Towards HLA epitope matching in clinical transplantation

Kramer, C.S.M.

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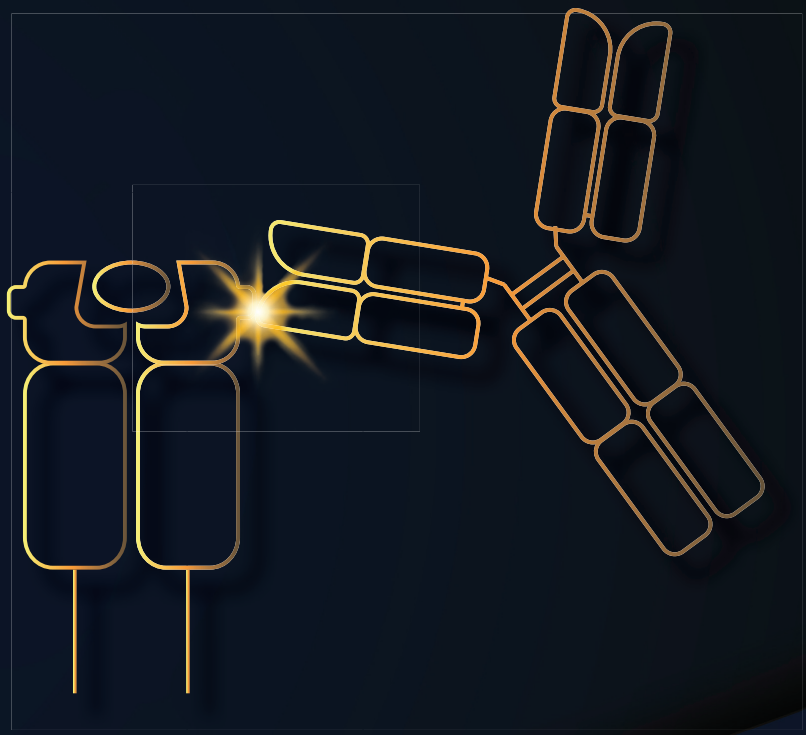

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Towards HLA epitope matching in clinical transplantation

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CHAPTER

1

GENERAL INTRODUCTION

The immune system

For humans to protect themselves against harmful pathogens it is crucial that the immune system can discriminate between self and non-self antigens. The first line of defence against foreign pathogens is the effective and rapid, but non-specific innate immune system that consist of the physiological epithelial barrier, plasma proteins such as those of the complement system, phagocytic cells like macrophages, mast cells, neutrophils, and natural killer cells that destroy cells expressing non-self patterns, and dendritic cells. These dendritic cells can also act as a bridge with the adaptive immune system.

This adaptive immune system is diverse and specific as it consists of the humoral immune response consisting of antibodies produced by B cells, and the cellular immune response that is mainly driven by T cells. Both these responses are only induced when specific antigens are recognised by the somatically rearranged receptors expressed on B and/or T cells. Once B or T cells have encountered an antigen for the first time, the primary response, a proportion of the naïve cells will differentiate into long-lived memory cells, which can induce a more rapid and effective response upon re-exposure to the same antigen, the secondary response.

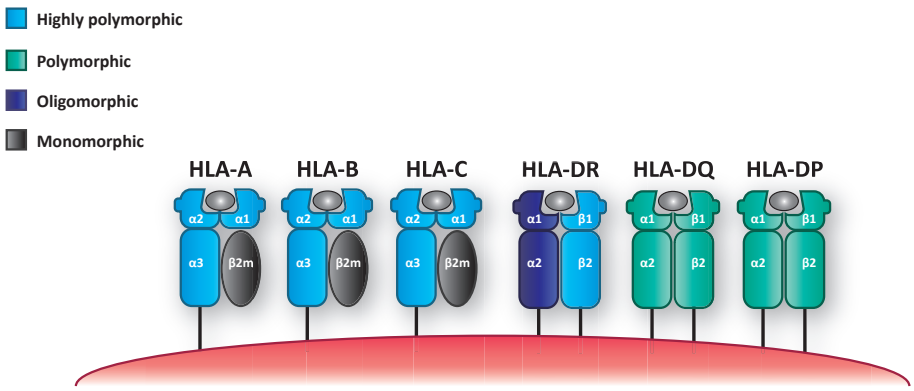

Figure 1: HLA class I and HLA class II molecules. HLA-A, HLA-B and HLA-C molecules have a highly polymorphic alpha chain in complex with a monomorphic β2-microglobulin. HLA-DR molecule has a highly polymorphic beta chain and an oligomorphic alpha chain. HLA-DQ and HLA-DP molecules consist of polymorphic alpha and beta chain.

HLA system

One of the key players of the human immune system are the human leukocyte antigens (HLA) of which there are two classes. HLA class I consist of the classical HLA-A, -B and -C antigens of which the genes encode for a polymorphic α-chain which forms a molecular complex with the monomorphic β2-microglobulin (Figure 1).[1,2] In contrast, the HLA class II HLA-DR, -DQ and

-DP genes code for two chains (Figure 1), the α- and β chains. Both chains are polymorphic for HLA-DQ and -DP whereas for HLA-DR only the β-chain is polymorphic and the α-chain oligomorphic. There are four HLA-DRB genes, namely HLA-DRB1, -DRB3, -DRB4, and -DRB5.[3-5] An individual inherits one HLA haplotype from the mother and one from the father and can thus express up to fourteen different HLA molecules.

HLA class I molecules are expressed on all nucleated cells and platelets, and their function is to present peptides derived from proteins inside the cell to cytotoxic T cells ($CD8^+$). In case these are non-self peptides (for example viral origin) this subsequently results in killing of the target cell. In contrast, HLA class II molecules are mainly expressed on antigen presenting cells, such as dendritic cells, monocytes, macrophages and B cells, and on activated endothelial cells. These HLA class II antigens present peptides derived from proteins outside the cell to helper T cells ($CD4^+$), which can activate macrophages, cytotoxic T cells, or B cells to secrete antibodies.[6] As different HLA antigens can present different peptides there are a wide variety of HLA alleles across individuals. This high polymorphism of HLA ensures that there is always an individual that can present a peptide of a particular pathogen, which protects the human population from new or mutated pathogens. So far 18.742 HLA class I and 7.060 HLA class II alleles have been identified, but this number is still growing mainly due to more sophisticated typing methods.[7]

Alloimmune response in transplantation

While the high diversity of HLA allele is beneficial for immunological defence against pathogens, it is unfavourable for (solid organ) transplantation. Already in 1960's it was observed that HLA matching is pivotal in kidney transplantation, as it was associated with a better allograft survival.[8] Donor HLA molecules of the graft can be recognised as foreign by the recipient's immune system, and the higher the number of HLA mismatches the higher the chance that this occurs. Early after transplantation recipient T cells can recognise intact HLA molecules expressed on antigen presenting cells of the donor, which can lead to cytotoxic T cell response. This phenomenon is known as direct allorecognition.[9,10] In addition, indirect allorecognition can occur when foreign HLA molecules of the donor are taken up and processed by recipient antigen presenting cells and subsequently presented as peptide in the context of self HLA to helper T cells.[10,11] Lastly, there is evidence that intact allogeneic HLA molecules can be expressed on recipient antigen presenting cells, known as semi-direct allorecognition.[10] While direct allorecognition is often associated with early graft rejection and indirect allorecognition with chronic rejection, the different pathways can interact and therefore can cause both acute and chronic rejection. Due to the crucial role of T cell

alloimmunity in graft rejection, the vast majority of current immunosuppressive treatment is aiming at suppression of T cell activation.[12]

During more recent years it became apparent that also the humoral alloimmune response, induced upon transplantation, plays an important role. The B cell receptor (BCR) on alloreactive recipient B cells recognise the donor HLA molecule as foreign (Figure 2) and produce HLA-specific antibodies. For a full-blown antibody response, B cells need to switch from IgM towards IgG antibody production which can be accomplished by the interaction with T helper cells that recognise T cell epitopes, derived from the donor HLA, presented by recipient's HLA class II molecules on the B cells (Figure 2).[13,14]

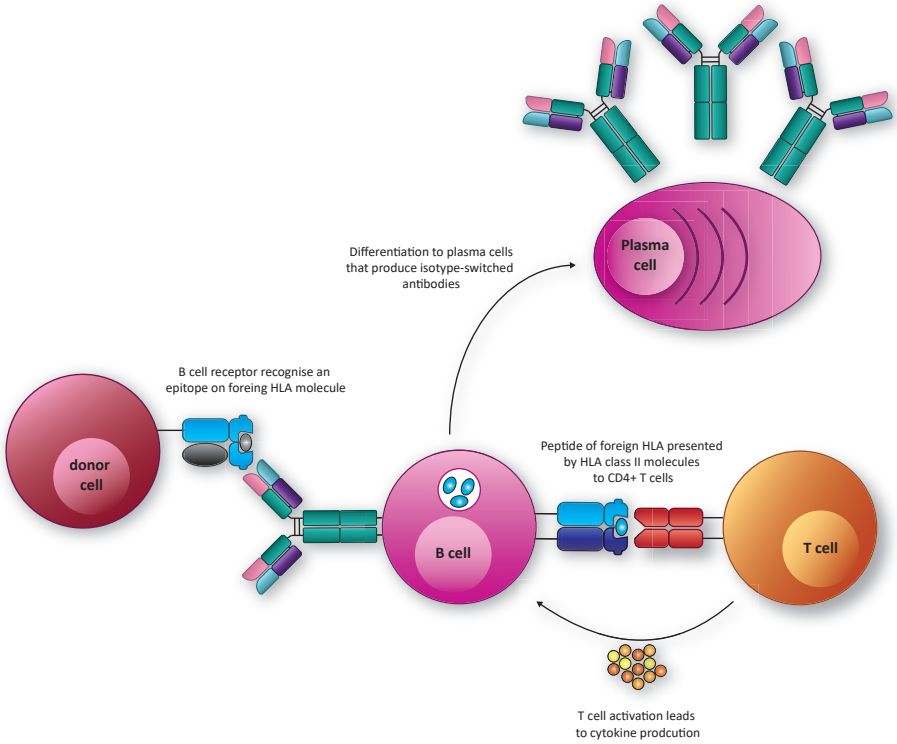

Figure 2: HLA-specific antibodies are produced after B cell receptor recognise donor HLA molecule as foreign. B cell receptor on alloreactive B cells recognise foreign epitope on donor HLA molecule. As a result, donor HLA molecule is internalised and degraded into peptides of which one is presented by recipient HLA class II molecules to CD4+ helper T cells. Subsequently, the helper T cell is activated and start producing cytokines and other immunoregulatory molecules that lead isotype-switched antibody production by plasma cells.

Indeed, recipients that receive an HLA mismatched graft can form *de novo* donor-specific antibodies (DSA) which are associated with inferior graft survival.[15-18] In addition, the presence of these DSA reduces the chance of repeat transplantation,[19] as these antigens are defined as unacceptable. This is done to reduce the risk of hyperacute rejection that may occur when antibodies already present before transplantation are able to react with donor HLA on a graft.[20,21] For that reason a serological or virtual crossmatch is performed prior to transplantation in order to exclude transplantation of a graft towards which the potential recipient has DSA. This matter is further complicated since DSA are not only specific for the mismatched HLA, the immuniser, but can also cross-react with other HLA antigens, due to the sharing of determinants or antibody epitopes between different HLA antigens.[22-24] These detrimental HLA antibodies are not only found after transplantation but can also be formed after pregnancy,[25] or blood transfusion.[26,27]

Outline of thesis

Despite the improved surgical techniques and more potent immunosuppression HLA matching on the antigen level is still beneficial for kidney transplantation.[28,29] However, due to the high polymorphism of HLA and scarcity of organs the chance of finding a fully HLA matched donor is slim. The development of high resolution HLA typing, luminex single antigen bead assay and HLA crystal structures has resulted in increased knowledge about HLA molecules. It has become clear that each HLA molecule consists of a unique set of polymorphic amino acid configurations, often referred to as epitopes, but an individual epitope can be shared by multiple HLA molecules.[30] As a result, the number of epitope mismatches for one HLA antigen mismatch depends on both the HLA phenotype of the recipient and the donor HLA mismatch (Figure 3). Recent studies suggest that epitope matching might be the basis of novel allocation strategies to prevent *de novo* DSA formation after transplantation.[31-34]

While the B cell epitope or structural epitope is the complete surface area of antigen that interacts with antibody, a small configuration of amino acids, the functional epitope, determines the specificity of antibody response via its interaction with complementarity-determining region 3 of the heavy chain (CDR-H3).[35-37] To prevent *de novo* DSA formation, it is pivotal to define the immunogenic polymorphic amino acids that can trigger an antibody response. Defining to which polymorphic amino acid configurations an antibody can actual bind to will contribute in defining acceptable and unacceptable HLA antigens for immunised recipients.

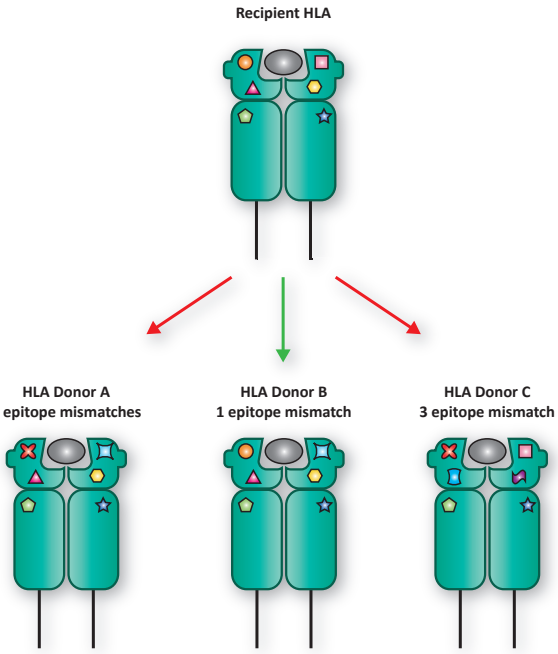

Figure 3: The number of epitope mismatches depends on epitope repertoire of recipient's HLA. From all three potential donors, donor B is the best option as it only has 1 epitope mismatch compared to recipient, while donor A has 2 epitope mismatches and donor C has 3 epitope mismatches and therefore are more likely to be immunogenic.

Various approaches have been introduced over the years to predict the immunogenicity of HLA molecules and subsequently the chance of developing *de novo* DSA. In **Chapter 2** and **Chapter 3**, we reviewed the strategies of these approaches to define immunogenicity, which all have been shown to associate with *de novo* DSA formation on the population level. In addition, the differences between immunogenicity and antigenicity is described, which is crucial for defining the clinical relevant epitopes (**Chapter 2**). We also highlight the differential immunogenicity of individual epitopes and that therefore novel matching strategies should not only be based on numbers (**Chapter 3**).

Eplets are one of these approaches and are theoretically defined configurations of surface exposed polymorphic amino acids within 3 to 3.5 Å radius. Eplet mismatches between donor and recipient have been associated with *dn*DSA formation,[32-34,38-41] transplant glomerulopathy,[42] and antibody-mediated rejection.[43-45] However, not every eplet mismatch will induce an antibody response, and the theoretically defined eplets require experimental verification to establish whether an antibody can actual bind to these residues. Human mAbs[46]

and absorption and elution studies[47,48] have shown to be useful for antibody-verification, but those of HLA class II are limited due to lack of HLA class II mAbs.[49,50] Therefore our aim was to develop recombinant human HLA class II mAbs to facilitate the definition of actual antibody epitopes. In **Chapter 4**, we used established human B-cell heterohybridomas to verify a method to generate recombinant human HLA mAbs of all four IgG subclasses. Next in **Chapter 5**, we used this method to generate recombinant human HLA-DR-specific mAbs from low frequency HLA-DR specific memory B cells that were isolated from peripheral blood of pregnancy immunised individuals using HLA-DR tetramers. Reactivity analysis of these generated HLA-DR mAbs was performed to define uniquely shared amino acid or amino acid configurations on the reactive HLA alleles, and those were mapped to the pre-defined eplets.

Another way to define the relevant epitopes is by identifying the immunogenic polymorphic amino acid to define relevant amino acid configurations. In **Chapter 6**, we describe the development of the software program HLA-EMMA to compare the amino acid sequence of the mismatched donor HLA allele with the amino acids sequences of the recipient of the same locus. HLA-EMMA provides not only the number of solvent accessible amino acid mismatches, but also the type and position of the mismatched amino acids. As *de novo* DSA directed against HLA class II are the most prominent after transplantation, we aimed to define the most immunogenic HLA class II amino acids. For this, we performed a pilot study of non-immunised male renal transplant recipients with at least one HLA class II antigen mismatch, who lost their graft due to immunological failure (**Chapter 7**). With HLA-EMMA the solvent accessible amino acid mismatches were determined and these mismatches were associated with *de novo* DSA formation.

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CHAPTER

2

DEFINING THE IMMUNOGENICITY AND ANTIGENICITY OF HLA EPITOPES IS CRUCIAL FOR OPTIMAL EPITOPE MATCHING IN CLINICAL RENAL TRANSPLANTATION

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ABSTRACT

Transplantation of an human leukocyte antigen (HLA) mismatched graft can lead to the development of donor-specific antibodies (DSA), which can result in antibody mediated rejection and graft loss as well as complicate repeat transplantation. These DSA are induced by foreign epitopes present on the mismatched HLA antigens of the donor. However, not all epitopes appear to be equally effective in their ability to induce DSA. Understanding the characteristics of HLA epitopes is crucial for optimal epitope matching in clinical transplantation. In this review, the latest insights on HLA epitopes are described with a special focus on the definition of immunogenicity and antigenicity of HLA epitopes. Furthermore, the use of this knowledge to prevent HLA antibody formation and to select the optimal donor for sensitized transplant candidates will be discussed.

INTRODUCTION

Human leukocyte antigen (HLA) matching in renal transplantation is an important factor influencing long-term graft survival.[1,2] The chance of finding an unrelated fully matched kidney donor is slim due to the high level of polymorphism of HLA antigens.[3] So far, matching in renal transplantation has mainly been done for HLA-A, -B, and -DR antigens and even then, most recipients receive a graft mismatched for at least one or more HLA antigens. Recipients of a mismatched graft have an increased chance to develop donor-specific antibodies (DSA), which are associated with poor allograft survival.[4-6] Furthermore, the development of DSA complicates repeat transplantation, a problem that is most obvious for highly sensitised patients, who generally experience long waiting times.

Immunisation by a single HLA antigen mismatch can result in antibodies directed against numerous other HLA antigens, which is called cross-reactivity.[7-9] It has previously been shown that this cross-reactivity is caused by sharing of determinants to which an antibody can bind, called epitopes, by various HLA antigens.[9-11] In the early 1990's, HLA antigens that shared an epitope were assigned to cross-reactive antigen groups (CREG)[12,13] and matching based on these CREGs appeared to be associated with a better graft survival.[14] The antibody-reactivity patterns observed in sensitised patients also indicated that a relatively small number of epitopes are involved in antibody induction and that the emerging antibodies are directed against common epitopes.[15,16]

Identifying antibody -or B cell- epitopes on HLA antigens and understanding their immunogenicity and antigenicity will be imperative for the development of novel matching strategies that aim at reduced antibody induction after transplantation, as well as identification of acceptable mismatches for highly sensitized patients. The application of high resolution molecular HLA typing has resulted in an increased knowledge of the amino acid sequences of HLA alleles, enabling the identification of polymorphic positions, as well as a better understanding of the quaternary structure of the HLA by modelling of the crystalline HLA molecule structures.[17-20] These tools have become very useful for defining polymorphic areas harbouring theoretical antibody epitopes. In addition, highly sensitive single antigen bead (SAB) based HLA antibody identification assays have been introduced, providing antibody-reactivity patterns with extensive specificity patterns on the allele level.[21] The latter is of great importance to determine the actual epitopes recognised by HLA antibodies. In this review, we will discuss the latest insights into antibody epitopes and the difference between immunogenicity and antigenicity. A proper definition of the immunogenic HLA

epitopes and understanding the interaction between HLA antigen and antibody is crucial for the improvement of matching strategies in clinical transplantation.

Box 1. Various definitions of epitopes
Eplet/functional epitope: polymorphic amino acid configuration that triggers an antibody response (defined by R. Duquesnoy)
Immunogenic epitope: polymorphism that triggers an antibody response
Structural epitope: all polymorphisms that are covered by an antibody footprint

STRATEGIES TO DEFINE HLA EPITOPES

Theoretical epitopes

Epitopes are defined as parts of an HLA molecule that are recognised by the immune system as foreign, which implies the involvement of amino acids (or amino acid sequences) not present on self-HLA antigens (Box 1). In case of antibody epitopes, the polymorphic amino acids must be at sites that are accessible by an antibody molecule. Antibodies recognise conformational epitopes, which can be both linear strands of amino acids (linear epitope), or amino acids in close proximity in the three-dimensional structure of a molecule (discontinuous epitope). In a pioneering attempt to define antibody epitopes on HLA molecules, Duquesnoy *et al.* used linear amino acid sequences of serologically defined HLA antigens together with known molecular three-dimensional structures to determine polymorphic amino acids at antibody accessible positions for each HLA class I locus.[22] These residues were proposed as being the critical components of an immunogenic linear epitope and resulted in the identification of linear sequences of maximal three polymorphic amino acids at the molecular surface that were referred to as triplets.[22,23]

As mentioned above, antibodies can also recognise epitopes formed by polymorphic amino acids from different parts of the molecule that come into close proximity due to the folding of the protein. Indeed, analyses of the available quaternary HLA molecule structures with Cn3D software (https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml)[19] clearly show that the triplet concept does not fully cover all theoretical epitopes, as discontinuous polymorphic amino acids form patches, with a radius of approximately 3 angstrom (Å), on or near the molecular surface. Consequently, a cluster of polymorphic amino acids, either linear or discontinuous, within a 3-3.5 Å radius of an antibody-accessible sequence position was defined as being capable of inducing an antibody response and was named an eplet.[24,25] So

far, many theoretical HLA class I and HLA class II eplets have been defined using this method and are listed in the HLA epitope registry (http://epregistry.ufpi.br/terms/index).[26,27]

It has become clear that each HLA allele consists of a unique combination of epitopes, but that the three classical HLA class I loci share certain epitopes. In addition, the location of the polymorphic amino acids of the three classical HLA class I loci turned out to be similar. Consequently, for epitope analysis, HLA class I alleles should not only be compared with alleles from the same locus, called intralocus, but also interlocus by which the alleles are additionally compared with the alleles of the other HLA class I loci. This principle has been applied for almost every HLA class I epitope study. In contrast, the epitopes of HLA class II loci are analysed intralocus due to the fact that HLA-DR has a highly polymorphic beta chain and an oligomorphic alpha chain, while HLA-DQ and HLA-DP molecules have two polymorphic chains. Therefore, the defined clusters of antibody accessible polymorphic amino acids are different between the three loci and thus HLA class II epitope should be analysed separately per locus. Regardless, further studies are necessary to prove that the use of interlocus comparison of HLA class I and intralocus comparison of HLA class II is the optimal way to define the number of eplet/epitope mismatches.

A computer algorithm named HLAMatchmaker was developed by Rene Duquesnoy, originally based on triplets, and later modified for eplets as the critical component of immunogenic epitopes that can elicit an antibody response (http://www.epitopes.net/). An important component of the algorithm is that antibodies cannot be induced against eplets present on a self-HLA antigen.[22,23,25,28] This algorithm can be used to determine for each mismatched donor HLA antigen which eplet is non-self, compared to the repertoire of self-eplets on the HLA antigens of the recipient, resulting in a quantification of eplet mismatches. Additionally, the algorithm can identify eplets involved in antibody-reactivity patterns detected in patient's serum.

Antibody-verified epitopes

Eplets have been defined based on sequence comparisons and available quaternary three-dimensional structures of HLA molecules. However, this does not mean that every eplet defined is indeed able to induce antibodies. These potential antibody-inducing patches need verification by laboratory tests showing antibody binding to these structures. Studies by various groups using monoclonal antibodies (mAbs) directed at defined HLA molecules showed that these reagents are very useful to identify shared antibody eplets between HLA antigens.[29,30] Especially human mAbs have been used in SAB assays to successfully verify

an extensive number of eplets.[31,32] Despite this, many eplets should for the moment be considered as theoretical eplets until it is proven that they are indeed being recognised by alloantibodies.

El-Awar *et al.* used an alternative approach to define antibody-verified epitopes.[33] Alloantibodies and mAbs were absorbed and eluted from recombinant single HLA antigen expressing cell lines and HLA antibody specificity was determined with SAB assays. The amino acid sequences of the reactive HLA alleles were compared and the exposed polymorphic amino acids within an antibody-binding region of approximately 700-900 Å, that were exclusively shared between the reactive HLA alleles, were considered to define the antibody epitope. The number of unique amino acids of these epitopes is between one and four, located in discontinuous positions. The epitopes defined by this method have been named Terasaki epitopes (TerEp).[34-38] As the TerEps are defined by one or a combination of amino acids it can occur that a residue at a specific position of an epitope on the immunising allele is also present on an allele of the recipient, yet this is not defined as a self-epitope as the combination of amino acids of the epitope is different.[39]

Similar to the mAb-verified eplets, for TerEps it has formally been proven that these are targets for antibodies. Likewise, for this method the limited number of mAbs and allosera studied so far makes it likely that the current list of TerEps is not complete. Comparative studies observed a huge overlap between eplets and TerEps, with only a small number of TerEps lacking a corresponding eplet.[40,41] Another limitation of the use of mAbs for the verification of antibody-verified epitopes is the restriction to common HLA alleles. As a consequence, the epitopes that are currently verified are mainly present on common HLA alleles, whereas epitopes on rare HLA alleles remain unverified. This poses a disadvantage for ethnic minorities as it will be more difficult to identify antibody-verified epitopes for these populations.

Other means of verifying epitopes on HLA antigens have been described, but one should be cautious to draw any definitive conclusions from these approaches. In a recent study, postpartum sera of women were used to screen for novel antibody-verified eplets.[42-44] Sera from women after one or two pregnancies were screened with SAB assays and the antibody-reactivity pattern was analysed for new antibody-verified epitopes. In contrast to the TerEp studies, no absorption and elution assays were performed. On basis of this type of reagents it is impossible to rule out that the antibodies are produced by more than one B cell clone, leaving the possibility that multiple epitopes are involved in the final antibody reactivity. Furthermore, analysis of sera obtained after a second pregnancy may be complicated by the

interference of antibodies induced after the first pregnancy by a different haplotype. This emphasises the importance of strict guidelines for verifying epitopes based on antibody-reactivity pattern observed in sera. In our opinion, the only way to formally verify an antibody epitope is by (human) mAbs and/or absorption-elution studies.

IMMUNOGENICITY OF AN ANTIBODY EPITOPE

For a proper use of epitope matching in clinical transplantation, the immunogenicity and antigenicity of epitopes should be defined first. Although these are different characteristics of an epitope, these terms are used often interchangeably, which is not correct. Immunogenicity is the ability to induce an antibody response while antigenicity is based on the actual interaction between an antibody and an antigen, which involves both the polymorphic amino acids comprising the immunogenic epitope and other crucial polymorphic amino acid configurations that act as contact sites.

Induction of antibodies

In theory, any amino acid on a particular position of a donor HLA molecule that is not present on the recipient's HLA molecules has the ability to trigger an antibody response. However, whether an epitope truly induces an antibody response is dependent on the total make up of HLA molecules of the recipient. Dankers *et al.* showed that HLA-A28 positive women who delivered an HLA-A2 positive child more often form antibodies against HLA-A2, while women who have HLA-A2 themselves and delivered an HLA-A28 child did not develop antibodies against this HLA antigen. These observations could be explained by an immunogenic epitope that is unique for HLA-A2. Thus, when HLA-A2 is a self-antigen, the unique immunogenic epitope is self and will not trigger an antibody, while in case of an HLA-A2 mismatch the epitope is more likely to trigger an antibody response.[45]

In population studies, various groups have shown (a) correlation between the number of epitope mismatches between donor and recipient, and DSA development, thus demonstrating a quantitative effect of mismatches on the immunogenicity of an HLA molecule. However, one or a few epitope mismatches can be sufficient to induce an antibody response.[46-48] This indicates that immunogenicity is not merely a quantitative issue. The characteristics of the amino acid substitution, in relation to the patient's own HLA molecules, are important for the induction of an antibody. A high number of epitope mismatches can be an indicator for the risk of forming an antibody because the chance is higher that one of these mismatched epitopes

is immunogenic. However, for an individual patient the nature of the epitope mismatch determines if an antibody response is actually triggered.

