Towards precision medicine in kidney transplantation: Epitope based HLA-matching and improved DSA diagnostics

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List of Abbreviations

3D	3-dimensional
Å	angstrom (1Å = 0.1nm)
AA	amino acid
AbVer	antibody verified
allo	allogenic (same species but antigenically distinct)
AMR	antibody-mediated rejection
APC	antigen presenting cell
ADCC	antibody-dependent cellular cytotoxicity
BCR	B-cell receptor
β2m	beta-2-microglobulin
C1q	(molecule that initiates the classical complement cascade)
CD	cluster of differentiation
CDC	complement dependent cytotoxicity
CSA	child specific HLA antibodies
DC	dendritic cell
de novo	newly formed
dn	*de novo*
dnDSA	*de novo* donor specific HLA antibodies
dnCSA	*de novo* child specific HLA antibodies
DSA	donor specific HLA antibody
EbHM	epitope based HLA-matching
Fc	fragment crystallizable
FcγR	Fc gamma receptor
Fab	fragment antigen-binding
GC	germinal centre
HLA	human leukocyte antigen
HR-2F	high-resolution 2-field HLA typing
IgA	Immunoglobulin A
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
mAb	monoclonal antibody
NGS	next generation sequencing
NK-cells	natural killer cells
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
SAB	single antigen beads
SOT	solid organ transplantation
TerEp	Terasaki epitope
TCR	T-cell receptor
Tfh	T follicular helper cells
vXM	virtual cross-match
XM	cross-match

Summary

Refinement of immunological pre-transplant risk assessment, specifically by improving the molecular compatibility between donor and recipient and by enhancing the characterization of the recipient's pre-established immunological memory, is key to transit to an improved organ allocation and a personalized immunosuppression in solid organ transplantation (SOT). For both of these diagnostic clarifications, novel conceptual and technological achievements have been attained, but still need to be perfected. This doctoral thesis is dedicated to both topics. Publication 1 presents a study on the immunogenicity of human leukocyte antigen (HLA) epitopes, the results and insights of which contribute to better prediction of newly formed (*de novo)* immune responses in allograft recipients. The study presented in Publication 2 addresses the question of which pre-transplant donor specific HLA antibodies (DSA) are clinically relevant and why. The results demonstrate how different antibody-IgG compositions affect complement activation, the most detrimental antibody effector function in relation to antibody-mediated rejection (AMR). Finally, Publication 3 reviews relevant issues in the context of DSA characterization by the Single Antigen Bead (SAB) assay, which has become the central assay for HLA-antibody characterization in transplant diagnostics.

Publication 1

To avoid a violent allogenic reaction of the recipient's immune system, the consequences of which lead to graft-dysfunction and -rejection, the structural dissimilarity between donor and recipient HLA must be minimized. The novel concept of 'Epitope based HLA-matching' (EbHM) serves as a bioinformatic instrument to implement this goal. It has significant advantages over the current HLA-matching approach, which simply counts HLA mismatch numbers. The prognostic potential of EbHM in predicting the development of *de novo* DSA (dnDSA) was demonstrated in several studies for (immunosuppressed) transplant recipients. The majority of these studies, however, focused on the quantitative effect of mismatched epitopes, considering each epitope as equally deleterious. Conclusive data on the quality of individual mismatched antigenic epitopes, potentially revealing their real immunogenicity, are not yet established. Subsequent extension of EbHM with epitope

immunogenicity scores could decisively strengthen the concept, as it potentially enhances its prognostic value with regard to dnDSA development.

The core study of this doctoral thesis was therefore to determine the immunogenic potential of functional elements on structural HLA epitopes, termed 'eplets'. For this I assessed all currently confirmed HLA class I eplets for their potential to induce child specific HLA antibodies (CSA) in previously non-sensitized mothers pregnant with their first child. The results of this comprehensive study revealed strongly immunogenic, barely immunogenic and non-immunogenic mismatched eplets. Subsequently, I rated their immunogenicity and visualized their localization on the topographic surface of HLA-A, -B, and -C molecules. Hence, this publication provides a valuable contribution towards the translation of the EbHM concept into clinical practice.

Publication 2

For patients pre-sensitized to donor HLA, as measured by the presence of DSA, pre-transplant risk assessment becomes more complex. Indeed, patients transplanted across DSA show various clinical outcomes, ranging from acute AMR followed by allograft loss to uneventful or even completely rejection-free courses. This indicates substantial differences with regard to the quality of the pre-established immunological memory and the effects of circulating DSA. If DSA features were found that accurately predict the net hazardous potential of their presence in the periphery, it would be possible to perform only those transplantations in which non-pathogenic DSA were involved. A number of research efforts to identify such DSA characteristics, however, have been unsuccessful to date.

The second publication of this thesis focused on one of the most detrimental effects of HLA antibodies, complement activation. We first developed an *in vitro* model to study C1q-fixation of target bound HLA antibodies. The results of our study demonstrated that dissecting patients' HLA antibodies into their IgG subclasses is necessary to understand the resulting net impact on complement activation. An excess of IgG2/IgG4 over IgG1/IgG3, as present in approximately a third of investigated genuine HLA-specific IgG subclass-mixtures from patients sensitized prior transplantation, exhibited an inhibitory effect on C1q-binding. By taking into account the epitope-specificity of the individual components of the antibody

mixture, it became apparent that binding of IgG2/IgG4 to an adjacent epitope can even synergistically enhance IgG1/IgG3-triggered C1q-binding.

Publication 3

Accurate determination of DSA is an integral part of pre-transplant risk assessment or post-transplant monitoring. The SAB assay, a solid-phase multiplex immunoassay, that uses the cutting edge xMAP® technology from Luminex, has become the new standard test to characterize DSA and has revolutionized HLA antibody diagnostics. SAB results are increasingly used for virtual crossmatching, for characterization of HLA epitopes by using monoclonal antibodies (mAb) or pre-absorbed/eluted allo-antibodies from patient sera, for determination of HLA antibody epitope-specificity, for assessment of DSA isotypes/subclasses, or for HLA antibody titre determination.

As the SAB assay is not without imperfections, and as the further improvement of mentioned applications rely heavily on SAB analyses, there is a significant need to better understand the assay in many important details. Indeed, the test format and the quality of recombinant HLA coated to beads lead to some considerable limitations and challenges that must to be considered when interpreting SAB results. The third publication of this thesis therefore reviews all important aspects of the SAB test, the Luminex platform, the handling of SAB multiplex results and their technical as well as clinical interpretation. We summarized the benefits, but also focused on the shortcomings of this assay and emphasized critical points for accurate test result interpretation. Furthermore, we suggested improvements for the future, especially in light of possible novel applications.

Outline of the doctoral thesis

This thesis is organized into eight chapters, including a general introduction (Chapter 1) in which I am highlighting the immunological aspects of SOT. Furthermore, Chapter 1 describes the existing knowledge gaps and associated diagnostic requirements with regard to the immunological risk stratification performed prior to a kidney transplantation. Chapter 2 contains the research objectives of my thesis as well as a list of my publications that are covered in Chapter 3, 4 and 5. Next, Chapter 3 presents the results of my core study, an assessment of the immunogenicity of HLA epitopes, explored in the pregnancy model. In this chapter I also describe the investigations regarding the methodological changes that were necessary to enable high-resolution 2-field HLA typing (HR-2F), which forms the basis to accurately define functional parts of donor HLA epitopes and to interpret HLA antibody epitope-specificity. Focusing on one specific HLA epitope, the experimental study presented in Chapter 4 demonstrates how DSA IgG-subclass compositions impact complement binding in different ways, and its clinical relevance for DSA positive pre-transplant patients. Chapter 5 comprises a review about the Single Antigen Bead (SAB) assay, which has become the gold standard test to characterize DSA, but where many important details need to be better understood. Besides summarizing the benefits of this assay, it especially highlights identified caveats and proposes improvements for the future. Lastly, Chapter 6 presents integrated discussions and conclusions of this doctoral thesis.

Chapter 1 General Introduction

1.1 Solid organ transplantation: success and burden

SOT has become the treatment of choice for end-stage organ failure and the continued improvement of transplant outcomes in SOT can be considered as a success story of modern medicine [1]. However, the downside of transplantation is the need to take immunosuppressive drugs. These medications, which suppress the recipient's immune system to prevent the recognition of foreign molecules on donor cells, must be taken daily and can cause side effects. The majority of such molecules are HLA glycoproteins [2, 3]. HLA molecules have been the focus of transplant diagnostics since it was recognized that their polymorphism is closely linked to graft rejection [4]. A strong risk factor for rejection episodes is the presence of DSA [5] which mediate effector functions that are deleterious for the transplanted donor tissue. The development of DSA can be promoted by suboptimal immunosuppression.

1.2 Biological function of HLA molecules and their role in the context of SOT

HLA proteins, encoded by the human major histocompatibility complex (MHC), a complex of highly polymorphic genes located on chromosome 6, are crucial players in adaptive immunity. Two of the three HLA classes are of high relevance in the context of transplantation: Class I (HLA-A, -B and -C) and class II (HLA-DPA1/DPB1, -DQA1/DQB1, -DRB1/DRA1, -DRB3/DRA1, -DRB4/DRA1, -DRB5/DRA1). The expressed molecules of both classes consist of highly variable extracellular domains but fairly constant transmembrane/cytoplasmic domains. The assembly of the extracellular units of the molecules is shown in **Figure 1** using one example for each of the two HLA classes.

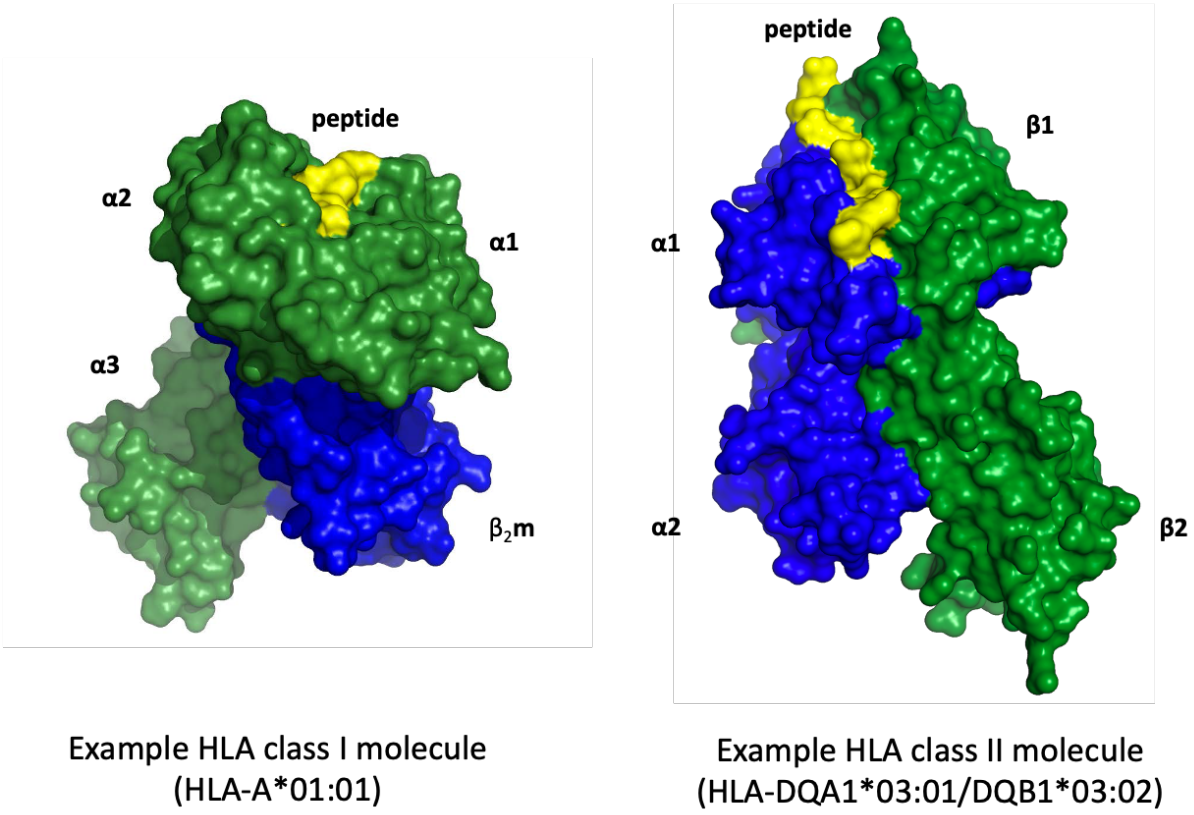

Figure 1. 3D view on extracellular domains of an HLA class I (left) and an HLA class II molecule (right). An HLA class I heterodimer consists of a heavy chain (green) comprised of three subunits (two peptide-binding domains α1 and α2, and the immunoglobulin-like α3 domain) and a non-covalently bound light chain, beta-2-microglobulin (blue). The peptide is shown in yellow. An HLA class II heterodimer consists of two non-covalently bound chains, the (blue) α- and (green) β-chain. Their α1- and β1-subunits form a membrane-distal peptide-binding domain (peptide indicated in yellow), while the remaining extracellular parts of the chains (α2- and β2-subunits) form a membrane-proximal immunoglobulin-like domain.

β2m: beta-2-microglobulin

Protein data bank (PDB; www.rcsb.org) codes of exemplary HLA: HLA-A*01:01: 3BO8; HLA-DQA1*03:01/DQB1*03:02: 4Z7U

The central biological function of HLA molecules is to bind processed antigenic peptides of intra-cellular proteins (HLA class I) or of proteins from extra-cellular spaces (HLA class II). The peptides are presented to the T-cell receptor (TCR) of CD8+ T-cells (HLA class I) or CD4+ T-cells (HLA class II), in order to promote antigen-specific immune-responses [6]. Indeed, the core component of both types of molecules resembles a pocket, in which peptides formed by degradation (HLA class I: lengths of 8-10 amino acids (AA) [2, 7, 8] and HLA class II: lengths of 12-25 AA [9-11] are embedded. Variation in AA composition within the peptide

pocket region, which is due to the exceptionally high mutation rate of HLA genes, can dramatically alter the binding properties towards peptides [2]. Indeed, continuous changes in HLA molecule composition ensure an ongoing presentation of novel peptides from pathogens that continuously mutate themselves. The diversity of HLA alleles [12, 13] is therefore the foundation of an efficient population-wide disease defence [14].

1.3 Allorecognition

While HLA variability is biologically immensely important, it is also responsible for allorecognition which potentially leads to graft rejection in the context of SOT. Indeed, the fact that these molecules are recognized as antigens by the recipient's immune system is reflected in the term: 'HLA' (human leukocyte **antigen**).

Allorecognition, the ability of the host organism to discriminate between self and non-self, occurs through different immunological pathways [15], but their detailed mechanisms are still the subject of research [16-18] (**Figure 2**). Briefly, three-dimensional (3D) structures of donor-HLA derived allo-peptides, presented by either donor- or recipient- antigen presenting cells (APCs), are recognized by allo-specific effector T-cells, which induces an allo-specific immune response.

In the '**direct pathway of recognition**', donor HLA:peptide complexes are directly recognized by the recipient's CD4+ T-cells on donor dendritic cells (DC). It appears that these donor DCs are transplanted as 'passengers' [19] with the graft, and migrate into the recipient's secondary lymph nodes where they present donor HLA derived peptides [20-22].

In the best researched pathway, termed '**indirect pathway of allorecognition**' [23], recipient DCs presenting donor HLA-derived peptides activate allo-specific T follicular helper cells (Tfh) [24], which in turn support the generation of allo-specific CD8+ effector T-cells and antibody producing B-cells [25-30].

The '**semi-direct pathway of allorecognition**' is the least established. The exchange of membrane proteins between immune cells [31] and/or the uptake of exosomes [32, 33] may result in the acquisition of allo-peptide presenting donor HLA molecules on recipient DCs, which may in turn stimulate the recipient's cytotoxic or helper T-cells via the two allorecognition pathways described above.

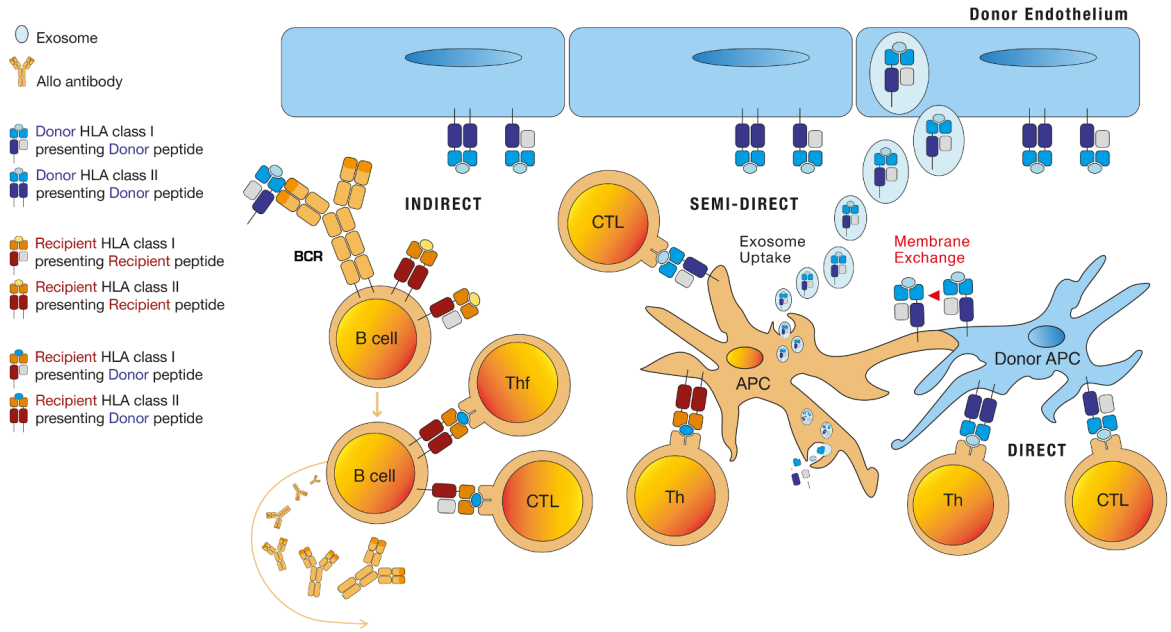

Figure 2. Indirect, semi-direct, and direct allorecognition. The figure is designed to accord with the review article from Hickey et al. [18]. Right: direct pathway, where structures of allogenic HLA class I and II on 'passenger' donor APCs, mainly dendritic cells, are directly recognized by recipient's cytotoxic or helper T-cells. Middle: semi-direct pathway, where donor HLA class I:peptide complexes on recipient APCs (uptaken through either endocytosed exosomes or membrane exchange) are recognized by $CD8^+$ T-cells, or processed donor-HLA-derived peptides are presented to helper $CD4^+$ T-cells. Left: indirect pathway, where donor allo-antigens are recognized by the specific BCR of B-cells, resulting in the presentation of donor-HLA-derived peptides which are recognized by cytotoxic T-cells and T follicular helper cells. The latter process leads to the generation of DSA.
Recipient cells: light brown, donor cells: light blue, HLA molecules recipient: dark and light brown, HLA molecules donor: dark and light blue, recipient-derived peptides: yellow, donor-derived peptides: blue.

APC: antigen presenting cell, BCR: B-cell receptor, CTL: cytotoxic $CD8^+$ T-cell, Thf: T follicular helper cell, Th: helper $CD4^+$ T-cell

1.4 Allo-specific immune response

The recognition of peptide structures derived from donor HLA proteins triggers both a cellular immune response as well as a humoral immune-response [4, 34, 35]. The specific effects of the thereby activated lymphocytes and their products, as well as additional effector mechanisms of macrophages and natural killer (NK) cells, lead to tissue injury and ultimately to graft-rejection, transplant glomerulopathy and subsequent allograft failure [36-38].

Undisputedly, the greatest damage is caused by the humoral arm of the immune response [39], through DSA produced after the interaction of naïve recipient B-cells with donor HLA and helper T-cells in germinal centres (GC) of the recipient's secondary lymphoid tissue [40, 41]. The effector functions of DSA substantially contribute to the clinical pathogenesis of AMR [4, 42]. The magnitude and durability of the humoral immune response is multi-factorial. Besides the central role of the recipient's repertoire of DSA and their functional diversity, the outcome is also impacted by effector cell characteristics of the recipient [i.e. cytotoxic/cytolytic capacity, and the type and density of specific receptors on the effector cells that bind to the fragment crystallizable (Fc) part of the antibodies (FcγR) [43, 44]].

1.5 Antibody-mediated rejection

Understanding the processes leading to AMR requires comprehension of the modes of action executed by antibodies. Indeed, the primary function of antibodies is to tag, block and/or crosslink the antigen, while their secondary function is to facilitate antigen destruction via pathways of the innate immune system [4]. In the transplant setting, the central destructive activities caused by DSA are

- **HLA crosslinking** on endothelial cells of the donor's graft (triggering endothelial cell activation, recruitment of leukocytes and inflammation) [45, 46]
- **C1q-binding** (causing complement activation, augmented leukocyte recruitment and T-cell allo-responses) [47, 48]
- **FcγR binding** (inducing antibody-dependent cellular cytotoxicity (ADCC) through NK-cells, monocytes, macrophages or neutrophils) [44, 49-51]

Because immunosuppression as currently applied does not always restrain DSA development well [5], AMR can occur at any time post-transplant [42, 52]. AMR is best defined in kidney [53], heart [54] and pancreas [55] transplantation. Some leading features of AMR include endothelial injury [54, 56], microvascular inflammation with subendothelial mononuclear cell infiltration [57, 58], presence of intravascular CD68+ macrophages and complement C4d-deposition [53].

1.6 HLA diversity: immunological hurdle for SOT

While HLA variability is appropriate to increase the probability of presenting antigenic variants of pathogens, it constitutes an immunological hurdle for SOT. Indeed, it is practically unfeasible to match all of the 11 highly polymorphic HLA genes (HLA-A, B, C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, DPB1) between (unrelated) donors and recipients. Moreover, the limited supply of organs adds to the difficulty [59]. Consequently, disparities among HLA molecules of donor and recipient are the leading cause for graft rejection in SOT [60-63]. In kidney transplantation, fifteen to twenty-five percent of non-sensitized patients will develop dnDSA after receiving the graft [64, 65]. These dnDSA increase the probability of clinical or sub-clinical AMR as well as allograft failure [64-66].

1.7 Individual HLA mismatches lead to different clinical manifestations

Overall, graft loss correlates with the degree of HLA mismatches between the recipient and the allograft-donor [67, 68]. However, a closer look reveals that individual HLA mismatches cause different clinical manifestations [69-72]. Nevertheless, most transplant centres do not modify the administered immunosuppression based on the number of HLA mismatches [61, 73]. A more accurate assessment of the structural HLA mismatch load and the co-consideration of immunogenic properties of specific structural dissimilarities promises a significant potential for patient specific finetuning of the applied immunosuppression.

1.8 From the traditional concept of 'HLA-matching' to the novel concept of 'Epitope based HLA-matching'

Graft survival, as is well documented especially for transplanted kidneys [74], has steadily improved over time. An important reason for this development, apart from medication, is that pre-transplant risk assessment has been steadily refined by virtue of technical and methodological enhancements supporting an accurate immunological risk prediction. Particularly noteworthy achievements in this regard are: improvement of HLA typing methods, enhancement of HLA antibody assessment, and application of virtual cross-matching [75, 76]. This process is ongoing. During the last decade, additional non-invasive technological and conceptual possibilities emerged out of the research realm with a high potential to take pre-transplant risk assessment to the next level. To be mentioned in this

regard are in particular full HLA gene sequencing by next generation sequencing (NGS), the translation of these sequencing data into the tertiary structure of the expressed HLA proteins, the breakdown of their 3D surface into structural epitopes, and the assessment of epitope-specificity of HLA antibodies.

The entire structural HLA epitope consists of several structural clusters of AAs positioned on the surface of the HLA molecule that can be accessed by complementarity determining regions (CDR) located in the fragment of antigen-binding (Fab) from B-cell receptors (BCR) or antibodies [77]. On any given HLA, multiple epitopes are present and many of them are shared between different HLA protein types [78]. Therefore, some HLA mismatches may represent many structural differences, while other HLA mismatches consist of relatively few structural dissimilarities [79].

Recognition of the latter has led in recent years to the development of a new HLA-matching concept for SOT. This approach is termed 'Epitope based HLA-matching' (EbHM) [80-82]. Here, HLA types present in the recipient and donor are compared at the structural level. Both similarities and differences are considered, in order to define those non-self 3D AA patches [83-85] and their physicochemical-properties [86] on donor-HLA that, in theory, may trigger an immune-response. At present, every HLA mismatch is classified with an equal risk. Contrarily, the EbHM concept calculates the real load of allo-epitopes on donor HLA of a given HLA mismatch [79]. The hope that this new approach will further enhance transplant outcome is supported by the results of retrospective studies, which demonstrate a clear association between the extent of epitope burden and both the development of *de novo* DSA [81, 87-89] and an adverse allograft outcome [81, 88-96]. Moreover, a statistical comparison of the current and the new approach clearly shows that the sum of HLA mismatches does not predict dnDSA, while the sum of eplet mismatches predicts dnDSA quite accurately. This justifies 'mismatched eplets' as an effective prognostic biomarker for dnDSA [79, 97, 98], but prospective interventional trials to determined their clinical utility are lacking [99]. A comparison between the current 'HLA matching' concept and the new 'EbHM' concept is given in an example in **Figure 3**.

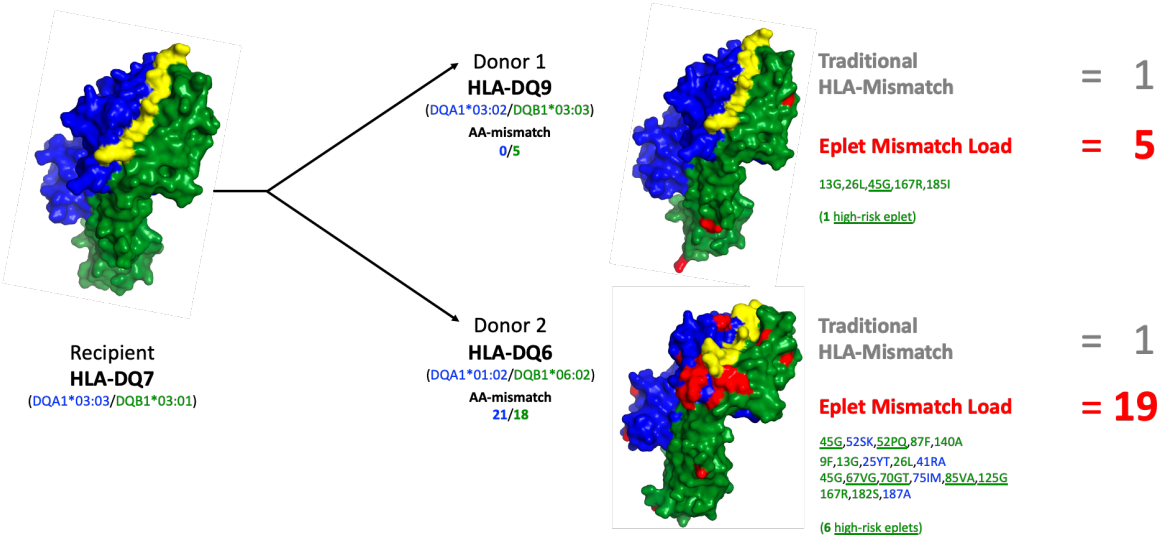

Figure 3. Example comparison between the current 'HLA matching' concept and the new 'EbHM' concept. For simplicity, only one HLA locus (HLA-DQ) is considered, assuming a recipient homozygous for HLA-DQ7 and, as an example, donors only mismatched at the HLA-DQ locus. The figure shows 3D images of the respective HLA-DQ molecules, each with a specific (blue) α- and (green) β-chain, and with the presented peptide (yellow) also visualized. According to the traditional HLA mismatch concept, both donors (donor 1 and donor 2) are considered as immunologically equal because their expressed HLA-DQ type differs from DQ7 (resulting in 1 mismatch for each of the two constellations). However, focusing on the real structural differences, DQ6 (donor 2) reveals a strikingly higher dissimilarity with DQ7 compared to DQ9 (donor 1). This difference is already clear from the amino acid (AA) mismatch score (only considering AAs of extracellular domains), indicated separately for each chain in the corresponding colour. All mismatched AAs located at the surface of the two donor HLA-DQ molecule types (top: DQ9; bottom: DQ6) are indicated in red, demonstrating a remarkable larger area with structural variation on DQ6 compared to DQ9. When assigning these structural differences to eplets present on the respective HLA-DQ type of each donor, it becomes obvious that the eplet load on HLA-DQ6 from donor 2 is almost 4-fold higher than that on DQ9 from donor 1. Finally, when focusing on the eplet-derived immunogenicity (determined using the method presented in Publication 1), the HLA-DQ type from donor 1 consists of only one high-risk eplet, while the HLA-DQ type from donor 2 reveals six high-risk eplets. This demonstrates that the EbHM concept decisively better reveals the effective structural differences between donor and recipient HLA.

PDB codes of exemplary HLA: 1UVQ, 4Z7U, 5KSA

1.9 Immunogenicity of HLA epitopes

One step further than just measuring the epitope load will be to co-account the individual immunogenicity of each epitope [100, 101]. Indeed, several observations have indicated that divergent allo-epitopes can elicit immune responses of different strengths [89,

102], but comprehensive studies are lacking. Both transplant scientists and transplant physicians therefore agree that the characterization of the immunogenicity of each identified allo-epitope is the next step forward [95, 103]. Assessment of epitope load combined with epitope immunogenicity represents a potentially feasible and clinically justifiable strategy to be used for organ allocation schemes and/or for better guidance towards tailoring the immunosuppression.

