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INCIDENCE OF IgA ISOTYPE OF HLA ANTIBODIES IN ALLOANTIGEN EXPOSED INDIVIDUALS

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OTKRIVANJE PRISUSTVA PROTUTIJELA HLA RAZREDA IgA U ISPITANIKA S PRETHODNIM IMUNIZIRAJUĆIM DOGAĐAJEM

Helena Car

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Kratki sažetak doktorske disertacije: Protutijela HLA razreda IgA mogu doprinijeti imunosnom odgovoru u primatelju presatka, no njihova uloga nije dovoljno jasna. Cilj ovog istraživanja bio je razviti test za otkrivanje protutijela HLA razreda IgA koristeći Luminex tehnologiju, utvrditi prisutnost protutijela HLA razreda IgA ovisno o načinu imunizacije te pokušati shvatiti njihovu ulogu na preživljavanje presatka u transplantaciji bubrega. Rezultati su pokazali da je modificirani test za otkrivanje prisustva protutijela HLA razreda IgA osjetljiv i pouzdan te da su protutijela HLA razreda IgA nisko zastupljena u serumima ispitanika koji su imali prethodni imunizirajući događaj.

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INCIDENCE OF IgA ISOTYPE OF HLA ANTIBODIES IN ALLOANTIGEN EXPOSED INDIVIDUALS

Helena Car

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Supervisor 1. Frans H.J. Claas, PhD, professor
Supervisor 2. Zorana Grubić, PhD, professor

Short abstract: IgA HLA antibodies may contribute to the transplant outcome but their role is not fully understood. Therefore, this study aimed to develop a Luminex based screening assay for IgA HLA antibody detection, explore their presence in groups of individuals with a different route of HLA alloantigen exposure and try to understand their role in kidney transplant outcome. Results show that the modified screening assay for IgA HLA antibody detection is sensitive and reliable and that the incidence of IgA HLA antibodies in alloantigen exposed individuals is low.

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1. INTRODUCTION

1.1. The immune system

The human immune system comprises a collection of cells, chemicals, and processes that function to protect the individual from harmful pathogens. To function properly, this defense system must be able to distinguish between self and non-self-antigens. The immune response is divided into innate immunity, which an organism is born with, and adaptive immunity, which an organism acquires during the lifetime. Despite the division into the innate and adaptive immune system, these two systems closely cooperate to defend the human organism against infection by the different pathogens [1]. The first line of defense to a foreign pathogen is provided by the innate immune response, a rapid antigen-independent (non-specific) defense mechanism without immunologic memory. The innate immune system includes the physiological epithelial barrier, phagocytic cells like macrophages and neutrophils, dendritic cells that capture and present antigens, cells that release inflammatory mediators such as mast cells and natural killer cells and the complement system [1]. The complement system contains over 20 different proteins and is named for its ability to complement the killing of pathogens by antibodies [2,3]. There are three pathways of complement activation: the classical pathway, which is triggered by antibody or by direct binding of complement to the pathogen surface; the MB-lectin pathway, which is triggered by mannan-binding lectin, a normal serum constituent that binds to the pathogen surface or antibody; and the alternative pathway, which is triggered directly on pathogen surfaces [4]. All of these pathways generate a crucial enzymatic activity that, in turn, generates the effector molecules of complement. The three main consequences of complement activation are opsonization of pathogens, recruitment of inflammatory cells, and direct killing of pathogens [2].

When pathogens are able to bypass innate immune defense, the adaptive immune system is activated [1]. The adaptive immune response is initiated when antigen-binding receptors on lymphocytes recognize a specific antigen. The two main types of lymphocytes that are involved in the adaptive immune system are the B lymphocytes and the T lymphocytes also called B cells and T cells. These cell types differ not only in the surface receptors that they possess, but also in their method of recognition of foreign antigen, and their effector mechanisms. The T lymphocytes mediate cellular immunity while B lymphocytes mediate humoral or antibody-mediated immunity.

1.1.1. Cellular and humoral immunity

The cell-mediated immune response is governed primarily by T cells. To be able to recognize intracellular antigens, T cells express a series of unique antigen-binding receptors (T cell receptors, TCR) on their cell membrane. T cell receptors can only recognize antigens that are bound to certain receptor molecules, called Major Histocompatibility Complex (MHC), positioned on the surface of antigen-presenting cells (APCs), such as dendritic cells, macrophages and B-cells [5]. When the TCR recognizes the antigen-MHC complex presented by APC, the T cell becomes activated and secretes cytokines which further stimulate the immune response [6]. The antigen presentation process stimulates T cells to proliferate and differentiate. Subsequently, T cells, which can be subdivided into CD8-positive (CD8+) T cells and CD4-positive (CD4+) T cells, become effector cells. Thereby, CD8+ T cells become cytotoxic T cells and CD4+ T cells differentiate into regulatory T cells or various types of helper T cells. Cytotoxic T cells can recognize an antigen-MHC class I complex and kill cells infected by foreign agents [7], whereas helper T cells recognize antigens in the context of MHC class II and provide help to cytotoxic T cells and B cells [8]. By providing help to B cells, helper T cells are responsible for amplification of the antibody response against the infecting pathogen. Regulatory T cells help suppress unwanted immune responses and keep the adaptive immune response under control. Once the infection is cleared, the majority of effector cells undergo apoptosis with a small percentage differentiating into effector memory T cells. These memory T cells can immediately respond upon re-exposure to the same antigen leading to a more rapid and effective secondary immune reaction [9].

Unlike T cells, B cells can directly recognize soluble antigens in body fluids through membrane-bound immunoglobulins on their surface that act as B cell receptors (BCR) for extracellular antigens [10]. All such receptor molecules on a single B cell are specific for one particular antigen. Still, the B-cell population as a whole represents a vast repertoire of receptors with different binding specificities. The immature B cell expresses immunoglobulin M (IgM) as BCR, while mature but naive B cells co-express IgM and IgD antibodies as BCR. When activated by foreign antigens to which they have an appropriate antigen-specific receptor, B cells undergo proliferation and differentiate. Subsequently, B cells become effector B cells called plasma cell which secrete soluble forms of immunoglobulins, known as antibodies [11]. During differentiation, a process called class switching takes place and some B cells can change from the production of IgM and IgD to other isotypes, such as IgG, IgA, and IgE [12]. This isotype/class switch changes the effector function of the antibody and improves its ability to

eliminate the pathogen that induced the response. A fraction of mature naive B cells do not differentiate into plasma cells but become memory B cells that use IgG, IgA or IgE as BCR and can respond rapidly when the antigen is encountered again [13]. Given their function in antibody production, B cells constitute the humoral immune response, a branch of the adaptive immune system [12].

1.1.2. Antibody structure and functions

An antibody or an immunoglobulin (Ig) molecule is a glycoprotein composed of one or more Y-shaped (monomer) units (Figure 1). The structural unit of an antibody is composed of four polypeptide chains, two heavy (H) chains and two light (L) chains joined together by disulfide bonds to form two symmetrical L-H pairs. There are two types of Ig light chains, namely lambda (λ) and kappa (κ), and five types of immunoglobulin heavy chains named by the Greek letters: α, δ, ε, γ, and μ. The amino-terminal ends of the polypeptide chains show considerable variation in amino acid composition and are referred to as the variable (V) regions to distinguish them from the relatively constant (C) regions. Each L chain consists of one variable domain, VL, and one constant domain, CL. The heavy chains consist of a variable domain, VH, and three constant domains CH1, CH2 and CH3. The variable regions of the light and heavy chains form the two antigen-binding sites which enable antigen binding [14].

Each Ig monomer contains two antigen-binding fragments (Fab2) and is said to be bivalent. The virtually infinite sequence diversity of the variable region is the basis for the great diversity of antigen-binding specificity that antibody has. A small (5-10 amino acids) region of each antigen-binding fragment (Fab) of an antibody forms an antigen-binding site or paratope. With his paratope, the antibody binds to the part of the antigen called an epitope. A single antigen may contain more epitopes that can be recognized by antibodies.

The hinge region is the area of the heavy chains between the CH1 and CH2 domains and is held together by disulfide bonds. If flexible, hinge region allows the distance between the two antigen-binding sites to vary. The CH2 and CH3 domains of the heavy chains form the Fc region which specifies the immunoglobulin class (IgG, IgA, IgM, IgD or IgE) and subclass (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2). Immunoglobulin classes differ in numbers of Y-shaped units that join to form the complete protein. For example, functioning IgG antibodies have one Y-shaped unit (monomer) and 2 antigen-binding fragments, while IgM antibodies have five Y-shaped units (pentamer) containing a total of 10 antigen-binding fragments. The

dominant class switching pathway leads from IgM/IgD to IgG3→ IgG1→ IgA1→ IgG2→ IgG4→ IgE→ IgA2. Specifically, IgM switches most commonly (approximately 85%) to IgG1, IgA1 and IgA2, while direct switches to IgE, IgG4 or IgA2 are rare (approximately 14%) and are most often produced indirectly through IgG1 and IgA1[15].

Antibody classes and subclasses differ in their physical appearance and their functions [16]. The diversification of antibody functionality via class switching is essential for mounting a protective response to different pathogens.

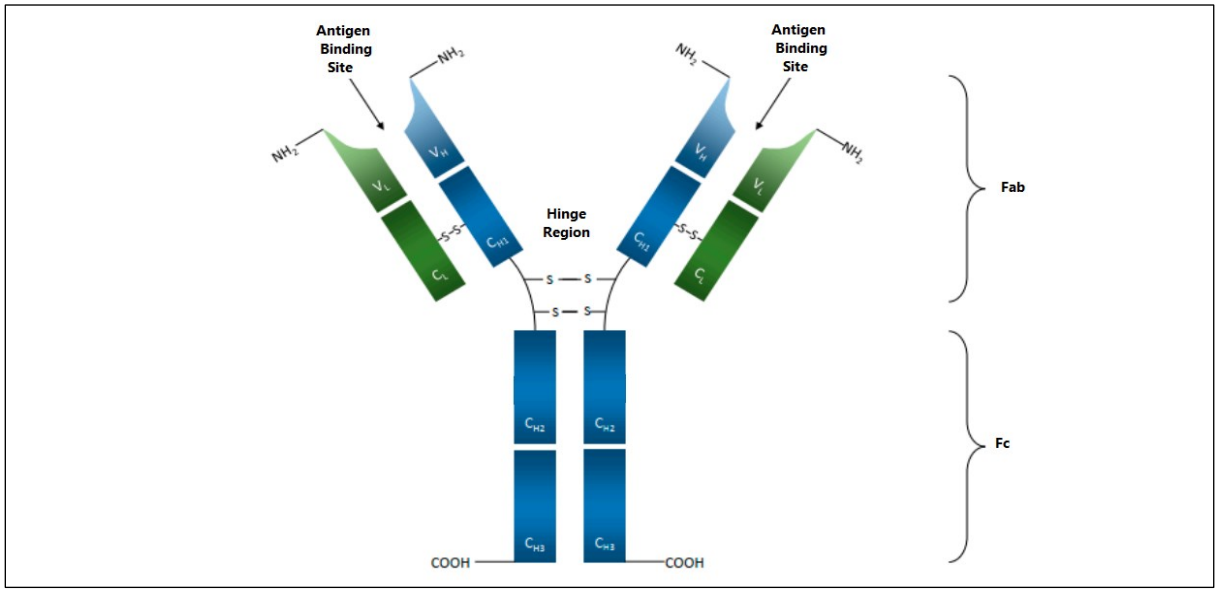


Figure 1. The basic structure of a monomer antibody. Adapted from reference [17].
Legend: C = constant domain, V = variable domain, H = heavy chain, L = light chain

IgM is the first Ig expressed during B cell development and appears as a B-cell receptor in monomeric form. Upon maturation and antigenic stimulation, mostly pentameric (970 kDa), rarely hexameric, IgM is secreted. IgM is associated with a primary immune response and frequently used to diagnose acute exposure to an immunogen or pathogen. It is the strongest inducer of classical complement activation. In adults, IgM accounts for 5-10% of total serum Ig.

Together with IgM, IgD (184 kDa monomer) acts as a receptor on the newly produced B lymphocytes. Circulating IgD is found in the serum, at very low levels. Functions of both the membrane-bound and circulating form of IgD are poorly understood.

IgG (150 kDa monomer) is the most extensively studied class of immunoglobulins. It is the predominant Ig isotype (70-75% of total Ig) in the serum and the only class of Ig that crosses

the placenta and provides passive protection during fetal and early post uterine life [18]. There are four IgG subclasses in humans (IgG1, IgG2, IgG3 and IgG4) and their effector functions are different. A key effector function of IgG antibodies is antibody-dependent cellular cytotoxicity (ADCC) in which antibody-coated antigens activate effector cells, such as natural killer cells or monocytes (eosinophils in IgA- and IgE-mediated ADCC), to destroy the antibody-coated target. IgG antibodies also contribute directly to an immune response including neutralization of toxins and viruses. Sublass IgG3, IgG1 and IgG2 are activators of the classical complement pathway, and with IgM, termed as complement-fixing antibodies.

IgA (160 kDa monomer, 400 kDa dimer) is synthesized in greater quantities than IgG, but due to its short half-life in serum (5 days versus 21 days for IgG), its concentrations are only 2-3 g/L. About 85-90% of the total amount of IgA is present in secretions (mucus, milk, saliva, tears) where it plays a role in the neutralization of microbes and their toxins. Approximately, 15% of serum Ig is IgA but the role of serum IgA is not clear yet [18]. More details on the IgA antibody are given in section 1.4. IgA antibody of the thesis.

Though it is present at the lowest serum concentration with the shortest half-life, IgE (188 kDa monomer) is a very potent immunoglobulin. It is associated with hypersensitivity and allergic reactions and defends the body against parasites [11].

1.2. Human leukocyte antigen system

One of the key players of the human immune system is the human MHC complex, also known as the Human leukocyte antigen (HLA) system. The HLA system was first described in the 1950s when Jean Dausset and Jan van Rood demonstrated that blood transfusions and pregnancies can induce the production of antibodies against antigens expressed on human leukocytes [19,20]. It is now known that the HLA system is involved in the recognition and presentation of self and foreign antigens and that the primary biological role of HLA molecules is in the regulation of immune response [21]. The role of HLA is particularly important in clinical transplantation where the immune response to non-self antigens (alloimmune response) can lead to graft rejection.

The HLA complex is a highly polymorphic genomic region on the short arm of chromosome 6 (6p21) and represents the most polymorphic genes of the human genome [22]. This means that many different gene variants of each locus are present in the human population, what is

illustrated by the number of alleles identified for each locus. Based on the latest World Health Organisation nomenclature update, 17.099 HLA class I and 6.695 HLA class II alleles have been described [23].

The HLA genomic region is 3.6 Mb big (about 0.1% of the human genome) and composed of three distinct regions, designated from the centromere to the telomere of the short arm as the HLA class II, class III, and class I region (Figure 2) [24].

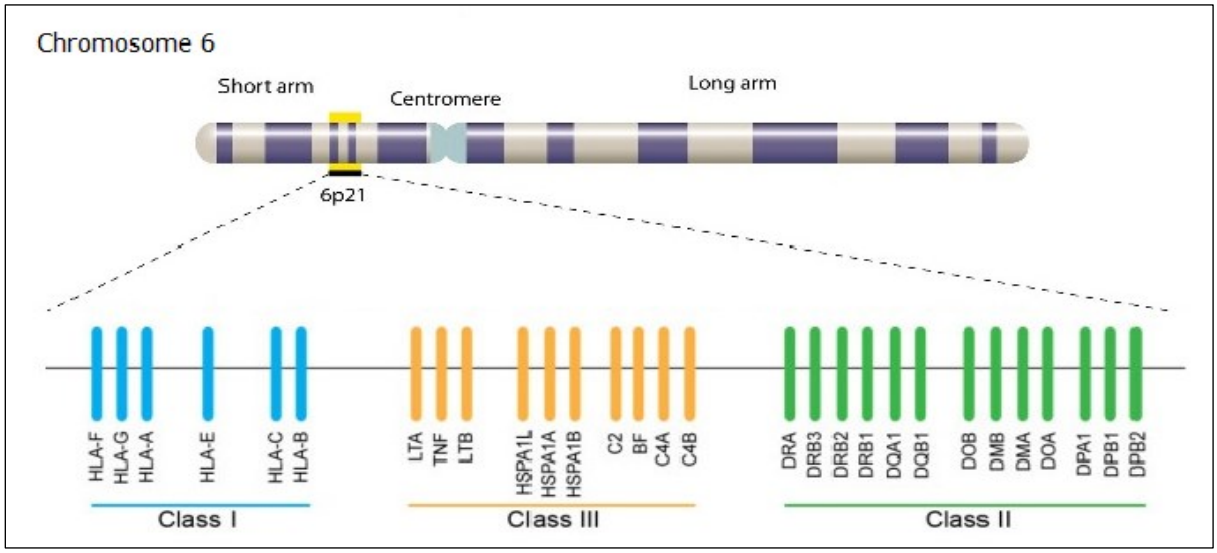


Figure 2. A simplified map of the HLA region. Adapted from reference [24].

The HLA class I region is positioned at the telomeric part of the HLA region. The HLA class I genes are classified into two groups, classical (HLA-A, -B, -C), and non-classical (HLA-E, -F, -G) HLA class I genes. In addition to the expressed HLA class I genes MIC (MHC-class I chain-related) genes are also mapped to this region. The classical HLA class I genes are highly polymorphic and their products (HLA class I molecules) are expressed on the surface of almost all nucleated cells and platelets. The main function of the classical HLA class I molecules is to present endogenous antigens to cytotoxic T cells [25].

The HLA class II region is located towards the centromeric part of chromosome 6. This region consists of subregions termed DR, DQ, DP, DM, DO that contain classical (HLA-DR, -DQ, -DP) and non-classical (HLA-DM, -DO) HLA class II genes. The LMP (Low Molecular weight Proteosome), TAP (Transport Antigen Processing) and Tapasin genes involved in the processing, transport and loading of HLA class I antigenic peptides are also located in this region. The classical HLA class II genes are very polymorphic. They encode HLA class II molecules which are expressed on APC such as dendritic cells, mononuclear phagocytes, some

endothelial cells, thymic cells and B cells [22]. Their main role is to present exogenous antigens to helper T cells, which can further activate macrophages, cytotoxic T cells, or B cells to secrete antibodies.

Within the HLA class III region, located between the HLA class I and HLA class II region, there are no HLA genes, but there are some genes involved in immune response like genes that encode complement factors, heat shock proteins and tumor necrosis factor [26].

1.2.1. HLA molecules

The HLA class I molecules consist of a polymorphic 45 kDa α or heavy chain, encoded by classical HLA class I genes (HLA-A, -B and -C) and a non-polymorphic β2 microglobulin (12 kDa) protein encoded by a gene on chromosome 15. The class I molecule is anchored to the cell membrane by the α-chain which is made up of three extracellular domains, α1, α2 and α3, a transmembrane region and a cytoplasmic chain. The α1 and α2 domains are folded together into a groove where peptides (8-10 amino acids long) can bind [27] (Figure 3A).

The HLA class II molecules are made up of two non-covalently associated chains, α (33-35 kDa) and β (26-28 kDa), both of which consist of two extracellular, transmembrane and cytoplasmic domains. Both, α and β chains are encoded by HLA class II genes (HLA-DR, -DQ, and -DP) and consist of two domains, α1 and α2 and β1 and β2. Together, domains α1 and β1 create a groove that is opened at both ends allowing longer peptides (15-25 amino acids long) to bind [28] (Figure 3B).

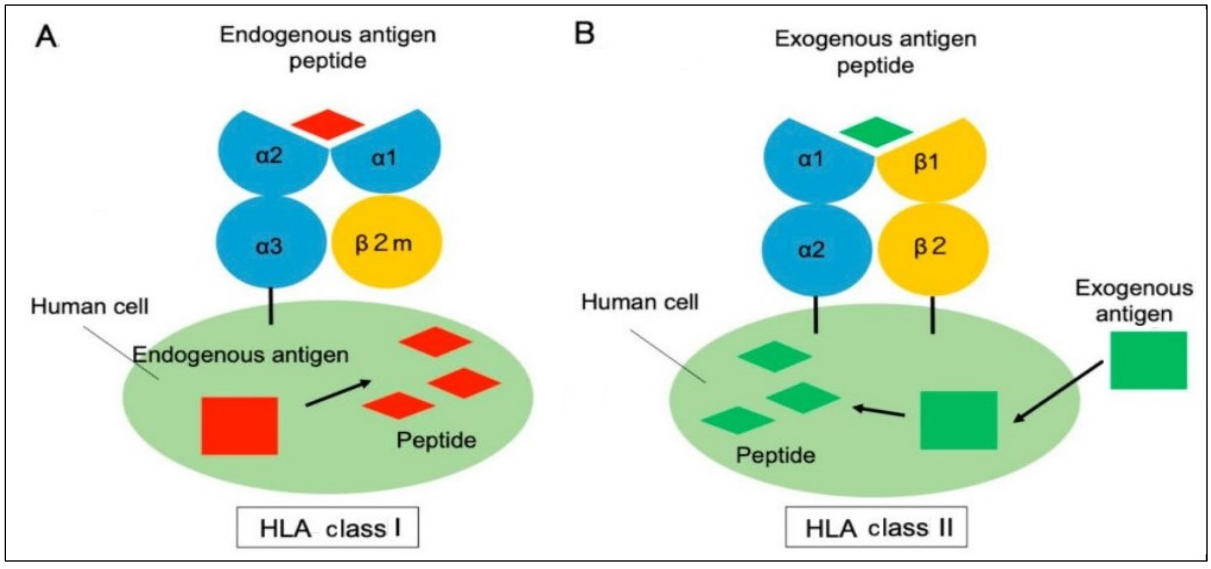


Figure 3. Schematic diagram of HLA class I (A) and HLA class II (B) molecules.
Adapted from reference [26].

The HLA molecules are a major part of the antigen-presenting complex on the cell surface, displaying their own and foreign peptides to lymphocytes. Therefore HLA molecules play an important role in the distinction between self and non-self and the proper functioning of the human immune system. Furthermore, there are numerous polymorphisms of HLA molecules in the human population. The major site of the structural polymorphisms in HLA class I and HLA class II molecules is in the region of the antigen-binding groove [27]. These polymorphisms provide diversity in peptide binding specificity and ensure that there will be always an individual that can present a peptide of a particular pathogen, which protects the human population from new or mutated pathogens.

1.2.2. HLA-specific antibodies

HLA-specific antibodies are produced as a response of the immune system to the foreign HLA antigens. The affinity (strength of one binding interaction with antigen), avidity (total affinity) and a class of the produced antibodies depend on various factors, including the route of immunization, the persistence and type of immunological challenge and the immune status of the host. The antigenic stimuli leading to HLA antibody formation in recipients may be presented during pregnancy [29], transplantation [30] or blood transfusion [31]. Still, some patients develop HLA antibodies in the absence of any of these sensitising events [32].

Previous pregnancy is one of the major causes of sensitisation and is due to the exposure of the mother to paternal HLA antigens present on the fetus [33]. Studies suggest that 50-75% of women develop antibodies against paternal HLA during pregnancy [34]. The produced HLA antibodies are normally multi-specific, in high titres, of high affinity and the IgG isotype. Although these IgG HLA antibodies can cross the placenta, there is no evidence that these antibodies are harmful to the fetus. In some of the women antibodies against paternal HLA antigens persist for long periods, while in other women these antibodies disappear early after delivery. In the case of transplantation of a woman where these antibodies had disappeared early after pregnancy, boostering of IgG antibodies directed against donor HLA antigens may occur as a result of presensitization through pregnancy [35].

In terms of blood transfusions, sensitisation occurs in around 20% of patients [36]. After renal transplantation, up to 60% of non-sensitised patients develop HLA alloantibodies for the first time [37]. Antibodies produced following transplantation are mostly IgG HLA antibodies, although rarely IgM HLA antibodies have been identified. In contrast, the majority of HLA

antibodies found in multi transfused patients are of IgM and IgG isotype. One or a few blood transfusions can induce broad and persistent HLA sensitisation in patients who have been previously exposed to HLA antigens through pregnancy, prior transplants, or massive transfusions [38].

HLA antibodies are significant risk factors for transplant rejection and failure and it has become evident that such antibodies bind to specific positions (epitopes) on the surface of HLA antigens [39]. Epitopes are conformational amino acid arrangements which may be present exclusively on a single HLA antigen (private epitope) or shared by multiple antigens (public epitope). In the early days of HLA, public epitopes were described based on serological cross-reactivity groups (CREGs) whereby antibodies against one specific antigen may bind to structurally similar antigens. Today, it is known that these antibodies correspond to distinct amino acid sequences in the HLA molecules [40]. The earlier strategy to define epitopes on HLA molecules considered each epitope as a string of continuous short linear sequences of a maximum of three polymorphic amino acid residues on the molecular surface called triplets. Later, a cluster of longer and often discontinuous sequences of polymorphic amino acids of an antibody-accessible position was defined as being capable of inducing an antibody response and was named an eplet [41].

To quantify triplet/eplet mismatches between the HLA antigens from a donor and a recipient, the HLAMatchmaker, a structurally-based computer algorithm, can be used. Thereby, it is possible to select a donor with a minimal number of HLA antigen mismatches and avoid induction of an antibody response. During the past years, several different approaches suggested that the number of foreign triplets, eplets, as well as physicochemical properties of mismatched amino acids, play a crucial role in the formation of antibodies against donor HLA antigens after transplantation [42]. Recently, it was demonstrated that in addition to B cells epitopes, T cells epitopes play a role as independent risk factors for the development of antibodies against donor HLA antigens [43]. With the purpose to identify T cell epitopes, a computer algorithm PIRCHE (Predictable Indirect ReCognizable HLA Epitopes) was developed. Yet, in addition to epitope determination, for proper use of epitope matching in clinical transplantation antigenicity as well as immunogenicity of epitopes should be defined. Thereby, it is important to differentiate between antigenicity, the capacity of specific antigen-antibody interaction, and immunogenicity, the ability of an antigen to induce an antibody response.

1.3. Clinical relevance of HLA antigens and HLA antibodies

High polymorphism of the HLA system has a beneficial effect on immunological response to pathogens but presents an obstacle for successful transplantation of organs, tissues or cells from a donor to a genetically different recipient.

There are two aspects to consider when assessing the role of HLA in clinical transplantation. Firstly HLA molecules expressed on donor cells are recognized as non-self by the recipient what induces an immune response in the recipient, against foreign HLA antigens on the donor cells [44]. Therefore, the main goal in transplantation is to match the HLA antigens of a donor and a recipient as best as possible. The second aspect is the role that antibodies against HLA class I and HLA class II molecules play in the rejection process in patients who have HLA antibodies formed before the transplantation, or who develop HLA antibodies after the transplantation.

The ability of recipient T cells to recognize HLA antigens of the donor is called allorecognition. There are three pathways of allorecognition: direct, indirect and semi-direct. In direct allorecognition recipient T cells can recognize intact HLA molecules on APC of the donor. In the indirect pathway, donor HLA molecules are taken up and processed by recipient APC and subsequently presented as allo-peptide in the context of self-HLA molecules to recipient T cells. In addition, semi-direct allorecognition can occur when recipient T cells recognize intact donor HLA molecules expressed on recipient APC [25].

1.3.1. T cell-mediated rejection

In all three pathways of allorecognition T cells play the central role. Once recipient T cells recognize alloantigen, they undergo clonal expansion, differentiate into effector subsets and take part in destroying the transplanted organ [45]. T cells produce cytokines which recruit inflammatory cells eventually leading to necrosis of the graft tissue. In addition, they can mediate B cell class switching to IgG antibodies [46].

1.3.2. Antibody-mediated rejection

Originally, only T cells were thought to mediate allograft rejection. Recent studies have shown that antibodies and the complement system also play an important role in allograft rejection [47,48]. When B cells are activated by exposure to a foreign antigen, with the help of T cells, they

proliferate and differentiate into plasma cells which secrete antibodies. Produced antibodies provide targeted immunity against specific foreign antigens. Moreover, they may activate the complement system and cause additional inflammation and graft injury [49].

Antibody-mediated rejection (ABMR) has been recognized as the leading cause of graft loss after kidney transplantation [50,51]. Antibodies that can mediate rejection include those against HLA molecules, endothelial-cell antigens and ABO blood-group antigens [52,53]. Still, the typical antibody-mediated rejection is caused by antibodies directed against HLA class I and HLA class II antigens [54]. Antibodies against HLA antigens produced in the recipient can be either present at the time of transplantation (pre-existing) in which case the patient is said to be sensitized, or can develop after transplantation (de novo) in response to foreign HLA antigens on the graft. Antibodies against donor HLA antigens, termed as donor-specific antibodies (DSAs), are one of the most important factors of both early and late graft dysfunction. The pre-existing and de novo developed DSA are risk factors for ABMR and potential graft loss [55,56].