A prerequisite for an epitope to be immunogenic is that the induced DSA reacts with that particular epitope. Upon transplantation, patients receive maintenance immunosuppression, which may interfere with DSA formation.[49,50] It is conceivable that not every epitope mismatch results in an antibody response due to the immunosuppressive medication. DSA detected in transplant recipient might be restricted to highly immunogenic epitope mismatches that escape the suppressive properties of the medication.[48]

As discussed above, both eplets and TerEps are polymorphic amino acid configurations on donor HLA alleles that are non-self. However, while amino acids on a specific position can be shared between donor and recipient, the combination of amino acids in a configuration can be mismatched. The question is whether such configurations are indeed necessary for antibody induction or if a single amino acid difference between recipient and donor on an antibody accessible position can already determine its immunogenicity. Possibly both scenarios are valid, since some of the eplets and TerEps consist of only a single non-self amino acid.[26,27,37] Recently, Kosmoliaptsis and colleagues compared the number of eplet mismatches and the number of HLA class I and II amino acid mismatches after intralocus and interlocus comparison between donor and recipient alleles as predictive parameters for DSA induction. This population study showed that both the number of eplet mismatches and the number of polymorphic amino acid mismatches are predictors of DSA formation. In this study, no advantage of the eplet approach over the number of amino acid mismatches was observed.[51-53]

Furthermore, the same group demonstrated that physiochemical properties of the amino acid substitution can predict immunogenicity. Each amino acid has unique characteristics, which are determined by the hydrophobicity and electrostatic charge of the side chains. In addition to the number and position of individual mismatched amino acids, the hydrophobicity and electrostatic charge can be determined for each mismatched amino acid, resulting in a hydrophobicity mismatch score (HMS) and electrostatic mismatch score (EMS). Both HMS and EMS appeared to be strong predictors of HLA class I and II *de novo* DSA formation after kidney transplantation.[51,53] The HMS and EMS had superior predictive value for DSA formation compared to eplet mismatches and amino acid mismatches, which warrants further studies.[52]

Nonself-self paradigm

The above-mentioned strategies define immunogenic epitopes as mismatched or foreign epitopes, which are absent on the HLA alleles of the recipient and trigger an antibody

response. Although HLAMatchmaker is also based on this principle, Duquesnoy more recently proposed a nonself-self paradigm to explain some antibody reactivity that otherwise remains unexplained. According to this paradigm, some antibody responses towards foreign HLA cannot solely be induced by an immunogenic eplet mismatch, but require a self-amino acid configuration to be present on the mismatched allele.[54,55] This theory is based on the assumption that B cells with low-affinity immunoglobulin receptors for self-HLA epitopes are present but their affinity for mere self-epitopes is too low to trigger B-cell activation and antibody production. These B cells do become activated when confronted with the combination of a few amino acids which are non-self, and a self-amino acid configuration. The hypothesis implies that the presence of a self-eplet is required to induce an antibody response for a certain group of epitopes. However, not every eplet present in the epitope registry meet the non-self-self criterion and many non-self-eplets are antibody verified.[26,27] While the non-self-self paradigm is interesting, it is difficult to conceive why HLA antibodies would have self-reactive properties. Unlike T cell receptors, B cell receptors are not selected on basis of a low affinity for self HLA antigens. So far, laboratory data confirming the presence of low affinity self-HLA reactive immunoglobulin receptors are lacking. An alternative explanation for the involvement of a self-amino acid configuration on the mismatched donor allele is that HLA molecules share many amino acids. If an amino acid or eplet induces an antibody response, a number of these adjacent nonpolymorphic amino acids will be shared with the patient's own HLA molecules.

T cell epitope

The immunogenicity of antibody epitopes becomes even more complex when considering the requirement for $CD4^+$ T cell help for a full-blown B cell response resulting in class-switched antibodies. Helper T cells play an essential role in the differentiation of B cells into IgG producing plasma cells.[56] After recognition by the B-cell receptor, the target antigen can be internalised and degraded into peptides, finally resulting in peptides being presented in context of self HLA class II on the B cell. The presence of peptides that can be presented by the particular self-HLA class II molecules determines whether B cell clones receive T cell help. Upon cognate interaction between B and T cell, enhanced CD40 ligand expression and cytokine production by the T cell results in B cell differentiation into plasma cells. Indeed, it has been shown that the HLA class II phenotype of the recipient determines the immunogenicity of HLA class I antigens of the donor.[57] For instance, the production of HLA-Bw4 antibodies preferentially occurred in HLA-DRB1*01 and HLA-DRB1*03 positive patients.[58]

Following this work, Spierings and colleagues investigated the role of donor HLA-derived T-helper epitopes in formation of *de novo* DSA *in silico*. To predict T-helper epitopes, an algorithm was developed to determine the number of HLA class II-restricted predicted recognizable HLA epitopes (PIRCHE-II) for each HLA class I antigen mismatch. PIRCHE-II are theoretical epitopes consisting of nine amino acids present on the mismatched HLA class I antigen and absent on all HLA class I antigens of the recipient, which are able to be presented in peptide binding groove of the HLA class II molecules of the recipient.

In a cohort study of non-immunised renal transplant patients, a correlation was observed between a low number PIRCHE-II, and the lack of *de novo* antibody responses against specific HLA class I mismatches.[59] The role of PIRCHE-II in antibody formation was subsequently confirmed in a pregnancy cohort.[60] Noteworthy, these studies indicate a predictive value of PIRCHE-II on the population level, but it does not show any clinical relevance for the individual patient. Furthermore, the PIRCHE-II algorithm likely overestimates the number over PIRCHE-II as it is unlikely that the proteasome indeed processes all these theoretical peptides. Additionally, the functional role of these T-helper epitopes with respect to the activation of $CD4^+$ T cells remains to be proven.

Interestingly, in both transplantation and pregnancy cohorts no correlation between the number of PIRCHE-II and eplet mismatches was found. This might be explained by the fact that the eplet model is restricted to polymorphisms at antibody accessible sites. Furthermore, this model does not take into account the restriction of presentation capacity by HLA antigen towards helper T cells. An alternative, yet not exclusive explanation could be the involvement of noncognate T-helper responses to multiple HLA disparities, in which B cells and T-helpers cell do not necessarily have to recognise the same antigen. The group of Pettigrew showed in murine models of transplantation that B cells specific for an alloantigen can receive help from T-helper cells with another allospecificity. Long-lasting humoral alloimmune responses can be explained by this mechanism as memory T-helper cells recognising one alloantigen can give CD4-independent help to B cells that are specific for another alloantigen.[61] To optimally predict the immunogenicity of an HLA mismatch for an individual patient, both antibody epitopes and T cell epitopes should be considered.

Immunogenicity of epitopes in clinical renal transplantation

The immunogenicity of epitopes in clinical renal transplantation has been extensively studied.[22,46-48,51-53] In these studies, the HLAMatchmaker computer algorithm was used to determine the epitope load of certain HLA mismatches in order to correlate these with

the development of *de novo* DSA. A significant correlation between the number of epitope mismatches between recipient and donor and the chance to develop *de novo* DSA was observed.

Wiebe *et al.* used HLAMatchmaker in a population study and observed no DSA production in recipients with 10 or less HLA-DR eplet mismatches and 17 or less HLA-DQ eplet mismatches.[48] In addition, recipients who were nonadherent to their immunosuppressive medication and received a transplant with an HLA class II eplet load above these thresholds were more likely to develop DSA, acute rejection and graft failure compared to adherent recipients with an epitope load beneath the thresholds, indicating a synergistic effect of nonadherence and HLA class II eplet load.[62] It is important to note that in this study the HLA class II eplet threshold was based on all potential eplets, rather than antibody-verified eplets. Using the same threshold another group found that paediatric recipients of a graft with a DQ eplet load below the threshold were at a low risk to develop both DR and DQ specific *de novo* DSA.[63]

As a possible consequence of the association between the number of HLA class I and II epitope mismatches and development of *de novo* DSA, the number of epitope mismatches appeared to be a predictive parameter for the outcome of graft survival and long-term outcome of renal transplant recipients.[62,64-67] Sapir-Pichhadze *et al.* even described an association between the number of HLA-DR and -DQ eplet mismatches and development of transplant glomerulopathy.[68] The sum of HLA-DR and -DQ eplet mismatches was found to be an independent risk for antibody mediated rejection and transplant glomerulopathy. Besides renal transplants, epitope matching appears also to be beneficial for other types of solid organ transplants. In both lung transplant recipients and heart transplant recipients the number of HLA eplet mismatches correlated with the development of DSA and consequently the outcome of the transplantation.[69,70]

Altogether, these studies indicate the higher the number of HLA class I and II epitope mismatches the higher the risk of developing *de novo* DSA, without yet considering the differential immunogenicity of the individual epitopes.

ANTIGENICITY OF AN ANTIBODY EPITOPE

Reactivity of existing antibodies

So far, we have discussed that the trigger for antibody production by a B cell is the presence of a foreign immunogenic epitope on a mismatched donor HLA molecule. Subsequently, the reactivity pattern of the induced antibody determines the antigenicity of an epitope. Not only the immunogenic epitope, but also other amino acids in its proximity are involved in the actual reactivity of the antibody and formation of the antigen-antibody complex.

Antigen-antibody complexes are formed by three complementary determining regions (CDR) on the variable heavy chain (VH) and three CDRs on the variable light chain (VL)[71] that bind to six contact sites on the immunising HLA molecule, forming an antibody 'footprint' of 700-900 Å on the molecular surface of an HLA molecule. The VH3 binds to the immunogenic epitope and is responsible for the strength and specificity of the antibody reactivity. The remaining CDRs are important for stability and affinity of the antibody binding and will bind to amino acids within the antibody footprint.

As mentioned, HLA molecules can share amino acid configurations and thus also the contacts sites involved in antigen-antibody complexes. This may explain the broad reactivity of antibodies observed in SAB assays when screening sera which not only contain DSA but also non-donor specific antibodies (NDSA).[72,73] All the reactive HLA molecules contain the immunogenic epitope of the mismatched HLA molecule that triggered the antibody response and often one or more critical amino acid configurations important for antibody binding (Figure 1). This immunogenic epitope and/or critical amino acid configurations are absent on the non-reactive HLA molecules. So, the actual reactivity of antibodies depends on both the immunogenic epitope and the other crucial amino acid configurations of the epitope that act as contact sites necessary to form a stable antigen – antibody complex.

Duquesnoy defines the antibody footprint as a structural epitope consisting of 15-22 amino acids. In the centre 2-5 amino acid residues reside of which at least one is non-self, also known as the eplet, or functional epitope, to which the VH3 of antibodies bind, similar to what we described above.[24,25,74]

Based on this antibody footprint, Duquesnoy analysed the broad reactivity observed in SAB assay of various mAbs with the HLAMatchmaker algorithm and proposed different reactivity patterns.[31,32,55] In one reactivity pattern, all the reactive HLA molecules contain the immunogenic eplet that induced the antibody response, which implies that the VH3 site

of antibody can bind to this eplet, while the non-reactive HLA molecules do not have this immunogenic eplet and as a result the VH3 has no binding site on these molecules.[26] In some cases, it appeared that the non-reactive HLA molecules do have the immunogenic eplet to which the VH3 can bind to, but lack additional crucial amino acid configurations within the antibody footprint that are present on the reactive HLA molecules (Figure 1). The need for two configurations to establish an antigen – antibody complex is designated as eplet pairs.[31,32,55]

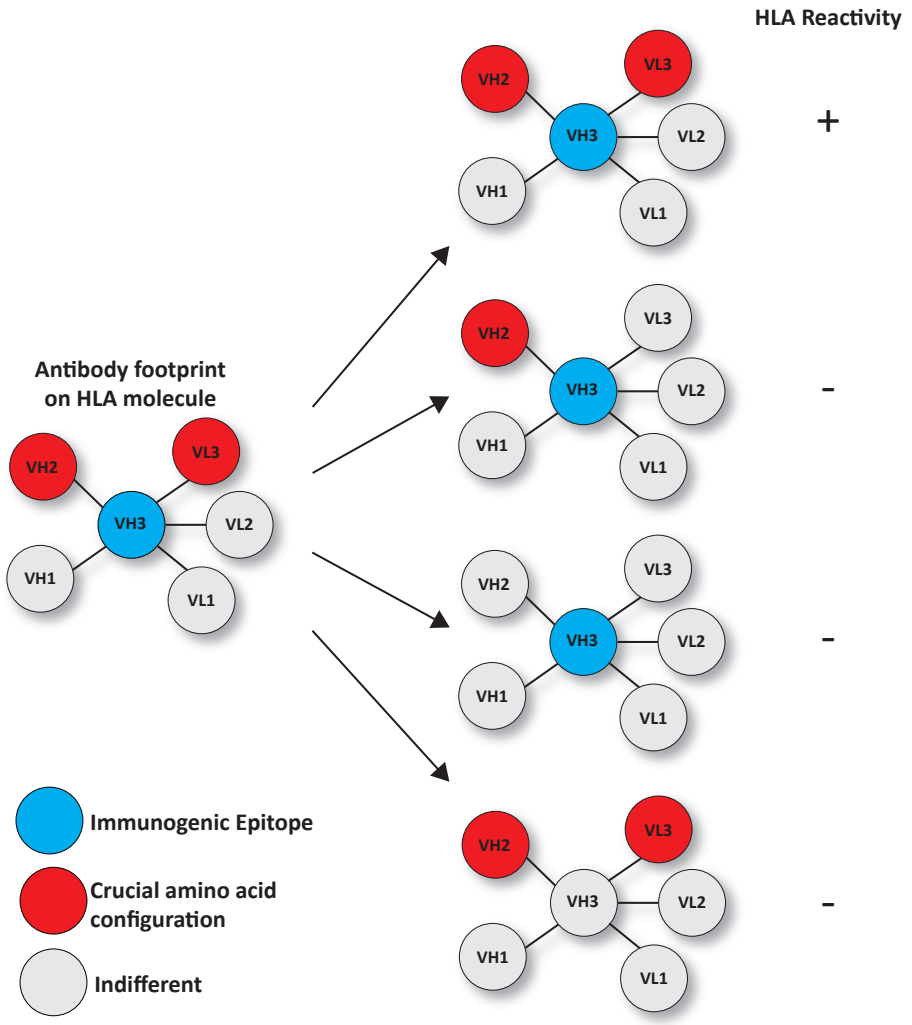

Figure 1. Crucial amino acid configurations determine the reactivity of an HLA antibody. Schematic representation of an antibody footprint present on an HLA molecule. The VH3 of the antibody binds to the immunogenic epitope (blue), while binding of other contact sites of the antibody to crucial amino acid configurations (red) is essential for reactivity. The remaining amino acid configurations within the antibody footprint are indifferent to the reactivity.

Additionally, Duquesnoy *et al.* showed different reactivity patterns detected when screening human HLA-specific mAbs with IgG SAB assays, C1q SAB assays, and complement-dependent cytotoxicity (CDC) assays.[75] Based on these data, they proposed that certain specific polymorphic amino acid configurations of structural epitopes are not only crucial for antibody binding, but also affect the release of free energy, which determines whether complement can be activated.

Complexity of HLA class II reactive antibodies

Defining epitopes of the HLA class II reactive antibodies is in its infancy, as mainly HLA class I-specific human mAbs are currently available. Moreover, epitopes on HLA-DQ and-DP are more complex due to the fact that both α and β chains are polymorphic. Tambur *et al.* have touched on this complexity and the importance of understanding the reactivity of HLA-DQ antibodies.[76,77] Upon analysis of HLA-DQ antibodies, immunogenic eplets are present on either the mismatched DQα or DQβ chain. The antibody footprint can cover an additional crucial polymorphic amino acid configuration on HLA molecule, as indicated in Figure 1, and depending on the location of immunogenic eplet these crucial configurations are located on either one or both DQ chains. As a consequence, it appears that antibodies can react with a self DQβ chain and a non-self DQα chain or vice versa in SAB assays, which complicates the analysis of the reactive antibodies. Tambur emphasises that for assigning the epitope specificity of HLA-DQ antibodies both the DQα and DQβ chains should be considered.[78,79]

Analysis of HLA-DQ antibodies becomes even more complicated when considering the two possible forms of the HLA-DQ antigens, which can be expressed on the cell surface. The first one is the cis-heterodimer, in which the β-chain and α-chain are derived from genes on the same chromosome, the second one is the trans-heterodimer, where the chains are derived from genes on two different chromosomes. Thus, HLA-DQ DSA can theoretically be formed against a DQβ-chain, DQα-chain, or both in either cis- or trans-heterodimer.[80] Extensive analysis of HLA-DP antibodies reactivity is lacking, but the structure of DPβ chain seems less complicated than that of HLA-DQ.

To simplify analysis of HLA class II antibody reactivity the previously mentioned computer model based on physiochemical properties of amino acids can be used.[51,53] With this computer model, high-resolution three-dimensional structural models of HLA class I alleles have been developed to understand the binding of antibodies to an epitope. These models can contribute to the prediction of HLA antigenicity by providing more insight in the antigen-antibody interaction.[81-83] This tool together with the antibody-reactivity patterns, especially

those of mAbs observed in SAB assays, will contribute to a better understanding of the role of both the immunogenic epitope and additional crucial polymorphic amino acid configurations in the antigen-antibody interaction.

USING EPITOPE KNOWLEDGE IN THE CLINIC

Epitope matching

Preventing DSA formation is pivotal in clinical transplantation and therefore it is important to avoid the antibody trigger, which can be any immunogenic epitope. This is especially important for paediatric patients who most likely need more than one transplant in their lifetime. Recently, Kausman *et al.* applied HLA eplet loads for the selection of donors in paediatric kidney transplantation.[84] This group used HLA class I <10 eplets and HLA class II <30 eplets as the threshold for each antigen. In the first year post-transplantation, an excellent early graft function and a low *de novo* DSA incidence was observed. This study indicates that paediatric patients benefit from epitope matching.

Implementation of an epitope matching algorithm for donor selection in renal transplantation may prevent the allocation of kidneys with highly immunogenic epitopes and prevent the induction of DSA. To achieve optimal epitope matching, a proper definition of the actual epitopes and their immunogenicity is of great importance.

Virtual crossmatching

As mentioned, the presence of DSA complicates repeat transplantation, especially for highly sensitised patients as the antibodies will cause a positive crossmatch with the majority of donors. The identification of acceptable and unacceptable HLA antigens on basis of extensive antibody screening[85,86] is currently used to predict beforehand which donor HLA antigens will result in a negative or positive crossmatch[87]: the so called virtual crossmatch.

However, this review shows that epitopes determine antibody reactivity. Therefore, future virtual crossmatching should be based on epitopes (Figure 2). Epitopes present on the non-reactive HLA molecules are instrumental for the definition of acceptable epitopes whereas unacceptable epitopes can be defined on basis of antibody reactivity. For virtual crossmatching, the combination of self-epitopes, acceptable epitopes, and unacceptable epitopes will determine the selection of a suitable donor. Suitable donors will have HLA molecules that consist only of self-epitopes and acceptable epitopes, as these will predict a negative crossmatch. The advantage of the use of acceptable and unacceptable epitopes compared to current strategies of virtual crossmatching, which are based on HLA antigens,

is the fact that one can even predict the antibody reactivity with HLA alleles, that are absent in antibody detection assays such as SAB assay, and thus have never been tested before.

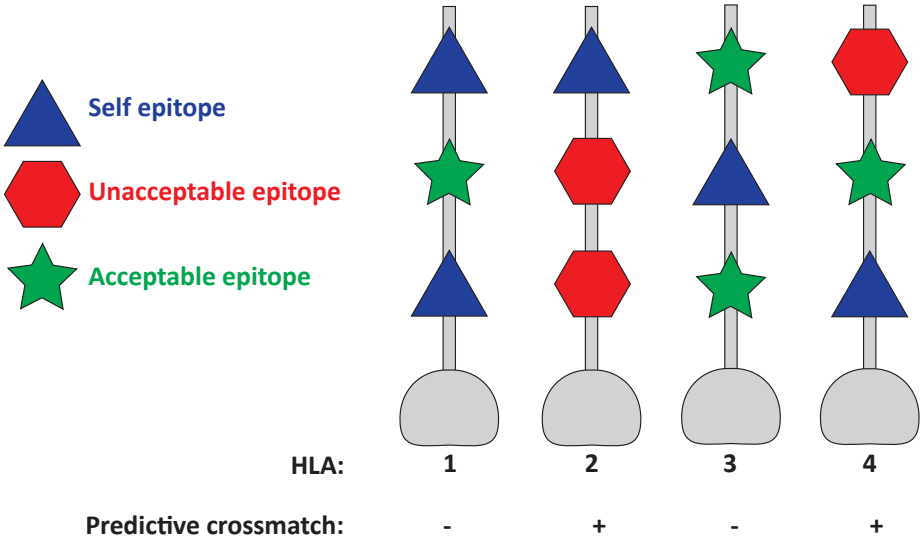

Figure 2. The use of epitope knowledge for virtual crossmatching. Each HLA molecule consists of a set of epitopes. HLA molecules with self-epitopes (blue triangles) and/or acceptable epitopes (green stars) will predict a negative crossmatch, while HLA molecules with unacceptable epitopes (red hexagons) will predict a positive crossmatch.

Highly sensitised patients

As indicated earlier, acceptable epitopes defined by the absence of antibody reactivity can be used to identify donors with acceptable HLA antigen mismatches for highly sensitised patients. The chance of finding a donor with HLA antigens towards which highly sensitized patients have no detectable circulating antibodies is very small. In order to increase this chance, Eurotransplant runs the Acceptable Mismatch (AM) program, in which the sera of highly sensitized patients are screened to determine the acceptable HLA mismatches to which the patient did not form any antibodies.[85,86] This knowledge is used for the selection of compatible donors. A donor kidney with an HLA type consisting of the combination of the patient's own HLA antigens and acceptable HLA mismatches will be mandatorily shipped to that specific highly sensitized patient. Nowadays, the AM program uses HLAMatchmaker to identify additional HLA class I antigens which are likely to be acceptable mismatches due to lack of antibody epitopes. This led to an increased number of HLA class I acceptable mismatches, and subsequently increases the chance of finding a suitable donor.[88-90] Altogether, defining

epitopes absent in antibody-reactivity patterns of sensitized patients will be beneficial for the selection of a donor with HLA molecules consisting of acceptable epitopes.

TOWARDS EPITOPE MATCHING

HLA epitope matching will be beneficial for preventing sensitization, selection of donors for highly sensitised patients, and improvement of transplant outcomes.

To identify the clinically relevant epitopes, cohort studies on antibody induction are extremely useful. However, many studies suffer from limitations, such as restricted numbers of patients included in the analysis. Additionally, the (partial) lack of second field HLA typing (current allocation is often based on intermediate typing of HLA-A, -B, and -DRB1) is an inhibitory factor. To circumvent this problem, HLAMatchmaker contains an algorithm that assigns most likely high-resolution typing based on race of the recipient and/or donor (http://www.epitopes.net/). When second field typing is available for HLA-A, -B, and –DR, the high-resolution typing of the other loci are assigned based on common associations of B-C, and DR-DQ antigens in the given population. While this approach is useful, to move the field forward, high resolution typing is pivotal.[91] While the antibody specificities from SAB assays are on the second-field level, it can appear that an allele-specific antibody is directed against a self-antigen, in case the HLA typing of the patient is only performed at first field level. This is due to differences in polymorphic amino acids that exist between alleles of the same serological antigen. Thus, for understanding the epitope that induced antibodies to a certain HLA mismatch and determining the relevance of these antibodies in transplantation, second field typing of the donor is an absolute requirement.

Most epitope studies used SAB assays for the identification of DSA in sera of recipients. However, analysis of multispecific sera is challenging, especially when more than one immunising event has occurred. In addition, when defining acceptable and unacceptable epitopes based on antibody reactivity observed in SAB analysis, it is essential that the data are interpreted with caution, and that a possible prozone effect is excluded.[92-97]

Several approaches are used to determine the immunogenicity of epitopes, but a systematic study on a large population of patients has not been performed. Large cohort studies combining the different approaches described in this review are essential to discriminate the immunogenic and non-immunogenic epitopes. For the patients carrying rare HLA alleles, it will be difficult to determine immunogenic epitopes, so for the time being these patients will have to take advantage of matching strategies based on theoretical epitopes.

Definition of antibody epitopes is one of the main subjects of the 17th International HLA and Immunogenetics Workshop (IHIWS) that will be held in Palo Alto, CA in September 2017. For this collaborative effort, (high resolution) HLA typing of recipient and donor, screening data from sera of recipient with various SAB assays, and graft outcome data from transplant centres all over the world are collected for various projects, including definition of the immunogenicity of individual epitopes (http://ihiws.org/).

Conclusion

The broad antibody reactivity observed in recipients after transplantation of an HLA antigen mismatched graft can be explained by the presence of mismatched epitopes on the HLA of the donor, which are shared with other HLA alleles. Identification of the immunogenicity of the individual epitopes and avoiding transplantation in the presence of highly immunogenic epitope mismatches will prevent DSA formation. In addition, understanding the complex interaction between the induced antibody and the reactive HLA molecules will contribute to the identification of acceptable mismatches and virtual crossmatching even for highly sensitised patients. It is to be expected that future HLA matching strategies will change from antigen or allele matching towards HLA epitope matching.

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Conflict of interest

The authors have declared no conflicting interests.

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CHAPTER

3

THE LONG AND WINDING ROAD TOWARDS EPITOPE MATCHING IN CLINICAL TRANSPLANTATION

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ABSTRACT

Recent data suggest that HLA epitope matching is beneficial for the prevention of de novo donor specific antibody (DSA) formation after transplantation. In this review, different approaches to predict the immunogenicity of an HLA mismatch will be discussed. The parameters used in these models are often called epitopes but the actual antibody epitope is far more complex. Exact knowledge of the antibody epitope is crucial if epitope matching is also used as a tool to select compatible donors for (highly) sensitized patients. Evidence is provided that it is not always possible to give an exact definition of an antibody epitope. We conclude that HLA "epitope" matching is superior over HLA antigen matching with respect to the prevention of de novo DSA formation and will enhance the prediction of acceptable HLA mismatches for sensitized patients. However, epitope matching at our current level of knowledge will not solve all histocompatibility problems as unexpected antibody reactivity still may occur.

INTRODUCTION

After the discovery that blood transfusion and pregnancy can lead to the induction of leucocyte-reactive antibodies,[1-3] it soon became clear that the antigens recognised by these antibodies played a pivotal role in the immune response leading to graft rejection after kidney transplantation. When donors and recipients had the same HLA type, graft and patient survival was significantly better than in case of HLA mismatched transplants.[4] These first results were obtained with transplants using living related donors, often siblings of the patient, which made the chance of transplanting an HLA identical graft relatively high. Selection of an HLA identical unrelated donor is far more difficult, due to the enormous polymorphism of the HLA system. Only in case a large pool of unrelated donors is available, full HLA matching on the serological level might become feasible. In order to reach this goal, the international organ exchange organization "Eurotransplant" was founded in 1967 by Jon van Rood.[5] By creating a common waiting list and a common donor pool of several countries in Europe, HLA matching became a realistic option, at least for a subpopulation of patients. It appeared that this initiative was successful, as about 20% of the patients within Eurotransplant were transplanted with an HLA-A, -B, -DR identical donor, which was associated with superior graft survival compared to HLA mismatched transplants.[6] Unfortunately, still the majority of patients were transplanted with a (partially) HLA mismatched graft. Nonetheless, it appeared that decreasing the number of HLA mismatches was already a tool to minimise sensitization and prevent early graft loss. During the years following, more efficient immunosuppression became available leading to prolonged graft survival, also in HLA mismatched transplants. Nevertheless, sensitization towards HLA still occurs, and those patients are at risk for early graft loss.[7] Moreover, once sensitized the chance of finding a suitable donor organ for a patient becomes far more difficult. Recently, several approaches of alternative HLA matching have been described to prevent the induction of DSA in order to improve graft survival. Knowledge of the exact amino acid sequence of the different HLA antigens appears to be crucial for the selection of an optimally HLA mismatched donor.[8] In this review, we will focus on the differential immunogenicity of epitopes, the requirement for T cell help and the difficulties in determining the exact binding determinants of HLA antibodies.

From HLA antigens towards HLA epitopes

Soon after the serological identification of HLA antigens it became clear that HLA molecules belonged to a highly polymorphic system. While the introduction of molecular typing was crucial for a more accurate definition of HLA antigens, it also resulted in an enormous increase

of the number of HLA alleles reported. At the moment, more than 15 000 alleles are known and it is to be expected that this number will increase far more by broader application of next generation sequencing as a tool for HLA typing. At first glance, these developments make the selection of an HLA mismatched donor organ that will not induce DSA a mission impossible.

Fortunately, knowledge on the exact amino acid sequence of the different HLA alleles has given more insight in the crucial positions on the HLA molecules for the induction and reactivity of allo-antibodies. Already in the early days of HLA, when the HLA antigens were still serologically characterised with allo-anti-sera, it became clear that the different HLA molecules share antigenic determinants that we now call epitopes[9]. Several CREGs (Cross REactive Groups) could be identified based on their shared reactivity with the same allo-antibodies.[10] However, for an optimal characterization of the epitopes expressed by the different HLA antigens, molecularly HLA typing at the allele level is crucial.[8] Nowadays it is clear that every HLA antigen consists of a unique set of antibody epitopes, while the individual epitopes can be shared by multiple HLA antigens.[11] The consequence is that the number of foreign antibody epitopes varies within the same level of HLA antigen mismatches (Figure 1). Some HLA mismatches have many epitopes not shared by the HLA antigens of the patient. These mismatches are likely to be more immunogenic compared to an HLA mismatch which shares most epitopes with the patient. Several tools have been developed to determine the relative immunogenicity of an HLA mismatch on basis of this principle.