1.10 Pre-sensitization to non-self HLA: another obstacle for SOT

Immune sensitization to HLA prior to transplantation mainly occurs after exposure to allogenic tissue, either through pregnancy, transfusion, or previous transplantation [4]. Roughly a quarter of healthy blood donors are pre-sensitized to foreign HLA structures, with a significantly higher sensitization rate in women [104-106]. For potential solid organ recipients, a similar prevalence is observed. If they receive an organ expressing HLA structures targeted by pre-formed HLA antibodies, they are at higher immunological risk. Unfortunately, allocation of a kidney free of DSA-antigen is not possible for all pre-sensitized allograft candidates. Consequently, a proportion of them must be transplanted across DSA. In Switzerland, the rate of such affected patients with regard to kidney recipients is currently nineteen per cent [107]. Alongside the standard immunosuppression, these recipients receive either plasmapheresis to decline present DSA and/or a special induction-therapy. The latter consists of T-cell depletion agents to stop T-cell help for DSA production and/or intravenous immunoglobulins to replace DSA with alternative antibodies [108, 109].

It is well established that a pre-transplant DSA status is associated with an increased risk for AMR [110, 111] and inferior graft survival [110-114]. In fact, up to half of DSA positive kidney recipients will experience subclinical or clinical AMR within the first year after transplantation [75, 115], but AMR can also occur at a later point in time. However, on the level of the individual patient, pre-existing DSA can show very different post-transplantation outcomes, from acute AMR to allograft loss or uneventful courses [75, 116-118]. With regard to chronic manifestations, pre-existing DSA and subsequent rejection episodes are strongly associated with a risk for chronic rejection and with late graft failure [52, 119-121]. Consequently, longitudinal monitoring of DSA after transplantation helps to identify patients at risk for chronic rejection [122] and to adjust immunosuppression accordingly.

1.11 Pre-sensitized patients and various post-transplant courses: Different pathogenicity of DSA present?

The fact that about half of patients transplanted across a DSA stay AMR negative post-transplantation[75, 115] raises important questions. Does the route and depth of pre-sensitization induce different stages of immunological memory? How could we better define the pre-established immunological memory and its pathological potential for the recall immune response? Why are not all DSA detrimental and which properties define their impact strength? Which additional factors not yet diagnostically detected are influencing the severity of the humoral recall immune response and the magnitude of the subsequent allograft rejection process in pre-sensitized patients? Do we need biomarkers beyond DSA or is the determination of additional DSA properties sufficient in distinguishing between a profound immunological memory and a weaker one?

One explanation for the incongruity between the DSA status (established using patient serum) of pre-sensitized patients and the clinical transplant outcome may be given by the fact that not all immunologically memorized antibody specificities may be represented by antibodies circulating in the periphery at the analysed time-point. Indeed, stimulation of circulating memory B-cells [123, 124] and subsequent analysis of the released antibodies potentially supplements the often incomplete HLA antibody profile of the serum, as has been recently shown [125-127]. An interesting approach to be applied in the near future for the identification of HLA-specific BCR and antibodies of pre-sensitized patients, is to combine the two techniques BCR-seq and Ig-seq [128].

The feasibility of sensitive HLA antibody detection by SAB has led to a continuous investigation of DSA properties, based on the intention to identify DSA features strongly linked with their pathogenicity. However, no such unique DSA property has yet been found. Currently intensively examined DSA properties potentially revealing new insights into the topic are DSA epitope-specificity [129], as well as DSA binding strength [130-133].

1.12 Definition of current knowledge gaps

In summary, we can state that the current model of pre-transplant risk stratification, which is mainly based on HLA mismatches and HLA pre-sensitization, represents a high

potential for improvement. The expanded concept, enabling personalized medicine in SOT, should be able to more deeply answer the following two immunological questions:

- **Patients without pre-sensitization**
    - What *de novo* DSA response is expected for a given recipient-donor HLA constellation?

- **Pre-sensitized patients**
    - Which recall immune response is expected for a given pre-sensitized patient?
    - Which pre-transplant DSA are relevant and why?

Once these knowledge gaps are closed, transplant recipients may be treated in accordance with the principle of precision medicine: the type and especially the dosage of immunosuppression could be adjusted on the basis of the individual risk score elicited. Experts agree the key to building this knowledge lies primarily in i) the immunogenic characteristics of divergent donor HLA structures which have to be evaluated at the tertiary protein structure level for each given donor/recipient HLA constellation [134, 135], and ii) the pathological potential of circulating DSA present [136] or of DSA obtained by stimulating circulating recipient memory B-cells [126, 137-140].

1.13 How the present thesis contributes to achieving parts of these goals

The fact that each HLA mismatch can promote a different immune response underlines the impact of specific structural differences between HLA molecules of the donor and recipient. My thesis is primarily dedicated to this topic. Based on specific *de novo* antibody responses, I was able to identify antigenic structures on expressed HLA molecules evoking different immune response reactivities, which allowed definition of their immunogenic potential (**Publication 1**). Thus, this work provides a valuable contribution towards the implementation of the EbHM concept [102] into clinical practice. As described in chapters 1.8 and 1.9, this new concept promises significant advantages over the current HLA-matching concept.

Immunological pre-sensitization presents a higher risk for transplantation. Unfortunately, the origin of sensitization (blood transfusions, pregnancies or previous transplantations [108]) is not helpful to better predict post-transplant outcomes of pre-sensitized patients. Furthermore, none of the so far available DSA test-derived main measures (DSA number, DSA HLA-class specificity, DSA HLA locus specificity and cumulative DSA test signal strength) allows discrimination between immunologically detrimental or presumably irrelevant DSA [108, 115]. The three most potent not yet routinely assessed features of DSA that may allow better definition of the hazardous potential of circulating DSA are their **target-specificity**, their **affinity/avidity** and their **effector-functionality**. With regard to the impact of the latter, **complement activation** appears to be of particular importance ([141, 142]. The ability to trigger the classical complement pathway is only given to certain antibody types [143, 144], but DSA isotype/subclass composition in patient sera is not yet routinely investigated. As part of my thesis, I therefore investigated the influence of different HLA-specific immunoglobulin G subclass mixtures on complement binding and the significance of the findings for a kidney transplant cohort **(Publication 2**).

In view of the importance to identify DSA in pre-sensitized patients [145] and the dominant role of DSA in allograft rejection [146], it is not surprising that HLA antibody assays are frequently used pre- and post-transplant. Due to the wide target specificities of emerged HLA antibodies, HLA antibody tests need to be performed in a multiplex format. Today, this is most elegantly realized by using HLA-coated Luminex Beads (SAB) [147]. Indeed, HLA-antibody assessments on the Luminex platform has become widely accepted in the transplant community, especially due to its high specificity, sensitivity and standardization [148]. Furthermore, SAB test results are increasingly used to define HLA epitopes [149-151] or to identify epitope-specificities of present DSA [152-156]. It is foreseeable that SAB assays will continue to play a decisive role in the future of transplant diagnostics and that supplementary DSA properties that may provide potentially revealing information about their pathological potential may also be discovered using SAB. Given these facts, it is highly crucial to have a comprehensive knowledge about the advantages, disadvantages and limitations of this central immunoassay. To complete my thesis, all important aspects of the SAB assay, the Luminex platform, the collection of SAB multiplex results and their technical as well as clinical interpretation were therefore summarized in a review (**Publication 3**).

Chapter 2 RESEARCH OBJECTIVES AND PUBLICATIONS OF THE THESIS

2.1 Research objectives of the doctoral thesis

A high degree of tissue-compatibility between recipient and donor is a leading goal for organ allocation. The extent of compatibility is traditionally calculated by summing up the number of HLA-matches. As HLA molecules differ greatly in terms of their epitope composition, moving from the HLA-matching approach to the new 'EbHM' concept, which accounts for the real structural differences between donor and recipient HLA, promises an improved solid organ allocation with subsequently better clinical outcome, and a patient-specific adjustment of immunosuppressive drugs. Nevertheless, EbHM has not been translated into clinical practice so far. One reason for the delay is that no specific immunogenicity has yet been assigned to the various epitopes, due to a lack of systematic studies. The core purpose of this doctoral thesis is therefore to contribute to the new concept by defining the immunogenicity of individual HLA epitopes based on pregnancy-induced allo-sensitization. **The hypothesis was that different HLA epitopes represent a different degree of immunogenicity.** This objective could only be achieved if HLA molecules were characterized at HR-2F. Therefore, an additional achievement of the presented work has been the establishment and validation of accurate HR-2F HLA typing by using an NGS approach.

Besides the assessment of the relative immunogenicity scores of the most prominent individual HLA epitopes, some of the specific objectives of the immunogenicity study were: i) to evaluate the degree of restriction with regard to the number of CSA targeted immunodominant epitopes ii) to define the range of epitope-reactivities, iii) to identify highly immunogenic (high risk) and lower immunogenic (low risk) HLA epitopes), iv) to study the location of different immunogenic epitopes on the tertiary structure of HLA class I molecules, and v) to examine topographic aspects of non-, low-, medium-, and high-immunogenic epitopes.

One limitation of the current DSA testing concept is the lack of IgG subclass discrimination. Another shortcoming is the relatively imprecise detection of the complement binding capabilities of DSA present by cellular assays. Another goal of my research was therefore to investigate different IgG subclass compositions of pre-transplant HLA antibodies

and their impact on the degree of complement activation by using a robust *in vitro* test system, free from non-specific reactions. **The hypothesis was that different ratios of IgG1/3 : IgG2/4 would exhibit different synergistic or inhibitory effects on complement-binding.** Some of the more specific questions that were addressed included: i) with regard to IgG subclasses targeting the same epitope, what proportion of non-complement binding IgG subclasses are necessary to suppress complement activation?, ii) what is the impact of HLA antibodies binding to additional epitopes in close proximity?, iii) to which extent do sensitized patients exhibit IgG subclass mixtures that critically diminish complement binding?, and iv) with respect to the identified IgG subclass mixtures of iii: what is the breadth of their HLA-specificity?

Accurate determination of DSA is an integral part of pre-transplant risk assessment and is mainly realized by using solid phase assays, especially the SAB test. This assay has revolutionized HLA diagnostics but it is not without imperfections. Moreover, the same test-type is now used for extended DSA characterization (e.g. determination of DSA epitope-specificity or DSA complement binding). We carried out a review of the utility, applications, practical aspects and clinical relevance of the currently used SAB test, which summarizes the benefits, and especially focuses on the shortcomings of this assay. This review also emphasizes critical points for accurate clinical test result interpretation. Some of the issues that we addressed in depth were: i) what is the clinical relevance of DSA tested by SAB?, ii) how can DSA testing by SAB enhance pre-transplant risk assessment and post-transplant monitoring?, iii) which technical aspects may impact the final test results?, iv) which are the existing test modifications and what is their purpose?, and v) what other SAB modifications could improve the characterization of pre-existing DSA or dnDSA?

2.2 List of publications within the doctoral thesis

[1] Hönger G, Niemann M, Schawalder L, Jones J, van Heck MR, van de Pasch LAL, Vendelbosch S, Rozemuller EH, Hösli I, Blümel S, Schaub S. Toward defining the immunogenicity of HLA epitopes: Impact of HLA class I eplets on antibody formation during pregnancy.

HLA. 2020 Nov;96(5):589-600.
doi: 10.1111/tan.14054. Epub 2020 Sep 10. PMID: 32829523.
Link: https://pubmed.ncbi.nlm.nih.gov/32829523/

[2] Hönger G, Amico P, Arnold ML, Spriewald BM, Schaub S. Effects of weak/non-complement-binding HLA antibodies on C1q-binding.

HLA. 2017 Aug;90(2):88-94.
doi: 10.1111/tan.13062. Epub 2017 Jun 5. PMID: 28585289.
Link: https://pubmed.ncbi.nlm.nih.gov/28585289/

[3] Wehmeier C, Hönger G, Schaub S. Caveats of HLA antibody detection by solid-phase assays.

Transpl Int. 2020 Jan;33(1):18-29.
doi: 10.1111/tri.13484. Epub 2019 Aug 16. PMID: 31359501.
Link: https://pubmed.ncbi.nlm.nih.gov/31359501/

My contribution to the work:

[1] Design of study and experiments; development of analytical method; performance of all experiments; data acquisition, evaluation and interpretation; statistical analysis; visualization of data; writing of manuscript; revision of manuscript

[2] Design of study and experiments; development of analytical method; performance of all experiments; analysis evaluation and interpretation of data; statistical analysis; visualization of data; writing of manuscript; revision of manuscript

[3] In-dept investigation and critical reviewing of a significant portion of the subjects; drafting a significant portion of the manuscript/figures/tables*

*45% of manuscript text (word count), 2/3 of figures, 1/2 of tables

Chapter 3 PUBLICATION 1

Towards defining the immunogenicity of HLA epitopes: Impact of HLA class I eplets on antibody formation during pregnancy

3.1 BACKGROUND

3.1.1 Immune recognition of non-self HLA structures at the molecular level

Due to their polymorphism, every individual's set of HLA molecules is almost unique. After the transplantation of foreign graft, the recognition of non-self HLA by the recipient's immune system is therefore almost inevitable. Thereby, the immune system applies very similar principles as it does when a foreign pathogen is detected. Of particular importance for the recognition of foreign HLA are recipient DCs, B-cells and T-cells. It is important to understand that the recognition mechanisms of the body's immune system are designed to interact with foreign molecular 3D structures, not with the entire foreign molecules. In the context of foreign tissue recognition, this means that surface exposed 3D structures of donor HLA molecules are recognized. Three pathways have been described for allo-recognition (see Chapter 1.3). They are termed 'direct', 'semi-direct' and 'indirect pathway of recognition' [18]. The latter is the process that predominates [6, 28, 32, 157]. Recipient B- and T-cells as well as recipient HLA class II molecules are the main actors of this pathway, of which the sequence of associated actions can be summarised as follows:

1. recognition and binding to surface exposed structural elements on allo-HLA by pre-formed BCRs of B-cells;
2. cellular uptake of the entire antigen by internalization of the BCR-antigen complex;
3. enzymatic digestion of the complex within lysosomes; subsequent transfer of resulting allo-HLA-derived peptides into endosomes; binding of these peptides

to HLA class II molecules; transfer of the formed HLA:peptide complexes via late endosomes to the cell membrane;

4. presentation of these allogenic peptides on the cellular surface of the B-cell;
5. recognition/binding of/to structural components of the allo-HLA-peptide by a specific TCR from a Tfh cell, followed by the upregulation of further ligands (e.g. CD40 ligand) on the corresponding T-cell which are recognized by the respective receptors on the B-cell and stabilizes B-cell : T-cell binding;
6. induction of cytokines within the involved Tfh cell;
7. secreted cytokines bind to the respective receptors on the B-cell, providing survival signals;
8. proliferation of activated B-cell and production of DSA.

The process is visualized in **Figure 4**.

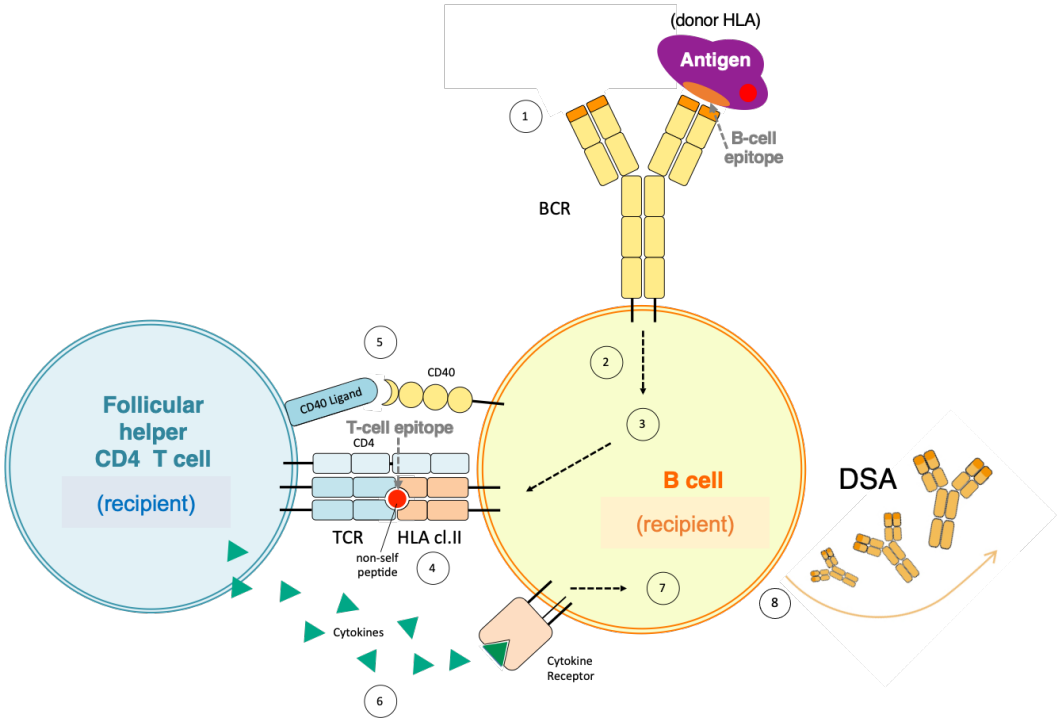

Figure 4. Individual elements and chronological sequence of the indirect allo-recognition pathway leading to the development of DSA. The process is explained above (3.1.1), using the same numbers for each intermediate step. Produced DSA consists of the exact same antigen-specific hypervariable region as the original BCR (orange). The presented peptide is not necessarily from the same region as the one detected by the BCR, which is the case in this example (therefore indicated in red).

TCR: T-cell receptor, BCR: B-cell receptor

The specificity of the emerged DSA is exactly identical to the specificity of the original BCR. Ultimately, such B-cells differentiate into both plasma cells and memory B-cells [158-163]. Plasma cells produce an even higher output of the specific antibody, and memory B-cells assure a rapid recall-response upon a future challenge with antigens consisting of the same allogenic structures [164].

Allorecognition is also subject to the variability of the recipient's pre-existing TCRs and BCRs. Indeed, the nature of random TCR and BCR gene rearrangement is responsible for an impressive diversity of TCRs and BCRs, and results in an individual- unique BCR- and TCR-repertoire [165-173]. In view of this fact, one may ask whether antigen recognition is guaranteed in each individual. The answer to this question is that the breadth of the individual BCR/TCR repertoire is indeed the assurance that virtually any antigen can be recognized [174]. We can therefore state that each individual TCR and BCR repertoire impacts a slightly different allorecognition, yet this diversity leads to a very similar result. Consequently, non-self HLA will in any case be specifically recognized and cellularly taken up by B-cells. The key question is rather which types of peptides are emerging after cleavage of the non-self HLA protein by enzymes like cathepsins [175] and if the recipient's individual HLA class II types are capable of properly binding these specific peptides so that they will be ultimately presented. If the affinity between a given peptide and the respective binding pocket of the recipient's specific HLA class II molecule is high enough, it ends up being presented [176] and will be recognized in any case by an individual's subpopulation of the Tfh cell pool. The outcome of the competition between several T-cells for access to the peptide presenting B-cell will ultimately decide about the immunodominance of the presented peptide [177, 178].

Due to the fact that BCR- and TCR-specificity for allo-HLA can be taken as given in each individual, the following remaining **2 variables** are particularly meaningful (and feasible) to determine, in order to estimate the risk of dnDSA production for a given donor-recipient pair:

- Variable 1 = **B-cell epitope**
  number and **kind** of **recognizable B-cell epitopes** on donor HLA
  (assumingly recognized by recipient BCR pool)

- Variable 2 = **T-cell epitope**
  number and **kind** of donor HLA derived **allo-peptides**
  properly binding/presentable in binding cleft of recipient HLA class II molecules
  (assumingly recognized by recipient TCR pool)

Separate bioinformatic algorithms for each variable are available. Importantly, the basis for accurate determination for these measures is HR-2F HLA typing of donor and recipient [179]. Publication 1 of this thesis deals with variable 1, the B-cell epitope, and the chapters following will consequently focus on this subject.

3.1.2 Antibody paratope and the targeted epitope on the antigen

Antibody-antigen interactions can only be truly studied in detail on the basis of 3D antibody-antigen complexes. Initial findings were gained with X-ray crystallographic assessments of antibodies interacting with egg white lysozyme [180-182], horse cytochrome c [183] or human interferon-gamma receptor [184]. These 3D images enabled study of the molecular size dimension of the antibody-antigen synapse, as well as the involved structural elements and their physicochemical binding properties. These pioneer establishments also helped to understand that antibody-binding is accomplished by the functionality of their variable regions, located on the Fab of the molecule [77]. The core structures of this region are termed **'complementarity determining regions' (CDRs)**. They consist of six hypervariable loops (three on the N-terminal of the heavy chain and three on the N-terminal of the light chain). The constitution of these CDRs is crucial to the diversity of antigen-specificities [77, 185]. Some of the involved AAs' atoms exert weak chemical forces of non-covalent nature, namely ionic interactions, hydrogen bonds, hydrophobic interactions or van der Waals forces [186]. The totality of these forces mediates the reversible binding to the antigen. Those CDRs (not necessarily all six) that participate in a specific antigen-binding represent the '*paratope*' of the antibody. The entire paratope represents a surface area of 500 to 900 square Å [77, 149, 187-192]. The corresponding structural complement on the targeted antigen, is termed as the antigenic determinant, or 'epitope' [186, 193-196]. **Figure 5** shows the 3D structure of an HLA-antibody bound to an HLA molecule and the spatial dimensions of the involved paratope and the epitope.

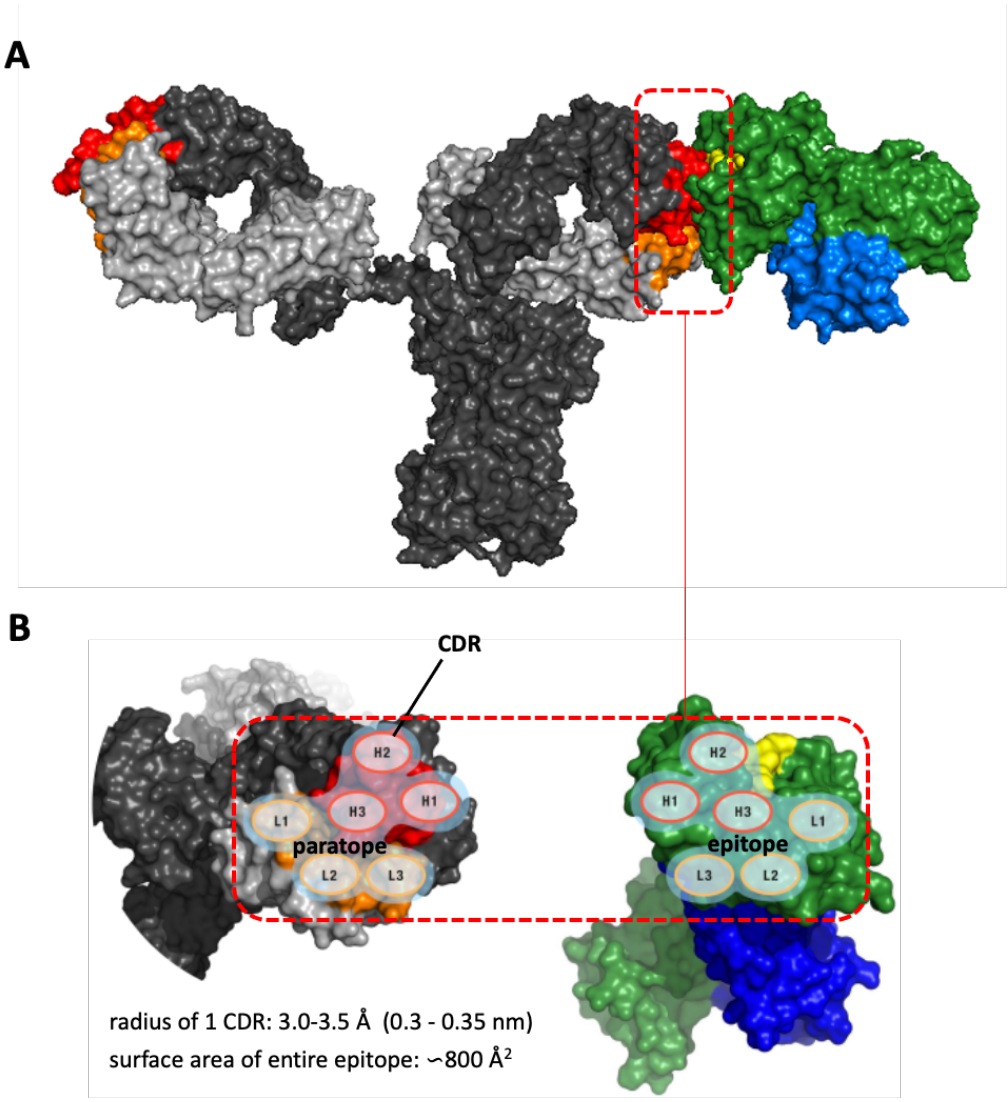

Figure 5. 3D structure of an HLA-antibody–HLA complex and the spatial dimensions of the involved paratope and epitope. A) The model shows an interaction between an IgG1 antibody molecule and an HLA-A*01:01 molecule and reveals their relative size relationship. The dashed red box indicates the region of the antibody-antigen synapse. Colour code for the HLA molecule: heavy chain in green, beta-2-microglobulin in blue, peptide in yellow. Colour code for the HLA antibody: heavy chains in black, light chains in grey, hyper-variable region on heavy chain in red, on light chain in orange. B) This image provides a view on the unravelled antibody-antigen synapse. Left: view on the area of the paratope located in the Fab, consisting of the hypervariable region formed by 6 CDRs [H1, H2, H3 at the N-terminus of the heavy-chain (red) and L1, L2, L3 at the N-terminus of the light chain (orange)]. The measured quantities indicated describe the binding span radius of 1 CDR and the approximate surface area of the targeted epitope. Right: view on the area of the epitope on the surface of the HLA molecule and the regions potentially interacting with corresponding CDRs.

Å: angstrom (1Å = 0.1nm), β2m: beta-2-microglobulin, CDR: complementarity determining region, Fab: fragment antigen-binding
PDB code of HLA molecule and Fab part of the antibody: 1W72

The structural conformations recognized by CDRs of BCRs or antibodies are either linear epitopes (residues from AAs on a linear AA strand) or discontinuous epitopes (residues from AAs that are disconnected from each other) at surface exposed sites on the antigen. Within the structural epitope, a small number of AA residues contribute to most of the free binding energy of the antibody-antigen interaction. These “hot-spots” or energetic residues predominantly determine the specificity and the affinity of the binding [197, 198] and interact almost always with the CDR3 loop of the BCR’s or the antibody’s heavy chain. This CDR represent the highest variability and is also termed ‘VH3’or ‘H3’, as it is the third CDR on the heavy chain, located in the centrum of the paratope [77, 84, 197] (**Figure 5**).

3.1.3 In silico definition of HLA epitopes

The first tertiary structure of an HLA class I molecule was published in 1987 [199]. It took eighteen more years, before a 3D model of a synapse between an HLA antibody and an HLA antigen became available [192]. This allowed study of the real binding interactions between HLA structures and CDR loops. The lack of more 3D data of HLA antibodies complexed with HLA molecules meant that the findings gained from alternative antibody-antigen complexes had to be transferred to the subject of HLA. Rene Duquesnoy, from the University of Pittsburgh (USA), was one of the pioneers in this regard. At the beginning of the new millennium, he defined surface positions on HLA class I molecules that are potentially antibody accessible. For this, he used a number of additional X-ray crystallized HLA molecules models that had become available in the interim. He then assigned these positions to all polymorphic AAs on the linear sequence of the most common (by this time only serologically defined) HLA class I proteins. He considered the resulting linear structural components as potential immunogenic functional parts of an HLA epitope and divided them into maximal 3 consecutive polymorphic AAs, termed ‘triplets’ [83, 200]. Intended to offer a tool to determine the degree of structural histocompatibility between recipient and donor and thereby to reach the higher aim of reducing allo-sensitization and enhancing transplant-outcome, Duquesnoy continued to develop the algorithm. In coordination with further published 3D HLA protein structures, he extended the approach to now include discontinuous epitopes [149]. The resulting final definition of the functional unit of a structural HLA epitope, capable of inducing a humoral immune response, was the following: a cluster of polymorphic

AAs, linear or discontinuous, lying within a 3-3.5 Å radius (the area of the binding span from one CDR) of an antibody-accessible position. Such AA patches were termed '**eplets**'. Duquesnoy also defined the antibody footprint consisting of the structural epitope involving 15-22 AAs, with 2-5 AA residues (where at least one must be non-self) located in the centre of the epitope and preferentially interacting with the VH3 of the antibody [84, 201, 202]. The international registry of HLA epitopes (www.epregistry.com.br) lists all currently defined eplets [203]. The structurally based histocompatibility algorithm, applicable by the 'HLAMatchmaker' software, identifies all mismatched eplets on donor HLA for a given recipient-donor constellation. To accurately define the mismatched eplets, HR-2F HLA typing data from both individuals are needed. The software is available at www.epitopes.net. Due to similarities with regard to the assembly of HLA class I molecules and partly HLA class II molecules, and the presence of polymorphic AAs at similar locations, the HLAMatchmaker software performs inter-locus comparisons between HLA-A, -B, and -C, as well as between HLA-DQ and HLA-DP heterodimers.

