Towards HLA epitope matching in clinical transplantation

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CHAPTER

5

GENERATION AND REACTIVITY ANALYSIS OF HUMAN RECOMBINANT MONOCLONAL ANTIBODIES DIRECTED AGAINST EPITOPES ON HLA-DR

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ABSTRACT

In kidney transplantation, eplet mismatches between donor and recipient have been associated with *de novo* donor-specific antibody development. Eplets are theoretically defined configurations of polymorphic amino acids and require experimental verification to establish whether they can be bound by allo-antibodies. Human HLA-specific monoclonal antibodies (mAbs) have been instrumental for this purpose but are largely lacking for HLA class II. In this study, we isolated single HLA-DR specific memory B cells from peripheral blood of immunized individuals (n=3) using HLA class II tetramers to generate recombinant human HLA-DR antigen-reactive mAbs (n=5). Comparison of the amino acid composition of the reactive HLA alleles in relation to the antibody reactivity patterns, led to identification of three configurations i.e. 70Q 73A, 31F 32Y 37Y, and 14K 25Q recognised respectively by HLA-DRB1*01:01, HLA-DRB1*04:01 and HLA-DRB1*07:01 antigen-reactive mAbs. The former two correspond to eplets 70QA and 31FYY and can now be considered antibody-verified. The latter indicates that eplet 25Q needs to be redefined before being considered as antibody-verified. Generation and reactivity analysis of human HLA-DR mAbs allowed for identification of amino acid configurations corresponding to known eplets, while the other patterns may be used to redefine eplets with similar, but not identical predicted amino acid composition.

INTRODUCTION

In kidney transplantation, mismatched donor human leucocyte antigens (HLA) can lead to formation of *de novo* donor-specific antibodies (*dn*DSA), which are associated with inferior graft survival.[1] These *dn*DSA are induced by polymorphic amino acid (AA) configurations on mismatched HLA molecules that have been theoretically defined as eplets,[2,3] which are listed in an online registry.[4,5] Eplets are defined as configurations of surface exposed polymorphic AA within 3-3.5 Å radius.[2,3] Various studies have shown an association between the number of eplet mismatches and *dn*DSA formation,[6-8] especially for HLA-DR and HLA-DQ.[9,10]

Eplets resemble functional epitopes but they are not necessarily identical. A functional epitope determines the specificity of an antibody through interaction, in most cases, with the complementarity-determining region 3 of the heavy chain (CDR-H3) of the antibody.[11-13] The complete surface area of an antigen that interacts with the paratope of an antibody is referred to as the structural epitope, which consists of additional AA configurations within 15 Å radius that are essential for binding and affinity.[14-16]

Since not every individual eplet is necessarily immunogenic due to the nature of the AA substitution, due to physiochemical properties, as well as the absence or presence of an accompanying T helper cell epitope, verification of the actual interaction of eplets with human antibodies is required to determine their clinical relevance.

For antibody-verification of eplets human HLA-specific mAbs[17-19] have been instrumental alongside HLA antibodies purified by absorption and elution from sera of alloimmunized individuals.[20-22] Several eplets have been listed as being verified based on mouse mAbs and/or polyclonal sera,[4,5,23-26] which, in our opinion, is not sufficient to determine whether a single human antibody can interact with an eplet. The limited array of available HLA class II-specific mAbs, hampers verification of many HLA class II eplets. Indeed, Sapir-Pichhadze *et al.* recently observed a strong effect of HLA class I antibody-verified eplet mismatches on graft survival, with no residual effect of HLA class I non-verified eplets. For HLA class II, a similar effect was shown, albeit with a residual effect of HLA class II non-verified eplets.[27] These data indicate that for HLA class II verification of additional eplets will allow for better risk stratification for individual patients.

Human mAbs can be generated from isolated antigen-specific B cells using recombinant technology.[28-30] Low frequency HLA-specific memory B cells in peripheral blood can be detected using flow cytometry and HLA-tetramers.[31-37] Here, we isolated HLA-DR specific memory B cells from peripheral blood using HLA-DR tetramers for the subsequent generation of recombinant human HLA-DR mAbs. Subsequently, uniquely shared AA within 3-3.5 Å radius were deducted from SAB reactivity patterns and referred to as functional epitopes. These were also mapped to eplets, from which the reactive AAs are theoretically pre-defined. Overall, we present five recombinant human HLA-DR mAbs and antibody-verification of three functional epitopes/eplets.

MATERIALS AND METHODS

Cells

Peripheral blood and serum samples were collected from healthy women (n=3) who had developed HLA class II antibodies due to pregnancy, as detected with luminex single antigen bead (SAB) assays. All samples were collected with informed consent under guidelines issued by the medical ethics committee of Leiden University Medical Centre (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (LUMC Pharmacy, Leiden, the Netherlands) density gradient centrifugation and kept frozen in liquid nitrogen until further use. HLA typed Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco Invitrogen, Paisley, UK) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Zwijndrecht, the Netherlands), 50 µM 2-mercaptoethanol (Sigma-Aldrich), 2mM L-glutamine (Gibco Invitrogen), and 100 U/ml penicillin with 100 µg/ml streptomycin (Gibco Invitrogen) in T75 flasks (Greiner, Frickenhausen, Germany).

HLA typing

All subjects were HLA typed for HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB1, -DQA1, -DPB1, and -DPA1 loci by next-generation sequencing (NGS). Genomic DNA was automated bead-based isolated from PBMC (Chemagen, Perking Elmer, Baesweiler, Germany). NGSgo-AmpX kit (GenDx, Utrecht, the Netherlands) was used for the amplification of HLA genes. Next, library and sequence preparation were performed with NGSgo-LibrX/IndX kit (GenDX) and subsequent sequencing was carried on an Illumina MiniSeq (Illumina, San Diego, CA, USA). NGS data were analysed with NGSengine software version 2.11.0 (GenDx).

HLA-DR specific memory B cell isolation and expansion

After thawing, B cells were enriched from 40-60x10^6 PBMC by negative selection using EasySep Human B cell enrichment kits (Stem Cell Technologies, Grenoble, France), according to the manufacturer's instructions (purity >95%). Enriched B cells were incubated for 45 min at 4°C with phycoerythrin (PE) and allophycocyanin (APC)-labelled HLA-DR tetramers (Table 1, ProImmune, Oxford, UK) and the following mouse anti-human antibodies: CD3 (pacific blue, SP34-2), IgD (PE-Cy7, IA6-2) (both from BD Biosciences, Breda, the Netherlands), and CD27 (fluorescein isothiocyanate, FITC, CLB-CD27/1, 9F4) (Sanquin, Amsterdam, the Netherlands). A FACSAria III sorter (BD Biosciences) was used to sort $CD3^{-}CD27^{+}IgD^{-}$ tetramer-APC^{+} and tetramer-PE^{+} cells at one cell per well in 96-well flat-bottom plates (Costar, Corning, NY, USA), containing 100,000 irradiated (50Gy) CD40L-expressing EL4-B5 cells.[38] B cells were expanded for thirteen days in IMDM containing 10% FBS, supplemented with 50 μM 2-mercaptoethanol, 2mM L-glutamine, 100 U/ml penicillin with 100 μg/ml streptomycin, 20 μg/ml insulin-transferrin-sodium selenite (Sigma-Aldrich), 50 ng/ml IL-21 (Gibco), 1 ng/ml IL-1β (Miltenyi, Leiden, the Netherlands), 0.3 ng/ml TNFα (Miltenyi), 0.5 μg/ml R848 (Toll-like receptor 7/8 agonist, resiquimod) (Sigma-Aldrich).[39]