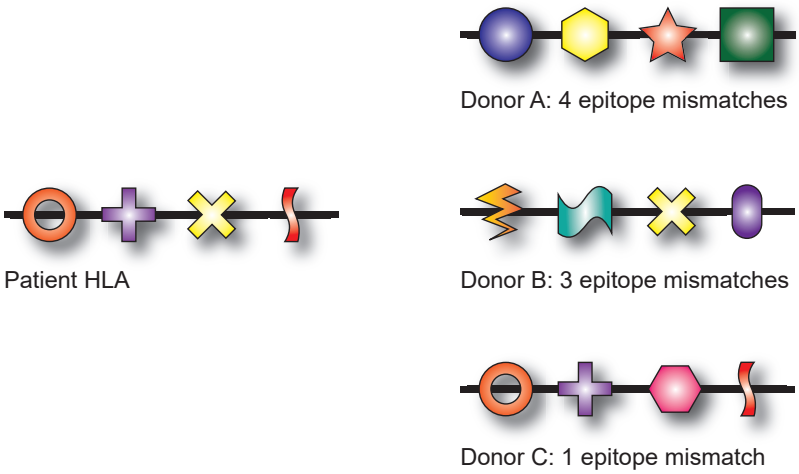

Figure 1: HLA alleles can be considered as a string of potential antibody epitopes. A specific HLA allele consists of a unique set of epitopes while the individual epitopes can be shared with other alleles. The consequence is that the number of foreign epitopes on an individual HLA mismatch can differ and depends on the HLA type of the potential antibody producer.

Tools to define the immunogenicity of an HLA mismatch

The first individual who realised that the patchwork character of HLA molecules with respect to antibody epitopes could provide a basis to determine the immunogenicity of foreign HLA molecules, was Rene Duquesnoy in Pittsburgh. He defined HLA molecules as strings of crucial amino acid polymorphisms, which potentially can trigger an antibody response.[12] Originally these polymorphisms were defined as consisting of three amino acids, adjacent to each other on antibody accessible sites of the molecule, called triplets. Later, Duquesnoy redefined the crucial polymorphism as amino acids within 3 Ångstrom radius, structurally near to each other on the tertiary conformation of the HLA molecule, called eplets.[13] The computer algorithm HLAMatchmaker made it possible to compare the triplets or eplets on a foreign HLA molecule with those present on the patient's own HLA molecules.[14] During the past years, several studies demonstrated clear associations between the number of triplets or eplets on a mismatched HLA antigen and the chance that a patient develops *de novo* DSA.[15-18] Both in case of mismatched HLA class I and HLA class II antigens, the incidence of antibody formation increases with the number of foreign triplets or eplets. Some studies even suggest that it is possible to define a threshold of a certain number of eplets, which predicts whether antibody production will occur.[19] However, even a very low number of mismatched eplets can already give rise to DSA formation as was already shown by the studies of Dankers et al., which demonstrated a clear association between the number of mismatched triplets and the chance that a patient will develop *de novo* DSA.[16] In case of 12 or more triplet mismatches 100% of the patients, who rejected their graft, had developed DSA but also in case of one or two triplet mismatches respectively 10% and 22% of the patients produced DSA. Furthermore, it remains to be established whether the definition of the number of foreign eplets is the optimal way to predict the immunogenicity of an HLA antigen. A similar predictive value has been demonstrated if one considers the total number of antibody-accessible amino acid substitutions of the mismatched HLA antigen in comparison with the own HLA antigens of the patient.[20]

A completely different approach has been developed by the Cambridge group. Their studies show a clear role of the physiochemical properties of mismatched amino acids. If the physiochemical properties of mismatched amino acids are very different from those of the own HLA antigens of the patients, induction of donor specific antibodies is far more likely than in case of similar properties.[20-22] They have validated a score system (EMS-2D), which determines the degree of foreignness of the physiochemical characteristics, both for the

induction of DSA after renal transplantation and after blood transfusion. The higher the score, the more likely it is that a patient will make antibodies.

The different approaches, which consider polymorphisms on antibody accessible sites of the donor HLA molecule, all suggest that quantitative aspects i.e. the number of foreign triplets, eplets, amino acids play a crucial role but do not consider a potential difference in the immunogenic properties of the individual polymorphisms.[23]

Differential immunogenicity of individual "epitopes"

Although the relationship between the number of triplet/eplet mismatches and the chance to develop DSA is well established, the immunogenicity of the individual triplets/eplets may vary. Not every polymorphic site has the same immunogenic potential and one could hypothesise that a higher number of triplet/eplet mismatches makes it likely that one of these is particularly immunogenic. This is also suggested by a study on the development of DSA associated with graft loss in a cohort of 1311 previously nonimmunized males, who returned on the waiting list of Eurotransplant after failure of their first transplant. This study, which focused on the development of antibodies against HLA-A and -B showed that some mismatched triplets led to antibody formation in about 50% of the cases whereas others led to a lower incidence of DSA or hardly induced any antibodies (Table 1). Similarly to the frequencies of HLA alleles, which differ amongst populations, the frequency of potential antibody epitopes will also be different between populations in the world (www.allelefrequencie s.net). The chance that a particular HLA allele mismatch will induce antibodies depends amongst others on the frequency of its most immunogenic epitope in the population. If this frequency is high, it is more likely that donor and recipient share this epitope, resulting in a low number of patients with that antibody specificity. If the frequency of the immunogenic epitope is low in a population, the incidence of antibody formation against that HLA allele in case that it is mismatched will be high. A preliminary study comparing the incidence of antibody formation in transplant recipients in Israel versus Eurotransplant confirms this hypothesis (Israeli et al. manuscript in preparation). These data suggest that future matching strategies should not only focus on the number of mismatched eplets, triplets or amino acids but, especially, on prevention of mismatches for highly immunogenic polymorphisms. In order to be able to reach this goal, it is crucial to identify the immunogenicity of the different polymorphisms with respect to the induction of allo-antibodies. This is one of the aims of the 18th International Histocompatibility workshop, which will take place in Amsterdam in 2021. By collecting information on the incidence of de novo DSA in a large group of high resolution typed donor recipient combinations from different populations, it should be feasible to identify the most

immunogenic antibody epitopes. In future allocation, these should be avoided as a mismatch in order to prevent the induction of DSA. The other side of the coin is the identification of polymorphisms, who do not lead to allo-antibodies in an individual patient and are likely to serve as acceptable mismatches.

Table 1. The immunogenicity of individual epitopes differs as demonstrated by analysing the epitope specificity of donor specific antibody (DSA) developed in previously nonsensitized males, who returned on the waiting list after failure of their first kidney transplant

Positions + amino acids	Yes DSA	No DSA	% DSA	Donor mismatches
79G 80T 81L	30	23	56.6	A23 A24 A25 A32
11A 12M 13S	55	49	52.9	B7 B18 B27 B37 B38 B39 B46 B48 B61
150A 151H 152V	104	95	52.3	A1 A3 A11 A23 A25 A26 A29 A30 A31 A32 A33
126L 127K 128E	102	94	52	A1 A3 A11 A25 A26 A29 A30 A31 A32 A33
130L 131S 132S	30	30	50	B7 B8
73I 74D 75R	14	47	23	A31 A33
73R 76E 77D	20	69	22.5	B27 B37 B47
185P 186R 187T	1	15	6.3	A33

Very immunogenic epitopes are associated with specific antibodies in more than 50% of the cases at risk whereas other epitopes are less immunogenic.

An additional role for T cell epitopes

The majority of the donor specific antibodies that develop after transplantation belong to the IgG class. Production of IgG antibodies is only feasible if $CD4^+$ T cells provide help to the B cells, as without such an interaction activation of B cells will only lead to IgM antibodies. T cell help is based on the recognition of peptides derived from allogeneic HLA molecules presented by the HLA class II molecules on the B cell (Figure 2). Whereas B cell epitopes solely reside on antibody accessible locations, polymorphisms throughout the HLA molecule can theoretically give rise to T cell epitopes. Indeed, studies on the location of B cell and T cell epitopes showed overlapping regions, as one would expect, but also the presence of T cell epitopes in areas where no B cell epitopes were present.[24] Identification of the actual peptides, which function as targets for this so called indirect allorecognition by $CD4^+$ T cells, has shown to be very difficult due to the low frequency of indirectly recognizing T cells.[25] Nonetheless, a computer algorithm has been developed, which calculates the number of potential allogeneic peptides derived from the mismatched HLA molecule that are able to bind to the HLA-DR molecules present on the B cells of the recipient.[26] This PIRCHE approach (Predictable Indirectly ReCognizable HLA Epitopes) has shown to be of additional value for the prediction of the chance that donor specific antibodies will be produced.[26-28] An increased

number of theoretical T cell epitopes is associated with a higher chance that a patient will start to make antibodies to a mismatched HLA antigen. A recent publication showed that the number of PIRCHE mismatches and the number of eplet mismatches were independent risk factors for both the development of DSA after transplantation, and graft outcome.[18] Similar to B cell epitopes, the challenge is to discriminate the actual immunogenic PIRCHEs from the non-immunogenic theoretical ones. It is to be expected that a combinatorial approach, focussing on the identification of both B cell and T cell epitopes, will be the optimal basis of future matching strategies.

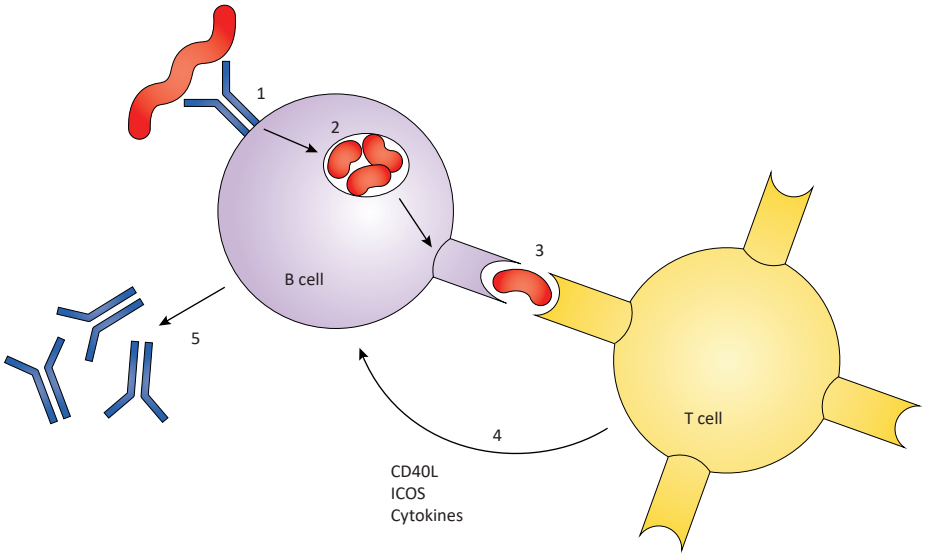

Figure 2: The production of IgG antibodies depends on a specific interaction between CD4+T cells and B cells. 1: The B cell receptor recognises an epitope on a foreign HLA molecule. 2: This leads to internalization of the target antigen, which is then degraded into peptides. 3: Some of these peptides bind to the HLA class II molecules on the B cell and the foreign (non-self) peptides are recognised by CD4+ T cells. 4: This leads to activation of the T cells associated with the production of immunoregulatory molecules. 5: These molecules trigger a class switch of the antibodies produced.

Immunogenic determinants versus actual antibody epitopes

So far, the immunogenicity of an HLA mismatch for the humoral immune response has been discussed with a special emphasis on the predictive value of polymorphic structures which serve as a trigger for, or are associated with, the induction of an antibody response. As discussed above, these structures are rather simple and consist of one or a few amino acids. However, the actual antibody epitope, which consists of the crucial contact sites on the HLA molecule necessary for the interaction with antibody molecule is far more complex. In principle, an antibody molecule has a paratope, consisting of six so called complementary

determining regions (CDRs), three on the heavy chain and three on the light chain, which interact with the HLA antigen recognised. The interaction of the epitope with these CDRs leads to a kind of footprint on the HLA molecule (Figure 3). The specificity of the antibody is determined by the amino acids on the HLA molecule, which interact with the CDR3 on the heavy chain of the immunoglobulin molecule. These amino acids include the triplets/eplets, which were responsible for the induction of the antibody response. The other contact sites contribute to the stability and avidity of the interaction between antibody and antigen. In order to be able to predict the HLA antigen reactivity pattern of an antibody, it is essential to determine which of these interactions are crucial for proper binding of the antibody. In some cases, antigens only sharing the polymorphism interacting with the CDR3 with the immunizing antigen, are already targets for an alloantibody. In other cases an additional requirement is that one or more other CDRs bind exactly the same amino acids as present on the immunizing antigen. The use of human HLA-specific monoclonal antibodies (mAbs) has been instrumental for the definition of actual antibody epitopes, while also absorption elution experiments are suitable to define the crucial polymorphisms defining an antibody epitope. A dedicated website has been developed to register the actual antibody epitopes on the different HLA antigens.[29,30] It is clear that some of the epitopes can be easily defined, while others are more complex, as shown below.

Definition of the actual HLA antibody epitope can be difficult, even with monoclonal antibodies

Both human mAbs and absorption/elution studies with alloantisera have been very instrumental for the definition of the actual antibody epitopes on the HLA molecules.[31-33] These verified epitopes are registered at the epitope registry (http://www.epregistry.com.br). As mentioned above, antigen-antibody interaction involves six CDRs of which the CDR3 binds to the immunogenic epitope and determines the specificity of the antibody. In most cases, the presence of this immunogenic epitope, alone or in combination with one or two other polymorphic sites, can explain the antibody reactivity pattern with the different HLA alleles (Table 2, Figure 3A, B, monoclonal antibodies MUS4H4 and VTM9A10). However, sometimes very complicated reaction patterns are observed, which make it virtually impossible to define an epitope that explains all antibody reactivities.

Here we describe an example of an HLA-A*11:01 induced mAb, WIM8E5, derived from a woman who became sensitised during pregnancy by paternal antigens. The trigger of the antibody response can be any foreign amino acid on the mismatched HLA, dependent on the phenotype of the mother. In this case, comparison of all the HLA class I molecules of

the mother with the immunising HLA-A*11:01, reveals that there are only two amino acid differences, which may have triggered the antibody response. However, if we compare only the HLA-A molecules of the mother with HLA-A*11:01, there are six amino acid differences.

When WIM8E5 was screened with single antigen bead (SAB) assays of two independent vendors, a broad HLA antigen reactivity was observed, including specificities of all three classical HLA class I loci. In contrast to other mAbs, the reactivity of WIM8E5 cannot be explained by the fact that the reactive alleles share the immunogenic epitope and some other crucial amino acid configurations, which are absent on the non-reactive antigens. Almost all HLA-A antigens are reactive, except for three, including the self-antigens. In addition, some HLA-B and HLA-C antigens were reactive. Extensive comparisons of the amino acid composition of the different reactive HLA antigens did not lead to a clear definition of an antibody epitope (Figure 3C).

Others have shown that dilution of serum samples can clarify the patterns observed in SAB assays.[34] Dilution of WIM8E5 led to a decrease in the breadth of reactive HLA antigens. One group of HLA-A antigens, including the immunising HLA-A*11:01, remained highly reactive in SAB assays upon dilution. The reactivity of the other HLA-A antigens was less consistent and decreased with each dilution step, while the reactivity of the HLA-B and HLA-C antigens decreased rapidly upon dilution.

Most of the HLA class I epitopes have been defined on the basis of interlocus comparisons of amino-acid sequences. However, for WIM8E5 the reactive HLA-C antigens do not have any amino acid configuration in common with the reactive HLA-A antigens. However, they do share a unique amino acid that is absent on the non-reactive HLA-C antigens. These data suggest that the induction of the WIM8E5 antibody was induced by an epitope mismatch specific for HLA-A and that the crossreactivity with HLA-B and HLA-C is not based on reactivity of the antibody with the same, HLA-A specific epitope.

This example shows that verification of HLA epitopes using mAbs involves more than screening the mAb with SAB assay, and just identifying the shared amino acid configurations of the reactive antigens. The different reactivity patterns upon dilution of WIM8E5 do support the conclusion that different epitopes are involved and that the affinity of the antibody for the different target antigens is different. An alternative explanation for the observed crossreactivity might be that the reactive antigens have similar physiochemical properties, despite amino acid variation within the critical contact site.

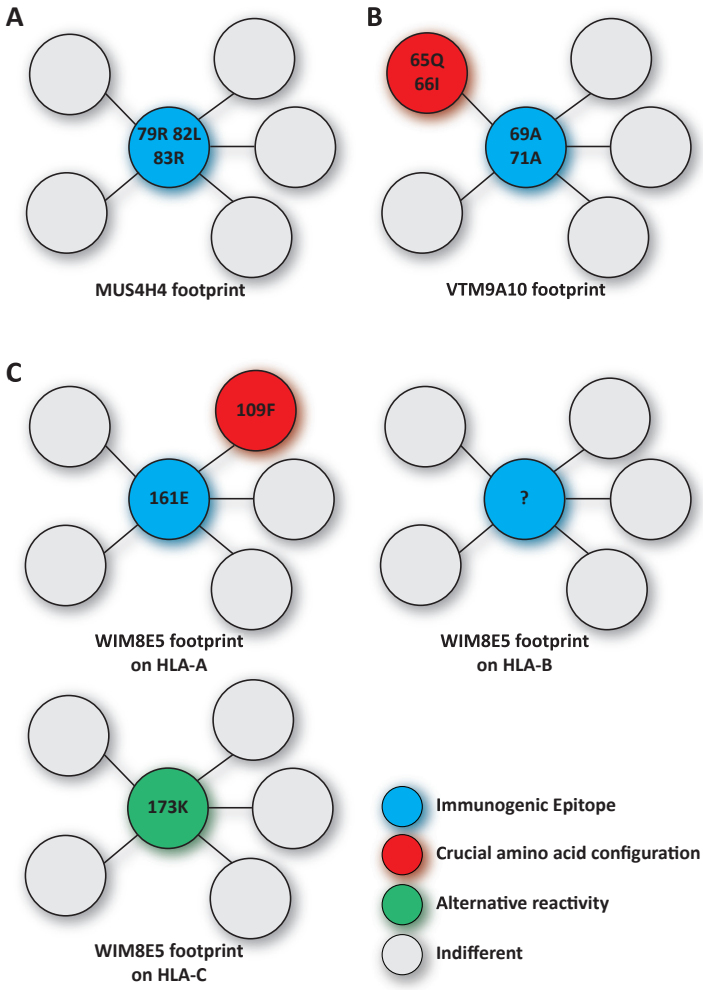

Figure 3: Footprints of human monoclonal antibodies on their target antigens. A) The reactivity of monoclonal MUS4H4 depends only on sharing of the immunogenic epitope with the HLA molecule, which has triggered the production of this antibody. B) For the reactivity of monoclonal antibody VTM9A10 sharing of both the immunogenic epitope and an additional contact site with the immunizing antigen is crucial. C) The reactivity of monoclonal antibody WIM8E5 is very complex. It appears that the reactivity with HLA-A antigens depends on sharing of the immunogenic epitope and an additional contact site. The observed cross reactivity with HLA-B and -C antigens has completely different requirements. The reactive HLA-C alleles have one particular polymorphic position in common (in green) whereas the basis of the reactivity with HLA-B targets remains unclear. Note: more details on the immunizing effect leading to the production of these antibodies is given in Table 2.

Table 2. HLA types of the antibody producer and immuniser and epitope specificity of the three monoclonal antibodies depicted in Fig. 3

Human mAb	HLA antibody producer	HLA immuniser	Epitopes on reactive HLA class I alleles		
			HLA-A	HLA-B	HLA-C
MUS4H4 (IgG)	A*02:01, A*26:01, B*39:01, B*41:01, C*12:03, C*17:01	A*24:02	79R82L83R	79R82L83R	-
VTM9A10 (IgG)	A*25:01, A*29:02, B*44:03, B*15:01, C*05:01, C*16:01	B*07:02	-	69A71A (65Q61I)	-
WIM8E5 (IgG)	A*03:01, A*03:02, B*47:01, B*51:01, C*06:02, C*15:02	A*11:01	161E(109F)	?	173K

? indicates the epitope recognised on HLA-B is unclear.

Concluding remarks

For many years, HLA matching strategies have focused on the selection of donors with a minimal number of HLA antigen mismatches. However, it is clear that the immunogenicity of mismatched HLA antigens can differ. If one would like to prevent the induction of *de novo* DSA, which is known to be associated with a poor outcome, then limiting the number of HLA antigen mismatches is not the optimal strategy. Our view on HLA antigens as possible targets for antibodies has been changed considerably over time. Antibodies are not specific for an HLA antigen but for an epitope present on the HLA molecule. Every HLA antigen can be considered as a string of antibody epitopes. A particular HLA antigen consists of a unique combination of epitopes while the individual epitopes can also be expressed on other HLA antigens. This is the reason why the number of foreign epitopes present on a mismatched HLA antigen will differ and depends on the HLA type of the recipient. Some HLA mismatches express many epitopes whereas others have only a few or even no foreign epitope. As a consequence, the presence of two HLA antigen mismatches on a donor organ may, for some recipients, be associated with fewer epitope mismatches than the presence of a single antigen mismatch. Therefore, novel matching strategies aim at the limitation of the number of foreign epitopes rather than the number of HLA antigen mismatches.

Current activities in the field of epitope matching can be divided in two categories. The first one is aiming at the prevention of *de novo* DSA formation by limiting the number of potentially antibody inducing polymorphisms on the mismatched HLA molecules. Indeed, several tools have been described, which can successfully predict the chance that DSA will

be induced.[23] These include the number of foreign eplets/triplet, amino-acids or PIRCHE's, and the physicochemical properties of the mismatched HLA molecule. Such parameters are already excellent predictors of the immunogenicity of an HLA mismatch and as soon as we are able to distinguish the very immunogenic polymorphisms from the non-immunogenic ones, their prediction will be even better. However, none of these parameters can be considered as the actual antibody epitope. Exact knowledge of the antibody epitope is crucial if one would like to introduce epitope matching as a tool for virtual crossmatching of (highly) sensitized patients. Human monoclonal antibodies and absorption/elution studies have been very helpful for the definition of several antibody epitopes, which are described in the HLA epitope registry. At the moment, this collection is far from complete and the example described in this review suggests that it will not always be feasible to explain the possible reactivity of an HLA antibody based on the knowledge of the amino acid sequence of the immunizing antigen.

In conclusion, "epitope" matching is superior over antigen matching with respect to the prevention of *de novo* DSA formation and will enhance the prediction of acceptable HLA mismatches for sensitized patients. However, one should realise that epitope matching at our current level of understanding will not solve all histocompatibility problems, as unexpected antibody reactivity still may occur.

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CHAPTER

4

RECOMBINANT HUMAN MONOCLONAL HLA ANTIBODIES OF DIFFERENT IGG SUBCLASSES RECOGNISING THE SAME EPITOPE: EXCELLENT TOOLS TO STUDY DIFFERENTIAL EFFECTS OF DONOR SPECIFIC ANTIBODIES

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ABSTRACT

In the field of transplantation, the humoral immune response against mismatched HLA antigens of the donor is associated with inferior graft survival, but not in every patient. Donor-specific HLA antibodies (DSA) of different IgG subclasses may have differential effects on the transplanted organ. Recombinant technology allows for the generation of IgG subclasses of a human monoclonal antibody (mAb), while retaining its epitope specificity.

In order to enable studies on the biological function of IgG subclass HLA antibodies, we used recombinant technology to generate recombinant human HLA mAbs from established heterohybridomas. We generated all four IgG subclasses of a human HLA class I and a class II mAb and showed that the different subclasses had a comparable affinity, normal human Fc glycosylation, and retained HLA epitope specificity. For both mAbs, the IgG1 and IgG3 isotypes were capable of binding C3d and efficient in complement-dependent cell lysis against their specific targets, while the IgG2 and IgG4 subclasses were not able to induce cytotoxicity. Considering the fact that the antibody-binding site and properties remained unaffected, these IgG subclass HLA mAbs are excellent tools to study the function of individual IgG subclass HLA class I and class II-specific antibodies in a controlled fashion.

INTRODUCTION

In the field of transplantation, the induction of a humoral immune response to mismatched HLA antigens on the donor kidney is associated with graft rejection and inferior graft survival, but only in a subpopulation of patients.[1-3] The various clinical effects may be caused by the (mixture of) IgG subclass of produced donor-specific antibodies (DSA).[4-7] Indeed, various patterns of IgG subclasses have been observed in sera of transplanted patients that developed *de novo* DSA. However, their relative contribution to graft damage remains elusive, due to conflicting results on their clinical significance.[7,8]

The pathogenicity of an HLA antibody is determined by both the affinity for the HLA epitope recognised by the Fab part and the effector function of the antibody, defined by the Fc part. Indeed, the degree of complement activation and the binding capacity to Fc gamma receptors (FcγR) differs per IgG subclass.[9-11] In renal transplantation, DSA capable of complement activation, e.g. IgG1 and IgG3, are associated with allograft loss.[7,12-14] However, other studies have implied that the presence of IgG2 and IgG4 can act either synergistically or inhibitory on complement activation, depending on the epitopes recognised.[15,16] Additionally, HLA IgG antibodies have been associated with graft damage independent of the complement cascade.[17-19] Binding of DSA to endothelial cells can lead to infiltration of macrophages causing antibody-mediated rejection, of which the severity is increased in case of IgG1 and IgG3 antibodies, due to their capacity to bind FcγR.[19] Furthermore, binding and crosslinking of HLA targets on endothelial cells can result in intracellular signalling, resulting in cell proliferation and initiation of coagulation.[18,20,21]

Thus, understanding the underlying mechanisms of IgG HLA antibody-mediated graft damage can contribute to the establishment of risk factors associated with antibody-mediated rejection. Several methodological studies on the effect of HLA antibodies in renal transplantation have been performed using human HLA monoclonal antibodies (mAbs).[16,19,22-25] However, these studies are restricted to the available human HLA mAbs, which are mainly of the IgG1 subclass. Therefore, we adapted a method to recombinantly generate and produce human mAbs of all four IgG subclasses, with the aim to generate HLA class I and class II-specific mAbs of all IgG subclasses recognising the same HLA epitope with the same affinity.

MATERIALS AND METHODS

B cell heterohybridomas

Human B cell heterohybridomas WIM8E5 (IgG1, κ) and RTLK1E2 (IgG1, κ), that had been established from two women who had been immunised during pregnancy by mismatched HLA-A*11:01 and HLA-DRB1*03:01 respectively, were used to generate recombinant human HLA class I and class II-specific mAbs.[25] Heterohybridoma cells were cultured in Iscove's modified Dulbecco's medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum, 200 mM L-glutamine (all Gibco, Invitrogen, Paisley, UK), 50 µM 2 mercapto-ethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands).

Generation of human recombinant IgG1 HLA mAbs

RNA was isolated from heterohybridoma cells using RNeasy mini kit (Qiagen, Hilden, Germany). Next, SMART cDNA synthesis was performed using PrimeScript Reverse Transcriptase (Takara, Saint-Germain-en-Laye, France), and variable heavy chain (VH) IgG1, and variable light chain (VL) kappa (k) or lambda (l) gene products were amplified by 5'-RACE PCR. The VH and VL PCR products were purified with QIAquick gel extraction kit (Qiagen) and treated with T4 DNA polymerase (Bioké, Leiden, the Netherlands). Subsequently, the VH and VL products were ligation-independently cloned into pcDNA3.3 expression vectors[26] with the constant domains of the human IgG1 (IGHG1*03), k (IGKC), or l (IGLC2*01). The vectors were used for transformation of One Shot MAX Efficiency DH5α-T1R competent cells (ThermoFisher Scientific, Waltham, MA, USA) by heat shock. The transformed cells were cultured on LB-agar plates supplemented with 50 µg/ml ampicillin (Sigma-Aldrich) and after overnight incubation at 37°C, multiple single colonies were picked and grown overnight in LB medium containing ampicillin. From the cultures, plasmids were isolated using either QIAprep Spin Miniprep kit (Qiagen) or NucleoBond Xtra Midi EF (Bioké). The plasmids were sequenced by Sanger sequencing (Macrogen, Amsterdam, the Netherlands) to verify the hybridisation of VH and VL products with the expression vector. All kits were used according to manufacturer's instructions.

Generation of human recombinant IgG subclass HLA mAbs

To generate recombinant IgG subclass HLA mAbs, the IgG1 plasmid was double digested with the appropriate restriction enzymes (Bioké). Simultaneously, pMK vectors containing IgG2 (IGHG2*02), IgG3 (IGHG3*01), or IgG4 (IGHG4*01) constant domains (ThermoFisher Scientific) were double digested the same way to obtain the constant domains. Next, the IgG subclass constant domain was ligated with the digested vector by T4 DNA ligase (Bioké). Subsequently,

plasmids were generated as described above. Plasmids were sequenced to verify ligation of constant domain with the vector and to check if any mutations had occurred. No adaptation was made to the light chain.