3.1.4 Experimental and bioinformatic confirmation of HLA epitopes

It is important to understand that eplets are computably determined functional elements of the structural HLA-epitope, not the entire epitope. Nevertheless, more than a quarter of all computable eplets have been experimentally confirmed by specific binding patterns of existing antibodies [203-205]. These eplets are termed 'confirmed' or 'antibody-verified' (AbVer). The method of choice to identify the eplet-specificity of an HLA antibody is to simultaneously incubate it with a set of HLA molecules and subsequently analyse and interpret the resulting binding pattern. Although originally developed to assign HLA-specificities of alloantibodies, the SAB assay, available since 2004 [147], is the best suited tool to perform such an epitope mapping. Consequently, the HLAMatchmaker software also conducts analysis and interpretation of HLA antibody binding patterns on SAB to identify epitope-specificity. Using this method, polymorphic AAs on or near the molecule's surface that are located within the radius of 15 Å (the average radius of the binding span of the entire paratope) from all positively reacting HLA types in the SAB assay, provide further information about crucial structural elements within the remaining area of the epitope that are stabilizing the binding and thereby enhancing the binding affinity [149, 200, 206]. Many of these findings

have been obtained from studying binding patterns of mAbs [149, 206-208] derived from human hybridoma cells (Epstein-Barr Virus transformed B-lymphocyte-clones from selected multiparous women) produced by the National reference centre (NRC) for histocompatibility research, Leiden, the Netherlands (https://nrc-hla.nl/).

An even more laboratory-based approach to define HLA epitopes was applied by team-members of the Terasaki Foundation Laboratory in Los Angeles, USA. They used cell lines expressing single recombinant HLA, to specifically absorb and elute alloantibodies, and subsequently analysed their binding patterns with first batches of SAB [147]. All AA sequences from SAB reactive HLA types were aligned to identify surface exposed polymorphic AA shared between the interacting HLA [150]. Consequently, these AA were considered as crucial elements of the epitope and termed 'Terasaki epitopes' (TerEp) [85, 150, 209-212]. A substantial part of TerEps were defined by additionally analysing SAB patterns of HLA-specific mAb generated by hybridoma cell lines [147, 156, 211]. Comparative studies revealed a huge overlap between TerEp and AbVer eplets [88, 213, 214].

The HLA Epitope Mismatch Algorithm (HLA-EMMA), avalable at www.HLA-EMMA.com, developed by Sebastiaan Heidt / Dave Roelen / Frans Claas from the Leiden University Medical Centre [215], is another algorithm in use to calculate a score for (B-cell) allo-HLA epitopes. The special feature of HLA-EMMA is the consideration of the relative solvent accessibility of polymorphic HLA protein structures, which implies the exclusion of inaccesible AA sequences.

As exemplified in 3.1.1, B-cells need T-cell help to start producing antibodies. In this context it is important to complete the list of HLA epitope prediction tools, by describing the existing prediction algorithm for T-cell epitopes (see 'variable 2' in Chapter 3.1.1), applicable for SOT. The algorithm is termed 'PIRCHE II' (Predicted Indirectly ReCognizable HLA class II Epitopes) and was developed by Eric Spierings of the University Medical Center Utrecht (The Netherlands). It allows counting relevant T-cell epitopes of peptides presented by donor HLA class II molecules [216]. This is realized by an *in silico* calculation of the binding-strength of each hypothetical mismatched donor HLA derived peptide to those HLA class II molecules expressed by the recipient. The algorithm uses donor and recipient HLA data and the peptide binding predictor netMHCIIpan [217]. It only considers allo-peptides revealing a certain level of binding-affinity. The sum of the applicable peptides reflects the PIRCHE II score, a proxy for

the T-cell epitope mismatch or the potential strength of the ‘indirect immune alloreactivity’ [218].

3.1.5 Antigenicity and immunogenicity of HLA epitopes

The quality of the HLA-epitope is decisive for the capability to interact with structural elements of the paratope (antigenicity) and for the inducement of an humoral immune response (immunogenicity) [202]. A solely antigenic epitope chemically interacts with the paratope but the total binding is insufficient to induce an immune reaction. In contrast, an immunogenic epitope will trigger an antibody response [202]. Investigating the feature ‘immunogenicity strength’ is therefore of paramount importance, but comprehensive studies on the specific immunogenicity risk potential of individual HLA epitopes are still lacking. Experts in the transplant community agree that only if such information is available, the previously described new concept of EbHM could be implemented into the clinical pre-transplant risk stratification progress [135].

Vasilis Kosmoliaptsis and colleagues from the University of Cambridge and NIHR Cambridge Biomedical Research Centre (United Kingdom) have developed a method to compare HLA structures between donor and recipient that partially reflects the antigenicity and immunogenicity of mismatched donor HLA structures [219]. The rationale behind the approach is that the quality of each HLA epitope is mainly impacted by physicochemical properties of the actual type of critical AA, more precisely their side chains that represent an individual hydrophobicity and an unique electrostatic charge. These characteristics can be measured and compared between donor and recipient HLA structures, and the calculated difference appeared to predict dnDSA development after renal transplantation [86, 220]. The two measures are termed ‘HMS’ (hydrophobicity mismatch score) and ‘EMS-3D’ (3D electrostatic mismatch score). In healthy female individuals receiving transfusion of lymphocytes from their male partners, HLA-DQ specific dnDSA was strongly associated with EMS-3D [221]. As the approach needs, however, X-ray crystallized HLA proteins for stereochemical modelling, it is not yet adapted to establish a wide data-set.

Another influence on the quality of an individual mismatched HLA epitope that cannot be neglected is the recipient's own HLA phenotype, as the total make up of recipient HLA proteins defines the degree of fine-structural difference on donor HLA [71, 72, 222]. This

makes consideration of the recipient's own HLA types – for any of the B-cell epitope prediction tools – all the more important.

3.1.6 The quantity of mismatched HLA epitopes correlates with dnDSA formation

In contrast to the few data available on the qualitative effects of HLA epitopes with regard to their impact on induction of a humoral immune response, many studies have been published that investigated the quantitative effect of mismatched epitopes on DSA induction. The common approach thereby is to simply sum up the number of mismatched HLA epitopes (calculated by means of the prediction models described above) for a given recipient-donor-pair and to compare this measure with dnDSA formation. Generally, it can be shown that a minimum epitope mismatch load reduces the risk for dnDSA formation and for antibody-mediated rejection. A selection of corresponding results for kidney transplantation or pregnancy, summarised according to the prediction algorithms, is given below.

Eplets:

Counting up all mismatched donor HLA derived eplets reveals the 'eplet-load' of a specific donor-recipient pair. Several studies confirmed a significant correlation between the eplet-load and the magnitude of dnDSA or dnCSA formation [81, 87, 98, 223-225]. For kidney-transplanted patients, it was shown that HLA class II eplet mismatch independently predicts development of dnDSA [79] and improves the prediction of AMR [97]. Moreover, for HLA-DR/DQ it has been demonstrated that the single molecule eplet mismatch score represents a precise and reproductible prognostic biomarker, potentially applicable for the tailoring of immunosuppression [98, 226].

HLA-EMMA:

As this prediction tool was developed only recently, there are as yet few studies on it. In a recently conducted investigation focusing on HLA-DQ mismatches in pregnancy and the presence of CSA at the timepoint of delivery, the mismatched epitope load on HLA-DQ heterodimers, as determined by HLA-EMMA, was significantly higher in CSA positive mothers [227]. In a retrospective study of a cohort representing non-sensitized simultaneous

pancreas-kidney recipients, no significant association between the degree of HLA-EMMA B-cell epitope load and dnDSA development was found [228].

PIRCHE II:

Several studies showed that the number of donor-derived HLA peptides presentable within the binding pockets of the recipient's HLA class II molecule repertoire, as predicted by the PIRCHE II algorithm, significantly predicts dnDSA development [87, 216, 224, 228-232].

3.1.7 Properties of *immunogenic* HLA epitopes

The results of the population studies described in the previous section demonstrate that over all, the higher the number of HLA class I and II epitope mismatches, the higher the risk of dnDSA development. However, these studies all have in common that individual epitope immunogenicity was not considered. Indeed, for a distinct patient, the quality of the specific epitope mismatch determines whether an antibody response will be truly induced or not.

Researchers from Manitoba, Canada, have plausibly demonstrated that at the patient level, the same number of HLA mismatches can cause a different magnitude of immune response due to the underlying eplet mismatch load [79, 81, 90, 98]. It was also shown that in some cases, one or few epitope mismatches can be sufficient to trigger antibody production [81, 233, 234]. In a few cases even a single AA mismatch difference could do so [85, 204, 205]. This is in agreement with the fact that some of the defined eplets, as well as TerEps, consist of a single non-self AA.

Moreover, by analysing mAb reactivity patterns established with solid-phase multiplex assays, Duquesnoy and colleagues noticed that – beside the principal binding component between the central eplet and presumably the VH3 site of the antibody's paratope – often an additional AA configuration, located within the antibody footprint, is necessary to stabilize the binding (eplet pairs) [149, 207, 235]. Other reactivity profiles revealed that certain polymorphic AA configurations on structural epitopes seem to impact the release of free energy, because the involved antibodies were capable of binding C1q and inducing complement dependent cytotoxicity (CDC) [206]. Another interesting finding was that in cases, where the structural difference between self- and non-self HLA was as little as one

single eplet, one or multiple self-eplets were contributing to the final binding [207]. This implies the existence of B-cell clones with weak binding to self-HLA structures, which only expand and maturate their BCR upon triggering by one or multiple self-AA configuration present on the mismatched HLA protein. Duquesnoy termed this principle the 'Non-self-self paradigm' [235, 236].

3.1.8 So far established models to determine HLA epitope immunogenicity

The degree of immunogenicity of an HLA epitope or its functional subunit is necessarily related to the frequency of the immune response against it. The most obvious approach to determine the degree of immunogenicity, namely to assess the immunogenicity of HLA epitopes within transplant cohorts, represents a complex challenge. First, each transplant pair reveals another immunological risk situation due to variable HLA mismatch constellations and possible pre-immunizing events of the recipient. Second, the fact that the immune system is suppressed after transplantation is not conducive to such a study. Furthermore, a slightly different induction therapy or post-transplant immunosuppression regimen would additionally confound findings. The latter would apply if a multi-centre study were conducted, which would be almost obligatory given the necessary number of cases.

A cleaner approach was applied by researchers of the Tambur group (Chicago, USA), who focused on transplants with exactly two HLA mismatches within the same HLA locus [237]. If dnDSA was detected only against one mismatch, structures on such donor HLA were regarded as more immunogenic. This is an elegant way to overcome the confounders described above, but the required number of similar cases to establish statistically stable declarations remains challenging.

3.1.9 Pregnancy as a model to study the immunogenicity of individual eplets

An alternative approach to study the impact of mismatched HLA-structures on the formation of specific antibodies is given by the pregnancy model, where mismatched child-HLA, inherited through paternal HLA alleles, potentially promotes the production of CSA [72, 223]. This model displays several advantages over the transplant model, of which four of the most important are i) the fact that only one HLA is mismatched per locus which facilitates the assignment of epitopes, ii) the development of CSA is not modulated by (exogenous)

immunosuppression, iii) full-term pregnancies have similar durations, and iv) a large cohort can be collected within a reasonable time frame.

Regarding the material to be studied in the pregnancy model, a single maternal blood-sample (whole blood for the isolation of peripheral blood mononuclear cells [PBMCs] to perform HLA typing of the mother with genomic DNA; serum or plasma to assess HLA-antibody analysis) and a small quantity of cord-blood to isolate child PBMC for genomic DNA isolation in order to perform HLA typing of the child) is required at the time-point of delivery.

3.1.10 Requirement of 2-field high-resolution HLA typing

A striking limitation in studies that investigated the impact of structural mismatches on the formation of dnDSA from pre-transplant non-sensitized patients, is the lacking of HR-2F HLA typing of donor and recipient [81, 87, 95, 98]. Indeed, a molecular HLA typing method that sequences all exons coding for extracellular HLA domains enhances the pre-transplant risk assessment [238, 239] and enables a more accurate eplet determination [240-243]. Moreover, antibody eplet-specificity results, determined by using SAB tests, are given at the allele level, thus HLA typing data should be made available at high resolution, too [244]. Therefore, for this work, the introduction, validation and utilization of a HR-2F HLA typing method was of high importance.

3.1.11 Establishment and validation of a 2-field high-resolution HLA typing method

An important difference between a low- and high-resolution HLA typing method is the resulting length of the amplified HLA gene [245]. Low-resolution molecular HLA typing methods commonly solely determines the core exons of HLA genes (exon 2 and 3 for HLA class I genes, and exon 2 for HLA class II alpha- and beta-chain coding genes), which often generates ambiguous results [244]. HR-2F HLA typing, appropriate for epitope analysis, resolves all exons that codes for extracellular domains of the expressed HLA protein (exons 2 to 4 for HLA class I genes and exons 2 and 3 for respective alpha- and beta-chain coding HLA class II genes). Translating of HR-2F HLA typing results firstly into the AA sequence of the respective HLA proteins and secondly into the tertiary protein structure on the basis of available 3D HLA models is the basis of HLA epitope assignment.

As such high-resolution HLA typing approach was not available locally at the timepoint of my doctoral thesis proposal, I planned to establish a sophisticated HLA typing method that pursued two goals: first, for it to be used for the described eplet immunogenicity project; second, for it to be applied as a new high-throughput allele-level HLA-genotyping method in the Laboratory for HLA Diagnostics and Immunogenetics in the Department of Laboratory Medicine at the University Hospital Basel.

Working with Regina Spinnler of the Laboratory for HLA Diagnostics and Immunogenetics, we decided to apply an NGS method. The workflow prior to the sequencing principally consisted of a long-range polymerase chain reaction (PCR), an amplicon pooling step, the enzymatic fragmentation of the amplicon, a ligation step, the indexing of the fragments, and a further pooling step. Prior to the sequencing, a clonal amplification was performed using either the Ion GeneStudio S5™ or the Illumina-MiSeq®. The sequencing itself was realized on the ION TORRENT™ or on the Illumina-MiSeq®. A total of 49 patient samples and 4 controls (2 internal controls from healthy donors and 2 external standards from the Fred Hutch Specimen Processing/Research Cell Bank) were selected to validate the method. The evaluated results were compared with Sanger-Sequencing results (performed at the Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Germany) and with results generated by two alternative NGS methods (both performed at Baylor University Medical Center, Dallas, USA [246]).

Since 2018, the established HR-2F HLA typing method has been routinely and successfully used in the Laboratory for HLA Diagnostics and Immunogenetics of the Department of Laboratory Medicine in the University Hospital Basel, and at this point an average of 16 high-resolution HLA typings are performed each week.

3.1.12 Aims of this study (Publication 1)

The aim of the study was to determine immunogenicity scores of all currently defined AbVer HLA class I eplets [203], by ranking the eplets according to their frequency of measured eplet-specific antibody-responses. For this we used a coherent cohort of 159 first pregnancy-mothers with no history of pre-sensitizing events [223]. Eplet-directed CSA responses were assessed at the time of delivery.

The data produced are intended to provide an initial basis for risk assessment of individual eplets. The risk scores will need validation in the clinical transplant setting. If further studies confirm the same eplets to trigger an enhanced DSA response in the post-transplant setting, they should be differently scored for pre-transplant risk assessment.

Another goal of the study was to visualize the eplets and their degree of immunogenicity on the tertiary protein structure of HLA class I molecules in order to facilitate the understanding of the results from a structural and topographical perspective.

This is the first study that systematically determines HLA class I eplet immunogenicity on the basis of HR-2F HLA typing and a coherent cohort of non-sensitized pregnant women. The main overall objective of this study is to strengthen the EbHM concept by providing immunogenicity scores of individual eplets, in order to enable an improved immunological pre-transplant risk assessment, which in turn will enhance kidney allocation and patient-specific tailoring of immunosuppression.

3.2 PUBLICATION 1

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ORIGINAL ARTICLE

HLA Immune Response Genetics WILEY

Toward defining the immunogenicity of HLA epitopes: Impact of HLA class I eplets on antibody formation during pregnancy

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Eplets are functional units of structural epitopes on donor HLA, potentially recognized by complementarity-determining regions of the paratope of the recipients' B-cell receptors or antibodies (Ab). Their individual immunogenicity is poorly described, yet this feature would be of clinical importance for pretransplant risk assessment. The aim of this study was to determine the relative immunogenicity of HLA class I eplets in the pregnancy setting, where mismatched eplets are present on paternal HLA antigens of the unborn child. One hundred fifty-nine predominantly Caucasian mothers giving birth at the University Hospital Basel and their first newborns were HLA-typed at high-resolution by next-generation sequencing (NGS) (NGSgo Workflow and NGSengine from GenDx; sequencing with a Miseq from Illumina) and eplets were determined using HLAMatchmaker. HLA class I specific IgG Ab was assessed in maternal sera drawn immediately after full-term delivery, by OneLambda LABScreen single antigen ibeads. The Ab profile was subsequently evaluated for eplet-associated patterns. All 72 currently Ab-verified HLA class I eplets were examined for their immunogenicity according to the frequency of child-specific HLA Ab (CSA) directed against their structures. Four hundred twelve of 477 (86.4%) paternal HLA-A, -B or -C alleles were mismatched. CSA were present in 46 mothers (28.9%), directed against 80 (19.4%) of these mismatches. The 10 most immunogenic eplets were 62GK, 145KHA, 144TKH, 62GE, 107W, 80I, 82LR, 41T, 127K, 45KE with immunogenicity rates between 45.8% and 27.3%. This pregnancy study also identified five non-reactive eplets: 62RR,

76ESN, 80TLR, 156DA, 163RW. Based on our results, immunogenic hot and cold spots on the surface of HLA class I molecules were localized and visualized on 3D models. This study strengthens the presumption that different eplets represent different immunogenic potentials. Validation of these results in the clinical transplant setting is an essential next step in identifying those eplets representing a particularly high-risk potential.

KEYWORDS

antibody-verified eplets, HLA class I, immunogenicity of eplets, pregnancy

1 | INTRODUCTION

Epitope-based HLA-matching has emerged as a promising strategy to improve solid organ transplant (SOT) outcome.[1,2] The goal of this concept is to allocate donor organs with minimal crucial structural differences to the recipient's HLA. Comparison of donor-HLA with the recipient's self-HLA repertoire is the basis of this approach. To accomplish this, two-field HLA-typing results must first be translated into the amino acid (AA) strings of the expressed HLA proteins. Next, an alignment of donor- and recipient-HLA allows to identify mismatched AAs. It was shown that the total AA-mismatch load is predicting the risk for the development of de novo donor-specific antibodies (DSA) more precisely than conventional HLA matching.[3] Another approach computable on the basis of AA sequences, useful for pre-SOT risk assessment, the Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE-II) algorithm, considers whether mismatched HLA is "presentable" as peptides within the binding cleft of the recipient's HLA class II of antigen-presenting cells (APCs) or B-cells in order to provide T-cell help for proper B-cell activation.[4]

A different level of analysis is reached by comparing tertiary protein structures of donor- and recipient-HLA because antigen allogenicity and immunogenicity are largely impacted by the three-dimensional (3D) conformation of critical AA patches. Resulting nonself 3D-structures on donor-HLA can be used to determine functional binding elements potentially targeted by structural B-cell receptors (BCR) and antibodies, termed eplets,[5] or to assess the degree of different electrostatic potentials (EMS-3D) between mismatched AA and AA-patches.[6]

Eplets are functional units of structural epitopes on donor HLA, potentially recognized by complementarity-determining regions (CDR) of the paratope of the recipients' BCR or antibodies (Ab). Many of these computable CDR-targeted eplets have meanwhile been experimentally confirmed by specific binding patterns of existing Ab from (a) allo-sera or their eluates, (b) rodent anti-HLA monoclonal Ab or (c) Ab derived from Epstein-Barr virus transformed B-cell lines. These eplets are termed "Ab-verified" and their investigative background is represented on www.epregistry.com.br. Recently, a study investigated the effect of Ab-verified and non-Ab-verified eplets on graft survival of a considerable cohort of >100 000 unsensitized first renal transplant recipients. For HLA class I, the results clearly showed that Ab-verified eplet mismatches predicted transplant glomerulopathy and graft failure.[7]

On a population level, the eplet load (ie, sum of mismatched eplets in donor-recipient constellations) is associated with the degree of DSA formation.[8,9] However, the clinical utility of immunological risk assessment based on eplet load for an individual patient is restricted, since not all eplets are generating a similar immune response.[10-12] At present, data about eplet-immunogenicity are limited. Studies that investigated the impact of structural mismatches on the formation of posttransplant de novo DSA from pretransplant nonsensitized patients can indicate risk epitope mismatches,[13] but are lacking of high-resolution HLA typing of donor and recipient. An inherent disadvantage, however, has to be considered for this kind of examination on transplant-cohorts: presence of exogenous immunosuppressive drugs within allograft recipients.

Pregnancy represents an attractive alternative model to study the impact of mismatched eplets on Ab formation. First, only one HLA haplotype—namely the paternal alleles of the child—represents mismatched HLA, which simplifies the analysis. Second, full-term pregnancies have similar durations. Third, Ab responses to HLA expressed by paternal alleles occur in the absence of exogenous immunosuppressive drugs. Fourth, serum specimens of the mother can be used to determine child-specific HLA antibodies (CSA).[14]

The aim of this study was therefore to assign the relative immunogenicity of individual Ab-verified HLA class I eplets in the pregnancy setting, by determining the frequency of eplet-specific Ab responses. On the basis of

these results, we further aimed to characterize and topographically visualize immunogenic and nonimmunogenic regions (hot and cold spots) on HLA class I molecules.

2 | MATERIALS AND METHODS

2.1 | Population and sample collection

This study was approved by the local ethics committee and performed with specimens from 159 women giving first full-term live birth at the University Hospital Basel between September 2009 and April 2011. All participants were healthy women without prior blood-transfusions, transplantations or miscarriages. The examined pregnancy was therefore assumed to be the only sensitization event. Their ethnicities (according to their country of origin) were as follows: 148 Caucasians (93.1%); 6 Hispanics (3.8%); 5 Asians/Pacific Islanders (3.1%).

Written informed consent was obtained from women enrolled in the study and written informed consent was obtained from women enrolled in the study. A blood sample was drawn from the mother between days 1 and 4 after delivery for high-resolution HLA-A/B/C typing and HLA antibody testing. Cord blood of the child was obtained immediately after delivery for high-resolution HLA-A/B/C typing.

2.2 | High-resolution HLA typing

High-resolution HLA typing of mothers and children was performed with white blood cell-derived genomic DNA by NGS using NGSgo Workflow from GenDx (www.gendx.com) for HLA amplification and library preparation, with subsequent sequencing on a MiSeq instrument from Illumina (www.illumina.com) using MiSeq V2 reagents with application of paired-end sequencing (2x151bp). The final HLA allele calling was done by NGSengine software from GenDx (www.gendx.com) on the basis of the database from IMGT/HLA database version 3.33.0.

2.3 | Determination of eplets

All 72 Ab-verified HLA-A, -B or -C (HLA-ABC) eplets of the HLA Epitope Registry version 3.0 (www.epregistry.com.br) have been considered for this study. Each mother-child combination was examined for the presence of relevant Ab-verified eplets. The analysis was performed across HLA-ABC (interlocus comparison): an eplet was called "present" if expressed on the paternally inherited mismatched HLA-ABC of the child and if the same eplet was not expressed by the mother's self HLA-ABC. No distinction was made whether an eplet was expressed by one or several mismatched child HLA loci.

2.4 | Identification of child-specific HLA antibodies

The postpregnancy sera were analyzed for child-specific HLA IgG antibodies (CSA) with LabScreen Single HLA Antigen Beads (SAB) for HLA class I (iBeads, lot 1; OneLambda ThermoFisher, Canoga Park, California) according to the manufacturer's instructions. CSA was assigned if the specific SAB representing the HLA protein of the mismatched paternal HLA allele was positive. Positivity was assigned if the mean fluorescence intensity (MFI) value of the respective HLA-bead was above the sample-specific biological cutoff (mean of all $SAB_{\text{self-HLA mother}}$ + 3 SD and >100 MFI). In cases where the two-field HLA typing result showed an HLA not represented within the iBead panel, the SAB with HLA of the highest AA compatibility was taken for CSA analysis.

2.5 | Eplet-specificity of CSA

To further assess eplet specificities of identified CSA we analyzed the multiplex (all SAB) reactivity pattern of each sample by using HLAMatchmaker HLA class I antibody analysis program (version 3.0, downloadable from www.epitopes.net). This approach first separates HLA-beads into two groups: negatively reacting beads (MFIs below mentioned cutoff) and positively reacting beads (MFIs abovementioned cutoff). All eplets expressed on beads of the negative group are then eliminated from the eplet analysis pool. Remaining eplets are subsequently analyzed for their Ab reactivity. This analysis implied that an eplet-specificity of a CSA was only assigned if all SABs representing this certain eplet were positive.

2.6 | Calculation of eplet immunogenicity scores

To assign the relative immunogenicity of identified mismatched eplets in an isolated way, they were ranked according to their frequency of measured eplet-specific Ab-response. For example, eplet 41T was mismatched in 22 pregnancies and 41T-specific CSA were assessed in six of these mothers, showing an immunogenicity of 27.3%

(immunogenicity score = 0.273). From a statistical point of view, scores derived from eplets that were mismatched less than 15 times (the mean number of cases per eplet) are less conclusive.

2.7 | Visualization of eplets and immunogenic hot spots on HLA class I molecules

Identified immunogenic single eplets were envisioned on appropriate 3D HLA molecules representing the relevant AAs at the respective positions from crystallized HLA data available on the protein data bank (www.rcsb.org[15]). If multiple eplets were visualized on the same molecule, surrogate *HLA-A*02:01* (protein data bank file 5f7d) was used or a crystallized HLA class I molecule with in silico modifications at relevant positions. The visualizations were realized using the cartoon representation view (for secondary protein structures) or the surface representation view (for tertiary protein structures) of the PyMOL software version 2.2 (https://pymol.org/2/).

To discriminate between study related hot and cold immunogenic spots on the HLA class I molecule, we ranked each AA position belonging to an Ab-verified eplet according to their direct (AA-type dependent for positions defined by only a single eplet) or cumulative (for positions being part of various eplets) study reactivity. The position with the highest cumulative immunogenicity was set to 1.00 and the cumulative reactions of all other positions were normalized to this reference, and termed "cumulative relative immunogenicity." This analysis allowed to draw a topographic map of the relative immunogenicity of different sites on the HLA class I molecule. Hot spots were defined as eplet-derived AA positions with a relative immunogenicity of >0.66.

2.8 | Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.3.1 (GraphPad Software, Inc., San Diego, California) and R software (R 3.6.1, R Foundation for Statistical Computing, Vienna, Austria). Interlocus eplet loads on CSA targeted and non-CSA targeted HLAs were compared using unpaired two-sided Student *t* tests. The relationship between the number of mismatched eplet cases and the eplet frequency in the maternal self-HLA compartment was investigated by means of Spearman's rank-correlation coefficient (rho). *P* values of less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Mismatched HLA class I alleles

Comparison of high-resolution HLA class I typing results for all 159 mothers and their children showed that 412 of the 477 paternal HLA-A/B/C-alleles (86.4%) were HLA-mismatched (132 HLA-A, 144 HLA-B and 136 HLA-C mismatches) and their expressed HLA proteins thus potentially capable to induce an immune response. The top five mismatched HLA alleles of each locus were: *HLA-A: A*02:01* (n = 19), *A*24:02* (n = 15), *A*01:01* (n = 13), *A*11:01* (n = 13), *A*03:01* (n = 11); *HLA-B: B*07:02* (n = 12), *B*35:01* (n = 12), *B*08:01* (n = 10), *B*51:01* (n = 10), *B*18:01* (n = 9); *HLA-C: C*04:01* (n = 20), *C*07:02* (n = 16), *C*06:02* (n = 14), *C*07:01* (n = 11), *C*12:03* (n = 11), reflecting a representative predominantly Caucasian population. HLA-A and -C mismatches (MM) were slightly but not significantly increased in CSA positive mothers as compared to CSA negative mothers (average of 0.89 vs 0.81 and 0.89 vs 0.84 MM, respectively), while a not significant decrease for HLA-B mismatches was apparent (0.87 vs 0.92 MM).

3.2 | Frequency and signal strengths of CSA

CSA was detected by Luminex technology in the postpartum sera of 46/159 (28.9%) mothers which were formed against 80/412 (19.4%) mismatched paternal HLA proteins. Stratification of HLA mismatches by locus showed CSA against 30/132 HLA-A (22.7), 32/144 HLA-B (22.2%) and just 18/136 (13.2%) HLA-C mismatches. Within CSA positive mothers, HLA-B mismatches indicated the most

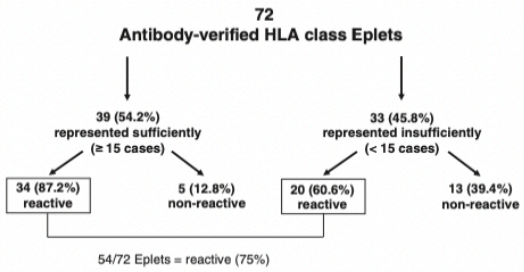

FIGURE 1 Reactivity of examined Ab-verified HLA class I eplets. The outline discriminates the assessed eplets into those represented at high numbers (left) and those underrepresented (right) and further differs between those targeted by CSA (reactive) and those not targeted by CSA (non-reactive). CSA, child specific HLA-antibodies

TABLE 1 Immunogenic eplets (eplets represented at case numbers ≥15)

Eplets	Polymorphic residues	CSA+	Total	I-Sc	Alpha domain	Eplet frequency
62GK	62G66K (74H77D)	11	24	0.458	1	0.472
145KHA	144K145H149A	7	18	0.389	2	0.572
144TKH	142T144K145H	7	20	0.350	2	0.572
62GE	62G63E	8	23	0.348	1	0.509
107W	107W	8	23	0.348	2	0.484
80I	80I	8	24	0.333	1	0.604
82LR	82L83R	6	20	0.300	1	0.736
41T	41T	6	22	0.273	1	0.484
127K	127K	6	22	0.273	2	0.742
45KE	45K46E	6	22	0.273	1	0.453
193PL	193P194L (273S)	5	27	0.185	3	0.465
65GK	65G66K	3	17	0.176	1	0.252
76ANT	76A77N80T	3	17	0.176	1	0.377
267QE	267Q268E	4	28	0.143	3	0.484
166DG	166D167G	3	22	0.136	2	0.472
90D	90D	2	16	0.125	1	0.483
163LW	163L167W	2	16	0.125	2	0.755
173K	173K	2	16	0.125	2	0.201
62EE	62E63E	2	17	0.118	1	0.252
71TTS	71T73T77S	2	18	0.111	1	0.698
80K	80K	3	27	0.111	1	0.660
76VRN	76V79R80N	2	21	0.095	1	0.767
163R	163R	2	22	0.091	2	0.503
73AN	73A77N	2	26	0.077	1	0.409
44RT	44R45T	2	30	0.067	2	0.503
163EW	163E167W	1	15	0.067	1	0.509
21H	21H	1	18	0.056	1	0.440
65QIA	65Q66I69A	1	18	0.056	1	0.258
180E	180E	1	18	0.056	2	0.428
62QE	62Q63E	1	19	0.053	1	0.723
144KR	144K145R	1	20	0.050	2	0.698
219W	219W	1	21	0.048	3	0.509
73TVS	73T76V77S	1	22	0.045	1	0.403
69AA	69A71A	1	23	0.043	1	0.333

Note: The eplets are listed according to their immunogenicity in a descending order. CSA+ indicates the number of CSA+ mothers with eplet specific antibodies. Total stands for the total of cases with the eplet present on child HLA. I-Sc represents the immunogenicity score. Furthermore, the location of the eplet on the HLA class I molecule is indicated and the frequency of the eplet in the self HLA repertoire (HLA-ABC) of the mothers.