Table 1: HLA-DR tetramers used for cell sorting

HLA Allele	Peptide	Peptide Sequence	Fluorochrome
DRB1*07:01 / DRA1*01:01	CMV	PDDYSNTHSTRYVTV	PE & APC
DRB1*01:01 / DRA1*01:01	Negative control / CLIP	PVSKMRMATPLLMQA	PE & APC
DRB1*04:01 / DRA1*01:01*	Negative control / CLIP	PVSKMRMATPLLMQA	PE & APC
DRB1*04:05 / DRA1*01:01*	Negative control / CLIP	PVSKMRMATPLLMQA	PE & APC

[*] DRB1*04:01 and DRB1*04:05 were used together in one sort

HLA-specific antibody detection

After expansion, supernatants were tested for the presence of IgG by ELISA, as previously described,[40] after which IgG positive supernatants were screened for the presence of HLA antibodies with Lifecodes Lifescreen Deluxe screening kit (LMX, Immucor Transplant Diagnostics, Stamford, CT, USA). The specificity of the HLA antibodies in positive supernatants was determined by Lifecodes HLA class II SAB assays (Immucor). Serum samples were treated with ethylenediaminetetraacetic acid (6% EDTA) prior to testing. Data were analysed with Match It! Antibody software version 1.3.0 (Immucor). The screening data were analysed using raw mean fluorescence intensity (MFI), while for the SAB data background corrected MFI (BCM) was used.

Production of recombinant human monoclonal antibodies

RNA was isolated from HLA-antibody positive B cell clones using TRIzol (ThermoFisher Scientific, Waltham, MA, USA). Next, the genes encoding the variable heavy chain (VH) and variable light chain (VL) were obtained and recombinant monoclonal antibodies (mAbs) were generated and purified as previously described.[41] Briefly, SMART cDNA synthesis and 5'-RACE PCR were performed to obtain the PCR products of VH and VL, which were cloned into pcDNA3.3 expression vectors containing the constant domains of human IgG1 (IGHG1*03), and kappa (κ) (IGKC) or lambda (λ) (IGLC2*01). Recombinant mAbs were expressed by transient co-transfection of heavy and light chain vectors of Expi293F cells with SV40-LT plasmid,[42] ExpiFectamine, Opti-Mem, and Expi293 expression medium (ThermoFisher Scientific). Further purification was done using Amicon ProAffinity Concentration Kit Protein G (Merck Millipore, Burlington, MA, USA). Concentrations of the purified mAbs were determined using the protein A280 protocol of Nanodrop2000 (ThermoFisher Scientific), yielding the molecular concentration of each mAb based on AA sequence.

Sequence analysis

Plasmids were sequenced by Sanger sequencing (Macrogen, Amsterdam, the Netherlands) to obtain nucleotide sequence data of VH and VL. The sequence data were analysed with IgBLAST[43] to define the V(D)J genes of the VH and VL domains and clonality of B cell clones.

Flow cytometric crossmatch assays

EBV-LCLs, $0.5x10^6$, were incubated with 25 µl mAb or PBS for 30 min at room temperature (RT). Cells were washed thrice with phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA, Sigma). Next, cells were stained with mouse anti-human CD3 (PE, SK7), CD19 (APC, HIB19, both from BD Biosciences), and rabbit anti-human IgG F(ab')2 (FITC, Dako, Leiden, the Netherlands) for 30 min at 4°C in the dark. After washing with 0.1%BSA/PBS, cells were fixed with 1% paraformaldehyde. Data were acquired using an Accuri C6 flow cytometer (BD Biosciences) and analysed using FlowJo V10 software (Ashland, OR, USA).

Complement-dependent cytotoxicity (CDC) assay

Terasaki plates (Greiner) were oiled and filled with 1 µl of supernatant containing the mAb of interest in triplicate. Then, 3000 EBV-LCLs were added to each well and incubated for 60 min at RT. Next, 5 µl rabbit complement (Inno-train, Kronberg, Germany) was added and incubated for 60 min at RT. To visualise cytotoxicity, 5 µl propidium iodide ink was added to each well, and after 15 min incubation in the dark at RT the plates were analysed using a Patimed (Leica Microsystems, Amsterdam, the Netherlands).

Antibody reactivity pattern analyses of mAbs

AA sequences of HLA alleles present in the Immucor SAB assay were obtained from IPD-IMGT/HLA (https://www.ebi.ac.uk/ipd/imgt/hla/ accessed on January, 2019), in order to define shared AA of the reactive HLA alleles. To determine the eplets present on the reactive HLA alleles, reactivity patterns were analysed with HLAMatchmaker (HLA DRDQDP Matching, version v2.0 and v3.0;

http://www.epitopes.net/). Eplet antibody-verification status was extracted from http://www.EpRegistry.com.br (accessed on July 15, 2019 and February 12, 2020).

The positions of uniquely reactive AA were visualised with Swissviewer[44] using the following HLA-DR crystal structures: PDB 3PDO and 4MD4 (downloaded from https://www.rcsb.org/ on February 4, 2019). Swissviewer allows for the distance between two atoms to be estimated as well as for the display of atoms that are at a certain distance from a specific atom. These options were used to determine whether AAs were within 3-3.5 Å or 15 Å radius of each other.

RESULTS

HLA-DR specific memory B cell clones isolated from peripheral blood

Flow cytometric cell sorting of HLA-specific memory B cells using HLA-DR specific tetramers (Figure 1A) yielded an average of 44 (range 9-88) single memory B cells. After B cell expansion, IgG could be detected in 50.7% (range 40.9%-66.7%) of sorted wells with a wide concentration range (Figure 1B). HLA class II antibodies were present in 36.8% (range 8.3%-68.8%) of the IgG positive B cell clones with a wide MFI range (MFI 811-18168) (Figure 1C). Subsequent SAB assays confirmed that the HLA-specific B cell clones produced antibodies with the same specificity as the tetramers used for cell sorting (Figure 1D). Eventually, from the total pool of memory B cells an average of 0.008% (range 0.002%-0.014%) HLA-specific B cells were acquired (Figure 1E) and 18.7% (range 3.4%-30.6%) of the sorted cells produced HLA antibodies after expansion (Figure 1F). Overall, an average of 5 (range 2-11) HLA antibody producing B cell clones were obtained, which is an average of 0.001% of memory B cells and 0.0002% of total B cells.

Recombinant human HLA-DR antigen-reactive mAbs generated from HLA positive B cell clones

From several HLA-specific B cell clones HLA-DR antigen-reactive mAbs were generated, and in this proof of principle study we describe one DR1 mAb (LB_DR1_B), one DR4 mAb (LB_DR4_A) and three DR7 mAbs (LB_DR7_A, B and D). The specificity of these mAbs was confirmed by SAB analysis (Figure 2). As expected, the HLA-DR antigen-reactive mAbs showed almost identical reactivity to the supernatants of the B cell clones they were derived from. Flow cytometric crossmatches and CDC assays with EBV-LCL lines expressing HLA alleles corresponding to the tetramers used for B cell isolation confirmed binding of the mAbs to their physiologically expressed HLA target (Supplementary Figure 1A-C), as well as their cytotoxicity capacity (Supplementary Figure 2A-C). Additionally, the mAbs also bound to other natively expressed HLA alleles that were reactive in SAB assays, while no binding was observed for non-reactive HLA alleles (Supplementary Figure 1).