Production of human recombinant IgG HLA mAbs

For recombinant mAb production, heavy and light chain containing vectors were used for transient co-transfection of Expi293F cells with ExpiFectamine, Opti-MEM, and Expi293 expression medium (ThermoFisher Scientific) according to the instructions provided by the manufacturer. After five days of culture, supernatants containing the recombinant mAbs were harvested and filtered. The presence of IgG was determined by total IgG ELISA, as previously described.[27] IgG specificity of the different subclasses was confirmed by a human IgG subclass ELISA kit (ThermoFisher Scientific).

Purification of recombinant IgG HLA mAbs

The recombinant mAbs were purified using Amicon ProAffinity Concentration Kit Protein G (Merck Millipore, Burlington MA, USA). A maximum of 1000 µg mAb was loaded onto 200 µl Protein G resin and incubated for 60 min at room temperature on a roller bench. After wash steps, mAb was eluted and neutralised. Next, the buffer was exchanged with phosphate buffered saline (PBS, B Braun, Melsungen, Germany) using a Slide-a-lyzer 0.5-3 ml dialysis cassette (ThermoFisher Scientific) by incubating the cassette in beaker with PBS for 21 h at 4°C, PBS was refreshed a couple of times during incubation. The concentrations of purified mAbs were measured using the protein A280 protocol of NanoDrop2000 (ThermoFisher Scientific), and molar concentration were calculated for each mAb.

HLA antibody detection

For verification of the IgG subclasses, the supernatants were screened with Lifecodes Lifescreen Deluxe screening kit (Immucor Transplant Diagnostics, Stamford, CT, USA) modified by using anti-IgG1 (10 µg/ml; HP6001), anti-IgG2 (2.5 µg/ml; HP6002), anti-IgG3 (10 µg/ml; HP6050), and anti-IgG4 (2.5 µg/ml; HP6025) PE-conjugated detection antibodies (Southern Biotech, Birmingham, AL, USA).

The HLA specificities of the recombinant mAbs were determined by screening the recombinant mAbs with Lifecodes HLA class I or II single antigen beads (SAB) using goat anti-human Pan-IgG PE-conjugated on a luminex platform (Immucor). The ability of recombinant mAbs to bind C3d was tested with Lifedcodes C3d detection (Immucor). Both Lifecodes kits were used according to manufacturer's instructions. The data was analysed with Match It! Antibody software version 1.3.0 (Immucor).

Bio-layer interferometry

Affinity of antibody to antigen was determined via bio-layer interferometry (BLI) using the Octet RED96 system (FortéBio, Fremont, CA, USA). HLA IgG subclasses from WIM8E5 were immobilised to anti-human IgG Fc kinetic biosensors with a response threshold of 0.6 nm. To determine the association phase, parallel sensors were dipped into wells containing soluble, recombinant HLA-A*11:01 in a two-fold titration from 200 nM to 6.25 nM for 300 seconds so an equilibrium could be reached. Next, sensors were placed into buffer alone-containing wells for a further 1000 seconds to determine the dissociation phase. Affinity values (K_D) were calculated via steady state analysis, where the response equilibrium (R_{eq}) was plotted against the HLA analyte concentration for each sensor and K_D values were measured as the HLA concentration of 50% of the overall calculated maximum response (R_{max}). All experiments were carried out using standard kinetic buffer (1x PBS, 0.1% bovine serum albumin, 0.02% Tween-20), at a temperature of 30°C and a constant plate shake speed of 1000 rpm.

Fc domain glycosylation profiling

Of WIM8E5 and RTLK1E2 IgG subclasses, 2 µg sample was added to a final volume of 20 µl PBS and affinity captured with ProtG beads. After desalting, the mAbs were eluted with 100 µl 100 mM formic acid and subsequently vacuum dried at 60°C. The dried samples were resuspended in 40 µl digestion solution consisting of 25 mM ammonium bicarbonate and 5 ng/µl sequencing grade trypsin and followed by overnight digestion at 37°C to obtain tryptic glycopeptides. Fc glycosylation was measured by nano liquid chromatography mass spectrometry of glycopeptides followed by data processing using LaCyTools as previously described.[28] From the relative abundances of the glycopeptides the levels hybrid-type, high-mannose, and complex-type Fc *N*-glycans as well as the level of galactosylation, fucosylation, bisection, and sialylation were calculated.

Cells

HLA-typed peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors after informed consent (Sanquin Blood Supply, Amsterdam, the Netherlands). PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and cryopreserved until further use. For the cytotoxicity experiment with RTLK1E2 mAbs, B cells were magnetically isolated from PBMCs using EasySep direct HLA cross-match B cell isolation kit (Stemcell Technologies, Köln, Germany) with a purity of >90%.

Complement-dependent cytotoxicity assay

Terasaki plates (Greiner) were oiled and filled with 1 µl of supernatant containing the mAb of interest in triplicate. Then, 3000 HLA typed PBMCs or B cells were added to each well and incubated for 60 min at 20°C. Next, 5 µl rabbit complement (Inno-train, Kronberg, Germany) was added and incubated for 60 min at 20°C. To visualise cytotoxicity, 5 µl propidium iodide ink was added to each well, and after 15 min incubation in the dark the plates were analysed with Patimed (Leica Microsystems, Amsterdam, the Netherlands).

Statistical analysis

The Kruskal-Wallis test was used for unpaired analysis and the Friedman test was used for paired analysis. Statistical level of significance was defined as $p<0.05$, and analyses were performed with GraphPad Prism, version 7.02 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Recombinant human IgG subclass HLA mAbs

Genes encoding the variable heavy chain and light chain domains were cloned into expression vectors, after which recombinant antibodies can be expressed by transient co-transfection of both vectors.[29-31] Here, we generated recombinant human HLA class I and class II mAbs, WIM8E5 and RTLK1E2 respectively, of all four IgG subclasses. To verify IgG subclass, the supernatant of all four IgG subclass mAbs were screened with IgG subclass ELISA and a modified luminex screening assay using detection antibodies specific for each IgG subclass. As shown in Figure 1, the specific IgG constant domains were recognised by the correct detection antibody, indicating that mAbs of all four IgG subclasses were produced.

To corroborate that HLA specificities remained unaffected by the recombinant technology, original hybridoma-generated mAbs and recombinant human IgG subclass mAbs were screened with HLA class I or II SAB luminex assay. Upon comparison of the background corrected mean fluorescence intensity (BCM) values of both WIM8E5 (Figure 2A) and RTLK1E2 (Figure 2B) mAbs, no difference in HLA specificities was observed with the original hybridoma-generated mAb for both recombinant IgG subclass HLA mAbs.

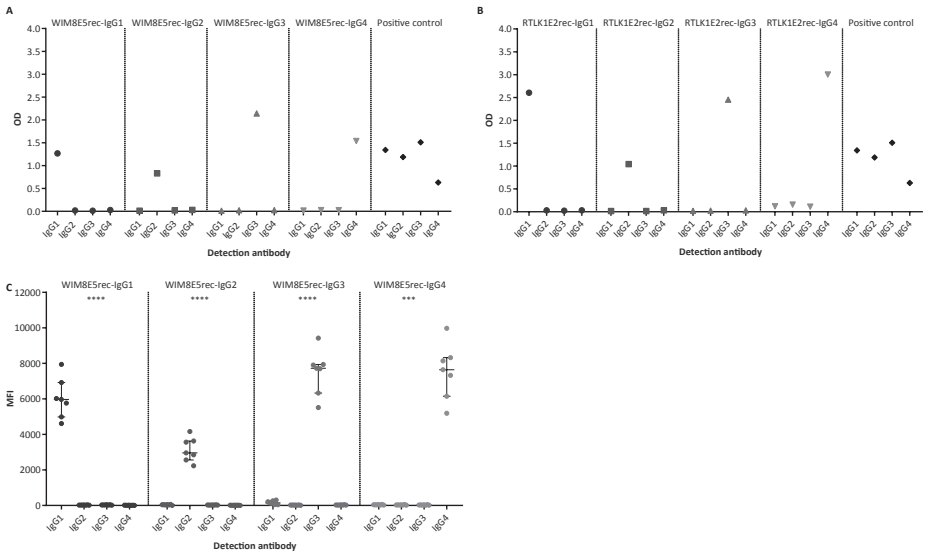

Figure 1: Recombinant IgG subclass HLA mAbs could be detected by the corresponding IgG-specific detection antibody. IgG subclass could be detected with IgG subclass ELISA kit for both WIM8E5rec-IgG mAbs (A) and RLTK1E2rec-IgG mAbs (B). Positive control is a human serum. (C) Similar findings were observed when screening recombinant WIM8E5 IgG subclass mAbs with Lifecodes Lifescreen Deluxe kit. The kit contains seven groups of HLA class I beads and each data point represents a single bead group. Kruskal-Wallis test was used to compare median of all four detection antibodies per IgG subclass mAb. Error bars represent median ± interquartile range. MFI is mean fluorescence intensity. OD is optical density. ***P<0.001, ****P<0.0001

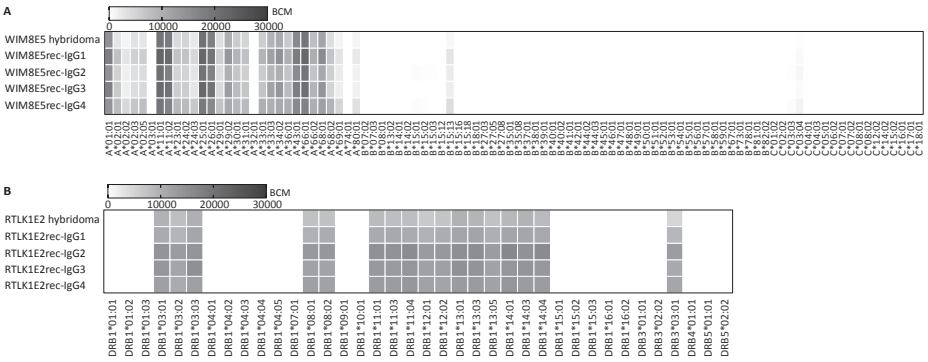

Figure 2: The same HLA epitope is recognised by the recombinant IgG subclass HLA mAbs. HLA specificities of recombinant IgG1, IgG2, IgG3, and IgG4 of WIM8E5 mAb (A) and RTLK1E2 mAb (B, only DRB beads shown as all other loci were negative) as detected by luminex SAB assay. Purified recombinant mAb concentration tested was 62.5nM. BCM is background corrected mean fluorescence intensity.

Affinity and Fc domain glycosylation is similar between IgG subclasses

As the recombinant IgG subclass HLA mAbs have the same HLA specificity, we next questioned whether these mAbs have the same affinity for the immunizing HLA allele. Therefore, the recombinant IgG subclass WIM8E5 mAbs were tested with bio-layer interferometry (BLI). The affinity values (K_D) observed for the target HLA-A*11:01 were in the range of 25-32 nM for all four IgG subclass WIM8E5 mAbs (Figure 3).

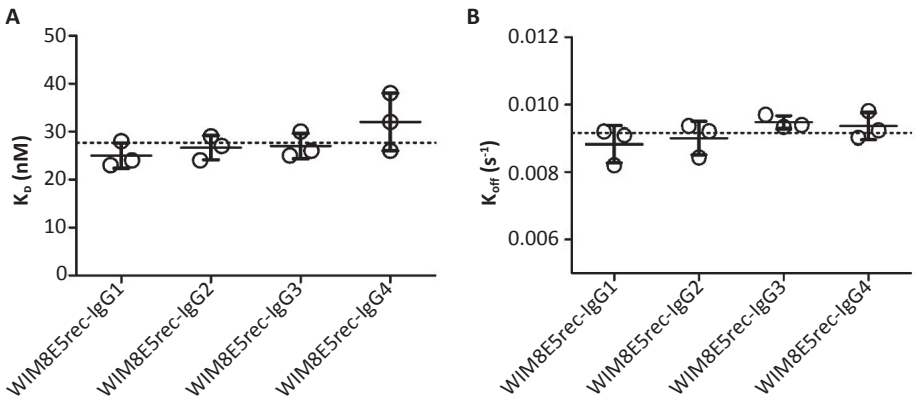

Figure 3: Recombinant IgG subclass HLA mAbs have similar affinity. The affinity (A) and dissociation rates (B) of recombinant IgG subclass WIM8E5 mAbs were determined via bio-layer interferometry. Calculated values are consistently similar across all IgG subclasses against the target HLA-A*11:01. The dotted lines represent the affinity (A) and dissociation constant (B) average across all four IgG subclasses. Error bars represent the mean ± standard deviation of 3 experiments.

For mAb production Expi293F cells are used, so we wanted to determine if the correct human glycosylation was present on the Fc part of the generated mAbs. The glycosylation characterisation of the generated recombinant IgG subclass HLA class I and class II mAbs showed that the IgG subclasses have a similar profile (Figure 4). In addition, the observed glycosylation traits of the mAbs are in accordance with those found on IgG in human serum using the same method.[32] However, the relative levels of bisection (the presence of a bisecting *N*-acetylglucosamine) and sialylation are lower on the mAbs, as compared to what is generally found on IgG in human serum, while the abundance of high mannose-type species is higher.[32] For IgG3, we detected partial occupancy of the *O*-glycosylation sites in the hinge region (data not shown), which is in line with the hinge region *O*-glycosylation of IgG3 from the human circulation.[33]

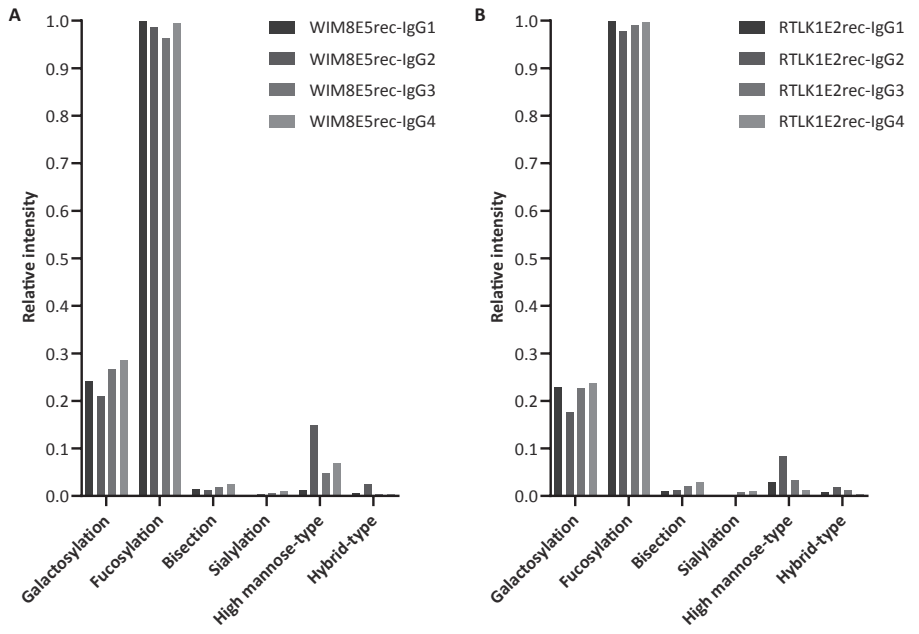

Figure 4: The glycosylation profile of recombinant IgG subclass HLA mAbs is similar. Relative intensity values of derived traits for the recombinant IgG1, IgG2, IgG3, and IgG4 of WIM8E5 mAb (A) and RLTK1E2 mAbs (B) are shown.

Cytotoxicity of recombinant human IgG subclass HLA mAbs

To determine whether the recombinantly generated IgG subclass HLA-specific mAbs showed the anticipated cytotoxicity patterns, we performed complement-dependent cytotoxicity (CDC) assays. Incubation of WIM8E5 recombinant IgG subclass mAbs with PBMCs expressing HLA antigens recognised by WIM8E5 (HLA-A11, -A1) showed that WIM8E5rec-IgG1 and -IgG3 mAbs were highly cytotoxic in a dose-dependent manner (Figure 5A). Both IgG2 and IgG4 subclasses did not show cytotoxicity. For the HLA class II mAb RTLK1E2 a CDC using purified B cells (HLA-DR17, -DR13) was performed. Both RTLK1E2rec-IgG1 and -IgG3 were highly cytotoxic (Figure 5B). While CDC with rabbit complement is standard practice in transplantation, it does not show if mAbs can also activate human complement. Testing the recombinant IgG subclass HLA mAbs with C3d SAB assay showed that only IgG1 and IgG3 mAbs are capable of binding human C3d (Supplementary Figure 1), suggesting that these mAbs can activate human complement.

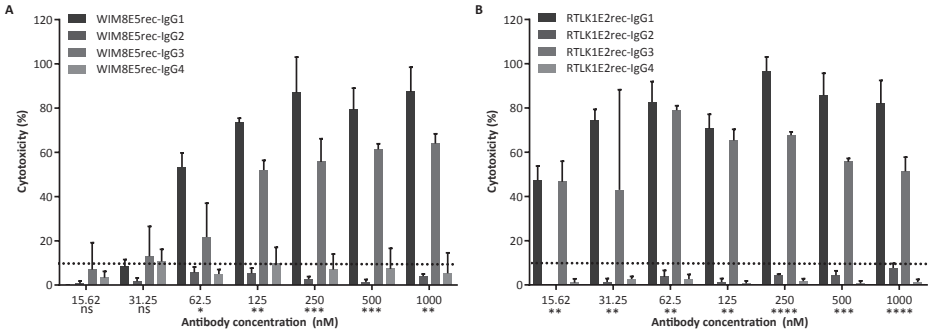

Figure 5: Recombinant IgG1 and IgG3 HLA mAbs are cytotoxic. (A) Recombinant IgG subclass WIM8E5 mAbs were incubated with PBMC expressing HLA-A1, -A11, -B8, -B55, -Cw3, and -Cw7. WIM8E5rec-IgG1 and -IgG3 induced cell lysis (>60%), while IgG2 and IgG4 were unable to induce complement cytotoxicity. (B) Recombinant IgG subclass RTLK1E2 mAbs were incubated with B cells expressing HLA-DR17, -DR13 and -DR52. RTLK1E2rec-IgG1 (>80%) and -IgG3 (>60%) induced cell lysis, while no cell lysis was observed for RLTK1E2rec-IgG2 and RTKL1E2rec-IgG4. mAbs were added in various concentrations (1000, 500, 250, 125, 62.5, 31.25, and 15.62nM). Error bars represent the mean ± standard deviation of triplicate wells. The Kruskal-Wallis test was used per dilution to compare the IgG subclass HLA mAbs. Dotted line indicates background. ****P<0.0001 ***P<0.001, **P<0.01, *P<0.05

DISCUSSION

In this study, we show the generation and production of recombinant human HLA class I and class II-specific mAbs of all four IgG subclasses from established B cell heterohybridomas. The generated recombinant HLA mAbs of all four IgG subclasses recognise the same HLA epitope with the same binding affinity. Currently, we were only able to determine the affinity for the HLA class I mAbs, due to lack of recombinant HLA class II. Furthermore, we show that all recombinant IgG subclass HLA mAbs have human-type Fc glycosylation and the glycosylation profiles were similar between the mAbs. The conserved *N*-glycans located at asparagine 297 of the Fc part play a role in the function of an antibody and the different levels of specific glycosylation traits could have pronounced effects on complement activation and FcγR binding.[34,35] Both IgG1 and IgG3 HLA class I and class II mAbs are capable of complement activation, while a weak or no cytotoxicity was observed for the IgG2 and IgG4 mAbs. Preliminary data suggest that IgG1 and IgG3 can induce antibody-dependent cell-mediated cytotoxicity (ADCC), but only a low percentage of cell lysis was observed (Supplementary Figure 2). This can be explained by the high levels of fucosylation (>96%) on the recombinant IgG subclass HLA mAbs, as it has been shown that high levels of fucosylation on IgG negatively influences ADCC activity.[36] Glyco-engineering of our recombinant IgG subclass HLA mAbs may further allow altering their functional properties.[37]

Currently available human HLA mAbs are mainly derived from multiparous women by Epstein-Barr virus transformation and electrofusion using mouse myeloma cell line, are primarily directed against HLA class I, and are restricted to an IgM or IgG1 isotype.[25,38,39] These human HLA mAbs have been widely used in various applications, such as flow cytometry assays,[40-42] B-cell ELISPOT assays,[43-45] blocking assays,[46] assays to determine HLA expression levels,[47-49] or functional assays of HLA antibodies.[16,19,22-24] Commercial chimeric IgG subclass HLA mAbs are available, but those have a mouse variable part, while W6/32 and F3.3 recognise all HLA class I molecules and majority of HLA class II molecules respectively.[50]

Recently, Gu *et al*.[51] elegantly characterised an HLA class I mAb generated by a germline phage display from a non-sensitised individual, resulting in an antibody that likely can be generated during an alloimmune response. In contrast we have produced human recombinant HLA mAbs generated from heterohybridomas that were derived from B cells of immunised individuals. Therefore, the mAbs we produced are truly representative of HLA antibodies produced through alloimmunisation. In addition, we generated both human HLA class I and class II mAbs of all four IgG subclasses. Especially the latter is unique, as there are only a limited number of HLA class II mAbs available and those are mainly IgG1.

Antibody effector function is determined by its isotype. As mentioned, complement binding HLA DSA is associated with graft loss, but in sera of renal transplant patients both complement binding, IgG1 and IgG3, as well as non-complement binding, IgG2 and IgG4 HLA antibodies are observed.[8,12-14] Previous studies have showed by mixing IgG subclass mAbs that IgG2 and/or IgG4 can inhibit complement activation of IgG1 and/or IgG3 when recognising the same epitope.[15,51] Others performed mixing experiments with human HLA mAbs directed against different epitopes of same HLA antigens and showed that combining these HLA mAbs promote complement activation, while individually the mAbs had no effect.[16] Additional mixture studies with human mAbs directed to HLA class I and class II molecules of different IgG subclasses, comparing different specificities and avidities, should be performed as this will allow greater understanding of the interaction of antibodies of different IgG subclasses recognising different epitopes on the same HLA molecules.

In addition, HLA mAbs have been used for functional assays to study HLA-antibody-induced graft damage independent of the complement cascade. HLA class I antibodies can cause crosslinking on endothelial and smooth muscle cells inducing intracellular signalling, resulting in inflammatory activation, and leukocyte recruitment such as P-selectin-induced monocyte adhesion.[17-19,52-54] Although crosslinking is irrespective of the IgG subclass, the level of P-selectin

on endothelial cells and the monocyte recruitment via FcγR mechanism are increased with IgG1 and IgG3 antibodies. The levels of P-selectin and FcγR-dependent monocyte recruitment have been well studied with human IgG1 HLA mAb,[19] but due to lack of IgG3, IgG2, and IgG4 HLA mAbs, the exact influence of antibodies with these isotypes, especially the latter two, is not fully clear.

Human HLA mAb do not represent the polyclonal response observed in sera. However, due to the mixture of antibody specificities, concentrations and isotypes of HLA antibodies present in sera it is difficult to study the role and function of HLA antibodies. By using human HLA mAbs, mixture experiments can be performed in a controlled manner, even with normal human serum as matrix. For future studies it is essential to extend the specificities of the available mAbs, since especially mAbs for HLA class II are currently lacking.

In conclusion, we show here that recombinant human HLA class I and class II mAbs of all four IgG subclasses recognising the same HLA epitope can be generated from established B cell heterohybridomas. This method enables us to generate more IgG subclass HLA mAbs recognising different epitopes on the same HLA antigen, which can be used in mixing experiments to study the role and function of HLA DSA of different IgG subclasses in a controlled fashion. These IgG subclass HLA class I and class II mAbs can provide mechanistic insights into the role of DSA in renal transplantation and in other clinical settings.

Acknowledgements

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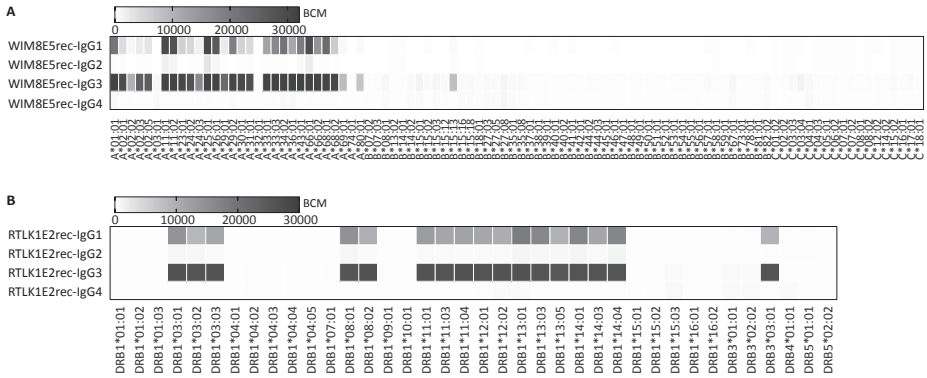
Data available on request from the authors

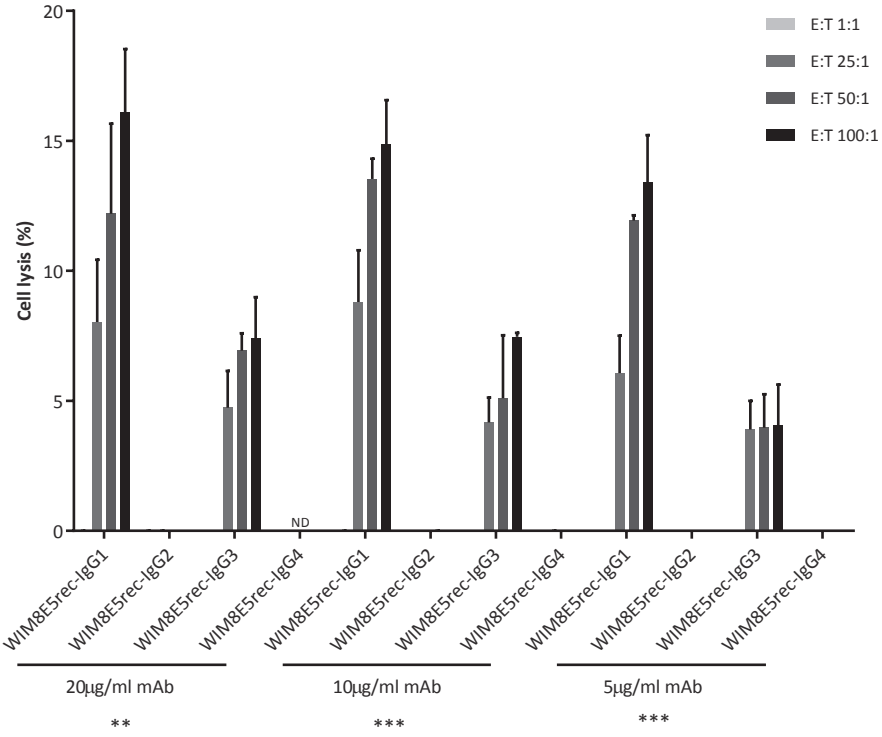
Conflict of interest:

Chalana C.C. Zilvold-van den Oever and Hendrik J. Rademaker are Genmab employees and own Genmab stocks.

SUPPLEMENTAL MATERIAL



Supplementary Figure 1: Human recombinant IgG1 and IgG3 HLA mAbs can bind to human C3d. Recombinant IgG subclass WIM8E5 mAbs (A) and RTLK1E2 mAbs (B, only DRB beads shown as all other loci were negative) were screened with Lifecodes C3d detection assay according to manufacturer's instructions. Purified recombinant mAb was tested with concentration 62.5nM. BCM is background corrected mean fluorescence intensity



Supplementary Figure 2: Human recombinant IgG1 and IgG3 HLA mAbs can induce antibody-dependent cell lysis. HLA-typed phytohemagglutinin (PHA) blast cells expressing HLA-A11, -A24, -B35, -B40, -Cw10, and -Cw4 were generated by culturing PBMCs for 7 days in RPMI 1640 medium (Gibco) containing 10% human serum S357, 200 mM L-glutamine, 10 CU/ml IL-2 (Proleukin Novartis, Arnhem, the Netherlands), and 4 µg/ml PHA (ThermoFisher Scientific). These PHA blasts were labelled with chromium-51 (^{51}Cr), and incubated with different concentrations non-purified WIM8E5rec-IgG1, -IgG2, -IgG3, and -IgG4 for 30 min at 37°C. Next, the effector cells, the HLA-typed PBMCs, were added and after 4 hours of incubation at 37°C, ^{51}Cr release was measured with γ-counter (PerkinElmer, Waltham, MA, USA). The percentage of cell lysis was calculated by the following formula: (experimental ^{51}Cr release – spontaneous ^{51}Cr release) / (maximum ^{51}Cr release – spontaneous ^{51}Cr release) x 100. ^{51}Cr-labeled PHA blasts incubated with medium alone gave spontaneous ^{51}Cr release and maximum ^{51}Cr release was determined by adding TritonX100. Experiment was performed at different effector:target (E:T) ratios. Cell lysis was only observed with WIM8E5rec-IgG1 and -IgG3 induced cell lysis. Per mAb concentration the Friedman test paired for E:T ratio was performed to indicate difference between the four IgG subclass mAbs. Error bars represent the mean ± standard deviation of triplicate wells. ND is not determined. ***P<0.001, **P<0.01, *P<0.05

ABBREVIATIONS

ADCC: Antibody-dependent cell-mediated cytotoxicity

BCM: Background corrected mean fluorescence intensity

CDC: Complement-dependent cytotoxicity

C1q: Complement component 1q

C3d: Complement component 3d

DNA: Deoxyribonucleic acid

DSA: Donor-specific antibodies

ELISA: Enzyme-linked immunosorbent assay

FcγR: Fc gamma receptors

HLA: Human leukocyte antigen

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL: Interleukin

LB: Lunia-Bertani

mAbs: Monoclonal antibodies

MFI: Mean fluorescence intensity

PBMCs: Peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCR: Polymerase chain reaction

PHA: Phytohemagglutinin

RACE: Rapid amplification cDNA ends

RNA: Ribonucleic acid

SAB: Single antigen beads

VH: Heavy chain variable domain

VL: Light chain variable domain

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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).

