The clinical importance of non-HLA specific antibodies in kidney transplantation

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To my parents, brothers and sisters, your support have sustained me throughout my whole life.

To my dear husband **Humaid Almahri**, you made me stronger with your words and advice.

Love you all!

The clinical importance of non-HLA specific antibodies in kidney transplantation

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Abstract

The clinical significance of human leukocyte antigen (HLA) antibodies (Abs) for hyperacute, acute and chronic antibody-mediated rejection (AMR) of kidney allografts has been clearly demonstrated. AMR occurs in the absence of donor-reactive HLA Abs. It is not known how common the problem of AMR by non-HLA Abs is because of lack of suitable assays for their detection. It is believed that the non-HLA Ab population, although heterogenic, is likely to target antigens on donor organ endothelial cells (ECs). We have been involved in the clinical introduction of a flow cytometric (FC) crossmatch (XM) test that permits the detection of Abs reactive with endothelial precursor cells (EPC) isolated from donor peripheral blood. In this context the EPCs may function as surrogates for mature vascular ECs.

The work in this thesis describes the adaptation of the EPCXM to detection of complement-fixing HLA and non-HLA Abs using complement fragment-specific antibodies and flow cytometry, describes the outcome of the EPCXM in relation to the conventional lymphocyte XM (LXM), degree of HLA sensitization and transplantation outcome in patients evaluated for living donor (LD) kidney transplantation (Tx), and assesses the long-term renal graft function in patients with a positive EPCXM pre-transplant.

In the first paper, we investigated whether EPCs could be used for detection of complement-fixing Abs and if complement factor and IgG deposition on co-purified T and B cells correlated to the outcome of the T- and B-cell complement-dependent

cytotoxicity (CDC) XM. Incubation of EPCs with HLA Ab-positive serum samples resulted in deposition of complement factors C3c and C3d, but not C1q nor C4d, on EPCs and co-purified lymphocytes. The amount of C3c deposition and IgG binding on EPCs and T cells, but not B cells, correlated. The specificity and sensitivity for C3d deposition on co-purified T cells *vs* the T CDC assay were 69% and 72%, while for B cells the sensitivity was considerably lower. In the second paper, we show that 32% of the LD patients had IgG and/or IgM-binding donor EPCs in their pre-Tx sera. Twenty-five percent of the patients were EPCXM IgM+. Of the patients with negative LXM tests, 25% had EPC Abs mainly of IgM class not reactive with HLA. There was no difference in rejection frequency or serum creatinine levels between the EPCXM positive and negative groups, which is in contrast to earlier published results. However, the clinical protocols used in the second paper included Ab pre-Tx treatments such as B cell depletion and Ab removal. The pre-Tx EPCXM positive group had significantly more patients with delayed graft function. In the manuscript we show that the difference in serum creatinine and glomerular filtration rates observed between EPCXM positive and negative groups at three and six months post-Tx disappears hereafter and during the four-year follow-up.

The detection of complement factors on EPCs and lymphocytes by flow cytometry allowing detection of complement-fixing non-HLA and HLA Abs widens the diagnostic repertoire that can be offered patients undergoing kidney transplantation and should thereby improve their clinical management. Prospective studies with appropriate control groups are needed to establish whether pre-treatments aiming at removing anti-EC Abs, as detected by the EPCXM pre-Tx, have a beneficial effect on short- and long-term graft survival.

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Populärvetenskaplig sammanfattning

Bakgrund: Patienter med kraftigt nedsatt njurfunktion behöver dialys för att överleva, ofta flera gånger i veckan. Genom njurtransplantation förbättras patientens livskvalitet och hen kan i allmänhet gå tillbaka till ett fullt yrkesverksamt liv. En fruktad komplikation vid njurtransplantation är avstötningsreaktionen, d.v.s. den reaktion där patientens immunsystem försöker stöta bort njuren. Denna reaktion orsakas bl.a. av antikroppar som känner igen de s.k. transplantationsantigenerna (HLA), vilket är strukturer som finns på alla kroppens celler och som oftast skiljer sig åt mellan olika individer. I en del fall kan antikroppar mot andra strukturer, vilka ofta sitter på kärlens insida på de s.k. endotelcellerna, än HLA orsaka antikroppsförmedlad avstötning.

Syfte: Arbetet i denna avhandling har syftat till att förfina en ny metod för att hos patienter inför njurtransplantation upptäcka antikroppar riktade mot den donerade njurens endotelceller, och att undersöka hur dessa antikroppar korrelerar till risken för avstötning och nedsatt funktion hos den transplanterade njuren.

Material och metoder: Patienternas HLA typ bestämdes med genetiska metoder (PCR) och om de hade HLA antikroppar eller inte avgjordes med cellbaserade metoder och solid fasmetoder. Förenlighet mellan patient och donator testades i s.k. korstester i vilka eventuella antikroppar i patientserum får binda till donatorns lymfocyter (en celltyp i blod som bär HLA antigen). Avläsningen av korstesten sker i mikroskop eller med s.k. flödescytometri. Den senare är en känslig metod för att undersöka om patientantikroppar bundit till donatorcellerna. Vidare användes och vidareutvecklades en ny korstestmetod som möjliggör detektion av antikroppar mot endotelcellsliknande celler från donatorn.

Resultat och diskussion: I det första arbetet vidareutvecklade vi det flödescytometriska korstestet som möjliggör identifiering av antikroppar mot donatorns endotelceller till att också identifiera de antikroppar som kan aktivera komplement. Denna typ av antikroppar är mer potenta och utgör en större risk för avstötning. I det andra arbetet visade vi att patienter med antikroppar detekterade i endotelcellskorstestet i högre grad hade njurar som kom igång senare efter transplantationen och t.o.m. ibland förlorades. I manuskriptet har vi följt njurtransplanterade patienter över tid för att se hur njurfunktionen hos de patienter som hade endotelcellsantikroppar utvecklades över tid. Den skillnad i njurfunktion vi såg tre och sex månader efter transplantationen mellan grupper med och utan endotelcellsantikroppar försvann från ett år efter transplantation och fortsatt under den fyraåriga uppföljningen.

Sammanfattning: Vi har vidareutvecklat ett test som möjliggör identifiering av en antikroppspopulation som tidigare ej kunnat identifieras och som kan bidra till försämrad funktion, och i värsta fall avstötning, av njurtransplantat.

List of publications

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Detection of complement-fixing and non-fixing antibodies specific for endothelial precursor cells and lymphocytes using flow cytometry. Ayeda AlMahri, Jan Holgersson, Mats Alheim. Tissue Antigens, 2012, 80, 404–415

II. The outcome of the endothelial precursor cell crossmatch test in lymphocyte crossmatch positive and negative patients evaluated for living donor kidney transplantation. Mats Alheim, Ayeda AlMahri, Jakob Nilsson, Gunnar Tydén, Jan Holgersson. Human Immunology, 2013, 74, 1437–1444

III. A pre-transplant positive endothelial precursor cell crossmatch does not imply reduced long-term kidney graft function. Markus Gäbel, Ayeda AlMahri, Lennart Rydberg, Jan Holgersson, Michael E. Breimer. (Manuscript).