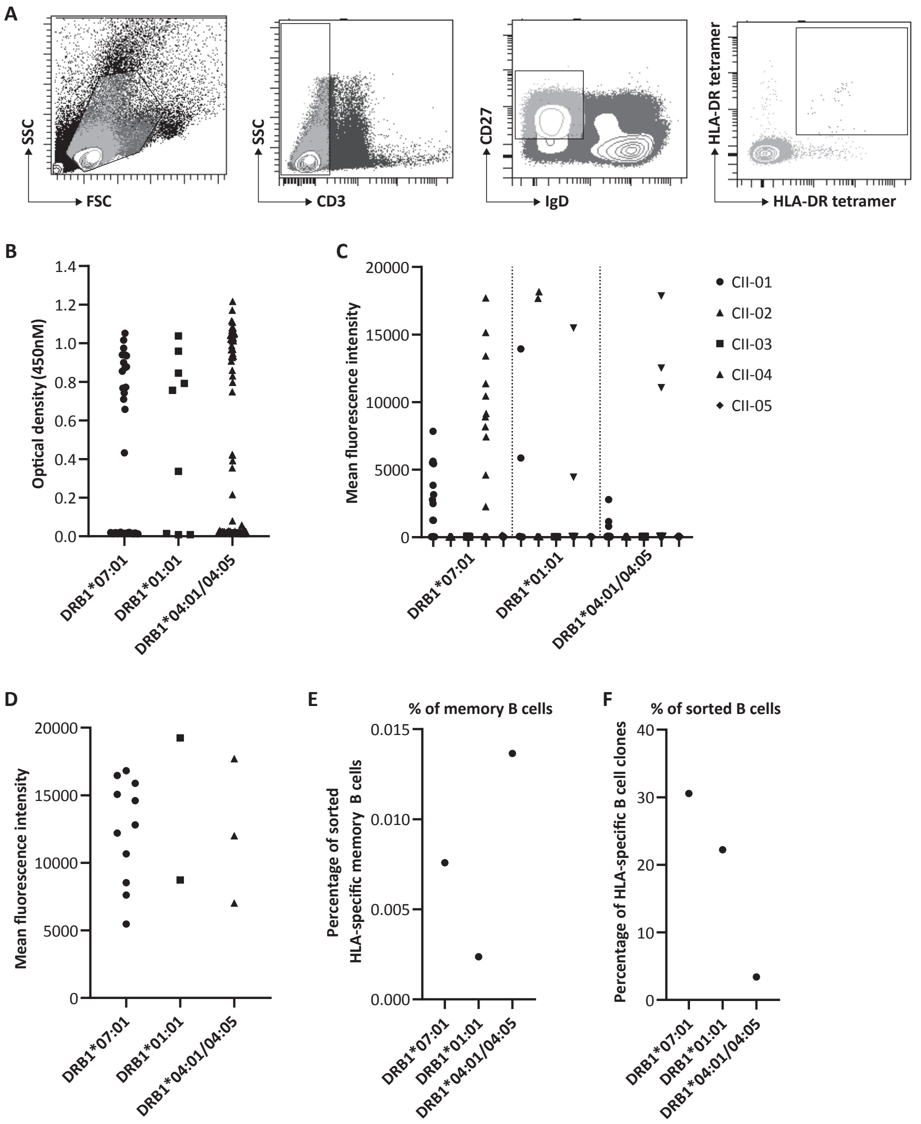

Figure 1: HLA-DR specific memory B cell clones isolated from peripheral blood. A) Representative example of three independent experiments depicting the flow cytometry gating strategy to live single cell sort CD3$^-$CD27$^+$IgD$^-$HLA-DR tetramer double positive B cells from PBMC. B) IgG antibody production by the clones was determined by ELISA. C) IgG positive clones were screened with HLA class II Lifecodes Lifescreen Deluxe kit to detect HLA antibody. The kit contains five groups of HLA class II beads and each data point represents a single bead group. D) HLA-specific B cell clones were tested with SAB assays to confirm tetramer specificity used for cell sorting. Each dot presents one clone and only MFI of bead with tetramer specificity is depicted. E) Percentage of sorted HLA-specific B cells from total memory B cells. F) Percentage of HLA antibody producing B cell clones from sorted B cells. On the x-axis are the specificity of the tetramers used depicted. OD: optical density MFI: mean fluorescence intensity.

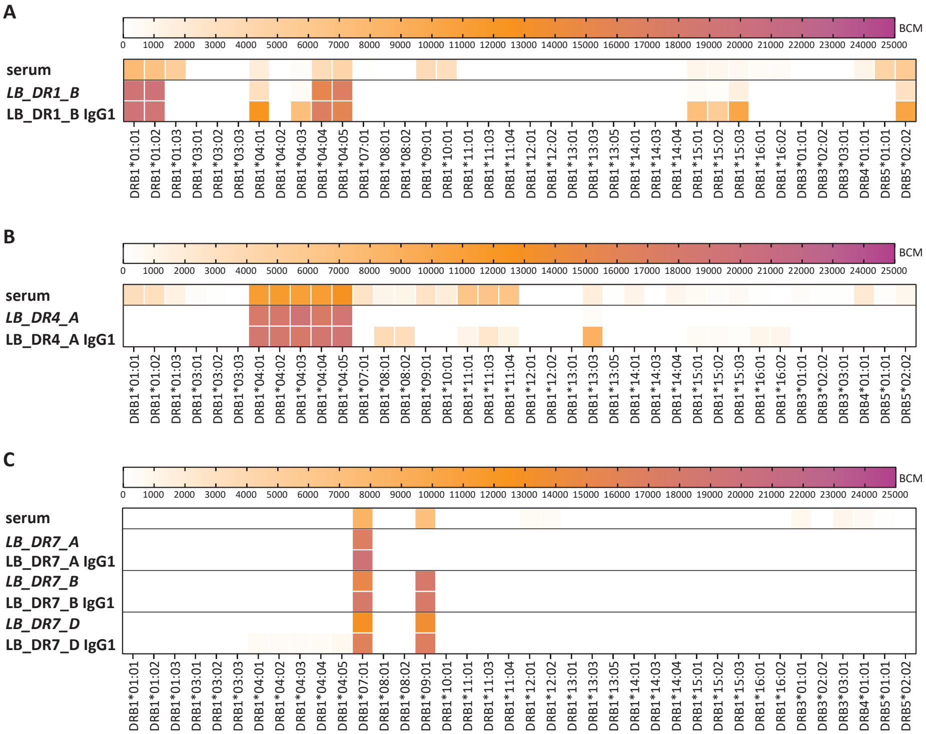

Figure 2: Recombinant human HLA-DR monoclonal antibodies have the same reactivity as the B cell clones. HLA-DR specificities in serum, supernatants of B cell clones (italic) and generated IgG1 mAbs. A) Serum of individual used for HLA-DRB1*01:01 sort and the generated LB_DR1_B mAb and with its respective B cell clone. B) Serum of individual used for HLA-DRB1*04:01/04:05 sort and the generated LB_DR4_A mAb and its respective B cell clone. C) Serum of individual used for HLA-DRB1*07:01 sort and the generated LB_DR7 mAbs with their respective B cell clones. Only DRB1/3/4/5 beads are shown as all other loci were negative for the B cell clones and mAbs. Purified recombinant monoclonal antibody concentration tested was 62.5nM. BCM: background corrected mean fluorescence intensity.