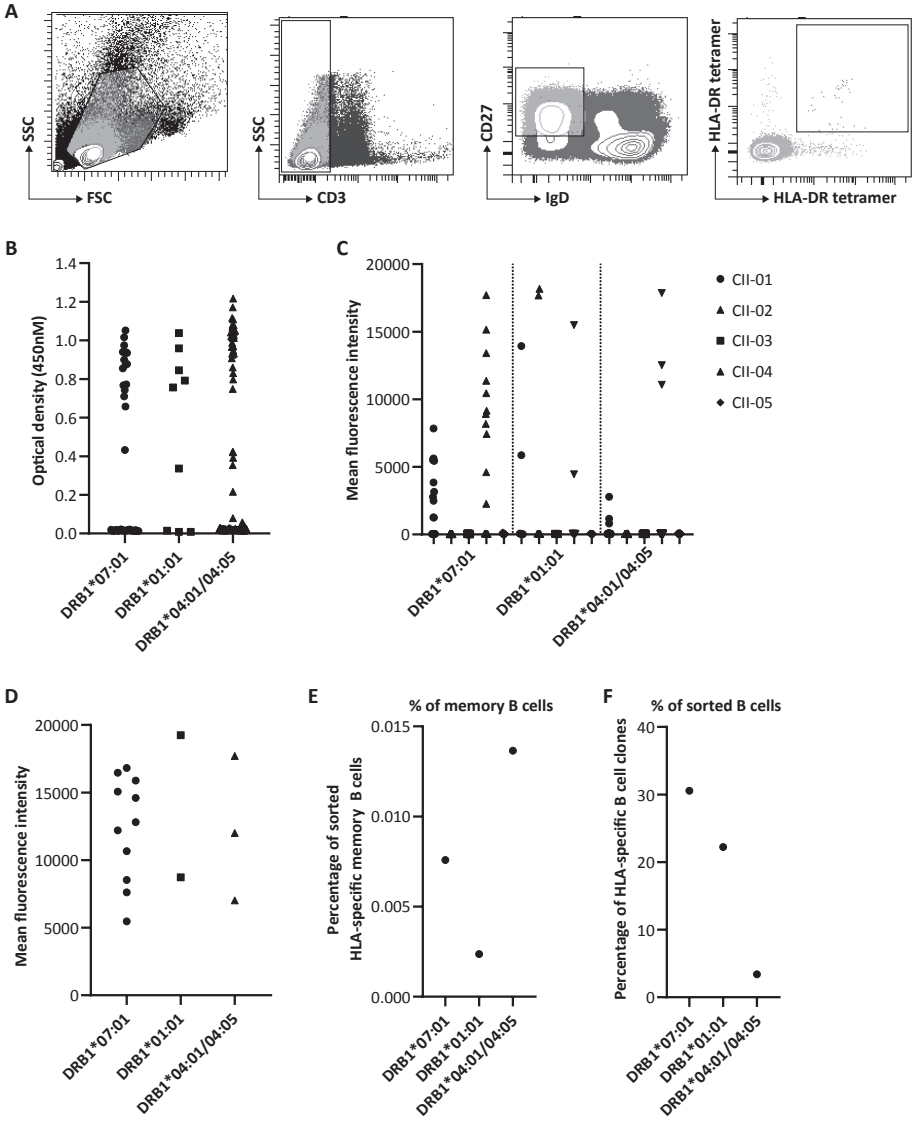

Figure 1: HLA-DR specific memory B cell clones isolated from peripheral blood. A) Representative example of three independent experiments depicting the flow cytometry gating strategy to live single cell sort $CD3^{-}CD27^{+}IgD^{-}$HLA-DR tetramer double positive B cells from PBMC. B) IgG antibody production 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.

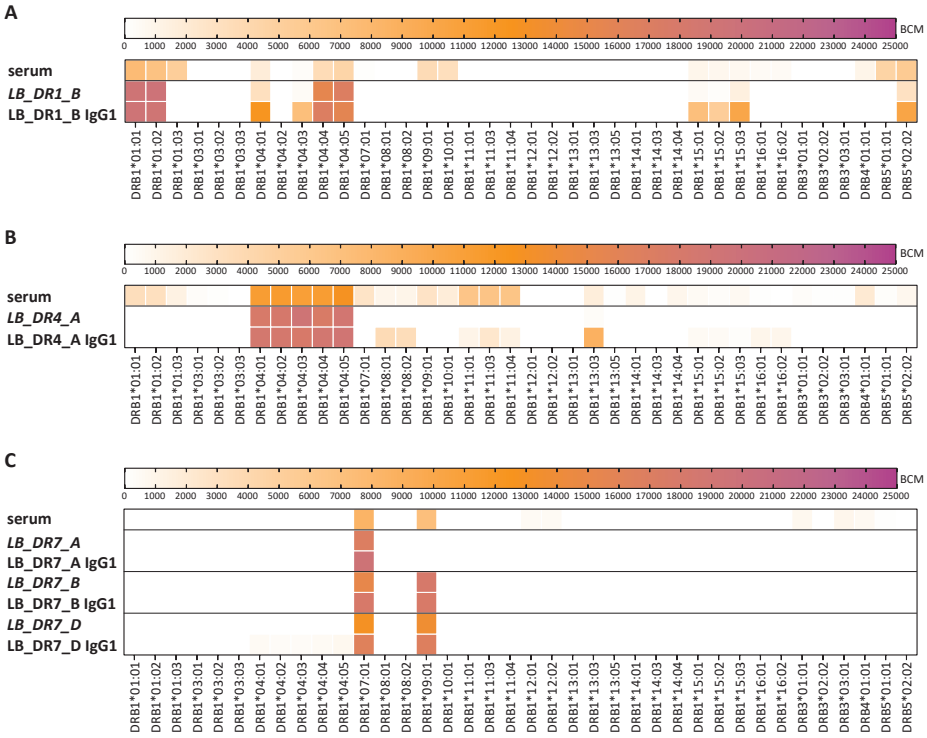

Figure 2: Recombinant human HLA-DR monoclonal antibodies have the same reactivity as the B cell clones. HLA-DR specificities in serum, supernatants of B cell clones (italic) and generated IgG1 mAbs. A) Serum of individual used for HLA-DRB1*01:01 sort and the generated LB_DR1_B mAb and with its respective 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
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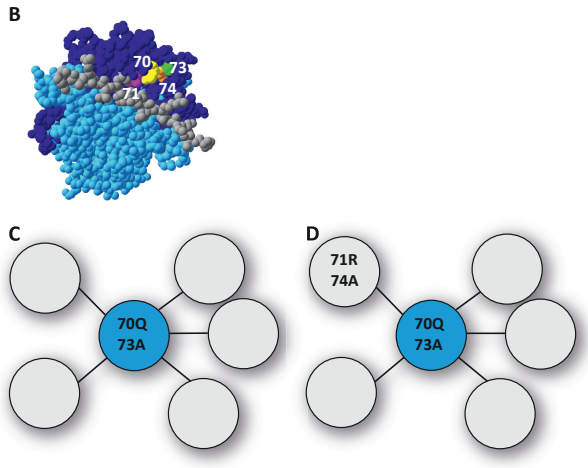


Figure 3: Reactivity analysis of LB_DR1_B monoclonal antibody. A) Comparison of the amino acid positions of interests of the reactive HLA-DR alleles of LB_DR1_B mAb and a selection of the non-reactive HLA-DR alleles. B) Locations of amino acid 70Q (yellow), 71R (magenta), 73A (green) and 74A (orange) 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						

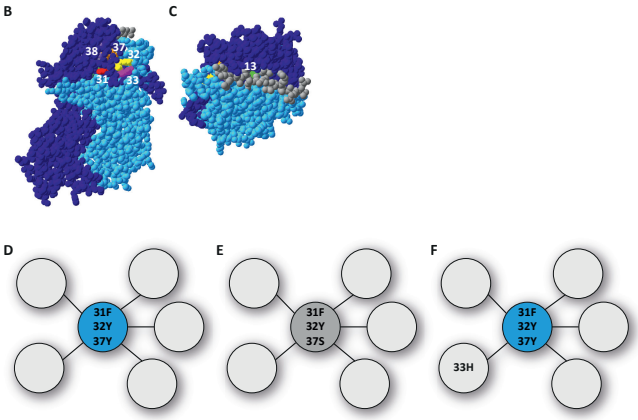


Figure 4: Reactivity analysis of LB_DR4_A monoclonal antibody. A) Comparison of the amino acid positions of interests of the reactive HLA-DR alleles of LB_DR4_A mAb and a selection of the non-reactive HLA-DR alleles. B) Locations of amino acid 31F (red), 32F (yellow), 33H (magenta), 37Y (orange), 38V (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.

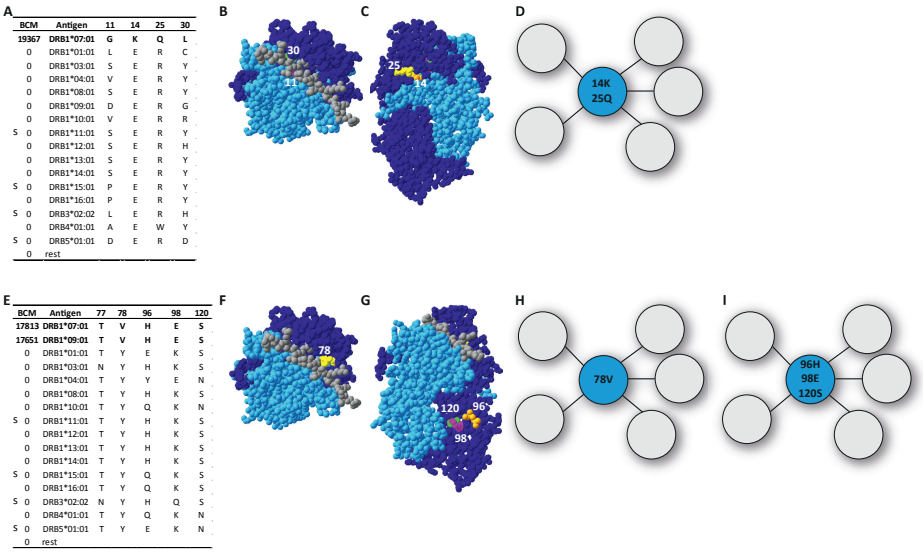
The stronger interaction observed for the DR4 alleles suggests involvement of AAs solely present on DR4 alleles, which are 96Y, 180 leucine (L), and histidine (H) on position 13 and 33. The latter two AAs are within 15 Å radius of 32Y, but only 33 is exposed and is therefore most likely involved in the 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.



	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				

	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

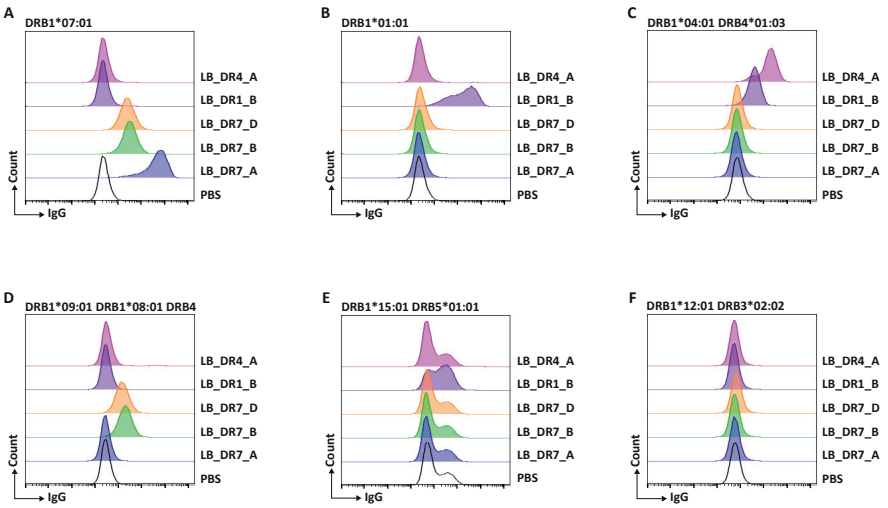
The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Data availability statement

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

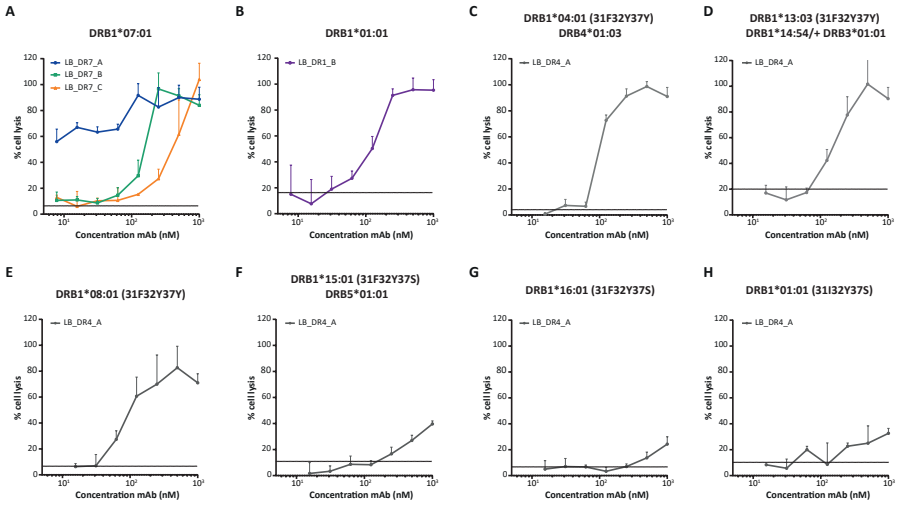
SUPPLEMENTAL MATERIAL

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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CHAPTER 6

HLA-EMMA: A USER-FRIENDLY TOOL TO ANALYSE HLA CLASS I AND CLASS II COMPATIBILITY ON THE AMINO ACID LEVEL

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ABSTRACT

In renal transplantation, polymorphic amino acids on mismatched donor HLA molecules can lead to the induction of *de novo* donor-specific antibodies (DSA), which are associated with inferior graft survival. To ultimately prevent *de novo* DSA formation without unnecessarily precluding transplants it is essential to define which polymorphic amino acid mismatches can actually induce an antibody response. To facilitate this, we developed a user-friendly software program that establishes HLA class I and class II compatibility between donor and recipient on the amino acid level.

HLA epitope mismatch algorithm (HLA-EMMA) is a software program that compares simultaneously the HLA class I and class II amino acid sequences of the donor with the HLA amino acid sequences of the recipient and determines the polymorphic solvent accessible amino acid mismatches that are likely to be accessible to B cell receptors. Analysis can be performed for a large number of donor-recipient pairs at once.

As proof of principle, a previously described study cohort of 191 lymphocyte immunotherapy recipients was analysed with HLA-EMMA and showed a higher frequency of DSA formation with higher number of solvent accessible amino acids mismatches.

Overall, HLA-EMMA can be used to analyse compatibility on amino acid level between donor and recipient HLA class I and class II simultaneously for large cohorts to ultimately determine the most immunogenic amino acid mismatches.

INTRODUCTION

In renal transplantation, human leukocyte antigen (HLA) antigen matching enhances long-term graft survival.[1,2] Nonetheless, most recipients receive a graft with one or more HLA antigen mismatches due to high level of polymorphism of the HLA system and scarcity of donor organs. In addition, even grafts that are matched on the antigen level can be mismatched at the allelic level and can therefore induce an alloimmune response.[3,4] The presence of mismatched HLA antigens on the donor graft can lead to the formation of *de novo* donor-specific HLA antibodies (DSA), which are associated with graft loss.[5,6] Moreover, sensitisation towards HLA significantly reduces the chance of receiving a repeat transplant.[7]

While current matching algorithms are mainly based on HLA-A, -B and -DR matching at the antigen level, one should realise that HLA antibodies are not specific for antigens, but recognise B cell epitopes present on HLA molecules.[8] In addition, the immunogenicity of HLA mismatches has been shown to be dependent on configurations of polymorphic amino acids on antibody accessible positions, which have been theoretically defined and are called eplets.[9,10] Indeed, several groups have shown that the chance of developing *de novo* DSA after transplantation increases with an increasing number of mismatched eplets.[11-13] However, not every eplet mismatch triggers an immune response, indicative of a difference in immunogenicity of individual eplet mismatches.[14] The immunogenicity of a mismatched HLA allele is, amongst others, dependent on the HLA class II phenotype of the recipient as it determines if a specific eplet mismatch will lead to a full-blown antibody response. B cells require $CD4^+$ T cell help to switch towards IgG antibody producing cells and this help depends on the recognition of T cell epitopes presented by the recipients HLA class II molecules on the B cells.[15,16] Furthermore, the type of amino acid substitution (i.e. difference in size, charge) can play a role in immunogenicity as it can affect the structure and physicochemical properties of an HLA molecule.[17] As eplets are theoretically defined, experimental verification is required to determine if an antibody can actually bind to an eplet, which has only been done for a limited number of eplets, mainly present on HLA class I.[18-20]

Other approaches based on amino acid mismatches and/or physicochemical scores have shown also to be useful to assess sensitisation risk of HLA allele mismatches on the population level.[12,21-23] While eplets are predefined entities that are still subject to change,[24] the amino acids that are the underlying basis of the eplets are fixed entities on HLA molecules. Therefore, we aim to define the immunogenicity of specific HLA mismatches based on polymorphic amino acids rather than eplets on HLA class I and class II molecules using large datasets of

donor and recipient pairs. Based on these mismatches and the information on *de novo* DSA formation, polymorphic amino acids crucial for the induction of an antibody response can be defined. For this purpose, we have developed a user-friendly software program, which analyses HLA class I and class II compatibility between donor and recipient on amino acid level focussing on the solvent accessible amino acid mismatches. For the analyses of large cohorts, a batch analysis option was incorporated into the software program.

METHODS

Development of HLA-EMMA

The HLA Epitope Mismatch Algorithm (HLA-EMMA) was developed in Microsoft Visual Studio and uses the .NET framework 4.6. It was written in VB.NET language. The software package is freely available for download (http://www.HLA-EMMA.com).

HLA amino acid sequences

All available HLA amino acid sequences were extracted from the IPD-IMGT/HLA database version 3.39 for HLA-A, -B, -C, -DRB1,3,4,5, -DQA1, -DQB1, -DPA1 and -DPB1 in January 2020,[25] and will be periodically updated in the software program. HLA alleles are included up to the second field typing resolution,[26] since higher resolution typing does not affect the amino acid sequence of the protein. Null alleles, such as DRB4*01:03N, are recognised by the software program and will not be considered for analysis as these HLA alleles are not expressed on cells. HLA-EMMA contains the amino acid sequences for position 1 to 275 for HLA class I, and position 1 to 226 for HLA class II, the beginning of the mature proteins and regions that are of interest for antibody induction. For some HLA alleles, amino acid data at the beginning and/or end of the sequence are lacking. These HLA alleles, often rare HLA alleles, are marked in the algorithm but not excluded from analysis.

Solvent accessible polymorphic positions

Solvent accessible polymorphic amino acid positions were determined using publicly available crystal structures and open source relative solvent accessibility prediction tools. HLA crystal structures were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB, https://www.rcsb.org/ accessed on February 4, 2019).[27] More than 690 PDB HLA structures were available, with the multiple structures of the same HLA allele. Therefore, the initial selection was based on previously described HLA structures used for modelling with accurate structural quality, based on parameters such as atomic resolution, R factor, total number of crystallographically resolved residues and stereochemical quality.[17,28]

Only HLA structures that are not in complex with a ligand and without any amino acid mutations were included. The list was extended with other, not yet included, HLA alleles of which structures are available with a correct amino acid sequence, not in complex with other ligands, and with finer atomic resolution (≤2.8Å). In case of multiple structures for a specific HLA allele the structure with highest atomic resolution was selected. This resulted in a total of 43 HLA class I crystal structures (Supplementary Table 1) and 20 HLA class II structures (Supplementary Table 2). Recently, an online database of HLA class I modelled structures of HLA molecules became available at https://www.phla3d.com.br/.[29] Here, HLA class I tertiary structures were predicted by homology modelling using the amino acid sequences and homologous HLA class I structures, and then refined to improve the quality of the structures. From these HLA class I modelled structures only HLA alleles that were missing from the PDB list were selected (database accessed on April 11, 2019), resulting in 72 modelled structures (Supplementary Table 3).

Open source tools NetSurfP2.0[30] and Porter Pale4.0[31] were used to predict solvent inaccessible amino acid positions. First, for each HLA structure the relative solvent accessibility of each amino acid positions was predicted using both tools. Next, if both tools predicted a relative solvent accessibility of lower than 25% for a specific amino acid position on all HLA structures of an HLA locus than this position was defined as solvent inaccessible. All the remaining positions were defined as solvent accessible. Only positions that are polymorphic within a locus and, in addition, solvent accessible are considered for defining solvent accessible amino acid mismatches.

Due to limited availability of structures for HLA-DR, the polymorphic solvent accessible positions of HLA-DR loci were defined by all positions that are not predicted as solvent inaccessible for the available DRB1, DRB3, DRB4, and DRB5 structures and if a position is polymorphic for at least one of the DRB1, DRB3, DRB4 or DRB5 amino acid sequences. In addition, the amino acid sequences of the HLA class II structures are incomplete, and as a result solvent inaccessible prediction was lacking for the positions near the end of the amino acid sequences (HLA-DR positions 198-226, -DQB1 positions 198-226, -DQA1 position 199-226, -DPB1 positions 190-226 and -DPA1 positions 183-226). Those positions are currently defined as solvent accessible if polymorphic.

Input and output of HLA-EMMA

The donor and recipient HLA typing input of HLA-EMMA is preferentially second field HLA typing, since this resolution describes the specific HLA protein. In case an HLA allele is entered

that is not present in the IPD-IMGT/HLA database, HLA-EMMA will show a warning. However, incomplete HLA typing information can be entered, e.g. if DQA1 typing is missing, an output will still be generated.

In case of serological typing or first field DNA typing HLA-EMMA will convert to the most likely second field typing, based on a panel of high-resolution typing results of a pre-defined population. Currently, conversion can be based on most common alleles of the population "the Netherlands, Leiden" (NL n=1305) (http://www.allelefrequencies.net), or most common HLA alleles of European Caucasians generated from the National Marrow Donor Program (EURCAU n=81106).[32,33] If required, upon request the conversion option can be extended to other populations of which high resolution typing data is available and published.

Besides manual entry, a batch analysis option is included for which the input format is a Microsoft Excel file. For comparing donor and recipient HLA, a file containing the HLA typing of an individual is present on each row, and each column represents an allele (Supplementary Figure 1). The order of recipient and donor in file is irrelevant, provided that each recipient-donor couple has a unique identification code, e.g. R1 and D1, for recipient and donor respectively.

Upon batch analysis, an export file .xml file is generated as an output file, which can be opened with Microsoft Excel (Supplementary Figure 2). While the output of the manual entry is generated and presented immediately, it can also be exported as .xml file for downstream application.

Study cohort for validation

To validate HLA-EMMA, we used a previously described lymphocyte immunotherapy study cohort (n=191).[17] Briefly, this cohort consists of women that received their first lymphocyte immunotherapy from their male partner in 2009 and 2010. The HLA type of the women and their partner was determined by genotyping array (Illumina, San Diego, CA, USA) and HLA imputation. In addition, reverse PCR sequence-specific oligonucleotide was used to type HLA-A and HLA-B that were used as quality control. Antibodies against donor antigen were identified by testing sera, obtained 5 weeks (median 33 days, SD 4.5) following lymphocyte immunotherapy, with luminex single antigen bead (SAB) assays (One Lambda, Canoga Park, CA, USA) and DSA were defined as MFI of ≥2000. HLA mismatches of which HLA second field typing could not be determined, towards which DSA were present before treatment, or that were not present in luminex SAB assay were excluded from analysis.

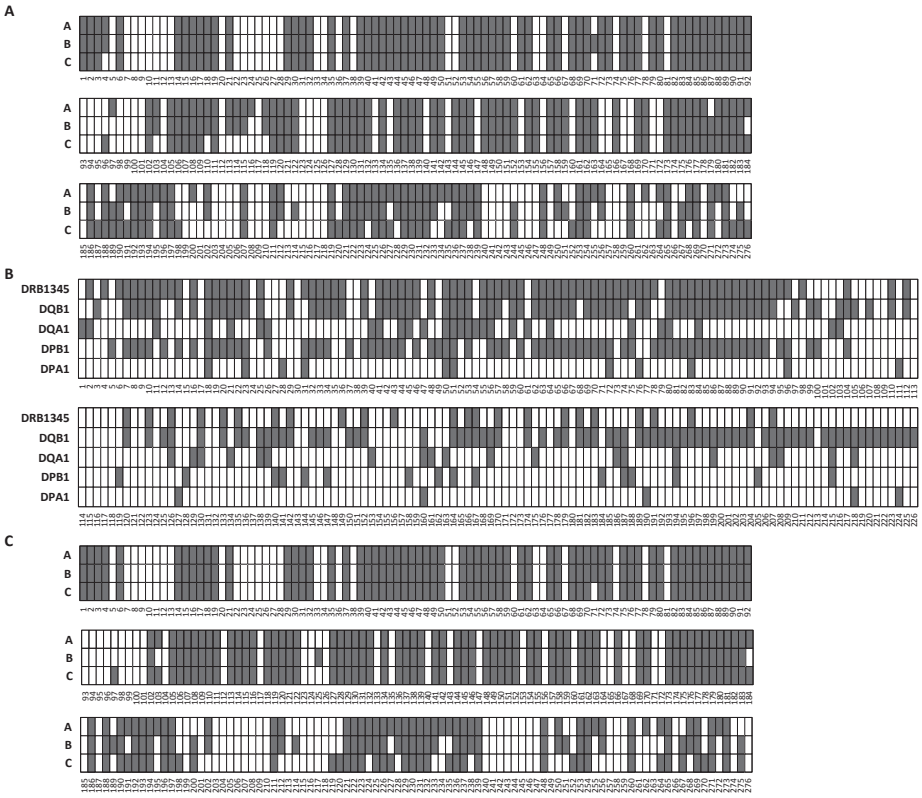

Figure 1: Polymorphic solvent accessible amino acid positions. For each locus the defined polymorphic solvent amino acid positions are indicated in grey for HLA class I (A) and HLA class II (B). C) The polymorphic solvent accessible positions defined by the HLA class I modelled structures.

RESULTS

Polymorphic solvent accessible amino acid positions

The main goal of HLA-EMMA is to analyse HLA class I and class II compatibility on the amino acid level for a large number of donor-recipient pairs. The software program is based on the hypothesis that any polymorphic amino acid exposed on the surface of an HLA molecule can trigger an antibody response. To this aim, the polymorphic solvent accessible amino acid positions were defined per HLA locus using all known HLA alleles to determine polymorphic positions, and available HLA crystal structures to predict the solvent inaccessible positions, used for deduction of solvent accessible positions. Overall, this led to identification of 174 polymorphic solvent accessible positions for HLA-A, 169 for HLA-B, 162 for HLA-C (Figure 1A), 106 for HLA-DRB1/3/4/5, 149 for HLA-DQB1, 48 for HLA-DQA1, 86 for HLA-DPB1 and 16 for HLA-DPA1 (Figure 1B). By analysing the HLA class I modelled structures, additional polymorphic solvent accessible positions were defined; 2 for HLA-A, 3 for HLA-B and 10 for HLA-C (Figure 1C).

HLA class I and class II solvent accessible amino acid mismatches between donor and recipient

HLA-EMMA compares the amino acid sequence of each donor HLA allele with the alleles from the same locus of the recipient, known as intralocus comparison, except for HLA-DRB1/3/4/5 which is interlocus compared. For HLA class I, the default setting is intralocus comparison, but interlocus option can be selected when required. Amino acid mismatches are calculated for: 1) each donor HLA allele by total amino acid mismatches irrespective of location on the molecule, and 2) amino acid mismatches that are solvent accessible. In case of an incomplete HLA allele, indicated by *, only amino acid mismatches are calculated for the known amino acid sequence.

An example of manual entry for defining HLA solvent accessible amino acid mismatches between donor and recipient is shown in Figure 2A. After computation, a table containing the number of amino acid mismatches per donor HLA allele is generated (Figure 2B). In addition, HLA-EMMA provides detailed information on the position and the type of amino acid that are mismatched for both total amino acid sequence and solvent accessible positions (Figure 2C).