Table of Contents

List of Abbreviations

Abs	Antibodies
AECA	Anti-endothelial cell antibodies
AMR	Antibody-mediated rejection
ATG	Anti-thymocyte globulin
ATN	Acute tubular necrosis
AT1R	Angiotensin Type 1 receptor
CAN	Chronic allograft nephropathy
CDC	Complement-dependent cytotoxicity
DSA	Donor-specific antibodies
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal disease
FACS	Fluorescence-activated cell sorting
HLA	Human leukocyte antigen
KTx	Kidney transplantation
LD	Living donor
mGFR	Measured glomerular filtration rate
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PRA	Panel-reactive antibodies
SCr	Serum creatinine
SSO	Sequence-specific oligonucleotides
SSP	Sequence-specific primer

TCMR	T-cell-mediated rejection
Tx	Transplantation
XM	Crossmatch

Introduction

I. Kidney Transplantation

Organ transplantation (Tx) is performed in order to replace a diseased or damaged organ with a healthy one. Patients with end-stage renal diseases (ESRD) are treated either by dialysis or kidney transplantation. ESRD occurs when both kidneys are no longer functional because of defective kidney filtering capacity leading to accumulation of waste products, perturbed salt balance and hormonal deregulation [1]. Symptoms of ESRD may remain mild or absent until kidney function drops to less than 20% of normal [2]. Symptoms can be significant and include, but are not limited to, weight loss, nausea or vomiting, general malaise, fatigue, headache, hiccups, itching, decreased urination, easy bruising or bleeding, lethargy, difficulty breathing, and seizures. Causes of ESRD include diabetes, high blood pressure and atherosclerosis, autoimmune diseases (*e.g.* lupus), genetic disorders (like polycystic kidney disease), infections, post-renal obstruction of the urinary tract, and exposure to toxic substances (*e.g.* antibiotics, chemotherapy, dyes used for contrast in radio imaging, analgesics, fungal toxins) [3].

Kidney transplantation is done in order to correct ESRD and in the majority of cases kidney transplantation allows the patient to return to a normal life and full time work [4]. The kidney donor can be living (*e.g.* a parent, sibling or a child of the recipient, a friend or spouse), or a deceased donor [5, 6].

In 1954 the first successful kidney transplantation was performed using an identical twin brother as donor. The graft functioned well without immunosuppressive drugs for 9 years until relapse of the underlying disease [7]. Complications sometimes seen following kidney transplantation include those related to the surgical procedure and

secondary complications caused by the life-long immunosuppressive treatment. They can also be classified as short term (vascular thrombosis, narrowing of the renal artery, obstruction of the ureter, urine leakage, acute rejection) or long-term (chronic rejection and negative effects caused by the immunosuppression including diabetes, high blood pressure, cancer and infections) complications [8-10].

Despite an ever increasing success rate of kidney transplantation which to a large part can be ascribed ever better and more effective immunosuppressive drugs, there is still room for improvements.

II. Renal allograft rejection

Increased serum creatinine post-transplant may suggest allograft rejection, but other conditions such as surgical complications, infections and drug toxicity can impair renal graft function and lead to a rise in serum creatinine [11]. Histopathological assessment of biopsies taken from the transplanted kidney is key to the diagnosis, and the morphology may influence the choice of therapy and subsequent prognosis [12, 13]. Besides evaluation of the histology of the biopsy, detection and specificity determination of donor-specific antibodies is important in order to determine whether the rejection is predominantly T-cell or antibody-mediated. Subclinical rejections, *i.e.* rejections not associated with a rise in serum creatinine, may be evident only upon examination of the biopsy.

Renal allograft rejection can be divided into acute T-cell mediated rejection (ACR), acute antibody-mediated rejection (AMR) and chronic rejection (CR) based on biopsy morphology and the presence of donor-specific antibody [14, 15]. AMR can be either hyperacute (HAR) occurring within minutes to hours, or acute occurring within days to weeks after transplantation. Donor-specific HLA antibodies have also been

implicated as a pathogenic factor in CR, which sometimes occur years after transplantation [16]. It should be emphasized though that many times a mixture of T-cell and antibody-mediated pathology contribute to rejection and that acute and chronic rejections may not be distinct events but rather represent a continuum of events [17]. The type of rejection of renal grafts can also be classified according to the histopathological picture found in the biopsy. This so called Banff classification (Table 1) of renal allograft biopsies grades the degree of interstitial infiltration of mononuclear cells, the number of mononuclear cells per tubular cross section, and the degree of arteritis in case of acute T-cell mediated rejection, and the degree of interstitial fibrosis and tubular atrophy in case of chronic rejection [17]. Complement factor C4d+ staining, the presence of circulating donor-specific antibodies (DSA) and morphologic evidence of acute tissue injury such as acute tubular necrosis (ATN)-like minimal inflammation, capillary and/or glomerular inflammation and/or thrombosis, or transmural arteritis are diagnostic criteria for AMR [18, 19].

Table 1 Banff 97 diagnostic categories for renal allograft biopsies—Banff'07 update

Banff's classification	Degree of rejection	Characteristics and subtypes
Normal		
Antibody-mediated rejection	Acute AMR	C4d+, DSA Type I: ATN-like minimal inflammation Type II: capillary and/or glomerular inflammation Type III: transmural arteritis
	Chronic active AMR	C4d+, DSA, glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries
Borderline	"Suspicious" TCMR	Tubulitis (t1, t2 or t3) with interstitial infiltration (i0 or i1) Interstitial infiltration (i2 or i3) with mild (t1) tubilitis
T-cell-mediated rejection	Acute TCMR	Type IA: i2 or i3 and t2 Type IB: i2 or i3 and t3 Type IIA: mild-to-moderate intimal arteritis (v1) Type IIB: severe intimal arteritis (v2) Type III: "transmural" arteritis and/or arterial fibrinoid change, necrosis and lymphocytic inflammation (v3)
	Chronic active TCMR	Arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima
Interstitial fibrosis and tubular atrophy (IFTA)		Interstitial fibrosis and tubular atrophy Grade I: mild Grade II: moderate Grade III: severe
Other	Changes not due to rejection	Chronic/sclerosing allograft nephropathy, recurrent diseases, toxic changes, and infection

Modified from Solez et al [19].

Acute cellular rejection

A rapid rise in serum creatinine may be caused by acute cellular rejection. Besides the increase in serum creatinine, patients may retain fluids, *i.e.* gain weight, develop fever and graft tenderness. The incidence of ACR is approximately 5-10% in the first year in unsensitized patients [20]. ACR is histologically characterized by an accumulation of mononuclear cells, mostly CD4+ and CD8+ T cells, in the intersititium, the tubules (causing tubulitis) and sometimes in the arteries (causing arteritis) [21, 22].

T cells cause cell damage by release of cytotoxic granules containing perforin and granzyme A and B, by engaging the Fas-FasL receptor pair and by releasing inflammatory cytokines (IFN-γ, TNF-α) and chemokines (CCL5/RANTES, CCL3/MIP-1) [23]. Tubulus infiltrating T cells and macrophages in tubulitis make tubular cells go into apoptosis as revealed by an increased number of TUNEL+ cells. Subendothelial and intimal infiltration of T cells and macrophages is characteristic of endarteritis, a hallmark of ACR [24] .The latter is detected in 25-40% of renal biopsies taken on the suspicion of ACR and is rarely found in stable grafts [25]. At times, also glomerulitis is found in ACR cases.

Antibody-mediated rejection

Antibody-mediated rejection can be divided into hyperacute (HAR) and acute antibody-mediated rejection (AMR) depending on the kinetics of the rejection.

HAR is a very dramatic response that occurs immediately after transplantation usually within the first hours and sometimes immediately after release of the vascular clamps. It is caused by pre-existing host antibodies that bind to antigens, commonly human leukocyte antigens (HLA) or blood group ABH antigens present on the graft

endothelium [26, 27]. Following antibody binding, complement is fixed, activated and the membrane attack complex (C5b-C9) deposited on the cell surface causing cell lysis/necrosis [28]. Platelet aggregation and the formation of microthrombi contribute to cessation of blood flow, and endothelial cell retraction cause leakage of red cells and fluid out in the interstitial tissue. The kidney becomes cyanotic and swollen. Grafts that have undergone HAR have to be removed and replaced with another graft. However, improvements in cross-matching techniques and specificity determinations of HLA antibodies have made HAR a rare event [29].