Based on the pathophysiologic event, rejection can be classified as cellular (T cell-mediated) and/or humoral (B cell-mediated) rejection, as described [57]. Based on the time occurrence, allograft rejection can be hyperacute, acute or chronic [49].

1.3.3. Hyperacute, acute, and chronic rejection

Hyperacute rejection occurs within minutes to hours after transplantation and is caused by the presence of pre-existing DSA in the recipient's serum [58]. Hyperacute antibody-mediated rejection most frequently occurs in sensitized patients or those with a history of a previously failed allograft [59,60]. It is usually mediated by the presence of DSA able to fix complement leading to rapid graft loss. In recent years the incidence of hyperacute rejection is reduced due to improved antibody detection methods and desensitization protocols [61].

Acute rejection occurs within the first weeks to several months after transplantation and usually affects every transplanted organ to some degree [62]. It is caused by the mismatch in HLA antigens and is mediated primarily by T cells. Acute antibody-mediated rejection is another form of acute rejection which is caused by DSA.

Chronic rejection develops slowly, within months to years after transplantation and is the major cause of long-term graft loss [62]. It is caused by several processes, particularly by repeated inflammation and injury from both immunological and non-immune mediated reactions [62]. As

well as acute rejection, chronic allograft failure due to immunological causes involves alloimmunity to the graft by T cells and/or antibodies [58]. Still, chronic cellular rejection is uncommon.

Several authors agree that HLA antibodies denote the highest risk for antibody-mediated rejection and graft loss if they are complement-fixing and de novo developed [63-66]. De novo developed HLA class I complement-fixing IgG subclasses are described in association with acute antibody-mediated rejection, while de novo developed HLA class II, commonly non-complement fixing IgG subclass antibodies, are associated with chronic antibody-mediated rejection [65-68].

In the past years, studies investigated the effect of low strength pre-transplant DSA detected by the Luminex single antigen bead assay (described further on) on acute rejection rate and found graft survival correlations between pre-existing DSA and acute rejection [69-71]. Moreover, there is growing evidence supporting the role of pre-existing and de novo developed DSA as independent risk factors for acute and chronic ABMR and graft loss [65,72-74].

On the other hand, some data indicate that patients with detectable DSA do not experience ABMR and demonstrate similar graft survival as patients without DSA who do not experience ABMR [75]. There are studies supporting the hypothesis that some HLA antibodies of IgG, as well as IgM and IgA isotype, may even favour graft acceptance [76-78].

1.4. IgA antibody

IgA antibody was first recognised as an immunoglobulin isotype at the end of the 1950s by Heremans and colleagues. In humans, IgA exceeds the daily production of all other immunoglobulins (~70 versus ~22 mg/kg/day of IgG and ~7 mg/kg/day of IgM). It is the second most prevalent immunoglobulin class after IgG and normally present in serum at concentrations of 2–3 mg/mL, while the concentration of IgG is about 12 mg/mL [79]. The lower serum level of IgA immunoglobulin compared with that of IgG is due to the shorter circulatory half-life of IgA (3-5 versus 21 days of IgG) and the fact that approximately two-thirds of produced IgA is selectively transported to external secretions [80,81]. Most of the IgA (~85%) is localised at the mucosal tissues, especially those of gastrointestinal, respiratory and genitourinary tracts [82-84]. In fact, IgA is the most abundant antibody class at mucosal surfaces [85,86]. It is also the predominant immunoglobulin in milk, colostrum, tears, and saliva.

1.4.1. Structure of the IgA antibody

Serum IgA antibody exists principally as a monomer (~80% in healthy individuals), while at mucosal surfaces, IgA predominates in a dimeric (dIgA) and secretory (SIgA) form [86,87]. Some higher molecular weight forms, mainly trimers and tetramers, are also present. Similar to the other immunoglobulin classes, IgA antibodies are based on a monomer unit comprising two heavy chains (in this case, α-chains) and two light chains (Figure 4A). Like other immunoglobulins, the heavy and light chains of IgA are arranged into two antigen-binding Fab regions that are linked through the hinge region to the Fc region. The IgA Fc structure resembles that of IgG, but the positions of oligosaccharides and disulphide bridge arrangements are different. At the C-terminus of the α-heavy chain of IgA, there is an 18 amino acid extension known as the tailpiece that enables IgA to polymerise. IgA polymerises primarily into dIgA. These consist of the two IgA1 monomer units and one additional glycoprotein of 15 kDa, termed J chain [88]. The SIgA is formed following the interaction of dIgA with the polymeric immunoglobulin receptor (pIgR) responsible for the transport of dIgA into the secretions. During the transfer of the dIgA-pIgR complex across the epithelium, the receptor is cleaved and a secretory component (SC), a polypeptide of ~80 kD extracellular portion of the pIgR, remains bonded to the dIgA serving as an integral component of SIgA [89,90].

IgA antibody exists in two subclasses, IgA1 and IgA2 (Figure 4B). The predominant isotype is IgA1 with approximately 85% of total IgA. Typical proportions of the two subclasses are around 90% IgA1 and 10% IgA2 in serum and 40% IgA1 and 60% IgA2 in the mucosal secretions [91]. Both isotypes can form polymers.

Monomeric IgA1 and IgA2 differ in their α-heavy chain constant regions which are encoded by separate Cα genes. Although numerous sequence differences are found in their heavy chain constant regions, the major difference is the presence of a 13-amino acid long hinge region in the IgA1 isotype. As a result, the tips of the Fab arms (i.e. antigen-binding sites) of IgA1 can be spaced at much greater distances apart than those of IgA2 [92]. Hence, IgA1 may be able to interact simultaneously with two antigen molecules separated by a considerable distance, while IgA2 may have a more limited capability in this respect. Such capacity may afford IgA1 advantages in higher avidity by recognition of repeated antigenic structures spaced along the surface of certain pathogens.

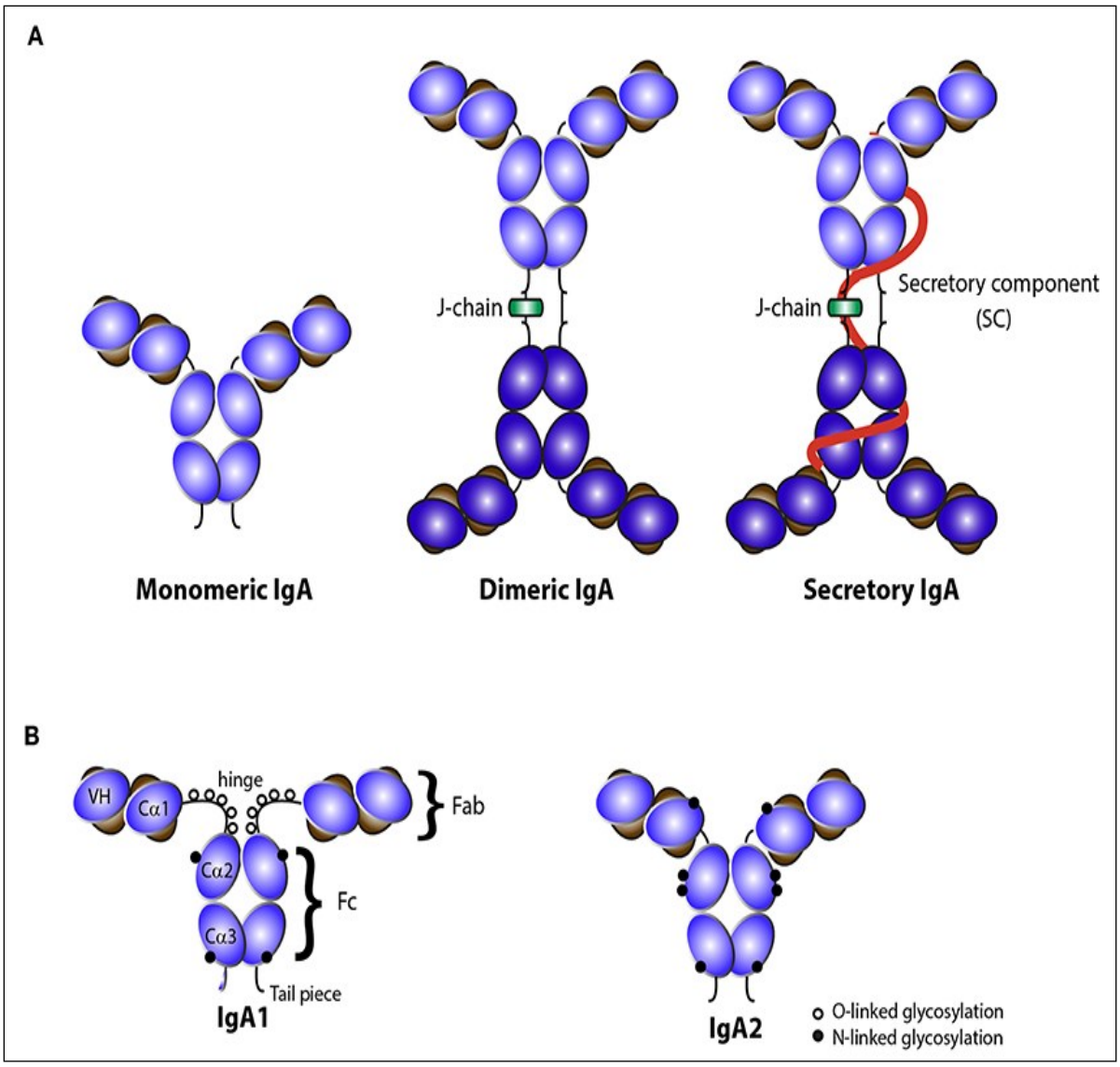


Figure 4. Schematic representation of the monomeric, dimeric, and secretory forms (A) and IgA1 and IgA2 isotypes (B) of human IgA antibody. Adapted from reference [86]

Until now, two monomeric IgA2 allotypes, named IgA2m(1) and IgA2m(2), have been well characterised in humans. In addition, a third possible allotype, IgA2n, has been described [92]. Although the IgA2m(1) and IgA2m(2) allotypes differ at just six amino acid positions in their heavy chain constant regions, there is a significant difference in the arrangements of their inter-chain disulphide bridges. While in IgA2m(2), the usual disulphide links between heavy and light chains are present, these are generally lacking in IgA2m(1). Instead, the light chains bond to each other and the association with the heavy chains are stabilized through non-covalent interactions.

Both, IgA1 and IgA2 subclasses carry N-linked carbohydrates, contributing to 6–7% of molecular mass in IgA1 and 8–10% in IgA2 [93]. In addition, IgA1 is characterised by the hinge

region that carries O-linked sugars [94,95]. The lack of this region in IgA2 makes it resistant to bacterial proteinases which cleave IgA1 in the hinge region and may play a role in the pathogenicity of the bacteria [84].

1.4.2. Production and function of IgA antibodies

Serum IgA antibody form is produced in the bone marrow, while the SIgA is produced locally by plasma cells situated at mucosal surfaces. As a result of these very different sites of production, different ways of immunisation can induce serum or secretory IgA responses or a combination of the two [96]. For example, oral immunisation with microbial antigens induces SIgA responses in external secretions but only low serum IgA responses. Conversely, systemic immunisation with antigens that induce dominant IgA responses in plasma does not induce strong IgA responses in mucosal secretions.

The function of IgA antibody secreted at mucosal surfaces throughout the body is well understood, while relatively little is known about the function of the serum form of IgA antibody. By direct interaction through their antigen-binding sites, mucosal IgA antibodies can neutralise the activity of pathogens and prevent attachment to host cells. In that way, they can provide effective protection against a range of invading pathogens including viruses, bacteria, and protozoa and their products such as toxins [97]. As well as this general protective role at mucosal surfaces, SIgA from human colostrum and milk has a specific role in the protection of the newborn [98]. In addition to the protective effects that SIgA can mediate after release into mucosal secretions, it has become apparent that it may fulfil defensive functions during its passage through the mucosal epithelium [99,100]. In secretion as well as in serum IgA has a dual function in immune responses, acting like a pro- and anti-inflammatory substance [84,101]. It is reported that both, serum and secretory IgA can mediate antibody-dependent cellular cytotoxicity [81,102]. Furthermore, IgA antibodies are able to block the IgG-mediated ADCC reaction if IgA and IgG antibodies recognize the same specific antigenic determinants [103].

Compared to IgG and IgM antibodies, IgA is unable to activate the classical complement pathway. Its ability to activate complement via the alternative pathway remains controversial [104]. Several reports have shown that in a situation where is a limited amount of antigens, IgA antibodies will inhibit the activation of the classical pathway by IgG or IgM antibodies recognizing the same antigens [105]. It has been reported that IgA binds mannose-binding lectin and activates complement via the lectin pathway of complement activation [106]. Lectin pathway

activation is mainly a function of dimeric and polymeric IgA. Further insights into the physiological significance of such activation are awaited since they may help resolve the controversy that has surrounded complement activation by IgA antibody.

1.4.3. HLA-specific IgA antibodies in transplantation

Due to their clinical relevance for transplant outcome, HLA-specific IgG antibodies are routinely determined in serum from transplant recipients, while screening of serum for the IgA isotype of HLA antibodies is not part of the routine in clinical practice. Yet, in earlier studies where enzyme-linked immunosorbent assay [78,107,108] and flow cytometry crossmatching [109,110] were used for IgA HLA antibody detection, as well as in recent studies, where the much more sensitive Luminex technology was utilized [111-114], IgA antibody subclasses against HLA were detected in the serum from transplant recipients collected before and after transplantation.

Studies indicated that kidney graft recipients have better graft survival if the IgA serum levels were high before the transplantation [115,116]. Moreover, superior graft survival was reported in kidney transplant recipients with IgA HLA antibodies [78]. Furthermore, *in vitro* studies performed on serum taken from patients with IgA nephropathy before the transplantation, showed an association between IgA HLA antibody and higher graft survival in those patients [117]. According to Lim et al., IgA HLA antibodies have a blocking effect on detrimental IgG HLA antibodies that cause a higher rate of survival in patients with IgA nephropathy than in other kidney transplant recipients. On the other hand, studies on samples from transplant recipients have also suggested IgA HLA antibody as a contributor to allograft rejection [107-111]. Besides, IgA HLA antibodies were found in elutes from explanted allografts and thereby they were indicated as a cause of rejection [118]. Evidently, the contribution of IgA antibodies against HLA in clinical transplantation is thus far not clear.

1.5. Detection of HLA-specific antibodies

In the field of organ transplantation, several methods for the detection of HLA antibodies have been developed. These are cell-based and solid-phase based assays which include the complement-dependent cytotoxicity test, flow cytometry crossmatch, enzyme-linked immunosorbent assay and more recently, Luminex bead-based assays [119].

1.5.1. Cell-based assays

Cellular assays for antibody detection in organ transplantation are crossmatching tests between donor lymphocytes and recipient serum. To perform the test viable donor cells are required. Cell-based assays are based on complement-dependent cytotoxicity and flow cytometry method.

1.5.1.1. Complement-dependent cytotoxicity test

The Complement-dependent cytotoxicity (CDC) method was pioneered by Terasaki and colleagues in the 1960s [120,121] and its modifications are still widely used [59,122]. The method involves incubation of test serum with lymphocytes of known HLA type. Rabbit serum as a source of complement is added and if the serum contains antibodies against a particular HLA, the complement cascade will be activated via the classical pathway resulting in lysis of the lymphocytes. The read-out of the test is the percentage of dead cells relative to live cells which can be visualized by staining and determined by microscopy. Result presented as a percentage of dead cells allows semi-quantitative analysis of the strength of the reaction.

Standard CDC test detects the presence of IgG1, IgG3 and IgM cytotoxic donor-specific antibodies. Antibodies that are detected by cytotoxicity are usually directed against HLA antigens but occasionally may be against non-HLA antigen [123]. Also, autoreactive cytotoxic donor-specific antibodies can generate positive CDC result. The majority of cytotoxic autoantibodies are IgM and their clinical role is doubtful. The problem of IgM antibody detection can be overcome to some extent with the use of 1,4-dithiothreitol (DTT) [124,125], although this can result in the loss of some IgG antibodies [126]. In addition, to establish if autoantibodies are responsible for the CDC result an auto-crossmatch can be performed. Over the years, various modifications of the CDC method including extended incubation, additional washings, and the addition of an antiglobulin reagent have been used to increase the sensitivity of the method [127-129].

There are two main purposes for applying the CDC method. First, this method can be used as an antibody screening assay in which the recipient's serum is incubated with a panel of HLA typed lymphocytes. By using a panel of accurate HLA phenotyped cells, it is possible to determine the HLA specificity of antibodies present in serum and to express the result as the percentage of cells in the panel giving a positive result, known as the percentage of panel reactive antibody (PRA) [130]. Second, it can be used as a crossmatch (CM) test in which the

recipient's serum is tested for the presence of complement binding antibodies against the HLA of a particular donor [131]. The test consists of incubating patient serum with lymphocytes from the specific donor to establish if the recipient has donor-specific HLA antibodies. A cytotoxic reaction (deemed positive) suggests the presence of DSA.

The CDC method reflects the situation *in vivo* more than any other method, the HLA antigens used as an antibody target are unaltered and have not been manipulated. Moreover, the clinical relevance of the CDC-detected antibodies has been established [132]. However, a considerable level of graft failure in recipients who were CDC-CM negative with their donors was reported, indicating that the sensitivity of the CDC method and its modifications were insufficient to detect low concentrations of DSA [120,133,125].

Availability of more sensitive solid-phase assays for antibody detection has resolved many of the problems of the CDC method and has basically replaced the CDC approach. Since the CDC is the only functional assay it is still used as a final test of pretransplant compatibility in the form of the CDC crossmatch [130].

1.5.1.2. Flow cytometry crossmatch

The flow cytometry crossmatch (FCXM) was introduced into clinical practice by Garavoy et al. in 1983 [134]. In this method, donor cells are incubated with recipient serum allowing the binding of the antibodies present in the recipient's serum to the target antigen on the donor cells. The bound antibody is then detected by using a secondary antibody against human immunoglobulin, labelled with a fluorescent marker such as fluorescein isothiocyanate or R-phycoerythrin. In cases where the secondary antibody is anti-human IgG, detection of the IgM isotype is excluded. Furthermore, the subclass of IgG can be elucidated by choosing a detection antibody that binds only to IgG1, IgG2, IgG3 or IgG4. By using a secondary antibody against cell-specific markers such as CD3 or CD19, it is possible to identify T cell or B cell reactivity. The test is read on a flow cytometer, and the degree of positivity is expressed as a channel shift. Since the level of fluorescence is proportional to the amount of bound antibody, the results can be interpreted as the percentage of PRA. The main advantages of the FCXM are the increased sensitivity when compared with CDC, and the possibility to detect non-complement-fixing antibodies [134,135]. Still, FCXM is not HLA specific and gives 5-10% false positive reactions [136].

1.5.2. Solid-phase assays

Solid-phase assays for antibody detection in organ transplantation use purified HLA antigens that are attached to polystyrene microplates or microbeads. These assays offer significantly higher sensitivities and specificities than cellular assays [64,137]. Available solid-phase assays are enzyme-linked immunosorbent assay and the Luminex bead-based assays.

1.5.2.1. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was the first solid-phase assay developed for antibody screening and specificity determination [138]. In this method, a pool of purified HLA antigens is immobilized on a microwell plate. HLA-specific antibodies bound to the immobilized antigens are then detected with an enzyme-linked secondary antibody which, upon addition of specific substrate, catalyzes a color change reaction that is detected in an ELISA reader. There are two main purposes for applying the ELISA method. First, purified, pooled HLA class I and HLA class II molecules bound to the wells are used for antibody screening/detection and PRA analysis. To define the antibody specificity, HLA molecules isolated from one individual cell or cell line are used to coat each well of the plate. By changing the isotype of the secondary anti-human antibody in the assay, it is possible to detect other isotypes of HLA antibodies, for instance, IgA[139].

The ELISA method is more sensitive than CDC in detecting HLA antibodies but still compared to CDC and FCXM has potential drawbacks. The natural conformation of the HLA molecules on the plate may be altered, which can result in false positive and false negative reactions of the test. Furthermore, ELISA can be limited with non-specific binding and increased background due to the large surface area of the individual ELISA microplate wells and the hydrophobic binding of the capture antibody [131].

1.5.2.2. Luminex bead-based assays for HLA antibody detection

The need to measure and quantify multiple analytes at the same time led to the development of bead-based flow cytometric assays. In the 1990s, Luminex Corporation developed the xMAP (Multi-Analyte Profiling) system and the Luminex flow analyser (Luminex, Austin, TX, USA). This technology has revolutionized the approach to HLA antibody analysis and resolved ambiguities associated with the interpretation of the CDC-CM and FCXM results.

Luminex microspheres or beads are highly uniform, polystyrene particles, 5.6 microns in size, impregnated with differing ratios of two fluorochromes resulting in a unique signal for each bead. In this way, 100 microspheres were created with a unique spectral signature. The surface of the beads allows coupling of DNA probes, antibodies or antigens. Upon reaction with the target analyte, the bead-analyte complex is made and further detected with the addition of R-phycoerythrin (PE)-conjugated detection antibody (Figure 5A). Beads are read on the Luminex flow analyser which provides a high signal-to-noise ratio for detection of low-level fluorescence and makes it very sensitive. Furthermore, there is no spectral overlap between the beads and the reporter molecules [140,141].

Commercial assay kits which consist of xMAP polystyrene beads coated with one or several types of HLA antigens are based on the incubation of beads with the patient's serum. If the serum contains HLA-specific alloantibodies, they bind to the beads with the compatible HLA antigens on the surface. Added secondary PE-conjugated anti-human antibody binds to the HLA antibody-antigen complex on the bead and gives a positive reaction.

For data acquisition and analysis, the Luminex flow analyser and HLA-visual software are used. The Luminex flow analyser is a flow cytometer with an excitation system that comprises two solid-state lasers that illuminate the beads as they flow through it in a single line. The red classification laser excites both the internal red and infrared dyes of the bead, allowing classification of the bead. The green reporter laser excites the fluorescence of the PE molecules bound to the HLA antibodies on each of the beads (Figure 5B). The fluorescent signal of the PE label, which is a measure for the amount of antibody bound to the bead, is expressed as mean fluorescence intensity (MFI) value per bead [131,142]. The combination of these signals defines the presence and specificity of the bound HLA antibody. The results of the analysis are interpreted by the HLA-visual software.

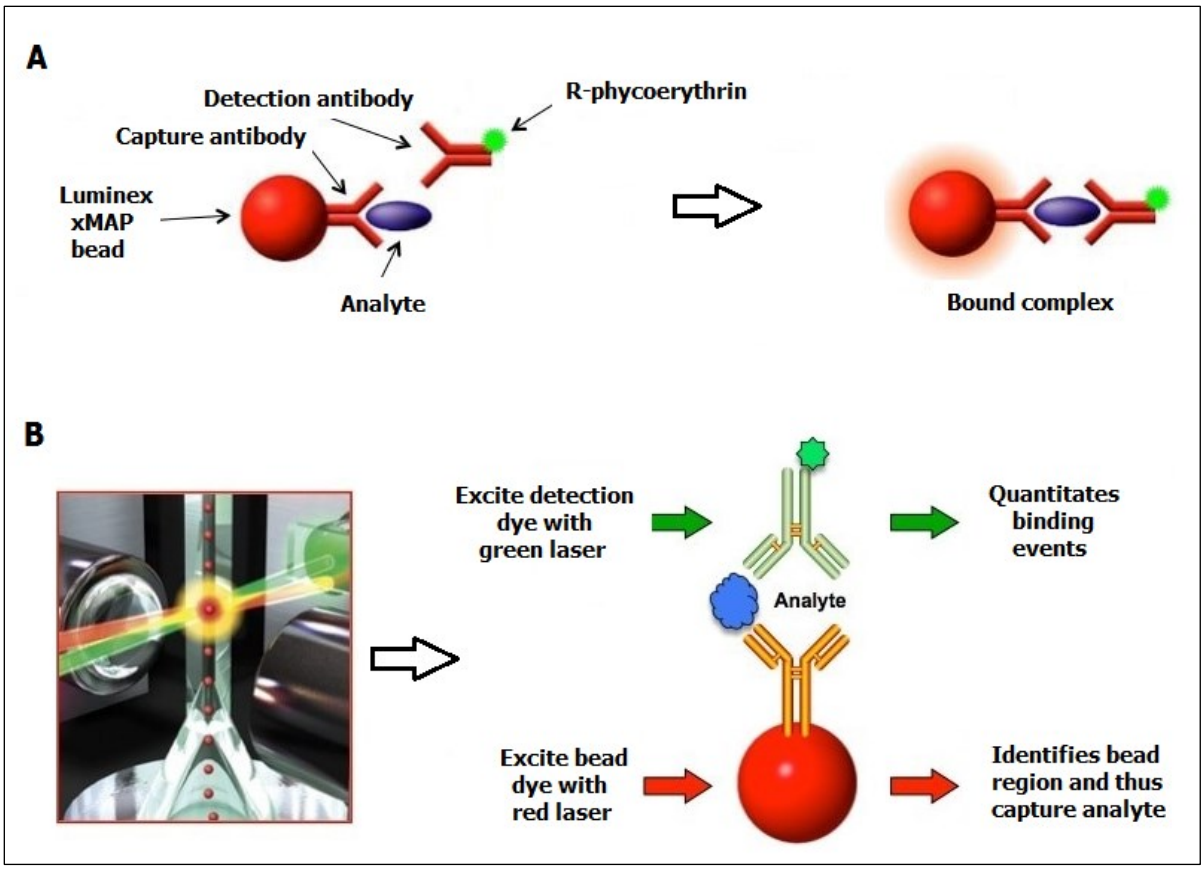


Figure 5. A general overview of the Luminex xMAP immunoassay detection scheme.
Adapted from https://www.thermofisher.com.

There are three commercially available variants for the detection of IgG HLA antibodies. The first variant consists of beads with a high number of HLA class I or class II antigens bound to the surface. The assay detects the presence of HLA antibodies and provides a negative or positive HLA class I and HLA class II test result. The second variant of testing determines the specificity of the HLA antibodies using beads coated with the phenotype equivalent of one cell, thus two HLA antigens present for each HLA locus. The result can be expressed as a percentage of PRA. The third variant of testing is in the single antigen bead (SAB) assay where the beads are coated with single recombinant antigens from transfected human cell lines, which allow accurate detection of the HLA class I or HLA class II antibody specificities present in the serum. All three variants of testing should be performed in a 96-well plate that allows simultaneous testing of 95 patient sera and one negative control serum. All bead mixtures contain a positive control bead coated with IgG and a negative control bead without HLA antigens attached to the surface. A negative control serum is provided for a run with every assay kit, to establish the background value for each bead in the mixture [130].

The considerably higher surface density of HLA on the microbeads compared to that on lymphocytes makes the Luminex SAB methodology extremely specific and highly sensitive. In the last decade, numerous reports have addressed the methodological aspects, clinical relevance, and standardization of the Luminex solid-phase SAB assay for the detection of antibodies [69,142-145]. The SAB methodological approach significantly improved antibody specificity analyses, particularly in highly sensitized patients [69,146,147]. Today, SAB is an integral component of clinical decision-making and pathological diagnosis of graft injury [142].

1.5.3. Benefits, limitations, and modifications of bead-based assays

The Luminex method represents the current highpoint in the evolution of HLA antibody detection. Existing differences between the antigen density on the coated bead and the cell surface result in differences in the level of sensitivity between Luminex antibody detection and the cell-based methods, CDC and FCXM. The additional sensitivity provided by Luminex method, particularly by SAB assay, has enabled the detection of HLA antibodies in the serum from potential transplant patients, which are not detectable by other means [145]. Moreover, increased sensitivity has enabled an improvement in the success rate of retransplant patients due to the detection of HLA presensitization. The advantage of the Luminex methodology is that by definition all detected antibodies are HLA-directed [131]. Moreover, Luminex enables the detection of antibodies directed at allelic specific differences within a patient's antigen group [131]. This fine level discrimination coupled with the HLAMatchmaker program has enabled the description of epitope sequences to which HLA antibodies are directed [39,148].