Reactivity analysis of LB_DR1_B mAb

Next, we analysed the mAb reactivity patterns to determine if the reactive HLA alleles in SAB assays uniquely share AA within a 3-3.5 Å radius acting as the functional epitope, determining the antibody specificity.[11-13] Furthermore, we analysed whether additional AA configurations within 15 Å radius of the functional epitope were an absolute requirement for the interaction between antibody and HLA alleles.[45]

The HLA type of the immuniser of LB_DR1_B was unknown (Table 2). This mAb has a broad reactivity pattern including DRB1*01:01 and DRB1*01:02, but not DRB1*01:03 (Figure 2A). Interestingly, no individual AA at a specific position was uniquely shared between reactive HLA alleles and absent on non-reactive HLA alleles, but the combination of 70 glutamine (Q), and 73 alanine (A) was only present on the reactive alleles (Figure 3A). Indeed, HLAMatchmaker v3.0 also showed that the reactive alleles share eplet 70QA (70Q 73A). These AAs are located on top of the HLA molecule (Figure 3B) and within 3 Å radius of each other, suggesting that 70Q and 73A are comprising the functional epitope (Figure 3C). This is in accordance with cellular assays, as LB_DR1_B binds only to cells expressing HLA alleles containing 70Q and 73A (Supplementary Figure 1).

Some of the reactive alleles showed a lower MFI in SAB analysis, suggesting that additional AAs are involved in binding and affinity. Indeed, the alleles showing the highest MFI values share arginine (R) on position 71 and 74A, which are located near positions 70 and 73, within the area of the functional epitope. DRB1*04:03 also harbours a 71R but lacks 74A, which might explain the lower MFI values against this allele.

Together, these data suggest that all four AAs are involved in binding of LB_DR1_B to HLA alleles with high MFI (Figure 3D). As the identified functional epitope corresponds to eplet 70QA, the latter can be considered as antibody-verified by LB_DR1_B.

Reactivity analysis of LB_DR4_A mAb

LB_DR4_A mAb showed a broad reactivity pattern in SAB assays with high reactivity observed for all included DR4 alleles, whereas eleven other alleles were reactive with low MFI values (Figure 2B and 4A). From the AA mismatches of the immunizing DRB1*04:04 with the HLA-DR constitution of antibody-producer (DRB1*03:01 DRB1*13:01 DRB3*01:01 DRB3*02:02), only tyrosine (Y) on position 32 was shared by all reactive HLA alleles. However, 32Y is also present on non-reactive HLA alleles, suggesting that other AAs are involved in interaction with this mAb (Figure 4A). HLAMatchmaker v3.0 identified eplet 37YV (37Y 38V), which was present on eleven out of sixteen reactive alleles. The other identified eplets were 96Y (96Y) present on all tested DR4 alleles, and 142M (142M) shared by five reactive HLA alleles with lowest MFI values in the positive range. These eplets are likely not involved in binding of this mAb since they are shared by a limited number of reactive alleles.

To identify the AA configuration involved, we analysed the DRB1*04:01 crystal structure and observed that 37Y is located within 4 Å radius of 32Y, while 38 valine (V) is 6 Å away from 32Y, and not surface exposed (Figure 4B). In addition, 31 phenylalanine (F) is located within 3.5 Å radius of 32Y, and also 37Y is located within 3.5 Å radius of 31F.

A

BCM	Antigen	70	71	73	74
19292	**DRB1*01:01**	**Q**	**R**	**A**	**A**
19091	**DRB1*01:02**	**Q**	**R**	**A**	**A**
16810	**DRB1*04:04**	**Q**	**R**	**A**	**A**
15554	**DRB1*04:05**	**Q**	**R**	**A**	**A**
12255	**DRB1*04:01**	**Q**	**K**	**A**	**A**
10191	**DRB5*02:02**	**Q**	**A**	**A**	**A**
10011	**DRB1*15:03**	**Q**	**A**	**A**	**A**
7071	**DRB1*04:03**	**Q**	**R**	**A**	**E**
7011	**DRB1*15:01**	**Q**	**A**	**A**	**A**
5227	**DRB1*15:02**	**Q**	**A**	**A**	**A**
0	DRB1*01:03	D	E	A	A
0	DRB1*04:02	D	E	A	A
0	DRB5*01:01	D	R	A	A
0	DRB1*03:01	Q	K	G	R
0	DRB1*07:01	D	R	G	Q
0	DRB1*08:01	D	R	A	L
0	DRB1*09:01	R	R	A	E
0	DRB1*10:01	R	R	A	A
0	DRB1*11:01	D	R	A	A
s 0	DRB1*13:01	D	E	A	A
0	DRB1*14:01	R	R	A	E
0	DRB1*16:01	D	R	A	A
s 0	DRB3*02:02	Q	K	G	Q
s 0	DRB3*03:01	Q	K	G	Q
0	DRB4*01:01	R	R	A	E
0	rest				

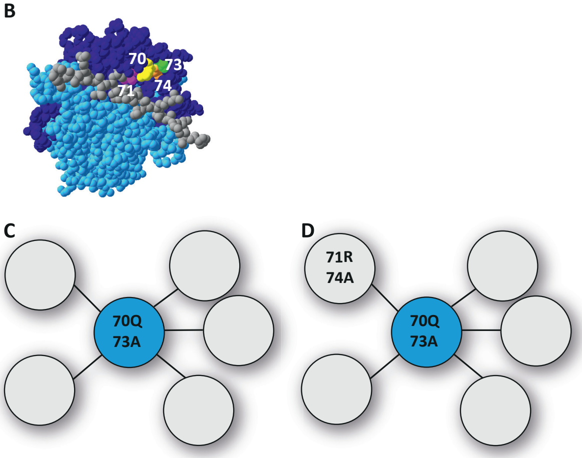

Figure 3: Reactivity analysis of LB_DR1_B monoclonal antibody. A) Comparison of the amino acid positions of interests of the reactive HLA-DR alleles of LB_DR1_B mAb and a selection of the non-reactive HLA-DR alleles. B) Locations of amino acid 70Q (yellow), 71R (magenta), 73A (green) and 74A (orange) are indicated on crystal structure of DRB1*01:01 (PDB: 3PDO). C) LB_DR1_B mAb interacts with HLA-DR alleles containing the functional epitope 70Q 73A. D) Schematic representation of the footprint of LB_DR1_B mAb that is highly reactive for HLA-DR alleles containing the functional epitope 70Q 73A (cyan) and additional amino acids 71R and 74A. In crystal structures the β chain is coloured dark blue, α chain light blue and peptide is grey. Purified recombinant monoclonal antibody concentration tested was 62.5nM. BCM: background corrected mean fluorescence intensity, negative values are presented as zero. s: self HLA alleles of antibody-producer.