Acute AMR is caused by antibodies binding to donor HLA or non-HLA expressed on endothelial cells. It is characterized by a rapid rise in serum creatinine which may occur days to weeks or even years after transplantation. It is believed that antibodies may contribute to acute rejection episodes in at least 25% of the cases [20]. Antibodies may act in concert with T-cells in an otherwise predominant ACR or may act alone in an AMR without clear signs of ACR. In sensitized patients, *i.e.* in patients previously transplanted, transfused or with earlier pregnancies, and in patients with poor compliance the humoral component may be even more significant.

Antibodies bound to the endothelium will activate complement causing endothelial cell injury, release of von Willebrand factor (vWF) and surface expression of P-selectin, which promote platelet aggregation and the formation of microthrombi. In addition, cytokines (IL-1α, IL-8), chemokines (CCL2), and the chemoattractants, C3a and C5a, which cause leukocytes to adhere to glomeruli (glomerulitis) or to dilated peritubular capillaries (margination) will be released [28]. The complement factors C4d, which is also a histopathological biomarker for AMR (see below), and C5b, which initiates the assembly of the membrane-attack complex, causes localized endothelial necrosis and apoptosis. In severe cases microthrombi, with hemorrhage

and arterial wall necrosis and infarction, occurs [28]. In order to rescue such grafts early diagnosis and treatment are necessary.

Deposition of the complement factor C4d, an inactive fragment of C4b, in the majority of peritubular capillaries in a ring formed pattern is a diagnostic hallmark of AMR. The recognition of the significance of C4d deposition and novel diagnostic tools for detection and specificity determination of DSA, have dramatically increased our ability to diagnose AMR [30]. Donor-specific class I or II Abs are present in around 90% of the patients with C4d deposition. In ABO incompatible transplantation C3d deposition is associated with acute inflammation, while C4d deposition in this patient group can be seen even in histologically normal grafts [31]. The ability of DSA to cause AMR is highly associated with its ability to fix complement. Thus, IgG3 and IgG1 DSA are more pathogenic than IgG2 and IgG4 DSA [32]. In this context novel diagnostic tools that enable identification of complement fixing DSA may become increasingly important [33].

Treatment options for AMR includes besides high dose steroids and proliferation inhibitors, removal of antibodies (plasmapheresis or immunoadsorption), immunoglobulin injections, B-cell depleting antibodies (anti-CD20), complement inhibitors such as the anti-C5 antibody (eculizumab) and proteasome inhibitors (bortezomib) [34, 35].

Chronic rejection

Cellular or humoral mechanisms or a combination of both may contribute to chronic rejection (CR), which occurs months or years after transplantation. The morphologic characteristics of chronic rejection can be seen in the glomeruli as glomerulopathy, in turn recognized ultra structurally as duplication or multilamination of the glomerular

basement membrane, and in the vessels as peritubular capillaropathy with features similar to those of the glomerulopathy and, in the arteries as transplant arteriopathy which is characterized by thickening of the arterial intima [36]. In addition, kidneys undergoing chronic rejection may develop interstitial fibrosis and tubular atrophy [37]. The majority of glomerulopathy cases are associated with HLA class II DSA in serum and between 30-50% of these have C4d deposition in the peritubular capillaries [28, 38]. When glomerulopathy is accompanied by DSA and C4d deposition it is diagnostic of a chronic humoral rejection (CHR) [36]. Early AMR in sensitized patients is a risk factor for later glomerulopathy and accumulation of mononuclear cells in peritubular capillaries as seen in CHR is also a risk factor for later graft failure [39]. Transplant arteriopathy may develop either as a consequence of a C4d+ or – CR. In the latter case macrophages and CD3+ T cells may be seen in the neointima [20].

III. Mechanisms of allorecognition

The alloreactive immune response is initiated by T cells recognizing foreign HLA antigens, which are widely expressed on different cell types. The importance of HLA for allorecognition and rejection is reflected in the fact that grafts from HLA identical siblings have significantly longer survival times than grafts from HLA non-identical donors [24, 40]. Host T cells can directly recognize donor HLA on graft cells (direct pathway of antigen presentation) or following processing in host antigen-presenting cells (APC; indirect pathway of antigen presentation) [20]. The TCR on CD4+ T cells bind peptides presented by HLA class II antigens, while TCRs on CD8+ T cells bind peptides presented by HLA class I [41]. Besides engagement of the T cell receptor, so called costimulatory molecules on the T cell need to be engaged by their cognate ligands, *e.g.* CD28/CTLA4:CD80/CD86, CD40:CD154, ICOS:ICOSL, OX40:OX40L, and CD27:CD70, in order for the T cell to be activated [42, 43]. Novel

immunosuppressive drugs inhibiting costimulatory receptors, *e.g.* CTLA4Ig, are currently being explored for use in transplant patients.

Because costimulation is needed for T cell activation to occur, APC, especially dendritic cells (DC), carrying ligands for T cell costimulatory molecules are essential triggers of the alloresponse [42]. T cell activation by DC takes place in the regional lymph nodes and spleen following migration of DC there from the graft [44]. Both donor and host DC can initiate the alloresponse [45]. The latter DCs migrate into the graft from the circulation and once they have taken up antigen, they migrate via the lymph to the draining lymph node; a process guided by chemokines (CCL19/CCL21:CCR7) [46].

CD4+ T cells develop into helper T cells (T_H cells) following activation. They help in the maturation of B cells into plasma cells (and production of DSA) and memory B cells as well as in the maturation of macrophages [47]. CD8+ T cells or cytotoxic T cells are important effector cells in ACR. They mediate cytotoxicity via release of cytotoxic granules containing perforin and granzyme A and B or via secretion of toxic cytokines such as TNF-α and β [48].

Cross-talk between the innate and adaptive immune systems is important for a potent alloresponse to occur. The inflammatory reaction caused by ischemia and reperfusion, and by the surgical trauma itself, potentiates the immune response by recruiting immune cells including APC to the graft [49]. Further, increased expression of ligands for toll-like receptors (TLR), damage-associated molecular-pattern (DAMP) receptors and other innate inflammatory molecules promote maturation and activation of dendritic cells [50, 51]. The complement system, in particular C3a and C5a, can directly activate intra-graft T cells and antigen-presenting cells (APC) [52-

55]. Donor-specific HLA antibodies may contribute to graft rejection not only by binding to graft cells and subsequent activation of complement, but also through binding of Fc receptors that may promote antigen uptake in APC and initiate antibody-dependent cellular cytotoxicity (ADCC) by NK cells [56].

IV. The HLA system

The major histocompatibility complex (MHC) on chromosome 6 in humans is a complex 4 Mb genetic region including more than 200 genes and encoding the human leukocyte antigens (HLA) [57]. HLA controls the activity of the immune system by presenting self and non-self peptides to the immune cells of the host. HLA class I antigens are found on all nucleated cells, while class II antigens are to be found mainly on APC. The former presents peptides generated inside the cell following digestion in the proteasome of for example viral antigens, while the latter presents antigens taken up from outside the cell [58]. Peptide loaded class I antigens are recognized mainly by the T cell receptor on CD8+ T cells and by the killer cell immunoglobulin-like receptors (KIR) of NK cells, while peptide-bearing class II antigens are recognized by the TCR mainly on CD4+ T cells [58]. The class III region of the MHC complex encodes, among other proteins, cytokines (*e.g.* TNF-α) and components of the complement system (C2, C4, factor B) [59]. The MHC class I region carries three loci encoding the HLA-A, -B, and C antigens, which are all structurally similar. HLA class I antigens are made up of a heavy alpha chain of 45kDa controlled by a gene in the relevant MHC locus (Fig. 1). It is associated with a smaller chain of 12kDa called β2-microglobulin. In July of 2014 there were approximately 2,800, 3,500 and 2,300 distinct alleles of HLA-A, -B and -C respectively [60]. There are three distinct HLA class II antigens, DR, DQ and DP, each composed of one α and one β chain (Fig. 1). There are four different DRβ genes, DRβ1, DRβ3, DRβ4 and DRβ5 [61].