Still, there are technical issues with the SAB assay that can render the interpretation of the data. Significant differences in detectability have been reported for the bead-based assays from two available manufacturers [149,150]. Furthermore, it is observed that antigen density between beads varies not only between different assays (SAB assay contains a higher density per bead of the given antigen than screening assay), then within a single assay. A lot to lot variability in bead reactivity, bead specificities, and reagents (including variability in detection antibody properties), has additionally been recognized as a confounder to intra- and interlaboratory reproducibility [146]. In addition, reported MFI differences may originate from differences in the used type of instrument and performed maintenance [144].

An unexpected low MFI signal obtained with SAB assay may be a result of the decreased density of antigens on the surface of the beads. By using the SAB assay, it has become clear

that many serum samples with multiple antibody specificities react with epitopes shared by several HLA molecules. Epitopes shared between different beads may dilute the amount of antibody bound to any single bead reducing the MFI signal of a single bead of interest [151].

Low MFI signal, observed mostly in high immunized patients, may also be detected due to a prozone effect or complement/IgM interference. It has been reported that the prozone effect caused by a high titre of antibodies may mask the detection of antibodies present in the serum [142]. Furthermore, IgM antibody of the same HLA specificity may have a blocking effect on the detection of the present anti-HLA antibodies [152]. Besides, complement interference may inhibit the detection of IgG HLA antibodies present in the serum [153]. Depending on the cause of the false negative SAB result, the problem can be overcome by serum dilution, pre-heating of the test serum, addition of ethylenediaminetetraacetic acid (EDTA) to the wash buffer or by the DTT treatment of the sera [154].

SAB assay may as well give false positive results. It has been noted that solid-phase technology can detect patient antibodies against denatured HLA molecules, as well as the native HLA molecules. Since single antigen beads are coated with HLA molecules produced by recombinant technology, a combination of native and denatured HLA molecules is expressed on the surface of the beads. The denatured molecules can express cryptic epitopes not normally accessible by antibodies and it is not possible to distinguish between these two types of antibodies [130]. Antibodies to these exposed cryptic epitopes on denatured HLA molecules have been detected in individuals including non-transfused males [32,155]. Furthermore, studies have demonstrated that the antibodies to denatured epitopes have no clinical impact in renal or heart transplantation [156].

Unexpected high MFI can be a result of non-specific binding of non-HLA antibodies to beads. Those antibodies may be related to medications such as intravenous Ig (IVIG), inflammation, or infection [157]. Patients that have antibodies to latex will react against the beads themselves, thus resulting in a positive shift of all beads, irrespective of the presence of HLA antibodies [157]. Strong background signals due to non-specific binding can be removed by serum pre-incubation with absorption reagent.

As demonstrated, the MFI signal of SAB assays has limitations and cannot be used as a quantitative metric of the antibody amount present in the serum. Therefore, there is no recommended cut-off value for MFI positivity. Most laboratories set their cut-off level for

positivity based on levels obtained with relevant controls and on experience gained from clinical results.

In general, bead-based assays use total anti-human IgG as secondary antibody and they do not discriminate between complement-fixing and non-complement-fixing antibodies. Because the clinical relevance of non-complement-fixing antibodies is still unclear, several modifications of the assay have been developed. Until now, a C1q [158] and C3d [159], as well as IgG subclass [111,160], IgM [161] and IgA [111] assay for the detection of HLA antibodies have been described.

2. AIMS OF THE STUDY

Until now, a few studies reported IgA HLA antibodies as contributors to the transplantation immune reaction and analysed their frequency among transplant recipients. Although the detection of IgA HLA antibodies was based on Luminex technology, we do not consider previously developed assays specific and sensitive enough. Moreover, the IgA HLA antibody prevalence analyses were based on the results gained with only one of two commercially available assay kits on transplant recipients exclusively. As known, IgA HLA antibodies may be formed in HLA alloantigen exposed individuals due to previous transplantation but also after a blood transfusion or pregnancy. Information on the occurrence of IgA HLA antibodies in the samples from HLA alloantigen exposed individuals can provide a more accurate insight into the role of these antibodies in the immune and more specific, transplant response.

Therefore, the specific aims of this study are:

1. To develop a sensitive and reliable Luminex technology-based screening assay to detect the presence of IgA HLA antibodies.

2. To analyse the frequency of IgA HLA antibodies in individuals exposed to HLA alloantigens through transplantation and pregnancy.

3. To determine the relation of IgA and IgG isotype of HLA antibodies in HLA alloantigen exposed individuals.

4. To elucidate the influence of IgA HLA antibodies on graft survival in kidney transplantation.

5. To compare the results of detection and specificity identification of IgA HLA antibodies.

6. To compare the results of IgA HLA antibody specificity identification performed with two commercially available single antigen bead assay kits.

3. MATERIALS AND METHODS

3.1. Study design

The study consisted of three parts. First, we designed human recombinant HLA-specific IgA1 and IgA2 monoclonal antibodies. Secondly, we modified the commonly used Luminex bead-based IgG HLA antibody detection (screening, LMX) and specificity identification (single antigen bead, SAB) assay into IgA HLA antibody detection and specificity identification assay by using human recombinant IgA HLA monoclonal antibodies (mAbs) as optimization and validation reagents. Thirdly, we tested serum samples from HLA alloantigen exposed and HLA non-exposed individuals by using IgA HLA antibody detection (IgA-LMX) and specificity identification (IgA-SAB) assay in addition to standard IgG HLA antibody detection and specificity identification (IgG-LMX and IgG-SAB) assays.

3.2. Study samples

Study samples included human HLA-specific monoclonal antibodies and sera collected from individuals with a different history of HLA alloantigen exposure.

3.2.1. HLA-specific monoclonal antibodies

For optimization and validation of the IgA-LMX and IgA-SAB assays, the following human HLA-specific monoclonal antibodies were used: B cell hybridoma derived mAbs (IgG, SN607D8 recognizing HLA-A2/A28 and MUS4H4 recognizing HLA-Bw4/A24/A32/A25), recombinant MUS4H4rec-IgG1, -IgA1 and -IgA2 mAbs and IgM blend, which is a mixture of 10 different mAbs of the IgM isotype together recognizing the complete panel of HLA class I coated beads included in the SAB assays. All HLA-specific IgG and IgM mAbs were part of the Leiden University Medical Center (LUMC, Leiden, the Netherlands) mAbs stock. MUS4H4rec-IgA1 and MUS4H4rec-IgA2 mAbs were produced for the purpose of this study. Monoclonal antibodies were either used as neat culture supernatants or in final concentrations within the range 0.06-4 μg/mL. When different isotypes of human recombinant MUS4H4rec mAb were compared in LMX and SAB assay, samples were used in equimolar (6.3 nM~1 μg/mL) concentrations.

3.2.2. Serum samples

A total of 312 serum samples from 307 individuals were obtained with informed consent as outlined in guidelines issued by the medical ethics committee of the LUMC. Samples were collected from individuals who have never been exposed to HLA alloantigens (N=18) and from individuals who had been exposed to HLA alloantigens via previous kidney transplantation (N=198) or pregnancy (N=91). Kidney transplant recipients were selected from the Eurotransplant database which includes patients of the eight-member states of Eurotransplant, resulting in samples from different geographical regions of Europe.

In the group of transplant recipients we included: a) kidney patients on the waiting list with a history of at least one previous transplantation (N=84) from whom 6 patients had IgA nephropathy, b) transplant recipients with functioning grafts at the time of sampling (N=109) and c) patients with biopsy-proven early ABMR (N=5) in the absence of IgG isotype of HLA antibodies. From 5 patients with ABMR serum was sampled before and after transplantation which led to in total 312 samples from 307 participants used for this study.

Women with a history of pregnancy were selected from previous LUMC studies. They were required to have had an uninterrupted pregnancy for at least 37 weeks and a minimum of one HLA-A, -B or -DR mismatch between the mother and the child.

For every sample in the study population groups, serum IgG HLA antibody status was determined and samples were further grouped into IgG HLA antibody positive and negative (Table 1).

For all HLA alloantigen exposed individuals available medical records and HLA typing were obtained from the LUMC information system. DSA status of women exposed to HLA alloantigens through pregnancy was determined by comparing the HLA typing of each woman with the HLA typing of her partner/s or offspring. To determine the DSA status of each kidney transplant recipient, HLA typing of the kidney transplant recipient was compared with the HLA typing of the corresponding donor/s. HLA typing of selected donors was performed by using serological or molecular HLA typing methods available at the time of transplantation. Depending on the applied HLA typing method, available donors' results were used in our data analysis as low or high-resolution HLA typing.

Table 1. Characteristics of the study population groups and their IgG HLA antibody status (N=307)

Study population group	IgG+ (N=121)	IgG- (N=186)	
Individuals not exposed to HLA alloantigens by any means (N=18)			
n	0	18	
F/M	0/0	5/13	
Kidney transplant waitlist patients with a history of tx (N=84)			
n	41	43	
F/M	18/23	21/22	
number of previous tx, median (range tx)	2 (1-5)	1 (1-2)	
time from last tx failure to sampling, median (range days)	917 (0-3940)	53 (2-2163)	
Transplant recipients with functioning grafts (N=109)			
n	25	84	
F/M	6/19	14/70	
time from tx to post-tx sampling, median (range years)	5 (1-11)	1 (1-14)	
Patients with biopsy-proven early ABMR without detected IgG HLA antibodies (N=5)		(pre-tx serum)	(post-tx serum)
n	0	5	5
F/M	0/0	5/0	5/0
time from tx to pre/post-tx sampling/ABMR diagnosis, median (range days)		7 (7-13)	7 (6-13)
Women with a history of pregnancy (N=91)			
n	55	36	
number of previous pregnancies, median (range pregnancies)	1 (1-2)	2 (1-7)	
time from last delivery to sampling, median (range days)	457 (3-8602)	1 (0-18)	

Legend: IgG+ = IgG HLA antibody positive, IgG- = IgG HLA antibody negative, N = Number of individuals, n = Number of samples, F = Female, M = Male, tx = Transplantation, ABMR = Antibody-mediated rejection

3.3. Study methods

3.3.1. Production and validation of human recombinant IgA HLA monoclonal antibodies

To produce human recombinant HLA-specific IgA1 and IgA2 mAbs, expression plasmids p33IgA1-MUS4H4 and p33IgA2-MUS4H4 (Genmab, Utrecht, the Netherlands) were used for transfection of Expi293F cells with ExpiFectamine, Opti-MEM, and Expi293 expression medium (Thermo Fisher Scientific, Waltham, MA USA) according to the instructions from the manufacturer. After 5 days of culture, supernatants containing recombinant mAbs were harvested and filtered.

To test the presence and concentration of MUS4H4rec-IgA1 and -IgA2 mAbs, supernatants were screened with the total IgA ELISA from Thermo Fisher Scientific. For the subclass verification, supernatants were screened with LIFECODES LifeScreen Deluxe Kit (Lifecodes-Immucor Transplant Diagnostics, Stamford, CT, USA) and PE-conjugated detection antibodies specific for each IgA subclass [(mouse anti-human-IgA1 (clone B3506B4) and -IgA2 (clone A9604D2), both from Southern Biotech, Birmingham, AL, USA)].

Selected detection antibodies were previously reported in publications on IgA HLA antibody detection. Due to the observed cross-reactivity between IgA2-PE (clone A9604D2) detection antibody and MUS4H4rec-IgA1 mAb, mouse anti-human IgA2-PE (clone IS11-21E11, MACS Miltenyi Biotec, USA) was used to accurately verify produced MUS4H4rec-IgA2 subclass. In the following SAB experiment, in order to determine the HLA specificities of the human recombinant MUS4H4rec-IgA1 and -IgA2 mAbs, LIFECODES LSA Class I Kit (Lifecodes-Immucor Transplant Diagnostics, Stamford, CT, USA), mouse anti-human IgA1-PE (clone B3506B4) and IgA2-PE (clone IS11-21E11) were used. Both Luminex assays were performed using Lifecodes kits following the instructions from the manufacturer and the protocol described for serum samples (Table 2). Finally, MUS4H4rec-IgA1 and -IgA2 mAbs were measured on Luminex flow analyser LABScan 100 (Luminex Corporation, Austin, TX, USA) and analysed with the compatible software, MatchIT! Antibody software version 1.3.0 (Lifecodes-Immucor Transplant Diagnostics, Stamford, CT, USA). Detected specificities of MUS4H4rec-IgA subclass mAbs were further compared with the IgG-SAB results obtained from the B cell hybridoma derived MUS4H4 IgG mAb.

The effector functions of the mAbs were tested with complement-dependent cytotoxicity assay. The Terasaki plates (Greiner Bio-One, Kremsmünster, Austria) were oiled and filled with 1 µL of MUS4H4rec-IgA1 or MUS4H4rec-IgA2 mAbs supernatants (prepared in the concentration range neat-1:64 dilution) in triplicate. Then, 3000 PBMCs with the HLA typing HLA-A1,A2;B8,B62 and HLA-A23, - ;B35,B49 were added and incubated for 60 min at 20°C. Next, 5 µL rabbit complement (Inno-train, Kronberg, Germany) was added and incubated for 60 min at 20°C. To visualise cytotoxicity, 5 µL propidium iodide ink was added to each well, and after 15 min incubation in the dark, plates were read using an automated fluorescence microscope Patimed (Leica Microsystems, Amsterdam, the Netherlands).

3.3.2. Detection of IgG HLA antibodies with Luminex bead-based assays

All serum samples were screened for the presence of IgG HLA antibodies using the LIFECODES LifeScreen Deluxe Kit (IgG-LMX). Samples found to be positive were further tested for IgG HLA antibody specificities with LIFECODES LSA Class I Kit and -Class II Kit (IgG-SAB) from Lifecodes, if necessary, following the described protocol (Table 2). Briefly explained, in IgG-LMX assay, serum samples were incubated with a mixture of beads conjugated with the purified HLA class I (7 groups) and HLA class II (5 groups) glycoproteins for 30 min and washed. If formed, the bead-antibody complex was detected after 30 min of incubation with goat anti-human IgG detection antibody conjugated to PE in the concentration known to Lifecodes (Table 2). In the IgG-SAB assay, a blend of beads each conjugated with a different single HLA class I or class II glycoproteins was used. The same detection antibody was used for IgG-LMX and IgG-SAB assay (Figure 6). Samples were measured on a Luminex flow analyser LABScan 100, assay signals were reported as mean fluorescence intensity (MFI) and assigned based on the MatchIT! Antibody software version 1.3.0 calculation**.**

Table 2. Protocol for Luminex IgG and IgA HLA antibody screening (LMX) and single antigen bead (SAB) assay with Lifecodes kits

Protocol step/Assay type	LMX (IgG and IgA)	SAB (IgG and IgA)
Serum pre-treatment (if required)	25 µL serum 5 µL serum cleaner incubation 30 min at 450 rpm spindown 7 min at 13000 g	25 µL serum 5 µL serum cleaner incubation 30 min at 450 rpm spindown 7 min at 13000 g
Serum pre-treatment (always required)	None	serum dilution 1:20 with EDTA for IgG-SAB
Filter-plate preparation	100 µL demineralized water incubation 2 min vacuum	100 µL demineralized water incubation 2 min vacuum
First incubation	24 µL wash buffer 7.5 µL serum 3 µL beads (vortexed) incubation 30 min at 600 rpm	30 µL beads (vortexed) 7.5 µL serum incubation 30 min at 600 rpm
Washing steps	1 x 100 µL wash buffer vacuum 2 x 200 µL wash buffer vacuum after every wash	1 x 100 µL wash buffer vacuum 3 x 200 µL wash buffer vacuum after every wash
Second incubation	30 µL detection antibody incubation 30 min at 600 rpm	37.5 µL detection antibody incubation 30 min at 600 rpm
Third incubation	80 µL wash buffer incubation 2 min at 600 rpm	100 µL wash buffer incubation 2 min at 600 rpm
Sample analyses	Luminex flow analyser xPONENT software MATCH IT! Antibody software	Luminex flow analyser xPONENT software MATCH IT! Antibody software

Legend: LMX = Antibody screening, SAB = Single antigen bead; The test was done at room temperature without exposing the plate to the light.

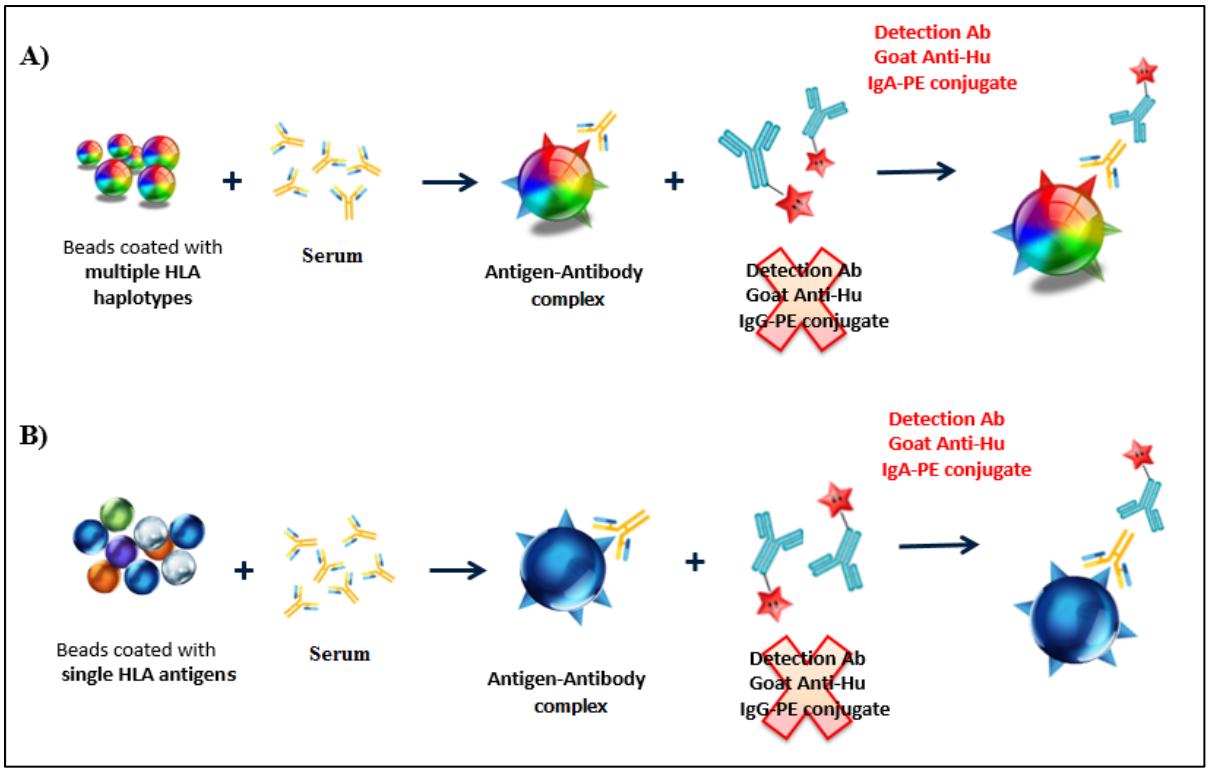


Figure 6. Schematic overview of the Luminex IgG HLA antibody screening (A) and IgG HLA single antigen bead (B) assay with the modification of assays for the IgA HLA antibody detection.
Legend: Ab = Antibody, Goat Anti-Hu = Goat anti-human, PE = R-phycoerythrin

3.3.3. Detection of IgA HLA antibodies with Luminex bead-based assays

For this dissertation, we modified the commonly used Luminex IgG HLA antibody screening (IgG-LMX) assay into IgA HLA antibody screening (IgA-LMX) assay and applied the developed assay on serum samples.

3.3.3.1. Development and optimisation of IgA HLA antibody screening assay

To modify the standard Luminex bead-based assays for IgA HLA antibody detection, replacement of standard goat anti-human IgG-PE secondary antibody with the IgA-specific detection antibody was necessary (Figure 6). Therefore, we tested the following IgA specific detection antibodies: mouse anti-human IgA1 (clone B3506B4, Southern Biotech, Birmingham, AL, USA; 10 µg/mL), mouse anti-human IgA2 (clone IS11-21E11, MACS Miltenyi Biotec, USA; 2.2 µg/mL) and goat anti-human IgA (heavy chain-specific, polyclonal, Southern Biotech, USA; 10 µg/mL), all conjugated to PE. To test the specificity and sensitivity of the above mentioned IgA detection antibodies, IgA1 and IgA2 isotype variants of human recombinant HLA-specific MUS4H4rec monoclonal antibodies were used.

Firstly, to be able to accurately detect IgA1 and/or IgA2 isotype of HLA antibodies we used IgA-PE subclass specific detection antibodies. Due to the obtained low MUS4H4rec MFI signals, subsequent experiments were performed with the polyclonal total IgA detection antibody, goat anti-human IgA-PE using IgA-LMX and IgA-SAB assay by the instructions and the described protocol from Lifecodes (Table 2).

To optimise the assay, the concentration of the selected goat anti-human IgA-PE was established. For that purpose, detection antibody (stock concentration 500 µg/mL) prepared in dilutions 1:10, 1:50 and 1:250 was applied on the human recombinant HLA MUS4H4rec-IgA1 and MUS4H4rec-IgA2 mAbs (1 µg/mL). MFI values were obtained from HLA class I bead groups, Pos Ctrl (detection antibody control) and CONs (background noise control) for each sample and further compared between the samples.

For the detection sensitivity test, we applied goat anti-human IgA-PE detection antibody in previously determined concentration on MUS4H4rec-IgA1 and -IgA2 mAb samples prepared in a range of 0.06-1 µg/mL final concentrations and measured the MFI signal strength for each sample.

3.3.3.2. Quality control of the developed IgA HLA antibody screening assay

The specificity of the selected polyclonal total IgA detection was tested in IgA-SAB assay by using IgM and IgG isotype of HLA-specific monoclonal antibodies and PBS (Phosphate-buffered saline) as samples. For the comparison of IgA and IgG HLA antibody detection sensitivity in screening and single antigen bead assays we applied isotype-specific polyclonal (IgA-PE or IgG-PE) detection on human recombinant HLA-specific MUS4H4rec-IgA1, -IgA2 and -IgG1 mAbs and analysed their isotype reactivity curves.

With the aim to test the detectability of the IgA isotype of HLA antibodies in the presence of IgG HLA antibodies, serum from HLA alloantigen non-exposed individual was spiked with mixtures of IgA1 (1 µg/mL) and IgG1 (1 µg/mL and 4 µg/mL) HLA mAbs recognizing the same epitope (IgA1 and IgG1 isotype variants of MUS4H4rec mAb) or different epitopes (MUS4H4rec-IgA1 and SN607D8-IgG1). Finally, samples from HLA alloantigen exposed (N=12) and HLA non-exposed individuals (N=18) were used to validate the modified LMX assay. Comparison of obtained MFI detection signal between the samples was assessed using the Mann-Whitney test.

3.3.3.3. Screening of serum samples for the presence of IgA HLA antibodies

All serum samples from HLA alloantigen exposed and HLA non-exposed individuals were screened for the presence of IgA isotype of HLA antibodies with the developed IgA-LMX assay. Screening results were further analysed in the same manners as for IgG HLA antibodies. The raw MFI values and the defined cut-off were used to assign samples positive or negative for the presence of IgA HLA antibodies.

3.3.4. Assigning the HLA antibody screening results

Due to a clear difference in IgA and IgG HLA antibody detection signal measured in screened serum samples, the threshold for positive assignment also differed for the two isotypes and the cut-off value to assign detected IgA HLA antibodies positive needed to be established.

3.3.4.1. Assigning the IgG HLA antibody screening results

In order to evaluate the samples, MatchIT! Antibody software version 1.3.0 was used. The software reports the result defined as positive or negative for the presence of IgG HLA antibodies according to the MFI value of 7 bead groups for HLA class I (marked CI-0X) and 5 bead groups for HLA class II (marked CII-0X) (Figure 7).

Class I/II LMX Results

Batch ID: MUS4H4rec-IgG1 screening_HC

Run Date: 07-10-2017 **Lot Number:** 3004989 3004922-LMX **Expiration Date:** 15-01-2018

Assignment Cutoff: 2,2 **Assignment Adjust:** 0.00,0.00

Supplemental Cutoff: 2,2 **Supplemental Adjust:** 0.00,0.00

Report By: Lab Supervisor **CON Ranges:** CON 1: 38 - 248; CON 2: 26 - 246; CON 3: 33 - 1674

Sample ID: IgGneat_IgG-PE
Patient Name: IgGneat_IgG-PE
Accession:
Draw Date:

	Class I Results								Class II Results						CONs	
	CI-01	CI-02	CI-03	CI-04	CI-05	CI-06	CI-07	Assignment	CII-01	CII-02	CII-03	CII-04	CII-05	Assignment	~~Pos Ctrl~~	
Raw	22026	22161	22847	22700	22872	22191	22451		139	266	139	184	118		18466	> Pos Ctrl
Adj 1	446.31	448.62	462.81	460.15	463.03	448.74	454.93		-1.33	-2.17	-2.28	0.16	-2.99		49	> CON1
Adj 2	211.90	212.55	219.46	218.68	219.84	212.20	215.78		-4.65	-7.09	-4.79	-3.24	-5.65		102	> CON2
Adj 3	116.04	116.26	120.25	119.92	120.09	116.26	118.28		-2.94	-4.76	-3.52	-2.38	-4.20		185	> CON3
Score	3	3	3	3	3	3	3	Positive	0	0	0	1	0	Negative		

BAF	CI-01	CI-02	CI-03	CI-04	CI-05	CI-06	CI-07	CII-01	CII-02	CII-03	CII-04	CII-05
CON1	3.194	3.638	3.456	3.104	3.741	4.138	3.251	4.162	7.593	5.117	3.600	5.401
CON2	4.040	4.705	4.535	3.869	4.391	5.356	4.324	6.016	9.694	6.151	5.040	6.810
CON3	3.018	3.523	3.247	2.777	3.540	3.692	3.072	3.694	6.200	4.272	3.379	4.837

Reviewer Comment:

Date: Completed By:

Date: Approved By:

Date: Reviewed By:

= Bead Count Failure

Immucor, Inc.

MATCH IT! Antibody v1.3.1

Figure 7. Illustration of the Luminex IgG HLA antibody screening report for the sample of MUS4H4rec-IgG1 monoclonal antibody.
Legend: CI-0X = HLA class I bead groups 01-07, CII-0X = HLA class II bead groups 01-05, Raw = Raw MFI value, Adj X = Adjusted ratio 1-3, Pos Ctrl = Positive control bead, CONX = Negative control bead 1-3, BAF = Background-adjusted factor

The positive assignment for HLA class I and/or HLA class II is based on at least one positive class I and/or class II bead group. To determine if the bead group is positive, adjusted MFI ratios 1, 2 and 3 (marked Adj X in Figure 7) were calculated and used to score the bead group 0-3. Except for group 01, which is positive with score 1, scores 2 and 3 represent positive assignment.