Interestingly, the previous version of HLAMatchmaker (v2.0) identified eplet 31FYY (31F 32Y 37Y) present on the same eleven reactive alleles as defined for eplet 37YV. Therefore, we deduced that positions 31F, 32Y, and 37Y together form the functional epitope, and indeed this configuration is unique for the reactive HLA alleles, but only for the highly reactive HLA alleles. Five of the lower reactive HLA alleles have a serine (S) instead of 37Y, and the combination of 31F, 32Y, and 37S is absent on the non-reactive HLA alleles. CDC assays showed that LB_DR4_A mAb can lyse cells expressing HLA alleles carrying 31F 32Y 37Y (Supplementary Figure 2C-D), whereas no specific lysis was observed for cells expressing HLA alleles with 31F 32Y 37S (Supplementary Figure 2F-H).

Since 32Y was the AA shared by all reactive HLA alleles, and mismatched with the antibody-producer, we deduce that the functional epitope of LB_DR4_A consists of 31F 32Y 37Y (Figure 4D) with the mAb weakly binding to HLA alleles containing 37S instead of 37Y (Figure 4E).

Table 2: Information of the five human HLA-DR monoclonal antibodies and description of the reactive HLA-DR alleles

					On reactive HLA DRB1/3/4/5 alleles		
Human mAb	HLA-DR antibody producer	HLA immuniser	HLA tetramer	Reactive HLA DRB1/3/4/5 alleles	Amino acids*	Eplet	TerEp
LB_DR1_B	DRB1*13:01 DRB1*13:02 DRB3*02:02 DRB3*03:01		DRB1*01:01	DRB1*01:01 DRB1*01:02 DRB1*04:01 DRB1*04:03 DRB1*04:04 DRB1*04:05 DRB1*15:01 DRB1*15:02 DRB1*15:03 DRB5*02:02	70Q 73A (71R 74A)	70QA	
LB_DR4_A	DRB1*03:01 DRB1*13:01 DRB3*01:01 DRB3*02:02	DRB1*04:04	DRB1*04:01/04:05	DRB1*04:01 DRB1*04:02 DRB1*04:03 DRB1*04:04 DRB1*04:05 DRB1*13:03 DRB1*08:01 DRB1*08:02 DRB1*11:03 DRB1*11:04 DRB1*11:01 DRB1*16:01 DRB1*16:02 DRB1*15:01 DRB1*15:02 DRB1*15:03	31F 32Y 37Y/S (*13H* 33H)	37YV	
LB_DR7_A	DRB1*11:01 DRB1*15:01 DRB3*02:02 DRB5*01:01		DRB1*07:01	DRB1*07:01	14K 25Q *11G 30L*	25Q	#1008, #1405, #1602
LB_DR7_B & LB_DR7_D	DRB1*11:01 DRB1*15:01 DRB3*02:02 DRB5*01:01		DRB1*07:01	DRB1*07:01 DRB1*09:01	78V 96H 98E 120S	77TV 98ES	#1029

*Amino acids in parentheses are present on the highly reactive HLA alleles, amino acids in italic are not-exposed on position 71 and 74A, which are located near positions 70 and 73, within the area of the functional epitope. DRB1*04:03 also harbours a 71R but lacks 74A, which might explain the lower MFI values against this allele.

A

	BCM	Antigen	13	31	32	33	37	38
	19022	**DRB1*04:05**	**H**	**F**	**Y**	**H**	**Y**	**V**
	18536	**DRB1*04:04**	**H**	**F**	**Y**	**H**	**Y**	**V**
	18293	**DRB1*04:01**	**H**	**F**	**Y**	**H**	**Y**	**V**
	18266	**DRB1*04:02**	**H**	**F**	**Y**	**H**	**Y**	**V**
	17788	**DRB1*04:03**	**H**	**F**	**Y**	**H**	**Y**	**V**
	8610	**DRB1*13:03**	**S**	**F**	**Y**	**N**	**Y**	**V**
	3547	**DRB1*08:01**	**G**	**F**	**Y**	**N**	**Y**	**V**
	3372	**DRB1*08:02**	**G**	**F**	**Y**	**N**	**Y**	**V**
	2351	**DRB1*11:03**	**S**	**F**	**Y**	**N**	**Y**	**V**
	1233	**DRB1*11:04**	**S**	**F**	**Y**	**N**	**Y**	**V**
	1006	**DRB1*11:01**	**S**	**F**	**Y**	**N**	**Y**	**V**
	834	**DRB1*16:01**	**R**	**F**	**Y**	**N**	**S**	**V**
	798	**DRB1*16:02**	**R**	**F**	**Y**	**N**	**S**	**V**
	467	**DRB1*15:01**	**R**	**F**	**Y**	**N**	**S**	**V**
	419	**DRB1*15:02**	**R**	**F**	**Y**	**N**	**S**	**V**
	195	**DRB1*15:03**	**R**	**F**	**Y**	**N**	**S**	**V**
	0	DRB1*01:01	F	I	Y	N	S	V
s	0	DRB1*03:01	S	F	H	N	N	V
	0	DRB1*03:02	S	F	H	N	N	V
	0	DRB1*07:01	Y	F	Y	N	F	V
	0	DRB1*09:01	F	I	Y	N	N	V
	0	DRB1*10:01	F	V	H	N	Y	A
	0	DRB1*12:01	G	F	H	N	L	L
s	0	DRB1*13:01	S	F	H	N	N	V
	0	DRB1*14:01	S	F	H	N	F	V
	0	DRB1*14:03	S	F	H	N	N	V
	0	DRB1*14:04	G	F	H	N	F	V
s	0	DRB3*01:01	S	F	H	N	F	L
s	0	DRB3*02:02	S	F	H	N	Y	A
	0	DRB4*01:01	C	I	Y	N	Y	A
	0	DRB5*01:01	Y	I	Y	N	D	L
	0	DRB5*02:02	Y	I	Y	N	N	V
	0	rest						

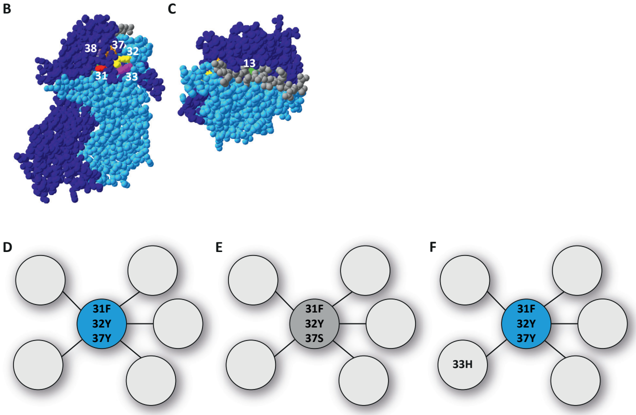

Figure 4: Reactivity analysis of LB_DR4_A monoclonal antibody. A) Comparison of the amino acid positions of interests of the reactive HLA-DR alleles of LB_DR4_A mAb and a selection of the non-reactive HLA-DR alleles. B) Locations of amino acid 31F (red), 32F (yellow), 33H (magenta), 37Y (orange), 38V (lilac) and C) 13H (green) are indicated on crystal structure of DRB1*04:01 (PDB: 4MD4). D) Schematic representation of the footprint of LB_DR4_A mAb interacting with the functional epitope 31F 32Y 37Y (cyan) E) LB_DR4_A mAb weakly binds to HLA alleles containing 31F 32Y 37S. F) Schematic representation of the footprint of LB_DR4_A mAb interacting with the highly reactive HLA-DR4 alleles. In crystal structures the β chain is coloured dark blue, α chain light blue and peptide is grey. Purified recombinant monoclonal antibody concentration tested was 62.5nM. BCM: background corrected mean fluorescence intensity, negative values are presented as zero. s: self HLA alleles of antibody-producer.