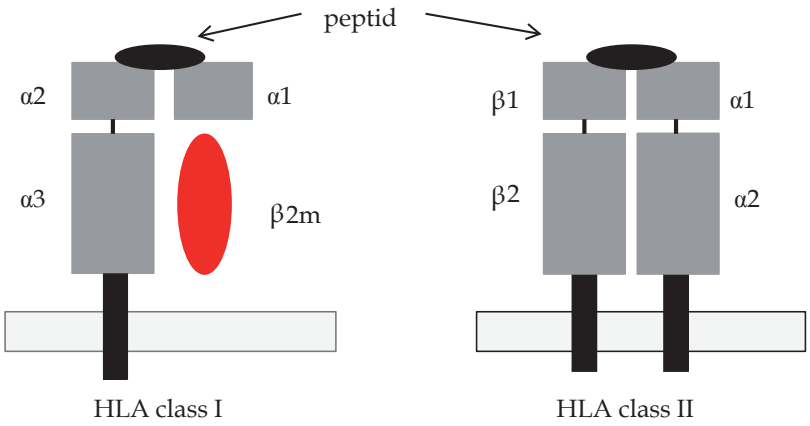

Fig. 1 The HLA class I and HLA Class II molecules

The strongest evidence that the HLA system is indeed the major histocompatibility system relevant for matching in transplantation comes from the fact that kidney or bone marrow grafts exchanged between HLA identical siblings survive almost as long as grafts between identical twins and far better than grafts exchanged between mismatched siblings or other relatives [62]. Advances in immunogenetics and histocompatibility testing have facilitated the clinical transplantation of solid organs and tissues. Improved definition of HLA antigens, alleles, and haplotypes has clarified the diversity of the HLA system among different racial/ethnic populations [63].

V. HLA antibodies

Antibodies, also called immunoglobulins, are large Y-shaped proteins, which function to identify and help remove foreign antigens or microbes such as viruses and bacteria (Fig. 2). Every different antibody recognizes a specific foreign antigen. This is because the two tips of its "Y" are specific to each antigen, allowing different antibodies to bind to different foreign antigens [64].

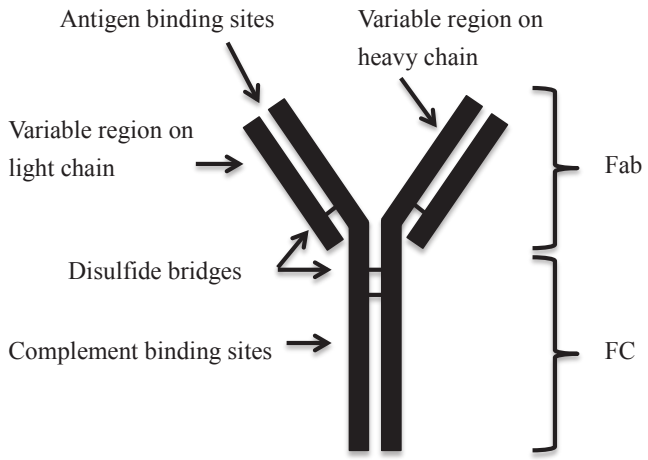

Fig. 2 Antibody structure

Antibodies are produced by the immune system in response to the presence of an antigen. Antigens can be carbohydrates like the blood group ABH antigens, lipids, *e.g.* phosphocholine or proteins. Antibodies are found circulating in blood, but are also present in the tissue interstitium and the various mucosae of the body [65]. There are five distinct classes of antibody namely IgG, IgA, IgM, IgD and IgE. They differ in size, charge, amino acid composition and carbohydrate content, but they share a similar basic structure [66]. The antibody molecule is bi-functional, the Fab component is used to bind antigen while the Fc region mediates the biological effect and is designated the effector region [67]. There are four IgG subclasses (IgG1, 2, 3, 4). IgG1 and G3 are most efficient activators of complement. IgG and IgM have been shown to mediate graft rejection [68]. Interestingly, IgA HLA antibodies may have a protective effect [69, 70].

Antigen binding by antibodies is their primary function and can result in protection of the host. Examples include neutralization of invading viruses or bacteria. Even though recent research has shown that binding of cell surface-expressed antigens, *e.g.* HLA, can directly activate cells [71], the most significant biological effects mediated by antibodies are a consequence of their effector functions mediated by the Fc part [72]. Usually the ability to carry out a particular effector function requires that the antibody bind to its antigen. The effector functions include complement fixation and activation, and Fc receptor binding [72, 73].

The immune system responds to HLA antigens that are non-self. Healthy individuals may carry anti-HLA antibodies as a consequence of sensitization via pregnancies, blood transfusions or a previous transplant. It has been claimed that 15-25% of women develop HLA antibodies after their first pregnancy and 50-60% after their second pregnancy [74]. Likewise, the frequency of patients acquiring HLA antibodies following blood transfusions increase with the number of transfusions and may approach 70% in patients having had 20 transfusions and more [74]. With increasing number of mismatches between donor and patient in their first transplant, patients have higher PRA levels at relisting [75]. Thus, the benefits of better HLA matching at first transplant on lifetime with graft function are significant [75].

Patients with HLA antibodies and a high PRA value are less likely to receive a transplant because of a positive pre-transplantation crossmatch, and may also reject their grafts more readily even if the pre-transplantation crossmatch is negative [76]. As described above, donor-specific antibodies (DSAs) to HLA antigens can cause acute AMR after kidney transplantation [75]. The clinical impact of HLA antibodies is related to their antigen specificity, complement fixing ability, immunoglobulin class and subclass, and titer [77]. Antibodies against HLA-A, -B, -Cw, -DRB1, -DRB3-5 and

–DQB1 can all cause AMR, while more studies are needed in order to clearly link HLA-DPB and –DQA specific antibodies to AMR [77]. Interestingly, class II specific antibodies have been associated with the development of transplant glomerulopathy [78]. The ability of DSA to fix complement as inferred by C4d deposition in biopsies appears to be a poor prognostic marker for graft survival such that patients with DSA and C4d deposition in their cardiac grafts had worse graft survival than patients with DSA and no C4d deposition or patients without DSA but with C4d deposition [79]. The ability of preformed, low-level, DSA to trigger C4d fixation *in vitro* on single antigen beads in patients with negative conventional crossmatch tests is predictive for AMR. Assessment of C4d deposition on single antigen beads is potentially a powerful tool for risk stratification prior to transplantation and may allow identification of unacceptable donor antigens, or patients who may require enhanced immunosuppression [76]. Stastny *et al* have presented data suggesting that the donor-specific HLA antibodies of IgM type is predictive of transplant rejection in renal transplant recipients and susceptibility to coronary arteriopathy in heart grafts [80]. The most disputed topic in HLA antibody diagnostics relates to the mean fluorescence intensity (MFI) in the single antigen bead assay and what MFI is clinically significant. It has been suggested that the outcome of the single antigen bead assay should be interpreted in light of the crossmatch results, clinical outcome data and the clinical protocol of each center [77].

A number of programs and protocols have been implemented in order to make highly sensitized patients transplantable. They include the acceptable mismatch program of Eurotransplant, which is an algorithm that matches donor kidneys based on their HLA type and the HLA antibody repertoire of the patient [81]. In order to increase the availability of live donors to sensitized patients whose donor is

unacceptable because of DSA, paired kidney exchange programs have been established. Thus, two or more donor-recipient pairs can crosswise offer each other matched kidneys [82]. In addition, various desensitization protocols have been proposed that involves removal of DSA by plasmapheresis or immunoadsorption followed by prevention of the resynthesis of HLA antibodies by administration of intravenous immunoglobulins (IVIg) [83].

New treatment options such as eculizumab, an inhibitor of terminal complement activation, decreases the incidence of early AMR in sensitized renal transplant recipients (ClincalTrials.gov number NCT006707) [84].

