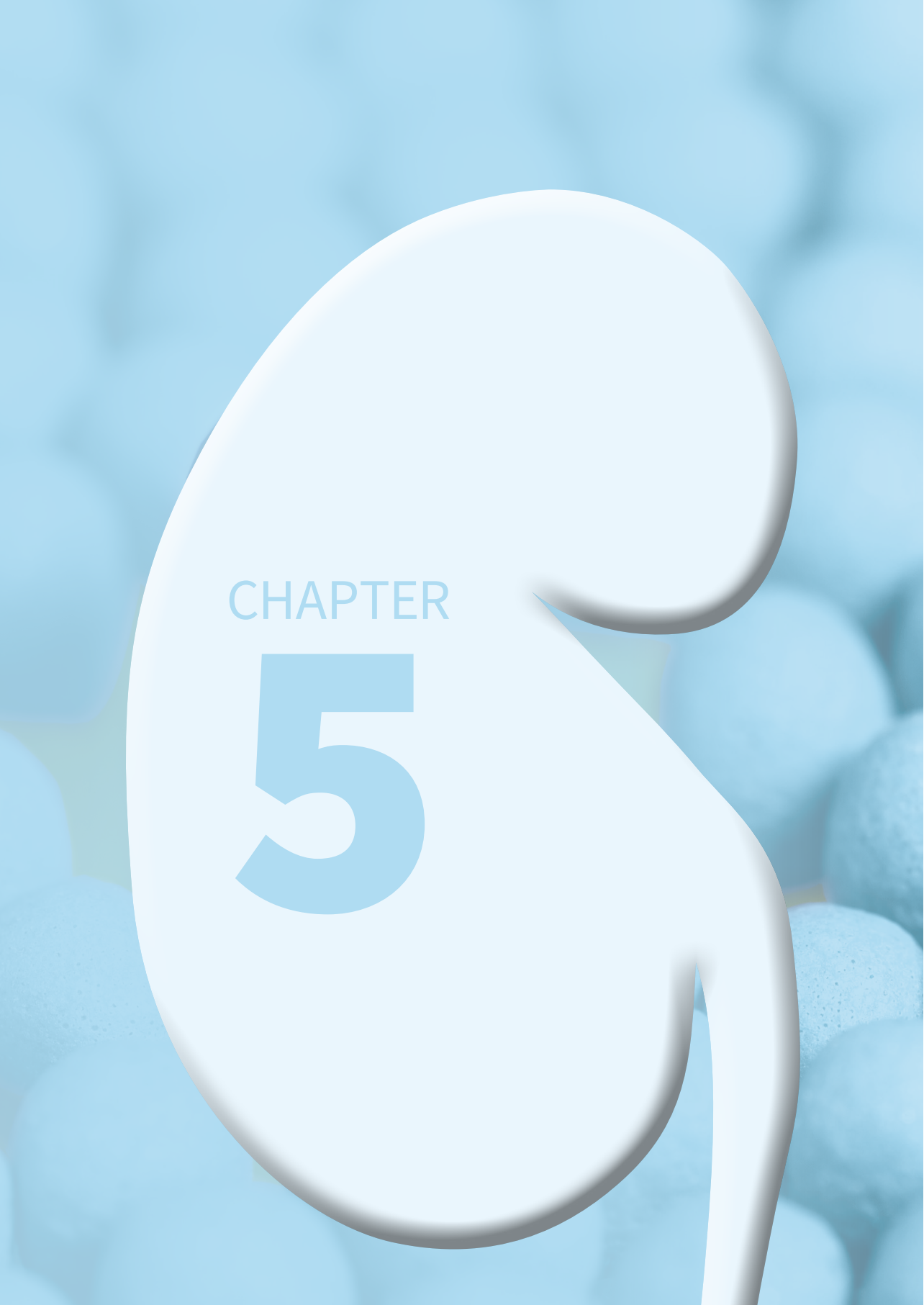
HLA epitopes in kidney transplantation: from basic science to clinical application

Bezstarosti, S.


CHAPTER
5

Site-directed mutagenesis of HLA molecules reveals the functional epitope of a human HLA-A1/A36-specific monoclonal antibody

Tina Meng[1], Suzanne Bezstarosti[2,3]
Ujjwala Singh[1], Michelle Yap[1]
Laura Scott[1], Naiiry Petrosyan[1]
Fred Quiroz[1], Ned Van Eps[1]
Eric Ka-Wai Hui[1], David Suh[1]
Quansheng Zhu[1], Rui Pei[1]
Cynthia SM Kramer[2], Frans HJ Claas[2]
David Lowe[1], Sebastiaan Heidt[2]

[1] *Department of Research and Development, One Lambda, Inc. (A Part of Thermo Fisher Scientific Inc.), West Hills, California, USA*
[2] *Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands*
[3] *Department of Internal Medicine (Nephrology), Leiden University Medical Center, Leiden, The Netherlands.*

Tina Meng and Suzanne Bezstarosti contributed equally to this work and share first authorship.