HLA-EMMA can be used to perform compatibility analysis for large numbers of donor-recipient pairs simultaneously in the form of a batch analysis. This requires uploading of an input file containing the HLA typing of the respective donor and recipient pairs (Supplementary Figure

1). HLA-EMMA generates an output file that consists of both the number of total and solvent accessible amino acid mismatches for each pair, as well as the position and the type of amino acid that are mismatched for each donor HLA allele (Supplementary Figure 2). In addition, the amino acids of the recipient's HLA on the corresponding positions are provided in a separate column. This output can then be used for further analysis.

Another option available in HLA-EMMA is an amino acid sequence overview of all HLA alleles (Supplementary Figure 3). With this overview, multiple HLA alleles can be compared, and it can also be used to consult which HLA alleles share a specific amino acid.

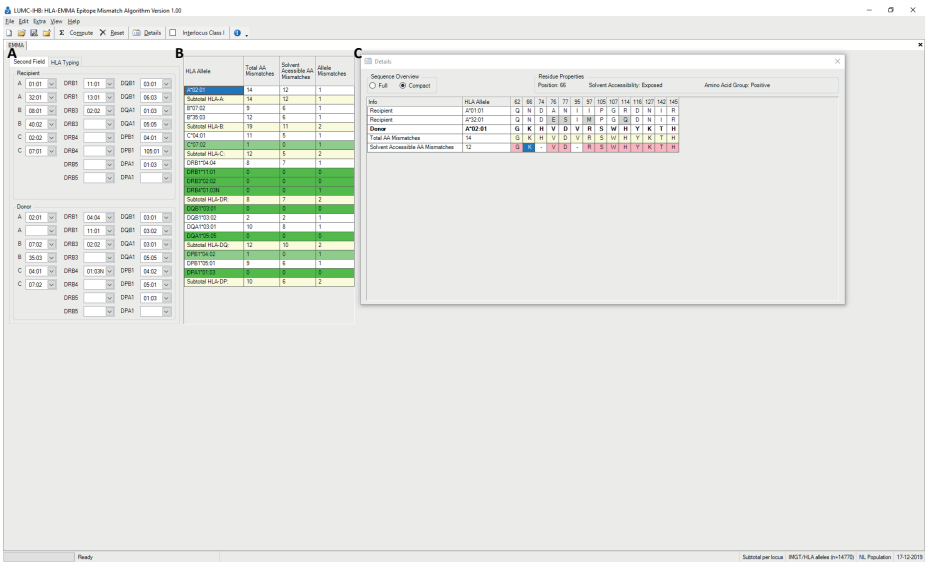

Figure 2: HLA-EMMA manual entry with an example of a donor-recipient couple. A) Input field for HLA typing of donor and recipient. B) After selecting compute, the number of amino acid mismatches are generated for each donor HLA allele and shown in the result table. C) Details of a mismatched donor HLA allele shown after selecting the donor HLA allele in result table. Here, the mismatched amino acids and positions are shown. Residue properties are shown when selecting a specific amino acid.

Proof of principle

For validation of HLA-EMMA we used a previously described cohort of which the HLA-specific antibody response was defined for women that received lymphocyte immunotherapy from their male partner.[17] Here, for each HLA-A, -B, -DRB1 and -DQ mismatch the number of solvent accessible amino acids was determined with HLA-EMMA using the default settings. For HLA-DQ the number of solvent accessible amino acids of DQA1 and DQB1 were combined (Figure 3). We determined how often an HLA mismatch with specific solvent accessible amino acid

mismatches resulted in DSA formation and observed that the proportion of HLA mismatches that resulted in DSA formation increased with higher number of solvent accessible amino acids. For HLA-A, -B, and -DQ mismatches, the incidence of DSA was 80% in the group with the highest number of solvent accessible amino acid mismatches.

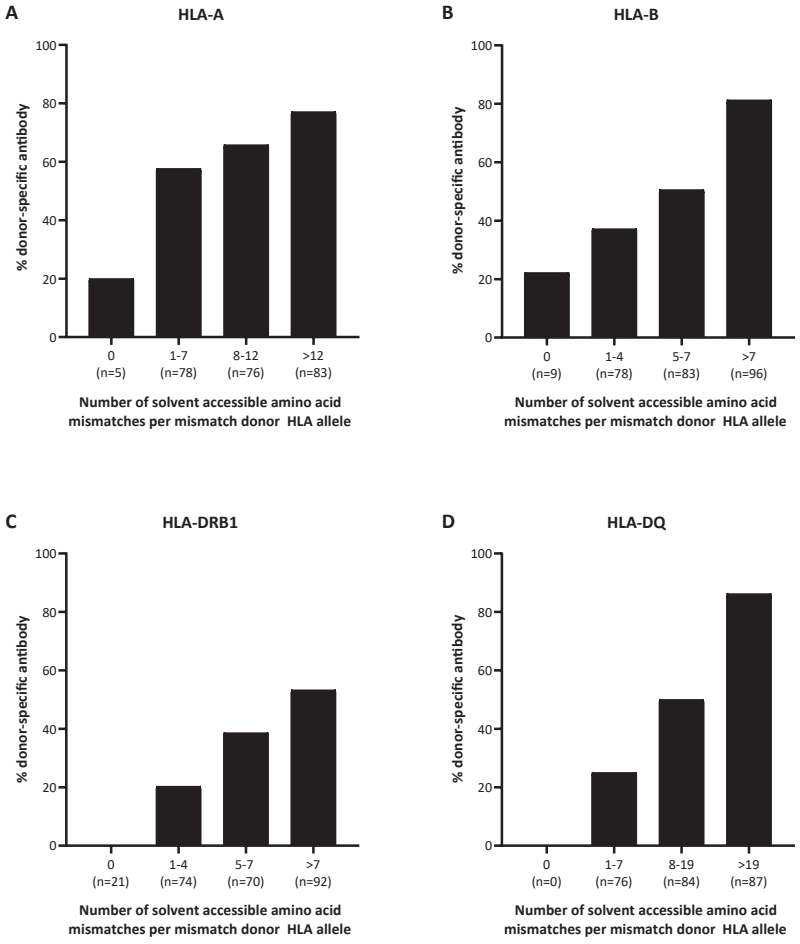

Figure 3: Association between DSA formation and the number of solvent accessible amino acid mismatches. The number of solvent accessible amino acid mismatches were defined and DSA were determined per mismatched donor HLA allele. For HLA-A (A), HLA-B (B), HLA-DRB1 (C) and HLA-DQ (D) an increased proportion of HLA mismatches formed DSA with higher number of solvent accessible amino acid mismatches.

DISCUSSION

Here, we present a software program HLA-EMMA that allows to determine the molecular HLA class I and class II compatibility between donor and recipient. Since amino acids are fixed entities on HLA molecules not dependent on assumptions or preconceptions, as well as the fact that single surface exposed amino acid mismatches can already be sufficient to induce an antibody response, HLA-EMMA was developed to analyse compatibility on the amino acid level. The software program focuses on solvent accessible amino acids mismatches, since B cell epitopes are known to consist of polymorphic amino acids that are surface exposed.[34-36] Data from recent studies showed no significant differences between eplet and amino acid mismatch scores for the prediction of DSA,[12,21] indicating that both strategies are potentially useful. The benefit of HLA-EMMA is that large datasets of donor-recipient pairs from diverse populations can be analysed for both HLA class I and class II simultaneously. Since the position and type of amino acid mismatches are provided these can be used to identify relevant mismatches that are associated with development of *de novo* DSA.

Currently, HLAMatchmaker is the main tool used to determine HLA compatibility on structural level by analysing eplet mismatches. Eplets are defined as patches of polymorphic amino acids on surface exposed areas of the HLA molecules. The definition of surface exposure in HLAMatchmaker is based on the analysis of polymorphic positions on a select number of HLA crystal structures with Cn3D structure viewer.[9,10] Surface exposure was labelled as prominent, readily visible or somewhat visible. In contrast, in HLA-EMMA, solvent accessible amino acid positions were defined per HLA locus and by excluding positions that were predicted by two validated tools to be solvent inaccessible for the available HLA structures per locus. These tools are neural network-based models trained to predict secondary structural features, such as relative solvent accessibility.[30,31] The reason to define accessibility in this way and not by predicted solvent accessibility is the fact that not for every HLA allele a crystal structure is available, which may result in an amino acid position being incorrectly classified as solvent accessible for a specific HLA allele. When more HLA crystal structures or models become available, the solvent accessible amino acid position database in HLA-EMMA will be updated.

Besides solvent accessible amino acid mismatches, HLA-EMMA also calculates the total number of amino acid mismatches. This is useful in cases where no solvent accessible amino acid mismatches are defined for a donor HLA antigen mismatch that has resulted in DSA. Such antibody responses may be explained by non-exposed amino acid mismatches as they could have been induced by surface changes due to buried amino acid polymorphisms.[28,37]

A previously described tool, the Cambridge HLA immunogenicity algorithm developed by Kosmoliaptsis and colleagues, also determines the number of total amino acids mismatches as well as hydrophobicity and electrostatic mismatch scores.[22,23]

The default setting of HLA-EMMA is intralocus comparison for HLA class I. This is in contrast with HLAMatchmaker that performs interlocus comparison to determine the eplet mismatches for HLA class I.[10,38] This difference in strategy is due to the fact that eplets are combinations of amino acids, while HLA-EMMA considers individual amino acids. A possible consequence of interlocus comparison on the individual amino acid level may be that polymorphic amino acids shared by HLA alleles are incorrectly classified as being compatible. With the interlocus comparison option for HLA class I the relevance of inter- versus intralocus comparison and antibody induction can be further investigated. In contrast, for HLA-DR the amino acid sequences are interlocus compared. This is due to the difference in expression of DRB3/4/5 molecules of which an individual can have no more than two of the three possible alleles. Thus, if a donor carries one of the DRB3/4/5 loci that the recipient lacks, all amino acids on this allele would be mismatched by intralocus comparison. This will result in an overestimation of the number of mismatches without any indication of the relevant mismatches.

A previously described clinical cohort was used to validate HLA-EMMA as a tool to determine the immunogenicity of HLA mismatches on basis of a large data set. As expected, this cohort showed a higher frequency of DSA induction with a higher number of solvent accessible amino acid mismatches, indicating the validity of the software. Strikingly, for two HLA class I mismatches DSA were observed while there were no amino acid mismatches at the solvent accessible level nor at the total amino acid level, when analysed in the default intralocus manner. Interestingly, these cases were analysed with HLAMatchmaker, no eplet mismatches were observed (data not shown). It is important to note that the HLA typing of the individuals of this cohort was not all based on HLA sequencing but was largely done by a genotyping array and HLA imputation, which may have led to false classification of the second field HLA data, potentially resulting in zero amino acid mismatches, whilst DSA were formed.

By using HLA-EMMA we aim to establish the ability of specific amino acid mismatches to induce an antibody response by determining the incidence of *de novo* DSA in case of a mismatch of that specific amino acid. Immunogenicity depends on the HLA phenotype of the recipient but also of the donor, and HLA allele frequencies differ between populations and even within regions.[39-41] This population difference is important to consider as amino acids that are highly immunogenic in one population, might be less immunogenic in another due to difference in

HLA allele distribution in the populations.[42] Therefore, HLA-EMMA is one of the tools that will be used during the 18th International HLA and Immunogenetics Workshop (IHIWS) to identify immunogenic amino acid mismatches for a large group of donor-recipient pairs from diverse populations with information on *de novo* DSA development.

Ultimately, defining the immunogenic polymorphic amino acids is just the beginning. Based on this knowledge, we want to define specific polymorphic amino acid configurations similar as what has been done for the definition of the eplets. The immunogenic polymorphic amino acids will serve as a basis for the definition of the relevant amino acid configurations involved in antibody binding. While single amino acid can induce an antibody response, indicating immunogenicity, configurations of amino acids are involved in antibody-antigen interaction, which is antigenicity.[43]

Preventing the induction of *de novo* DSA formation after transplantation is essential for maximizing graft survival and the chance of potential repeat transplantation and therefore of utmost importance in paediatric setting where children will certainly need a repeat transplant. With HLA-EMMA, we developed a software program to perform HLA class I and class II compatibility analysis on amino acid level for recipient and donor couple individually and for large population studies that will contribute to the identification of these immunogenic polymorphic amino acid mismatches.

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ABBREVIATIONS

DSA: Donor-specific antibodies

HLA: Human leukocyte antigen

HLA-EMMA: HLA epitope mismatch algorithm

IHIWS: International HLA and Immunogenetics Workshop

PDB: Protein Data Bank

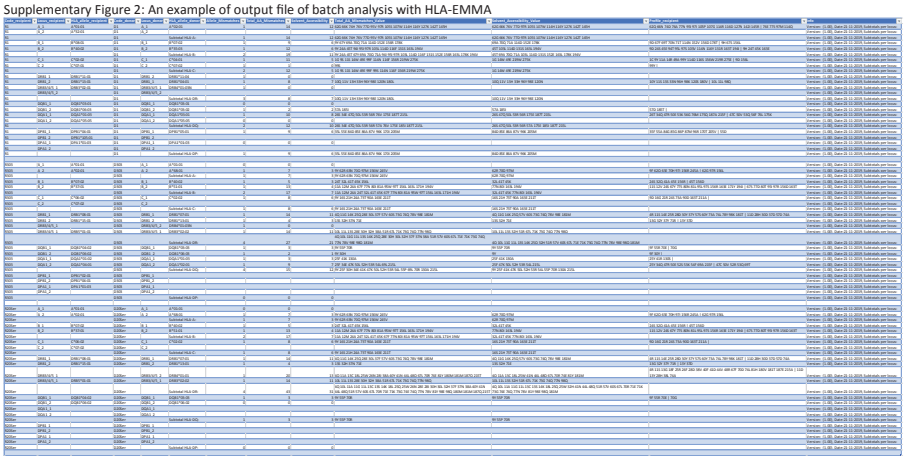
SUPPLEMENTAL MATERIAL

Supplementary Figure 1: An example of HLA typing input file for batch analysis with HLA-EMMA

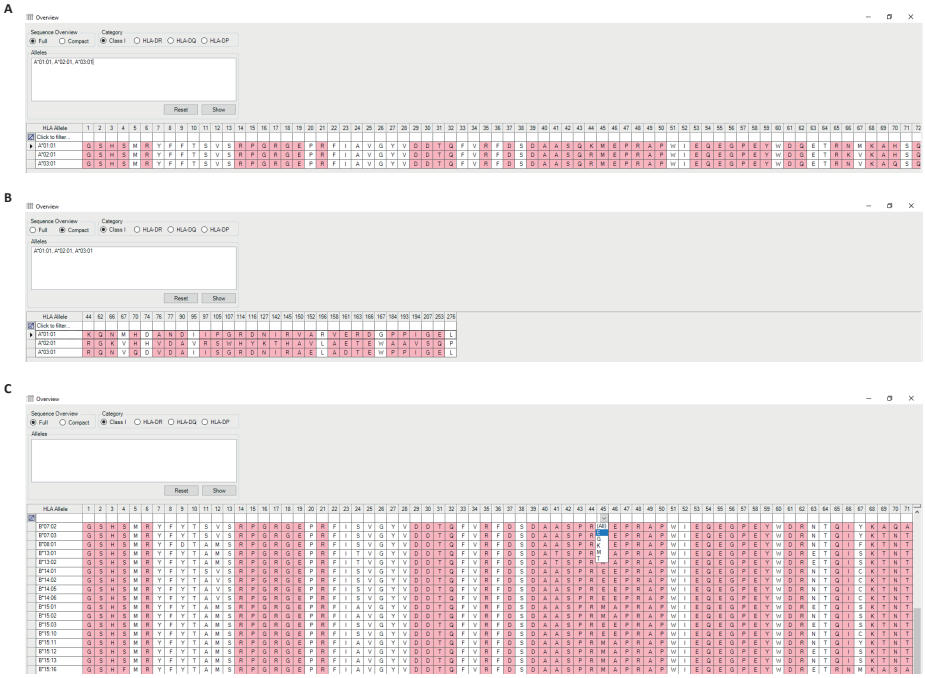
Code	A_1	A_2	B_1	B_2	C_1	C_2	DRB1_1	DRB1_2	DRB3/4/5_1	DRB3/4/5_2	DQB1_1	DQB1_2	DQA1_1	DQA1_2	DPB1_1	DPB1_2	DPA1_1	DPA1_2
R1	A*01:01	A*32:01	B*08:01	B*40:02	C*02:02	C*07:01	DRB1*11:01	DRB1*13:01	DRB3*02:01		DQB1*03:01	DQB1*06:03	DQA1*01:03	DQA1*05:05	DPB1*04:01	DPB1*105:01	DPA1*01:03	
R303	A*01:01	A*02:01	B*07:02	B*37:01	C*06:02	C*07:02	DRB1*08:01	DRB1*15:01	DRB5*01:01		DQB1*04:02	DQB1*06:02	DQA1*01:02	DQA1*04:01	DPB1*02:01	DPB1*04:01	DPA1*01:03	
D1	A*02:01		B*07:02	B*35:03	C*04:01	C*07:02	DRB1*04:01	DRB1*11:04	DRB4*01:03N		DQB1*03:01	DQB1*03:02	DQA1*03:01	DQA1*05:05	DPB1*05:01	DPB1*05:01	DPA1*01:03	
D303	A*01:01	A*68:01	B*40:02	B*51:01	C*02:02		DRB1*07:01	DRB1*13:01	DRB3*02:02	DRB4*01:03N	DQB1*03:03	DQB1*06:03	DQA1*01:03	DQA1*02:01				
R20Ser	A1	A2	B7	B37	Cw6	Cw7	DR8	DR15	DR51		DQ4	DQ6						
D20Ser	A1	A68	B61	B51	Cw2		DR7	DR13	DR52	DR53	DQ9	DQ6						

Supplementary Figure 1: An example of an HLA typing input file for batch analysis with HLA-EMMA. For batch analysis the input format is a Microsoft Excel file according to the template as indicated in the figure. Donor and recipient pairs are indicated by corresponding codes, here indicated by same number, thus compatibility analysis between D1 and R1 will be performed and same applies for D303 and R303, and D20Ser and R20Ser. Both second field typing, and serological typing can be entered, as shown for D20Ser and R20ser.

Supplementary Figure 2: An example of output file of batch analysis with HLA-EMMA



Supplementary Figure 2: An example of output file of batch analysis with HLA-EMMA. The output file of HLA-EMMA batch analysis is a Microsoft Excel file. Here the output file of input file from supplementary figure 1 is shown. In the output file the HLA alleles of donor and recipient are presented. The first six columns show the information of the input file, thus code_recipient, Locus_recipient, HLA_allele_recipient, code_donor, Locus_donor, and HLA_allele_donor. Then each column provides information of the analysis. Allele_Mismatches column indicates if the donor allele of that specific row is mismatched with recipient by the number 1 and if the donor allele is matched than a 0 is given. Next, in Total_AA_mismatches the number of total amino acid mismatches is provided for the donor allele, and the number of solvent accessible amino acid mismatches can be found in Solvent_Accessibility column. The Total_AA_Mismatches_Value and Solvent_Accessibility_Value columns list the positions and the type of amino acid that are mismatched. Next column, Profile_Recipients, shows the amino acids of the recipient's HLA allele on the mismatched positions, divided by | to separate each HLA allele within the locus. The HLA-EMMA version, run date, and other additional information can be found in the last column.



Supplementary Figure 3: Amino acid sequence overview. HLA-EMMA also provides all the amino acid sequences in an overview. This can be used to compare different alleles on full amino acid sequence (A) or compact and therefore only showing the positions that are different (B). In addition, filter option is present to filter on HLA alleles or on a specific amino acids (C) to view only the HLA alleles with that specific amino acid.

Supplementary Table 1: HLA class I crystal structures used to predict solvent accessibility

PDB	Resolution	Antigen
3BO8	1.8Å	A*01:01
1I4F	1.4Å	A*02:01
3OX8	2.16Å	A*02:03
3OXR	1.7Å	A*02:06
3OXS	1.75Å	A*02:07
3RL1	2Å	A*03:01
1X7Q	1.45Å	A*11:01
3WL9	1.66Å	A*24:02
4HWZ	2.397Å	A*68:01
4HX1	1.802Å	A*68:02
4U1H	1.59Å	B*07:02
3SPV	1.3Å	B*08:01
3BXN	1.864Å	B*14:02
1XR9	1.788Å	B*15:01
4XXC	1.426Å	B*18:01
5DEF	1.6Å	B*27:04
2A83	1.4Å	B*27:05
5DEG	1.83Å	B*27:06
1K5N	1.09Å	B*27:09
2CIK	1.75Å	B*35:01
3BWA	1.3Å	B*35:08
6MT6	1.31Å	B*37:01
4O2C	1.802Å	B*39:01
5IEK	1.8Å	B*40:02
3LN4	1.296Å	B*41:03
3LN5	1.9Å	B*41:04
4U1N	1.77Å	B*42:02
1M6O	1.6Å	B*44:02
1N2R	1.7Å	B*44:03
1SYV	1.7Å	B*44:05
4LCY	1.6Å	B*46:01
1E27	2.2Å	B*51:01
3W39	3.1Å	B*52:01
1A1O	2.3Å	B*53:01
3VRI	1.6Å	B*57:01
2BVP	1.35Å	B*57:03
5VWH	1.648Å	B*58:01
5IND	2.132Å	B*58:11
4U1S	1.76Å	B*81:01
1QQD	2.7Å	C*04:01
5VGD	2.4Å	C*05:01
5W6A	1.74Å	C*06:02
4NT6	1.84Å	C*08:01

Supplementary Table 2: HLA class II structures used to predict solvent accessibility

PDB	Resolution	Antigen
3PDO	1.95Å	DRB1*01:01
1A6A	2.75Å	DRB1*03:01
4MD4	1.95Å	DRB1*04:01
4MD5	1.65Å	DRB1*04:04
4MDJ	1.7Å	DRB1*04:02
6BIR	2.3Å	DRB1*04:05
6CPN	2Å	DRB1*11:01
6ATF	1.9Å	DRB1*14:02
5V4M	2.1Å	DRB1*15:01
6CPO	2.4Å	DRB1*15:02
2Q6W	2.25Å	DRB3*01:01
3C5J	1.8Å	DRB3*03:01
1FV1	1.9Å	DRB5*01:01
1S9V	2.22Å	DQB1*02:01 / DQA1*05:01
1JK8	2.4Å	DQB1*03:02 / DQA1*03:02
2NNA	2.1Å	DQB1*03:02 / DQA1*03:01
1UVQ	1.8Å	DQB1*06:02 / DQA1*01:02
3WEX	2.4Å	DPB1*05:01 / DPA1*02:02
4P5M	1.7Å	DPB1*02:01 / DPA1*01:03
4P57	2.6Å	DPB1*105:01 / DPA1*01:03

Supplementary Table 3: HLA class I modelled structures

PDB used for modelling	Resolution	Antigen
3BH9	1.7Å	A*02:02
3HH9	1.7Å	A*02:05
2HN7	1.6Å	A*11:02
4F7T	1.7Å	A*23:01
4F7T	1.7Å	A*24:03
1X7Q	1.5Å	A*25:01
2HN7	1.6Å	A*26:01
3LR1	2.0Å	A*29:01
3LR1	2.0Å	A*29:02
2HN7	1.6Å	A*30:01
1X7Q	1.5Å	A*30:02
3LR1	2.0Å	A*31:01
5E00	1.7Å	A*32:01
4HWZ	2.4Å	A*33:01
4HWZ	2.4Å	A*33:03
4HX1	1.8Å	A*34:01
1X7Q	1.5Å	A*34:02
3BO8	1.8Å	A*36:01
1X7Q	1.5Å	A*43:01
1X7Q	1.5Å	A*66:01
4HWZ	2.4Å	A*66:02
4WJ5	1.6Å	A*69:01
3LR1	2.0Å	A*74:01
1X7Q	1.5Å	A*80:01
4U1H	1.59Å	B*07:03
4JQX	1.9Å	B*13:01
1M6O	1.6Å	B*13:02
3BXN	1.9Å	B*14:01
1XR9	1.8Å	B*15:02
1XR9	1.8Å	B*15:03
3C9N	1.9Å	B*15:10
3C9N	1.9Å	B*15:11
1XR9	1.8Å	B*15:12
1XR9	1.8Å	B*15:13
3VRI	1.6Å	B*15:16
3C9N	1.87Å	B*15:18
5IB2	1.44Å	B*27:03
1UXS	1.5Å	B*27:08
4O2C	1.8Å	B*38:01
3LN4	1.3Å	B*40:01
5IEK	1.8Å	B*40:06
3LN4	1.3Å	B*41:01
4U1J	1.4Å	B*42:01
3LN4	1.3Å	B*45:01
4JQX	1.9Å	B*47:01
4UIS	1.8Å	B*48:01

Supplementary Table 3: (Continued)

PDB used for modelling	Resolution	Antigen
3L3I	1.7Å	B*49:01
5IEH	1.5Å	B*50:01
1E27	2.2Å	B*51:02
4U1N	1.8Å	B*54:01
4U1J	1.4Å	B*55:01
4U1J	1.4Å	B*56:01
1E27	2.2Å	B*59:01
4O2C	1.8Å	B*67:01
1OGT	1.5Å	B*73:01
1E27	2.2Å	B*78:01
4U1J	1.4Å	B*82:01
4NT6	1.8Å	C*01:02
4NT6	1.8Å	C*02:02
4NT6	1.8Å	C*03:02
4NT6	1.8Å	C*03:03
4NT6	1.8Å	C*03:04
4NT6	1.8Å	C*04:03
4NT6	1.8Å	C*07:02
4NT6	1.8Å	C*08:02
4NT6	1.8Å	C*12:02
4NT6	1.8Å	C*12:03
4NT6	1.8Å	C*14:02
4NT6	1.8Å	C*15:02
4NT6	1.8Å	C*16:01
4NT6	1.8Å	C*17:01
4NT6	1.8Å	C*18:02

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CHAPTER

7

TOWARDS THE DEFINITION OF THE MOST IMMUNOGENIC HLA CLASS II AMINO ACID MISMATCHES USING HLA-EMMA

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ABSTRACT

In kidney transplantation, polymorphic amino acid configurations on mismatched donor HLA molecules can lead to the development of *de novo* donor-specific antibodies (*dn*DSA). These *dn*DSA are mainly directed against HLA class II, and especially HLA-DQ, and are associated with graft loss. Defining which polymorphic residues are able to induce an antibody response and which not, is pivotal for the development of strategies to prevent *dn*DSA formation. Therefore, the aim of this study was to define the most immunogenic HLA-DQ polymorphic amino acid mismatches in a cohort of kidney transplant recipients.

From multiple Dutch transplant centres we selected non-immunised male recipients that received their first kidney transplant with at least one HLA class II antigen mismatch and subsequently lost their graft due to immunological failure (n=79). Donor and recipient HLA typing was performed at high resolution using next generation sequencing (NGS) and the number of solvent accessible amino acid mismatches was established with HLA-EMMA. Formation of *dn*DSA was determined by screening recipients' sera collected upon graft failure with Luminex single antigen bead assays.

HLA-DQ-specific *dn*DSA were most prevalent, occurring in 35% of the 79 recipients. Increasing numbers of solvent accessible amino acid mismatches resulted in a higher frequency of HLA-DQB1 or HLA-DQA1 *dn*DSA. However, we also observed that a single solvent accessible amino acid mismatch on HLA-DQB1 or HLA-DQA1 was sufficient for the induction of *dn*DSA. In this pilot study with a limited number of cases, it was not yet possible to identify specific amino acid positions or types, which were significantly more immunogenic than others.

Overall, we showed that within this cohort the chance of HLA-DQ-specific *dn*DSA increased with more solvent accessible amino acid mismatches, although on individual level a single amino acid mismatch can be sufficient to trigger an antibody response. This latter observation supports the importance to define the most immunogenic residues, which requires a much larger and diverse cohort.

INTRODUCTION

Human leukocyte antigen (HLA) matching can prevent the occurrence of an alloimmune response. However, most grafts are transplanted with one or more HLA antigen mismatches, which can result in immunisation of the recipient as reflected by the formation of donor specific HLA antibodies. These *de novo* donor-specific antibodies (*dn*DSA) are strongly associated with graft loss,[1-3] and reduce the chance of repeat transplantation.[4] Therefore, it is of utmost importance to prevent the development of *dn*DSA after transplantation.

Induction of *dn*DSA can be triggered by polymorphic amino acid configurations on mismatched HLA antigens and are mainly directed against HLA class II, and more specifically HLA-DQ.[1,2,5] There are several ways of defining and analysing these polymorphic residues. One of these is used by the HLAMatchmaker algorithm, which defines patches of (dis)continuous polymorphic amino acids, called eplets, that theoretically can induce an antibody response.[6,7] Indeed the number of eplet mismatches between donor and recipient have been associated with *dn*DSA formation [8-11] as well as transplant glomerulopathy.[12] In addition, the immunogenicity of HLA mismatches on the population level have also been assessed based on amino acid mismatches and/or physiochemical disparity scores.[13,14] While these approaches provide mismatch scores that are good predictors of sensitisation risk on population level, it remains to be determined which mismatched amino acids triggered the formation of *dn*DSA in an individual patient.