Abbreviations: CSA, child-specific HLA antibodies; I-Sc, immunogenic score.

pronounced Ab-response (80%), followed by HLA-A (73.2%) and HLA-C (43.9%). The median of the MFI values of all CSA was 5318 (range 103-11 998) and highest for HLA-B (7229), followed by HLA-A (3334) and HLA-C (3214). Overall, rather low noise-signals on self-HLA-beads (mean = 64 MFI; median = 10 MFI) were observed. For 29 of the 46 CSA-positive postdelivery sera (63.0%) the cut-off for positive signals was therefore 100 MFI.

3.3 | Mismatched eplets represented on expressed child HLA proteins from paternally inherited HLA alleles

Our eplet analysis consisted of all currently described 72 Ab-verified HLA class I eplets. Eplets expressed on HLA of the unborn child but not expressed on any maternal HLA-ABC were called "mismatched eplets" and considered as potentially immunogenic. Indeed, all 72 Ab-verified eplets were mismatched between 4 and 30 times on mentioned 412 expressed child HLA proteins of paternally inherited HLA-alleles. The average HLA-ABC eplet load was higher on HLAs targeted by CSAs as compared to HLAs not targeted by CSAs (6.26 vs 5.51, $P = 0.25$).

3.4 | Immunogenic and potential immunogenic HLA class I eplets

Thirty-nine of the Ab-verified HLA-ABC eplets (54.2%) were represented between 15 to 30 times (Figure 1), allowing to allocate a reliable immunogenicity-score (Figure 1). Analysis of the relevant maternal Ab-patterns from the time of delivery indicated that 34 of these eplets (87.2%) were targeted by CSA, showing immunogenicity-scores between 0.043 and 0.458 (Table 1).

Twenty of these 34 eplets were located on the α1- (58.8%), 11 on the α2- (32.4%) and 3 on the α3-domain (8.8%). The following 10 eplets showed an immunogenicity above 25%: 62GK (45.8%), 145KHA (38.9%), 144TKH (35.0%), 62GE (34.8%), 107W (34.8%), 80I (33.3%), 82LR (30.0%), 41T (27.3%), 127K (27.3%), 45KE (27.3%). Six of these eplets (62GK, 145KHA, 144TKH, 62GE, 80I and 82LR) are located on the α1- or α2-domain forming the edges of the peptide-binding groove, but four of them are situated at accessible locations outside the binding groove: 107W and 41T are both on loops (between the first and second β-strand on α2 and between the third and fourth β-strand on α1, respectively), whereas 127K and 45KE are parts of β-strands (end of third β-strand on α2 and beginning of fourth β-strand on α1, respectively). Locations and configurations of a selection of immunogenic eplets based on availability of crystallized HLA data, are shown in Figure 2. On the relatively conserved α-3 domain, the segment which represents significant AA sequence homology to immunoglobulin constant domains, only six Ab-verified eplets are defined. Five of them showed positivity: 193PL (5 positive cases/27 total cases), 219W (1/21), 248M (1/4), 253Q (1/9) and 267QE (4/28). With the exception of positions 267/268 located in the loop between β-strand 6 and 7, these eplets are situated at relatively close proximity to the transmembrane-domain.

Twenty mismatched HLA class I Ab-verified eplets (27.8%) were also associated with Ab reactivity, however, the total of the cases was below 15 (Table 2). Despite their low frequency and statistical uncertainty, such eplets (mismatched at fewer numbers, but Ab reactive) must also be regarded as potentially considerable immunogenic, because they are typically less represented in the self-HLA repertoire of the recipient population. Indeed, in our study 15/20 (75%) of mentioned underrepresented but reactive mismatched eplets were present at relatively low frequencies (<0.30) on maternal HLA as visualized in the dotplot of Supporting information, Figure S1, which visualizes the relationship between case numbers, frequencies and immunogenicity scores of each investigated mismatched Ab-verified eplet.

Together, in the cohort examined, 54/72 (75%) Ab-verified HLA class I eplets appeared to be reactive (Figure 1).

Of note, striking differences were found regarding the reactivity of eplets of similar composition which only differ at one involved position by the type of AA (62EE/62GE/62QE, 80I/80K/80N, 149TAH/150AAH, 163EW/163LW/163RW, 163RG/163RW, 163LW/163LG/S, 193PL/193PV).

3.5 | Nonimmunogenic HLA class I eplets

Eighteen eplets were not associated with Ab responses, although up to 23 (range 4-23) times present (Table 2). Thirteen of these eplets (72.2%) were present at low case-numbers (range 4-13) and may have become positive upon higher case numbers.

The five remaining eplets, namely 163RW (23 cases), 62RR (20 cases), 156DA (20 cases), 80 TLR (18 cases), 76ESN (15 cases), respectively, were present at least 15 times but not targeted in any case by CSA. It can be concluded that their immunogenicity must be very low. A common property of them is their localization within the α-helices of the α1- or α2-domain at positions in relatively close proximity to the presented peptide (Figure 3).

3.6 | Location of study-derived hot and cold immunogenic spots on the HLA class I molecule

Figure 4 illustrates an overview of the degree of the cumulative (specific AA positions are shared by various eplets) relative immunogenicity from Ab-verified eplet-derived positions on the secondary and tertiary structure of an HLA class I molecule. The color levels reflect

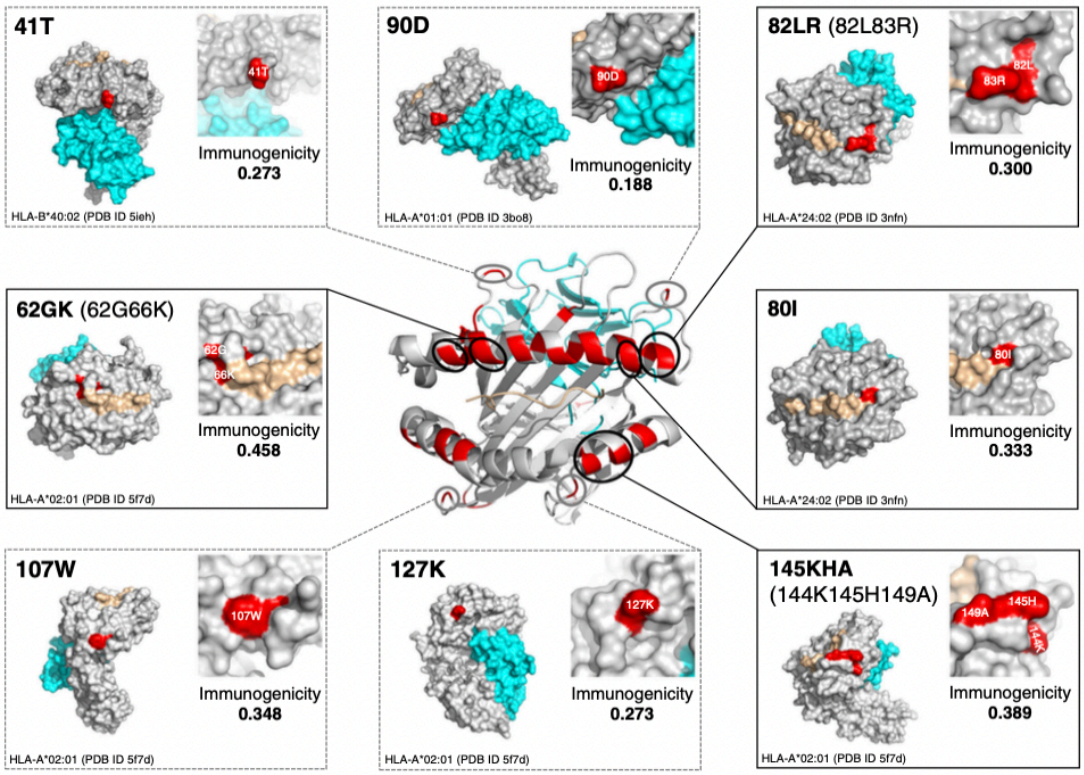

FIGURE 2 Localization and configuration of immunogenic eplets. The molecule in the center of the figure represents the secondary protein structure of an HLA class I molecule (surrogate *HLA-A*02:01*; PDB ID 5f7d) at top view. The positions colored in red indicate the locations of those study-derived 34 eplets with calculable immunogenicity due to sufficient high case numbers. The eight surrounding boxes typify chosen eplets. They are shown on the tertiary protein structure of HLA molecules representing the appropriate AAs of the eplet, in full view on the left and in a zoomed view on the right. Black boxes represent eplets located in close proximity to the peptide binding groove, grey boxes in dashed lines represent eplets located outside the peptide-binding groove. The study- derived immunogenicity scores of the individual eplets are displayed below the zoomed image and the PDB file source of used X-ray crystallographic HLA data is given at bottom left of each box. Alpha chains are displayed in grey, β2-m in cyan and the peptide in light orange. β2-m, beta-2 microglobulin; 3D, three dimensional; AA, amino acid; PDB, protein data bank (www.rcsb.org)

different immunogenic potentials of accessible AA positions if the respective Ab-verified eplets were mismatched.

This analysis showed seven hot spots (cumulative relative immunogenicity of >0.66). Five of them are situated on the α2-domain, precisely between positions 106 and 145, the region consisting of a loop between the first and second β-strand followed by β-strands 2 to 4 and continued by two and a half turns within the first α-helix segment. Interestingly, most of involved positions are in far proximity to Beta-2 microglobulin (β2-m). Another hot spot is represented by the relatively exposed buldge at position 41 on the α1-domain and another one is situated in the lower segment of the α3-domain at position 248, only about 9 Å apart from where the α3-domain transits to the transmembrane-domain.

Identified cold spots (non or low immunogenicity) are predominantly situated on the upper side of the molecule, on the α1- and α2-domain beside or near the peptide.

Of note, for positions belonging to several eplets, the cumulative degree of immunogenicity was calculated, as described in the methods section. By contrast, positions defined by only one eplet, indicate the direct and AA-type dependent immunogenicity. In this context it is important to mention that five of the seven described hotspots (namely positions 41, 107, 127, 131 and 248) are represented by a single eplet only, while cold spots (non or low

TABLE 2 Reactive but underrepresented eplets (<15 cases) (potentially immunogenic but established on less conclusive case-numbers; I-Sc is therefore given in brackets)

Eplet	Polymorphic residues	#CSA+	#Total	I-Sc	Alpha domain	Eplet frequency
131S	131S	2	7	(0.286)	2	0.943
248M	248M(6K)(99C)	1	4	(0.250)	3	0.088
144QL	144Q145L	1	4	(0.250)	2	0.069
71ATD	71A73T77D	1	4	(0.250)	1	0.025
161D	161D	3	14	(0.214)	2	0.208
62LQ	62L63Q	1	5	(0.200)	1	0.025
76EG	76E79G	1	5	(0.200)	1	0.031
56R	56R	2	12	(0.167)	1	0.126
62GRN	62G65R66N	1	6	(0.167)	1	0.076
143S	143S	1	6	(0.167)	2	0.126
44KM	44K45M	2	13	(0.154)	1	0.289
163RG	163R167G	2	13	(0.154)	2	0.289
70IAQ	66I69A70Q	2	14	(0.143)	1	0.214
71SA	70S71A	1	8	(0.125)	1	0.094
253Q	253Q	1	9	(0.111)	3	0.912
69TNT	69T70N71T	1	9	(0.111)	1	0.937
138K	138K	1	11	(0.091)	2	0.176
138MI	138M142I	1	12	(0.083)	2	0.918
44RMA	44R45M46A	1	13	(0.077)	1	0.283
150AAH	149A150A151H	1	13	(0.077)	2	0.881
Non-reactive eplets						
Eplet	**Polymorphic residues**	**#CSA+**	**#Total**	**I-Sc**	**Alpha domain**	**Eplet frequency**
*163RW**	163R167W	0	23	0.000	2	0.252
*62RR**	62R65R	0	20	0.000	1	0.245
*156DA**	156D158A	0	20	0.000	2	0.346
*80TLR**	80T82L83R	0	18	0.000	1	0.308
*76ESN**	76E77S80N	0	15	0.000	1	0.811
151AHA	150A151H152A	0	13	0.000	2	0.151
65RNA	65R66N69A	0	13	0.000	1	0.849
177KT	177K178T	0	12	0.000	2	0.239
76ESI	76E77S80I	0	11	0.000	1	0.120
149TAH	149T150A151H	0	11	0.000	2	0.101
163LS/G	163L-167G/S	0	10	0.000	2	0.208
145RT	145R149T	0	10	0.000	2	0.101
193PV	193P194V	0	9	0.000	2	0.925
80N	80N	0	9	0.000	1	0.918
79GT	79G80T	0	6	0.000	1	0.950
144K	144K	0	6	0.000	2	0.937
65QKR	65Q66K69R	0	5	0.000	1	0.956
158T	158T	0	4	0.000	2	0.101

Note: Those marked with an asterisk = high case numbers = can be considered as non-immunogenic. Those with low case numbers may have become positive upon higher case numbers.

Abbreviations: CSA, child specific HLA antibodies; Eplet frequency, frequency in self HLA repertoire (HLA-ABC) of the mothers; I-Sc, immunogenic score.

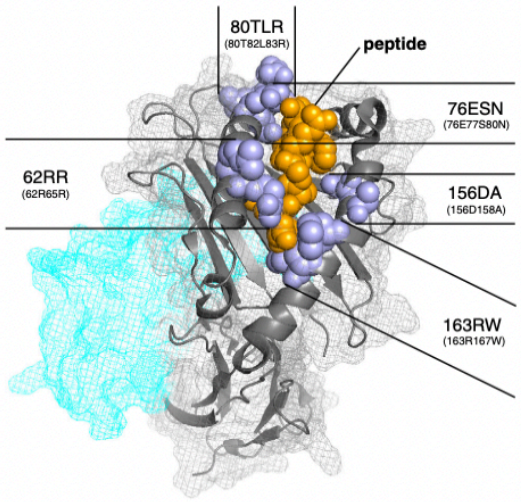

FIGURE 3 The five nonimmunogenic eplets. Top/lateral-view on a modified *HLA-B*44:02* molecule, representing all appropriate AAs (PDB file 3l3d was modified in silico [using Pymol software] at positions 65, 77, 163 and 167). The identified nonimmunogenic eplets, indicated in light blue spheres, are situated in the α-helix of α1- (left) or α2-domain (right). The rest of the α-chains are depicted in gray-cartoon/mesh and the β-chain (β2-m) in cyan-mesh style, while the presented peptide is indicated in yellow orange spheres. β2-m, Beta-2 microglobulin; AA, amino acid; PDB, protein data bank (www.rcsb.org)

immunogenicity) are comprising positions mainly represented by various eplets.

4 | DISCUSSION

Eplets represent crucial structural components of HLA epitopes. In the transplant setting they can be targeted by DSA,[16,17] but their individual immunogenicity has not been profoundly examined. In the present study, first pregnancies were analyzed to determine the relative immunogenicity of HLA class I eplets. Importantly, all mothers were free of presensitizing events. We focused on all currently defined 72 Ab-verified HLA class I eplets. All of them were mismatched several times on child HLA, indicating comparability with a Caucasian transplant cohort. The immunogenicity of an individual mismatched eplet was determined as its percentage eliciting an eplet-specific Ab response.

About a third of the mothers developed IgG-type CSA, directed against approximately a fifth of mismatched HLA-class I, measured at the time of delivery. Evaluation of the eplet-specificities of these CSAs showed that several eplets triggered a more pronounced humoral immuno-response than others equally represented. This supports the presumption that 3D elements on donor HLA molecules represent different immunogenic potentials.

Even though the data should be considered preliminary, it suggests a new approach to be incorporated into the calculation of eplet load for mismatched HLA alleles in SOT: a numerical value for each eplet indicating the relative degree of immunogenicity. The sum of these scores might represent a more accurate assessment of the eplet-repertoire presented by mismatched HLA alleles. Information provided about the immunogenicity of mismatched eplets can therefore potentially improve the approach of considering epitope-mismatches for pretransplant risk assessment of individual patients, because each eplet could be scored according to the respective detrimental nature. Results of running studies (https://www.ihiw18.org/component-epitopes/) evaluating the impact of HLA epitopes on the production of posttransplant de novo Ab will indicate to which degree this dataset conforms with results of the transplant setting.

One of our findings was that certain Ab-verified eplets did not induce an Ab production at all, although they were adequately represented on mismatched child HLA. These identified nonimmunogenic HLA class I eplets (62RR, 76ESN, 80TRL, 156D, 163RW) may represent structural HLA elements of less biological relevance, at least in the pregnancy-setting. Molecular topographic considerations indicate that they are homing locations on the α1- or α2-domain in a rather close proximity to the presented peptide, which may potentially diminish the accessibility by CDRs of B-cell receptors and antibodies. However, other eplets located within a similar proximity to peptide residues showed a striking immunogenicity. It remains to be seen, why these differences exist.

Our results also suggest a possible impact of distance/proximity to β2-m: the fact that most patches of highest cumulative relative immunogenicity are located on the side front of the α2-domain, precisely on the opposite side of β2-m (visualized on Figure 4), may underline the importance of unhindered accessibility to large antigenic areas for B-cell receptors during the initiation of the humoral response.[18] On the other hand, our analysis showed two eplets of remarkable immunogenicity located in vivo very closely to the cell membrane. This finding may have a different clinical manifestation because complement binding antibodies directed against membrane proximal nonself AA-patches may be more efficient at inducing the membrane-attack-complex.

Beside these topographic considerations it is important to note that the reactivity of mismatched eplets or of

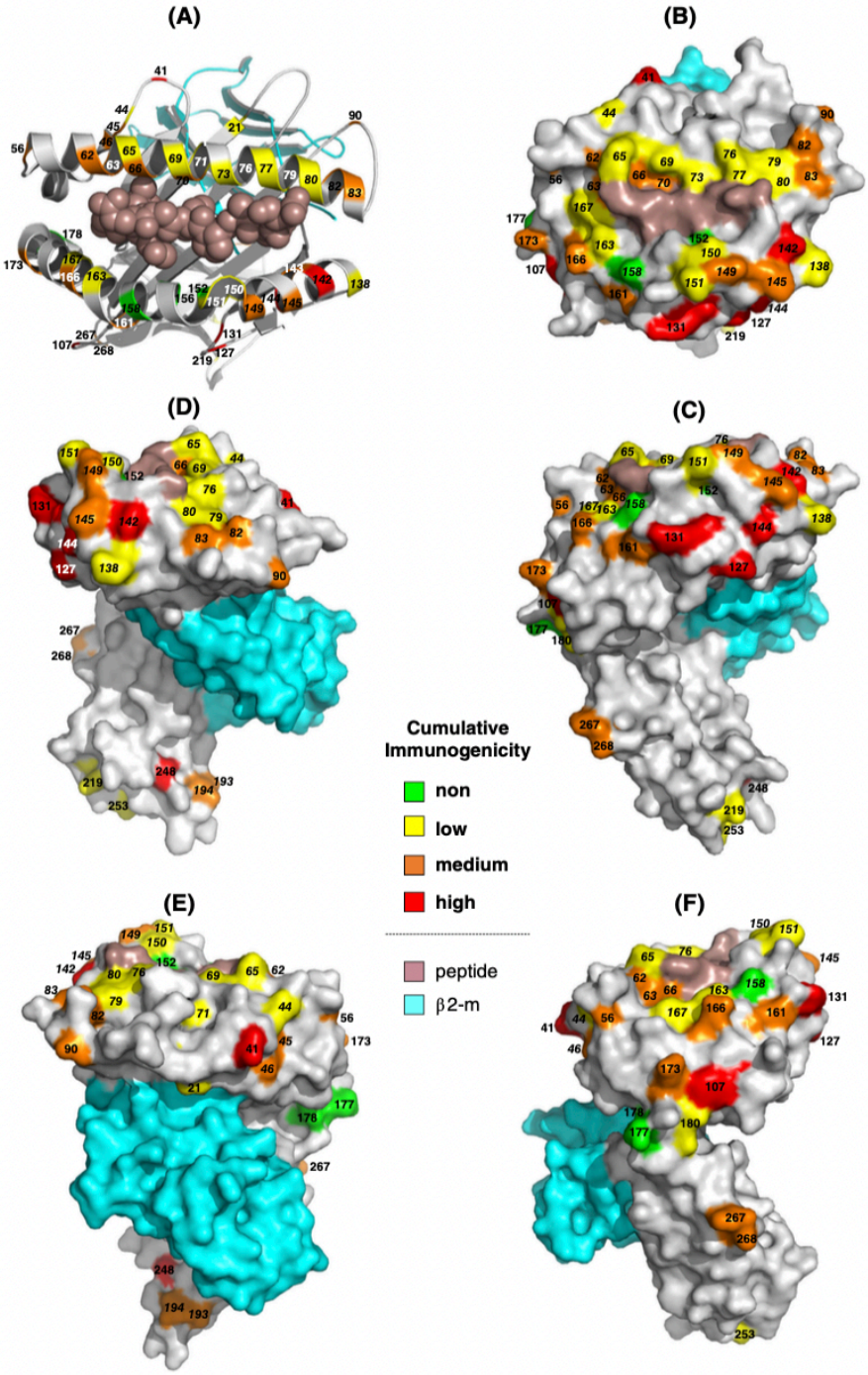

FIGURE 4 Legend on next page.

certain mismatched AA positions which are represented by several eplets was clearly influenced by the type of AA, which confirms the strong impact of physichochemical properties of different AA on the immunogenicity.[19]

As described in a recent review by Kramer et al,[20] immunogenicity of structural patches is also impacted by their frequency: immunogenic structures located on HLA types of lower frequency are generally showing a higher incidence of Ab formation if such an HLA is mismatched. Indeed, eplets that induced Ab production although they were rather underrepresented in our study were predominantly represented by HLA types of relatively low frequency.

Clearly, investigating the immunogenicity of structural molecular mismatches is population dependent and impacted by additional variables (eg, BCR-repertoire, TCR-repertoire, PIRCHE II[21-23]).

The HLAMatchmaker algorithm assesses key immune-determinant structures but not the entire epitope that can be targeted by alloantibodies.[24] In addition, the pregnancy setting may show a slightly different outcome as compared to the transplant setting. Moreover, the overall eplet-reactivity in the pregnancy setting appears rather low, possibly impacted by immunoregulatory processes active during pregnancy. Any remaining reactive eplets may therefore represent the most deleterious immunogenic 3D-structures because they have evoked an antibody response despite above mentioned physiological suppression. It is also very likely that some antibodies may have emerged after the time-point of delivery. Another limitation of this study is given by the inherent fact that the composition of the SAB panel—originally designed for the assessment of HLA-specificities—does not represent a balanced mix of eplets (ie, underrepresentation of private HLA epitopes). In consequence, some reaction patterns can hardly be attributed to single eplet mismatches, independent on structural proximity. Due to the relatively small case-numbers, certain immunogenicity-scores may also be over- or under-estimated. Since SABs are also susceptible for denaturation, which can potentially alter eplet-analysis-results, CSA binding patterns from this study were established using HLA class I iBeads, which are largely devoid of such denaturation.[25,26]

To the best of our knowledge, this is the first comprehensive investigation of HLA class I eplet immunogenicity. The results are established on high-resolution HLA typing and provide information about the immunological responsiveness of individual Ab-verified eplets in the pregnancy setting by means of the degree of emerged eplet-directed Ab of IgG-type at the time of delivery. The established immunogenicity scores are intended to improve epitope-based HLA matching, by weighting each eplet according to its hazardous nature. Validation of these data in various clinical transplant settings is necessary to definitely identify the true deleterious eplets representing a clinically high risk.

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CONFLICT OF INTEREST

This study was approved by the local ethics committee, and written informed consent was obtained from women enrolled in the study. M.R.v.H., L.A.L.v.d.P., S.V., E.H.R. all work at GenDx, supplier of HLA typing reagents and software. Matthias Niemann works for PIRCHE AG, which runs the PIRCHE webservice. The other authors of this manuscript have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

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

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FIGURE 4 Study-derived hot and cold immunogenic spots. Topographic visualization of the cumulative relative immunogenicity on the structure of an HLA class I molecule (*HLA-A*02:01* was taken as surrogate molecule; PDB ID 5f7d). Red, orange, yellow or green patches represent mismatched Ab-verified eplet-derived positions. The peptide is indicated in dark salmon and β2-m in cyan. A, Secondary protein structure, top-down view. B, Tertiary protein structure, top-down view. C-F, Tertiary protein structures, lateral views. To discriminate between study related hot and cold immunogenic spots, each eplet-derived AA-position is ranked according to their study related cumulative immunogenic reactivity (some specific positions are part of different eplets; such position numbers are indicated in *italic*). The results were normalized in relation to the position of the highest cumulative immunogenicity and the calculated degree of this "cumulative relative immunogenicity" was visualized according to the provided color code (green: 0%; yellow: 1-33%; orange: 34-66%; red: 67-100%). β2-m, beta-2 microglobulin; Ab, antibody; AA, amino acid; PDB, protein data bank (www.rcsb.org)

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Chapter 4 PUBLICATION 2

Effects of weak/non-complement-binding HLA antibodies on C1q-binding

4.1 BACKGROUND

4.1.1 Are pre-transplant DSA clinically relevant?

In certain cases, especially for hypersensitized recipients, transplantation across low level DSA is unavoidable. To accommodate such higher immunological risk constellation, most transplant centres apply an enhanced induction immunosuppression [76]. Despite this special treatment, some DSA-positive recipients show a very unfavourable post-transplant course. Conversely, other DSA positive patients experience uneventful and rejection-free courses [75, 116-118, 225, 247-249]. Much research has been carried out in the hope of identifying relevant factors or biomarkers capable to predict the severity of circulating DSA present and the expected recall immune response of pre-sensitized patients. Neither the route of sensitization (blood-transfusions, pregnancies, previous transplantations) was found to be indicative for a stronger or weaker response, nor did the four readily available pre-transplant DSA characteristics (DSA number, DSA HLA-class specificity, DSA HLA locus specificity and cumulative DSA test signal strength) predict their pathogenicity [75, 108, 115, 250]. What has become clear, is that not the breadth of sensitization but the donor specificity of pre-existing antibodies contributes to the immunological risk [251].

The answer to the title's question is therefore yes, some pre-transplant DSA are clinically relevant, yet we do not sufficiently know why.

4.1.2 Unrevealed DSA properties may indicate the real injury potential of circulating DSA

In order to distinguish between harmful and presumed relatively irrelevant DSA, additional knowledge of yet to be considered antibody properties, may therefore be beneficial. Learning from other subject areas in the field of diagnostics, where antibody-features play a central role (i.e. determination of evidence of immunity to certain pathogens

or monitoring of the immune response to vaccination), I see three DSA features that have the potential to sustainably improve risk prediction for pre-transplant DSA patients: **DSA target-specificity**, **DSA affinity/avidity** and **DSA effector-functionality**. Importantly, these antibody properties are all interrelated and must therefore be interpreted together. Below, I devote a section to each of these three characteristics.

DSA target-specificity: The relationship between epitope specificity of pre-existing DSA and the severity of the immune response after transplantation across DSA has not yet been investigated. In this context it is worth mentioning that both commercial providers of HLA antibody multiplex assays, the companies OneLambda and Immucor, have recently integrated epitope assignment algorithms into their latest version of HLA antibody test evaluation software. As a consequence, all interested HLA laboratory customers worldwide are now capable of assessing DSA epitope-specificity, and to integrate this information into their centre-specific immunological pre-transplant risk stratification. However, it remains to be considered that patient antibodies are potentially polyclonal, resulting in complicating binding patterns. Without purification of the patient serum by absorption using the appropriate donor HLA of interest and subsequent elusion followed by reanalysis of the eluate's binding profile, there may be a high likelihood of incorrect epitope-specificity assignment.

DSA affinity/avidity: It is generally assumed that precise determination of DSA binding strength could be a key determinant to estimate the potential for AMR and to adjust immunosuppression accordingly [78, 130]. Indeed, antibodies are continuously developing their binding-strength in a process termed 'affinity-maturation', which takes place in secondary lymphoid tissues [252-255]. From studies in infectious diseases, evidence exists that antibody binding strength, as determined by antibody avidity assays, correlates with B-cell clone maturation and consequently with the magnitude of a future memory response [256-258]. Several avidity assays have been developed to improve sero-diagnosis for various infectious diseases [257-265]. Furthermore, antibody avidity assessment has enabled discrimination between primary and secondary infections, and monitoring of vaccination efficacy [256, 266-268]. The development of a feasible rapid assay for routine DSA affinity/avidity testing of patient serum samples is, however, associated with a number of difficulties that are time-consuming to resolve. It is therefore not surprising that the few

publications currently available on the topic of HLA antibody affinity/avidity are still almost exclusively limited to HLA-specific mAb [133, 269, 270].