HLA. 2023 Feb;101(2):138-142.

ABSTRACT

Eplet 44KM is currently listed in the HLA Epitope Registry but does not adhere to the eplet definition of an amino acid configuration within a 3.5 Ångstrom radius. Eplet 44KM has been previously redefined to the antibody-verified reactivity pattern 44K/150V/158V, based on reactivity analysis of monoclonal antibody VDK1D12. Since the three residues are always simultaneously present on common HLA alleles, methods to define which residue is crucial for antibody-induction and binding are limited. In this proof-of-concept study, we performed site-directed mutagenesis to narrow down the antibody-verified reactivity pattern 44K/150V/158V to a single amino acid and defined 44K as the eplet or functional epitope of mAb VDK1D12.

1 INTRODUCTION

Antibody-mediated rejection is one of the major causes of graft failure in kidney transplantation and is caused by pre-existing or *de novo* donor-specific antibodies (dnDSA) mainly directed against a mismatched HLA molecule [1, 2]. Increasing evidence indicates that HLA mismatch analysis on the amino acid or eplet level is superior in predicting dnDSA development compared to the HLA antigen level[3]. HLA eplets are configurations of polymorphic amino acids within a 3.5 Ångstrom radius and resemble the functional epitope that interacts with the complementarity-determining region 3 on the heavy chain of the B cell receptor[4]. Eplets have been theoretically defined based on the amino acid sequences of HLA alleles, necessitating experimental antibody verification to confirm their actual interaction with anti-HLA antibodies. Reactivity pattern analysis of an HLA-specific human monoclonal antibody (mAb) tested in a single antigen bead (SAB) assay is an excellent method for antibody-verification of eplets[5, 6]. However, occasionally SAB analysis of a mAb identifies multiple distant amino acids that are uniquely shared by the reactive HLA isoforms but cannot be part of the same eplet because the residues are not located within 3.5 Ångstrom from each other. Currently, a number of eplets listed in the HLA Epitope Registry do not adhere to such spatial definition, and therefore we recently introduced the term antibody-verified reactivity pattern to describe these eplets[7].

One of the antibody-verified reactivity patterns for HLA class I, 44K/150V/158V, has been verified using the HLA-A1/A36-specific human mAb VDK1D12. Residues 44K, 150V and 158V are all uniquely shared by HLA-A*01:01 and HLA-A*36:01 and are always simultaneously present on common HLA isoforms, limiting the possibilities to investigate which of these three residues is actually crucial for antibody binding using SAB or cellular assays[7]. In this proof-of-concept study, we performed site-directed mutagenesis on the HLA-A*01:01 molecule to investigate whether a point mutation of single amino acids can impede the binding of the VDK1D12 mAb to its target HLA molecule. We show that this approach can be used to narrow down the functional epitope or eplet of an HLA-specific mAb to a single amino acid, contributing to our understanding of HLA immunogenicity and antigenicity.

2 METHODS

2.1 Single antigen bead assay

The human IgM mAb VDK1D12 was previously produced by a cloned B cell hetero-hybridoma derived from a pregnancy-immunized individual, as described by Mulder et al.[8] and was tested on combined LABScreen (One Lambda, West Hills, CA, USA) LS1A04 and LS1AEX01 SAB using FLEXMAP 3D System (Luminex, Austin, TX, USA). Anti-human IgM-PE (Jackson ImmunoResearch, Ely, Cambridgeshire, UK, Code Number 709-116-073) was used as detection antibody. Data were analyzed with HLA Fusion version 4.6 (One Lambda).

2.2 Expression constructs and transfection

cDNA of HLA-A*01:01 based on IPD-IMGT/HLA Database (https://www.ebi.ac.uk/ipd/imgt/hla/) and individual mutants with single amino acid mutations (K44A, K44R, V150A or V158A) were synthesized and cloned into an OLI expression vector, sequence confirmed, and plasmid prepped by GeneArt (Thermo Fisher Scientific, Waltham, MA, USA). Mutants K44R, V150A and V158A were selected because these are the self-residues of the antibody producer. Host cells derived from a human B cell lineage lacking endogenous HLA Class I expression were cultured in RPMI1640 plus 10% fetal bovine serum. The Neon™ Transfection System (Thermo Fisher Scientific) was used to electroporate expression plasmid into host cells following the manufacturer's instructions. Part of the transfected cells were harvested on day 3 post transfection for transient expression analysis by flow cytometry; the remainder of the cells were placed on antibiotic selection to establish a stable pool following standard recombinant cell line generation protocols.