The stronger interaction observed for the DR4 alleles suggests involvement of AAs solely present on DR4 alleles, which are 96Y, 180 leucine (L), and histidine (H) on position 13 and 33. The latter two AAs are within 15 Å radius of 32Y, but only 33 is exposed and is therefore most likely involved in the interaction with the antibody (Figure 4F). The functional epitope, 31F 32Y 37Y, corresponds to eplet 31FYY and thereby this eplet can be considered as antibody-verified by LB_DR4_A.

Reactivity analysis of LB_DR7 mAbs

LB_DR7 mAbs were obtained from an individual of which the immunizing event was unknown. We analysed three LB_DR7 mAbs from which the variable domains were acquired by sequencing (Table 3), showing different V(D)J usage, and unique VH and VL clonotypes. This indicates that memory B cells with BCRs recognizing different AA configurations can be isolated with one tetramer specificity.

LB_DR7_A is only reactive with HLA-DRB1*07:01 (Figure 2C), strongly suggesting that this was the immunizing allele. Upon comparing the AA sequence of DRB1*07:01 with the non-reactive HLA-DR alleles present in SAB assay, glycine (G) on position 11, lysine (K) on position 14, Q on position 25, and L on position 30 were identified as unique AAs for DRB1*07:01 (Figure 5A). Three of these AAs, are present within the eplet 25Q (25Q 30L 14K), which is also the eplet determined upon analysis with HLAMatchmaker v3.0. The four unique AA correspond to TerEp #1602[22,46] and have been previously described for mouse mAbs.[47-50] Positions 11 and are 30 located at the bottom of the peptide-binding groove (Figure 5B), while 14K and 25Q are surface exposed and within 3.5 Å radius of each other (Figure 5C). Due to location of 11G and 30L and as neither are within 3.5 Å radius of 14K and/or 25Q, it is unlikely that those form the functional epitope. Additionally, mutation assays with mouse mAbs showed that only mutation of 14K and 25Q affected binding.[47-50] Altogether, we suggest that 14K and 25Q comprise the functional epitope without 30L being involved (Figure 5D).

LB_DR7_B and LB_DR7_D bind to DRB1*07:01 and DRB1*09:01 in both SAB assays (Figure 2C) and flow crossmatch, whereas HLA alleles with low reactivity to LB_DR7_D in SAB did not react with natively expressed alleles in flow (Supplementary Figure 1). DRB1*07:01 and DRB1*09:01 share a valine (V) on position 78, which is absent on all non-reactive HLA alleles (Figure 5E) and located on top of the molecule (Figure 5F) and correspond to TerEp #1029. Concomitantly, eplets 77TV (77T 78V) and 98ES (98E 120S) were identified as unique for the reactive alleles. Analysis with HLAMatchmaker v2.0 suggested that 96H could potentially be involved, based on previously listed eplet 78V2, which was the predecessor of 77TV and 98ES. Interestingly, while the individual AAs 96H, 98E, and 120S are present on non-reactive HLA alleles, including self, the configuration of the three is only present on DRB1*07:01 and DRB1*09:01. Since this configuration is exposed but not within 15 Å radius of 78V (Figure 5G), either 78V (Figure 5H) or 96H 98E 120S (Figure 5I) act as contact site for the CDR-H3 of LB_DR7_B and/or LB_DR7_D.

A

	BCM	Antigen	11	14	25	30
	19367	**DRB1*07:01**	**G**	**K**	**Q**	**L**
	0	DRB1*01:01	L	E	R	C
	0	DRB1*03:01	S	E	R	Y
	0	DRB1*04:01	V	E	R	Y
	0	DRB1*08:01	S	E	R	Y
	0	DRB1*09:01	D	E	R	G
	0	DRB1*10:01	V	E	R	R
S	0	DRB1*11:01	S	E	R	Y
	0	DRB1*12:01	S	E	R	H
	0	DRB1*13:01	S	E	R	Y
	0	DRB1*14:01	S	E	R	Y
S	0	DRB1*15:01	P	E	R	Y
	0	DRB1*16:01	P	E	R	Y
S	0	DRB3*02:02	L	E	R	H
	0	DRB4*01:01	A	E	W	Y
S	0	DRB5*01:01	D	E	R	D
	0	rest				

E

	BCM	Antigen	77	78	96	98	120
	17813	**DRB1*07:01**	**T**	**V**	**H**	**E**	**S**
	17651	**DRB1*09:01**	**T**	**V**	**H**	**E**	**S**
	0	DRB1*01:01	T	Y	E	K	S
	0	DRB1*03:01	N	Y	H	K	S
	0	DRB1*04:01	T	Y	Y	E	N
	0	DRB1*08:01	T	Y	H	K	S
	0	DRB1*10:01	T	Y	Q	K	N
S	0	DRB1*11:01	T	Y	H	K	S
	0	DRB1*12:01	T	Y	H	K	S
	0	DRB1*13:01	T	Y	H	K	S
	0	DRB1*14:01	T	Y	H	K	S
S	0	DRB1*15:01	T	Y	Q	K	S
	0	DRB1*16:01	T	Y	Q	K	S
S	0	DRB3*02:02	N	Y	H	Q	S
	0	DRB4*01:01	T	Y	Q	K	N
S	0	DRB5*01:01	T	Y	E	K	N
	0	rest					

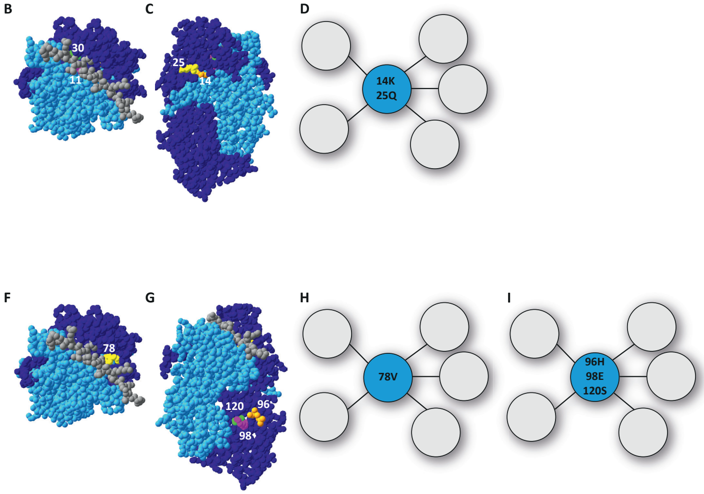

Figure 5: Reactivity analyses of LB_DR7_A, and LB_DR7_B and LB_DR7_D monoclonal antibodies. A) Comparison of the amino acids of the reactive HLA-DR alleles of LB_DR7_A mAb with non-reactive HLA-DR alleles. B) Positions 30 (green), and 11 (magenta) and C) 25 (yellow), and 14 (orange) are indicated on the crystal structure of DRB1*01:01 (PDB: 3PDO). D) A schematic representation of the footprint of LB_DR7_A mAb with 14K 25Q as functional epitope (cyan). E) Comparison of the amino acids of the reactive HLA-DR alleles of LB_DR7_B and LB_DR7_D with the non-reactive HLA-DR alleles, of which only a selection is shown. Only BCM of LB_DR7_B are depicted. F) Location of position 78 (yellow) and G) of 96 (orange), 98 (magenta) and 120 (green) on the DRB1*01:01 crystal structure. H) A schematic representation of LB_DR7_B and LB_DR7_D footprint with 78V or I) with 96H 98E 120S as the functional epitope (cyan) of the mAb. In crystal structures the β chain is coloured dark blue, α chain light blue and peptide is grey. Purified recombinant monoclonal antibody concentration tested was 62.5nM. BCM: background corrected mean fluorescence intensity, negative values are presented as zero. s: self HLA-DR alleles of antibody-producer.