Adjusted ratio calculation:

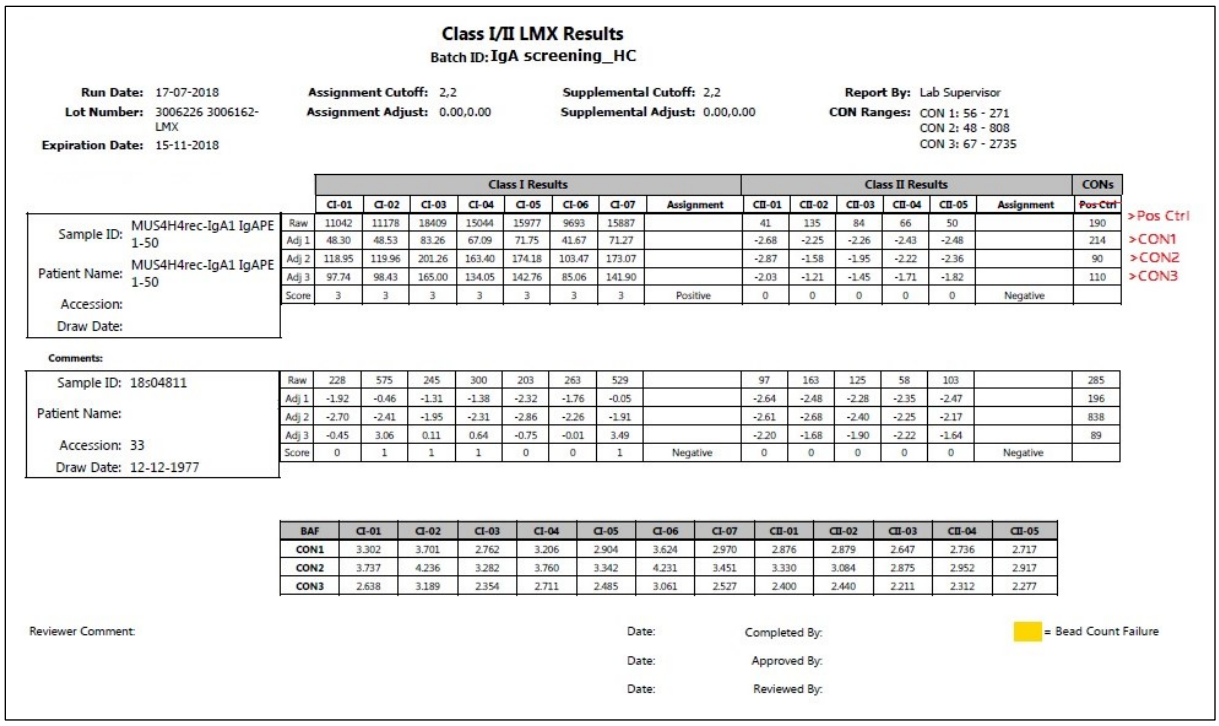
$$\text{Adjusted ratio} = \frac{\text{Individual bead MFI}}{\text{CON MFI}} - (\text{BAF})$$

MFI: Mean fluorescence intensity
CON: Negative control bead
BAF: Background-adjusted factor

Provided assay controls, Pos Ctrl (Positive control bead) which is coated with IgG HLA antibody, confirms that the IgG conjugate is added and CONs (Negative control beads) determine the background and normalize the results. In contrary to Pos Ctrl which should be high, CONs should be low and fall into a specified range. To compensate for the background noise due to the bead variation, a pre-determined MFI ratio for each bead/CON combination (BAF) is given with the lot number of each LMX kit. For each screening assay kit, there is a lot-specific cut-off provided by the manufacturer. In our study, the lot-specific cut-off value for IgG HLA antibodies was 1500 MFI.

3.3.4.2. Assigning the IgA HLA antibody screening results

To determine if a test sample is positive for IgA HLA class I and/or class II antibodies, raw MFI values with the applied arbitrary cut-off of 500 MFI were used. Thereby, the software scores and assignments were not taken into account. Pos Ctrl and CONs, which should remain low for IgA detection, were used as control parameters (Figure 8).



Class I/II LMX Results
Batch ID: IgA screening_HC

Run Date: 17-07-2018 | Assignment Cutoff: 2,2 | Supplemental Cutoff: 2,2 | Report By: Lab Supervisor
Lot Number: 3006226 3006162-LMX | Assignment Adjust: 0.00,0.00 | Supplemental Adjust: 0.00,0.00 | CON Ranges: CON 1: 56 - 271; CON 2: 48 - 808; CON 3: 67 - 2735
Expiration Date: 15-11-2018

Sample ID: MUS4H4rec-IgA1 IgAPE 1-50
Patient Name: MUS4H4rec-IgA1 IgAPE 1-50
Accession:
Draw Date:

	Class I Results								Class II Results						CONs
	CI-01	CI-02	CI-03	CI-04	CI-05	CI-06	CI-07	Assignment	CII-01	CII-02	CII-03	CII-04	CII-05	Assignment	Pos Ctrl
Raw	11042	11178	18409	15044	15977	9693	15887		41	135	84	66	50		190
Adj 1	48.30	48.53	83.26	67.09	71.75	41.67	71.27		-2.68	-2.25	-2.26	-2.43	-2.48		214
Adj 2	118.95	119.96	201.26	163.40	174.18	103.47	173.07		-2.87	-1.58	-1.95	-2.22	-2.36		90
Adj 3	97.74	98.43	165.00	134.05	142.76	85.06	141.90		-2.03	-1.21	-1.45	-1.71	-1.82		110
Score	3	3	3	3	3	3	3	Positive	0	0	0	0	0	Negative	

>Pos Ctrl
>CON1
>CON2
>CON3

Comments:

Sample ID: 18s04811
Patient Name:
Accession: 33
Draw Date: 12-12-1977

Raw	228	575	245	300	203	263	529		97	163	125	58	103		285
Adj 1	-1.92	-0.46	-1.31	-1.38	-2.32	-1.76	-0.05		-2.64	-2.48	-2.28	-2.35	-2.47		196
Adj 2	-2.70	-2.41	-1.95	-2.31	-2.86	-2.26	-1.91		-2.61	-2.68	-2.40	-2.25	-2.17		838
Adj 3	-0.45	3.06	0.11	0.64	-0.75	-0.01	3.49		-2.20	-1.68	-1.90	-2.22	-1.64		89
Score	0	1	1	1	0	0	1	Negative	0	0	0	0	0	Negative	

BAF	CI-01	CI-02	CI-03	CI-04	CI-05	CI-06	CI-07	CII-01	CII-02	CII-03	CII-04	CII-05
CON1	3.302	3.701	2.762	3.206	2.904	3.624	2.970	2.876	2.879	2.647	2.736	2.717
CON2	3.737	4.236	3.282	3.760	3.342	4.231	3.451	3.330	3.084	2.875	2.952	2.917
CON3	2.638	3.189	2.354	2.711	2.485	3.061	2.527	2.400	2.440	2.211	2.312	2.277

Reviewer Comment:
Date: Completed By:
Date: Approved By:
Date: Reviewed By:
= Bead Count Failure

Figure 8. Illustration of the Luminex IgA HLA antibody screening report for the sample of MUS4H4rec-IgA1 monoclonal antibody and a sample of HLA immunized individual.
Legend: CI-0X = HLA class I bead groups 01-07, CII-0X = HLA class II bead groups 01-05, Raw = Raw MFI value, Adj X = Adjusted ratio 1-3, Pos Ctrl = Positive control bead, CONX = Negative control bead 1-3, BAF = Background-adjusted factor

In order to establish a cut-off value for positive responses in IgA-LMX assay, serum samples from individuals who have never been exposed to HLA alloantigens by any means and hence having no HLA antibodies (N=18) were screened for IgG and IgA HLA antibodies and Tukey's extreme outlier calculation [third quartile + (3x interquartile range)] was applied to the recorded MFI values. After the calculation approach was confirmed on IgG-LMX assay results, an arbitrary cut-off value (250 raw MFI) was set for all HLA class I and/or class II bead groups in IgA-LMX assay.

To validate the cut-off value for the IgA-LMX assay, IgA-SAB assay was performed on serum samples from HLA alloantigen exposed individuals with a history of transplantation (N=12), selected based on their IgA-LMX results (raw MFI values for the highest-ranked HLA class I or HLA class II bead group in a range 100-700). For each sample, IgA-LMX result was compared with donor-specific IgA HLA antibody (IgA DSA) status. Based on the analyses on 12 selected samples, the cut-off value for IgA-LMX was redefined to 500 raw MFI and further used throughout the study.

3.3.5. Comparison of IgA HLA antibody screening and single antigen bead assay results

For all IgA-LMX positive samples, IgA-SAB assay with Lifecodes kit was performed (protocol in Table 2) and their IgA-LMX and IgA-SAB results were compared. For that purpose, IgA-SAB results were used without assigning results positive or negative based on the cut-off MFI value. Instead, for each sample, IgA-LMX result was compared with the MFI value of the highest-ranked bead in the IgA-SAB assay and the DSA, non-DSA or NS (not specified, due to the missing HLA typing data) status of the bead (Figure 9).

Class I Single Antigen Results
Batch ID: LSA_33_HC_13032018-a

Patient Name: mIgA1 plus IgA-PE **Patient DOB:** **Draw Date:** **Donor Number:** **Expiration Date:** 15-10-2018
Sample ID: mIgA1 plus IgA-PE **Lot ID:** 3006129 3006116-SA1 **Donor Center #:** **Accession #:**
Patient HLA Type: **Report By:** Lab Supervisor
Method Type: Legacy Calculation

Positive CON MFI: 7123 **Negative CON MFI:** 113 **Analysis Mode:** Manual **% PRA:** 23

Bead	Bead Count	Raw Value	BCM	BCR	AD-BCR	Assignment	A	B	C	Bw	A Serology	B Serology	C Serology	Epitopes
115	84	20336	20184	708.19	681.61	Positive	A*25:01			Bw4	A25(10)			82LR
144	68	20096	19929	424.01	445.86	Positive		B*15:13		Bw4		B77(15)		82LR
145	87	20043	19876	422.89	382.71	Positive		B*15:16		Bw4		B63(15)		82LR
154	78	19911	19755	420.31	389.54	Positive		B*38:01		Bw4		B38(16)		82LR
174	88	19899	19743	420.06	403.52	Positive		B*57:01		Bw4		B57(17)		82LR
112	112	19896	19742	692.68	743.22	Positive	A*23:01			Bw4	A23(9)			82LR
166	99	19857	19705	419.26	393.67	Positive		B*49:01		Bw4		B49(21)		82LR
114	88	19858	19700	691.23	665.92	Positive	A*24:03			Bw4	A2403			82LR
121	79	19815	19660	689.82	706.06	Positive	A*32:01			Bw4	A32(19)			82LR

Figure 9. Illustration of the IgA HLA single antigen bead assay report for the sample of MUS4H4rec-IgA1 monoclonal antibody.
Legend: Raw Value = Raw MFI value, BCM = Background corrected MFI, BCR = Background corrected ratio, AD = Antigen density; The highest-ranked bead specificity (HR) for the sample MUS4H4-IgA1 is A*25:01.

3.3.6. Comparison of IgA HLA antibody single antigen bead assay results acquired with two commercially available assay kits

Until now, all published data on the prevalence of IgA HLA antibodies was reported exclusively based on the applied LABScreen™ Single Antigen, HLA class I and LABScreen™ Single Antigen, HLA class II kits (both from One Lambda-Thermo Fisher Scientific Inc., West Hills, CA, USA). Therefore, we performed IgA-SAB assays with Lifecodes and One Lambda kits and goat anti-human IgA-PE detection antibody (conc. 10µg/mL) applied on the IgA-LMX positive samples from transplant recipients (N=8) (protocol in Table 3). For each sample, Lifecodes and One Lambda IgA-SAB assay results were compared based on the raw MFI values of patient's IgA DSAs and the highest-ranked bead in the IgA-SAB assays report. Furthermore, for comparison of Lifecodes and One Lambda IgA-SAB assay kits, bivariate

correlations were performed using Person's correlation coefficient and linear regression on all the beads of the assay kits. In addition, Bland-Altman plots were used to compare MFI values for paired antigens between the two kits.

Table 3. Comparison of Lifecodes and One Lambda IgA-SAB protocol

Protocol step/Assay type	Lifecodes IgA-SAB	One Lambda IgA-SAB
Serum pre-treatment (if required)	25 µL serum 5 µL serum cleaner incubation 30 min at 450 rpm spindown 7 min at 13000 g	25 µL serum 5 µL serum cleaner incubation 30 min at 450 rpm spindown 7 min at 13000 g
Filter-plate preparation	100 µL demineralized water incubation 2 min vacuum	250 µL demineralized water incubation 10 min at 450 rpm vacuum
First incubation	30 µL beads (vortexed) 7.5 µL serum incubation 30 min at 600 rpm	4 µL beads (vortexed) 20 µL serum incubation 30 min at 450 rpm
Washing steps	1 x 100 µL wash buffer vacuum 3 x 200 µL wash buffer vacuum after every wash	1 x 200 µL wash buffer vacuum 4 x 250 µL wash buffer vacuum after every wash
Second incubation	37.5 µL detection antibody incubation 30 min at 600 rpm	100 µL detection antibody incubation 30 min at 450 rpm
Washing steps	none	1 x 150 µL wash buffer vacuum 4 x 200 µL wash buffer vacuum after every wash
Third incubation	100 µL wash buffer incubation 2 min at 600 rpm	85 µL wash buffer incubation 2 min at 450 rpm
Sample analyses	Luminex flow analyser xPONENT and MATCH IT! Antibody software	Luminex flow analyser xPONENT and HLA-Fusion software

Legend: SAB = Single antigen bead; The test was done at room temperature without exposing the plate to the light.

3.4. Statistical analysis

All analyses were performed with GraphPad Prism, version 7.02 (GraphPad Software, La Jolla, CA, USA). Throughout the whole study, the MFI signal was expressed as raw values.

Tukey's extreme outlier calculation was used to define the cut-off value for the IgA HLA antibody screening assay. The normality of data distribution was tested with the D'Agostino-Pearson test.

Accordingly, non-parametric tests were used in the analyses. The Kruskal-Wallis test was used for unpaired analysis between two groups and the Mann-Whitney test for unpaired multiple analyses, while the Wilcoxon test was applied for paired analysis between two groups and the Friedman test for paired multiple analyses.

In addition, parametric tests were applied in the analyses, Pearson's correlation test and Linear regression for data comparison. Bland-Altman plots were used to quantify bias (mean difference) and the range (95% confidence interval; 95% CI) in paired analyses.

Statistical level of significance was defined as $p<0.05$ [162].

4. RESULTS

4.1. Human recombinant IgA HLA monoclonal antibodies

Concentrations for human recombinant HLA mAb supernatants MUS4H4rec-IgA1 (6.88 µg/mL) and MUS4H4rec-IgA2 (4.49 µg/mL) were determined based on the total IgA ELISA. Furthermore, for both MUS4H4rec-IgA1 and -IgA2 supernatants, with the modified Luminex screening assay, mAb subclass was verified as each IgA subclass mAb was accurately recognized by IgA subclass specific detection antibody.

For that purpose, we first used the mouse anti-human-IgA1 (clone: B3506B4) and -IgA2 (clone: A9604D2) PE-conjugated detection antibodies in dilution range 1:5-1:1000 (stock concentration 100 µg/mL). When applied on MUS4H4rec-IgA1 supernatant, a median MFI for HLA class I beads with the values of 5909 (IgA1-PE) and 5306 (IgA2-PE) as the highest detection signals were observed in prepared dilution range 1:5-1:1000. That suggested cross-reactivity between IgA2-PE detection antibody and IgA1 supernatant (Figure 10A). The same IgA antibody detection clones applied on MUS4H4rec-IgA2 supernatant showed the highest median MFI values of 62 (IgA1-PE) and 2189 (IgA2-PE) and thereby the cross-reactivity between IgA1-PE antibody detection and IgA2 supernatant was excluded (Figure 10B).

After that we used another clone of IgA2-PE detection antibody (clone: IS11-21E11) in dilution range 1:5-1:1000 (stock concentration 11 µg/mL) and detected MUS4H4rec-IgA2 mAb (median MFI: 2282) without any cross-reactivity with the MUS4H4rec-IgA1 mAb (median MFI: 9), assuring an accurate IgA1 and IgA2 isotype control of HLA antibodies in produced supernatants (Figure 10C).

In the IgA subclass verification experiments, human recombinant monoclonal antibodies were used as neat supernatants MUS4H4rec-IgA1 (conc. 6.88 µg/mL) and MUS4H4rec-IgA2 (conc. 4.49 µg/mL). As a negative mAb control sample, with each clone of detection antibodies, PBS was used and gave low median MFI values of 31 (B3506B4), 13 (A9604D2) and 4 (IS11-21E11).

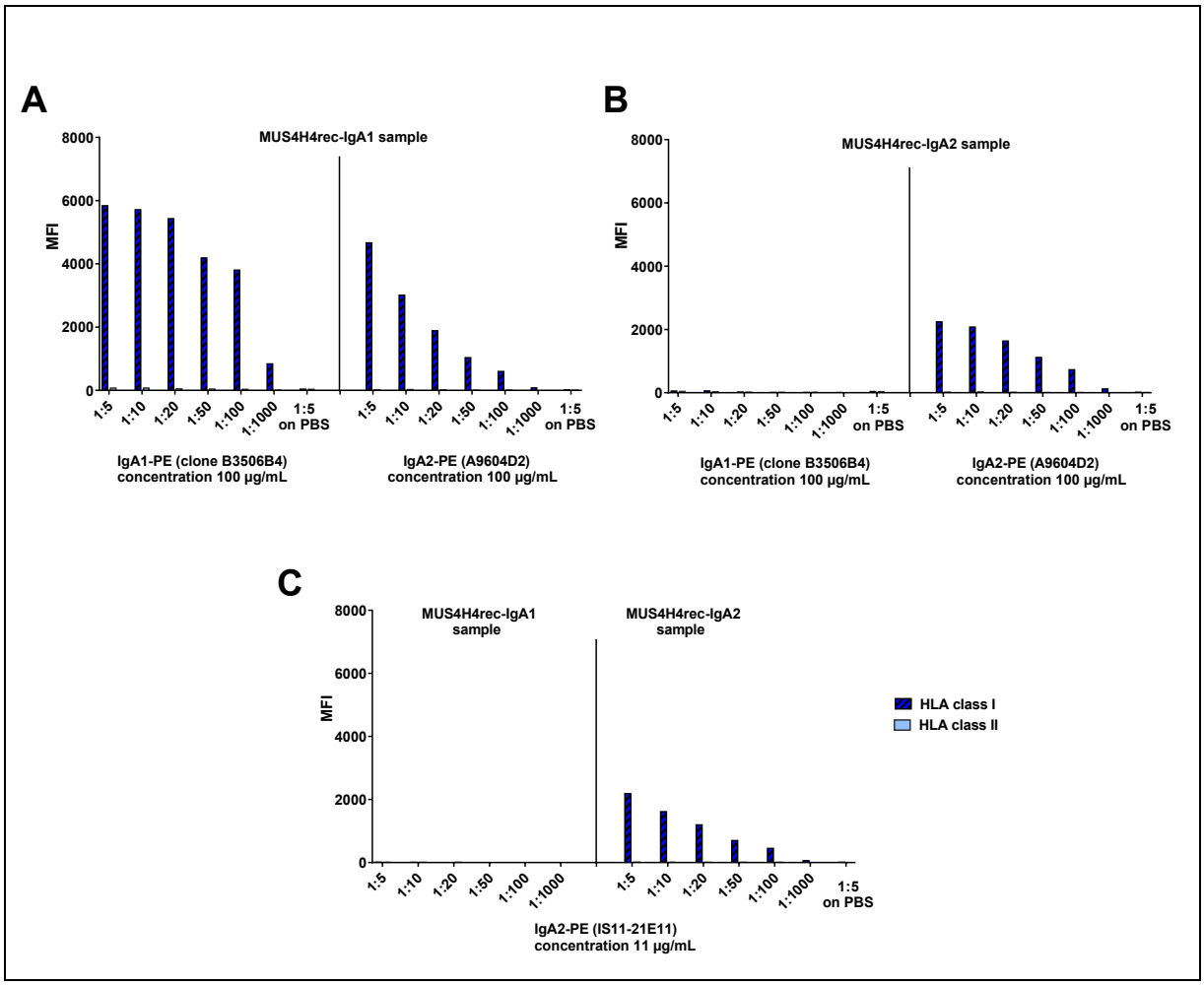


Figure 10. Subclass confirmation for the produced human recombinant monoclonal antibodies performed by applying IgA1-PE (clone: B3506B4) and IgA2-PE (clone: A9604D2) detection antibody on MUS4H4rec-IgA1 (A) and MUS4H4rec-IgA2 (B) sample as well as applying IgA2-PE (clone: IS11-21E11) detection antibody on both MUS4H4rec-IgA1 and –IgA2 samples (C).
Legend: MFI = Mean fluorescence intensity, PBS = Phosphate-buffered saline; All MFI values for HLA class I and HLA class II are presented as mean values of all the bead groups.

The SAB assay results from human recombinant mAb supernatants confirmed the presence of all 22 MUS4H4 specificities in MUS4H4rec-IgA1 (conc. 6.88 µg/mL) (median MFI: 12216, range: 8532-22293) and MUS4H4rec-IgA2 (conc. 4.49 µg/mL) (median MFI: 4134, range: 3133-4511) samples, which were identical to the MUS4H4 IgG original hybridoma (conc. 45.4 µg/mL) (median MFI: 19986, range: 17173-21313) specificities (Figure 11). Computed Pearson's correlation test confirmed the strong association between MUS4H4 IgG hybridoma and produced human recombinant MUS4H4rec-IgA1 ($r=0.9801$, $r^2=0.9607$, $p<0.0001$) and MUS4H4rec-IgA2 ($r=0.9969$, $r^2=0.9939$, $p<0.0001$) monoclonal antibodies.

As expected, when MUS4H4rec-IgA1 and MUS4H4rec-IgA2 mAbs recognizing HLA-Bw4/A24/A32/A25 were tested with the CDC test, cytotoxicity was not observed in the reaction between mAbs and PBMCs with the HLA typing HLA-A1,A2;B8,B62 nor between mAbs and PBMCs with the HLA typing HLA-A23, - ;B35,B49.

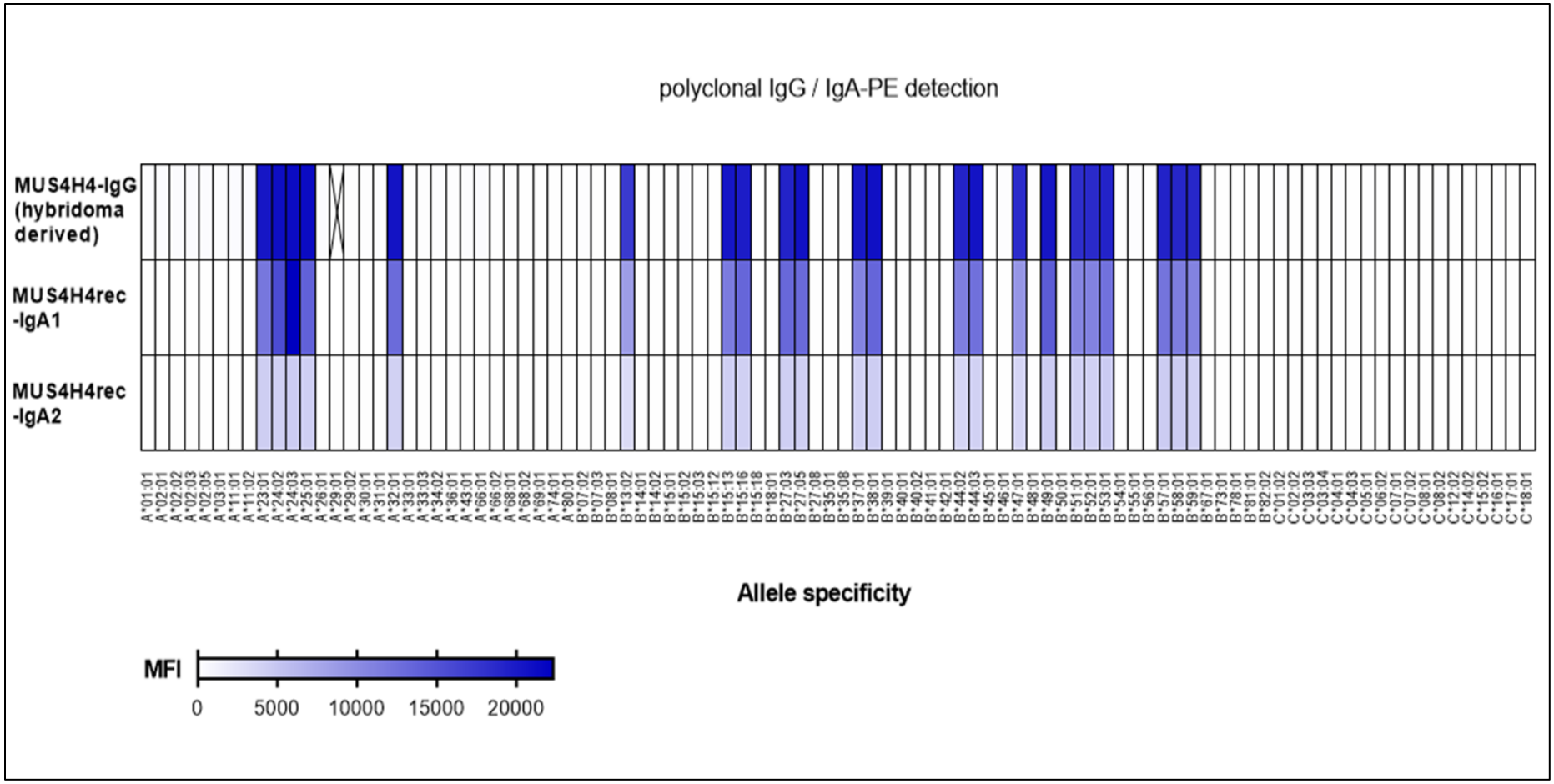


Figure 11. Specificities confirmation for the produced human recombinant IgA HLA antibodies.
Legend: MFI = Mean fluorescence intensity; Monoclonal antibodies were used as neat supernatants with the specific detection.

4.2. Luminex bead-based assay for IgA HLA antibody detection

Development of the Luminex IgA HLA antibody screening assay included a selection of the detection antibody which was further confirmed in specificity, sensitivity and IgA HLA antibody detectability test.

4.2.1. Selection of the antibody detection for IgA HLA Luminex bead-based assays

In order to screen for IgA1 and IgA2-specific HLA antibodies separately in a sample, we first used the mouse anti-human-IgA1 (clone: B3506B4, conc. 10 µg/mL) and -IgA2 (clone: IS11-21E11, conc. 2.2 µg/mL) PE-conjugated detection antibodies on human recombinant HLA class I-specific MUS4H4rec-IgA1 and -IgA2 mAbs (both 1 µg/ml). Detection antibody IgA1-PE specifically detected IgA1 mAb (median MFI: 4376, range: 2234-5400) without any cross-reactivity when tested on IgA2 mAb (median MFI: 36, range: 7-52) (Figure 10A, left panel). With the IgA2-PE used on IgA1 and IgA2 mAbs, IgA2 mAb (median MFI: 1544, range: 630-2068) was detected and cross-reactivity with IgA1 mAb (median MFI: 21, range: 18-31) was excluded (Figure 12A, right panel). Still, compared to IgA1-PE applied on IgA1 mAb (median MFI: 4376, range: 2234-5400), IgA2-PE applied on IgA2 mAb (median MFI: 1544, range: 630-2068) gave lower detection signal.

Considering that a polyclonal detection antibody for IgA may amplify the MFI signals by binding multiple epitopes on IgA antibody and hence increase the detectability, we tested the specificity and sensitivity of a polyclonal IgA detection antibody goat anti-human IgA-PE (conc. 10 µg/mL) on human recombinant MUS4H4rec-IgA1 and -IgA2 mAbs (Figure 12B). Polyclonal detection antibody provided a higher MFI signal when applied on IgA1 mAb (median MFI: 15049, range: 9874-18220) and IgA2 mAb (median MFI: 12089, range: 7741-14546) antibody samples in a concentration of 1 µg/ml (Figure 12B, left panel). When the polyclonal IgA detection antibody was used on 10 fold-lower concentrations of IgA1 and IgA2 mAbs (0.1 µg/ml), measured detection signal was still high for IgA1 mAb (median MFI: 9701, range: 6312-13415) and IgA2 mAb (median MFI: 9539, range: 5912-11946) (Figure 12B, right panel).

Comparison of the MFI signal obtained from monoclonal and polyclonal IgA-PE detection antibodies applied on MUS4H4rec-IgA1 and -IgA2 mAbs, showed a significant difference (Wilcoxon test, p=0.0156) between the monoclonal and polyclonal detection (Figure 12C).

Moreover, utilization of the goat anti-human IgA-PE detection antibody resulted in higher MFI values indicating the superior sensitivity of polyclonal IgA detection.