2.3 Flow cytometry

Cells were stained with primary and secondary antibodies according to standard flow cytometry protocols. Viability dye 7-AAD (Sigma-Aldrich, St. Louis, MO, USA) was added to gate out dead cells. Cells were acquired by an Accuri C6 Plus (BD Biosciences) flow cytometer and analyzed using FlowJo Software version 10.8.1 (BD Biosciences, Ashland, OR, USA). Pan anti-HLA class I mouse IgG mAb FR3315 (One Lambda, West Hills, CA, USA) was used as a positive control. Fluorescence labeled secondary Abs anti-human IgM-PE (Code Number 709-116-073) and anti-mouse IgG-FITC (Code Name 115-095-072) from Jackson ImmunoResearch laboratories were used to detect VDK1D12 and FR3315 respectively. Human IgM (Catalog Number 31146, Invitrogen) and IgG isotype Ab (Catalog Number 555742, BD Pharmingen) were used as the negative control.

3 RESULTS

3.1 Specificity of VDK1D12

Previously published SAB data of VDK1D12 tested with Lifecodes (Immucor) showed strong reactivity against HLA-A*01:01 and HLA-A*36:01. Subsequent analysis with HLA-EMMA[9] demonstrated that three amino acids were uniquely shared by the reactive HLA isoforms: 44K, 150V and 158V[7], corresponding to eplet 44KM in the HLA Epitope Registry[10]. Here, VDK1D12 was tested in the LABScreen SAB assay and showed a similar reactivity pattern, with strong reactivity against HLA- A*01:01, HLA-A*01:02 and HLA-A*36:01 (Figure 1). Similar to the previously published SAB results, there were several lower reactive beads. Since these HLA isoforms were negative in previously performed complement-dependent cytotoxicity assays and became negative upon dilution in SAB, they were not considered in this study[7,11].

	HLA Allele	MFI	44	150	158
I	**A*01:02**	**11215**	**K**	**V**	**V**
I	**A*01:01**	**10646**	**K**	**V**	**V**
	A*36:01	**10102**	**K**	**V**	**V**
	A*69:01	3398	R	A	A
	A*33:01	2550	R	A	A
	A*66:01	1257	R	A	A
	A*68:02	1121	R	A	A
	B*15:11	1078	R	A	A
	A*26:02	1030	R	A	A
	A*34:02	935	R	A	A
	A*66:02	573	R	A	A
	A*25:01	543	R	A	A
	A*26:01	479	R	A	A
	A*33:03	446	R	A	A
	A*80:01	395	R	A	A
	A*02:01	282	R	A	A
	A*68:01	249	R	A	A
S	A*03:01	55	R	A	A
S	C*04:01	11	R	A	A
S	A*31:01	6	R	A	A
S	B*35:01	3	R	A	A
S	B*35:03	1	R	A	A
	Other class I beads	<200	R	A	A/T

Figure 1. Reactivity analysis of mAb VDK1D12 tested in single antigen bead assay. Amino acid residues on position 44, 150 and 158 are depicted for the HLA class I isoforms of interest. Beads in bold are considered positive and amino acids in bold are uniquely shared by the reactive beads. The immunizer's typing was HLA-A1. The mAb was tested in a concentration of 0.25 ug/ml. I, immunizer; MFI, mean fluorescence intensity; S, self HLA of antibody producer.

3.2 Mapping the crucial amino acid for binding of VDK1D12 by mutagenesis

In order to elucidate which of the three residues was crucial for antibody binding, four HLA-A*01:01 mutants were synthesized; K44A, K44R, V150A and V158A. Transient and stable pool expression of HLA-A*01:01 and mutants were confirmed by flow cytometry with pan anti-HLA Class I mAb FR3315 resulting in almost identical profiles, indicating that the residue changes had little impact on expression levels and global conformation. In contrast, binding to VDK1D12 was disrupted when lysine (K) at position 44 was replaced by either arginine (R) or alanine (A), while valine (V) substitution with alanine at position 150 or 158 had no impact on binding to VDK1D12 (Figure 2). This identifies residue 44K as the crucial amino acid for VDK1D12 to bind to its target HLA. (Figure 3).