DSA effector functionality: The central effector functions of DSA leading to the manifestation of AMR, as described in the Introduction Chapter of this thesis, are **HLA crosslinking** (triggering endothelial activation and recruitment of leukocytes [45], **C1q-binding** (causing complement activation, augmented leukocyte recruitment and T-cell allo-responses [47, 48, 271], and FcγR-**binding** (causing ADCC) through FcγR expressing NK-cells, monocytes, macrophages or neutrophils) [49-51, 271]. While HLA-crosslinking through DSA is substantially influenced by the antigen (i.e. HLA density [272] and their epitope constitution) and by the concentration and affinity of DSA [18, 46, 273], C1q- and FcγR-binding are strongly impacted by the stereo-chemical structure of the antibody-isotype/subclass itself and by their specific glycosylation profiles [274-277]. Knowing the isotype/subclass-composition of DSA is therefore essential to estimate the types of effector-function that can be executed by the DSA present. However, DSA often consists of all four IgG subclasses [278-281] and frequently contains immunoglobulins of isotype M (IgM) [278, 280, 282], or of isotype A (IgA) [280, 283-285] and even of isotype E (IgE) [280].

Methodologically, the breakdown of DSA into their isotype(s)/subclass(es) is feasible by simply replacing the commonly used reporter-antibody (anti-IgGpan) with isotype/subclass-specific reporter-antibody-conjugates [279, 280]. Our group has previously established a standard operational procedure to simultaneously determine IgG1, IgG2, IgG3 and IgG4 of HLA antibodies, using SAB technique [279, 286]. Other groups have developed similar protocols to assess DSA of IgA- [284] and of IgM-origin [278, 282]. Importantly, correlation of Ig isotypes/subclasses of pre-transplant DSA with clinical outcome has revealed inconsistent results (reviewed in [287]), indicating that without knowing the DSA components' individual affinities or their epitope-specificities, their isotype/subclass composition is not sufficiently predictive for post-transplant events.

A more direct approach to assess the mediated biological activity of a given DSA mixture is to measure the triggered net effect by using functional assays. This applies in particular to the immune effector mechanism of complement activation, as mutual promotion or inhibition can occur depending on the specific isotype/subclass mixture. The result of the cellular CDC-test (described in Chapter 5) describes a functional property of DSA,

namely the capability to induce cytotoxicity. Beside the CDC assay, which lacks of specificity and sensitivity, cell-free assays to specifically measure antibody-dependent complement binding have been developed and used: C1q-binding [141, 286, 288, 289], C3d-deposition [290, 291], or C4d-fixation [292, 293] can be assessed by using a modified SAB assay protocol. Hope prevailed that the complement activation potential of the DSA as measured by using these modified tests, would better predict the clinical outcome [141, 294, 295]. However, more in-depth studies have shown that due to the predominance of complement-binding IgG1 and IgG3 in DSA, the level of the SAB test signal is directly related to the strength of complement binding and that the need for such additional complement-tests is therefore not compelling [131, 286, 296].

4.1.3 IgG subclasses and their effector functions

Even though DSA consists of other isotypes in addition to IgG, the isotype G usually represents the largest amount [280] and is clinically the most significant [285]. IgG consists of four subclasses, IgG1, IgG2, IgG3, and IgG4, each of which can initiate different effector functions [287]. In terms of design and structure, the 4 subclasses differ in i) the length and flexibility of their hinge region [297], ii) their type of N-glycans in their C_H-2 domain [298] and iii) certain AA at several positions in their C_H-1, C_H-3 and upper part of C_H-2 domains [299]. Binding sites for both, C1q- and FcγR overlap in the C_H-2 domain of IgGs. The mentioned structural differences between subclasses imply different mediation of effector cells, differences with regard to the phagocytosis of antigen-antibody complexes, and variable efficacy in turns of complement activation [143, 276]. A schematic representation of the structure of an IgG antibody and its domains is shown in **Figure 6**.

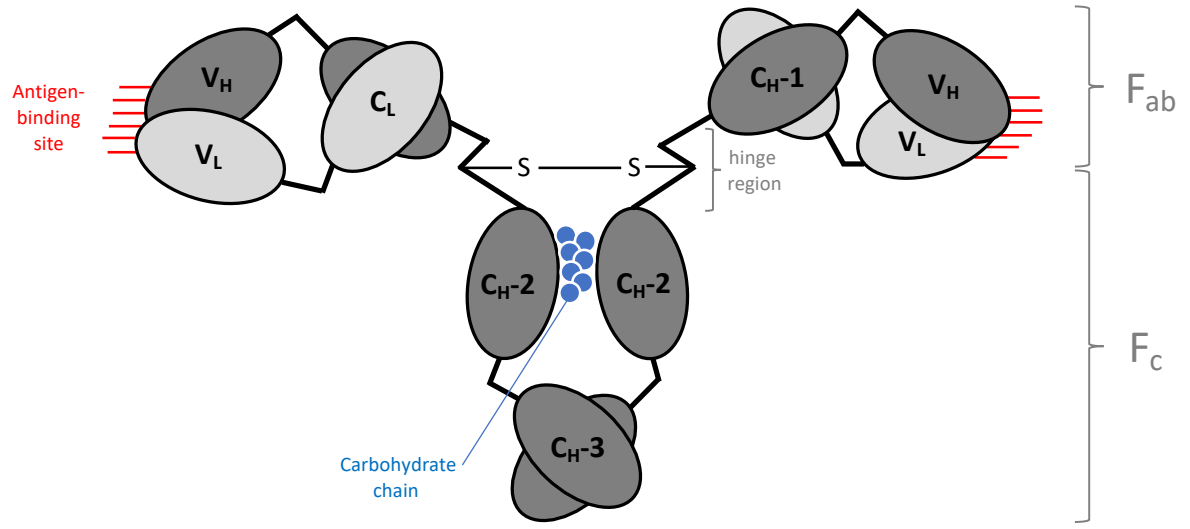

Figure 6. Schematic structure of an IgG Antibody. The figure is designed to accord with the publication from Silverton et al. [300]. The schematic cartoon shows the construction of an IgG antibody, the domains and their names from the two heavy- and light chains, the location of the hinge region and the F_c-attached carbohydrate chain.

F_{ab}: fragment antigen-binding, F_c: fragment crystallizable, C_H-1: constant domain 1 of heavy chain, C_H-2: constant domain 2 of heavy chain, C_H-3: constant domain 3 of heavy chain, C_L: constant domain of light chain, V_H: variable domain of heavy chain; V_L: variable domain of light chain, -S—S-: disulfide bond

A vital function of IgG is its capability to bind to FcγR on effector cells, specifically FcγRIII expressed on eosinophils, neutrophils, macrophages, mast cells and especially on NK-cells. Engagement of target bound IgG1 or IgG3 with FcγRIII on NK-cells induces ADCC, which leads to the killing of the targeted cells [49, 50, 301, 302].

Antigens consisting of protein often evoke the development of IgG1 and IgG3 subclasses, promoted through T-cell help after presentation of antigen peptides through HLA class II on the respective B-cell [276]. IgG2 is the dominant subclass involved in responses to capsular polysaccharide antigens of bacteria [303, 304]. The relevance of this property for DSA of subclass IgG2 targeting epitopes in the glycosylated regions of HLA glycoproteins [305, 306] remains to be determined. Recent studies have also shown that IgG2 can exchange disulfide-linkages and that one of the three resulting isoforms (IgG2-B) provides an extended flexibility of the Fab-arms (achieving a ‘T’ form, compared to the conventional ‘Y’ form) [307]. Another unique behaviour is displayed by the Ig subclass of type 4: the relatively unstable

hinge region of IgG4 (mainly due to AA Serine at position 228) facilitates heavy-chain swapping. Therefore, most IgG4 of human serum are monovalent bispecific molecules, no longer capable of binding antigen with avidity or of crosslinking their target [276]. Moreover, antibodies of subclass IgG4 exhibit a high affinity to the inhibitory receptor FcγRIIb. IgG4 may therefore serve to prevent an excessive immune response and is often termed a 'blocking antibody' [276, 308].

Complement activation by the classical pathway is considered the most important biological function of IgG and is considered to be a major contributor to the clinical manifestation of AMR [47]. The next section is dedicated to this topic.

4.1.4 Complement activation by IgG

Activation of the classical complement pathway is initiated by binding of the globular heads of C1q to distinct structural variations (AA 285−292 and 317−340) in the hinge region of the IgG's C_H-2 fragment [309]. When C1q approaches these sites, the mentioned structural variations induce steric interference by the Fab arms, which impacts C1q-binding capability and subsequently leads to different complement reactivity profiles (IgG3 > IgG1 > IgG2) [309, 310]. C1q to IgG3 binding is about 40 times greater than to IgG2, while IgG4 does not show appreciable C1q-binding *in vitro* [309, 311]. Binding to multiple Fc segments (at least two) in close proximity (30-40nm) is a requirement for stable C1q-binding [299, 312, 313], underlining an enhanced complement mediated cytotoxicity by repetitive structural patterns in close proximity (or antigens clustered on a mobile membrane, as is the case for cell membrane embedded HLA). In a survey where IgG binding to clustered antigens on liposomes was studied, the most efficient C1q-binding was observed upon formation of IgG hexamers [314]. The end products of the classical complement cascade are membrane attack complexes, which trigger cell lysis, which in turn promotes inflammation and – in the context of transplantation – eventual rejection of the graft [42].

4.1.5 Aims of this study (Publication 2)

DSA consists of a complex mixture of different isotypes/subclasses, present at different concentrations and binding strengths [133, 280], together targeting multiple epitopes. In the present study, two of these complexities, 'epitope-specificity' and 'antibody

affinity', were first excluded. For this purpose, IgG1/2/3/4 HLA antibodies of unique epitope-specificity were used, namely chimeric mAb targeting a public epitope on HLA-DR and HLA-DP. These mAbs, one of each subclass (IgG1, IgG2, IgG3 and IgG4), were originally engineered to treat B-cell lymphoma [315]. These two simplifications allowed us to study the impact on C1q-binding solely by the type or concentration of a certain IgG-subclass or an IgG-subclass mixture. The aim of this investigation was to reveal the **net C1q-binding effect** induced by different IgG subclass mixtures targeting the exact same HLA epitope.

Next, for a subsequent series of experiments the factor 'epitope-specificity' was reintroduced. This was done to measure and compare C1q-binding of HLA antibodies from pre-transplant patient sera. The patient serum samples were used neat, or spiked with the model antibody described above (interacting with a public epitope, thus not competing with an epitope targeted by the patient antibodies). The goal of this investigation was to clarify to which extent HLA antibodies of different IgG subclasses targeting adjacent epitopes may impact C1q-binding.

The third aim of the study was devoted to genuine HLA-specific IgG subclass antibody mixtures: HLA antibodies present in 72 pre-transplant patient sera were examined for their *net* C1q-binding and in parallel for their exact IgG subclass composition. This analysis was to verify which subclass compositions promote or inhibit complement activation in the clinical setting.

4.1.6 Used *in vitro* model

In order to achieve the first two of the objectives described above, an *in vitro* model was established. The model allowed deliberate study of C1q-binding on a single HLA-molecule type. The chosen HLA-class II heterodimer (DRB1*07:01/DRA1*01:01), coated on Luminex xMAP®-beads, expressed an epitope that was specifically targeted by each of the four applied IgG subclasses of the chimeric human/mouse mAb clone F3.3 [315-317]. While the constitution of their hinge region in the Fc part is subclass-specific, they all consist of the exact same hypervariable region in their Fab. Consequently, they all interact with the exact same epitope and their affinity is per definition identical. The mentioned single HLA-beads were subsequently incubated with either i) a separate mAb IgG subclass, or ii) a specific IgG subclass mixture, or iii) a human pre-transplant serum spiked with/without mAb. Thereafter, either

recombinant human C1q together with fluorochrome conjugated C1q reporter antibody, or specific IgG subclass conjugated reporter antibody was added for the secondary incubation. Ultimately, the HLA-beads were analysed for their reporter antibody signal on a Luminex instrument, to quantify both net degree of C1q-binding and IgG1/2/3/4 content, respectively. **Figure 7** visualizes the used approach.

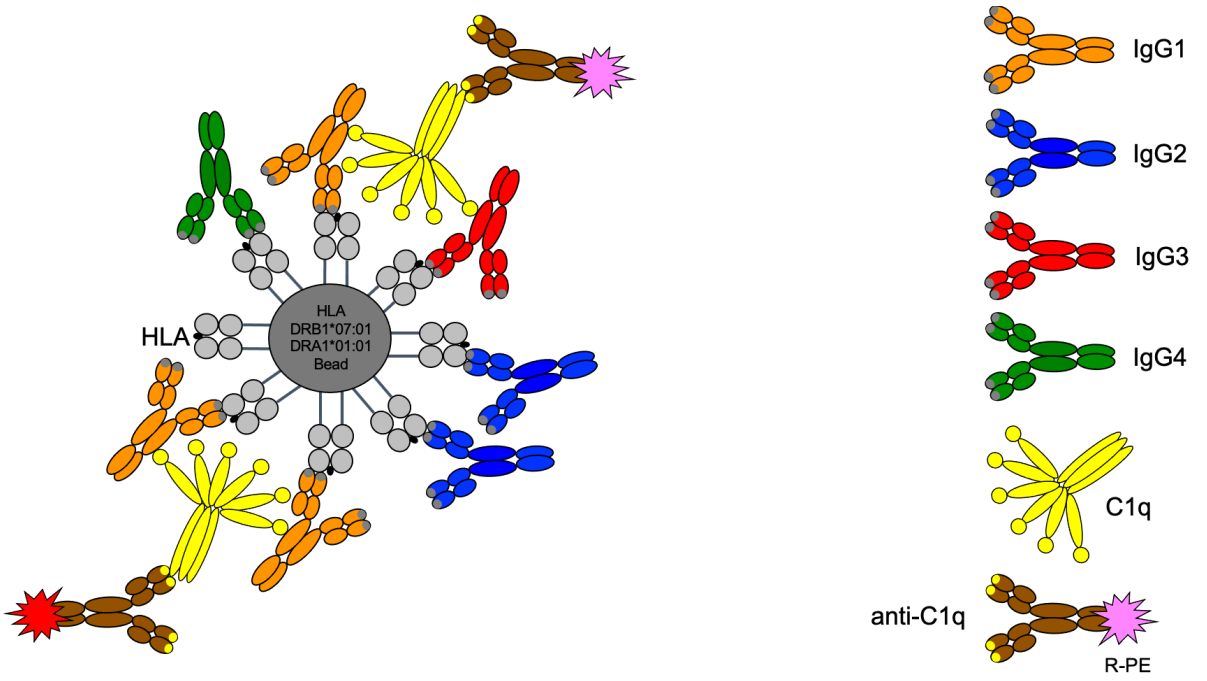

Figure 7. Visualization of the model assay used and its components.
HLA-DRB1*07:01/DRA1*01:01 molecules (light grey), immobilized on the surface of a Luminex-bead (dark grey), targeted by specific HLA antibodies of different subclasses (IgG1: orange, IgG2: blue, IgG3: red, IgG4: green). The complement binding capability of the given HLA-specific antibody IgG subclass mixture is assessed by C1q (yellow), which solely binds to IgG1 and IgG3, but requires them to be clustered in close proximity. Bound C1q is quantified via a C1q specific reporter antibody (brown) conjugated to the fluorochrome R-PE (pink).
R-PE: R-Phycoerythrin, a fluorescent red protein-pigment complex

Another important feature of the model was the paratope constitution of the chosen mAb used and the expression of the corresponding targeted epitope on the model HLA-protein used. Important to achieve the second objective of this study (impact on C1q-binding by different IgG-subclasses that interact with adjacent epitopes), no concurrent epitope

specificity by patient antibodies was allowed to be present. Only when this was guaranteed could the spiking experiments (patient serum samples mixed with model antibodies) provide the requested information. By choosing a highly conserved epitope motive, targeted by the mouse F(ab') fragments present in the chimeric antibody construct F3.3 [318], this condition was fulfilled. There is no published description of the structural epitope of F3.3, but our independent analysis of the mAb's binding profile on LabScreen™ HLA class II multiplex SAB identified a glutamic acid at position 46 expressed on the β1-domain of positively reacting HLA-DRB1/3/4/5, and HLA-DQB1, or at position 44 for HLA-DPB1 molecules, as the most probable predominant antigenic binding motif. For HLA-DR and -DP, the AA polymorphism in the immediate vicinity of this position is less pronounced compared to other surface areas on the β1-domain. Therefore, the patient antibodies, directed against polymorphic AA clusters, did not compete for the exact same binding site as the spiked model mAbs, but assumingly interacted with structures in a certain spatial distance.

4.2 PUBLICATION 2

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WILEY HLA Immune Response Genetics

ORIGINAL ARTICLE

Effects of weak/non-complement-binding HLA antibodies on C1q-binding

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It is unknown under what conditions and to what extent weak/non-complement (C)-binding IgG subclasses (IgG2/IgG4) can block C1q-binding triggered by C-binding IgG subclasses (IgG1/IgG3). Therefore, we investigated *in vitro* C1q-binding induced by IgG subclass mixtures targeting the same HLA epitope. Various mixtures of HLA class II specific monoclonal antibodies of different IgG subclasses but identical V-region were incubated with *HLA DRB1*07:01* beads and monitored for C1q-binding. The lowest concentration to achieve maximum C1q-binding was measured for IgG3, followed by IgG1, while IgG2 and IgG4 did not show appreciable C1q-binding. C1q-binding occurred only after a critical amount of IgG1/3 has bound and sharply increased thereafter. When both, C-binding and weak/non-C-binding IgG subclasses were mixed, C1q-binding was diminished proportionally to the fraction of IgG2/4. A 2- to 4-fold excess of IgG2/4 inhibited C1q-binding by 50%. Very high levels (10-fold excess) almost completely abrogated C1q-binding even in the presence of significant IgG1/3 levels that would usually lead to strong C1q-binding. In sensitized renal allograft recipients, IgG subclass constellations with ≥ 2-fold excess of IgG2/4 over IgG1/3 were present in 23/66 patients (34.8%) and overall revealed slightly decreased C1q signals. However, spiking of patient sera with IgG2 targeting a different epitope than the patient's IgG1/3 synergistically increased C1q-binding.
In conclusion, if targeting the same epitope, an excess of IgG2/4 is repressing the extent of IgG1/3 triggered C1q-binding *in vitro*. Such IgG subclass constellations are present in about a third of sensitized patients and their net effect on C1q-binding is slightly inhibitory.

KEYWORDS
allo-reactivity, C1q-binding, HLA antibody, IgG subclass, *in vitro*

1 | INTRODUCTION

Pre-formed donor specific HLA antibodies (DSA) are a risk factor for transplantation. They can induce different effector mechanisms such as complement activation, antibody-dependent cellular cytotoxicity and agonistic signaling.[1] Complement (C) activation is driven by various properties of the DSA themselves, such as the amount, the affinity and the IgG subclass composition, but also by the density and the conformational changes of the target antigen.[2,3]

Recently, a new assay (C1qScreen) was introduced to assess the first step of the C-reaction, the formation of stable C1q-antibody complexes.[4,5] The required stability is only accomplished by multivalent binding, meaning several heads of the hexameric glycoprotein C1q molecule must join with multiple CH2 domains of different target-bound antibodies (Ab). This process is strongly dependent on the

proximity of immunoglobulin molecules bound to the antigen—here HLA.[6]

Complement activation triggered by monoclonal antibodies (mAb) of individual IgG subclasses have been well studied in vitro.[7–9] Based on accessibility of C1q to the different hinge-regions, IgG subclasses can be subdivided into C-binding (IgG1 and IgG3), weak- (IgG2) or non-C-binding (IgG4). HLA-antibodies in humans are often polyclonal and contain mixtures of IgG subclasses. Indeed, most DSA contain IgG1/3 as the dominant subclass, and are therefore C-activating.[10–12] About half of DSA, however, simultaneously contain IgG2 and/or IgG4.

The aim of this study was to investigate the *in vitro* C1q-binding induced by IgG subclass mixtures targeting the same HLA epitope. In addition, we analyzed C1q-binding induced by different IgG subclass mixtures in patient sera.

2 | MATERIALS AND METHODS

2.1 | LABScreen single antigen beads (*HLA-DRB1*07:01*)

LABScreen singles coated with recombinant human *HLA-DRB1*07:01* of uniform HLA-density (ie, same Lot) were used throughout the study (OneLambda, Canoga Park, California). In order to adjust and keep the number of these single antigen beads (SAB) identical between assays (ie, 500 beads/reaction), the concentration of the SAB stock solution was determined using the handheld particle counting device Scepter 2.0 (Millipore, Billerica, Massachusetts). This device produced stable measurements with an intra-assay coefficient of variation of 1.44% ($n = 6$; data not shown). Prior to each experiment, the designated concentration was adjusted by diluting the SAB-solution with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA).

2.2 | Monoclonal HLA class II antibodies

HLA class II specific monoclonal chimeric human/mouse Ab of each IgG subclass were used.[13] They contain HLA class II specific variable light and heavy chain sequences from murine F3.3 hybridoma and constant human light/heavy chains of different hinge-regions. They recognize all HLA-DR-, all HLA-DP- and partially HLA-DQ-molecules, but their precise epitope-specificity is unknown.[14] They were provided as supernatants of BHK21(Baby Hamster Kidney)-cells transfectoma in IMDM (Iscove Modified Dulbecco Medium) and contained 10% heat inactivated fetal calf serum (FCS) & Peniciline/Streptomycin. The monospecificity of these mAb towards HLA class II molecules (here *HLA-DRB1*07:01*) can be regarded as constant. Subsequently, their concentration was assessed by using the Easy-Titer Human IgG gamma chain Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts). Whenever IgG3 was used in a mixture, 1.16-fold increased concentrations were applied (molecular weight of IgG3 = 170 000 g/mol vs 146 000 g/mol for IgG1/2/4), to assure comparable stoichiometric proportions. However, the dimension for the concentration was kept in "ng/mL" rather than "molecules/mL". A single isolated IgG subclass or a mixture of 2 different IgG subclasses was applied in a total volume of 25µL, consisting of 5µL bead-solution (containing 500 SAB) and 20µL mAb solution (both in PBS/1%BSA). Beads and mAb were incubated for exactly 30 minutes at room temperature.

2.3 | Reporter antibodies

We used polyclonal phycoerythrin (PE)- conjugated goat anti-human IgGpan reporter Ab (LS-AB2; OneLambda, Canoga Park, CA) and monoclonal PE-conjugated human IgG subclass specific reporter Ab (anti-IgG1/2/3/4) from Southern Biotec (Birmingham, Alabama). We previously established the specificity and crossreactivity of the latter antibodies.[10] However, the formerly used clone 31-7-4 for anti-IgG2 was replaced with clone HP6002, which showed negligible crossreactivity with the other IgG subclasses (≤2%; data not shown). To assess the level of bound C1q, PE-conjugated anti-C1q from OneLambda's C1qScreen kit was applied. Further details (e.g. applied concentration) of the mentioned reporter Ab are summarized in Table 1.

2.4 | C1q-binding Assay using SAB

Detection of HLA Ab triggered C1q-binding was performed with the C1qScreen (OneLambda) assay.[4] We modified the assay as follows: per 1 well of a V-bottom polystyrene 96 well plate (Whatman, GE Healthcare Lifesciences,

TABLE 1 Used PE-conjugated reporter-antibodies

	Anti-IgG1	Anti-IgG2	Anti-IgG3	Anti-IgG4	Anti-IgGpan	Anti-C1q
Company	Southern Biotec	Southern Biotec	Southern Biotec	Southern Biotec	OneLambda	OneLambda
Article-number	#905409	#907009	#921009	#920009	#LS-AB2	#PEPAC1Q
Clone	HP6001	HP6002	HP6050	HP6025	(Polyclonal)	(Polyclonal)
Concentration of stock solution (mg/mL)	0.5	0.1	0.1	0.1	0.5	(No information)
Applied concentration (µg/mL)	1.6	1.6	1.6	1.6	8	—
Applied volume (µL per well)	15	15	15	15	15	1 in 15
Applied amount (ng per well)	24	24	24	24	120	—
Applied amount (pg per bead)	48	48	48	48	240	—

Freiburg, Germany), 500 *HLA-DRB1*07:01* LABScreen Singles (5µL) were incubated with 20µL of single mAb solution (diluted in PBS/1%BSA) or a mixture of 2 mAb solutions. Bead-Ab-solutions were incubated for 30 minutes in the dark, rotating 500 rpm on a IKA MS3 digital plate rotator (Guangzhou, China) at room temperature. Following incubation, the beads were washed and spun 3 times with 200µL wash buffer (OneLambda; centrifugation: 1800*g* for 3 minutes). SAB with bound Ab were then incubated with either

- 15µL of a cocktail containing 1µL C1q working solution (recombinant human C1q stock solution 1:5 diluted in HEPES buffer; both OneLambda) + 1µL PE-conjugated anti-C1q (OneLambda) + 13µL wash buffer (OneLambda), or
- 15µL of IgG subclass reporter Ab (anti-IgG1-PE or anti-IgG2-PE or anti-IgG3-PE or anti-IgG4-PE) (Southern Biotec; diluted in wash buffer), or
- 15µL of diluted (wash buffer) PE-conjugated polyclonal goat anti-human IgGpan reporter Ab (OneLambda).

Finally, SAB were aquired on a LABScan 100 instrument (Luminex Corp., Austin, Texas) and median fluorescence intensity (MFI) from at least 50 beads were analyzed. A concentration of 60 ng IgG/mL led to SAB-HLA occupation near saturation. In the IgG subclass mixing experiments, the C1q signal (i.e. triggered by IgG1 or IgG3) of this concentration was set to 100% and the results were normalized to this C1q-binding level (cutoff for positive result = 10%).

2.5 | Clinical samples

IgGpan-, IgG subclass- and C1q-data (LABScreen Single Antigen Beads OneLambda; HLA class I Lot 8, HLA class II Lot 9) of pre-transplant sera from 72 sensitized patients receiving a renal allograft at the University Hospital Basel were retrospectively analyzed.[15] Only SAB representing anti-HLA IgG subclass mixtures of at least one C-binding (IgG1/3) and at least one weak/non C-binding (IgG2/4) subclass were evaluated. For each of these SAB, a retrospective although restricted quantitative estimation (missing information regarding Ab affinity) for each IgG subclass was performed as follows: The results (achieved within the same assay) of serial dilutions of each of the previously described chimeric mAb were used to generate a standard curve (function: *one site binding with Hill slope*; more details in 2.7). From this curve, the quantity of each allo IgG subclass was inferred and used to group the beads into those with [IgG2/4] / [IgG1/3] ≥2 and those with [IgG2/4] / [IgG1/3] <2.

2.6 | Patient sera: spiking with IgG2

Four sera, positive for *HLA-DRB1*07:01* antibodies (IgGpan MFI signals > 15 000), from patients with known IILA-DR7-sensitization, were heat-inactivated and characterized for their anti *HLA-DRB1*07:01* IgG subclass composition. The serum samples were then spiked with the previously described anti-HLA class II mAb of subclass IgG2 as following: 10µL undiluted serum was mixed with 10µL of (1) PBS 1% BSA, (2) 15 ng IgG2/mL, or (3) 60 ng IgG2/mL. These mixtures were incubated with 500 *DRB1*07:01* beads. Subsequently, the degree of C1q-binding, with/without IgG2 spiking was assessed and compared.

2.7 | Statistical analysis

Prism Version 7.0a (Graph Pad, SanDiego, California) and JMP software version 12.0 was used for statistical analysis. For categorical data, χ^2 test was used. Ab concentrations were assessed by plotting a standard curve and by applying nonlinear regression curve fitting with the function of *one site specific binding with Hill slope* [$y = B_{max} * x^h / (Kd^h + x^h)$], where B_{max} = maximal binding; K_d = equilibrium dissociation constant between the HLA Ab and the antigen (HLA); h = Hill slope.

3 | RESULTS

3.1 | C1q-binding of isolated HLA Ab IgG subclasses

Each HLA class II specific IgG subclass was titrated separately to the *HLA DRB1*07:01* beads and resulting C1q-binding was monitored. These experiments revealed different capabilities for C1q-binding for each IgG subclass (Figure 1). IgG1- or IgG3-induced C1q-binding was detectable after a critical amount of Ab has bound, and abruptly increased thereafter. Less IgG3 was required to reveal similar proportions of C1q-binding, and the slope of C1q-increase was steeper for IgG3 compared to IgG1. For IgG2 and IgG4, even 10-fold higher concentrations were applied, but only small C1q-binding occasionally occurred, however clearly dose independent.

Importantly, all IgG subclass-specific monoclonal reporter Ab (ie, applied at the same concentration) as well as the polyclonal IgGpan reporter Ab (applied at the concentration recommended by the test provider) revealed different binding signals, presumingly due to their different affinities towards each IgG subclass. Of note, the standard IgGpan Ab poorly detected IgG4.