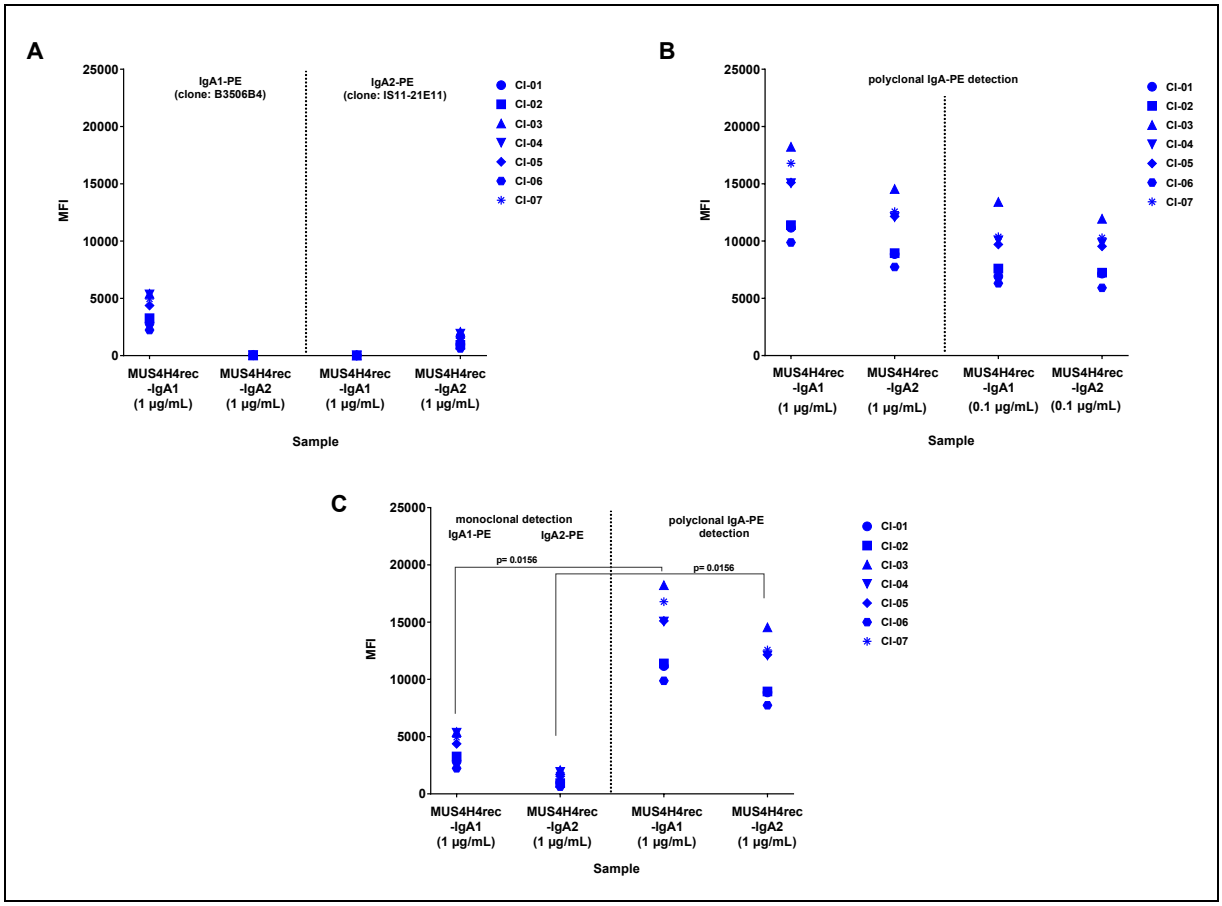


Figure 12. Luminex screening assay for the IgA HLA antibody detection modified with monoclonal (A) and polyclonal (B) IgA detection antibodies and comparison of monoclonal and polyclonal IgA detection (C).
Legend: MFI = Mean fluorescence intensity, CI-0X = HLA class I bead group; Every sample is represented with 7 symbols (bead groups) for HLA class I.

4.2.2. Concentration determination for the selected IgA detection antibody

The final dilution 1:50 (concentration 10 µg/mL) of the selected goat anti-human IgA-PE (IgA-PE) detection antibody for the IgA-LMX assay was determined based on the comparison of MFI signal measured after applying IgA-PE detection in dilutions 1:10, 1:50 and 1:250 on human recombinant HLA-specific MUS4H4rec-IgA1 and -IgA2 mAbs in the Luminex screening assay. Significant difference was observed (Friedman test, p=0.0169 for IgA1 mAb and p=0.0023 for IgA2 mAb) between the signals obtained with detection dilutions 1:10 (median MFI: IgA1-12456, IgA2-6098), 1:50 (median MFI: IgA1-15044, IgA2-11521) and 1:250 (median MFI: IgA1-12505, IgA2-9631) (Figure 13). Thereby, the strongest detection

signal was measured when the IgA-PE detection was applied in dilution 1:50. Furthermore, MFI values for Pos Ctrl (raw MFI: IgA1-190, IgA2-251) and CONs (mean MFI: IgA1-138, IgA2-156) were low, excluding cross-reactivity of detection antibody with IgG HLA antibody coating on the positive control bead as well as the influence of background noise on results.

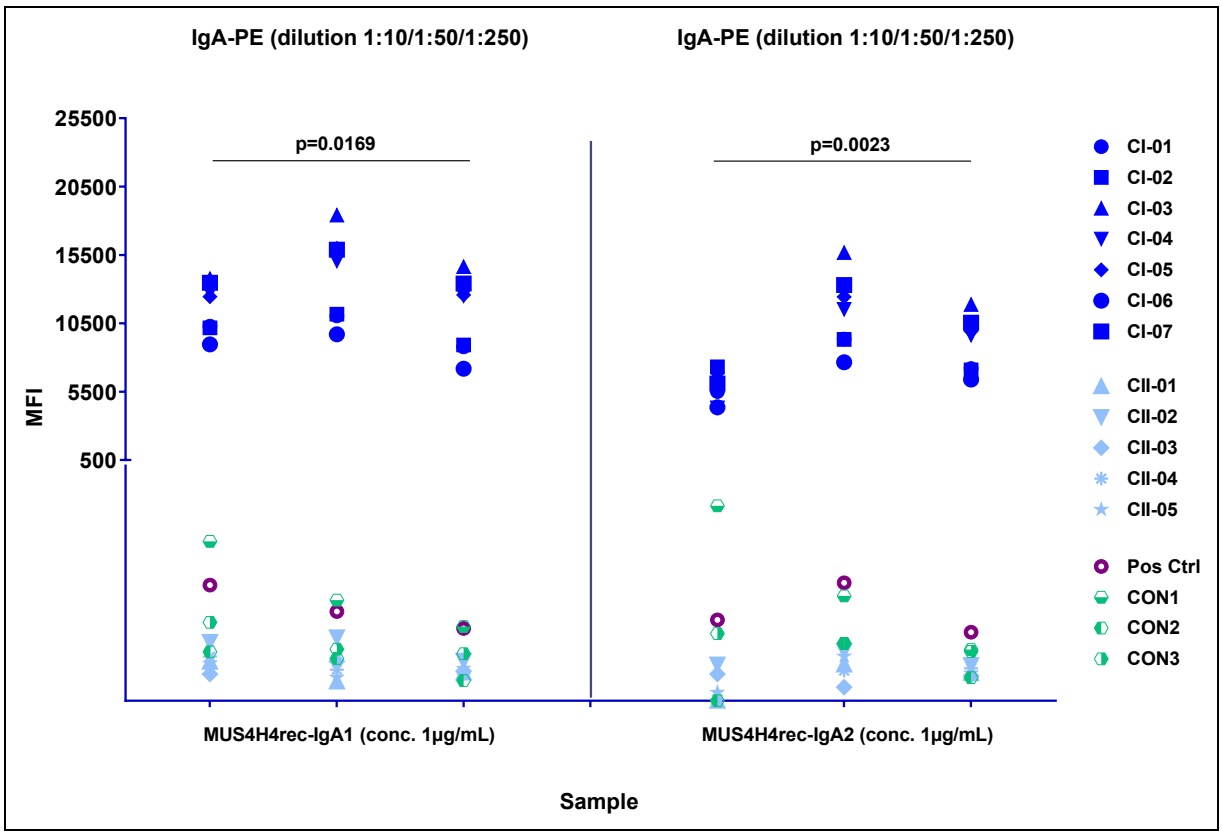


Figure 13. Determination of the detection antibody dilution (concentration) to be used in the IgA HLA antibody screening assay.
Legend: MFI = Mean fluorescence intensity, CI-0X = HLA class I bead group, CII-0X = HLA class II bead group, Pos Ctrl = Positive control bead, CON = Negative control bead; Every sample is represented with 7 symbols (bead groups) for HLA class I and 5 symbols (bead groups) for HLA class II.

Polyclonal IgA-PE detection used in the above-selected dilution 1:50 (conc. 10 µg/mL) showed high sensitivity in IgA-LMX assay. When applied on samples of human recombinant HLA-specific MUS4H4rec-IgA1 and MUS4H4rec-IgA2 mAbs prepared in a concentration range 0.06-1 µg/mL, the measured MFI signal was high and comparable in mAb samples prepared in the concentration 1 µg/mL (median MFI: IgA1-15049, IgA2-12089), 0.5 µg/mL (median MFI: IgA1-13970, IgA2-11543) and 0.25 µg/mL (median MFI: IgA1-13060, IgA2-10911). Even in a very low concentration of 0.06 µg/mL both MUS4H4rec-IgA1 (median MFI: 6565) and MUS4H4rec-IgA2 (median MFI: 7365), mAbs were detectable, showing selected dilution of polyclonal IgA-PE detection reliable for IgA screening assay (Figure 14).

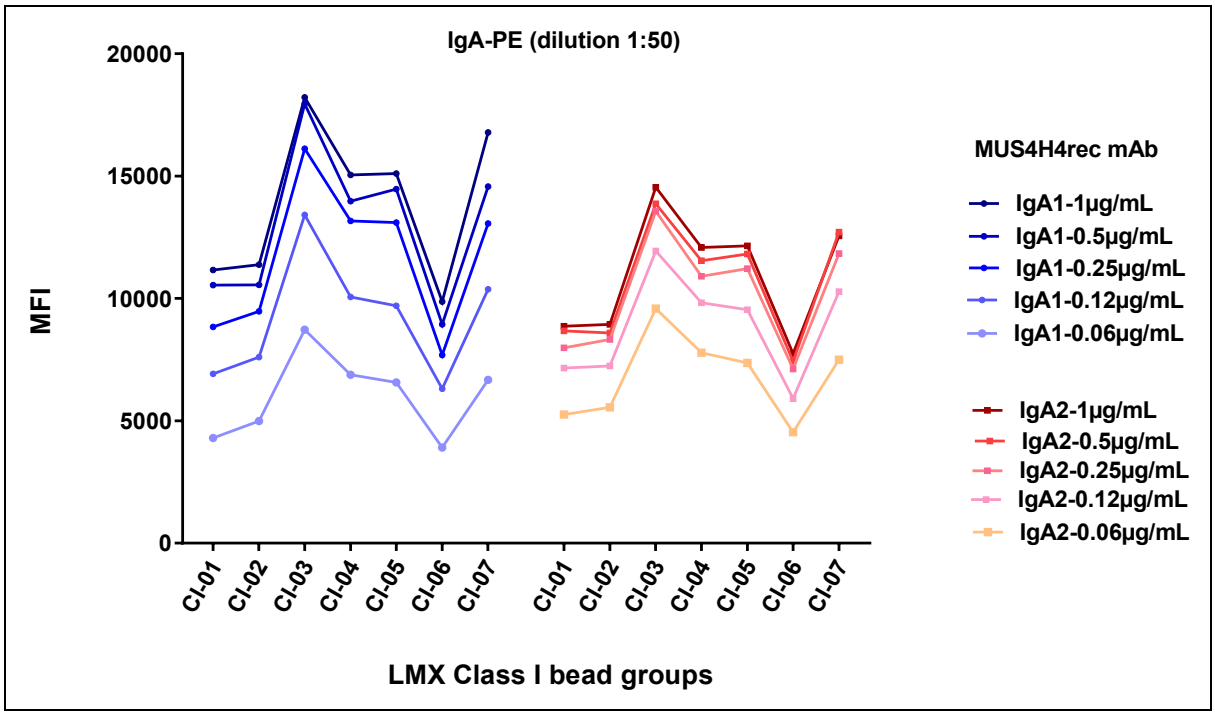


Figure 14. Sensitivity test for the selected dilution 1:50 (conc. 10 µg/mL) of the polyclonal IgA-PE detection antibody to be used in IgA HLA antibody screening assay.
Legend: MFI = Mean fluorescence intensity, CI-0X = HLA class I bead group; Every sample is represented with 7 dots (bead groups) for HLA class I.

4.2.3. Specificity test for the selected IgA detection antibody

The specificity of the polyclonal IgA-PE detection (conc. 10 µg/mL) was confirmed in the IgA-SAB assay where the IgA detection was applied on the HLA-specific MUS4H4rec-IgA1, -IgA2 and -IgG1 mAbs, blend of B cell-derived IgM mAbs and PBS sample. When IgA detection antibody was added on IgA1 and IgA2 mAbs (conc. 1 µg/mL), measured detection signal for IgA1 (MFI range: 29-20336) and IgA2 (MFI range: 33-18725) mAb samples gave very high MFI values (marked in blue) for 22 HLA class I specificities with which these antibodies are determined. When IgA-PE detection was applied on IgG1 (neat, conc. 60 µg/mL) and IgM (neat, conc. 40 µg/mL) mAb samples, low MFI values (marked in orange) observed for IgG (MFI range: 35-116) and IgM (MFI range: 41-115) mAbs demonstrated the absence of cross-reactivity between IgA-PE detection antibody and IgG and IgM mAb isotypes. Non-specific binding of the IgA-PE detection antibody to the beads was excluded based on the low MFI values (marked in orange) obtained when PBS (MFI range: 34-100) was used as a sample (Figure 15).

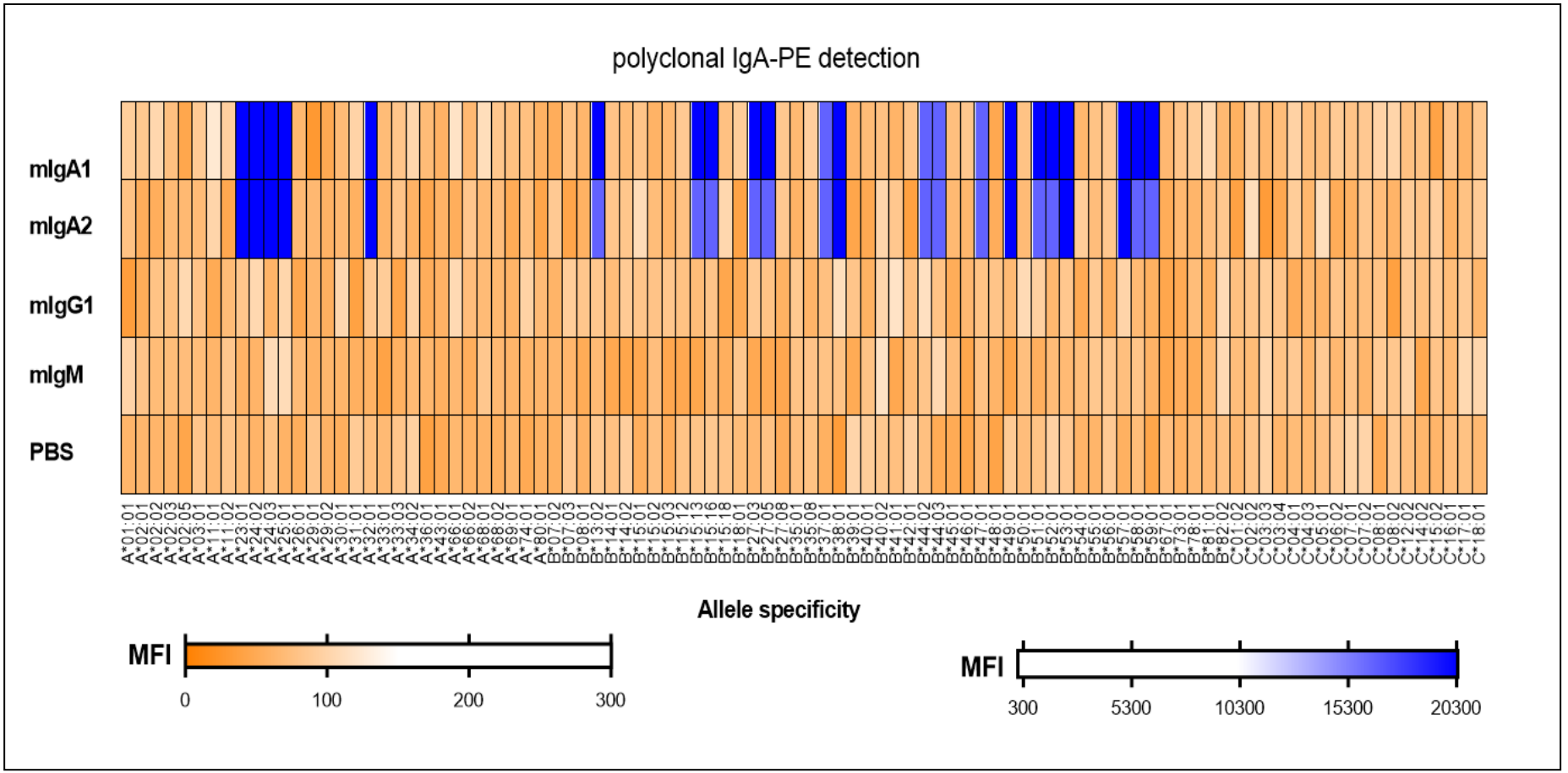


Figure 15. Polyclonal IgA-PE detection antibody cross-reactivity and non-specific binding test performed in the IgA HLA antibody single antigen bead assay.

Legend: m = Monoclonal, PBS = Phosphate-buffered saline, MFI = Mean fluorescence intensity

4.2.4. Comparison of IgA and IgG detection antibody sensitivity

The sensitivity of the polyclonal IgA-PE detection antibody was further compared to standard IgG detection by testing IgA1, IgA2 and IgG1 isotype variants of the same human recombinant HLA class I-specific MUS4H4rec mAb at the same equimolar concentrations of 6.3 nM~1 µg/mL. When polyclonal IgA detection antibody was used to detect MUS4H4rec-IgA1 (MFI range: 9693-18409) and MUS4H4rec-IgA2 (MFI range: 7655-15670) mAbs, MFI values comparable to MUS4H4rec-IgG1 (MFI range: 9474-16384) detected with IgG detection were obtained in the LMX assays. Since only HLA class I-specific mAbs were tested, HLA class II reactivity was not detected, as expected. There was no significant difference in the detection signal (Kruskal-Wallis test, p=0.2314) between IgA and IgG HLA antibody screening assay (Figure 16A). In the IgA-SAB assay, all 22 MUS4H4 HLA class I specificities were detected in IgA1 (MFI range: 15926-18651) and IgA2 (MFI range: 13837-18529) mAbs with similar but significantly different (p=0.0110) MFI values to detected IgG1 isotype (MFI range: 15189-20453) in IgG-SAB assay (Figure 16B).

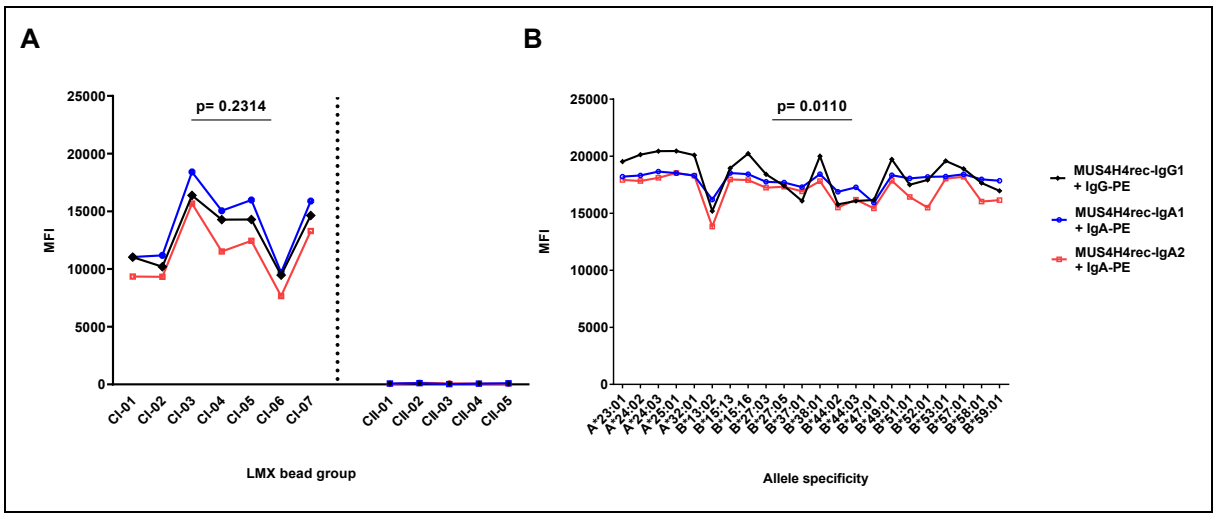


Figure 16. Comparison of polyclonal IgA-PE and IgG-PE detection sensitivity in Luminex HLA antibody screening (A) and single antigen bead (B) assays.
Legend: MFI = Mean fluorescence intensity, CI-0X = HLA class I bead group, CI-0X = HLA class I bead group

4.2.5. Detectability of IgA HLA antibody in serum environment

Spiking experiments were performed to test the detectability of the IgA HLA antibody in the serum environment when surrounded by IgG HLA antibodies that recognize a different or the same epitope.

In serum from HLA non-alloantigens exposed individual spiked with HLA-specific IgA1 (MUS4H4rec) and IgG1 (SN607D8) mAbs in 1:1 and 1:4 IgA1/IgG1 ratio, detectability of IgA1 (median MFI: 20954; range: 18722-21576) was not affected by the presence of IgG mAb in 1:1 mAbs ratio (median MFI: 21187, range: 17464-21668) nor in 1:4 mAbs ratio (median MFI: 21157; range: 17226-21979), recognizing a different epitope (Figure17A). The Mann-Whitney test indicated no significant difference between the MFI signal obtained from MUS4H4rec-IgA1 and MUS4H4rec-IgA1/SN607D8-IgG1 1:1 mAb ratio solution (p=0.1250) nor from MUS4H4rec-IgA1 and MUS4H4rec-IgA1/SN607D8-IgG1 1:4 mAb ratio solution (p=0.0500).

Similarly, when HLA-specific IgA1 and IgG1 (both MUS4H4rec) mAbs were mixed in a serum sample, MUS4H4rec-IgA1 mAb was still detectable, although with relatively lower MFI values in 1:1 (median MFI: 15386; range: 11449-19075) and 1:4 (median MFI: 9683; range: 6669-13476) MUS4H4rec-IgA1/MUS4H4rec-IgG1 mAbs ratios compared to serum spiked solely with MUS4H4rec-IgA1 mAb (median MFI: 20954, range: 16044-20336) (Figure 17B). The Mann-Whitney test showed a statistically significant difference (p<0.0001) in the MFI signal when the MUS4H4rec-IgA1 sample was compared with both, 1:1 and 1:4 MUS4H4rec-IgA1/MUS4H4rec-IgG1 mAb ratio solutions.

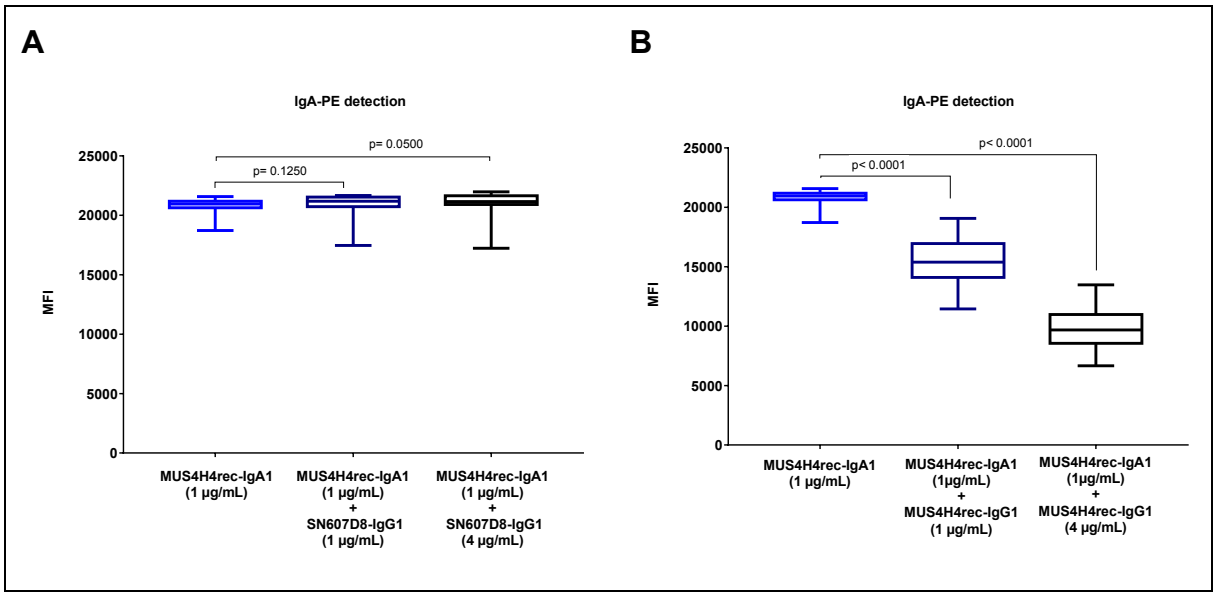


Figure 17. Detectability of IgA HLA antibody in the IgG HLA antibody enriched serum tested in the IgA HLA antibody single antigen bead assay.
Legend: MFI = Mean fluorescence intensity; In sample comparison, only positive MUS4H4 mAb HLA specificities were taken into account.

4.3. Validation of the IgA HLA antibody screening assay on serum samples and cut-off determination

To validate the developed IgA-LMX assay, sera from HLA alloantigen non-exposed individuals with IgG-LMX negative status (N=18) were selected and screened for the presence of IgA HLA antibodies. Measured MFI values in samples from HLA alloantigen non-exposed individuals, for HLA class I (median MFI: 38, range: 0-119) and HLA class II (median MFI: 40, range: 0-131), were in a range of MFI values obtained from PBS, for HLA class I (median MFI: 42, range: 16-91) and HLA class II (median MFI: 66, range: 38-75). This confirmed the absence of IgA HLA antibodies in the serum.

The threshold for positive IgA-LMX responses was defined based on Tukey's extreme outlier calculation applied to the IgA and IgG HLA antibody screening results from 18 HLA alloantigen non-exposed individuals. When Tukey's extreme outlier calculation was applied to the IgG-LMX results, thereby defined cut-off for IgG HLA antibodies (extreme MFI: HLA class I-1242, HLA class II-1666) was in line with the cut-off provided by the manufacturer (Lifecodes) for IgG-LMX kit (1500 MFI). In this way, we confirmed that Tukey's extreme outlier calculation can be used to define the cut-off value for the IgA HLA screening assay. Based on the values for IgA HLA antibodies (extreme MFI: HLA class I-206, class II-166) an arbitrary cut-off (250 MFI) was set for HLA class I and HLA class II beads in the IgA-LMX assay (Figure 18).

To validate the established Tukey's arbitrary cut-off (250 MFI), we performed IgA-SAB assay on serum from HLA alloantigen exposed individuals with a history of transplantation (N=12), selected based on their IgA-LMX results (raw MFI values for the highest-ranked HLA class I or HLA class II bead group in a range 100-700) and compared their IgA-LMX results with the detected IgA DSAs. As a result, we were able to define the highest-ranked specificities in IgA-SAB assay as DSA in 2 out of 12 samples. Specifically, the IgA DSAs were detected only in samples with raw MFI>500 of the highest-ranked bead group in IgA-LMX assay, in sample number 11 (#11) with 508 MFI and sample #12 with 633 MFI (Table 4). Therefore, based on the IgA DSA status of tested samples, the cut-off value for IgA-LMX assay was redefined and samples with raw MFI≥500 for any of the HLA class I and/or HLA class II bead group were considered positive throughout the study.