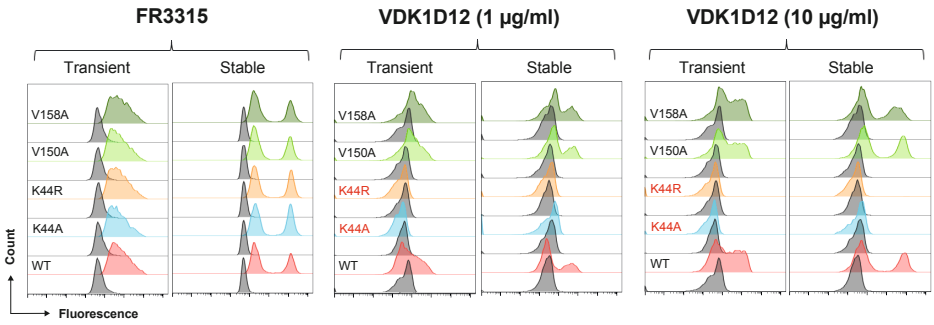


Figure 2. Transient and stable expression patterns of HLA-A*01:01 wildtype and mutants detected by FR3315 and VDK1D12. Flow cytometry analysis demonstrated that K44A and K44R mutants lost signal, while V150A and V158A did not, when stained with VDK1D12 at both 1 ug/ml and 10 ug/ml.

4 DISCUSSION

While accumulating evidence demonstrates the association between HLA eplet mismatches and transplant outcomes, identification of clinically relevant eplets using antibody-verification is still an uncompleted endeavor[3, 12]. Previous studies have shown that human mAbs are excellent tools for this purpose[5, 6]. However, occasionally, multiple uniquely shared residues that are > 3.5 Ångstrom apart are identified, which means that these residues cannot be part of a single eplet[6, 7]. As these residues are always simultaneously present on the reactive HLA isoforms, it is not possible to determine which of these residues are truly crucial for antibody-binding, because of the limitations of the SAB panels or cellular experiments with natively expressed HLA. In this study, we show that site-directed mutagenesis of a wildtype HLA molecule is an excellent approach to determine which amino acid is crucial for binding of a mAb to its target HLA.

In mutation studies, a positive control antibody is essential to confirm that the expression profile is not grossly affected simply due to the introduced amino acid changes. Although the targeted epitope of the pan anti-HLA class I mAb FR3315 is not known, it is likely to be a conserved epitope distinct from the region where residues 44, 150 and 158 are located, as evidenced by the preserved binding upon mutation. Binding to FR3315 also indicates that the local conformational change introduced by the specific mutation does not cause a global conformational change that could non-specifically affect the binding to the antibody. In addition, this cell-based approach not only bypasses the time and efforts required for extensive cell culturing, antigen purification, and bead coating steps to prepare single antigen beads, but also provides confidence that the antigen expressed on the cell surface adopts its native conformation. Furthermore, binding profiles comparing wildtype and mutants from transient expression can be obtained within one week (upon the availability of expression constructs), which makes this approach feasible for high throughput investigation of HLA reactivity patterns and eplets.