VI. The complement system

Complement activation can occur in three ways by the classical pathway, the lectin pathway, and by the alternative pathway. Both the classical and alternative pathways converge on the activation of the C5 convertase, the activity of which results in the production of C5b [85]. Antibody-mediated cytotoxicity is enhanced through activation of complement via the classical complement pathway, which is initiated by the binding of the C1q component to the Fc portion of IgM, IgG1 or IgG3. The final product of complement activation, the membrane attack complex composed of C5b-C9 subunits, creates a pore in the cell membrane resulting in cell lysis and death [86, 87]. Besides the presence in the circulation, complement factors such as C3 may be produced locally in the graft, for example in tubular epithelial cells, and contribute to both humoral and cellular rejection [88]. Complement activation is an important contributor to AMR, and C4 deposition in the graft as revealed by histological examination of a biopsy is an essential diagnostic criteria for AMR [89].

VII. Alloantigens beside HLA (non-HLA) including endothelial cell antigens

The fact that kidneys transplanted between HLA identical siblings can be lost in HAR or AMR suggests that other antigenic systems besides HLA may be important targets of DSA [90]. Further support for this idea comes from patient cases experiencing AMR despite negative lymphocyte crossmatch tests [77], which is the standard test for detection of donor-specific HLA antibodies. In addition, it has been shown that there is a correlation between increased PRA % and poor long term renal graft survival in recipients of grafts from HLA identical siblings, which suggests that non-HLA immunity contributes to poor graft survival [91, 92]. Because endothelial cells of the graft are important targets for donor-reactive antibodies, it is likely that autologous or allogeneic endothelial cell antigens mediate this interaction [93]. Non-HLA of potential clinical importance for organ allograft rejection include, but are not limited to, major histocompatibility complex (MHC) class I chain-related antigens A/B (MICA/B) [94], the angiotensin II type 1 receptor [95], the endothelin type A receptor [96], and the cytoskeletal elements vimentin, actin, tubulin and cytokeratin (reviewed in [77, 97]). Antibodies specific for MICA/B have been claimed to be associated with increased renal graft loss by some investigators [98-100]), while Scornik *et al* found no correlation between anti-MICA antibodies and C4d+ renal rejection [101]. The antigen is expressed on endothelial cells and monocytes [92]. An association between agonistic angiotensin II type 1 (AT1)-receptor (AT_1R) antibodies and corticosteroid-resistant vascular rejection along with malignant hypertension has been shown [102]. However, their exact pathogenic role is unclear [102].

Assays by which anti-endothelial cell Abs (AECAs) can be detected have in general been laborious and time-consuming, which has made them impractical for clinical use

[103-107]. Patients who had experienced antibody-mediated rejections despite negative LXM had in high frequencies Abs against EPCs isolated from the blood of a panel of third party donors by paramagnetic beads carrying Abs specific for the Tie-2 receptor [98]. In a prospective, multicenter kidney transplantation (KTx) trial, it was shown that the presence of donor-reactive anti-EPC Abs (AEPCA), as detected by the use of a novel crossmatch (XM) kit (XM-ONE®; AbSorber AB, Stockholm, Sweden) based on this method, was strongly associated with acute rejections and increased serum creatinine levels at 3 and 6 months post-Tx [108].

VIII. Immunological evaluation

HLA typing

Currently, typing of the HLA-A, -B and -DRB1 loci is required in solid organ transplantation by the national Organ Procurement and Transplant Network in the U.S. and by the European Federation for Immunogenetics in Europe. Within Scandiatransplant, a Nordic organ exchange organization, the centres are required to type also the HLA-C and –DQB1 loci. For solid organ transplantation, typing on the antigen level (low resolution) is usually sufficient even though improved solid phase immunoassays (SPI) now allows detection of allele-specific antibodies which in turn may necessitate typing with high resolution in order to say whether the antibody is donor-specific or not.

Serological typing used to be the method of choice for determining the HLA type of a patient or donor. Serological typing was usually done in a microcytotoxicity format with target T or B cells from the individual to be typed and a panel of sera (also

monoclonal antibodies were used) with HLA antibodies of known specificity. The read-out was done in the fluorescence microscope following addition of rabbit serum as a source of complement and the fluorochromes, acridine orange and ethidium bromide [109]. Serological typing was cheap and gave information on expression levels of the different HLA, but was limited by the lack of resolution (rare antigens and alleles were not resolved) and availability of typing sera [109].

Currently, molecular techniques have completely taken over and replaced the serological techniques much thanks to the introduction of the polymerase chain reaction (PCR). The techniques in use are sequence specific primer (SSP)-PCR, reversed sequence specific oligonucleotide (rSSO)-PCR and sequence based typing (SBT) that relies on the classic chain termination technique according to Sanger [109]. Novel next generation sequencing (NGS) techniques are slowly being introduced and are expected to resolve the problem of typing ambiguities and offer increased high throughput [109]. SSP-PCR offer quick turnaround times and does not require expensive equipment [110]. It relies on primer extension using primers which in their 3′ end hybridizes to an antigen- or allele-specific polymorphic sequence. A set of primer pairs is used to obtain allele resolution. The readout is accomplished by agarose gel electrophoresis and allows detection of the specific amplicon - which varies in size - expected out of each reaction [109]. A reaction control is included and is comprised of a primer pair annealing to non-polymorphic sequences. In rSSO-PCR the specific oligonucleotide probes are presented by the solid phase, most commonly the microbeads of the Luminex™ platform [111, 112]. PCR-amplified and biotinylated DNA corresponding to exon 2 and 3 for class I and exon 2 for class II are then allowed to hybridize to the specific probes coated on the colour-coded beads [109]. Colour-coded beads with hybridized amplicons are detected by streptavidin-phycoerythrin

in the Luminex™ fluoroanalyzer [109]. A clear advantage of the rSSO-PCR method is that it can be automated and easily adapted for high throughput typing, while high resolution typing is limited by the high number of probes needed [109]. SBT is the most robust method by which high resolution typing can be achieved [109]. Usually it starts by amplifying exon 2 and 3 for class I and exon 2 for class II, after which the sequencing is performed with sequence-specific primers, DNA polymerase and a mixture of deoxynucleotides and the four dideoxynucleotides each labelled with a distinct fluorochrome [109]. Following the sequencing reaction, generated DNA fragments of different size are separated and identified by capillary electrophoresis [109]. The sequence of new alleles can be obtained (which is not the case for the SSP-PCR and rSSO-PCR methods), but SBT is suffering from cis/trans and phase typing ambiguities [109]. Such ambiguities can be resolved by combining different techniques or by using additional, group-specific primers [109]. All three methods need powerful, user-friendly softwares in order to interpret the raw data and require access to an updated HLA allele database [109].

HLA antibody detection and specificity determination

The detection of HLA antibodies in patients awaiting an organ transplant is crucial to the risk assessment performed on each patient pre-transplantation. HLA antibody detection and specificity-determination is either accomplished by the use of T and B cell panels from donors well-typed with regard to their HLA phenotype, or by solid phase immunoassays (SPI). In the latter, pooled or single HLA antigens are coated/bound to a solid surface for example an ELISA plate or a microbead [109].

Prior to the introduction of SPI, patient serum was investigated with regard to the presence of HLA antibodies by the use of complement-dependent cytotoxicity (CDC) on panels of cells from well characterized donors in terms of their HLA type [109]. The term panel reactive antibodies (PRA) refers to the width of reactivity of the patient serum; *i.e.* a serum reacting with 10 of 40 cells in a panel would have a PRA of 25%. A disadvantage of the cell-based serologic assessment of PRA is the difficulty associated with finding a representative cell panel also containing cells from donors with rare HLA types known to be present in the population [109]. It is cheap, but labour intensive. Detecting DSA with the cell panel suggests a patient at higher risk than a patient having antibodies only detected in the SPI, because only antibodies that can fix complement and that are present in sufficient amounts will give a positive response in the CDC panel [109]. HLA class I antibodies are identifiable by the microcytotoxicity assay, but HLA class II antibodies often remain unidentified due to the overlapping distribution of MHC class I and II antigens [109].