It has been shown that not every eplet mismatch is equally immunogenic[15] and *dn*DSA are observed even when only a low number of triplet, predecessor of eplet, mismatches are present on the mismatched donor HLA.[16] In addition, as mentioned, eplets are theoretically defined and require experimental verification to determine if an antibody can actually bind to these polymorphic residues.[17,18] While data of a recent study showed that both HLA class I and class II antibody-verified eplets were good indicators for risk of graft loss for individual patients, especially for HLA class II additional verification of eplets is required to further improve this risk stratification.[11] Thus, the current list of potential immunogenic polymorphic amino acid configurations is still incomplete.

Recently, we developed a software program, HLA-EMMA, to analyse the compatibility between donor and recipient HLA class I and class II molecules on the amino acid level.[19] The benefits of HLA-EMMA are that large cohorts of donor-recipient pairs from diverse populations can be analysed for HLA class I and class II simultaneously and, in addition, it provides the type and position of the amino acid mismatches. Using this software program, we aim to define

the most immunogenic polymorphic amino acids that ultimately form the basis of specific polymorphic amino acid configurations involved in antibody binding.

As mentioned, the most frequently observed *dn*DSA after transplantation are directed against mismatched HLA-DQ. To prevent HLA-DQ-specific *dn*DSA formation it is essential to determine the immunogenic polymorphic amino acids on the HLA-DQB1 and HLA-DQA1 molecules. To this aim, we performed a pilot study using a kidney transplant cohort study of non-immunised male transplant recipients with at least one HLA class II antigen mismatch, who lost their graft due to immunological failure.

MATERIALS AND METHODS

Study population

From multiple Dutch transplant centres, we selected non-immunised male recipients that received their first renal transplant with at least one HLA class II antigen mismatch, and who subsequently lost their graft due to immunological failure. From Leiden and Rotterdam transplant centre we selected recipients that underwent their first kidney transplantation between 1992 and 2015 (n=40) (Figure 1). Subsequently, the cohort was extended with patients from the PROCARE consortium (PROfiling Consortium on Antibody Repertoire and Effector functions) database with recipients that received their first kidney transplantation between 1995 and 2005 (n=129).

HLA typing

DNA samples were collected from recipients and donors for HLA typing by next-generation sequencing (NGS) of HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB1, -DQA1, -DPB1, and -DPA1 loci. 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 subsequently sequencing was carried on Illumina MiSeq or MiniSeq (Illumina, San Diego, CA, USA). NGS data was analysed with NGSengine software (GenDx).

Ambiguities at the second field level were still observed for several HLA alleles (n=6 DRB1, n=3 DQB1, n=17 DPB1) after NGS typing. Therefore, we selected the first allele of the ambiguity group.

Definition of donor-specific antibodies

Recipients' sera before transplantation and after graft failure were collected for screening for the presence of HLA antibodies with Lifecodes Lifescreen Deluxe screening kit (LMX,

Immucor Transplant Diagnostics, Stamford, CT, USA). Subsequently, positive serum samples were treated with ethylenediaminetetraacetic acid (6% EDTA) and tested with Lifecodes HLA class I and class II single antigen beads (SAB) (Immucor Transplant Diagnostics). Data was analysed with Match It! Antibody software version 1.3.0 (Immucor Transplant Diagnostics). The screening data were analysed using raw mean fluorescence intensity (MFI) and for the SAB data background corrected MFI (BCM) as provided by software were used. For defining DSA from the SAB data of each individual both the software positive assignments and the BCM of recipient' alleles were considered. For HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5 donor alleles with BCM > 1000 were defined as DSA. For HLA-DQB1 and HLA-DQA1 DSA was assigned if the actual mismatched HLA-DQB or HLA-DQA donor allele present on a bead in combination with respectively the self HLA-DQA or HLA-DQB allele of the recipient was positive, BCM > 1000. If the mismatched allele was not present on a bead in combination with a recipient allele, then DSA was assigned if all beads with mismatched allele were positive and the reactivity was not caused by the other allele on the bead. In case donor HLA allele was not present in SAB assay, no *dn*DSA could be defined (n=3 DRB1, n=1 DRB4, n=1 DQB1, n=1 DQA1) and these cases were excluded from analysis.

Solvent accessible amino acid mismatches

HLA-EMMA software program version 1.0 was used to define the solvent accessible amino acid mismatches between donor and recipient.[19] With this software program the amino acid sequences of the donor HLA alleles are compared with the recipient' sequences, interlocus for HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5, and intralocus for HLA-DQB1 and HLA-DQA1. HLA-EMMA identified and quantified the position and type of solvent accessible amino acid mismatches of each donor allele.

Visualising solvent accessible amino acid positions

HLA-DQB1*03:02-DQA1*03:02 crystal structure PDB 1JK8 (downloaded from https://www.rcsb.org/ on January 16, 2020) was used to visualise solvent accessible amino acid positions with Swissviewer.[20]

Statistical analysis

SPSS statistics version 25 (IBM, Armonk, NY, USA) was used to perform statistical analysis. Binary logistic regression was used to assess the significance of antigen, allele, or solvent accessible amino acid mismatches as predictors of *dn*DSA formation. P values of <0.05 were considered statistically significant.

Table 1: *dn*DSA formation in study cohort

*dn*DSA	N (missing*)	% of 79
HLA class I	13 (2)	16.5
HLA-DRB1	10 (4)	12.7
HLA-DRB3/4/5	8 (8)	10.1
HLA-DQB1	26 (1)	32.9
HLA-DQA1	22 (1)	27.8

*HLA alleles that are not present in single antigen bead assay

RESULTS

Study cohort

The selection criteria of the study cohort were non-immunised first kidney transplant male recipients with at least one HLA class II antigen mismatch with graft failure due to immunological failure. This resulted in a study cohort consisting of 79 donor-recipient couples for analysis (Figure 1).

Overall within this cohort, 39% of recipients developed HLA class II *dn*DSA (n=31) of which 45% recipients (n=14) also had HLA class I *dn*DSA (Table 1). HLA-DQB1 and/or HLA-DQA1-specific *dn*DSA were most prevalent in the recipients (35% n=28) and 11 of those recipients also developed HLA-DRB1/3/4/5 specific *dn*DSA, while HLA-DRB1/3/4/5 *dn*DSA were only observed in 3 recipients.

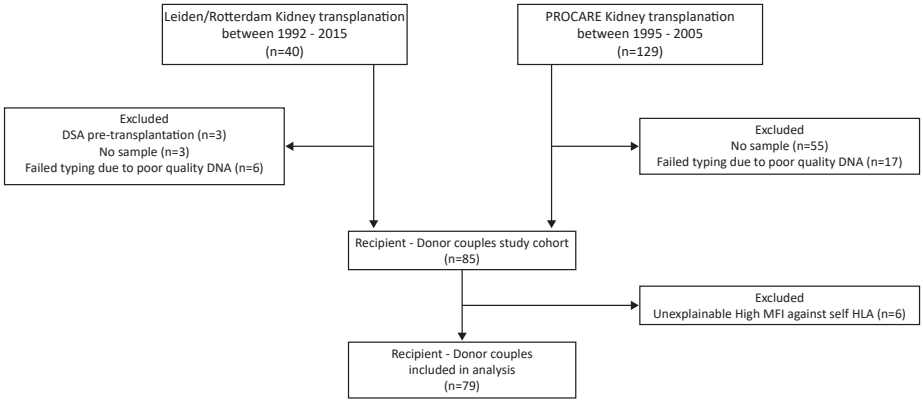

Figure 1: Flow chart of inclusions and exclusions for the study cohort.

Solvent accessible amino acid mismatches are associated with dnDSA formation

Per individual we determined if a HLA-DQB1 and/or HLA-DQA1 *dn*DSA was formed and observed that for HLA-DQB1 the chance of antibody response was higher for two HLA-DQB1 antigen mismatches, based on serological split typing, compared to one antigen mismatch although this was not significant (Table 2). Interestingly, the chance of *dn*DSA formation was lower for 2 HLA-DQB1 or HLA-DQA1 allele mismatches compared to 1 allele mismatch (Tables 2 and 3), although this was only significant for HLA-DQB1. However, when defining the sum solvent accessible amino acid mismatches for HLA-DQB1 and HLA-DQA1 per individual we found that the chance of *dn*DSA formation was higher for solvent accessible amino acid mismatches above the mean, 12 for HLA-DQB1 and 10 for HLA-DQA1. This was further supported when comparing the number of HLA-DQB1 and HLA-DQA1 solvent accessible amino acid mismatches of one allele mismatch with two allele mismatches (Figure 2). For many double allele mismatches the sum of solvent accessible amino acid mismatches was very low and often lower than the number of solvent accessible amino acid mismatches of several single allele mismatches.

Table 2: HLA-DQB1 mismatches predicting *dn*DSA using logistic regression analysis

	OR (95% C.I.)	p value	No DSA	DSA
DQB1 antigen 1 mm (ref)			33	23
DQB1 antigen 2 mm	2.323 (0.575-9.381)	0.237	10	3
DQB1 allele 1 mm (ref)			30	22
DQB1 allele 2 mm	0.260 (0.078-0.865)	0.028	21	4
DQB1 SA AA mm <=12 (ref)			35	11
DQB1 SA AA mm >12*	2.983 (1.123-7.927)	0.028	16	15

OR = Odds Ratio, C.I. = Confidence Interval

* grouped based on mean

Table 3: HLA-DQA1 mismatches predicting *dn*DSA using logistic regression analysis

	OR (95% C.I.)	p value	No DSA	DSA
DQA1 allele 1 mm (ref)			37	18
DQA1 allele 2 mm	0.514 (0.150-1.762)	0.290	16	4
DQA1 SA AA mm <=10 (ref)			39	8
DQA1 SA AA mm >10*	4.875 (1.686-14.097)	0.003	14	14

OR = Odds Ratio, C.I. = Confidence Interval

* grouped based on mean

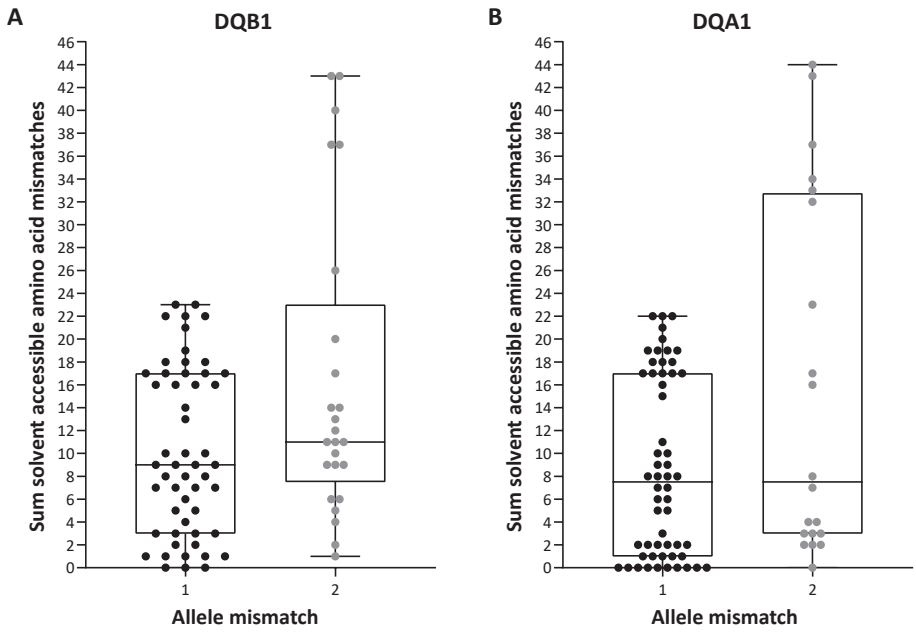

Figure 2: HLA-DQB1 and HLA-DQA1 solvent accessible amino acid mismatches and association with *dn*DSA formation. The number of solvent accessible amino acid mismatches were defined and *dn*DSA per mismatched donor allele for HLA-DQB1 (A) and HLA-DQA1 (B). A higher frequency of *dn*DSA formation is observed with increased number of solvent accessible amino acid mismatches. Groups were equally divided in tertiles and zero amino acid mismatches was set as separate group.

Next, we defined the number of solvent accessible amino acid mismatches for each mismatched donor allele, and we analysed whether an antibody response was formed against that donor allele. Mismatched donor HLA alleles that were not present in SAB assay were not included in the analysis. For HLA-DRB1/3/4/5, HLA-DQB1 and HLA-DQA1 we determined whether a correlation between the number of solvent accessible amino acid mismatches and *dn*DSA existed. For HLA-DR, no association was observed between *dn*DSA formed and number of solvent accessible amino acid mismatches due to low number of pairs (data not shown). For both HLA-DQB1 and HLA-DQA1 a higher frequency of *dn*DSA formation was observed with increasing number solvent accessible amino acid mismatches (Figure 3). The HLA-DQB1 and HLA-DQA1 solvent accessible amino acid mismatched were grouped based on tertiles, and this resulted in odds ratio (OR) 3.788 (1.1912 – 7.504) and OR 3.677 (1.908 – 7.086), respectively. The risk of *dn*DSA formation was also associated with an increased number of solvent accessible amino acid mismatches with OR 1.121 (1.049 – 1.198) for HLA-DQB1 and OR 1.138 (1.064 – 1.217) for HLA-DQA1 if no groups were defined on basis of tertiles.

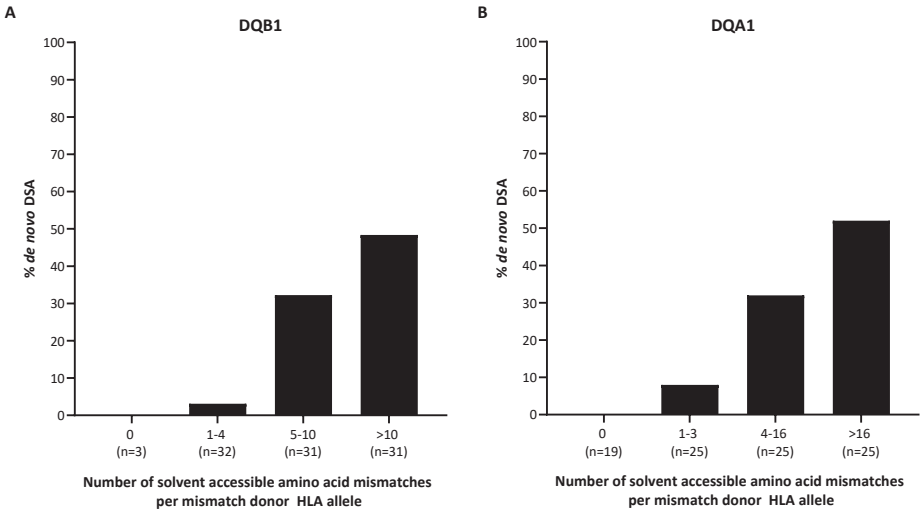

Figure 3: HLA-DQB1 and HLA-DQA1 allele mismatch was associated with a wide range of solvent accessible amino acid mismatches. Each data point represents the sum of solvent accessible amino acid mismatches for HLA-DQB1 (A) or HLA-DQA1 (B). The boxplot indicates the median with minimum and maximum values.

A single HLA-DQB1 or HLA-DQA solvent accessible amino acid mismatch can already be sufficient to induce dnDSA

In many publications a specific cut-off of epitopes or eplets is used to stratify patients into low or high risk to develop *dn*DSA. Also in our analysis above, we could identify a cut-off for HLA-DQB1 and HLA-DQA1 that correlated with a high risk of *dn*DSA development. However, low numbers of solvent accessible amino acid mismatches can already result in *dn*DSA formation. Upon in-depth analysis, we found that for one mismatched donor HLA-DQB1 allele and for one mismatched donor HLA-DQA1 allele there was only a single solvent accessible amino acid mismatch leading to *dn*DSA formation. For the HLA-DQB1 allele, DQB1*03:02, the mismatch was alanine on position 57 (Figure 4A), which is located on the top of HLA-DQ molecule (Figure 4B). In contrast, the amino acid aspartic acid on position 160 located near the bottom of the HLA-DQ molecule was the only mismatched amino acid of HLA-DQA1*03:03 but is nonetheless still regarded as solvent accessible (Figure 4C and D).

A

		Amino acid on position 57	Highest BCM in SAB
Recipient	DQB1*03:19	Aspartic acid	n.d.
	DQB1*05:01	Valine	0
Donor	**DQB1*03:02**	**Alanine**	**6894 (in combination with donor DQA1)**

C

		Amino acid on position 160	Highest BCM in SAB
Recipient	DQA1*03:01	Alanine	273
	DQA1*05:05	Alanine	44
Donor	**DQA1*03:03**	**Aspartic acid**	**1426 (in combination with self DQB1)**

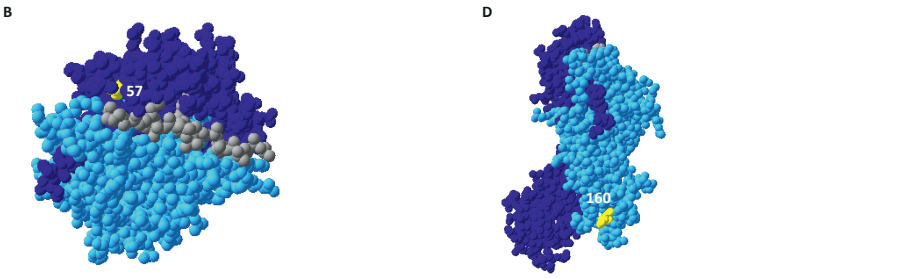

Figure 4: A single solvent accessible amino acid mismatch is sufficient to induce *dn*DSA formation. The amino acid alanine on position 57 was the only mismatch between donor HLA-DQB1*03:02 and recipient's HLA-DQB1 alleles (A), but still resulted in *dn*DSA formation. Position 57 is located on the top of the molecule (B). Aspartic acid on position 160 was the only mismatch between donor HLA-DQA1*03:03 and recipient HLA-DQA1 alleles (C), but position 160 is located near the bottom of the molecule (D).

No specific amino acid position is preferentially associated with dnDSA formation

Next, we determined whether in this pilot study it was already possible to define specific amino acid positions or type preferentially resulting in *dn*DSA formation. To this aim, we calculated how often specific position or type occurred as mismatches and how often that specific position or type was mismatched on a mismatched donor HLA-DQ allele that resulted in *dn*DSA formation. So far, no specific amino acid position could be identified for HLA-DQB1 (Figure 5A), nor for HLA-DQA1 (Figure 5B), as the average frequency of *dn*DSA of positions is 39% (range 15-53%) and 40% (range 0-57%), respectively. Interestingly, in the current cohort no mismatches were observed for 116 solvent accessible amino acid positions of HLA-DQB1 and for 17 positions of HLA-DQA1. In addition, when further looking into the type of mismatched amino acids, no specific solvent accessible amino acid type resulting more frequently in *dn*DSA formation could be identified (data not shown).

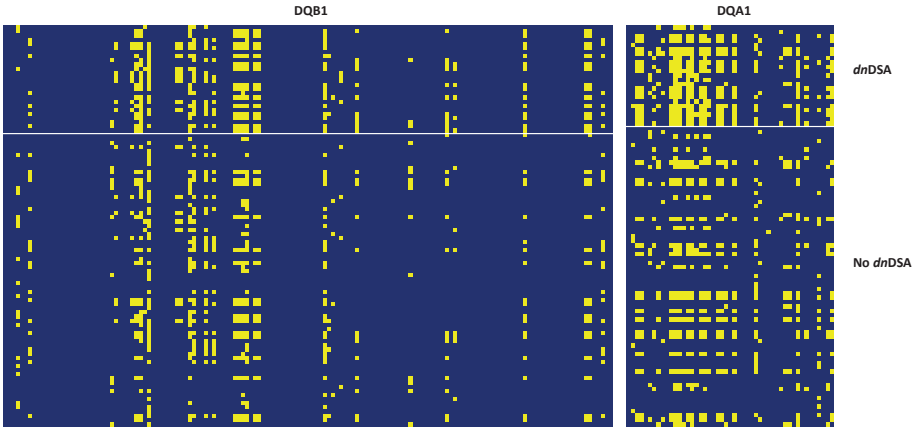

Figure 5: Overview of all mismatched solvent accessible amino acid positions. All solvent accessible amino acid positions of HLA-DQB1 and HLA-DQA1 are depicted in the columns, and each row is a mismatched donor allele. Yellow indicates the positions that were mismatched between donor and recipient and blue are the matched positions. *Dn*DSA was observed against the donor HLA alleles above the line and no *dn*DSA was observed for mismatched donor alleles below the line.

DISCUSSION

The development of *dn*DSA after transplantation is associated with inferior graft survival and reduces the chance of repeat transplantation. Interestingly, since the introduction of the sensitive Luminex SAB assays it is appreciated that *dn*DSA are often directed against HLA-DQ.[1-5] In the current study, we showed that the formation of HLA-DQB1 and HLA-DQA1-specific *dn*DSA is associated with the number of solvent accessible amino acid mismatches on mismatched HLA-DQ antigens. These findings are in accordance with previous studies based on eplet, amino acid, or physiochemical properties mismatches.[8,9,14,21] However, in the present study no association was observed between the number of HLA-DR solvent accessible amino acid mismatches and *dn*DSA formation, which is probably due to exclusion of large number of donor-recipient pairs (n=30) as the mismatched HLA alleles were not present in SAB assay used in this study resulting in low number of pairs and subsequently allele mismatches for analysis.

In addition, *dn*DSA formation, albeit at lower frequency, was observed for low number, or even a single, solvent accessible amino acid mismatches on both HLA-DQB1 allele or HLA-DQA1 alleles, similar as recently observed for HLA-DQ molecule mismatches.[22] One should take this into consideration, both in donor selection and risk estimation, and not only trust on the definition of eplet loads that have been proposed as good indicators for sensitisation

risk[9,21,23] and have already been applied in kidney allocation to paediatric patients.[24] These data support the notion that with an increasing number of amino acid differences, the chance that an immunogenic disparity is present increases, but that in individual cases, a single amino acid can already be immunogenic.[25]

On the other hand, not every mismatched donor HLA allele with a high number of solvent accessible amino acid mismatches resulted in *dn*DSA formation, as in this group the *dn*DSA frequency was 48% and 52%, for HLA-DQB1 and HLA-DQA1 respectively. The data suggest that for some allele mismatches the amino acids mismatches that are present are of low immunogenicity.[26] The HLA phenotype of the recipient itself plays a major role in immunogenicity. So is class switching to IgG antibody producing cells dependent on the T cell epitopes presented by the recipients' HLA class II molecules of the B cell.[27,28] However, in current study, we could not find an association between the recipients' HLA-DR phenotype and *dn*DSA HLA-DQ-specific formation could be defined (data not shown), also likely due to limited sample size. Besides T cell dependency for IgG antibody formation, also the type of amino acid substitution plays a role in immunogenicity, because if the size and charge are like those of the recipients than the HLA molecules structure and physiochemical properties can be similar.[29]

Defining either an immunogenic amino acid position or type of the HLA-DQB1 and HLA-DQA1 alleles was not reached. This is probably due to the relatively low number of donor-recipient pairs. While the cohort consist of high number of HLA-DQB1 and HLA-DQA1 allele mismatches and HLA-DQ-specific *dn*DSA formation, the strict selection rules and the requirement of presence of DNA samples for NGS-based HLA typing resulted in a low number of donor-recipient couples. In addition, the numbers for analysis were even more reduced as not all HLA alleles obtained with NGS typing were present in SAB assay used in this study and therefore were excluded from analysis.

Nonetheless, high resolution typing is essential as it provides optimal accuracy in compatibility analysis on the amino acid level, and while for certain populations translation from low resolution to high resolution typing is possible,[30] for individuals with rare alleles this does not apply. High resolution HLA typing was also pivotal for assigning *dn*DSA towards HLA-DQ. In this study, we analysed HLA-DQB1 and HLA-DQA1 separately, and while indeed antibodies can be clearly specific for one chain, it is also possible that an antibody is specifically directed against the whole HLA-DQ molecule.[31,32] This is further complicated by the fact that certain HLA-DQB1 and HLA-DQA1 alleles can not only occur in cis form but also as trans-encoded

heterodimers,[33] and *dn*DSA against trans-encoded heterodimers have been observed.[34] Thus, further studies are required to fully understand what the HLA-DQ antibodies recognise.

To facilitate the analysis of HLA-DQ immunogenicity, it has been proposed that a cohort consisting of patients that have been transplanted with two HLA-DQ mismatches an developing *dn*DSA to only one of the mismatches can be useful, as well as recipients homozygous at the HLA-DQ locus.[22] This will be one of the components of the 18th International Immunogenetics and Histocompatibility Workshop (IHIWS) (https://www.ihiw18.org/). Our study indicates the need to perform larger and more diverse studies, such as the ones proposed in the IHIWS, to identify the most immunogenic HLA-DQ amino acid mismatches. In the current, relative small cohort, we observed that for the HLA-DQB1 locus most of polymorphic solvent accessible amino acid positions were never mismatched, because HLA-EMMA software includes the polymorphic positions for all HLA alleles in IMGT database, even rare alleles. The reason why these positions were never mismatched in the current study may be due to their monomorphic character in our study population, which does not exclude that these positions may be relevant in other study populations with other ethnicities.

Eventually, identification of the immunogenic polymorphic amino acids should lead to defining the relevant amino acid configurations to which an antibody can actual bind, similar as has been done for eplets. These configurations can then be used as parameters for allocation to prevent *dn*DSA formation after transplantation. In addition, amino acid configurations can be used for highly sensitised patients to define acceptable and unacceptable HLA alleles.[26,35]

In summary, in this pilot study we showed that even though the chance of HLA-DQ-specific *dn*DSA is higher with more solvent accessible amino acid mismatches, on individual level a single amino acid mismatch can already be sufficient to trigger an antibody response. Thus, to prevent *dn*DSA formation the most immunogenic residues must be avoided during allocation rather than the HLA alleles with high number of polymorphic amino acid mismatches with relative low immunogenicity. To accomplish this, it is pivotal to define the most immunogenic residues, especially for HLA-DQ, in larger and more diverse cohorts.

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CHAPTER

8

GENERAL DISCUSSION

Since the beginning of solid organ transplantation HLA matching has been shown to be beneficial for graft survival,[1] which is still the case even in the modern era of immunosuppressive drugs. However, due to high polymorphism of the HLA system and scarcity of organs, most recipients receive a (partially) HLA mismatched graft. The allogeneic HLA molecules of the donor can be recognised as foreign by the immune system of the recipient, which may result in the development of antibodies directed against donor HLA, known as donor-specific antibodies (DSA). These *de novo* DSA can develop early or late after transplantation,[2,3] and the presence of these antibodies often leads to graft injury and eventually rejection.[4] In addition, the presence of DSA severely impacts the chance of finding a suitable donor for repeat transplantation.[5] This is also the case for patients on the transplant waiting list that developed HLA antibodies upon pregnancy or blood transfusion.

Towards definition of immunogenic amino acid configurations (epitopes)

Interestingly, not every HLA antigen mismatch leads to a humoral alloimmune response. As reviewed in **Chapter 2** and **Chapter 3**, while each HLA antigen consists of unique set of epitopes, or configurations of polymorphic amino acids, epitopes can be shared by several HLA antigens. Therefore, the number of foreign antibody epitopes present on mismatched donor HLA antigen varies and depends on the HLA phenotype of the recipient. In addition, the type and physicochemical properties of the amino acid substitution, as well as the presence of accompanying T helper cell epitopes contribute to the immunogenicity of a mismatched antibody epitope. These aspects have been implemented in several algorithms aiming at the prediction of the immunogenicity of a mismatched HLA antigen, such as eplets,[6,7] amino acid mismatches, electrostatic mismatch scores,[8,9] and Predicted Indirectly ReCognizable HLA Epitopes presented by recipient HLA class II (PIRCHE-II).[10,11] Indeed, mismatch scores based on these approaches have shown to predict the chance of *de novo* DSA formation on the population level, while none of these algorithms is superior over the others.[12,13] However, we argue that on the level of the individual patient it is not a numbers game, as a single amino acid or configuration present on a mismatched HLA allele can already be sufficient to induce an antibody response. This was observed previously for HLA class I configuration (triplet) mismatches,[14] and confirmed in our cohort study on amino acid mismatches for HLA class II (**Chapter 7**).

Therefore, as highlighted in **Chapter 2** and **Chapter 3**, it is essential to define immunogenic configurations of amino acids (Figure 1), eplets, or single amino acids so that these can be avoided during donor selection in order to prevent *de novo* DSA formation. With HLA-EMMA software, we developed a tool that enables analyses on amino acid compatibility between

donor and recipient (**Chapter 6**). This tool can perform analyses for large number of donor-recipient pairs at once due to batch option, and for HLA class I and HLA class II simultaneously, which is either very laborious or impossible with the other available algorithms. Additionally, HLA-EMMA considers all available HLA alleles from the IMGT, so there are no restrictions in analysing donor-recipient pairs with rare HLA alleles. Therefore, HLA-EMMA is a very useful and user-friendly tool, which can be used in cohort studies to define the relevant/immunogenic amino acids and positions. This software was further validated in a cohort study of non-immunised male recipients of a first renal allograft (**Chapter 7**).