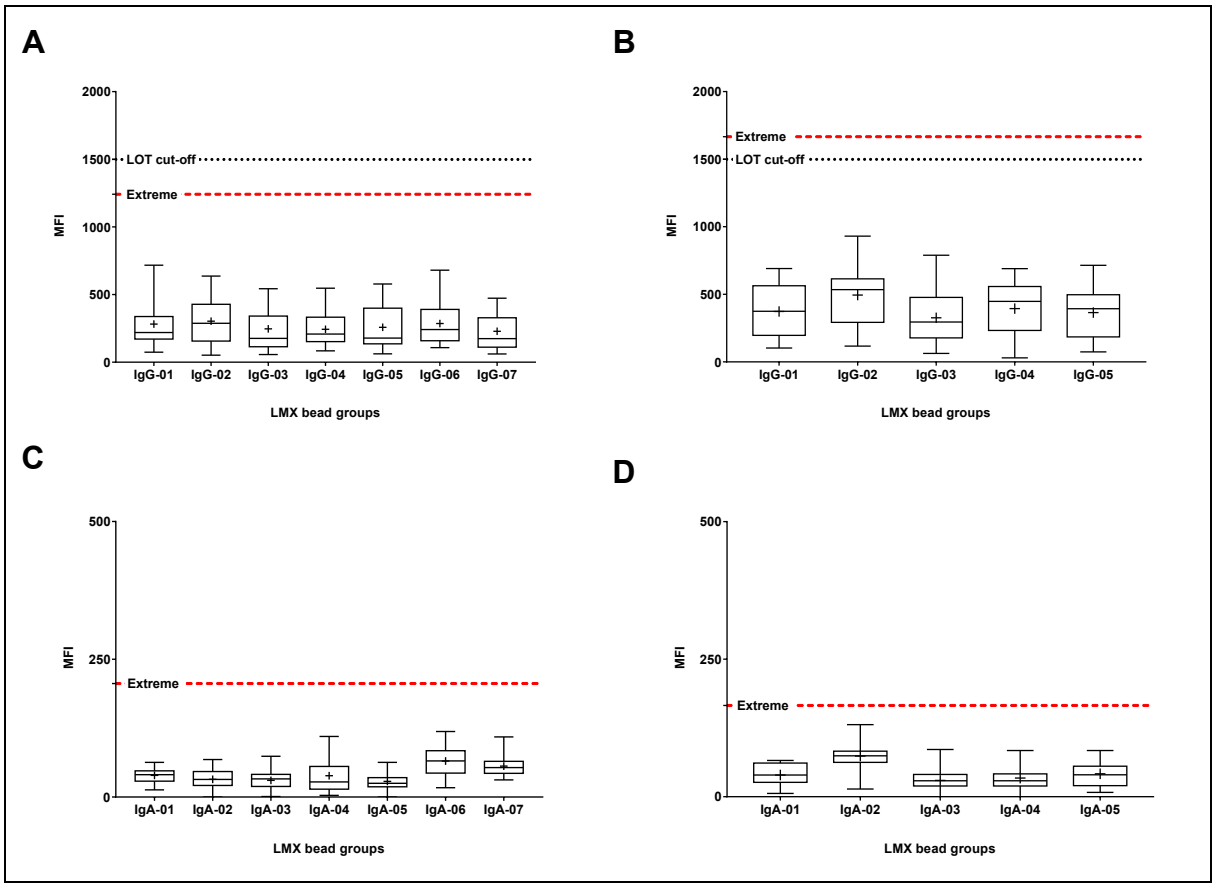


Figure 18. Threshold determination for the IgG HLA class I (A), IgG HLA class II (B), IgA HLA class I (C) and IgA HLA class II (D) antibody positive screening responses based on Tukey's extreme outlier calculation applied on screening results from HLA non-immunized individuals. The box depicts the median and the first and third quartiles and the "+" symbol represents the mean value. The vertical red line represents the highest extreme value for the LMX group of class I/II beads and the blue line demonstrates the cut-off value provided by the manufacturer of the LMX assay kit.

Table 4. Validation of the Tukey's based cut-off for IgA HLA antibody screening results performed on serum samples from HLA alloantigen exposed individuals

Sample number	MFI value of HR HLA bead group in IgA-LMX class I/class II		MFI value of HR HLA class I bead in IgA-SAB	HLA class I IgA DSA		MFI value of HR HLA class II bead in IgA-SAB	HLA class II IgA DSA	
				specificity	MFI		specificity	MFI
1	**131**	100	96	A11	66,82	105	DR53 DQ7 DQ8	60 67,55,63,25 66,35,61
2	**137**	87	144	B*35 C*01:02 C*04:01	59,53 75 102	142	DRB1*11 DQB1*03:01	60,56,77 70,103,56,46
3	**180**	110	119	A*24:02 A*24:01 B7 B60 C*03:03 C*03:04 C*07:01 C*07:02	102 81 74 81 108 73 113 96	204	DR4 DR15 DQ8 DQ6	75,75,77,67,67 84,89,84 141,97,100 91,77,97,80, 73,127
4	**204**	110	136	A*03:01 B*07:02 B*44:02 B*44:03 C*07:02	90 69 83 88 87	130	DQ4	71,64,58,39,36
5	**228**	96	117	B*15:01 C*03:04	76 85	140	DRB1*01 DQB1*05:01	63,56,44 63,56
6 *	**254**	135	455	B8	51	150	DR17 DQ2	37 94,77,72,10,54
7 *	**267**	136	137	A*03:01 A*30:01 B*35:01 C*04:01 C*17	80 73 90 64 89	238	no MM in HLA typing between patient and donor	
8 *	**302**	199	115	A*31:01	86	162	no MM in HLA typing between patient and donor	
9 *	**324**	156	128	A*30 B*44:02 C*05	86 91 80	117	DRB1*12 DQB1*03:01	74,72 71,72,75,78
10 *	**489**	284	398	A1 B39	144 121	186	DR8 DQ4	39,60 125,139,169, 119,165,136
11 *	227	**508**	439	B*40:01 B*44:02 C*03:04 C*05:01 C*07:01	118 406 77 102 76	405 DSA	DRB1*04 DRB1*13:01 DQB1*03:01 DQB1*06:03 DQB1*06:09	47,52,50,49, 74,80 44 405,373, 353,291 79 NA
12 *	366	**633**	730	A*33:01 A*68:01 B*14:02 C*08:01	13 175 156 77	255 DSA	DRB1*01:02 DRB1*04:01 DQB1*05:01	209 67 255,163

Legend: HR = Highest-ranked, IgA-LMX= IgA HLA antibody screening, IgA-SAB = IgA HLA single antigen bead, IgA DSA = Donor-specific IgA HLA antibody, MFI = Mean fluorescence intensity, MM = Mismatch, NA = No specific allele in the assay, * IgA-LMX sample assigned positive based on Tukey's cut-off of 250 MFI; The relevant MFI values for sample selection are shaded and bolded and all MFI values in the table are given as raw values. For HLA antigen specificities present in the assay kit on more than one bead and/or as a part of broad specificity, raw MFI values for all beads/specificities were noted. Specificities belonging to the same broad specificity are given by rising specificity numbers.

4.4. Detection of IgA HLA antibodies in study population samples

Having developed a reliable IgA-LMX assay, all 289 HLA alloantigen exposed individuals were screened for the presence of IgA HLA antibodies. Screening results were further analysed and IgA-LMX positive samples were tested with two commercially available single antigen bead assay kits.

4.4.1. Frequency of IgA HLA antibodies in HLA alloantigen exposed individuals

To determine the IgA HLA antibody frequency related to a different route of HLA alloantigen exposure, we analysed 175 selected samples from kidney transplant waitlist patients with a history of transplantation (N=84) and women immunized through pregnancy (N=91). In the serum from transplant recipients, IgA HLA antibodies were detected in eight samples (9.52%). Detected IgA HLA antibodies were directed against HLA class II in seven samples, while in one sample directed against HLA class I and HLA class II antigens. Among samples from women with a history of pregnancy, IgA antibodies were observed in two samples (2.20%), directed against HLA class I in one sample and HLA class II antigens in another sample. In all samples, IgA HLA antibodies were detected exclusively in the presence of IgG isotype of HLA antibody.

In summary, 10 samples (5.71%) from 175 individuals with a different history of HLA alloantigen exposure contained IgA HLA antibodies. When combined HLA IgA and anti-HLA IgG (IgA±/IgG±) antibody frequencies between the two groups were compared, no significant difference was observed (Mann-Whitney test, p=0.9445) (Figure 19).

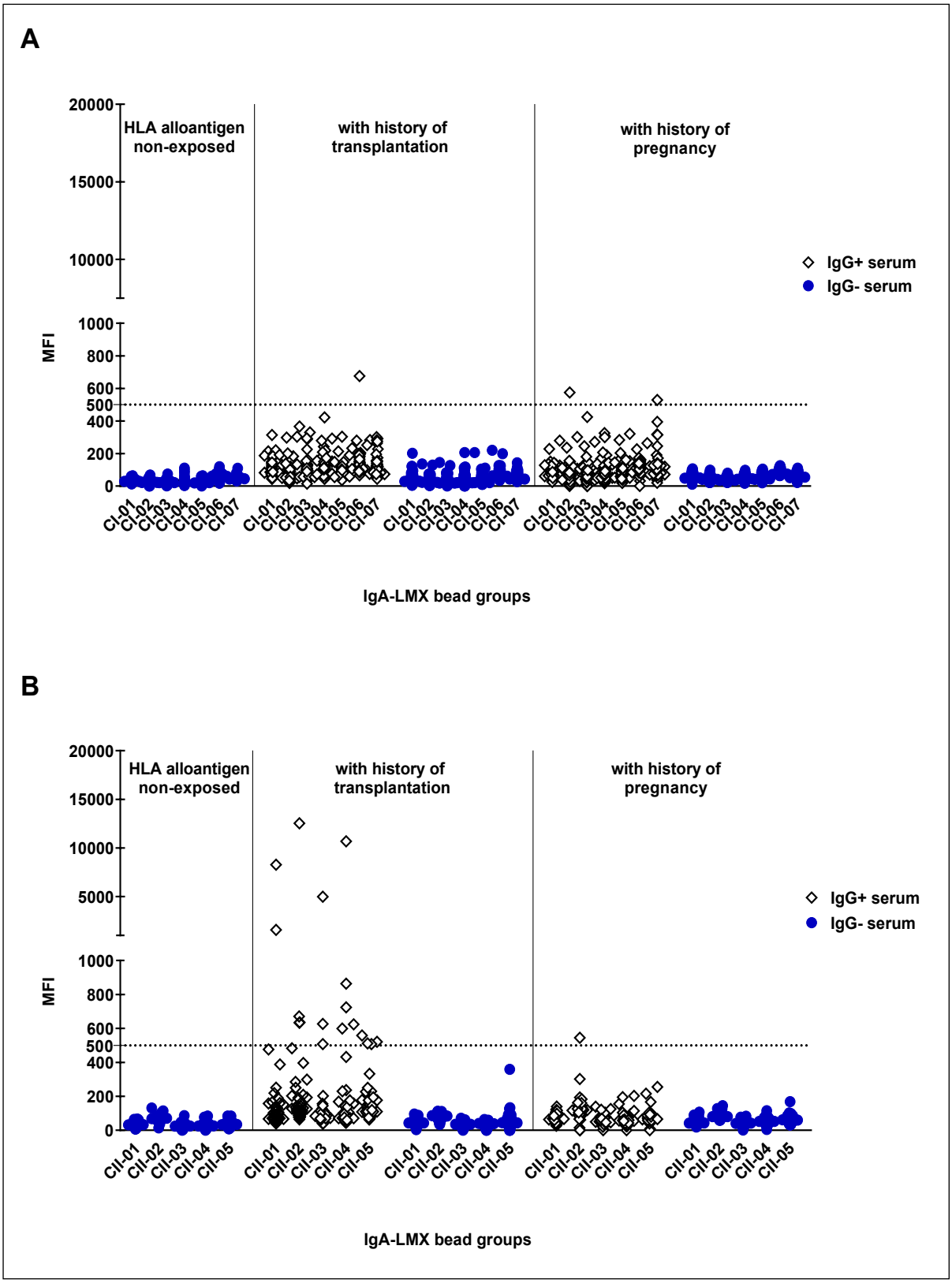


Figure 19. IgA HLA class I (A) and IgA HLA class II (B) antibody screening results from individuals with a different history of HLA alloantigen exposure (N=175).

Legend: IgG- = IgG HLA antibody negative, IgG+ = IgG HLA antibody positive; Every individual is represented with 7 symbols (bead groups) for HLA class I and 5 symbols (bead groups) for HLA class II. The arbitrary cut-off of 500 MFI (represented with dotted line) for any of HLA class I and/or HLA class II bead groups was used to assign samples as IgA-LMX positive.

Since the presence of IgA HLA antibodies in kidney transplant recipients is correlated with a better graft outcome, post-transplant serum samples from 109 kidney transplant patients with functioning grafts were screened for the presence of IgA HLA antibody. In samples from these patients, IgA HLA antibodies were not observed. Considering that IgA HLA antibodies may have a role in ABMR, pre- and post-transplantation samples (10 samples in total) from 5 patients who experienced ABMR were screened for the presence of IgA HLA antibody but did not contain any IgA HLA antibodies.

The overall frequency of IgA HLA antibody among all 289 HLA alloantigen exposed individuals was low (Table 5).

Table 5. Overview of detected IgA HLA antibodies in serum from all HLA alloantigen exposed individuals included in this study (N=289)

Study population group	Detected IgA HLA antibodies					
	F	M	HLA class I	HLA class II	HLA class I+II	DSA status
Kidney transplant waitlist patients with a history of tx (N=84)						
IgG+ (N=41)	2/18	6/23	0	7	1	5 DSA, 1 non-DSA, 3 NS
IgG- (N=43)	0/21	0/22	IgA HLA antibody not detected			
Transplant recipients with functioning grafts (N=109)						
IgG+ (N=25)	0/6	0/19	IgA HLA antibody not detected			
IgG- (N=84)	0/14	0/70	IgA HLA antibody not detected			
Patients with biopsy-proven early ABMR without detected IgG HLA antibodies (N=5)						
IgG+ (N=0)	0	0	IgA HLA antibody not detected			
IgG- (N=5)	0/5	0	IgA HLA antibody not detected			
Women with a history of pregnancy (N=91)						
IgG+ (N=55)	2/55	0	1	1	0	1 DSA, 1 NS
IgG- (N=36)	36	0	IgA HLA antibody not detected			

Legend: N = Number of individuals, IgG+ = IgG HLA antibody positive, IgG- = IgG HLA antibody negative, ABMR = Antibody-mediated rejection, F = Female, M = Male, DSA = Donor-specific antibody, non-DSA = Non-donor-specific antibody, NS = Not specified (typing not available); DSA status is reported for the higest-ranked IgA-SAB specificity. IgA-LMX assay was applied for antibody detection and IgA-SAB assay for specificity identification.

IgA HLA antibodies were detected in ten out of 294 samples (3.40%), directed mostly against HLA class II (8; 2.72%) (Figure 20).

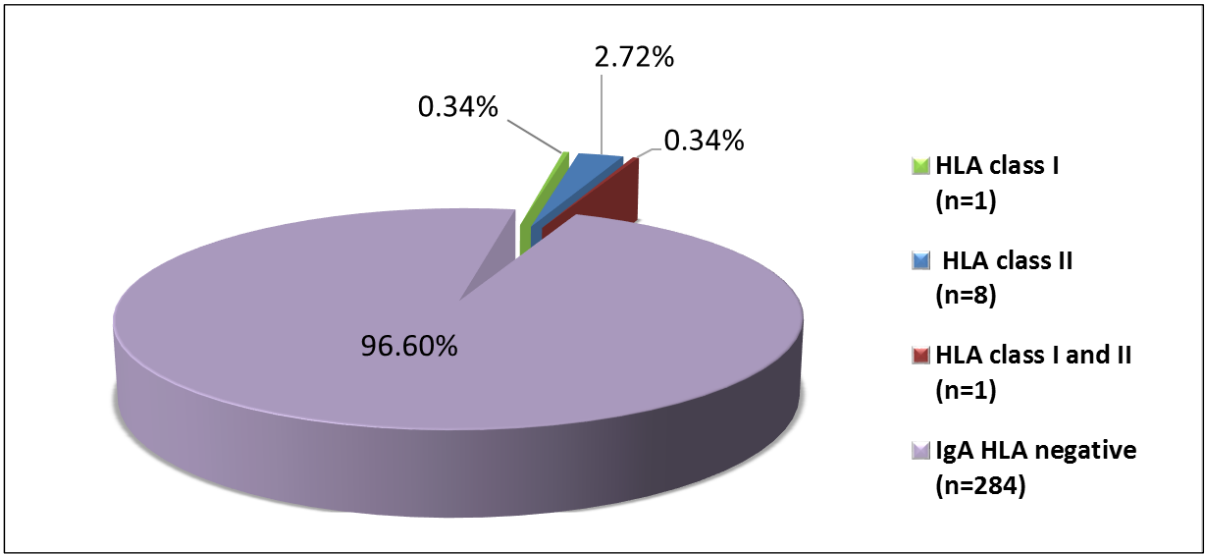


Figure 20. Incidence of detected IgA HLA antibody in serum from HLA alloantigen exposed individuals (N=289). Luminex IgA HLA antibody screening assay was applied.

4.4.2. Comparison of IgA and IgG HLA antibody screening results from HLA alloantigen exposed individuals

When IgA and IgG HLA antibody screening results from HLA alloantigen exposed individuals were compared, we observed IgA HLA antibodies always followed by IgG isotype. IgA HLA antibodies were found in IgG HLA antibody positive (IgG+) samples from kidney re-transplant waitlist patients (8/41; 19.51%) and women with a history of pregnancy (2/55; 3.64%) while among IgG HLA antibody negative (IgG-) transplant recipients (N=42) and women with a history of pregnancy without IgG HLA antibody isotype (N=36), IgA HLA antibodies were not found. Among kidney re-transplant waitlist patients having IgG HLA antibodies, IgA HLA antibodies were more prevalent in male (6/23; 26%) than in female patients (2/18; 11%). Despite their IgG HLA antibody status, in samples from 109 (83 IgG- and 24 IgG+) kidney transplant recipients with functioning graft and 5 patients who experienced ABMR (IgG- pre- and post-transplantation), IgA HLA antibodies were not observed. Upon the antibody status stratification into four mutually exclusive subgroups IgG+/IgA+, IgG-/IgA+, IgG+/IgA- and IgG-/IgA-, observed overall incidence of IgG+/IgA+ subgroup was low when antibodies were directed against HLA class I (0.68%) and HLA class II (3.06%) antigens (Figure 21).

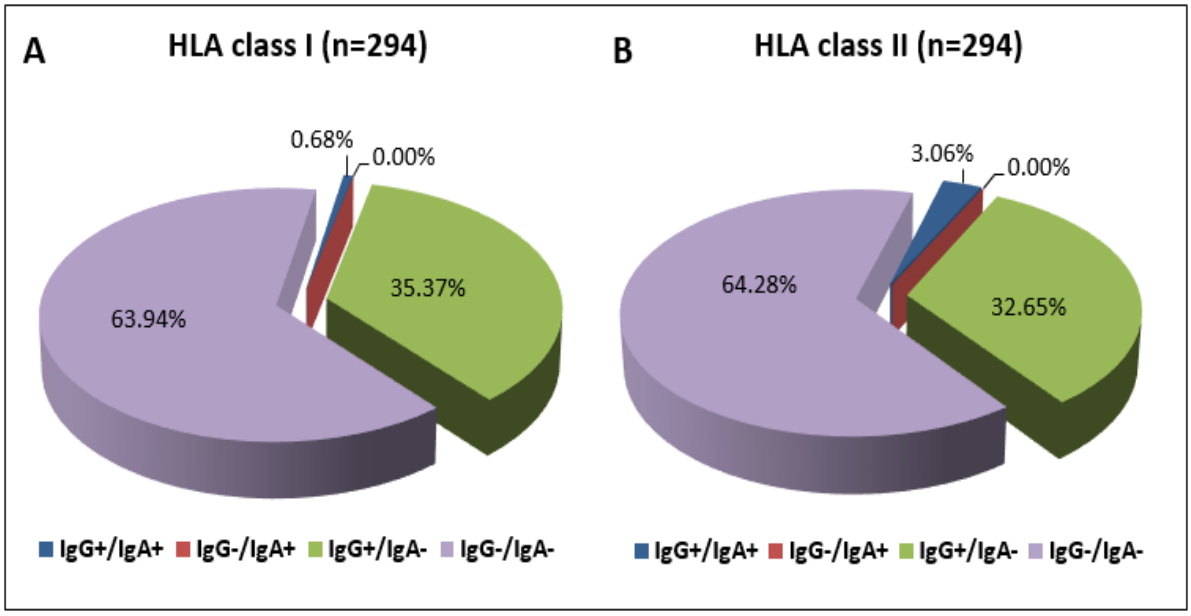


Figure 21. Incidence of IgA HLA class I (A) and IgA HLA class II (B) antibodies dependent on the presence of IgG HLA antibody in serum from HLA alloantigen exposed individuals (N=289).
Legend: IgG- = IgG HLA antibody negative, IgG+ = IgG HLA antibody positive,
IgA- = IgA HLA antibody negative, IgA+ = IgA HLA antibody positive; Luminex IgA HLA antibody screening assay was applied.

4.4.3. Comparison of IgA HLA antibody screening and single antigen bead assay results from IgA-LMX positive samples

In IgA-LMX positive individuals immunized through pregnancy (N=2) and transplantation (N=8), IgA antibodies were directed against HLA class I in one sample and HLA class II in eight samples, while one sample had IgA antibodies against HLA class I and class II. Detected IgA HLA antibodies had low MFI signal (median MFI: 877, range: 230-9178) and were related to DSA in six samples (MFI range: 255-9178) and non-DSA in one sample (MFI 877), while for four samples (MFI range: 230-1666) antibodies were not specified (NS) due to the missing HLA typing data. Detected IgA DSA were directed against HLA class I (N=2) and HLA class II (N=4) antigens. Further epitope analysis showed that IgA non-DSA directed against HLA class II in sample #1 recognizes the same epitope as IgA DSA detected in the same sample but with a lower MFI value. In three out of four samples with NS antibodies, detected antibodies were directed against HLA DP antigens (Table 6). Finally, self-antigens were observed with low MFI values (MFI range: 16-178) and among lower-ranked HLA specificities.

Table 6. Overview of the IgA HLA antibody screening and single antigen bead assay results from IgA HLA antibody positive screening samples (N=10)

Sample number	Route of immunization	Gender	IgA-LMX positive	IgA-SAB HR bead specificity	IgA-SAB HR bead specificity raw MFI	Status of the HR IgA-SAB specificity
1	tx	M	HLA class I	B*08:01	1602	DSA
			HLA class II	DRB1*11:01	877	non-DSA
2	tx	M	HLA class II	DRB1*04:05	9178	DSA
3	tx	M	HLA class II	DRB4*01:01	661	DSA
4	tx	F	HLA class II	DQA1*05:01 DQB1*03:01	405	DSA
5	tx	M	HLA class II	DQA1*01:02 DQB1*05:01	255	DSA
6	tx	M	HLA class II	DPA1*02:02 DPB1*28:01	230	NS
7	tx + pr	F	HLA class II	DPA1*02:01 DPB1*04:01	1479	NS
8	tx	M	HLA class II	DPA1*02:01 DPB1*04:01	1666	NS
9	pr	F	HLA class I	A*32:01	1145	DSA
10	pr	F	HLA class II	DRB1*11:01	244	NS

Legend: IgA-LMX = IgA HLA antibody screening, IgA-SAB = IgA HLA antibody single antigen bead, HR = Highest-ranked, MFI = Mean fluorescence intensity, tx = Transplantation, pr = Pregnancy, neg = Negative, pos = Positive, DSA = Donor-specific antibody, non-DSA = Non-donor-specific antibody, NS = Not specified (typing not available); Lifecodes kits were applied in IgA-LMX and IgA-SAB tests.

4.4.4. Comparison of IgA HLA antibody single antigen bead assay results from IgA-LMX positive samples obtained with two commercially available assay kits

Single antigen bead assay results from IgA HLA class I (N=1) and IgA HLA class II (N=8) antibody positive screening samples selected from transplant recipients showed an existing difference when two commercially available SAB assay kits (Lifecodes, LC and One Lambda, OL) were used. Comparison of MFI values of donor-specific IgA HLA class I antibodies detected with LC (median MFI: 109, range: 33-1602) and OL (median MFI: 227, range: 47-

5690) IgA-SAB assay kit, showed higher detection signal (ratio OL/LC=2.94) when OL kit was used. When we compared MFI values of donor-specific IgA HLA class II antibodies detected with LC (median MFI: 233, range: 38-9178) and OL (median MFI: 530, range: 20-8871), we again noted a higher detection signal (ratio OL/LC=1.81) when OL kit was applied (Figure 22). Self-antigens from eight tested patients were observed with low MFI values when IgA-SAB assay kit from LC (MFI range: 16-178) and OL (MFI range: 19-465) were applied.

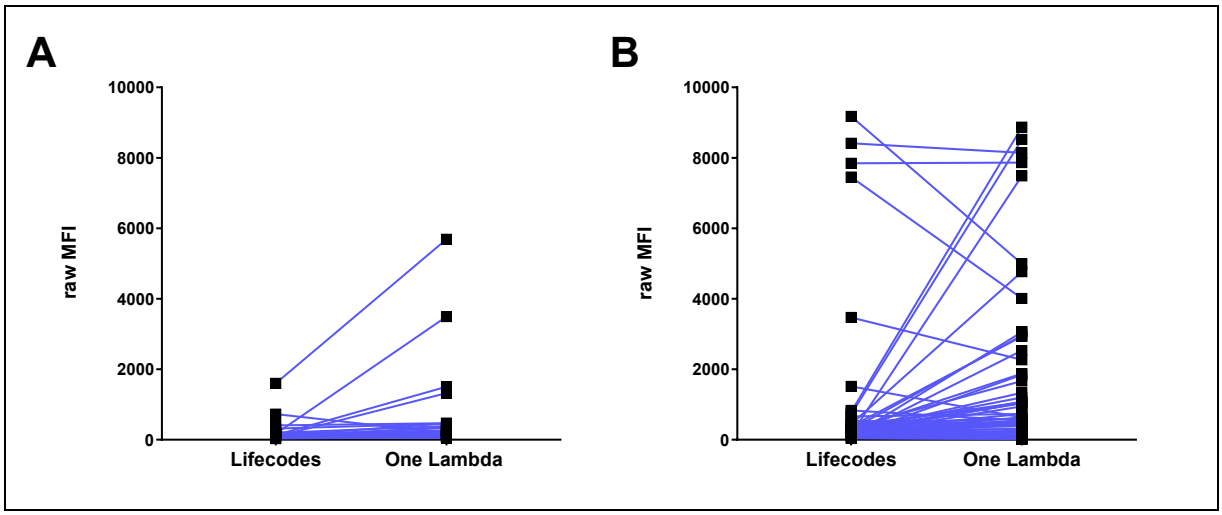


Figure 22. The difference in MFI signal obtained for donor-specific IgA HLA class I (A) and IgA HLA class II (B) antibodies detected with two commercially available IgA single antigen bead assay kits.

In order to compare the IgA-SAB results obtained with LC and OL kit more accurate, IgA-LMX positive samples were grouped based on their IgA HLA class I/II antibody positive and negative screening results (Table 6 and Table 7).

Among IgA-LMX positive samples from transplant recipients, one patient had IgA antibodies directed against HLA class I and HLA class II, while seven patients had only IgA HLA class II antibodies (Table 7). The highest-ranked bead specificities in the IgA-SAB assay reports from these samples were defined as DSAs in five (four common and one different) samples with LC and OL kits. IgA non-DSA related highest-ranked bead specificity was detected with LC kit in one sample (#1), while with OL kit in three (#1, #5 and #7) samples. Epitope analysis for sample #1 showed that IgA non-DSA (DRB1*11:01-LC and DRB1*13:03-OL) related to the highest-ranked bead in IgA-SAB assay reports recognize the same epitope as DSA DRB1*13:01 which is present in the sample but with lower MFI value. The highest-ranked bead specificity was related to the HLA-DP region in three samples when LC and one sample when OL IgA-SAB

assay kit was used. Due to the lack of HLA-DP typing for this patient, we were not able to specify the detected antibodies as IgA DSA or IgA non-DSA.

When we analysed IgA-SAB reports from IgA-LMX positive transplant recipients with negative assigned HLA class I screening result, we were able to relate the highest-ranked bead specificities in the IgA-SAB assay reports to IgA DSA in one sample with LC and two samples with OL kit (Table 8). For the rest of the samples, the highest bead specificity was related to IgA non-DSAs which were detected in six samples with LC and five samples with OL kit. When the MFI signal of the highest-ranked bead specificities defined as IgA DSA and IgA non-DSA detected with LC (DSA MFI: 316; non-DSA MFI range: 181-620) and OL (DSA MFI range: 1126-1325; non-DSA MFI range: 682-4984) IgA-SAB kit were compared, we noticed that some of the highest-ranked bead specificities related to IgA non-DSA had higher MFI values than the IgA DSAs. Furthermore, much higher raw MFI values were observed for the highest-ranked bead specificities defined as IgA non-DSA when OL (MFI range: 682-4984) instead of LC (MFI range: 181-620) assay kit was applied.

