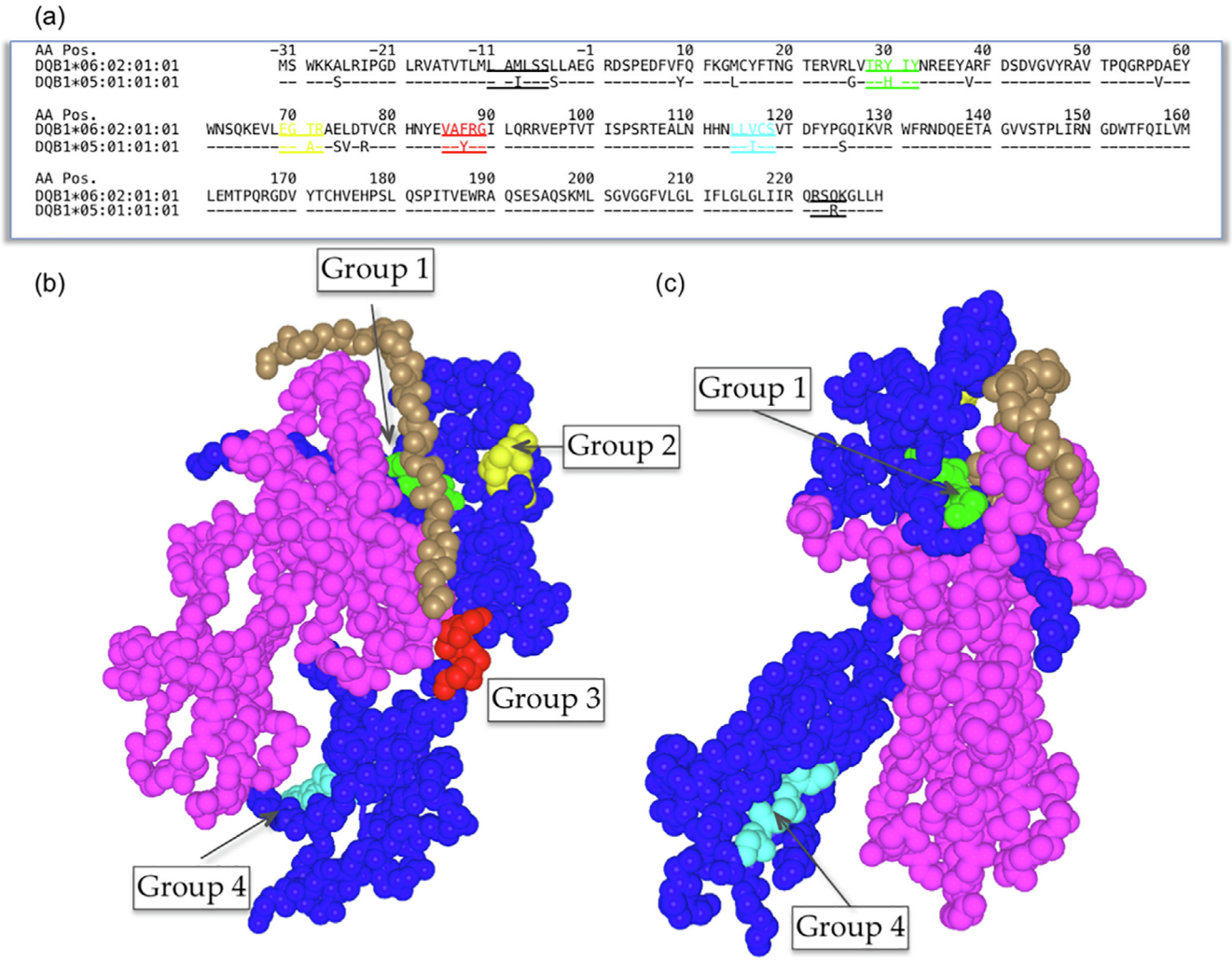


Fig. 4. Potential epitope regions corresponding to the example in Fig. 3 (a) The PDB ID for the closest 3D structure to DQB1*05:01 is 1UVQ and the corresponding allele is DQB1*06:02. We underlined and colored the bracketed regions of Fig. 3c on the alignment from the IMGT database. (b) For the allele DQB1*05:01, we colored the donor specific AA sequences of Group 1 to Group 4 in Fig. 3c on the 3D structure of PDB 1UVQ with two viewpoint angles. Pink = alpha chain, Blue = beta chain, Brown = peptide. (c) Another angle of view.

determine if they are ancillary in some manner. Consensus obtained from multiple approaches will provide a solid foundation for the development of a standardized nomenclature for epitopes.

Declaration of interest

None.

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