While the aim of defining the most immunogenic HLA class II amino acids and/or positions was not accomplished in this cohort study due to small numbers, we did observe that a high number of amino acid mismatches is not always a guarantee for the induction of an antibody response. The latter suggests that not all mismatches are immunogenic, as previous mentioned (**Chapter 2** and **Chapter 3**), but also that not all defined polymorphic solvent accessible positions included in HLA-EMMA are equally important for the induction of an antibody response. Narrowing down the solvent accessible positions to only those that are proven to be able to induce an antibody response can be achieved by using human HLA-specific monoclonal antibodies (mAbs), as reactivity analysis of these mAbs allows for identification of amino acids and/or positions that are involved in antibody binding (**Chapter 5**), and thus amino acid and/or positions that can be immunogenic. Including only confirmed immunogenic or relevant polymorphic amino acid and/or positions will improve the prevention of DSA formation without unnecessarily preventing allocation based on non-immunogenic epitope mismatches.[15] Additionally, amino acids or amino acid configurations associated with *de novo* DSA formation identified in clinical cohort studies can be verified by human HLA mAbs, using methods described in **Chapter 5**.

Upon transplantation antibodies directed against HLA class II and more specifically against HLA-DQ are most prominent. Therefore, understanding of the immunogenicity of especially HLA-DQ is of great interest. One of the features of HLA-DQ is that both the alpha and beta chain are polymorphic. Therefore we analysed the HLA-DQ chains separately with respect to their ability to induce an antibody response (**Chapter 7**), in contrast to previous studies that consider the whole HLA-DQ molecule.[16-19] Of course, once an antibody is formed the whole HLA molecule should be considered as the antibody-footprint could cover both alpha and beta chain of the HLA-DQ molecule. However, we observed that a single amino acid mismatch on either the alpha or beta chain was sufficient to induce an antibody response that was clearly directed against that specific chain. In addition, we often detected an antibody response in

case only the HLA-DQB1 or HLA-DQA1 allele was mismatched. Important to note that this analysis was performed on first transplant recipients that did not have any detectable HLA antibodies prior to transplantation by sensitive single antigen bead assays. This indicates that for predicting immunogenicity not the whole HLA-DQ molecule should be considered, because then recipients can be incorrectly classified as being at low risk for allloimmunisation.[17,19]

One could argue that our tools are of no additive value, as on the group level eplets have shown to be good predictors of sensitization,[18,20-22] graft damage,[23] and subsequently rejection.[24,25] This has resulted in the introduction of cut-offs of numbers of eplets to identify alloimmunisation risk,[17,18,26,27] which has even been applied in allocation strategy for paediatric patients.[28] However, as discussed in **Chapter 2** and **Chapter 3** eplets are theoretically defined and not every eplet mismatch is immunogenic.[29] Furthermore, eplets require experimental verification to establish if an antibody indeed can bind to the eplet. Both absorption and elution studies[30,31] as well as human mAbs[32,33] have been shown to be very useful for antibody-verification of eplets.[34,35] However, we emphasised that there is a need for HLA class II eplet verification, and as shown in **Chapter 5** the newly generated human HLA-DR mAbs contribute to antibody-verification of eplets. More importantly, our antibody reactivity analysis also highlighted that the current list of eplets on HLA Epitope Registry contains inaccuracies and is subject to change without valid reasoning and validation. In addition, when defining polymorphic solvent accessible positions to incorporate in HLA-EMMA (**Chapter 6**) we observed discrepancies between our definition of surface exposed positions and those considered for defining eplets,[6,7,36] This clearly indicates there is a need for standardisation for defining antibody-verified eplets. Both HLA-EMMA and human HLA mAbs will contribute to define the immunogenic polymorphic amino acids and subsequently immunogenic or relevant amino acids configurations in a more standardised and validated manner.

Our cohort study already highlighted that for defining immunogenic polymorphic amino acids a large number of donor-recipient pairs of diverse population is required (**Chapter 7**). The latter is essential, as currently the proposed cut-offs are based on Caucasian population studies, but just like HLA allele frequency the frequency of the most immunogenic epitope can differ between populations (**Chapter 3**). One of such studies will be the upcoming International Immunogenetics and Histocompatibility Workshop, in which not only HLA-EMMA but all factors that regulate antibody induction will be included, such as T cell epitopes (PIRCHE-II) and physicochemical properties (EMS-3D), for a comprehensive analysis.

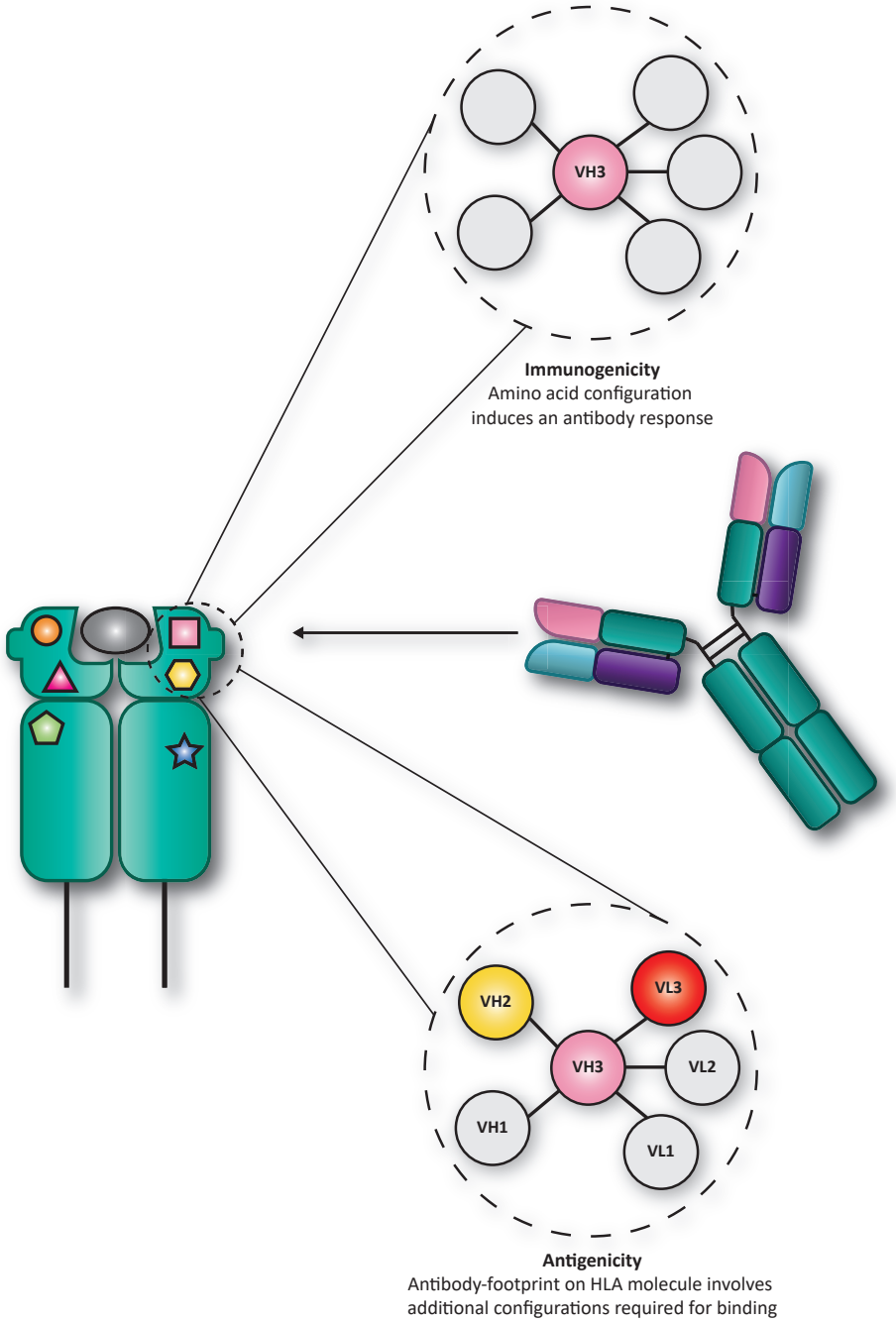

Figure 1: Immunogenicity and antigenicity. An immunogenic amino acid configuration (epitope) on mismatched donor HLA induces alloantibody response and determines the specificity of antibody as it interacts with CDR-H3 of the antibody. However, the antibody-footprint on HLA molecules involves additional configurations required for binding, which is the antigenicity of HLA antibody.

Antigenicity of formed HLA antibodies

Besides immunogenicity, definition of relevant amino acid configurations is also essential for understanding the antigenicity of HLA antibodies (Figure 1), which is imperative for sensitised patients to determine acceptable and unacceptable HLA antigen mismatches to predict a negative crossmatch (**Chapter 2**). HLAMatchmaker was developed to identify uniquely shared eplets by reactive HLA antigens in single antigen bead assay and used for reactivity analysis of mAbs[32,33] and sera[37-40] for antibody-verification of eplets. In recent years, eplets have been incorporated in analysis software of both single antigen bead assay used by HLA laboratories, and also a software tool to perform epitope analysis and virtual crossmatching based on eplets was developed.[41] However, as depicted in **Chapter 2**, reactivity of HLA antibodies is determined by crucial amino acid configurations in addition to the eplet or functional epitope. While some antibody reactivity patterns can indeed be explained by a single eplets/amino acid configuration, which is the functional epitope as it determines the specificity of antibody, this is not always the case as shown for both human HLA class I (**Chapter 3**) and HLA class II (**Chapter 5**) mAbs. In addition, not every reactive HLA antigen detected with single antigen bead assay is relevant, as mAbs can bind to HLA expressed on beads, but not to natively HLA expressed on cells (**Chapter 5**). Many HLA laboratories assign every HLA allele with a mean fluorescence intensity (MFI) value in single antigen bead assay as unacceptable, but our data shows that not every reactive HLA allele is relevant.

In addition, polyclonal serum consists of multiple antibodies, including HLA antibodies recognising different epitopes on same HLA antigen. This is nicely illustrated by our findings on mAbs directed against different epitopes on same HLA antigen that were generated from single memory B cell clones isolated from one individual with one tetramer specificity. The different memory B cell clones obtained showed different V(D)J usage indicating that the clonotypes are unique and not caused by somatic hypermutation (**Chapter 5**). Functional assays with cell expressing the target HLA showed differences in binding strength and differential efficiency in complement mediated cell lysis of the generated mAbs and therefore we surmised that the antibodies had different affinity for target HLA. Overall, this indicates that the abovementioned methods to interpret single antigen bead assay data of neat serum based on shared eplets is not so straightforward, as multiple factors such as immunising event, both immunogenic/functional epitopes and additional crucial configurations, and dilutions[42] should be taken into account to determine the true and relevant HLA alleles.

MFI values are often interpreted as being indicative of the relative concentration of HLA antibodies. However, the different level of reactivity of HLA antigens observed for mAbs

could also reflect the affinity the mAbs for specific HLA alleles (**Chapter 5**). It has been shown that the affinity for immunising antigen is often higher than for other antigens,[43] which might be due to the presence of the optimal set of crucial amino acid configurations. Additionally, amino acid substitution within the structural epitope can affect the affinity as well,[44] on the other hand if amino acid substitution does not affect surface area structure due to similar electrostatic potential, hydrophobicity or size than binding ability most likely remains the same.[45,46] Defining the affinity of HLA antibodies for various reactive HLA alleles will both elucidate on the interaction between antigen and antibody and the corresponding crucial additional contact sites and thus the antibody reactivity patterns observed in single antigen bead assays. Additionally, it will contribute in the understanding of the differential pathogenicity of HLA antibodies. This is essential because while *de novo* DSA are associated with graft rejection, not every recipient with detectable *de novo* DSA has clinical signs of rejection.[3,47] In addition, antibody-mediated rejection is mainly associated with complement activation,[48-50] but also complement independent graft injury has been observed.[51-54] Human HLA class I mAbs have shown to be useful for methodological studies of HLA antibodies to establish the clinical effect of IgG isotype and epitopes recognised.[51,52,55-57] However, as mentioned HLA class II antibodies are the dominant type of antibody to develop upon transplantation and recent studies demonstrated that non-human pan-HLA class II antibodies can induce endothelial cell damage independent of complement system.[53,54,58] The newly generated human HLA class II mAbs can be used to more specifically study the effect of HLA class II antibodies on graft injury, especially the effect of mixture of antibodies recognising different epitopes on same HLA antigen. Additionally, the role of different IgG subclasses can be studied as this method allows for the generation of mAbs of all four IgG subclasses, fully human glycosylated, recognising the same epitope with identical binding affinity (**Chapter 4**).

Besides these methodological studies, crystal structures of antigen-antibody complex are pivotal for studying both the pathogenicity of HLA antibodies as well as defining antigenicity of HLA antibodies. These structures will provide insight on how paratope of the antibody exactly binds to the epitope on the HLA antigen and so which amino acid configurations interact with the different complementary-determining regions of the antibody.[55] This will be especially of interest for HLA-DQ antibodies and how they bind to HLA-DQ molecule. Currently, we are working on isolating HLA-DQ-specific memory B cells similar as described in Chapter 5 but using a sorting strategy with HLA-DQ monomers instead.[59] Preliminary data implies that specificities of memory B cell clones are often directed to one chain, which supports our

HLA-DQ *dn*DSA findings in clinical cohort study (**Chapter 7**), but we suspect to isolate B cell clones that produce antibodies direct against both chains as well.

Clinical application

Once immunogenic epitopes are defined these can be avoided during allocation of kidneys to prevent the formation of *de novo* DSA after transplantation (**Chapter 2** and **Chapter 3**). By basing allocation only on truly immunogenic epitopes, patients will not be denied an organ based on non-immunogenic polymorphisms. Besides allocation, immunogenic epitope mismatch scores can also be used for personalised medicine as these may serve as a biomarker for alloimmunisation risk.[17,19] For individuals at low risk the immunosuppressive drugs can potentially be lowered, which will diminish the risk of side effects.[60] Personalised medicine based on immunogenic epitope mismatch scores will not only apply to kidney transplantation, but also to liver,[61,62] lung,[63] and heart transplant.[64-66] While allocation based on avoiding the most immunogenic epitopes may not always be feasible for these organs due to the necessity of short cold ischemia times and the lower number of available donor organs, immunogenic epitope mismatch scores may be used as indicator of the risk for alloimmunisation and therefore as a parameter upon which treatment can be adjusted.

As discussed, the presence of HLA antibodies complicates repeat transplantation, especially for highly sensitised patients. The Eurotransplant Acceptable Mismatch program has shown to be successful in determining acceptable mismatches to which the patient did not form antibodies, and which are used for selection of compatible donors.[67,68] Highly sensitised patients transplanted through this program had a superior graft survival compared to patients transplanted based on merely avoiding unacceptable mismatches.[69,70] In this program epitope analysis to define acceptable/unacceptable mismatches has already been incorporated, but will benefit from an inventory of well-defined immunogenic HLA class I and HLA class II epitopes. However, while it is important to note that for defining acceptable and unacceptable HLA mismatches understanding the exact antibody-antigen interaction and the crucial configurations involved are essential, this is extremely complex and requires additional research. Eventually, this knowledge can be used to define acceptable and unacceptable epitopes to be used for virtual crossmatching, as described in **Chapter 2**. Importantly, this approach allows for defining acceptability of HLA alleles not present in single antigen bead assays. Currently, we are working on implementing defining acceptable and unacceptable HLA mismatches in HLA-EMMA.

While HLA epitope matching is becoming a hot topic in the transplant community and clinicians are eager to start epitope matching, more research is required to introduce HLA epitope matching properly. This thesis forms the basis for these additional studies to be performed.

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CHAPTER

9

NEDERLANDSE SAMENVATTING

Het immuunsysteem

Het immuunsysteem beschermt de mens tegen schadelijke ziekteverwekkers (pathogenen) en daarvoor is het van belang dat het onderscheid kan maken tussen lichaamseigen en lichaamsvreemde antigenen. Het verdedigingssysteem doet dit in eerste instantie door middel van de aspecifiek aangeboren immuniteit die effectief en snel het pathogeen vernietigt. Het adaptieve (verworven) immuunsysteem is specifieker maar ook langzamer en bestaat uit antistoffen geproduceerd door B-cellen, de humorale immuunreactie, en T-cellen die zorgen voor een cellulair immuunreactie. De receptoren op zowel B-cellen als T-cellen moeten eerst een specifiek antigeen herkennen voordat de cellen differentiëren in zowel effector cellen die het pathogeen vernietigen als in langlevende geheugen cellen die een snelle en effectieve reactie kunnen veroorzaken bij nieuwe blootstelling aan hetzelfde antigeen.

HLA systeem

De humane leukocyten antigenen (HLA) spelen een belangrijke rol in het immuunsysteem. Het HLA-systeem bestaat uit HLA klasse I moleculen, HLA-A, -B en -C, en HLA klasse II moleculen, HLA-DR, -DQ, en -DP. HLA klasse I moleculen komen voor op alle cellen behalve rode bloedcellen en presenteren peptiden afkomstig van lichaamsvreemde eiwitten in de cel aan cytotoxische T-cellen ($CD8^+$) die vervolgens die cel doodt. HLA klasse II moleculen komen vooral tot expressie op antigeen presenterende cellen (APC) en geactiveerde endotheel cellen. Deze cellen nemen eiwitten op uit de omgeving en de HLA klasse II moleculen en presenteren die als peptiden aan T-helper-cellen ($CD4^+$) die vervolgens andere cellen activeren, zoals B-cellen om antistoffen uit te scheiden. Verschillende HLA moleculen presenteren andere peptiden. Er zijn inmiddels heel veel HLA moleculen geïdentificeerd terwijl bij elk individu verschillende, erfelijk bepaald, HLA moleculen op de cellen aanwezig zijn. Deze extreme polymorfisme van HLA zorgt ervoor dat er altijd een individu is dat een peptide van een bepaalde pathogeen kan presenteren waardoor de menselijke populatie altijd beschermd is tegen nieuwe of gemuteerde pathogenen.

Allo-immuun reactie in transplantatie

Waar het HLA polymorfisme voordelig is voor bescherming tegen pathogenen, is het ongunstig voor (orgaan) transplantaties. Al in de jaren 60 bleek dat HLA matchen cruciaal was voor niertransplantatie omdat donor nieren met dezelfde HLA als ontvanger een betere overlevingskans hadden. HLA moleculen van de donor kunnen namelijk worden herkend als lichaamsvreemd door het immuunsysteem van de ontvanger. Zo kunnen T-cellen van de

ontvanger zowel de intacte HLA moleculen op donor APC herkennen, directe herkenning, als peptiden afkomstig van een donor HLA molecuul gepresenteerd in HLA moleculen van ontvanger, indirecte herkenning. Beide reacties induceren immuunreacties die leiden tot acute en chronisch afstotingen. Transplantatie patiënten moeten levenslang afweer onderdrukkende medicijnen nemen en de meerderheid daarvan is dan ook gericht op het onderdrukken van T-cel activatie.

Maar ook de B-cel receptoren op de B-cellen van de ontvanger kunnen de intacte HLA moleculen van de donor herkennen als lichaamsvreemd. Vervolgens gaan de B-cellen HLA-specifieke IgG antistoffen produceren waarbij ze geholpen worden door T-helper-cellen, die peptiden herkennen die afkomstig zijn van het donor HLA molecuul gepresenteerd door HLA klasse II moleculen op de B-cellen van de ontvanger.

Antistoffen specifiek gericht tegen donor HLA gevormd na transplantatie worden donor-specifieke antistoffen (DSA) genoemd. De aanwezigheid van deze DSA is geassocieerd met een verhoogde kans op afstoting van de getransplanteerde nier. Daarnaast zorgen deze DSA ervoor dat het moeilijker is om een geschikte donor te vinden voor een nieuwe transplantatie, omdat de HLA antigenen waartegen de al voor transplantatie antistoffen aanwezig zijn, worden beschouwd als onacceptabel. Dit is nodig om te voorkomen dat een nier na transplantatie meteen wordt afgestoten omdat er antistoffen aanwezig zijn die de donor HLA op het orgaan kunnen herkennen. Deze ongewenste DSA kunnen ook worden gevormd na een zwangerschap of bloedtransfusie. Een bijkomend probleem is dat DSA niet alleen specifiek zijn voor het gemismatchte donor HLA, maar ook kunnen kruisreageren met andere HLA moleculen, die een beetje lijken op het donor HLA.

Dit proefschrift

Ondanks de verbeterde chirurgische technieken en de betere immunosuppressiva is HLA matchen nog steeds essentieel voor niertransplantatie. Maar door de hoge mate van HLA polymorfisme en schaarste van organen is de kans op het vinden van een HLA identieke (gematchte) donor klein. Nu blijkt dat niet elke HLA antigeen verschil (mismatch) een humorale allo-immuunreactie induceert. Ontwikkeling van nieuwe technieken, waarbij de genen die de erfelijke informatie bevatten voor de HLA moleculen in detail worden geanalyseerd, hebben geleid tot een betere kennis over de HLA moleculen. Het is inmiddels duidelijk dat elk HLA molecuul bestaat uit een unieke set van polymorfe aminozuur configuraties, vaak aangeduid als epitopen, maar dat individuele epitopen gedeeld kunnen worden door verschillende HLA

moleculen. Recente studies hebben aangetoond dat het aantal epitoop verschillen tussen donor en ontvanger geassocieerd is met vorming van DSA na transplantatie.

Daarom is het interessant om nieren te alloceren op basis van epitoop matchen in plaats van HLA antigeen matchen om DSA vorming na transplantatie te voorkomen. Hiervoor is het van belang om in kaart te brengen welke epitopen een antistofreactie kunnen veroorzaken. Het blijkt dat een configuratie van een paar aminozuren, ook wel functioneel epitoop genoemd, op het HLA molecuul verantwoordelijk is voor de inductie van antistoffen en ook de specificiteit van het antistof zal bepalen. He vermogen om een antistof reactie op te wekken wordt immunogeniciteit genoemd. Voor de binding van antistof aan antigeen zijn meerdere aminozuur configuraties betrokken en die bepalen de affiniteit en sterkte van binding. Dit laatste wordt aangeduid als de antigeniciteit van een HLA molecuul. Het complete gebied van antigeen waaraan antistof bindt wordt ook wel B-cel epitoop of structureel epitoop genoemd.

In **hoofdstukken 2** en **3** beschrijven wij verschillende benaderingen die zijn geïntroduceerd om de immunogeniciteit van HLA moleculen te voorspellen en daarmee ook de kans op DSA vorming. Daarnaast beargumenteren wij dat DSA vorming niet enkel afhankelijk is van het aantal gemismatchte epitopen, maar dat de immunogeniciteit van individuele epitopen wellicht nog meer bepalend is (**hoofdstuk 3**). Het verschil tussen immunogeniciteit en antigeniciteit wordt ook uitgelegd (**hoofdstuk 2**). Dit is belangrijk omdat DSA vorming voorkomen kan worden door immunogene aminozuur configuraties, of epitopen, te vermijden tijdens allocatie van organen. Als DSA al gevormd zijn, zoals bij hoog geïmmuniseerde patiënten, is het van belang om de antigeniciteit in kaart te brengen teneinde de onacceptabele en acceptabele configuraties, of epitopen, te bepalen en op basis daarvan geschikte donoren te vinden.

De meeste bestudeerde benadering om de kans op immunisatie te bepalen is op basis van gemismatchte eplets. Eplets zijn theoretisch gedefinieerde configuraties van aan de oppervlakte van het HLA molecuul gelegen polymorfe aminozuren. Van veel van deze eplets moet nog worden vastgesteld of een antistof er inderdaad aan kan binden. Dit kan worden gedaan met behulp van humane monoklonale HLA antistoffen (mAbs). Dit is zeer succesvol gebleken voor de HLA klasse I moleculen maar deze zijn nauwelijks aanwezig voor het in kaart brengen van de relevante polymorfisme op HLA klasse II moleculen. Daarom hebben we een techniek opgezet om HLA klasse II mAbs genereren om zo eplets te verifiëren waaraan een antistof kan binden. In **hoofdstuk 4** gebruiken wij bestaande B-cel hybridoma's om een methode te verifiëren voor het genereren van recombinant humaan HLA mAbs van alle vier de IgG subklassen. Vervolgens hebben wij deze methode gebruikt in **hoofdstuk**

5 om recombinant humaan HLA-DR-specifieke mAbs te genereren uit geheugen (memory) B-cellen die door middel van HLA-DR tetrameren waren geïsoleerd uit perifeer bloed van zwangerschap geïmmuniseerde individuen. Reactiviteit analyse van deze mAbs hebben geresulteerd in de identificatie van uniek gedeelde aminozuur of aminozuur configuraties op de reactieve HLA allelen. Sommige van de geïdentificeerde configuraties kwamen overeen met bestaande eplets en daarmee bevestigden wij dat een antistof kan binden aan deze eplets. Andere geïdentificeerde configuraties toonden aan dat andere eplets niet helemaal juist waren gedefinieerd.

Een andere manier om de relevante epitopen te definiëren is door de immunogene polymorfe aminozuren te identificeren. In **hoofdstuk 6** beschrijven wij de ontwikkeling van een gebruiksvriendelijke software HLA-EMMA dat aminozuur sequenties van gemismatchte donor HLA allelen vergelijkt met de aminozuur sequenties van dezelfde locus van de ontvanger. HLA-EMMA bepaalt de aminozuur verschillen van zowel de hele sequentie als enkel die posities die gedefinieerd zijn als toegankelijk voor antistof. Naast de verschillen geeft HLA-EMMA ook de soort en positie van de gemismatchte aminozuren.

Aangezien DSA specifiek voor HLA klasse II voornamelijk worden gevormd na transplantatie, wilden wij de meest immunogene HLA klasse II aminozuren identificeren. Hiervoor analyseerde wij in **hoofdstuk 7** niet-geïmmuniseerde mannen die voor het eerst een niertransplantatie hadden ondergaan met minimaal één HLA antigeen mismatch maar die uiteindelijk hun transplantaat verloren als gevolg van immunologisch falen. Met HLA-EMMA bepaalde wij de “solvent accessible” aminozuur verschillen voor HLA-DR en HLA-DQ allelen mismatches. In deze kleine studie zagen wij al voor het aantal gemismatchte HLA-DQ aminozuren een associatie met nieuwgevormde DSA. In tegenstelling tot andere studies, analyseerden wij de HLA-DQ ketens (alfa en bèta keten) afzonderlijk met betrekking tot hun vermogen om een antistofreactie te induceren en toonde aan dat één aminozuur mismatch op de alfa of op de bèta keten al voldoende kan zijn om een antistofreactie te veroorzaken.

Dit bevestigde onze hypothese dat niet alleen het aantal epitoop mismatches maar ook de immunogeniciteit van de individuele epitopen bijdraagt aan de inductie van HLA antistoffen.

HLA-EMMA en de door ons ontwikkelde humane HLA mAbs zullen beiden bijdragen aan de identificatie van immunogene en daarmee de relevante epitopen die de basis moeten vormen van toekomstige strategieën gebaseerd op epitoop matchen om zo DSA vorming te voorkomen en om niet onnodig een orgaan af te wijzen op basis van niet-immunogenen epitopen. Aanvullende studies met de recombinant humaan HLA klasse II mAbs zullen zeker

ook meer inzicht geven in de interactie tussen antistof en HLA antigeen. Daarnaast kunnen methodologische studies worden uitgevoerd met de recombinant humaan HLA mAbs vooral omdat we alle vier de IgG subklassen kunnen genereren en dit zal bijdragen aan het begrijpen van de differentiële pathogeniteit van HLA antistoffen die gevormd worden na transplantatie.

In conclusie, dit proefschrift vormt een goede basis voor aanvullende studies die nodig zijn om HLA epitoop matchen te introduceren in de praktijk.

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CURRICULUM VITAE

Cynthia Silvia Maria Kramer werd geboren op 20 maart 1989 te Amersfoort. In 2007 behaalde ze haar VWO diploma aan het Corderius College in Amersfoort. Vervolgens ging ze voor een jaar naar de EF International Language Schools in Londen, Engeland. In 2008 begon ze aan de opleiding Moleculaire levenswetenschappen aan de Radboud Universiteit Nijmegen waar ze zowel haar Bachelor als Master of Science diploma's behaalde. Tijdens deze studie liep ze stages bij laboratorium Hematologie van de Radboud UMC, waar ze onder ander onderzoek deed naar *in vitro* genereren van minor histocompatibiliteit antigeen-specifiek stamcel geheugen T-cellen. Haar afstudeerstage liep ze bij de Hepatic Fibrosis groep van de Queensland Institute of Medical Research in Brisbane, Australië. Hier deed ze onderzoek naar galzuur geïnduceerde chemokine expressie in lever progenitorcellen van patiënten met galgangatresie en cystic fibrosis leverziekte. In mei 2015 startte ze met haar promotieonderzoek in transplantatie immunologie op de afdeling Immunohematologie en Bloedtransfusie van de Leids Universitair Medisch Centrum onder begeleiding van prof. dr. Frans Claas, dr. Sebastiaan Heidt en dr. Dave Roelen. De uitkomsten van het promotieonderzoek zijn beschreven in dit proefschrift. Sinds 2020 werkt ze als researcher bij de afdeling Immunohematologie en Bloedtransfusie.

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