3.2 | C1q-binding of mixtures of C-binding and weak/non-C-binding IgG subclasses

We measured C1q-binding after incubation of *HLA DRB1*07:01* beads with the following mixtures of IgG subclasses: IgG1 + IgG2, IgG1 + IgG4, IgG3 + IgG2, IgG3 + IgG4. Importantly, the applied IgG concentrations were sufficient to almost saturate SAB-HLA thereby

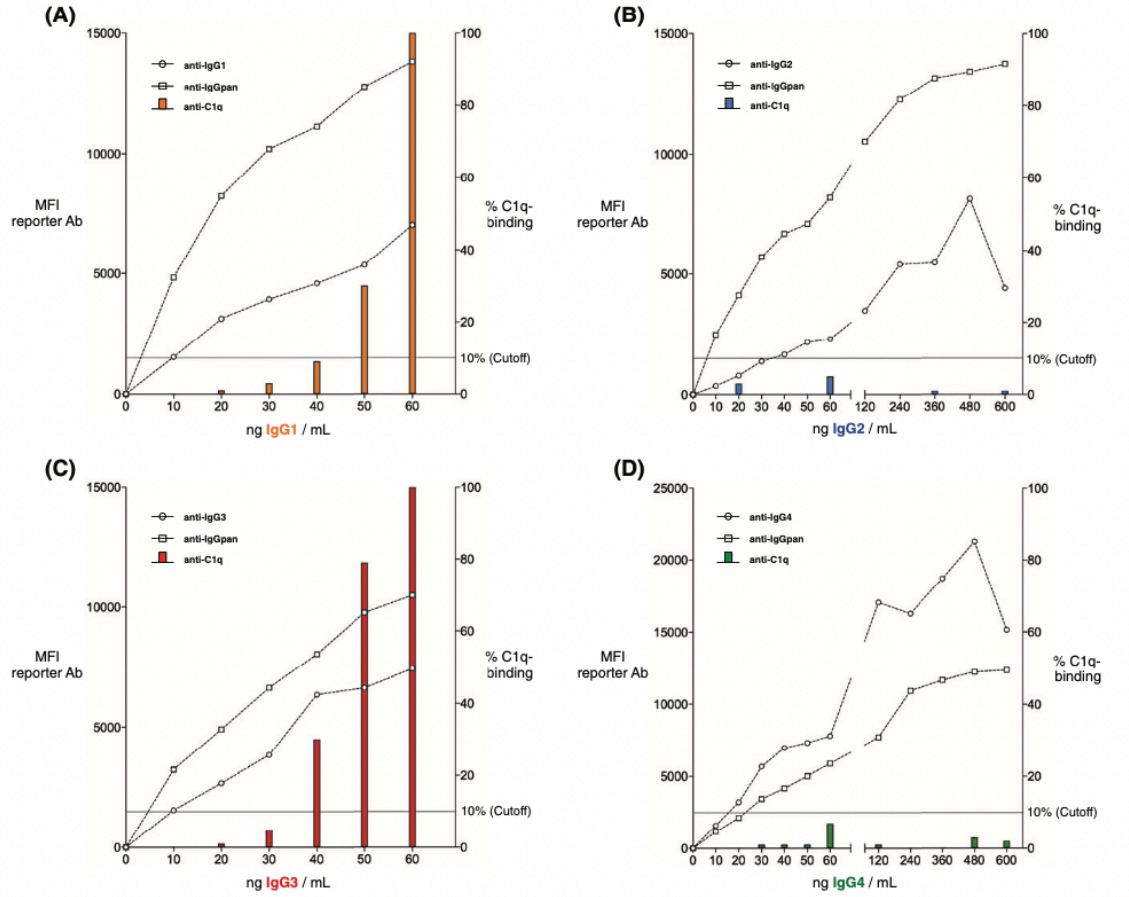

FIGURE 1 Percental C1q-binding (bars, scale on right *Y*-axis) of each HLA Ab IgG subclass within the concentration range (*X*-axis) between 0 and 60 ng/mL (IgG1 and IgG3) or 0 and 600 ng/mL (IgG2 and IgG4). A, IgG1. B, IgG2. C, IgG3. D, IgG4. Curves represent staining signal of monoclonal PE-conjugated anti-IgG subclass reporter Ab (circle) or polyclonal PE-conjugated anti-IgGpan reporter Ab (rectangle). The line at 10% C1q-binding represents the cutoff for C1q-positivity. The mean of 2 experiments is shown.

allowing us to observe competition for available antigen binding places between the applied IgG subclasses. These findings are presented in Figure 2. Generally, the presence of weak/non-C-binding IgG subclasses inhibited C1q-binding. The degree of blocking was linear to the fraction of IgG2/4 that was present. IgG4 induced a slightly stronger blocking effect. A 2- to 4-fold excess of IgG2/4 inhibited C1q-binding by 50%. At an explicit high degree of excess (10-fold), the blocking effect was almost complete, but more pronounced for IgG4 (IgG4: ≥ 96% blocking; IgG2: ≥ 90% blocking).

3.3 | C1q-binding of high IgG2/4 HLA Ab in clinical samples

Next, we retrospectively analyzed IgG pan-, IgG subclass- and C1q-data from clinical pre-transplant serum samples of 72 sensitized patients. Sixty-six patients (92%) revealed IgG subclass mixtures with at least 1 C-binding IgG subclass (IgG1/3) and at least 1 weak/non C-binding IgG subclass (IgG2/4) on 1618 individual SAB. These beads were divided according the quartiles of their IgGpan MFI and further subdivided into those with an excess of IgG2/4 (at least twice as much IgG2/4 as IgG1/3) and those without. Twenty three of 66 patients (34.8%) had an excess of IgG2/4 on 216/1618 SAB (13.3%). Next, we compared the degree of C1q positivity (cutoff for positive result = 300 MFI) between these groups (Figure 3). The percentage of C1q positive reactions was lower on SAB with an excess of IgG2/4 across all IgGpan MFI quartiles: −1%, −12%, −6%, −4%. These results suggest a small but consistent C1q inhibition effect of IgG2/4 in clinical patient serum samples with IgG subclass mixtures.

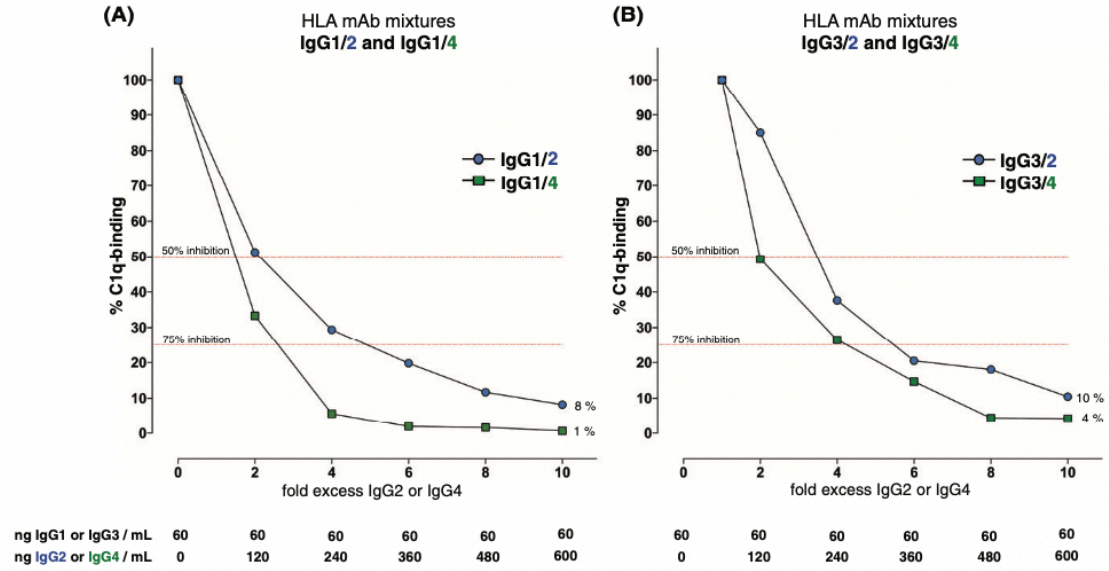

FIGURE 2 Percental C1q-deposition of mixtures of C-binding IgG subclasses (IgG1 or IgG3) and weak/non-C-binding IgG subclasses (IgG2 or IgG4) in relation to 60 ng IgG1 or IgG3 / mL alone (=100%). *X*-axis reflects "fold excess" of the weak/non-C-binding opponent (ratio of [IgG2/4]/[IgG1/3]) as well as the concentration of each IgG subclass. A, Mixtures of IgG1/2 and IgG1/4. B, Mixtures of IgG3/2 and IgG3/4. Dotted red lines indicate 50%- and 75%-inhibition.

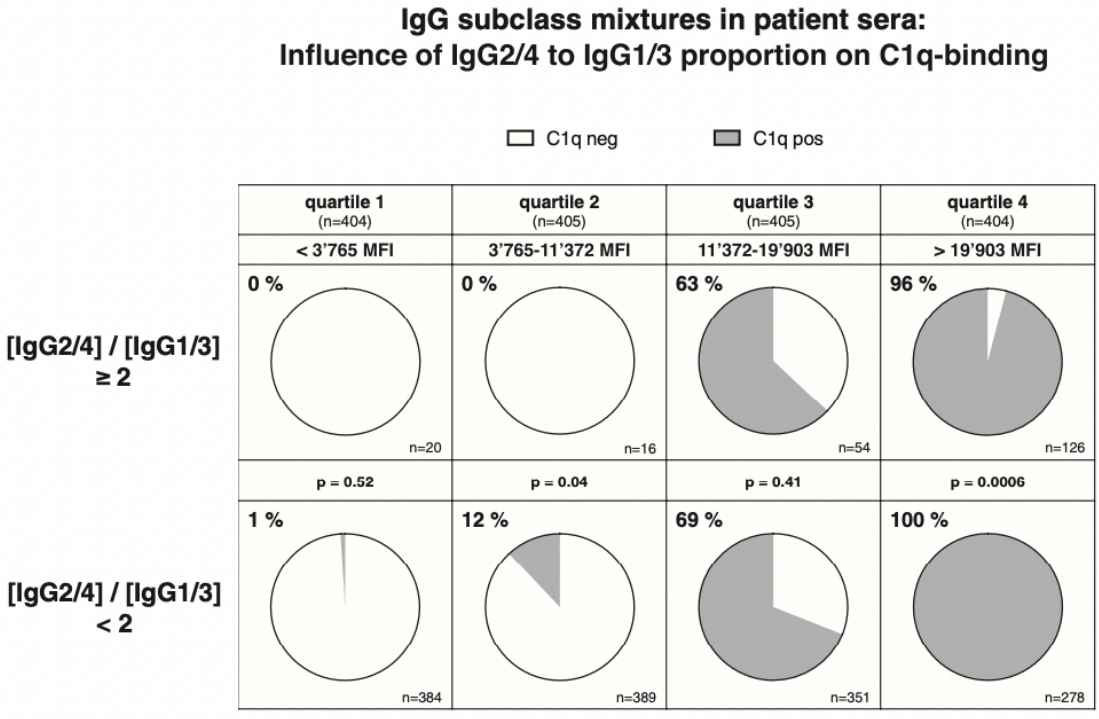

FIGURE 3 Pie charts representing the percentual proportion of positive (gray area) C1q reactions (≥ 300 MFI) in sera with HLA antibody IgG mixtures. Grouping into the 4 quartiles of their IgGpan signal. Further separation into mixtures with [IgG2/4]/[IgG1/3] ≥2 (upper pies) or mixtures with [IgG2/4]/[IgG1/3] <2 (lower pies). *P*-values from χ^2 test. MFI, mean fluorescence intensity.

3.4 | Spiking of DR7 Ab positive patient sera with IgG2 mAb anti-HLA class II

Finally, 4 *HLA-DRB1*07:01*-antibody positive sera from patients with known HLA-DR7-sensitization were chosen which fulfilled the following criteria: high IgGpan MFI, high C1q MFI signals, and an IgG subclass pattern negative for IgG2/4.

The serum samples exhibited IgGpan signals between 15 600 and 21 100 MFI and C1q signals between 6 100 and 17 000 MFI. IgG1 was predominantly present in all 4 sera. These sera were spiked with 2 concentrations (15 and 60 ng/mL) of the described HLA class II targeting mAb of subclass IgG2. Of note, applied IgG2 targets a public epitope present on all HLA-DR molecules, which by definition must be different from the patient derived HLA antibodies.

If the sera were spiked with the lower amount of IgG2, C1q-binding increased in 3 of 4 samples. When spiking with the higher amount of IgG2, all samples showed an elevated C1q-signal (delta change to baseline C1q signal: +7%, +19%, +71%, +58%). Details are provided in Figure 4.

4 | DISCUSSION

The key finding of this study is that IgG2 and IgG4 inhibits *in vitro* C1q-binding when competing with IgG1 and/or IgG3 directed against the same HLA epitope. The blocking effect of IgG4 was slightly stronger compared to IgG2.

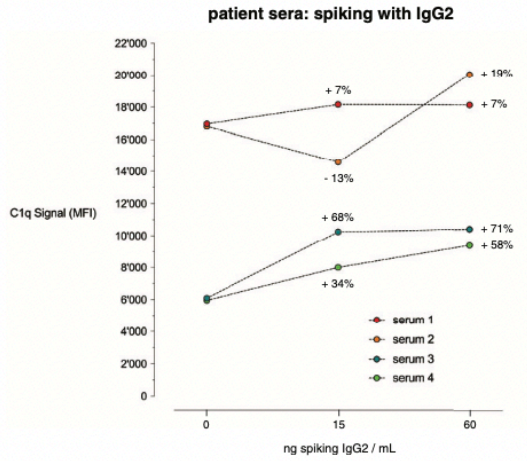

FIGURE 4 Spiking of 4 patient sera with IgG2 targeting a public HLA class II epitope and resulting C1q-binding change on *s* beads. First dot (left): C1q-level with unspiked serum, second dot (middle): C1q-level of serum spiked with low IgG2 concentration (15 ng/mL), third dot (right): C1q-level of serum spiked with high IgG2 concentration (60 ng/mL). The percentage at each dot indicates the delta change to the baseline C1q signal. MFI, median fluorescence intensity.

In the mechanistic part of the present study we kept all experimental conditions except the concentration of the applied IgG subclasses constant. Specifically, all reactions were performed on a single HLA-molecule level, the applied IgG subclasses had the same epitope-specificity/affinity, and the assay was strictly standardized. This standardized experimental system enabled us to attribute differences of measured C1q-binding directly to the concentration of the applied IgG subclasses. As anticipated, a slightly lower concentration of IgG3 was sufficient to initiate C1q-binding compared to IgG1, confirming the better accessibility of C1q to the larger hinge region present on IgG3.[16]

Mentioned standardized experimental system is helpful to understand the principal biological processes. However, patient samples are often much more complex. Human HLA-antibodies can be polyclonal and may contain IgG subclass mixtures, often with variable epitope specificities and different affinities. If IgG2/4 are directed against a different epitope on the same molecule, they can even synergistically enhance IgG1/IgG3-triggered C1q-binding.[17,18] Unfortunately, we could not explore this synergistic effect *in vitro*, because the required human IgG subclasses targeting 2 different epitopes on the same HLA molecule are currently not available. However, we retrospectively assessed C1q-binding of different IgG subclass mixtures in patient samples, where C1q-blocking effects and synergistic C-activating can coexist depending on the epitope specificities of the IgG subclasses. This analysis revealed that about a third of sensitized patients exhibits an excess of allo IgG2/4 over IgG1/3 (the constellation that led to C1q-blocking in the *in vitro* assays). Indeed, the latter patient Ab-mixtures showed slightly less C1q positive reactions on their SAB. This proposes a predominance of inhibitory effects over synergistic effects of clinical IgG2/4 HLA antibodies.

By contrast, when patient sera with exclusively C-binding Ab were spiked with IgG2 antibodies targeting a public and therefore different epitope (not located in the polymorphic region of the HLA molecule's peptide binding groove), C1q-binding mostly increased. The extent of this effect was variable from serum to serum and changed with different ratios of [spiking-IgG2]/[patient-IgG1/3], but not in a clear dose dependent manner, underlining the polyclonal complexity of each serum. These experiments highlight the influence of the kind, number and location of epitopes and their inter-epitope distances on antibody mediated complement-binding/activating. Clearly, further investigations are required to comprehensively study these relationships.

Taking together, if IgG2/4 is targeting the *same* epitope, an inhibitory effect can be expected. If they target a *different* epitope, synergistic effects are possible. In our patient population, the overall effect was slightly inhibitory. However, on the individual patient level, some IgG subclass/

epitope-specificity constellations might result in a synergistic net effect. In this context, it is intriguing that patients having DSA containing all IgG subclasses—and especially with the presence of IgG4—seem to be associated with an increased risk for antibody-mediated rejection and/or inferior graft survival.[11,19] Clearly, more studies are required to better understand the biological role of IgG2/4 subclasses directed against HLA molecules in organ transplantation.

We and other groups have reported a close relationship between high MFI of HLA-antibodies and C1q-positivity.[15,20–22] The finding of the present study further supports this observation. Low amounts of SAB-bound HLA-antibodies were not sufficient to result in a minimal Fc-residue proximity to form a stable binding to the C1q-molecule's globular heads (proximity of 30-40 nm according to Cooper[23]). By increasing the concentration, this requirement got suddenly fulfilled and the C1q assay turned positive.

In conclusion, if targeting the same epitope, the presence of an excess of IgG2/4 inhibits the extent of IgG1/3 triggered C1q-binding in the used *in vitro* system. Such IgG subclass constellations are present in about a third of sensitized patients and overall slightly reduce C1q-binding. By contrast, when spiking allo HLA antibodies with IgG2 targeting a different epitope, C1q-binding synergistically increases.

Conflict of interest

The authors have declared no conflicting interests.

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Chapter 5 PUBLICATION 3

Caveats of HLA antibody detection by solid-phase assays

5.1 BACKGROUND

5.1.1 History of HLA antibody testing

In the landmark study carried out by Patel and Terasaki [319], a significant finding was that kidney recipients with preformed cytotoxic DSA have a very high prevalence for immediate graft failure. As a consequence, kidney recipients were tested prior to transplantation by incubating their serum with donor lymphocytes and complement, followed by an analysis of the proportion of lysed cells. This test was termed 'complement dependent lymphocytotoxicity-test' because it permitted the exploitation of the complement activation function of DSA [319, 320]. For many years, this test was the reference standard to assess preformed cytolytic DSA. From the mid-nineties on, availability of recombinant HLA facilitated HLA antibody determination with higher specificity and sensitivity by an enzyme linked immunosorbent assay (ELISA) [321-323]. This solid-phase assay additionally detected the presence of non-complement activating HLA antibodies. Furthermore, the identification of the antibodies' HLA-specificities could now be assessed non-cellularly, although this required multiple test reactions. Already before the turn of the millennium, flow cytometry became the technique of choice in leading HLA laboratories to assess HLA antibodies at even lower detection levels. For this purpose, HLA coated latex microspheres, termed 'Flow PRA® beads', were used to characterize patient's HLA antibodies by flow cytometry [324-327]. Incubation of patient serum with a panel of HLA-beads, each presenting a cell-line specific set of HLA, enabled the simultaneous assessment of the presence of HLA antibodies and their specificities within a single test reaction. Shortly thereafter, it became evident that recombinant HLA, coated to xMAP® Beads and acquired at the Luminex platform, revealed an even better performance to assess HLA antibodies and allowed for better standardization [147].

5.1.2 HLA coated Luminex beads and their diagnostic relevance in the transplant setting

Luminex HLA antibody testing became the new gold standard for sensitive HLA-antibody detection in patient sera. The format of the test is a multiplex immuno-assay: patient serum is first incubated with up to 100 various fluorescent polystyrene microspheres (Luminex xMAP®-beads), each coated with a different HLA-type [termed 'Single Antigen Beads' (SAB)], to capture HLA antibodies [328]. Bound antibodies are subsequently visualized by means of a fluorochrome conjugated reporter-antibody. During the acquisition stage at the Luminex instrument, each HLA-bead is excited by two lasers in order to i) directly identify the HLA-type (on the basis of the emitted fluorescence, which is specific for each bead) and to ii) indirectly assess the degree of bound HLA antibodies (on the basis of the emitted fluorescence from bound fluorochrome conjugated reporter-antibodies).

Given the highly specific and highly sensitive results obtained by SAB assays, a debate arose within the transplant community, about whether pre-transplant cross-match (XM) **tests**, routinely performed so far in many HLA diagnostics laboratories, were still necessary [329, 330]. XM-tests are applied pre-transplant and simulate the transplantation *in vitro*. Importantly, XM-tests use donor surrogate cells (providing the donor HLA) and serve to indicate whether circulating DSA are present or not. Ideally lymphocytes are used for the most common XM-tests, the flow-cytometric XM and the CDC-XM, as they express HLA and can be drawn non-invasively. While the flow-cytometric XM assay [331, 332] is a classical antibody binding test, the CDC-XM assay is considered a functional test [319]. For both tests, donor T-cells (representing HLA class I) and B-cells (representing HLA class I and II) are isolated from whole blood and incubated with recipient serum (potentially containing HLA antibodies). Next, the extent of DSA binding to donor HLA is either measured directly by recording the signal of a fluorochrome-conjugated reporter antibody (flow-cytometric XM assay), or visualized indirectly by assessing the degree of antibody triggered complement reaction (CDC-XM assay). The problem of these XM-tests is their rather low specificity due to nonspecific binding/reaction.

After the successful introduction of the SAB test, which is largely free of such nonspecific reactions, and after the clinical relevance of the test results was proven, many kidney transplant centres changed their approach to pre-transplant risk assessment by switching from 'wet' (physical) XM testing to the 'virtual' XM (vXM) test [73, 75, 109, 333].

The vXM simply combines SAB results and HLA donor typing information: the presence of DSA can be virtually assigned, as long as the SAB panel consists of all HLA types determined for the corresponding donor [334]. Consequently, in the case of a negative vXM (meaning that no DSA are present), the wet XM test can be omitted [75, 335-338].

HLA antibody assessment by using the SAB technique is at present very common [109] and vXM has significantly improved pre-transplant risk stratification [75, 76, 330, 333]. Furthermore, additional features of HLA antibodies, for example IgG-subclass composition or complement-binding, have been explored mainly on the basis of SAB test modifications [279, 284, 286, 288, 290-293, 339].

5.1.3 Limitations of HLA antibody assessment using HLA coated polystyrene Beads

Several critical issues must be considered when Luminex SAB test results are clinically interpreted. For example, recombinant HLA molecules are very susceptible to conformational changes during their isolation from the cell surface and/or their immobilization to solid phases [340-342]. Such denaturation can cause false positive SAB test results due to binding of antibodies to neo-epitopes present. Another example concerns the fact that similar HLA-epitopes are present on multiple HLA-beads, resulting in a decreased signal on donor-relevant HLA-beads for SAB multiplex tests (‘shared epitope phenomenon’ [131, 343]). Moreover, special attention must be taken when drawing conclusions with respect to the test signal level. Indeed, the United States Food and Drug Administration (FDA) has declared the SAB test a qualitative test only. Still, the test results are often interpreted quantitatively, assuming a superior clinical relevance of high mean fluorescence intensity (MFI) signals. However, the results of several studies have demonstrated that the correlation between SAB signal strength and the clinical outcome is far from perfect [344-346] and that sera of hypersensitized patients often generate false low MFI signals [78, 131, 132, 347], assumedly due to an excessively high antibody concentration, leading to steric hindrance.

5.1.4 Aims of this review (Publication 3)

This review on SAB testing summarizes both the strengths and flaws of the test. It addresses the issue of the clinical relevance of SAB test results, the possible uses of the assay within the pre-transplant risk stratification and the post-transplant monitoring, as well as the

technical and clinical interpretation of SAB data. Furthermore, it suggests improvements for the future.

Specifically, we have aimed to explain:

- the factors that are contributing to the final test signal
- why the SAB test signal should not erroneously be associated solely with HLA antibody concentration
- why DSA strengths are potentially underestimated
- two different approaches on how to set the (technical) SAB assay cutoff
- the discrimination between the technical and the clinical cutoff
- the main reasons for false-positive and false-negative SAB test results
- which endogenous molecules of the patient's undiluted serum have the potential to generate nonspecific positive test reactions
- the impacts of high titre HLA antibody sera on SAB test results
- that the SAB test is very susceptible for antibody-mediated complement accumulation and how this can be prevented
- which/how medical drugs can impact SAB results
- the advantages and disadvantages of HLA presented on SAB as compared to native HLA on the cellular surface
- the occurrence and the impact of slightly denatured HLA on SAB and how antibody binding to neo-epitopes can be recognized
- that SAB antibody reactivity pattern are the result of HLA epitope binding
- that HLA epitopes are unevenly distributed across the SAB
- why HR-2F donor HLA typing is important in case of allele-specific DSA
- why Ethnicity-adapted SAB panels might more accurately assess DSA epitope-specificities
- the value of SAB assay modifications and how their application complete the picture of clinical DSA parameters
- that single SAB, beside the SAB panel, could specifically serve to absorb/elute/re-analyze DSA from highly poly-specific HLA antibody sera

- that the availability of an IgG/IgM/IgA-specific reporter antibody cocktail potentially enhances DSA testing
- what guidelines should be considered for pre-transplant DSA screening by using SAB

5.1.5 Placement of Review

The Review was published in **Transplant International**, the official journal of the European Society for Organ Transplantation (ESOT), the European Liver and Intestine Transplant Association (ELITA) and the German Transplantation Society (DTG). It is one of the premier European-based scientific journals in the field of transplantation and aims to promote transplantation research and to distribute subject-specific information. All manuscripts are peer-reviewed. Transplant International reaches a wide international readership, mainly clinical professionals who are primarily responsible for the accurate interpretation of SAB test results. The latter is the main reason why we have chosen this journal to publish our review.

5.2 PUBLICATION 3

Transplant International

REVIEW

Caveats of HLA antibody detection by solid-phase assays

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SUMMARY

Solid-phase assays for human leukocyte antigens (HLA) antibody detection have clearly revolutionized the field of HLA diagnostics and transplantation. The key advantages are a high sensitivity and specificity for detection of HLA antibodies compared with cell-based assays, as well as the potential for standardization. Solid-phase assays enabled the broad introduction of tools such as "virtual crossmatching" and "calculated panel reactive antibodies," which are essential components in many organ allocation systems, kidney-paired donation programs, and center-specific immunological risk stratification procedures. The most advanced solid-phase assays are the so-called single antigen beads (SAB). They are available now for more than 15 years, and the transplant community embraced their significant advantages. However, SAB analysis and interpretation is complex and many pitfalls have to be considered. In this review, we will discuss problems, limitations, and challenges using SAB. Furthermore, we express our wishes for improvements of SAB as well as their future use for immunological assessment and research purposes.

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Key words
HLA antibody, solid phase assays, transplantation, single antigen beads

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Solid-phase assays: a revolution in HLA antibody diagnostics

Solid-phase assays for HLA antibody detection have clearly revolutionized the field of HLA diagnostics and transplantation. The key advantages are a high sensitivity and specificity for detection of HLA antibodies compared with cell-based assays, as well as the potential for standardization [1,2]. Solid-phase assays enabled the broad introduction of tools such as "virtual crossmatching" and "calculated panel reactive antibodies" (cPRA), which are essential components in many organ allocation systems, kidney-paired donation programs, and center-specific immunological risk stratification procedures [3–7]. In addition, a much more accurate assignment of donor-specific HLA antibodies (DSA) improved the diagnosis and classification of antibody-mediated rejection (ABMR) processes in various transplanted organs [8,9].

The most frequently used system for HLA antibody detection by solid-phase assays is the Luminex platform. Two vendors offer different products ranging from rather less expensive screening assays containing a collection of HLA molecules on their surface to more expensive single HLA antigen beads (SAB) to define the specificity of HLA antibodies. In this review, we will discuss problems, limitations, and challenges using SAB on the Luminex platform. Furthermore, we will express

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our wishes for the improvements of SAB as well as their future use for immunological assessment and research purposes.

Readout of the Luminex platform

Most commercial Luminex tests for serological approaches do not exploit the full potential of the xMAP® technology, yet the SAB assay that uses up to 100 differently coated beads does. This panel of HLA-coated SAB is incubated with the patient serum, followed by a second incubation with the detection antibody conjugate. Each SAB carrying an individual HLA protein is identified by the first laser of the Luminex instrument. The second laser excites the fluorochrome of the detection antibody. The emitted fluorescence thus reports the number of bound HLA antibodies. To define negative and positive results, a two-step procedure has to be applied.

In a first step, the signal must be technically adjusted for background fluorescence signals. This adjustment can be achieved either "classically" by subtracting the signal of the negative control bead and a negative serum, or "biologically" by subtracting the average signal of those SAB representing self-HLA of the recipient [10]. The remaining signal is reported as baseline subtracted trimmed (removal of upper and lower 5% extreme values) mean fluorescence intensity (MFI). It represents the "technical" cutoff. A second step determines which level of MFI indicates clinically relevant HLA antibody strength (i.e., predicting ABMR and graft survival). This "clinical" cutoff should be set in accordance with the collaborating transplant center and is discussed later.

Technical challenges and limitations

Due to the complexity of the SAB analysis, false-positive and false-negative results can occur and should be carefully evaluated (Table 1). Here, we will describe the most important technical challenges and current limitations in the SAB analysis potentially leading to false-positive and false-negative results.

Quantification

While the multiplex assay principle is suitable to qualitatively assess DSA according to their HLA specificity, their quantification is inappropriate due to the intrinsic characteristics of both SAB and DSA. First, precise quantification would require each SAB to be present in the same proportion and coated with the same quantity and quality of HLA molecules. Even though these bead properties were improved over time, conformity over such a high number of SAB will always remain challenging and a standard assessment for determining HLA molecule concentration on SAB is not established yet. Second, in the majority of cases DSA react to public epitopes present on several SAB [11]. Binding distribution of such DSA will subsequently diminish MFI on the SAB of interest (representing the HLA allele of the donor), and DSA strength will thus be underestimated.

Erroneously, MFI units are commonly associated with the concentration of HLA antibodies. It is important to realize that MFI reflects just a proportion of all HLA antibodies present in the serum, namely the rate of antibodies capable of binding to SAB in a stable manner under the applied assay conditions (i.e., short incubation time). The assessed proportion is, therefore, not only triggered by the antibodies' concentration but also by their affinity, a characteristic that greatly differs between monoclonal HLA antibodies, as accurately shown by surface plasmon resonance [12]. Furthermore, the measured MFI represents only the fraction of isotypes that are targeted by the applied detection antibody conjugate. Indeed, coexistence of IgM and IgA DSA has been described [13–18]. In an own evaluation of 26 renal allograft recipients having only one HLA class I IgG DSA, we found coexisting IgM DSA in 31% and IgA DSA in 33%, respectively (unpublished data). Independent of their clinical relevance, these antibodies—not monitored by the standard anti-IgG_{pan}—may have an influence on MFI, as they are competing for binding on SAB and thus may decrease IgG-derived MFI [18,19]. Moreover, the final level of MFI is influenced by concentration, affinity, and incubation time of the used detection antibody conjugate [20].