Table 3: V(D)J usage of the different HLA-DR monoclonal antibodies

		Heavy chain				Light chain			
Clone	HLA	V gene	D gene	J gene	CDR3	κ or λ	V gene	J gene	CDR3
LB_DR1_B	DRB1*01:01	4-59	3-10	6	ARRNLTIDRGGDYGMDV	λ	3-1	2 or 3*	QAWDSNTYVV
LB_DR4_A	DRB1*04:01/04:05	3-48	3-16 or 6-6*	4	ARDGGLNRPPD	λ	1-40	1	QSYDISLSGPYV
LB_DR7_A	DRB1*07:01	4-61	6-13 or 6-25 or 6-6*	4	ARDLAADH	λ	2-14	2 or 3*	SSYTSSSTLVV
LB_DR7_B	DRB1*07:01	3-30	3-10	4	AKDLPRYELPVQGDY	κ	1-5	1	QQYKSYPWT
LB_DR7_D	DRB1*07:01	3-30	6-19	6	ARDGGYRSGWSLTKGYYYGVDV	κ	1-16	2	QQYKNYPHT

*Multiple equivalent top matches valine (V) on position 78, which is absent on all non-reactive HLA alleles (Figure 5E) and located on top of the molecule (Figure 5F) and correspond to TerEp #1029. Concomitantly, eplets 77TV (77T 78V) and 98ES (98E 120S) were identified as unique for the reactive alleles. Analysis with HLAMatchmaker v2.0 suggested that 96H could potentially be involved, based on previously listed eplet $78V_2$, which was the predecessor of 77TV and 98ES. Interestingly, while the individual AAs 96H, 98E, and 120S are present on non-reactive HLA alleles, including self, the configuration of the three is only present on DRB1*07:01 and DRB1*09:01. Since this configuration is exposed but not within 15 Å radius of 78V (Figure 5G), either 78V (Figure 5H) or 96H 98E 120S (Figure 5I) act as contact site for the CDR-H3 of LB_DR7_B and/or LB_DR7_D.

DISCUSSION

Increasing numbers of HLA class II eplet mismatches are associated with the development of *dn*DSA,[6-8] which led to the hypothesis that eplet mismatch loads can be used as predictor of DSA occurrence.[9,10] However, eplets have been theoretically defined and for several eplets it remains to be established whether they are indeed reactive with antibodies, and thus clinically relevant. Therefore, eplets require experimental verification, either by human mAbs or absorption and elution studies,[4,5,19-21] to establish if interaction with an antibody can occur. This is of importance for the implementation of eplet matching in allocation systems aiming at prevention of *dn*DSA development. By performing eplet matching solely on relevant functional eplets, patients will not be denied an organ offer based on irrelevant eplet disparities with the donor.

In this study, we isolated HLA-DR specific memory B cells from peripheral blood of immunised individuals using HLA-DR specific tetramers. While tetramers have been used to detect and isolate HLA class I-specific B cells,[35,37,51] to our knowledge this study is the first to use tetramers for the isolation of HLA class II-specific memory B cells. Here, we describe generation of five HLA-DR mAbs with four different specificities: DR7, DR7/DR9, DR1/DR9/DR10/DR51 and DR4/DR1303/DR8/DR11/DR15/DR16.

Overall, the specificity of the generated mAbs resembled the antibody repertoire observed in the serum. For LD_DR7_D additional reactive HLA alleles were observed, albeit with very low MFI. A possible explanation is that the memory B cell compartment may contain a broader repertoire than that of circulating antibodies.[52-54] However, the additional reactive HLA alleles could not be confirmed with flow cytometric crossmatch assays using natively expressed HLA alleles (Supplementary Figure 1). Therefore, the additional reactivity for the mAbs appears to be due to non-specific binding in SAB assays or due to the mAb concentration used.

Based on both SAB and cellular data presented here, the eplets 70QA and 31FYY, corresponding to AA configurations 70Q 73A and 31F 32Y 37Y, have been antibody-verified by the human mAbs LB_DR1_B and LB_DR4_A, respectively, despite the limitation of missing HLA typing of the immuniser for LB_DR1_B. Eplet 31FFY was previously registered as antibody-verified based on reactivity analysis of mouse mAbs[5,55] and pregnancy sera.[25] Peculiarly, this eplet is no longer present on the HLA Epitope Registry as it was deemed redundant. The data presented here suggest that this eplet does actually exist and should be relisted on the Registry.

For the narrow reactivity patterns of LB_DR7_A, LB_DR7_B and LB_DR7_D HLAMatchmaker defined eplets 25Q, and 77TV and 98ES, respectively, to be uniquely shared. For eplet 25Q the current description of the AAs involved exceeds the original definition of an eplet since the suggested residues are not located within 3.5 Å radius of each other. The same applies to eplet 37YV, defined by HLAMatchmaker v3.0 for LB_DR4_A. Provided that eplet 25Q (25Q 14K 30L) is to be redefined as 14K and 25Q, mAb LB-DR7_A allows for antibody verification of this eplet.

Eplet 98ES is currently listed as antibody-verified based on pregnancy sera, but our reactivity analysis shows that either 78V or 96H 98E 120S can act as contact site for mAb reactive for HLA-DRB1*07:01 and HLA-DRB1*09:01. In contrast to HLAMatchmaker v2.0, the newly defined 98ES no longer includes residue 96H in v3.0. Reactivity analysis of the HLA-DR mAbs showed that AAs on reactive HLA alleles not always correspond to pre-defined eplets identified by HLAMatchmaker. For some of these mAbs we were able to identify different eplets on basis of version 2.0 of HLAMatchmaker, indicating that the list of eplets in this program and on the HLA Epitope Registry is subject to change without broadly accepted and validated arguments showing the need to install an international nomenclature committee for the definition of antibody-verified eplets and/or epitopes. Overall, the data presented herein indicates that the current, widely used list of eplets contains inaccuracies. Furthermore, our results show that performing reactivity analysis of human HLA mAbs based on AA rather than on pre-defined eplets may be more useful in defining the relevant AA configurations, and this will require several mAbs.[18,56]

Antibody reactivity analyses based solely on SAB assay can be complex and can benefit from additional functional assays to determine true reactivity. AA substitutions within the functional epitope which do not affect binding of mAbs to an HLA allele in SAB assay may affect the ability to induce complement-dependent cytotoxicity,[17] as was the case for LB_DR4_A. In addition, MFI values can reflect differential affinity of the mAbs for specific HLA alleles[57] and AA substations within the structural epitope can lead to lower affinity, which can be reflected in the MFI values.[58] Mutation studies have been informative on determining the involvement of single AAs in the interaction between the HLA molecule and antibody.[48,49,59] However, it is important to realise that AA substitutions can affect the tertiary structure and surface electrostatic potential of the HLA molecule.[60,61]