SPI has revolutionized HLA antibody detection and specificity-determination. They usually come in three formats. HLA antibody screening assays containing pools of different HLA class I and II antigens from multiple individuals are used to detect the presence or absence of HLA antibodies in a rapid and specific manner, and do not typically provide any information on the HLA specificity of the detected antibodies [109, 113]. In the second format single HLA class I or class II haplotypes from a panel of well-typed individuals are selected in order to obtain a broad representation of HLA antigens [109]. The antibody response in this format has been shown to correlate well with crossmatch results [114]. In the third format, single HLA class I or II antigens purified from cell lines expressing it are coated on the solid phase surface [109]. This format has become a very powerful tool for specificity determination of

individual HLA antibodies. A disadvantage is its lack of robustness with inter-lab, day-to-day, inter-technician and lot-to-lot variation [109]. As mentioned above different platforms can be used in the SPI; ELISA plates, microbeads or glass slides as used in an array format. The Luminex™ multiplex platform has become particularly popular and offers a high throughput format with a high resolving power based on the ability of the fluoroanalyzer to distinguish 100 or more distinct microbeads based on their fluorescence. SPI can simultaneously detect and distinguish MHC class I and class II antibodies, thus providing information that was previously problematic to obtain. The FlowPRA test consists of a pool of microparticle beads that are coated with a full HLA class I or class II phenotype, and which can be analyzed in a conventional flow cytometer [115, 116]. The percentage of panel-reactive antibodies (PRAs) can be determined by calculating the percentage of beads that react positively with patient sera.

The crossmatch test

Through the introduction of the cytotoxic crossmatch test in the 1960s the hyperacute rejection with its rapid and irreversible destruction of the graft could be avoided [117]. Hyperacute rejections are therefore rare today. However, also levels of donor-specific antibodies lower than those detected by the CDC crossmatch can cause acute and chronic AMR, so more sensitive crossmatch techniques are currently used in the clinic in order to identify patients at risk for immune antibody-mediated graft damage [118]. Donor T- and B-cells are used in both the CDC and flow cytometric (FC) crossmatch tests in order to detect both class I and class II DSA.

Crossmatch tests are used primarily for organ transplant candidates to assess the suitability of a potential donor. They may also be used for platelet refractory patients

and hematopoietic stem cell transplantation candidates with aplastic anaemia who may have developed anti-HLA antibodies because of repeated blood transfusions.

Complement-dependent cytotoxicity crossmatch

The CDC crossmatch is usually performed in a microcytotoxicity format using immunoisolated T and B cells from the donor, patient serum, rabbit serum as a source of complement, and with readout in a fluorescence microscope following addition of the vital dye ethidium bromide and acridine orange [117]. Antibodies specific for class I antigens will result in a positive T and B cell CDC crossmatch, while antibodies specific for class II will give rise only to a positive B cell CDC provided there are no class I specific antibodies present. It is important to note that cell-based crossmatch techniques detect also antibodies specific for other antigens besides HLA. Therefore, the outcome of the crossmatch should be interpreted in light of the HLA antibody analyses. Because patients transplanted following a negative CDC crossmatch can still develop acute AMR, the more sensitive FC crossmatch technique was introduced.

The flow cytometric crossmatch

The flow cytometric crossmatch is more sensitive than the CDC crossmatch for detection of donor-specific HLA antibodies [109]. It detects IgG antibodies irrespective of whether they are complement-fixing or not. Usually a multiplex format is used by which T and B cells can be detected by CD3 and CD19 specific monoclonal antibodies carrying different fluorochromes, while detecting bound IgG with a secondary anti-IgG antibody conjugated to third fluorochrome [109]. In

general the strength of the reaction is expressed as the median channel shifts (on a linear or logarithmic scale) between the sample and a negative control serum. In order to accomplish better accuracy between tests the response can be expressed as molecules of equivalent soluble fluorochrome (MESF) by comparing the sample fluorescence with that of a standard curve obtained with a set of beads with known fluorochrome amounts [109].

Testing for Abs against non-HLA including anti-endothelial cell antibodies

As discussed above, antibodies against non-HLA can also cause AMR. Because there is no consensus around which antigen is the most important non-HLA, there is no standard protocol or test available for detection of all clinically important non-HLA antibodies. Besides the commercially available endothelial precursor cell crossmatch (described below), there is a Luminex™-based assay available for detection of anti-MICA antibodies and an ELISA for semi-quantification of AT_1R antibodies [119].

Aims of the thesis

1. To adopt the endothelial precursor cell crossmatch test for detection of antibody-induced complement factor deposition on EPCs and co-purified lymphocytes by multicolour flow cytometry.
2. To correlate the outcome of the endothelial precursor cell crossmatch in relation to the conventional lymphocyte crossmatch, the degree of HLA sensitization and transplantation outcome in patients evaluated for living donor kidney transplantation.
3. To assesses the long-term renal graft function in patients with a positive endothelial precursor cell crossmatch pre-transplant.

Methodological considerations

This part of the thesis presents additional information and methodological considerations regarding some novel experimental procedures used in paper I-III. Further experimental details can be found in *Materials and Methods* for each paper.

Endothelial precursor cell crossmatch assay

In the late 1990's a 10-year old patient at the Tx-center of Karolinska, Huddinge unexpectedly lost three renal grafts early after transplantation despite negative lymphocyte crossmatch tests before transplantation [120]. The patient was found to have anti-endothelial cell antibodies (AECA) in pre- and post-Tx serum samples as determined by flow cytometric staining of cultured human umbilical vein endothelial cells (HUVEC).

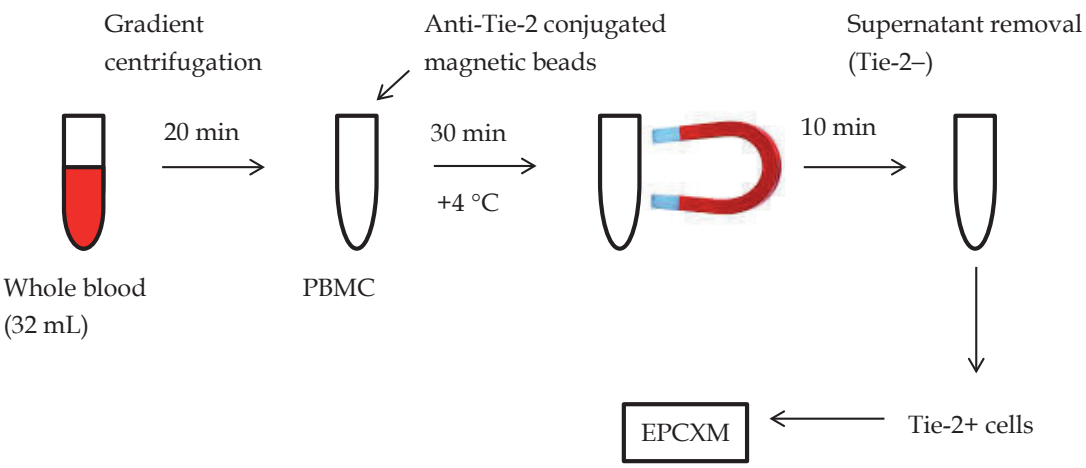

Fig. 3 Isolation of Tie-2+ EPC from peripheral blood using XM-ONE® kit.

This dramatic event was the starting point for the development of routine assay for detection of AECA [98]. The key step in this method is the immuno-magnetic isolation of Tie-2 positive endothelial precursor cells from the peripheral blood of the donor (Fig. 3).