Interference of serum components

The serum matrix of the probe is a major interfering factor and also the most uncontrollable one. Indeed, multiple endogenous molecules that potentially cross-react with assay components or disturb the outcome of immuno-assays have been described [21]. Since SAB assays are using undiluted serum—in contrast to the majority of solid-phase immuno-assays—high quantities of albumin, irrelevant immunoglobulins, haptoglobin, transferrin, antitrypsin, and fibrinogen can interfere in two ways: (i) by masking the binding of specific HLA antibodies and (ii) by nonspecific binding especially to

Table 1. Main reasons for false-negative and false-positive reactions in the IgG SAB assay.

False-negative	False-positive
• "Dilution" of the MFI signal across multiple beads sharing the same epitope • Serum matrix masks binding of HLA antibodies • High amount of bound HLA antibodies leading to accumulation of complement components, which interfere with binding of the detection antibody (i.e., complement interference) • Massive excess of HLA antibodies leading to steric hindrance of binding (i.e., hook or prozone effect) • IgA and/or IgM antibodies competing for binding sites of IgG antibodies • Rabbit anti-HLA antibodies present in medical products (e.g., polyclonal anti-thymocyte globulin) competing for binding sites of human HLA antibodies • IgG antibodies with low concentration or affinity unable to sufficiently bind during the 30 min incubation time	• Exposure of neo-epitopes • Unspecific binding of serum matrix components • HLA antibodies present in medical products (e.g., polyclonal anti-thymocyte globulin)

the negative control bead [22]. The latter effect can be partially removed by Adsorb Out™ beads [23].

By far the most significant disturbing interference, however, is caused by complement components, which accumulate after binding of high amounts of complement activating HLA-specific IgM/IgG1/IgG3 on SAB. Indeed, if enough Fc domains of adjacently bound antibodies are in close proximity, C1q can initiate the complement cascade [24–26]. The complement component C3 was identified as the main cause for steric hindrance of binding of the antibody detection conjugate, resulting in low MFI [27]. The formation of the C1 complex can be inhibited by pretreatment of the blood sample with heat, dithiothreitol (DTT), or ethylenediaminetetraacetic acid (EDTA) [28,29]. Using plasma samples or adding EDTA to the patient serum sample has meanwhile become the preferred option of many HLA laboratories to prevent this so-called "complement interference" [30], which often is still erroneously termed "prozone effect" as pointed out by Berth [31]. Some sera do—uniquely or additionally—exhibit a prozone or hook effect, which describes the phenomenon of a low signal because of an oversaturation with the investigated molecules in an immuno-assay (here HLA antibodies). It is important to note that dilution diminishes both complement interference and prozone effect.

Interference of medicinal drugs

Polyclonal anti-thymocyte globulin preparations (Gravalon, Neovii, and Thymoglobuline, Sanofi-Aventis) contain rabbit anti-human HLA antibodies, which can partly be detected by the anti-human IgG [32,33]. Since Thymoglobuline is produced by immunization with human thymocytes of several individuals, multiple HLA class I and II specificities can be present. In the case of Gravalon (immunization with a single Jurkat cell line expressing HLA-A3, A32, B7, B35), the specificities are better defined, and thus, false-positive results are easier to identify [34]. In addition, these rabbit HLA antibodies compete with human HLA antibodies for binding to the SAB and can therefore diminish the MFI signal.

Intravenous immunoglobulin potentially contains human HLA antibodies of any specificity [35]. However, as this product consists of IgG from thousands of plasma donors, HLA-specific antibodies are very likely diluted below the limit of SAB detection [33,36]. It has been observed that high concentrations of intravenous immunoglobulin can significantly increase the background fluorescence making interpretation of the SAB assay impossible (personal communication with Howard Gebel, Emory University, Atlanta, USA).

Denatured HLA molecules

Positive SAB results may be caused by so-called "natural" antibodies and do not necessarily refer to an HLA antibody specificity being present *in vivo*. In 2008, Morales *et al.* [37] found repeatedly detectable HLA antibodies in sera of nonsensitized men. While these specificities were mostly directed against rare alleles of the general population, similar observations have also been made for more frequently existing specificities [38,39]. In the meantime, it became clear that SAB can carry denatured HLA antigens exposing cryptic, usually nonaccessible epitopes. These neo-epitopes originate from the SAB production process leading to some denaturation of HLA class I molecules, lacking β2

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microglobulin and/or the peptide in the binding groove. Furthermore, the transmembrane region and the cytoplasmatic tail of HLA molecules can be exposed on SAB, while these structures are embedded into the plasma membrane *in vivo*. Figure 1 schematically visualizes potential neo-epitopes on SAB class I. Although less data are available, HLA class II molecules, composed of two noncovalently associated polypeptides (α-chain and β-chain), can be denatured as well.

The clinical significance of these "natural" antibodies is a matter of debate and studies revealed conflicting results, even though the clinical impact might be rather limited [40–42]. Interestingly, one study reported that antibodies against denatured HLA antigens may also be able to bind to intact HLA molecules [43].

To reliably evaluate SAB test results, it is of critical importance to know the used bead sets. Since 2015, our laboratory monitors each new SAB lot with continuously obtained sera from nonsensitized males in order to identify "problematic" beads. The top 10 of these "problematic" beads from the three last lots for classes I and II are listed in Table 2. Some specific patterns such as DP1/DP5/DR53 and DR1/DR4/DR16/DQ7/DP19 were repeatedly observed. Notably, denatured HLA on SAB can be confirmed by parallel testing of suspected sera with acid-treated SAB [40].

Beads versus cells as HLA antibody targets

It is important to be aware of some fundamental differences between the physiological presence of HLA on the surface of surrogate cells (e.g., on T and B cells) and their rather artificial coating on beads. HLA molecules embedded in the lipid bilayer of the plasma membrane are mobile, highly accessible and tend to build clusters [44]. The precise organization of HLA antigens on SAB has not been explored, but they are fixed, and might be evenly distributed but arranged rather irregularly over the entire bead surface. Another striking difference between SAB and cells concerns the HLA molecule density. T and especially B cells show quite variable allelic HLA expression, while the HLA density on SAB is constant and two- to threefold higher (own estimation), which can lead to discordant results between SAB and crossmatch tests [45–48]. For example, if a patient serum with high amounts/affinities of DSA is incubated with donor cells having a low HLA expression, the complement-dependent cytotoxicity (CDC) crossmatch remains negative [48].

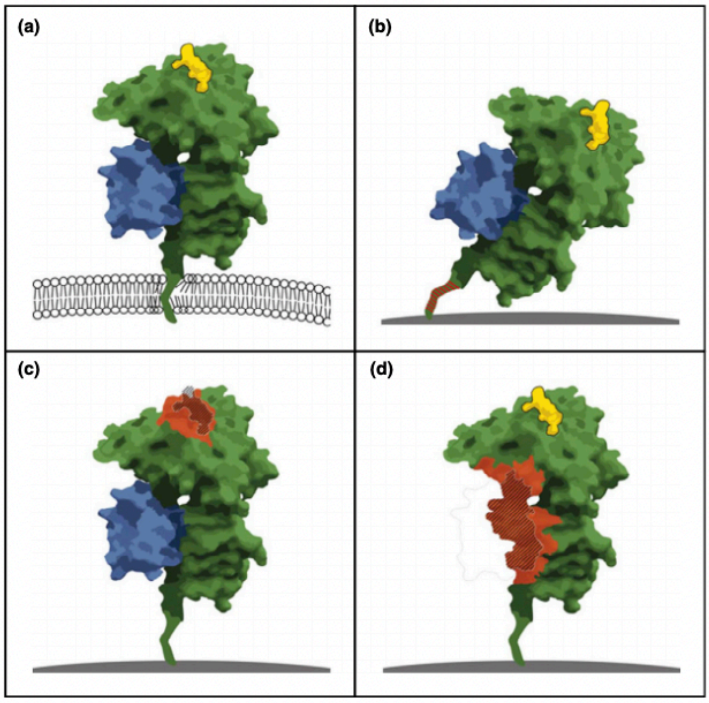

Figure 1 Potential neo-epitopes of SAB class I. Potential neo-epitopes are indicated by dashed lines and a hypothetical surrounding area in red color. (a) HLA class I molecule (α-chain in green, β2 microglobulin in blue, peptide in yellow) in its natural configuration embedded into the plasma membrane. (b) HLA molecule coated on single antigen beads exposing neo-epitopes in the tail structure. (c) Neo-epitopes in the area of the peptide binding groove at the α1 and α2 domains after loss of the peptide during the production process. (d) Neo-epitopes in the area of the α3 domain after loss of β2 microglobulin during the production process.

Table 2. Top 10 of positive SAB results in nonsensitized men.

Class I									
Lot 9 123 analyses from 95 nonsensitized men			Lot 10 133 analyses from 115 nonsensitized men			Lot 11 135 analyses from 111 nonsensitized men			
Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)	
B76 (B*15:12)	11.4	1824 (6726)	B76 (B*15:12)	14.3	1708 (6454)	B76 (B*15:12)	9.6	1327 (3010)	
B8 (B*08:01)	8.9	1547 (3995)	Cw17 (C*17:01)	8.3	1629 (5945)	Cw12 (C*12:03)	7.4	2981 (8733)	
Cw17 (C*17:01)	8.9	1568 (3295)	A66 (A*66:02)	7.5	1557 (3432)	Cw17 (C*17:01)	7.4	1846 (4480)	
Cw4 (C*04:01)	8.1	1054 (2476)	B37 (B*37:01)	6.8	2413 (6499)	B63 (B*15:16)	6.7	1932 (4947)	
A34 (A*34:01)	7.3	668 (829)	B57 (B*57:03)	6.8	1756 (4410)	Cw4 (C*04:01)	6.7	1339 (4854)	
A66 (A*66:02)	7.3	1170 (1902)	B73 (B*73:01)	6.0	1160 (2201)	A80 (A*80:01)	5.2	1343 (3067)	
B46 (B*46:01)	7.3	968 (3094)	Cw12 (C*12:03)	6.0	1661 (3490)	B44 (B*44:02)	5.2	1443 (3500)	
B57 (B*57:01)	7.3	1308 (5460)	B75 (B*15:11)	5.3	1115 (2457)	B45 (B*45:01)	5.2	2276 (4950)	
B73 (B*73:01)	6.5	1064 (1933)	Cw4 (C*04:01)	5.3	1026 (1581)	A11 (A*11:02)	4.4	2107 (3362)	
A11(A*11:02)	6.5	2543 (6659)	A25 (A*25:01)	4.5	1607 (5903)	B8 (B*08:01)	4.4	1983 (5074)	
Class II									
Lot 11 181 analyses from 118 nonsensitized men			Lot 12 220 analyses from 147 nonsensitized men			Lot 13 39 analyses from 38 nonsensitized men			
Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)	
DP1 (B*01:01/A*01:03)	12.2	1719 (4286)	DP1 (B*01:01/A*02:01)	18.6	1253 (4701)	DP1 (B*01:01/A*02:01)	15.4	1944 (4587)	
DP5 (B*05:01/A*02:02)	11.6	1364 (3398)	DP5 (B*05:01/A*02:02)	16.8	1285 (4660)	DR53 (DRB4*01:01)	15.4	855 (1662)	
DR4 (DRB1*04:04)	11.0	1047 (2856)	DP1 (B*01:01/A*01:03)	15.0	1257 (4269)	DP5 (B*05:01/A*02:02)	12.8	1212 (2748)	
DP1 (B*01:01/A*02:01)	7.7	1238 (2589)	DP19 (B*19:01/A*01:03)	15.0	915 (2202)	DR4 (DRB1*04:01)	12.8	917 (1578)	
DP11 (B*11:01/A*02:02)	7.7	1026 (1750)	DR16 (DRB1*16:02)	13.2	1120 (2482)	DP19 (B*19:01/A*01:03)	10.3	801 (1221)	
DR16 (DRB1*16:02)	7.7	1078 (2155)	DR4 (DRB1*04:04)	12.3	913 (2031)	DP3 (B*03:01/A*01:03)	7.7	724 (927)	
DR16 (DRB1*16:01)	7.2	1376 (2903)	DQ4 (B*04:01/A*02:01)	10.5	1090 (3771)	DQ7 (B*03:01/A*05:03)	7.7	787 (926)	
DQ6 (B*06:03/A*01:03)	6.6	1334 (2894)	DP14 (B*14:01/A*02:01)	9.1	1291 (3625)	DQ7 (B*03:01/A*05:05)	7.7	862 (1109)	
DR18 (DRB1*03:02)	6.1	958 (1837)	DQ7 (B*03:01/A*05:03)	9.1	3271 (9998)	DR103 (DRB1*01:03)	7.7	795 (909)	
DQ2 (B*02:01/A*05:01)	5.5	1756 (7551)	DR53 (DRB4*01:01)	9.1	1070 (2671)	DR4 (DRB1*04:02)	7.7	1033 (2026)	

From November 2015 to April 2019, three different lots of LabScreen™ SA beads class I/II (OneLambda) were used. Beads with an MFI > 500 were considered positive.

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In this context, it is important to discriminate between binding and functional assays. Binding assays such as the SAB or the flow crossmatch are very sensitive and can detect low amounts of HLA antibodies. By contrast, functional assays such as the C1qScreen™ test or the CDC crossmatch will only turn positive, if a high density of HLA antibodies is reached on the SAB or cell surface enabling C1q binding. Figure 2 visualizes the impact of the HLA molecule density and the amount/affinity of HLA antibodies on the most commonly used assays.

Critical issues for data interpretation

Once acquired, SAB data interpretation is challenging and requires careful contextual evaluation including the patient's HLA typing and sensitization history, the donor's HLA typing, as well as the awareness of technical caveats as discussed above [49].

An important aspect in interpretation of SAB results is the antibody reactivity pattern, which was recognized as cross-reactive groups already several decades ago [50,51]. Over the last years, the understanding of shared antigenic determinants among HLA molecules, so-called epitopes, has emerged and resulted in the availability of a tool (HLA Matchmaker) that aids in identifying those patterns (reviewed in [52]). Beyond commonly used MFI cutoffs, an antibody reactivity pattern belonging to an epitope being clearly present in a recipient's serum should be considered as relevant, even in case of a weak or borderline signal and especially if the epitope has been a mismatch in a previous transplantation.

With the increasing use of SAB, it has become clear that HLA antibodies target not only public epitopes, but also private epitopes present on one or just a few HLA alleles. This has important implications for DSA assignment. If HLA antibodies detected by SAB are present, the donor has to be typed for the corresponding HLA antigen/alleles to facilitate an accurate DSA assignment. This might include typing for DQA1 and DPA1, as well as high-resolution typing, if allele-specific HLA antibodies with potential donor specificity are present.

The current SAB products consist of close to 100 different HLA molecules for class I and II. However, more than 20 000 HLA alleles have been reported, and around 600 have a frequency of >1:100 000 persons in a predominantly Caucasian population from Germany [53]. The question arises, whether the SAB products can ever cover this enormous polymorphism of the HLA molecules. Luckily, the HLA antibodies target epitopes and there are very likely fewer HLA epitopes than HLA alleles. The HLA Matchmaker version 2 has a repertoire of 132 eplets for class I (derived from 561 alleles) and 279 eplets for class II (derived from 290 alleles). The current SAB panels cover 98.5% of these eplets (LabScreen™, OneLambda). Therefore, the eplet/epitope coverage is very high, but there is still a small chance to miss some HLA antibodies. Ethnicity-adapted SAB panels might further increase the overall eplet/epitope coverage.

Clinical impact of pretransplant DSA defined by SAB

The MFI cutoff for a clinically relevant positive SAB result is widely debated [54]. We believe that it should be defined together with the transplant center in the context of an overall risk stratification concept. It is beyond the scope of this review to provide a detailed assessment of all available data for all organs.

On a population level, pretransplant DSA defined by SAB are widely accepted as a risk factor for ABMR and inferior allograft survival for most solid organs [55–57]. However, on an individual patient level, the clinical impact of pretransplant DSA is highly variable ranging from uneventful courses to early ABMR with allograft loss [58]. There are many factors that influence the pathogenicity of DSA, which are still largely unexplored (e.g., epitope specificity of the DSA, magnitude and durability of the memory response, density of the antigen expression in the transplanted organ, regulation of effector functions) [59–62]. Indeed, many DSA might be rather unique for a given donor–recipient constellation, which makes it challenging to define universal risk factors with a high predictive value.

Modifications of the generic SAB assay

In an attempt to better predict the risk associated with DSA, the generic SAB assays have been modified. One approach is to assess the capability of DSA to bind C1q or C3d on SAB (reviewed in [63]). Notably, about 98% of DSA contain either IgG1 or IgG3 subclasses and hence have the intrinsic capability to bind complement [64]. The major determinant of C1q or C3d binding on SAB is the amount of DSA, which can be approximated by the MFI value [20,64]. It is not surprising that most studies found a very strong correlation of the MFI value and C1q or C3d binding [65–67]. While some studies including a recent meta-analysis found that C1q or C3d

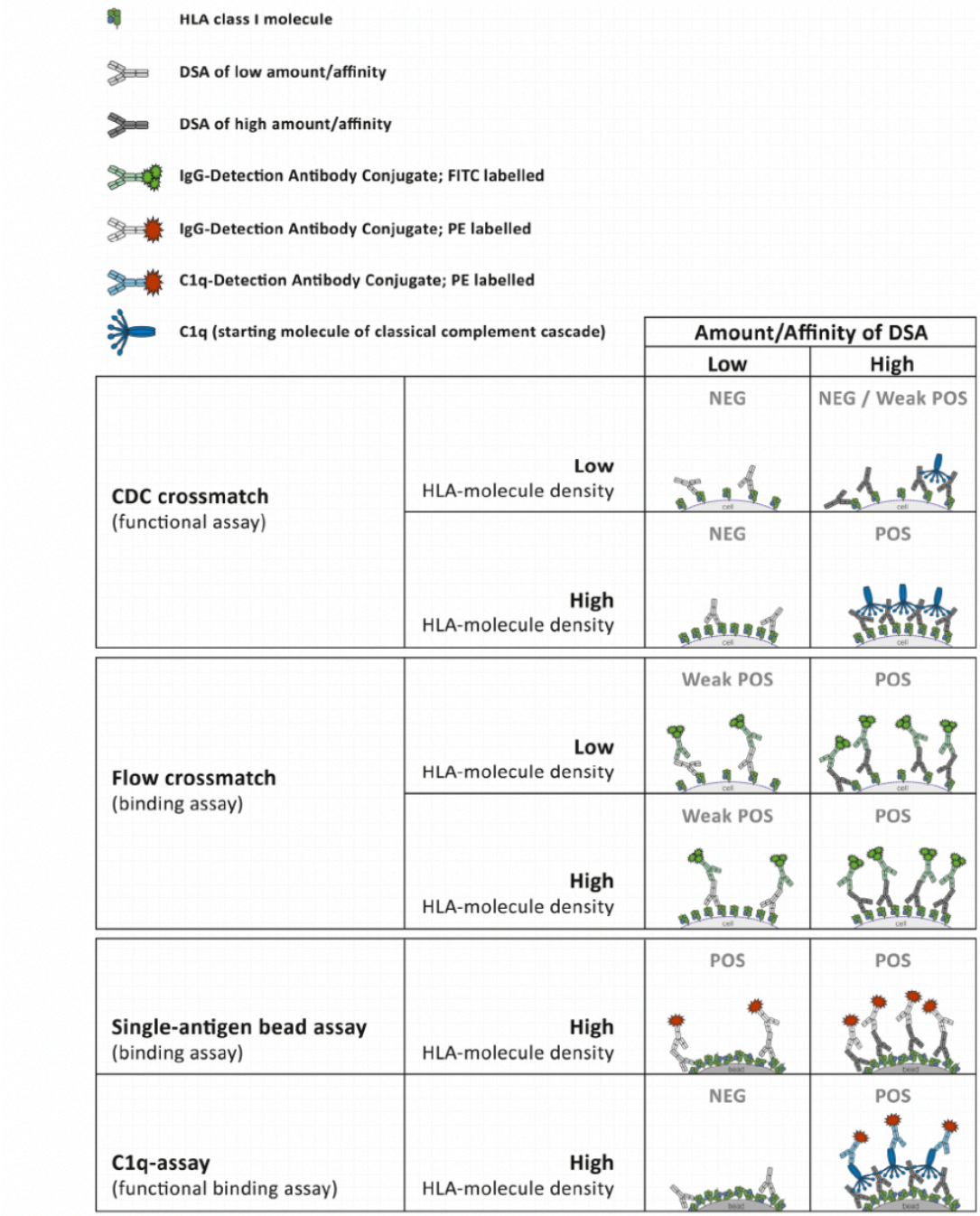

Figure 2 Impact of HLA molecule density and amount/affinity of HLA antibodies on test results. Based on the assay principle, the assays can be separated into binding assays (single antigen beads, flow crossmatch) and functional assays (C1q assay and CDC crossmatch). The anticipated results for all assays are given depending on the HLA molecule density and the amount/affinity of DSA. Overall, low amount/affinity DSA are still detectable in binding assays irrespective of the HLA molecule density. Functional assays require high amount/affinity DSA and a high HLA molecule density to become positive. Notably, single antigen beads have by default a high HLA molecule density.

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binding assays add some prediction beyond the MFI value, other studies did not [65,68–70].

Another approach is to assess the IgG subclass composition of DSA enabled by IgG subclass-specific secondary antibodies (reviewed in ref. [71]). The basic assumption is that strong complement binding IgG subclasses (i.e., IgG1 and IgG3) are more detrimental than weak or noncomplement binding IgG subclasses (IgG2 and IgG4). Unfortunately, the biology is much more complex [71]. High amount of IgG2 and/or IgG4 can block the effect of IgG1 and/or IgG3 subclasses if the antibodies target the same epitope. By contrast, these subclasses can act synergistically if they target different epitopes on the same HLA molecule [20,72,73]. It has also been shown that an expansion of DSA to IgG2/IgG4 subclasses rather indicates an advanced immune response [74]. A clear picture how to best use IgG subclass assays has not emerged yet [71].

The vast majority of studies used secondary antibodies specific for IgG isotypes. As mentioned previously, it is known that HLA antibodies can also contain IgA and IgM isotypes. Due to the predominance of IgG isotypes, it is difficult to decipher the precise contribution of other isotypes in the process of organ rejection. Generally spoken, it seems that the presence of IgA isotypes reflects a broader and more mature immune response, while IgM isotypes could indicate an ongoing recruitment of naive B cells [14,17,75].

As already discussed above, quantification of DSA by means of the MFI value can be misleading. Titration studies are the best method to capture the true DSA strength (reviewed in [76]). Alternatively, the results of flow or CDC crossmatches are used in many transplant centers to define thresholds as transplantation barriers [77].

Overall, it is still a matter of debate whether the modified SAB assays (C1q/C3d-binding; IgG subclasses; IgA/IgM; titration studies) enhance risk prediction beyond the generic SAB assay in a clinically significant way. It is conceivable that they provide very important information in specific cases, but their general application might not be necessary and they are currently expensive as well as labor-intensive.

Screening for HLA antibodies

The development of a pre- and post-transplant HLA antibody screening protocol can be deemed a transplant center-specific process that should take various factors into account, such as the local performance of the assay, own clinical experiences, consensus guidelines, and cost-related issues [78,79].

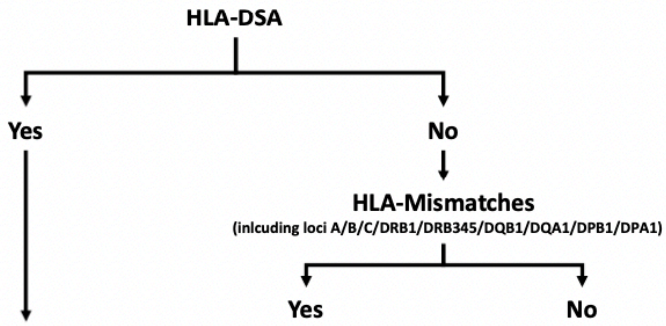
For patients on the waiting list, a regular screening strategy with additional testing following immunizing events or clinical decisions such as withdrawal/reduction of immunosuppression or allograft nephrectomy in previously transplanted patients should be established. To the best of our knowledge, changes in SAB-defined HLA antibody profiles in waitlisted kidney allograft recipients without interfering sensitizing events have not been studied. In our experience, HLA antibody profiles in such patients are quite stable, allowing to reduce the screening frequency to once a year, if a well-functioning reporting system for sensitizing events is in place [5,80].

Compared to pretransplant, there is even more controversy within the transplant community on when and how frequently a transplant recipient should be screened post-transplantation. In general, screening of DSA by SAB in the setting of allograft dysfunction with biopsy-proven microvascular inflammation or C4d deposition suggesting ABMR can be regarded as advisable at any time point, even though absence of DSA detection presumably because of absorption by the graft may occur [81]. As demonstrated by Sis *et al.* [82], DSA may even be absent in the context of ABMR with (severe) microvascular inflammation. This should not preclude its diagnosis and has also been acknowledged in the Banff 2017 meeting report [83]. Routine screening in the absence of allograft dysfunction is, however, more debatable and cannot, in terms of cost-benefit considerations, be commonly recommended within the first year post-transplant. Wiebe *et al.* [84] have nicely shown that the frequency of *de novo* DSA in the first year is only 2%, but steadily increases over the subsequent years at a rate of about 2%/year.

But how to deal with the detection of *de novo* DSA in a transplant recipient with stable allograft function beyond the first year post-transplant? Unfortunately, in case of evidence of allograft injury in a biopsy following *de novo* DSA detection, therapeutic options are currently very limited as neither the proteasome inhibitor bortezomib nor a combination of intravenous immunoglobulin and rituximab was effective in two randomized-controlled trials [85–87]. Detection of *de novo* DSA might, nevertheless, still be helpful to identify patient having insufficient immunosuppression (e.g., nonadherence or physician-induced minimization) and to tailor immunosuppression on an individual basis [88].

The future of SAB

Single antigen beads have been used in research and clinics for more than 15 years. They must be considered



Primary type of immune response	Memory immune response against HLA	*De novo* immune response against HLA	No immune response against HLA
Current risk assignment	High risk	Standard risk	Low risk
Key question for better risk stratification	• Magnitude/durability of memory immune response? • Epitope target of DSA?	• Immunogenicity of mismatched HLA-epitopes?	

Figure 3 General risk stratification based on presence/absence of donor-specific HLA antibodies and HLA compatibility.

a very important milestone in transplant diagnostics. The HLA and transplant community embraced their significant advantages, learnt to deal with their limitations, and developed skills for more accurate interpretation.

How can SAB be further improved and how can they contribute to a better immunological risk stratification? Immunological risk stratification is mainly based on the presence/absence of DSA and the number of HLA mismatches (Figure 3). If DSA are present, a memory immune response can be expected, but its magnitude and durability is currently not predictable. Furthermore, the targeted epitopes of the DSA might be an important determinant for the clinical impact, but this is also often unknown. In case of a *de novo* immune response, the load of mismatched epitopes—and even more their individual immunogenicity—might be the driving force for rejection. Novel "memory" assays and sophisticated software algorithms (i.e., HLA Matchmaker, PIRCHE II) have been developed to address these gaps [89–95]. All these tools and their further improvement rely heavily on SAB analyses. Detailed epitope analyses of DSA will often require some absorption/elution studies, which can be facilitated by individual SAB that are not compiled in a full SAB panel (so-called "singles") [11,96]. "Singles" were once on the market, but are currently not commercially available anymore. In addition, "singles" are also a valuable tool to study the affinity/avidity as well as the quantity of DSA.

Our wish list for future SAB products includes (i) SAB carrying only a minimal amount or no denatured HLA molecules, (ii) renewed availability of "singles", (iii) ethnicity packages covering the most frequent alleles and DQB1-DQA1/DPB1-DPA1 dimers for a given ethnic group, and (iv) a cocktail of secondary antibodies allowing for simultaneous detection of different immunoglobulin isotypes and IgG subclasses.

The HLA community will need to improve standardization of SAB across different providers and different HLA laboratories. Clinicians and researchers are called to fill the above-mentioned knowledge gaps in a common effort. May the SAB be with us!

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Conflicts of interest

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Chapter 6 FINAL DISCUSSION AND PERSPECTIVES

This section summarizes the main results of Publications 1 and 2 based on the aims and hypotheses addressed in Chapter 2. Furthermore, the findings of all three publications, their relevance and future aspects are critically discussed.

PUBLICATION 1

Alloimmunity in transplantation is the result of molecular differences between donor and recipient. The new concept of EbHM ensures an enhanced donor-recipient compatibility at the molecular level and represents an efficient strategy to reduce immune risk and its consequences, as demonstrated by a recent study involving a large number of patients investigated [129]. However, before the new concept will become part of clinical routine, important shortcomings need to be addressed.

It is important to realize that, given the large number of identical epitopes on different HLAs, it will never be possible to achieve complete epitope matching. It is the immunogenicity of eplets that must be given more importance with the aim of circumventing high-risk eplets. However, to date, there is scant information on the immunogenic risk of single HLA epitopes. Co-consideration of eplet immunogenicity strength will therefore make the HLA-epitope matching concept decisively stronger, as allo-sensitization and humoral rejection after SOT will be potentially reduced. It will promote acceptable HLA mismatch constellations for a given recipient and thereby identify suitable donors, currently considered as poor matches. Moreover, EbHM including eplet immunogenicity may guide physicians towards patient-specific tailoring of immunosuppression.