Table 7. Comparison of Lifecodes and One Lambda IgA-SAB assay results obtained from IgA HLA antibody positive screening transplant recipients with IgA HLA class I/II antibody positive screening results (N=8)

Sample number	Sample IgA DSA	IgA DSA raw MFI values with LC kit	HR bead in IgA-SAB with LC kit		IgA DSA raw MFI values with OL kit	HR bead in IgA-SAB with OL kit	
			DSA / non-DSA (specificity)	Raw MFI		DSA / non-DSA (specificity)	Raw MFI
1	B*08:01 C*07:01	1602 181	DSA (B*08:01)	1602	5690 NB	DSA (B*08:01)	5690
	DRB1*13:01 DQB1*06:03	722 273	non-DSA (DRB1*11:01)	877	8525 7490	non-DSA (DRB1*13:03)	8871
2	DRB1*11:01 DRB1*04 DQB1*03:02	1517 8017,3663, 7648,8414, 9178 92,135,88	DSA (DRB1*04:05)	9178	634 7865,2267, 4008,8141, 4992 2533,223,85	DSA (DRB1*04:04)	8141
3	DRB1*07 DRB1*11:02 DR1 DR2 DRB4 DQ1	115 NB 400,380 127,112,120, 123,132 661 236,247,183, 162,138,134, 233,150,124, 131	DSA (DRB4*01:01)	661	205 NB 508,477,581 156,138,136 733,606 552,208, 530,255,449, 95,1336,463	DSA (DQA1*01:03 DQB1*06:03)	1336
4	DRB1*13:01 DQB1*06:03 DQB1*06:09 DRB1*04 DQB1*03:01	44 79 NB 52,49,74, 50,80 405,353,373, 291	DSA (DQA1*05:01 DQB1*03:01)	405	20 48 19 32,52, 42, 38, 26 1058,1666,29 31,2959,3069	DSA (DQA1*06:01 DQB1*03:01)	3069
5	DRB1*01:02 DRB1*04:01 DQB1*05:01	209 67 255,163	DSA (DQA1*01:02 DQB1*05:01)	255	517 75 1032	non-DSA (DQA1*01:02 DQB1*05:02)	1037
6	DRB1*13:04	NB	NS (DPA1*02:02 DPB1*28:01)		NB	NS (DPA1*02:02 DPB1*11:01)	230
7	DRB1*04:01 DQB1*03:01	501 283, 278, 259,210	NS (DPA1*02:01 DPB1*04:01)	1479	945 301,385,731, 635,1019	non-DSA (DQA1*01:01 DQB1*05:01)	1465
8	DRB1*15 DQB1*06	255, 183,116 831,428,471, 472,375,539	NS (DPA1*02:01 DPB1*04:01)	1666	1083,1054, 1845 546,463,480, 600,403,467	DSA (DRB1*15:03)	1845

Legend: LC = Lifecodes, OL = One Lambda, IgA DSA = Donor-specific IgA HLA antibody, HR = Highest-ranked, MFI = Mean fluorescence intensity, NB = No bead, NS = Not specified (typing not available); For HLA antigen specificities present in the assay kit on more than one bead and/or as a part of broad specificity, raw MFI values for all beads/specificities were noted. Specificities belonging to the same broad specificity are given by rising specificity numbers. If the highest-ranked bead specificity in the IgA-SAB assay report was related to IgA DSA of the sample it is marked by shading.

Table 8. Comparison of Lifecodes and One Lambda IgA HLA class I SAB assay results obtained from IgA-LMX positive transplant recipients with IgA HLA class I antibody negative screening results (N=7)

Sample number	Sample IgA DSA	IgA DSA raw MFI values with LC kit	HR bead in IgA-SAB with LC kit		IgA DSA raw MFI values with OL kit	HR bead in IgA-SAB with OL kit	
			DSA / non-DSA (specificity)	Raw MFI		DSA / non-DSA (specificity)	Raw MFI
2	A*01:01 A*33:01 A*32:02 B*49:01 C*07:01	33 75 170 132 107	non-DSA C*08:01	263	71 351 3493 1505 NB	non-DSA C*08:01	4984
3	A*30:01 B*13:02 Cw10 (C*03:04) Cw6 A2 A3 B62 B35 Cw3 (C*03:03) Cw4	85 106 113 60 100,104,92 90 112 91,74 88 66	non-DSA B*07:02	181	80 230 249 224 131,231,98 47 463 113 1325 230	DSA C*03:03	1325
4	A*32:01 B*40:01 C*03:04 B*44:02 C*05:01 C*07:01	113 118 77 406 102 76	non-DSA B*44:03	439	168 336 98 471 266 NB	non-DSA A*01:01	682
5	A*33:01 A*68:01 B*14:02 C*08:01	130 175 156 77	non-DSA A*43:01	549	90 1126 136 262	DSA A*68:01	1126
6	B*58:01 C*03:02	116 NB	non-DSA A*24:02	385	182 108	non-DSA A*69:01	822
7	A*01:01 B*44:02 C*05:01	316 208 192	DSA A*01:01	316	458 190 387	non-DSA A*30:01	1817
8	A*02 B*07	574,640, 666,728 65,59	non-DSA A*66:02	620	188,250,227 140	non-DSA B*73:01	1100

Legend: LC = Lifecodes, OL = One Lambda, IgA DSA = Donor-specific IgA HLA antibody, HR = Highest-ranked, MFI = Mean fluorescence intensity, NB = No bead, NS = Not specified (typing not available); For HLA antigen specificities present in the assay kit on more than one bead and/or as a part of broad specificity, raw MFI values for all beads/specificities were noted. Specificities belonging to the same broad specificity are given by rising specificity numbers. If the highest-ranked bead specificity in the IgA-SAB assay report was related to IgA DSA of the sample it is marked by shading.

Due to the observed difference in the MFI signal obtained for IgA DSAs when LC and OL kit were used, we compared the two IgA-SAB assay kits based on the MFI signal obtained for all the beads in kits. For that purpose, we again used IgA-SAB results from IgA-LMX positive samples (N=8). Bivariate correlations were performed using the Person's correlation coefficient and linear regression.

For IgA HLA class I antibody values, there was a moderate positive correlation between two assay kits ($r=0.5245$, $r^2=0.2751$, $p<0.0001$) (Figure 23A). As shown in the scatter plot, MFI values for paired IgA HLA class I antibody specificities were high when detected with OL (up to 6000 MFI) and low with LC (<1000 MFI) kit. Detection signal observed for a group of outliers (raw MFI: OL>2000, LC<1000) clearly demonstrated higher sensitivity of OL, when compared to LC IgA-SAB assay kit.

Correlation and regression analysis for IgA antibodies directed against HLA class II showed a positive correlation of the same strength between the MFI values obtained with two assay kits ($r=0.5306$, $r^2=0.2815$, $p<0.0001$) (Figure 23B). Moreover, depicted outliers demonstrated a higher MFI signal measured for antibodies detected with OL kit. Group of outliers (raw MFI: OL>6000 and LC<4000) were related to DSAs of a specific sample or in another case, non-DSAs that recognise the same epitope as DSAs of a specific sample.

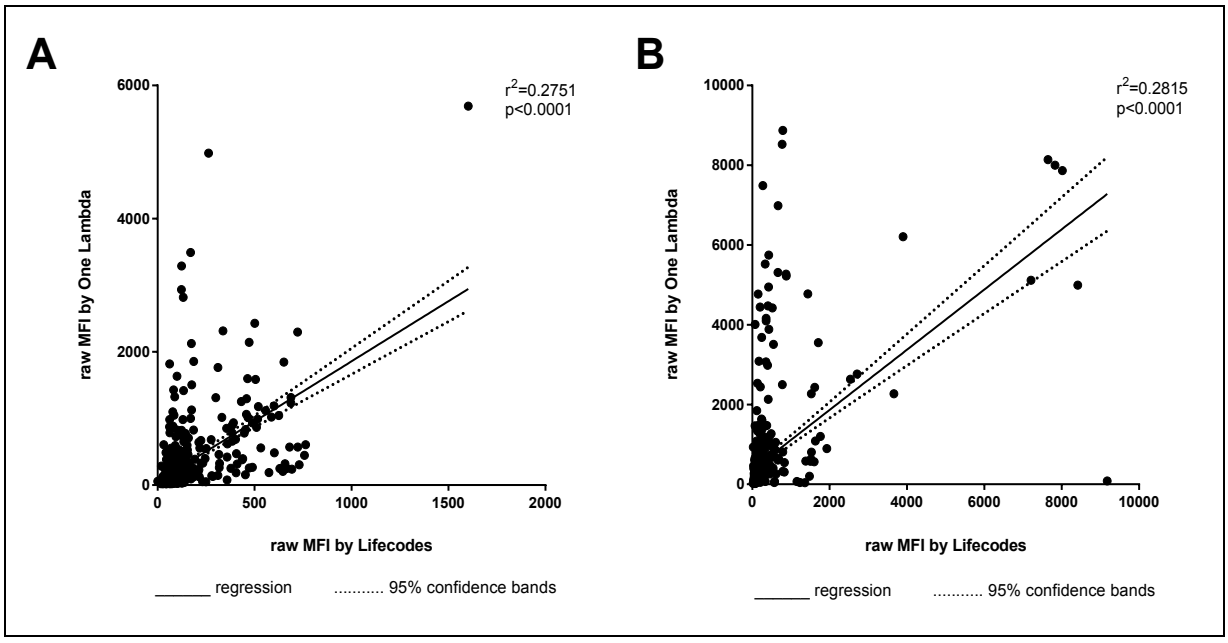


Figure 23. Correlation between One Lambda and Lifecodes IgA HLA class I (A) and IgA HLA class II (B) antibody signal from IgA-LMX positive samples (N=8).

Bland-Altman plots were used to quantify bias and the range of the MFI values for paired antigens between the Lifecodes and One Lambda assay kit. For IgA HLA class I antibody MFI values, MFI bias (-158 Mean Difference) with a range from -1007 to 691 (95% CI) between the two kits was observed. For IgA HLA class II antibody MFI signal, between the two kits, we noted bias (-280 Mean Difference) ranging from -2512 to 1951 with a broad 95% CI (Figure 24).

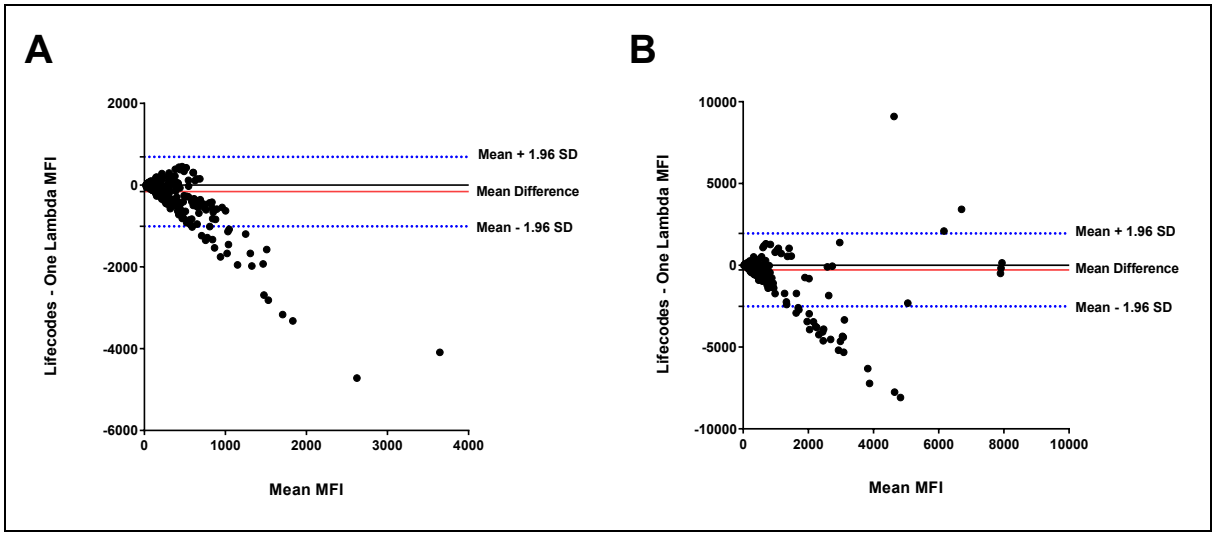


Figure 24. Bland-Altman comparison of MFI values obtained for HLA class I (A) and HLA class II (B) IgA antibody detection with Lifecodes and One Lambda IgA-SAB assay kits. Red and blue lines represent the mean of MFI difference and 1.96 mean standard deviation, respectively.

5. DISCUSSION

5.1. Development and validation of screening assay for IgA HLA antibody detection

Based on the humoral theory of transplantation, the presence of IgG isotype of HLA antibodies in the serum from graft recipient is associated with the transplantation outcome [163-167]. The role of IgG HLA antibodies in the immune response by the recipient of graft is very well studied and the presence of mismatched DSAs in the circulation of the recipient before and after transplantation is considered to be a risk factor for the development of antibody-mediated rejection and inferior allograft survival [164,167-173]. Therefore, screening and identification of the IgG DSAs is a part of the standard protocol before and after the transplantation. However, not all patients with IgG DSAs experience poor graft survival [174-176]. In contrast, ABMR may develop in the absence of IgG DSAs in some cases [69,75,177-179]. A possible explanation could be the contribution of other isotypes of HLA antibodies, such as IgA, to the alloimmune response.

Compared to IgG antibodies which represent 80% of the total serum immunoglobulins and may cause complement-mediated lysis resulting with ABMR, IgA isotype of antibodies are represented with only 15-20% of the total amount of immunoglobulins in the serum [82,120,180]. Moreover, IgA is considered a poor complement binding isotype and therefore is not routinely determined in clinical practice nor well studied within the transplantation setting [181]. As a result, the clinical relevance of IgA HLA antibodies in transplantation is still unclear.

Earlier studies on the presence of IgA HLA antibodies in transplant recipients used ELISA [78,107,108] and flow cytometry [109,110], antibody detection methods with lower sensitivity. In the later studies, very sensitive Luminex technology was used for the detection of IgA HLA antibodies in serum from transplant recipients. However, assays were modified with the monoclonal IgA1- and IgA2-PE detection and validated by using chimeric mouse/human HLA-specific IgA1 and IgA2 monoclonal antibodies (chF3.3) as controls [111-114,160]. The binding activity and specificity of these antibodies were confirmed by ELISA and Luminex screening assay. Since published chimeric F3.3-IgA1 and IgA2 mAbs had mouse variable part to recognise the majority of HLA class II molecules, we could not consider them as a proper reflection of human allorecognition [182-184].

For that reason, we produced human recombinant HLA-specific MUS4H4rec-IgA1 and -IgA2 mAbs by using expression plasmids generated from heterohybridomas that were derived from B cells of immunised individuals, what makes them truly representative of human HLA antibodies produced through alloimmunisation. All 22 MUS4H4 specificities were confirmed

by very sensitive Luminex single antigen bead assay and cytotoxicity was excluded by CDC assay.

Furthermore, in the previous Luminex based studies on the IgA HLA antibody detection, PE-conjugated mouse anti-human IgA1 (clone B3506B4) and IgA2 (clone A9604D2) detection antibodies were used. When we applied previously reported detection antibodies on supernatants of our human recombinant HLA class I-specific MUS4H4rec-IgA1 and MUS4H4rec-IgA2 monoclonal antibodies, the detection signal obtained from the reaction between IgA1-PE detection antibody and IgA1 mAb sample was low (median MFI: 5909) and between IgA2-PE detection antibody and IgA1 mAb sample occurred cross-reactivity with a strength of 5306 median MFI.

Because of the above-mentioned absence of appropriate control antibodies and detection reagents, we considered previously used Luminex assays for IgA HLA antibody detection not reliable nor sensitive enough. With the aim to develop a reliable and highly sensitive screening assay for IgA subclass HLA antibody detection, we first used PE-conjugated monoclonal antibodies IgA1 (clone B3506B4) and IgA2 (clone IS11-21E11) for HLA antibody subclass detection, previously verified in our laboratory. When applied on target MUS4H4rec-IgA1 and -IgA2 mAbs, we observed low reactivity of IgA1 (median MFI: 4376) and IgA2 (median MFI: 1544) mAbs. Compared to monoclonal antibody detection, polyclonal detection reagent offers greater sensitivity for detecting antibodies, especially the ones present in low quantities in a sample, as IgA HLA antibodies [185]. Next, we developed IgA HLA antibody screening assay with polyclonal detection antibody, goat anti-human IgA-PE and compared detection signal when polyclonal and monoclonal detection antibodies were used on the same target MUS4H4rec-IgA1 and -IgA2 mAbs. As expected, the Wilcoxon test showed significantly lower sensitivity ($p=0.0156$) when monoclonal reagents were used. In addition to higher sensitivity, the ability to detect multiple epitopes gives polyclonal detection antibody higher overall affinity against the target antibodies and more robust detection [186]. Therefore, we decided to further develop and optimise a Luminex bead-based IgA HLA antibody screening assay that utilizes polyclonal IgA-PE detection. Availability of IgA and IgG isotype of HLA-specific human recombinant monoclonal antibodies enabled us to properly validate the assay.

Our validation experiments showed a few points as follows:

1) concentration of 10 µg/mL for polyclonal IgA-PE detection antibody was optimal for saturation of the assay beads, giving high HLA class I detection signal for MUS4H4rec-IgA1

(median MFI: 15049) and MUS4H4rec-IgA2 (median MFI: 12089) mAbs samples, while low detection signal was observed for IgG antibody-coated positive control bead (raw MFI: IgA1-194; IgA2-230) and negative control beads (IgA1 MFI range: 62-196, IgA2 MFI range: 85-187), as expected;

2) performed sensitivity test demonstrated the ability of the assay to detect IgA1 and IgA2 mAbs in a very low concentration of even 0.06 µg/mL;

3) there was no significant difference (p=0.2314) in detectability between IgA HLA antibody screening assay and IgG HLA antibody screening assay when mAbs were used in the same concentration of 1 µg/mL;

4) cross-reactivity of the IgA-PE detection antibody with other isotypes of HLA antibodies as well as non-specific binding to the beads were excluded in the single antigen bead assay for IgA HLA antibody detection.

With the above described, we confirmed that the generated screening assay for IgA HLA antibody detection is very sensitive and specific and can be used for screening of serum samples.

When the developed IgA HLA antibody screening assay was applied on serum samples from HLA alloantigen exposed and non-exposed individuals, IgA HLA antibodies were detected only in samples from HLA alloantigen exposed individuals. Still, an overall frequency of detected IgA HLA antibodies was low (3.40%).

For further validation of the IgA HLA antibody screening assay, a bigger cohort should be tested in multiple independent laboratories with the same here described protocol to minimize the test variability. Thereby, to be able to compare the frequency of IgA HLA antibodies, all laboratories should use the same sample selection and the proposed threshold for positivity. Additional robust multicenter studies are required to standardize this newly developed assay and establish the clinical utility of testing for IgA HLA antibodies in the transplantation setting.

5.2. Frequency of detected IgA HLA antibodies

It is well established that HLA antibodies may develop as a result of alloimmunization through transplantation, blood transfusion, and pregnancy [136,187,188]. Based on the Luminex SAB assay results in the recent studies, 50-75% of women have developed IgG HLA antibodies at the time

of delivery, and the rate of pregnancy-induced sensitization increases with the number of pregnancies [189,190].

It is clear that the gender of the donor and organ recipient play a role in transplantation event. Globally, it is estimated that females constitute 65% of all living kidney donors but only 35% of all kidney recipients [191,192]. It is well documented that female patients gaining access to the transplant waitlist and receiving a transplant after being waitlisted is less likely than male patients [193-199]. Based on data from the United States, Wolfe et al. reported a 16% lower rate of women being admitted to the waiting list compared to men and a 14% lower likelihood for women to receive a kidney transplant after being waitlisted [195]. Analysed serum from kidney transplant waitlist patients showed that 53% of women have pre-formed IgG HLA antibodies versus 32% of men [200]. Due to a higher level of IgG HLA antibodies, a higher proportion of women is excluded from receiving living donor transplants [201]. Moreover, pregnancy-induced HLA antibodies may reduce graft survival in female transplant recipients [202-204].

In order to determine the prevalence of IgA HLA antibodies related to a route of HLA alloantigen exposure, a total of 175 samples were screened for the presence of IgA HLA antibodies. IgA HLA antibodies were detected in samples from kidney transplant recipients (N=8) and women immunized exclusively through pregnancy (N=2), demonstrating that the exposure to HLA alloantigens via organ transplantation and pregnancy may induce the development of IgA isotype of HLA antibodies. In all analysed samples, IgA HLA antibodies were detected exclusively in the presence of the IgG isotype of HLA antibodies. When an overall frequency of IgA±/IgG± HLA antibody status between kidney transplant waitlist patients and women immunized exclusively through pregnancy was compared, the Mann-Whitney test showed no statistically significant difference ($p=0.9445$) between these two groups.

Among IgG HLA antibody positive samples from kidney waitlist transplant recipients (N=41), IgA HLA antibodies were more prevalent in male (6/23; 26%) than in female patients (2/18; 11%). This is an interesting observation since, in that group of transplant recipients, 89% of female patients were exposed to HLA alloantigens through pregnancy and transplantation and; therefore, represent individuals with two different sensitizing events. As known, the risk of sensitization increases as there is exposure to more than one sensitizing factor [205]. Moreover, among our selected samples, an average number of transplantations before sampling was higher in female (2.27) than in male (1.89) patients.

It has been reported that female transplant recipients have a significantly higher percentage of detected IgG HLA antibodies than male recipients [206]. Furthermore, IgG HLA antibodies showed higher production levels and significantly higher MFI values after re-transplantation than after the first transplantation [206]. Finally, in the study from Higgins et al., higher levels of IgG HLA antibodies were detected upon transplantation in transplant recipients were antibodies were previously stimulated by pregnancy than in patients with previous transplantation [207].

An explanation for the occurrence of higher prevalence of IgA HLA antibodies in male than in female kidney waitlist transplant recipients, we were not able to give. Between compared groups, we did not observe any difference in the MFI signal measured for detected IgA HLA antibodies. Based on a small number of IgA HLA antibody positive samples we cannot draw any conclusion about the influence of the route of HLA alloantigen exposure on the development of these antibodies.

To the best of our knowledge, this is the first study reporting on the frequency of transplantation and pregnancy-induced IgA HLA antibodies detected with Luminex technology. Therefore, by providing preliminary data on the frequency of IgA HLA antibodies developed through the two mentioned routes of HLA alloantigen exposure, this study represents a notable contribution to the field of the immune response in pregnancy and transplantation.

This study was limited, when attempting more elaborate analyses, with the missing detailed medical documentation for transplant recipients and the HLA typing for partners of all women with a history of pregnancy. This is partially due to the sample selection from the Eurotransplant database and studies on pregnancy focused only on mother-child mismatch. This should be taken into consideration in future studies, to provide more detailed commentary on a specific query on the relation between HLA antibodies development and route of HLA alloantigen exposure.

In addition to the 175 samples, transplant recipients with functioning grafts or diagnosed with ABMR were also screened for IgA HLA antibodies, for the purpose of this study. IgA HLA antibody screening results for a total of 294 samples showed the presence of IgA HLA antibodies in only 10 samples. Interestingly, our study showed 10-11 fold lower overall frequency (3.40%) for detected IgA HLA antibodies than reported (31-43%) in the previous Luminex technology based studies by Arnold et al. [111,112,114].

5.3. Discrepancy between the present and published frequency of IgA HLA antibodies

One of the reasons for the different IgA HLA antibody frequency between the present and previous studies could be the applied detection antibody. In the previous studies, IgA2-PE (clone: A9604D2) detection antibody was used and detected IgA1 HLA antibodies and IgA2 HLA antibodies were summarized and presented as IgA HLA antibodies. Taking into account our finding on cross-reactivity between IgA2-PE (clone: A9604D2) detection and MUS4H4rec-IgA1 mAb, it may be possible that the incidence of IgA HLA antibodies in the previous studies by Arnold et al. is overestimated [111,112,114].

The second possible explanation for the difference between our findings and those reported in studies by Arnold et al. could be the discrepancy in the composition of the study cohorts. While Arnold et al. used samples from waitlist transplant recipients, in our study 31.49% of samples belonged to women exposed to HLA alloantigens through pregnancy without a history of transplantation. It is reported that pregnancy-induced HLA antibodies may decrease or completely disappear over time [35]. In the study where almost 4000 serum samples from women with a history of previous pregnancy were tested 10 years, between 10-20, 20-30, and more than 30 years after delivery, the sensitisation rates of IgG HLA antibodies found by Luminex bead-based assay declined from 31% to 26%, 22%, and 18%, respectively [190]. In our study, the sample collection time point for women exposed to HLA alloantigens exclusively through pregnancy was from 8 days to 24 years from the last delivery. Indeed, by taking into consideration reports on the decrease of IgG HLA antibodies which happens years after pregnancy, there may be a possibility that IgA HLA antibodies in our study were not detected due to their loss over time.

Besides, 37.72% of study samples were from transplanted patients with at least 1-year graft survival for whom the information on provided immunosuppression regime was not available. As known, immunosuppression may affect the HLA antibody frequency [208]. However, even when only transplant waitlist patients from our study population were tested, the frequency of IgA HLA antibodies was still lower (8/84; 9.52%) than previously published.

Third, the difference in frequency may be attributed to the different definition of positivity and/or utilization of Luminex bead-based kits from different vendors. The first Luminex based study on the detection of IgA HLA antibodies in sera from transplant recipients reported the presence of IgA HLA antibodies up to 43% of serum samples by using panels of individual

HLA class I and HLA class II phenotypes (LABScreen PRA kit) from One Lambda-Thermo Fisher Scientific Inc. [111].

In the later studies, Arnold et al. reported a frequency of 31% for IgA HLA antibodies based on the IgA HLA antibody screening assay results, further confirmed with IgA HLA antibody single antigen bead-based assay, both performed with OL kits [112,114]. The threshold for the positive screening results was calculated according to the relative ratio between the patient sample and negative control and set at a ratio of two, while the cut-off for positivity in single antigen bead assay was not specified in the publications.

In the present study, we used kits from Lifecodes-Immucor Transplant Diagnostics and for the positivity of IgA HLA antibody, we applied an arbitrary cut-off value for any of class I and/or class II LMX bead groups set at raw 500 MFI. To test the influence of the cut-off value on our result, we lowered our IgA-LMX threshold value to raw 250 MFI. That resulted in a higher IgA HLA antibody frequency of 19% among our samples, but still not in the range of previously published 31%.

As known from the literature, because of variation in antigen integrity and density on antigen-coated beads, it is not uncommon to have discrepant results between detection and specificity identification assay. Based on more than 300 samples tested simultaneously with the IgG HLA antibody detection and specificity identification assay with the same applied threshold for positive assignment, Attas et al. reported an overall discordance of 32% between the two assays from the same vendor [209]. Since the type of detection assay may influence the percentage of detected IgA HLA antibodies, we decided to test the difference between results acquired from our samples with screening and single antigen bead assay kit, both from LC.

For that purpose, we compared our Luminex IgA-LMX and IgA-SAB assay reports from ten IgA-LMX positive samples. Screening samples assigned positive by the arbitrary cut-off of raw 500 MFI, had a median of 877 MFI for the highest-ranked bead specificities in the IgA-SAB assay which is in line with the previous reported existing difference in antibody reactivity between LMX and SAB assay found for IgG HLA antibodies [209]. In all previous studies on the IgA HLA antibody detection with Luminex bead-based assays, OL kits were used while we used LC kits. As shown in IgG HLA antibody analyses, kits from two selected vendors differ in sensitivity, specificity and antigen panel composition [149,210,211]. Moreover, a comparison of OL and LC bead-based assays showed OL assay as having greater reactivity to HLA antibodies with lower MFI values [210]. With the aim to test the difference between results obtained when

two commercially available assay kits from two different vendors are used, serum samples from eight transplant recipients assigned positive for IgA HLA antibodies by using IgA-LMX assay supplied from LC were analysed in a more sensitive IgA-SAB assay with LC and OL kit. A sample was considered IgA-LMX positive if IgA HLA IgA class I and/or HLA class II positive. When IgA-SAB assays were performed, the same concentration of IgA-PE detection was used for both, OL and LC kit.

5.4. Discrepancy between IgA-SAB assay results acquired with two commercially available assay kits

Single antigen bead assay results from IgA-LMX positive transplant recipients, acquired by using LC and OL IgA-SAB assay kits, revealed discrepant measurements when considering raw MFI values of the detected donor-specific IgA HLA antibodies. Both, donor-specific HLA class I (ratio OL/LC=2.94) and donor-specific anti-HLA class II (ratio OL/LC=1.81) IgA antibodies we detected with higher MFI signal when OL kit was used.