In the present study we obtained multiple B cell clones from one individual with subtle differences in specificity reflecting the polyclonal reactivity of serum. These observations substantiate the notion that antibody-verification of eplets should only be done by using human mAbs or absorption and elution studies,[22,62-64] and not based on sera of women after first or second pregnancy, as is currently done for various eplets.[24-26] While in this proof of principle study, we present HLA-DR mAbs obtained from three subjects, the inventory of HLA-DR mAbs will expand soon, which will result in identification and antibody-verification of additional relevant AA configurations. In addition, we are developing methods to utilise HLA-DQ monomers[33] to isolate HLA-DQ-specific memory B cells for the generation of recombinant HLA-DQ mAbs and subsequently reactivity analysis to verify HLA-DQ eplets, since HLA-DQ DSA are most prevalent after transplantation and associated with rejection.[8,9,65-67]

These human HLA class II mAbs can be used in functional studies to provide more insight in the respective roles of HLA-specific IgG antibodies in causing graft damage,[68-72] with the possibility of all IgG subclasses to be generated.[41] In addition, as shown for LB_DR7, distinct B cell clones with different levels of affinity maturation, as suggested by the different binding strengths and efficacy in cell lysis, can be obtained from a single individual. Thus, the method described herein can contribute to understanding the development of the HLA-specific memory B cell compartment[73] besides their use in eplet verification.

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Disclosure

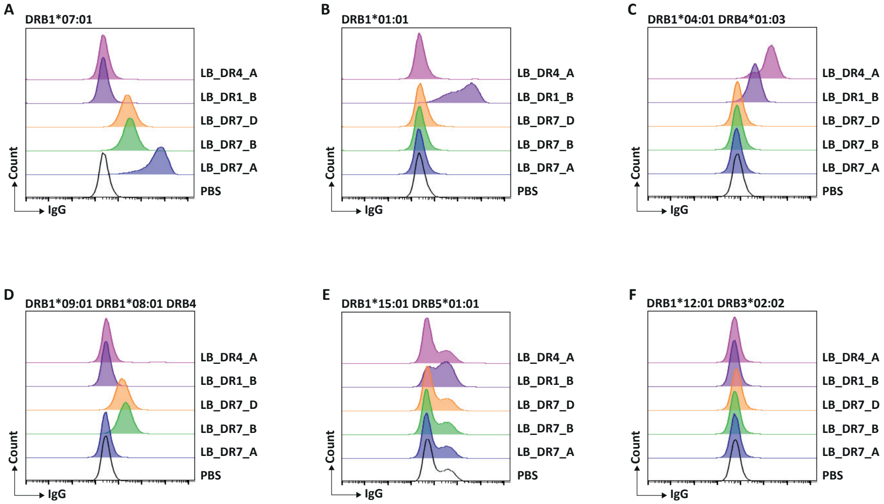
The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Data availability statement

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

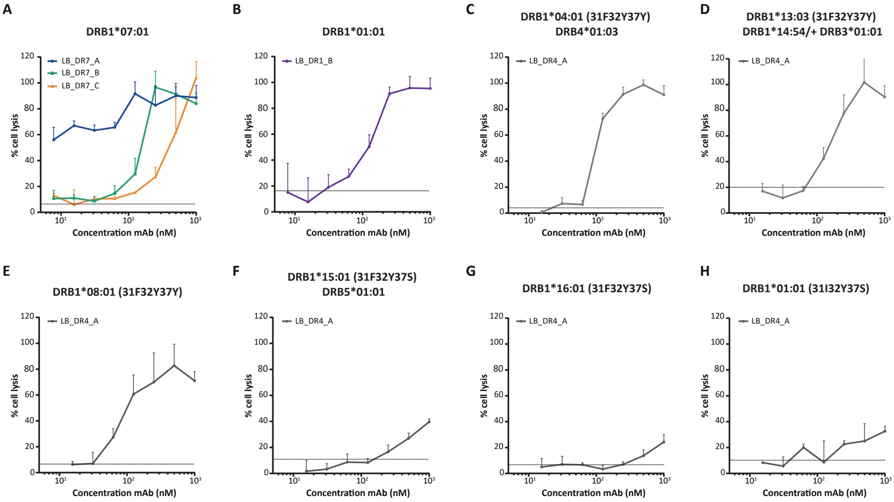
SUPPLEMENTAL MATERIAL

Supplementary Figure 1



Supplementary Figure 1: Recombinant human HLA-DR mAbs bind only to reactive HLA expressed on cells. Flow cytometry crossmatches on EBV-LCL lines were performed with all five DR mAbs to show that each mAb binds its respective natively expressed target HLA, as well as other reactive HLA, and does not bind to non-reactive HLA. Concentration mAbs used 62.5nM. HLA-typed DRB1*07:01, DQB1*02:01, DPB1*15:01 (A), DRB1*01:01, DQB1*05:01, DQA1*01:01, DPB1*04:01 (B) and DRB1*04:01, DRB4*01:03, DQB1*03:02, DQA1*03:01, DPB1*03:01, DPB1*04:01 (C), DRB1*09:01, DRB1*08:01, DRB4 (D), DRB1*15:01, DRB5*01:01, DQB1*06:02, DQA1*01:02, DPB1*02:01 (E) and DRB1*12:01, DRB3*02:02, DQB1*03:01 (F) EBV-LCL lines were used.

Supplementary Figure 2



Supplementary Figure 2: Cytotoxicity reactivity of recombinant human HLA-DR mAbs. Complement dependent cell lysis was observed in a dose-dependent manner for LB_DR7 IgG1 mAbs (A), LB_DR1 IgG1 mAb (B), and LB_DR4 IgG1 mAb (C). LB_DR4_A mAb only induced CDC reactivity for cells expressing HLA molecules DRB1*04:01 (C), DRB1*13:03 (D) and DRB1*08:01 (E) all having 31F 32Y 37Y, while similar background was observed for cells expressing DRB1*15:01 (F) and DRB1*16:01 (G) (31F 32Y 37S), and cells expressing non-reactive DRB1*01:01 (31I 32Y 37S) (H). CDC assays were performed with HLA-typed EBV-LCL lines and various concentrations of mAbs were added (1000, 500, 250, 125, 62.5, 31.25, and 15.62 nM. Additional concentration 7.8nM was used for A-C. Data point with error bars represent the mean ± standard deviation of triplicate wells. Lysis is relative to positive control (pan-HLA class II antibody).

ABBREVIATIONS

Å: Angstrom

Amino acid: AA

APC: Allophycocyanin

BCM: Background corrected mean fluorescence intensity

BCR: B cell receptors

BSA: Bovine serum albumin

CDC: Complement-dependent cytotoxicity

cDNA: Complementary Deoxyribonucleic acid

CDR: Complementarity-determining region

DSA: Donor-specific antibodies

EBV-LCL: Epstein-Barr virus-transformed lymphoblastoid cell line

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

FITC: Fluorescein isothiocyanate

HLA: Human leukocyte antigen

IgG: Immunoglobulin G

IL: Interleukin

IMDM: Iscove's modified Dullbecco's medium

mAbs: Monoclonal antibodies

MFI: Mean fluorescence intensity

OD: Optical density

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PE: Phycoerythrin

RACE: Rapid amplification cDNA ends

RNA: Ribonucleic acid

SAB: Single antigen beads

TerEp: Teresaki epitope

VH: Heavy chain variable domain

VL: Light chain variable domain

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