These cells express a panel of endothelial markers such as von Willebrand factor (vWF) and vascular endothelial growth-factor receptors (VEGF R1 and R2) [98]. They also express HLA class I and II antigens which is of relevance for the interpretation of EPCXM outcome as discussed further below. In addition to EC markers, a subpopulation of Tie-2+ cells also expresses the monocytic marker CD14 [121]. The Tie-2+ cells constitute ~ 2% of peripheral blood mononuclear cells (PBMC) and display functional capacity *in vivo* as shown by Nowak *et al* [122]. Although the Tie-2+ cells isolated by the commercial kit, XM-ONE® (AbSorber AB, Stockholm, Sweden), do express several EC markers (but not all) it is still unknown how well they represent the kidney endothelium and whether they display clinically relevant antigens [123]. For instance it is not known whether the Tie-2+ cells can be used for identification of antibodies directed against known EC targets such as MICA, vimentin, collagen V and the angiotensin II type I receptor [124]. Tie-2+ EPC express low levels of MICA and it is unclear whether these levels permit detection of those antibodies [98].

The endothelial crossmatch assay (EPCXM) is performed as described in figure 4a (left panel). The identification of Tie-2+ cells in the flow cytometric assay is based on forward and side scatter gating as shown in figure 5a. Occasionally, the EPC population appears elongated and its position is shifted upwards (Fig 5b). The reason for this cell population shift is not clear. Interestingly, in two patients the shift was undetectable in serum collected after immunoadsorption.

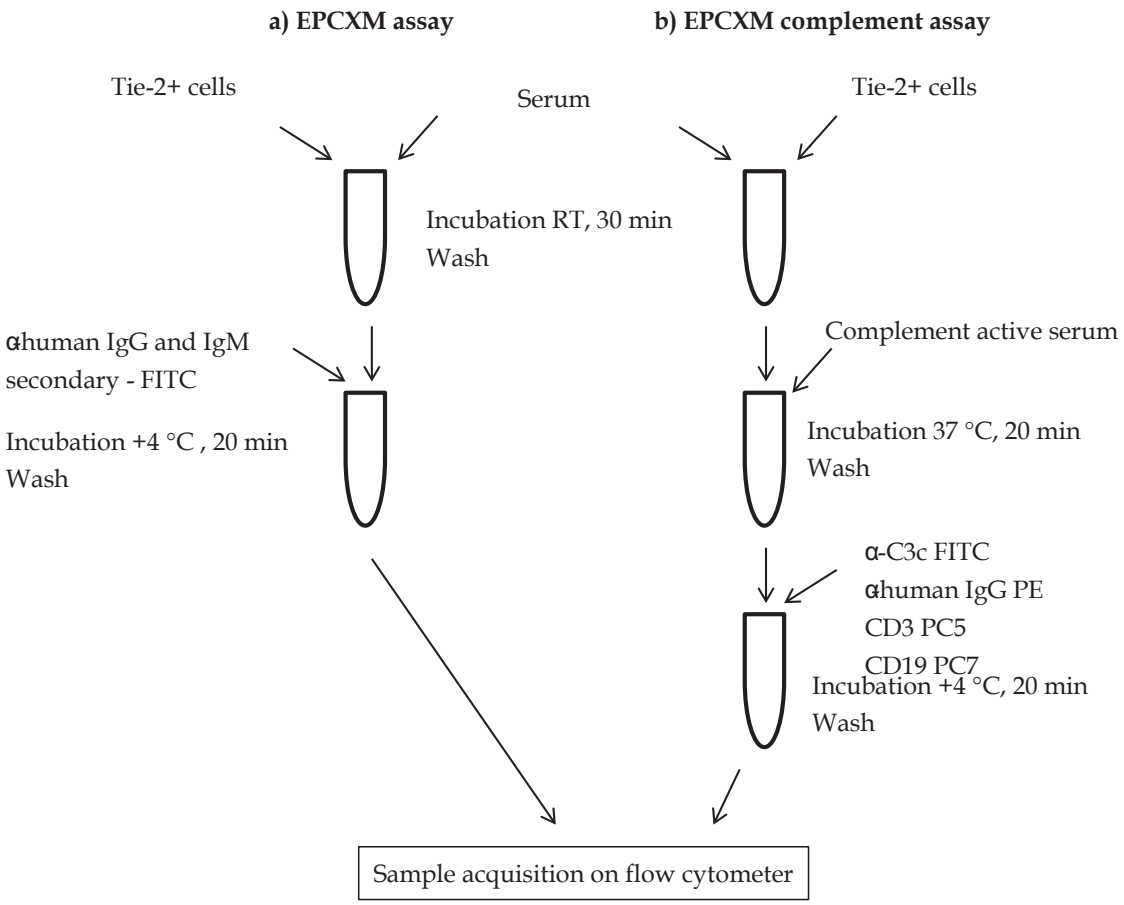

Fig. 4 Schematic description of EPCXM and EPCXM complement assay

Besides the isolated EPCs, a second population of cells with lower FSC and SSC characteristics is co-purified (Fig 5a, indicated with an arrow). These cells are lymphocytes (e.g CD3+ T and CD19+ B cells) as described previously [125]. The frequencies of co-isolated lymphocytes constitute around 10-20% of the isolated cells. Importantly, the Tie-2 antigen is not expressed on lymphocytes [121]. The isolation of lymphocytes is Tie-2 independent and can be reduced by additional washing steps [125]. For some applications, this rather high number of "unwanted" cells obtained

after immuno-magnetic separation can be problematic. However, here in this application it is advantageous since it enables simultaneous crossmatching of both EPCs and lymphocytes [125]. This finding form an important basis for the work described in Paper I as discussed in detail below.

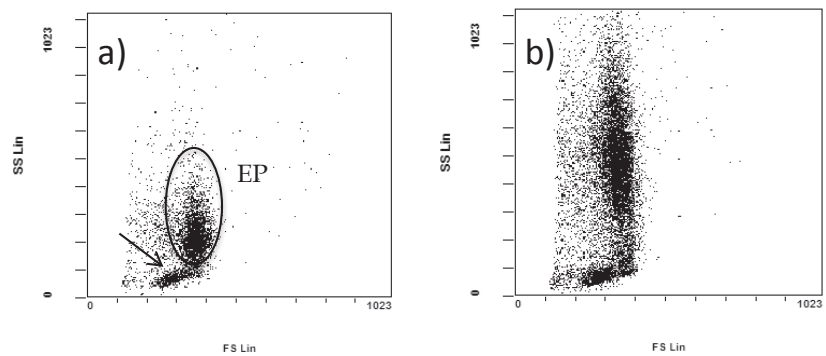

Fig. 5 Identification of EPC by FSC/SSC gating (a) and a shifted EPC population as sometimes seen in the FSC/SSC dot plot (b)

Flow cytometric complement deposition assay

The lymphocytotoxicity assay developed in the mid 1960's has been the assay of choice ever since for detection of complement-fixing HLA antibodies [117]. The assay detects only HLA antibodies with high enough avidity and IgG subclass to induce cytolysis. This type of assay read-out may thus result in lack of detection of less favorable antibodies unable to fully activate lysis. In particular, anti-EPC antibodies, which have been shown to be of predominately IgG2 and IgG4 subclass, may thus be missed [126].

An alternative strategy to identify complement-fixing antibodies besides cell lysis observed under the microscope, is to measure complement fragment deposition by

flow cytometry [127]. In this assay, complement factors (e.g C1, C4 and C3) are deposited (bound) on the cell surface as a result of initial activation by IgG or IgM antibodies. This concept was picked-up by Scornik *et al* who presented exciting data on its potential usefulness in assessment of HLA antibodies in the context of kidney transplantation [128]. Apart from these two publications no follow-up studies have to our knowledge been performed to further optimize this assay.