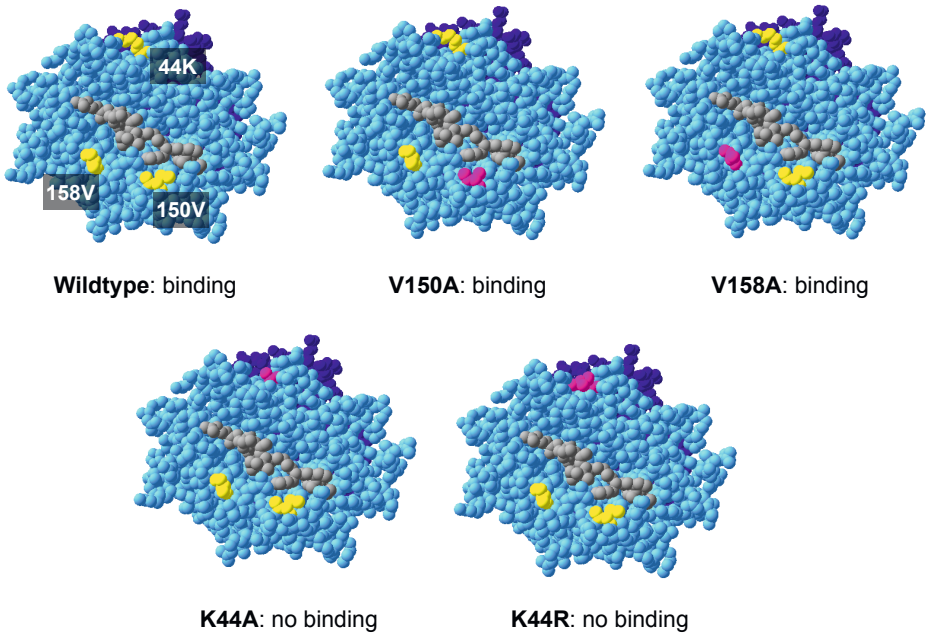


Figure 3. Mutation of residue 44K prevents binding of mAb VDK1D12 to HLA-A*01:01. Location of residues 44K, 150V and 158V on the A*01:01 molecule (PBD: 3BO8) visualized in Swissviewer version 4.1 (https://spdbv.unil.ch/). Residues of interest are mutated using the Swissviewer software. The HLA molecule is viewed from the top, with the α chain depicted in light blue, the β chain in dark blue, and the peptide in grey. Yellow color indicates the residues of interest and pink indicates the mutated residue. A, alanine; K, lysine; R, arginine; V, valine; PBD, Protein Data Bank.

Through this method of site-directed mutagenesis, we were able to narrow down the antibody-verified reactivity pattern 44K/150V/158V to a single amino acid and define 44K as the functional epitope of mAb VDK1D12. Currently, the corresponding eplet 44KM is defined in the HLA Epitope Registry as 44K 45M (149A 150V 151H 152A) (158V). We request the recently established HLA Epitope Registry Oversight Panel to redefine eplet 44KM to the antibody-verified eplet 44K and the non-verified eplets 150V and 158V.

We are of the opinion that the current method should be incorporated in the HLA Epitope Registry as a minimal requirement to elucidate antibody reactivity patterns and eplet pairs. This streamlined approach will be further used to determine functional epitopes of the previously described HLA-DQ reactivity patterns[6], and will allow for the further characterization of factors influencing antibody induction and antibody affinity.

Disclosure

This work was funded by the National Reference Center for Histocompatibility Research, Leiden University Medical Center, the Netherlands and One Lambda, Inc. (a part of Thermo Fisher Scientific Inc.).

REFERENCES

1. Mayrdorfer M, Liefeldt L, Wu K, et al. Exploring the Complexity of Death-Censored Kidney Allograft Failure. *J Am Soc Nephrol*. 2021;32(6):1513-1526.
2. Loupy A, Lefaucheur C. Antibody-Mediated Rejection of Solid-Organ Allografts. *N Engl J Med*. 2018;379(12):1150-1160.
3. Bezstarosti S, Kramer CSM, Claas FHJ, et al. Implementation of molecular matching in transplantation requires further characterization of both immunogenicity and antigenicity of individual HLA epitopes. *Hum Immunol*. 2022;83(3):256-263.
4. Duquesnoy RJ. A structurally based approach to determine HLA compatibility at the humoral immune level. *Hum Immunol*. 2006;67(11):847-62.
5. Kramer CSM, Franke-van Dijk MEI, Bakker KH, et al. Generation and reactivity analysis of human recombinant monoclonal antibodies directed against epitopes on HLA-DR. *Am J Transplant*. 2020;20(12):3341-3353.
6. Bezstarosti S, Kramer CSM, Franke-van Dijk MEI, et al. HLA-DQ-Specific Recombinant Human Monoclonal Antibodies Allow for In-Depth Analysis of HLA-DQ Epitopes. *Front Immunol*. 2021;12:761893.
7. Bezstarosti S, Bakker KH, Kramer CSM, et al. A Comprehensive Evaluation of the Antibody-Verified Status of Eplets Listed in the HLA Epitope Registry. *Front Immunol*. 2021;12:800946.
8. Mulder A, Kardol MJ, Arn JS, et al. Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. *Mol Immunol*. 2010;47(4):809-15.
9. Kramer CSM, Koster J, Haasnoot GW, et al. HLA-EMMA: A user-friendly tool to analyse HLA class I and class II compatibility on the amino acid level. *HLA*. 2020;96(1):43-51.
10. Duquesnoy RJ, Marrari M, Mulder A, et al. First report on the antibody verification of HLA-ABC epitopes recorded in the website-based HLA Epitope Registry. *Tissue Antigens*. 2014;83(6):391-400.
11. Fernandez-Vina M, Maiers M, Gutierrez M, et al. 13th IHWS Serology and HLA Phenotypes Joint Report. In: Hansen JA, ed. *Immunobiology of the Human MHC - Proceedings of the 13th International Histocompatibility Workshop and Conference - Volume I*. IHWG Press; 2006:890-931.
12. Heidt S, Claas FHJ. Not all HLA epitope mismatches are equal. *Kidney Int*. 2020;97(4):653-655.