Although there is wide acceptance that the way to an improved HLA matching follows the epitope track [348], to date the new concept is not routinely used: neither for risk stratification, nor for organ allocation or immunosuppression fine-tuning. How can this reluctance be understood? It is not only the as yet imperfect definition of individual eplet immunogenicity, but rather the awareness that a final classification of the immunological risk will have drastic implications: underestimation of eplet-immunogenicity would lead to more

rejections and acceleration of graft failure, whereas overestimation of eplet-immunogenicity would prevent transplantation inadvertently. These considerations underline the great responsibility involved in establishing applicable HLA epitope characteristics.

Given this responsibility, our study results were established in an immunologically coherent cohort of non-sensitized healthy women that were exposed to a similar immunological event, namely pregnancy with their first child. It is well established that during pregnancy, paternal HLA can trigger CSA production [223, 233]. In comparison to transplant recipients, which are often pre-sensitized, represent varying HLA mismatches with their respective donor, as well as being immunosuppressed by various medications, the pregnancy model offers several advantages to study allo-immunization on the basis of generated CSA: i) time and type of HLA exposure are similar, ii) children are haplotype-mismatched only, and iii) HLA antibody development is not impacted by exogenous immunosuppression [349].

Our study was established on highly resolved mother/child HLA as well as highly-resolved antibody-specificity data from maternal serum at the time-point of delivery. This is of critical importance. Most eplet-DSA association studies published so far are based on presumed 2-field high-resolution genotypes translated from low-resolution HLA genotypes. As recently demonstrated by Senev at al., such inference produces notable misclassification of DSA [244]. Consequently, using incorrectly inferred recipient- or donor-HLA genotypes falsifies the assignment of eplets and DSAs' eplet-specificities. Our CSA study data do not suffer from these shortcomings as we established HLA-genotyping data of mothers and children on the allelic level, which required full validation and standardisation of a sophisticated NGS HLA typing method. The results thus enabled the characterisation of 3D structural differences between donor- and recipient-HLA and assured accurate eplet-calling as well as exact eplet-specificity assignment of CSA. Furthermore, our study used iBeads, a special version of SAB, which have been shown to be largely devoid of denatured HLA class I molecules [342, 350-352].

Multiple studies have linked eplet load and graft outcome, but this comparison is rather indirect. Another strength of our study is the fact that we investigated the impact of eplet-mismatches directly on the emergence of *de novo* CSA. Through our study results, we gained four main insights: i) the observed child-specific immunization was limited in its breadth, ii) the individual mismatched eplets responded differently, iii) highly reacting eplets were localised at specific sites on the molecule and often represented a special

geomorphological shape, and iv) although classified as AbVer, some eplets were unresponsive.

Referring to i) the narrow breadth of epitope-specificities from arisen CSA, our results present signatures of classic humoral immune responses. Epitope-mapping of the analysed CSA generally revealed rather low numbers of targeted epitopes and allowed for a relatively clear analysis. The narrow binding-specificity of analysed CSA indicates an expansion of only a minority of the significant numbers of existing B- and T-cell clones [175]. This observation is in line with the performance characteristics of an alloimmune response, generally directed against a restricted number of immunodominant epitopes [20, 178, 353], at the expense of other existing epitope-specific B- and T-cell clones [178, 354]. It is well established, that, within the 'indirect pathway of allorecognition', applicable for DSA (or CSA) development, T-cell epitope immunodominance is highly dependent on the affinity between antigenic structures on presented peptides and TCRs of T helper cells [176].

With regard to ii), concerning the different reactivity of individual eplets, our study results strengthen the presumption that different AbVer eplets represent dissimilar immunogenic potentials. The study's main target was to classify the immunogenic risk of AbVer HLA class I eplets. The established immunogenicity scores of the 72 currently defined AbVer class I eplets represent a first guideline with regard to low-, medium- and high-risk HLA epitopes for Caucasian donor-recipient constellations. All eplets were present in the population studied. Calculation of immunogenicity scores on statistically sufficiently high number of mismatched cases was, however, applicable for 34 AbVer eplets (47.2%) only. To include risk potentials of rarer eplets, larger studies are needed. The immunogenic reactivity evaluated for the 34 AbVer eplets ranged from 4 to 46 per cent, and ten eplets revealed reactivities above 25 per cent. Although the data should be considered preliminary, the established numerical immunogenicity scores can be regarded as a proxy for the detrimental nature of the respective mismatched HLA structures, and are proposed to be incorporated into centre-specific pre-transplant risk stratification algorithms.

Clearly, the established results must be validated in the transplant setting. We cannot exclude a slightly different recognition of HLA epitopes induced by immunoregulatory processes active during pregnancy [349]. Indeed, the fact that only about a third of the examined women developed IgG-type CSA, directed against structures on approximately a fifth of mismatched HLA, indicates a rather suppressed immune-recognition.

In reference to iii), the preferred shape and localization of highly reacting eplets, our observation reflects the role of the molecule's geomorphic landscape in the context of CDR accessibility [355]. Some examples are the protruding shapes represented by eplets 41T, 107W or 90D, which are all situated at flank positions of outward AA loops and revealed an accentuated immunodominance. The latter eplet was recently reported as the putative immunodominant determinant targeted by an alloantibody binding to HLA-A*11:01. The binding interactions involved between 90D and the respective antibody's CDR were stereo-chemically described from the respective X-ray crystallographic data [356]. Another occurrence was that although the investigated eplets are fairly homogeneously distributed over the lateral surface of the α1- and α2- subunits of the HLA class I heavy chain, eplets on α2, located on the opposite side of the nonpolymorphic beta-2-microglobulin (β2m), revealed higher reactivities. This underlines the importance of unhindered accessibility of BCRs' or antibodies' CDRs to the antigen surface. Nevertheless, it is important to note that, as exemplified in the publication mentioned above [356], monomorphic β2m structures are not always excluded as CDR targets. For other reactive eplets, however, no similar explanations could be found. If our findings would have been analysed with regard to physicochemical properties of eplets, we hypothesize that this could have potentially provided further clarifications in that regard. Indeed, the two most instructive features in this context are hydrophobicity of the involved mismatched AA side chains and their electrostatic charge [357, 358]. Two algorithms, HMS and EMS-3D (described in Chapter 3.1.5) focus on these characteristics and promise enhancement of humoral alloimmunity risk prediction [221], but this sophisticated approach needs further development.

The observation iv) that some AbVer eplets were non-responsive (i.e. five eplets in our study), shows that the classification 'AbVer eplets' does not automatically mean noticeable activity in the general human population. It is important to remember that such classification is allocated upon a single proof of an HLA-specific antibody binding pattern. Four of the five eplets that did not induce a humoral immune response in our setting, although present at high case numbers, were originally determined using the absorption/elusion technique and did reveal an equivalent TerEp, according to the information provided by the HLA epitope register. *Vice versa* and in line with other studies [95, 359], our data indicate some relevance with regard to CSA-development for eplets classified as non-AbVer (data not shown). Generally, and given the growing interest in including epitope compatibility for patient care,

it is essential to establish a standardised and reproducible procedure for justifying additions/changes/deletions of epitopes from the HLA epitope register in the future. This progress and the maintenance of a list with clinically relevant epitopes needs active effort of the HLA community.

With regard to further enhancements of current epitope matching concepts, I believe that previously established T-cell memory (Tmem) [360] and shared T-cell epitopes (STEP) [361] will gain in importance. Consequently, 'linked T and B-cell recognition' [348, 362] will be key. From an immunobiological point of view, chaining the B-cell and T-cell epitope is indispensable. Current epitope matching models, however, ignore this link. They only focus on B-cells (HLAMatchmaker, HLA-EMMA, EMS-3D) or on T-cells (PIRCHE), and they disregard the other component. Indeed, any DSA response is due to linked B- and T-cell recognition. Without T-cell confirmation, the B-cell response is less pronounced. *Vice versa*, without B-cell recognition, PIRCHE II scores are irrelevant, as no allo-HLA is internalized into B-cells. It was also shown that high HLA mismatch degrees lead to accelerated DSA formation, because 'linked recognition' works very efficiently. By contrast, if only few eplet mismatches (and high PIRCHE II) are present, or few PIRCHE II but a high eplet load, the feedback mechanism is weaker [354, 363]. As a follow-up of this doctoral thesis project, we consequently analysed the contribution of T-cell epitopes to CSA production, using data of the very same pregnancy cohort [364]. By linking B-cell and T-cell epitopes, we identified mismatched HLA derived peptides that contributed strongly to antibody formation in the presence of highly immunogenic eplets, indicating the potential for a refined aggregation of eplet and PIRCHE II matching beyond numeric chaining.

Another significant optimization potential concerns the composition of the SAB panel. The large number of identical epitopes on different HLAs in the current panel, originally composed to assess HLA-specificities of antibodies, often results in overlapping binding patterns and the calling of multiple eplet-specificities. Every SAB based eplet-specificity analysis suffers this deficiency, including ours. A more balanced HLA set (i.e. including rare HLA alleles) would reduce such ambiguous results. Importantly, assumption of multiple eplet-specificities becomes a disadvantage for DSA positive patients, as it proposes to avoid certain HLA mismatches. This, however, is the opposite of the new concept's intended goal, namely to identify non-targeted eplets and low-risk eplets, aiming to raise more donor opportunities. We have addressed this issue in the review about SAB-derived antibody characterization in

Publication 3. Moreover, in the follow-up study of Publication 1, where we focused on immunodominant HLA class II eplets, we described this phenomenon in detail [227].

Our study represents considerable restrictions: Firstly, the investigated number of first-pregnancies was rather low and only HLA class I eplets were evaluated. Secondly, we did not perform absorption/elusion experiments, which could have potentially helped to confirm or rule out certain CSA eplet-specificities. The reason for this omission is that relatively rare HLA molecule types (either soluble or solid phase bound) would have been necessary to separate antibodies of slightly different specificities. Unfortunately, such HLAs are currently not available. Thirdly, our study lacks in longitudinal observation. Especially post-delivery samples could have provided valuable information on whether pre-delivery non-responding mothers develop dnCSA post-delivery, or how CSA properties change over time. However, recalling the mothers back to the hospital for blood drawing is challenging. Fourthly, our study, as with most eplet studies so far, disregards inter-individual aspects of the hosts' immune system, which potentially alter the immune-response, and may therefore give an incomplete picture of eplet immunogenicity [365]. Finally, the available study material did not contain infantile or paternal cells to perform epitope-specificity profiles of CSA on natural HLA glycoproteins consisting of fucosylated biantennary structures (HLA class I) or bi-, tri- and tetra-antennary or high-mannose structures (HLA class II) [305, 306]. To date, no studies have examined to what extent glycosylation differs between recombinantly produced HLA on SAB and native cellular HLA. It remains to be elicited to which degree N-glycans on HLA, bound in close proximity to certain eplets impact CDR binding of BCR and the resulting generation of CSA/DSA.

In summary, our study data suggests a clear hierarchy between currently defined AbVer HLA class I eplet mismatches with regard to their capability to individually trigger child-specific alloimmune responses. After validation in the transplant setting, consolidated immunogenicity risk scores for individual eplets can be applied for organ allocation for patient specific immunosuppression in order to minimize the risk for dnDSA development and graft loss by avoiding high risk HLA epitope mismatches in SOT.

PUBLICATION 2

In Switzerland, about twenty per cent of kidney recipients receive transplants across DSA [107]. To meet their increased immunological risk, the composition and dose of their (induction-) immunosuppression is adapted. However, a better definition of the individual pre-established B-cell memory of each of these patients would help to personalize the immunosuppression. DSA features not yet considered might provide more insight into the preformed immunological memory and predict the severity of the effector functions of circulating DSA present. Since one of the most detrimental effects of DSA is complement activation, this study focused on complement binding capabilities of pre-established DSA. Indeed, complement binding represents an effector mechanism of DSA that substantially participate in AMR and subsequently to allograft damage.

In the first part of this research study, we focused on the functional capability of DSA interacting with C1q, the molecule that initiates the classic complement reaction. This mechanistic investigation required the establishment of a robust *in vitro* assay. This assay served as a model to study the impact of different antibody mixtures (simulating DSA mixtures) on C1q-binding and thus on complement activation. The results generated were devoid of interference from serum components, as all reactants were dissolved in buffer with low bovine serum albumin (BSA) content. Moreover, the assay was strictly standardized (constant experimental conditions with regard to the total amount of beads, reaction-volumes, incubation times, temperature, concentration of reporter antibody, etc.). Finally, using a definite HLA (DRB1*07:01/DRA1*01:01) and specifically reacting mAb allowed us to directly attribute differences of measured C1q-binding solely to the 'concentration' of the applied model antibody or model antibody mixture. The four model antibodies (IgG1, IgG2, IgG3, IgG4) all targeted the exact same (public HLA-DR/DP-) epitope. Indeed, the assay output was very informative with regard to the determined IgG-subclass complement-activation thresholds and the assessed dose responses (dose: IgG subclass concentration; response: C1q-binding). For example, we observed a slightly lower concentration of IgG3 (as compared to IgG1) necessary to initiate C1q-binding, which is in line with previously published data [143, 366] and indicates high accuracy of our *in vitro* model.

The main purpose of the functional assay was, however, to simulate different DSA constitutions and to measure the resulting net complement reaction effect. The most striking finding of this research was that HLA-specific IgG2 and – to an even higher extent – IgG4, when competing with IgG1/3 for binding against the same HLA target, were capable of strongly inhibiting C1q-binding. While it is well established that IgG2/4 *per se* exhibit low/no complement-binding performance [143, 312, 366], our results demonstrate their effect on coexisting IgG1/3: High concentration of IgG2/4 can abolish the effects of coeval strong complement-binders IgG1/3. Specifically, a two-fold excess of IgG4 over IgG3 or IgG1 already resulted in a decrease of C1q-binding of fifty to sixty-five per cent, while a six-fold excess exhibited an inhibition rate of eighty per cent or higher, and a ten-fold excess almost completely abolished complement binding. This suggests an interesting general (i.e. not restricted to HLA-specific antibodies) regulatory mechanism of IgG2/4: by class switching to IgG2/4, followed by high proliferation of corresponding IgG2/4 clones, an IgG1/3-inducced over-reaction can be diminished or corrected.

Given the fact that the IgG1/2/3/4 used were composed of the exact same hypervariable Fab region and thus presented the exact same affinity profile [315], measured C1q-binding could be solely attributed to the parameter 'concentration' of applied IgG subclass/(-mixture). Unfortunately, we could not examine the impact of 'affinity' on C1q-binding, since mAb targeting the exact same epitope but differing in their affinity are barely available. However, we speculate that HLA antibody affinity exerts an even enhanced effect on complement binding. Another missing clarification is whether or not *in vivo* IgG2/4 DSA have an increased affinity. Contrarily to IgG3/1, *in vivo* IgG2 and IgG4 often appear during a later phase of the humoral immune-response and some may emerge from cell clones with a history of several class switches. However, such cell clones do not necessarily produce antibodies with a comparably higher affinity, since immunoglobulin class switching [367-369] and affinity maturation [174, 370, 371] are independent processes [367, 372]. Our results indicate that IgG2 and especially IgG4, even if present at equal affinity, inhibit IgG1/3 triggered C1q-binding very efficiently, suggesting a protective mechanism of these two more terminal subclasses against complement-induced damage [311]. It is crucial to realize that these conclusions are only applicable to antibody mixtures targeting the same epitope.

By contrast, clinical samples often present HLA antibodies mixtures consisting of various affinities and epitope-specificities. In the context of the latter, complement responses

may be critically influenced by the spatial distances between the DSA targeted epitopes. Our results reflect the requirement of a critical distance between Fc residues of bound IgG molecules and C1q for an efficient C1q fixation [312, 314]. By increasing the concentration of IgG1 or IgG3 in our *in vitro* model, the constraint of critical proximity of bound antibody molecules were suddenly fulfilled, manifested by a rapid increase of C1q-binding. Furthermore, and in agreement with the conclusions of a mouse study conducted by Murata et al. [47], our spiking experiments demonstrated that IgG2/4 can synergistically enhance C1q-binding when they bind to epitopes adjacent to IgG1/3 targeted epitopes. This finding emphasizes the importance of HLA epitope location and the spatial distance between HLA epitopes. It also reassures of the benefit of DSA epitope-specificity analyses as conducted in Publication 1.

After having gained the described insights into the model experiments, we explored the IgG subclass distribution of HLA antibodies in genuine pre-transplant patient sera. The rational of this investigation was to assess the extent of antibody mixtures representing elevated IgG2/4, capable of critically modulating the complement reaction. By measuring the resulting C1q-binding on each relevant targeted HLA in the SAB panel, we explored the impact of IgG2/4 containing HLA antibody mixtures on complement activation. This retrospective analysis indicated that slightly more than a third of the investigated sensitized patients indeed exhibited HLA antibody mixtures with an excess of IgG2/4. Such mixtures, however, only targeted about thirteen per cent of HLA present in used HLA class I and II SAB panels. While IgG2/4 in some of these mixtures contributed to an increased complement reaction, they demonstrated an inhibitory effect in others. Overall, the net complement binding effect of high content IgG2/4 HLA antibodies was slightly inhibitory. Importantly, these measurements were realized for all HLA antibodies present in pre-sensitized patients. Only a proportion of these antibodies are of clinical relevance, namely those with Fab against structures on mismatched donor HLA. Therefore, justifying the introduction of subclass examinations and C1q tests for all patients, with an ultimate benefit for only a small proportion of patients, remains difficult. However, while we have shown here only the value of DSA subclass information in the context of the expected complement response, DSA subclass information was shown to be quite conclusive in terms of FcγR binding and the associated recruitment of immune effector cells [44, 51, 373].

With regards to the restrictions of this study, we are aware that the C1q-binding capability of single IgG subclasses does not necessarily fully reflect an equivalent ability to activate the classical complement cascade. For example, although IgG3, in comparison to IgG1, binds C1q more efficiently, it was demonstrated that IgG1 is more effectual in triggering the complement reaction towards cell lysis, compared to IgG3 [143, 366]. Another limitation of the study is given by the fact that although the model IgG subclasses used were unique with regard to their equal Fab constitution, the very same restriction did not allow further exploration of the reported synergistic effect of IgG2/4 *in vitro*. To continue studying binding competition and the resulting effector function output of different co-existing HLA antibodies targeting different epitopes, I am currently specifying further HLA-specific mAb for their epitope-specificity, as well as for their affinity (manuscript in preparation).

In summary, our study has shown that DSA IgG subclass composition provides information on whether complement binding is triggered or suppressed. Our established *in vitro* model confirmed the following C1q-binding capability of each single IgG subclass: IgG3 > IgG1 > IgG2/4. An interesting finding was that IgG2 and especially IgG4 inhibited IgG1/3-induced C1q-binding when competing for binding to the same epitope. By way of contrast, IgG2/4 synergistically enhanced C1q-binding if bound to an epitope adjacent to bound IgG1/3. Our analysis of sera from sensitized pre-transplant patients identified HLA antibody mixtures with an excess of IgG2/IgG4 over IgG1/IgG3, present in about a third of pre-transplant patients and directed against thirteen per cent of Caucasian HLA-specificities. These clinical HLA antibody mixtures exhibited a slightly inhibitory effect overall on C1q-binding.

The scientific achievements from Publication 1 and 2 lead to several propositions that may potentially contribute to a personalized pre-transplant risk assessment. However, the benefit of the improoved diagnostic procedures (summarised in **Figure 8**) may only become apparent after an evaluation phase.

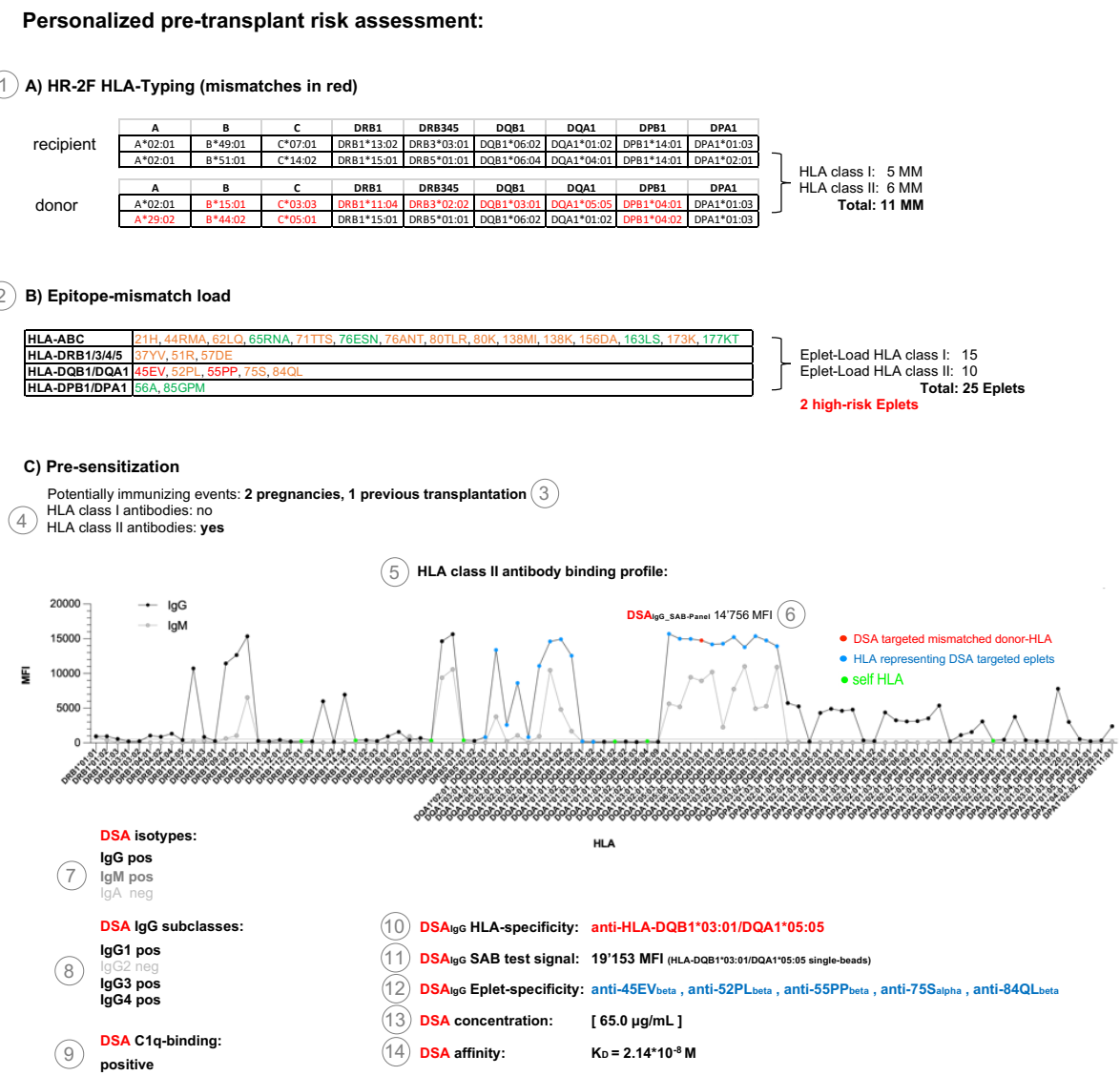

Figure 8. Personalized pre-transplant risk assessment. Using an example of a recipient with pre-established DSA, this overview shows the most important elements (and results) of an improved pre-transplant risk assessment. Points 1 and 2 are concerned with the immunological risk in the context to the structural mismatch constellation given by the potential donor, whereas the remaining points describe features of the circulating DSA present. Points 1,2,5,7,8,9,11,12 are elements that constitute the content of this doctoral thesis and are proposed to be potentially used in order to enhance the immunological risk assessment prior to a transplantation of DSA positive recipients. The text below provides additional information or explains the purpose of each investigation.

1) **HR-2F HLA-Typing** provides the basis to accurately determine the structural differences between donor and recipient. Mismatched donor HLA appears in red.

2) **Epitope-mismatch load** quantifies the burden of epitope subunits. The three colours indicate the immunological risk based on the work from Publication 1 and from Ref 227 (green: low, medium: orange, high: red).

3) **Number and route of pre-sensitization**: helps to explain a pre-existing immunological memory or indicates potential immunological memory no longer measurable.

4) **HLA class specific pre-sensitization**: indicates the HLA class specificity of diagnostically assessed circulating HLA antibodies

5) **HLA antibody binding profile**: according to the results from point 4, only HLA class II specific antibodies of present Ig isotypes (point 8) are shown here, to visualize the extent of pre-sensitization. In the context of a transplantation with the given donor, only antibodies that are targeting structures on mismatched donor HLA are relevant. The results of this epitope-mapping (as applied in Publication 1) indicate the eplet-specificities given under point 12. A notable part of the pattern is due to the presence of HLA molecules carrying the eplets (blue dots) present on the relevant mismatched HLA-DQB1*03:01/DQA1*05:05 (red dot). Of note, this example indicates a high similarity between the IgM- and IgG-profile.

6) **Resulting MFI value of DSA_{IgG} from the Luminex bead coated with the mismatched HLA**. As explained in 5.1.3, this value should not be overinterpreted, as the SAB_{panel} (multiplex)-test result is not suitable for quantification.

7) **DSA isotypes**: the presence of IgA and/or IgM indicates a broader immunological memory but also impacts the MFI signal in IgG tests (binding competition) as pointed out in Publication 3.

8) **DSA IgG subclass distribution**: Presence of IgG2 and/or IgG4 in the DSA mixture can impact the complement binding behaviour, as demonstrated in Publication 2.

9) **DSA C1q-binding status:** the test result indicates the net impact of the DSA mixture on complement-binding as illustrated in Publication 2.

10) **DSA_{IgG} HLA-specificity**: indicates the mismatched donor-HLA that represents the DSA_{IgG}-targeted eplets

11) **DSA_{IgG} SAB test signal**: The MFI value generated by using solely the SAB coated with the donor-HLA of interest (SAB_{mono}), represents a more reliable quantitative measure, because – in contrast to the SAB_{panel} – the 'shared epitope phenomenon' (explained in 5.1.3) does not apply.

12) **DSA_{IgG} Eplet-specificity**: provides the information about the eplets that are targeted by circulating DSA

13) **DSA concentration** is a not yet determined measure that provides the information about the current DSA production activity of present HLA-specific plasma cells.

14) **DSA affinity:** is one of the three features (see chapter 4.1.2) that has potential to better predict the hazardous potential of circulating DSA. We have recently developed a method to quantify DSA and to determine their affinity (manuscript in preparation). The serum of this example is one of which both characteristics have been determined. DSA affinity has substantial diagnostic potential and holds great promise to provide a novel clinically relevant assessment of transplant patients.

PUBLICATION 3

Microparticles coupled with recombinant HLA have completely overtaken traditional determination of HLA antibodies in coated ELISA plate wells. The SAB assay is considered the standard of care for the assignment of DSA in sensitized patients prior to transplantation [73] or to monitor DSA post-transplant [115].

Beside their simple determination, DSA can also be examined for their epitope-specificity [147] on the basis of the SAB binding pattern, if properly separated from third party HLA antibodies by absorption/elusion [155]. Thanks to their availability in transplant laboratories and their ease of use, SAB assays have steadily been used to characterize additional DSA qualities or to expand DSA measurements. As an example, SAB testing has been proposed to assess HLA antibodies obtained by *in vitro* activation of circulating memory B-cells [123, 124] from sensitized recipients to complete HLA antibody analysis from serum, in order to detect pre-established DSA no longer measurable by circulating antibodies anymore [126, 127, 374].

These multiple applications, as well as potential future purposes, give an immense importance to SAB tests. This makes it all the more important to know exactly the up- and downsides of the test method, to use it professionally and to assess SAB test results with the right expertise. Having been working with this assay since its birth, I have a clear awareness of the benefits but also the downsides of SAB tests. By writing this review, I and my author colleagues have taken on the responsibility to clearly explain the shortcomings of SAB testing and to propose appropriate improvements for the future.

The review first highlights the benefits of SAB testing. Clearly, HLA antibody detection has become much more standardized and sensitive by using SAB. Moreover, virtual cross-matching, as well as calculation of the sensitization broadness by means of cPRA (calculated panel reactive antibodies) has been highly promoted by the SAB technique and are very beneficial especially for pre-transplant risk assessment and organ allocation. Furthermore, SAB tests – in combination with HR-2F HLA typing – are of particular value for accurate DSA assignment and thus are of great value to prevent or to diagnose AMR.

The review continues to describe the Luminex bead technology, the reading platform, and how the test signal is generated. We explain the two thresholds that can be applied (the technical and the clinical cutoff) in order to discriminate positive from negative reactions.

Based on this, we highlight technical issues that critically impact the test result and we describe important limitations in the context of SAB testing. We further elucidate why the currently used test format does not reveal quantitative antibody results and we provide all known factors that impact the test outcomes towards false positive as well as false negative results. At the same time, we provide concrete advice on how to circumvent certain interfering factors.

Another major theme that we raise in the review concerns the presence of abnormal HLA-variants on SAB and the fact that recombinant HLA coated to SAB reacts slightly different in comparison to HLA expressed on cells. We have illustrated the differences between bead-based and cell-based tests and their behaviour in various situations (e.g. low/high HLA antibody concentration or low/high antibody affinity), as these different tests are often used and evaluated in parallel when clarifying the sensitization status of the patient.

Another topic of the review concerns SAB test modifications in order to discriminate between Ig isotypes or IgG subclasses of HLA antibodies or to measure antibody binding induced complement factors. We discuss the clinical relevance of these properties when used to characterize existing DSA.

At the conclusion of the review, we made various suggestions regarding the future of SAB testing, knowing that the technology will be increasingly used to further characterize DSA. Our propositions aim to significantly improve the use of SAB for HLA antibody epitope-specificity and to expand the measurement of all DSA (e.g. all isotypes/subclasses) in the near future.

The review was written especially for transplant medicine physicians who are involved in transplant diagnostics. It provides them with a guide to better understand the SAB test, its position in comparison to other existing HLA antibody tests and, in particular, helpful explanations regarding the test results' interpretation. We have received some appreciative feedback from the HLA community on this piece of work and we are pleased that this review seems to be fit for purpose.

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