Furthermore, among eight IgA HLA antibody positive samples with HLA class I and/or class II positive IgA-LMX reports, the highest-ranked IgA-SAB specificity was related to IgA DSA in five samples and IgA non-DSA in one sample (both with OL and LC) which recognize the same epitope as the lower-ranked DSA in that sample. Besides, the highest-ranked bead specificities related to IgA non-DSAs were detected in two more samples exclusively with the OL kit.

When HLA class I IgA-SAB assay was applied on IgA-LMX positive samples with HLA class I negative IgA-LMX reports, detected highest-ranked bead specificity was defined as DSA in one sample with LC and two samples with OL IgA-SAB assay kit. Comparison of the MFI signal measured from the highest-ranked bead specificities defined as IgA DSA and IgA non-DSA detected with LC (DSA MFI: 316; non-DSA MFI range: 181-620) and OL (DSA MFI range: 1126-1325; non-DSA MFI range: 682-4984) IgA-SAB kit, showed that some of the highest-ranked bead specificities related to IgA non-DSA had higher MFI values than the IgA DSAs, which may have been in these samples easily perceived as false positive results acquired with both kits. Finally, in one of the samples with IgA HLA class I antibody negative screening result, applied OL IgA-SAB assay kit revealed the MFI value of 4984 for the highest-ranked bead specificity (Cw8) related to non-DSA, which can be explained as a reaction to recently published denatured HLA antigens in the OL SAB kit [212].

Since we observed a difference between IgA-SAB assay results obtained with LC and OL kit when only the highest-ranked bead specificities were compared, we decided to address the question of the existing difference between SAB assay kits from two vendors based on all IgA-SAB assay bead specificities. Single antigen bead kits from both vendors had a similar number of coated beads for HLA class I (LC/OL=96/97) and HLA class II (LC/OL=96/95), but antigen panel between LC and OL differed, making 89% HLA class I and 68% HLA class II antigens common for both assay kits. Furthermore, LC and OL provide different protocols for their respective product. In our laboratory, OL and LC IgG-SAB assay protocols were standardized for routine patient serum analysis, but they differed in the beads/serum ratio and the amount of added detection antibody. In order to perform IgA-SAB assays with LC and OL kits, in the present study beads/serum ratio standardized for IgG HLA antibody detection with LC (30µL/7.5µL=4.3) and OL (20µL/4µL=5) SAB assay was used and the same dilution (1:50) of the IgA-PE detection was applied on each sample (0.75µL-LC; 2µL-OL).

To assess the degree of agreement between the MFI values obtained from LC and OL IgA-SAB assay, we considered all IgA HLA antibody paired values and thereby observed a moderate positive correlation for HLA class I ($r=0.5245$, $p<0.0001$) and HLA class II ($r=0.5306$, $p<0.0001$). Both HLA class I and HLA class II MFI values displayed the same strength of the relationship ($R^2=0.28$). Bland-Altman plots showed notable constant variation in antibody intensity between the two assays. Analysis considering all HLA class I and class II loci revealed a bias (Mean Difference HLA class I= -158; HLA class II= -280) with LC MFI values being lower than values obtained with OL.

This finding of higher MFI signal obtained with OL kit is in accordance with the reports for IgG HLA antibodies [210]. Therefore, when interpreting the IgA HLA antibody results, the same consensus guidelines recommended for IgG isotype by Tait et al. should be followed for IgA isotype and rather than assigning IgA HLA antibodies as positive based solely on MFI values, the individual cut-off for positivity in correlation to previous sensitizing events and HLA epitope analysis should be applied to samples [144,213]. Our data suggest that the difference in composition of study cohorts, as well as utilization of different cut-off values for positivity and different Luminex bead-based kits from different vendors, may all be explanations for the much higher incidence of IgA HLA antibody detected in Arnold et al. studies [111,112,114].

5.5. Further explanation for the low incidence of IgA HLA antibodies

As noted, serum IgA occurs in the monomeric and polymeric, usually dimeric form [84]. Monomeric IgA has only two antigen-binding sites whereas dimeric IgA has four, which may influence binding the IgA HLA antibody to the assay beads and further detection of the antibody. Due to a low level of dimeric IgA in serum, this is less likely to have an impact on the observed frequency of IgA HLA antibodies but could be a possible confounding factor.

The observed low incidence of IgA HLA antibodies may be as well caused by the competition for the binding sites on HLA molecules, existing between IgG and IgA isotype of HLA-specific antibodies. Significantly higher serum levels of IgG HLA than IgA HLA antibodies should be here also taken into account [84]. With the objective to test the existence of competition between IgG and IgA isotype of anti-HLA antibodies as a confounding variable in the detection of IgA HLA antibodies, we performed spiking experiments. In serum spiked with HLA-specific IgA1 (MUS4H4rec) and IgG1 (SN607D8) mAbs of a different epitope, Mann-Whitney statistical test showed no significant difference between the MFI signal obtained from MUS4H4rec-IgA1 and 1:1 MUS4H4rec-IgA1/SN607D8-IgG1 mAbs ratio solution ($p=0.1250$), or MUS4H4rec-IgA1 and 1:4 MUS4H4rec-IgA1/SN607D8-IgG1 mAb ratio solution ($p=0.0500$). Therefore, the detectability of IgA HLA antibody was not affected by increasing concentrations of IgG isotype of an HLA antibody recognizing a different epitope.

However, when HLA-specific IgA1 and IgG1 (both MUS4H4rec) mAbs recognized the same epitope, MUS4H4rec-IgA1 antibody was still detectable albeit with significantly lower ($p<0.0001$) detectability in both, 1:1 and 1:4 MUS4H4rec-IgA1/ MUS4H4rec-IgG1 mAbs ratio solutions. MFI signal obtained from 1:1 (median MFI: 15386) and 1:4 (median MFI: 9683) MUS4H4rec-IgA1/MUS4H4rec-IgG1 mAbs ratio solutions was 1.36 and 2.16 times lower than the signal detected from serum spiked exclusively with MUS4H4rec-IgA1 mAb (median MFI: 20954) which demonstrated a dose-dependent lower detectability of the IgA1 in the presence of IgG1 variant. This confirmed the presence of competition for the binding sites on HLA molecules existing between IgA and IgG isotype of HLA antibodies that recognize the same epitope. Indeed, the lower titer of IgA HLA antibodies in serum combined with lower detectability of IgA HLA antibodies when surrounded with IgG isotype of HLA antibodies recognizing the same epitope can contribute to the observed lower frequency of IgA HLA antibodies.

Furthermore, it is well known that after the initial immunoglobulin class switch from IgM to IgG, the IgG fraction first comprises the IgG3 and IgG1 subclasses. A further expansion to IgA1, IgG2, IgG4 and IgA2 is possible, as well as a complete switch to these non-complement-fixing antibodies [15]. Several studies have previously confirmed the presence of different IgG or IgG and IgA subclass mixtures of HLA antibodies in serum from kidney waitlist patients [113,214,215]. It is possible that in our study IgA HLA antibodies in serum from HLA alloantigen exposed individuals were developed, but due to the IgG3→ IgG1→ IgA1→ IgG2→ IgG4→ IgA2 class switching, present IgA and IgG isotypes were competing for the same epitopes and therefore the detectability of IgA was too low for the screening (detection) assay. Since IgA2 comes at the end of class switching it is also possible that IgA2 HLA antibodies were not developed at the time of sample collection.

In our study population groups, IgA HLA antibodies were found exclusively in the presence of IgG HLA antibodies. This finding differs from the latest findings for transplant recipients from Arnold et al., where IgA HLA antibody occurred independently from the IgG isotype of HLA antibody [112,114]. When we further analysed samples assigned positive in IgA HLA antibody detection assay with the IgA and IgG specificity identification assays, in one sample we observed non-overlapping specificities for IgA and IgG isotype of HLA antibodies.

Thereby, four highest-ranked IgA antibody specificities in the IgA-SAB assay report were the lowest in IgG-SAB assay report (DPA1*02:01DPB1*04:01: IgA-1479, IgG-131; DPA1*01:03DPB1*04:02: IgA-1359, IgG-123; DPA1*01:03DPB1*18:01: IgA-1308, IgG-156 and DPA1*01:03DPB1*02:01: IgA-1232, IgG-177). All four highest-ranked IgA antibody specificities were related to the same epitope 85GPM. Unfortunately, HLA typing for that specific sample was not available for the HLA-DP region and we were not able to relate these specificities to DSAs, non-DSAs or self-antigens from the patient. Therefore, this finding should be interpreted with caution.

5.6. The role of IgA HLA antibodies in kidney transplantation

One of the aims of this study was to elucidate the influence of IgA HLA antibodies on graft survival in kidney transplantation. Earlier studies have proposed that the IgA isotype of HLA antibodies is a competitive inhibitor of deleterious IgG isotype of HLA antibodies and suggested a beneficial effect of IgA HLA antibodies on the kidney graft survival [78,107,117]. Our spiking experiment demonstrated the existence of competition between IgA and IgG HLA

antibodies for the same binding sites on the HLA molecules. Based on that, IgA isotype can block IgG isotype of HLA antibodies but the presence of IgA isotype may not always have a blocking effect.

In the study from Lim et al., it is suggested that IgA antibodies directed against the HLA class I molecules formed before the transplantation block IgG isotype of HLA antibodies and in that way contribute to higher 1-year kidney graft survival in IgA nephropathy (IgAN) patients [117]. The report was based on 27 patients suffering from IgAN from whom 16 serum samples had IgA HLA antibodies. The proposal that the IgA antibodies act as blocking agents, is in line with studies on the association between the IgA antibody deposits in kidneys after transplantation and the allograft survival in IgAN patients. In those studies, allograft survival was better during the first 5 years after transplantation [216-218], comparable at 10 years [216-219] and worse after 12 years [220] compared to a control group of kidney recipients with other primary diseases than IgAN.

On the other hand, Heinemann et al. reported the occurrence of IgA HLA antibodies in rejected and explanted kidneys from transplant recipients and associated IgA HLA antibodies with early (within 2 years) graft loss [118]. In the mentioned study, among 94 samples nine were positive for IgA HLA antibodies and one sample belonged to a patient diagnosed with IgAN but the corresponding kidney eluate had no IgA HLA antibodies. However, our analysis specifically focuses on IgA HLA antibodies detected in serum. In serum from our IgAN positive kidney waitlist patients, we did not detect any IgA HLA antibodies.

The role of serum IgA HLA antibodies as contributors to the development of early acute rejection was also suggested in the study from Karuppan et al. [110]. IgA HLA antibodies were detected in two samples from kidney transplant patients with early acute rejections (N=25) within one to two weeks post-transplant but not in samples from patients having functional grafts for at least one year (N=12). In comparison to the study from Karuppan et al. in the present study, we did not detect any IgA HLA antibodies in samples from patients with ABMR collected before (N=5) and after (N=5) the transplantation. Furthermore, we analysed a higher number of serum samples from transplant recipients with functional grafts (N=109). Consistent with previous reports, no patient with functional graft had IgA HLA antibodies, regardless of the time of sampling (1-14 years after the transplantation).

5.7. Difficulties encountered when comparing our results with published data

There are some difficulties encountered when comparing our results with published data including a small number of studies, variety in the method of sample selection and/or the usage of a wide range of methodological approaches for IgA HLA antibody detection.

In the previous studies ELISA, flow-cytometry and biopsy were used and all recent Luminex based data came from studies published by Arnold et al. In the Luminex based studies from Arnold et al., pre-transplant serum samples were analysed but none of the patients tested for the presence of IgA HLA antibodies was diagnosed with an IgAN or the information on their IgAN status was not provided [111-114]. Furthermore, the ABMR and graft survival status were presented only for a limited group of samples. Specifically, in the study from 2014 Arnold et al. reported 31 out of 274 IgA HLA antibody positive samples but only seven samples with defined IgA DSA status were further analysed for the transplantation outcome [112]. Thereby, four out of seven samples with IgA DSAs were from patients with diagnosed ABMR and in two of those four samples, graft failure occurred. In addition, three out of seven patients with IgA DSAs had functional grafts. In the last study from 2018 where Arnold et al. reported that the presence of- IgA HLA antibody is associated with a higher risk for early graft rejection, samples from kidney re-transplant patients were tested and a median time to the first dialysis after previous transplantation was used as a measure for graft failure [114]. Their dataset did not include the cause for the loss of graft function such as ABMR.

In the present study, we had a limited number of analysed samples from kidney transplant recipients diagnosed with IgAN (N=6) and ABMR (N=5) what may be the reason why IgA HLA antibodies were not found in those groups of patients. Unfortunately, due to the selection criteria, we were not able to collect more samples from IgAN patients on the kidney transplant waitlist with a history of transplantation and patients with biopsy-proven early ABMR without detected IgG HLA antibodies.

Future studies could overcome this problem and apply our IgA HLA antibody screening assay on additional samples from these patient groups. Furthermore, it would be interesting to conduct a follow-up study on 1-, 5-, 10- and 12-years post-transplantation graft survival in IgAN patients to examine the association between the IgA antibody deposition in kidneys and the presence of IgA HLA antibodies in serum from kidney transplant patients. Although this study examined samples from patients with different transplantation histories and outcomes, it was

limited with a small number of samples containing IgA HLA antibodies and lack of detailed transplantation history including immunosuppression regiment and graft status for all tested transplanted patients. Unfortunately, we did not find evidence to support any of the hypothesis concerning the potential role of IgA HLA antibodies in kidney transplantation reaction.

Until recently all studies on the presence and/or the role of serum IgA HLA antibodies in transplantation, except the study from 2013 published by Arnold et al., focused on kidney transplant recipients. In the study from 2013, Arnold at al. provided data for the presence of IgA HLA antibodies in samples from the kidney, liver and thoracic re-transplant candidates [112]. Thereby, IgA HLA antibodies alone were detected in the kidney (31/755; 4.10%) and thoracic (2/10; 20%) transplant recipients, while in serum from 18 liver recipients IgA HLA antibodies were not detected. Due to the restriction on patient selection, we focused solely on kidney transplant recipients. However, to determine the role of IgA HLA antibodies in transplantation in general, samples from patients before and after non-renal solid organ transplantation should be analysed in future studies.

In summary, we established a Luminex IgA HLA antibody screening assay and demonstrated that this screening assay can be reliably used for the detection of HLA-specific antibodies of the IgA isotype. The current IgA-LMX assay can serve as a cost-effective method to screen for IgA HLA antibodies before utilizing expensive single antigen bead kits. Using this assay, IgA HLA antibodies were detected among individuals exposed to HLA alloantigens via different routes of sensitization but with very low frequency. We were not able to correlate the presence of IgA HLA antibodies in patient sera with the transplant outcome. Our study was limited with missing medical data for the analysed samples and a small number of samples from IgAN patients and patients with early ABMR without IgG HLA antibodies in the serum. The application of this modified screening assay on a larger study population comprising of the same population groups as in this study may facilitate the research on the development and the role of IgA HLA antibodies in immune response and more specifically kidney transplantation reaction.

6. CONCLUSIONS

This study on the IgA HLA antibodies could be summarized into several key points that are related to the incidence and the role of IgA HLA antibodies in HLA alloantigen exposed individuals, which are as follows:

1. The developed Luminex technology-based screening assay for IgA HLA antibody detection is sensitive and reliable.

2. IgA HLA antibodies were detected in 10 out of 294 (3.40%) samples from individuals exposed to HLA alloantigens through transplantation (2.72%) and pregnancy (0.68%).

3. In HLA alloantigen exposed individuals IgA HLA antibodies were detected exclusively together with the IgG HLA antibodies; in total 90% of them were against HLA class II.

4. Due to a low number of samples containing IgA HLA antibodies, it was not possible to draw a definitive conclusion about the role of these antibodies on graft survival in kidney transplantation.

5. Specificity identification of IgA HLA antibodies performed in ten IgA HLA antibody screening detection positive samples defined detected antibodies as IgA donor-specific in six samples, IgA non-donor-specific in one sample and not specified in four samples.

6. Comparison of IgA HLA antibody specificity identification results showed an existing difference in the antibody detection signal and the highest-ranked antibody specificity status against the donor when two commercially available single antigen bead assay kits were used.

7. This modified screening assay can serve as a cost-effective method for IgA HLA antibody detection and could facilitate the research on the role of IgA HLA antibodies in kidney transplantation.

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8. SUMMARY

Antibodies specific to allogenic HLA may be induced through pregnancy, blood transfusion or previous transplantation. In organ transplantation, the presence of donor-directed IgG HLA antibodies in serum from transplant recipient is strongly associated with an increased risk of graft failure. Therefore, serum samples from kidney transplant recipients are regularly screened for IgG isotype of HLA antibodies before and after transplantation. However, other isotypes of HLA antibodies, such as IgA may also contribute to the alloimmune response. Here, we modified the commonly used Luminex screening assay for IgG HLA antibody detection from Lifecodes into a sensitive and reliable IgA HLA antibody detection assay by replacing the detection antibody. Optimization and validation were performed by using IgG, IgA1 and IgA2 isotype variants of the HLA-specific recombinant human monoclonal antibody MUS4H4 (recognizing Bw4/A24/A32/A25). Specificities found for the IgA1 and IgA2 isotype mAbs were identical to those of the IgG isotype. Observed reactivity pattern of IgA1 and IgA2 isotype mAbs in IgA HLA antibody screening assay were identical to those of IgG isotype in IgG HLA antibody screening assay. Cross-reactivity of detection antibody with IgG and IgM antibodies and non-specific binding to the assay beads were excluded. Further, assay verification performed on serum from individuals without any HLA alloantigen exposure showed no presence of IgA HLA antibodies, as expected. To detect IgA HLA antibodies and compare their presence with IgG isotype of HLA antibodies, serum from individuals exposed to HLA alloantigens through transplantation and pregnancy was tested (N=294). IgA HLA antibodies were detected in kidney recipients (N=8) and women immunized through pregnancy (N=2). In total 3.40% of samples had IgA HLA antibodies, all containing IgG HLA antibodies, 90% of them being against HLA class II. We were not able to correlate the presence of IgA HLA antibodies in serum from transplant recipients with transplant outcome. Our results suggest that the IgA HLA antibody detection assay can reliably be used as a cost-effective screening assay and may facilitate the research on the role of IgA HLA antibodies in kidney transplantation.

9. SAŽETAK

Protutijela HLA mogu se razviti kao reakcija na trudnoću, transfuziju krvi ili transplantaciju. Jedan od glavnih uzroka nepovoljnog ishoda liječenja transplantacijom organa jest odbacivanje presatka uzrokovano protutijelima HLA razreda IgG uperenim protiv davatelja. Stoga je probir seruma bolesnika za prisustvo protutijela HLA razreda IgG metodom mikrokuglica obloženih s antigenima HLA (tzv. Luminex metoda) rutinski imunogenetski test koji se koristi prije i poslije transplantacije. Međutim, istraživanja uloge protutijela HLA drugih razreda imunoglobulina pokazala su da i protutijela HLA razreda IgA, mogu utjecati na ishod transplantacije. Do danas nije razvijen pouzdan test za otkrivanje prisustva protutijela anti-HLA razreda IgA korištenjem Luminex tehnologije. Postojeća Luminex metodologija određivanja protutijela HLA razreda IgA temeljena je na korištenju monoklonskih detekcijskih protutijela, a specifičnost vezanja testirana je na uzorcima kimeričkih protutijela što je ukazalo na nužnost modificiranja testiranja.

Cilj ovog istraživanja bio je modificirati Luminex probirni test za otkrivanje protutijela HLA razreda IgG (LMX-IgG) proizvođača Lifecodes-Immucor Transplant Diagnostics, Stamford, CT, USA (Lifecodes) u osjetljiv i pouzdan test za otkrivanje prisustva protutijela HLA razreda IgA (LMX-IgA), utvrditi prisutnost protutijela HLA razreda IgA ovisno o načinu imunizacije te pokušati razjasniti njihovu ulogu na preživljenje transplantiranog bubrega.

Luminex metoda određivanja protutijela HLA razreda IgA razvijena je uz korištenje poliklonskog detekcijskog protutijela i rekombinantnih humanih HLA specifičnih monoklonskih protutijela kao reagensa za optimizaciju i validaciju. Specifičnost vezanja sekundarnog protutijela potvrđena je u prethodno razvijenom testu određivanja specifičnosti protutijela HLA razreda IgA (IgA-SAB) na monoklonskim protutijelima HLA razreda IgG i IgM, kao i na za svrhu ovog istraživanja proizvedenim HLA rekombinantnim humanim monoklonskim protutijelima MUS4H4 razreda IgA1 i IgA2 poznate specifičnosti vezanja (HLA-Bw4/A24/A32/A25). Nespecifično vezanje detekcijskog protutijela za mikrokuglice isključeno je testiranjem na uzorku fiziološke otopine. Stupanj osjetljivosti razvijenog testa IgA-LMX u razini je sa IgG-LMX testom što je utvrđeno usporedbom krivulja reaktivnosti specifičnih rekombinantnih humanih monoklonskih protutijela HLA podrazreda IgA1, IgA2 i IgG1. Validacija testa provedena je testiranjem seruma skupine ispitanika bez prethodnog HLA imunizirajućeg događaja što je ujedno iskorišteno za određivanje vrijednosti praga za definiranje

rezultata testa IgA-LMX kao pozitivnih i negativnih za prisustvo protutijela HLA razreda IgA.

Standardni testovi određivanja protutijela HLA razreda IgG (IgG-LMX i IgG-SAB) i modificirani testovi određivanja prisutnosti (IgA-LMX) i specifičnosti (IgA-SAB) protutijela HLA razreda IgA primjenjeni su na uzorcima primatelja bubrega s različitim ishodima transplantacije i uzorcima seruma žena imuniziranih isključivo tijekom trudnoće. Od 294 uzorka seruma, protutijela HLA razreda IgA pronađena su u 3,40% uzoraka, kod osam primatelja bubrega i dvije žene imunizirane isključivo kroz trudnoću. Sva protutijela HLA razreda IgA pronađena su isključivo u uzorcima koji su sadržavali protutijela HLA razreda IgG, pri čemu je 90% njih bilo usmjereno protiv molekula HLA razreda II. Zbog malog broja uzoraka seruma primatelja bubrega s različitim ishodima transplantacije u kojima je otkrivena prisutnost protutijela HLA razreda IgA nismo bili u mogućnosti donijeti zaključak o ulozi protutijela HLA razreda IgA u transplantaciji. Primjena IgA-SAB testa na uzorcima seruma pozitivnim u IgA-LMX testu pokazala je prisutnu razliku u osjetljivosti između dva navedena testa i nizak detekcijski signal za protutijela HLA razreda IgA izmjeren pri IgA-SAB testiranju. Za svrhe našeg istraživanja modificirali smo testove proizvođača Lifecodes dok je u svim do sada objavljenim radovima koji su uključivali detekciju protutijela HLA razreda IgA korištena Luminex tehnologija uz primjenu testova proizvođača One Lambda-Thermo Fisher Scientific Inc., West Hills, CA, USA (One Lambda). Testiranje seruma ispitanika IgA-SAB testom oba proizvođača pokazalo je postojanje višeg detekcijskog signala kada je primjenjen test proizvođača One Lambda. Kada su među rezultatima IgA-SAB testova uspoređeni detekcijski signali protutijela HLA uperena protiv donora uočene su sličnosti, ali i razlike između rezultata testova dvaju proizvođača.

Rezultati ovog istraživanja pokazuju da smo uspjeli razviti visoko osjetljiv i specifičan Luminex test za otkrivanje prisustva protutijela HLA razreda IgA te da su protutijela HLA razreda IgA nisko zastupljena u serumu ispitanika koji su imali prethodni HLA imunizirajući događaj. Test IgA-LMX je brži i financijski povoljniji u usporedbi s testom IgA-SAB i može biti značajna nadopuna rutinskom testiranju seruma bolesnika u programu transplantacije bubrega na protutijela HLA razreda IgG te olakšati buduća istraživanja protutijela HLA razreda IgA. Ovdje predstavljeni rezultati doprinijeti će boljem razumijevanju uloge protutijela HLA razreda IgA u transplantaciji.

10. ABBREVIATIONS

TCR:	T cell receptor
BCR:	B cell receptor
MHC:	Major histocompatibility complex
APC:	Antigen-presenting cells
CD4+:	CD4-positive
CD8+:	CD8-positive
Ig:	Immunoglobulin
H:	Heavy
L:	Light
V:	Variable
C:	Constant
Fab:	Antigen-binding fragment
ADCC:	Antibody-dependent cellular cytotoxicity
HLA:	Human leukocyte antigen
MIC:	MHC-class I chaine relate gene
LMP.	Low molecular weight proteasome
TAP:	Transport antigen processing
CREG:	Cross-reactivity groups
PIRCHE:	Predictable indirect recognizable HLA epitopes
ABMR:	Antibody-mediated rejection
DSA:	Donor-specific antibody
Non-DSA:	Non-donor-specific antibody
dIgA:	Dimeric form of IgA
SIgA:	Secretory form of IgA
pIgR:	Polymeric immunoglobulin receptor
SC:	Secretory component
m:	Monoclonal
ELISA:	Enzyme-linked immunosorbent assay
CDC:	Complement-dependent cytotoxicity
DTT:	1,4-dithiothreitol
CI-0X:	HLA class I bead groups 01-07
CII-0X:	HLA class II bead groups 01-05
FCXM:	Flow cytometry crossmatch
CM:	Cross-match
PRA:	Percentage of reactive antibodies
PE:	R-phycoerythrin
MFI:	Mean fluorescence intensity
LMX:	Antibody screening
SAB:	Single antigen bead
EDTA:	Ethylenediaminetetraacetic acid
IVIG:	Intravenous immunoglobulin
mAb:	Monoclonal antibody
OL:	One Lambda
LC:	Lifecodes
PBMC:	Peripheral blood mononuclear cell
Raw:	Raw MFI value
Adj X:	Adjusted ratio
CON:	Negative control bead
Pos Ctrl:	Positive control bead
BAF:	Background-adjusted factor
PBS:	Phosphate-buffered saline
Conc.:	Concentration
N:	Number of individuals
n:	Number of samples
F:	Female
M:	Male
HR	Highest-ranked
BCM:	Background corrected MFI
BCR:	Background corrected ratio
AD:	Antigen density
NA:	No specific allele in the assay
MM:	Mismatch
IgG-:	IgG HLA antibody negative
IgG+:	IgG HLA antibody positive
IgA-:	IgA HLA antibody negative
IgA+:	IgA HLA antibody positive
IgAN:	IgA nephropathy
tx:	Transplantation
pr:	Pregnancy
neg:	Negative
pos:	Positive
NS:	Not specified
NB:	No bead
CI:	Confidence interval
IgA DSA:	Donor-specific IgA HLA antibody
IgG DSA:	Donor-specific IgG HLA antibody

11. CURRICULUM VITAE

I was born on June 5, 1983 in Vinkovci, Croatia. After graduating from Gymnasium M.A.Reljković in 2001, I started my Masters in Education in Biology and Chemistry at the University J.J. Strossmayer Osijek, Croatia. In the period 2002-2005, I worked as an Assistant Student in Cell Biology Practicum and Genetics Practicum courses at the Department of Biology. I completed my Master thesis project "Polymorphism of microsatellite loci within the human tumor necrosis factor in Croatian population" at the University Hospital Center Zagreb, Croatia under the supervision of Prof. Dr. Zorana Grubić. The work there allowed me to become proficient in performing standard molecular biology techniques and developed my interest in molecular biology and population genetics. From my graduation in 2006 until 2016, I worked as a Biology and Chemistry Teacher and for a while as a German Language Teacher. While pursuing my teaching career I worked on projects focused on the application of technology in the learning process and development of learning strategies. Throughout this period, I received education in ICT and project management for EU funded projects. Gained project management knowledge I further used for leading the Young Nature Guards, a section of the Croatian Society of Natural Sciences. Furthermore, I worked as an expert associate for several national institutions related to science and education. The knowledge and experience I gathered during my teaching career I introduced in a handbook for biology teachers Biologija 8, ALFA which was published in 2014.

In the same year, I started the University Postgraduate Interdisciplinary Doctoral Study Molecular Biosciences, Module Biomedicine and Health in Croatia. In 2016, I got the opportunity to work as a visiting PhD student at the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center in the Netherlands, under the supervision of Prof. Dr. Frans H.J. Claas. My research interest has been focused on the detection, role and function of HLA antibodies in organ transplantation. During my stay in Leiden, I enhanced my laboratory skills, gained additional knowledge in immunology and conducted my PhD project "Incidence of IgA isotype of HLA antibodies in alloantigen exposed individuals".

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