Inspired by the work of Scornik and colleagues we here describe flow cytometric measurement of complement deposition on cells isolated with the XM-ONE® kit (Fig. 4b). Cells were stained with negative and positive control serum, and test serum for 30 minutes. Thereafter cells were incubated with normal human serum (devoid of HLA antibodies) for 20 minutes at 37 °C as the source of complement. It is important to choose a serum potent enough to induce sufficient levels of complement deposition. We used serum prepared, and stored in aliquots at -70 °C. A new aliquot of frozen serum was thawed for each new experiment. We noticed differences in activity between different serum donors (data not shown). Screening of serum for complement activity can either be performed on a panel of cells from different donors or by measurement of complement activity by ELISA [129]. This step is of considerable importance for assay standardization before implementation as clinical routine test. Another crucial step in the optimization of the assay is the choice of anti-complement binding antibody. As described in more detail in the *Results and Discussion* section below we found that antibodies specific for C3 fragments were the best choice to obtain a sufficient level of staining.

As shown in paper I, 30-50% of lymphocytes (but not EPCs) were found to be non-viable (*i.e.* propidium iodine positive) upon binding to HLA antibodies. Note that gating of T and B cells were based on CD3/SSC and CD19/SSC gating including both

viable and non-viable cells. As suggested by Spiller, cell lysis can be prevented by using complement-active serum lacking the terminal components C5 to C9 [127].

The main obstacles that we experienced with EPCXM complement deposition assay were i) few B cells after Tie-2+ isolation, ii) high complement factor background staining on EPCs and B cells, iii) occasionally undetectable C3 deposition for unknown reason(s) even with positive control serum. We do not know whether the latter occurs due to technical failures, variability among donor cells regarding expression of complement inhibitory factors or lack of the appropriate HLA antigens for complement activation. It is, however, advisable to screen the positive control serum with alternative techniques such as CDC or the Luminex®-based C1q assay [130, 131]. In addition, isolation of cells from HLA typed donors would obviously facilitate result interpretation. None of these "confirmatory" analyses were performed here (paper I).

The EPCXM complement deposition assay enable, as described in paper I, simultaneous detection of IgG binding and complement factor deposition on the cell surface. The IgG binding ratio (MFI test serum/MFI negative serum) was generally high using positive control serum. We noticed though that the signal-to-noise ratio was increased upon heat inactivation of the serum complement (data not shown). The reason for this is not known but one explanation could be that complement proteins (e.g C1) partially block the IgG binding by secondary antibodies. Heat inactivation may abrogate this blockage and enable proper IgG binding. A similar mechanism has been proposed as an explanation for the prozone effect seen in single antigen bead assays [132].

Results and discussion

Paper I

Here in paper I we describe the development of a novel flow cytometric assay for determination of complement fixing and non-fixing antibodies. This crossmatch assay is based on the use of donor Endothelial Precursor Cells (EPCs) isolated by commercially available XM-ONE® kit. Furthermore, T and B cells co-isolated with EPC, as described previously [108] were included as a second and third type of donor cells. Initial experiments on PBMC stained with CD3 and CD19 formed the basis for additional validation of the assay with specific focus on the use of the XM-ONE® kit. The initial experiments with PBMC were promising, and supported previously published data on the deposition of complement on T cells derived from PBMC [128]. Deposition of complement on B cells, which has not been shown previously, was also demonstrated. These experiments show that presence of complement fixing HLA class II antibodies in patient serum could be detected with this methodological approach. However, the level of specific complement deposition was considerably lower than on T cells. There was substantially higher background staining on B cells than on T cells. It means that on B cells there is less difference in signal strength between HLA antibody positive and negative serum samples. High level of IgG background staining on B cells is also well documented in the conventional B cell crossmatch assay [128]. The presence of Fc-receptors on B cells is thought to contribute to this background. Treatment of PBMC, including both T and B cells, with the enzyme pronase can reduce this background considerably. It is not known whether this could be a potential way to circumvent high background staining for

complement factors on B cells. Notably, it has been suggested that treatment with pronase may enhance the level of sensitivity for lysis by reducing complement regulatory proteins [133, 134]. It may also affect the degree of complement factor deposition. Although the use of pronase is beneficial for interpretation of the B cell crossmatch assay there are concerns regarding its effect on other cell surface proteins apart from CD20 and Fc-receptors [128]. HLA expression and/or structure may also be affected by pronase treatment, which could potentially induce incorrect crossmatch interpretation [135].

Next, we addressed whether EPC and co-purified lymphocytes could be used as targets for determination of complement factor deposition. These experiments showed that certain anti-complement factor antibodies worked better than others. For instance the C1q and C4d antibodies did not result in any clear deposition on lymphocytes. Lack of C1q deposition on lymphocytes has previously been shown by Watanabe and Scornik [128]. However, they could demonstrate deposition of C4d. The discrepancies between our results may lie in the choice of complement factor antibodies or other assay differences. In contrast, C3c and C3d antibodies were found to give strong signals on EPC T cells and EPCs, with somewhat lower signal on B cells. The reason for differences in staining levels between C3c/d and C1q (and C4d) may be explained by amplification of C3 signal that occur within the complement pathway through the action of the C3 convertase [127]. It is therefore of importance, as demonstrated here, to put emphasize on the choice of complement factor antibodies, both with regard to clonal origin and specificity, in order to optimize the performance of the assay further.

The deposition of C3 is an early step in the complement pathway which ends in the generation of lytic components, which form pores in the cell membrane and disrupt

cell integrity [127]. Propidium iodine (PI) is one of several markers that can be used for assessment of cell viability [136]. PI efficiently penetrates non-viable cells and bind to DNA. We found that lymphocytes were efficiently stained with PI (alongside with C3c and C3d deposition) whereas no PI+ EPCs were detected (see figure 2D, paper I) despite high levels of C3c and C3d. This suggests that EPCs have the ability to withstand the complement-induced lysis. The mechanism behind this lack of EPC lysis was not analyzed further in this paper. However, it could at least partly be due to high levels of complement inhibitory receptors (*e.g.* CD46 and CD55) acting downstream of C3c/d deposition. Interestingly, human umbilical vein endothelial cells (HUVEC) have been shown to express as much as four times more CD59 than lymphocytes [133]. It could thus be of interest to determine the level of these inhibitory receptors on EPCs and on co-purified lymphocytes in relation to complement deposition. Preliminary data from our group indicate that CD55 (but not CD 46 nor CD59) is expressed at considerably higher levels (~ 4-5 times) on EPC compared to T cells (M. Alheim, unpublished). However, additional experiments need to be performed to verify these initial findings.

It is common practice as part of validation of new assays to compare with currently available techniques. Therefore our next step was to compare the above described flow cytometric complement deposition assay with the conventional complement dependent cytotoxicity assay (CDC). It is important to emphasize, already at this stage, that several differences between CDC and the EPCXM complement assay exists including isolation of targets, kinetics and assay read-out. In addition, the source of complement in these two assays differs considerably.

We determined the outcome of the CDC test vs C3d complement deposition on EPCs as well as on T and B cells co-isolated with EPC in the XM-ONE® test. In a previous

study we had shown that T and B cells isolated with XM-ONE® and T and B cells within bulk PBMC express similar levels of HLA class I and II molecules [125]. Importantly, there was a correlation between lymphocyte XM (LXM) and XM-ONE® LXM. This shows that T and B cells co-purified with XM-ONE® can be used for crossmatching. In this study complement factor deposition (C3d) on EPCXM T cells and CDC T cell (lysis) correlated; the specificity and sensitivity being 69% and 72%, respectively. However, as shown in Table 2 (paper I) discrepant results between T/B CDC crossmatch and EPCXM T and B cells were observed. In 41% of the T and/or B cells CDC+ crossmatches no C3d+ EPCXM T or B cell could be detected. The reason for this high percentage of CDC+/EPCXM- crossmatch tests is not clear but several non-mutually exclusive explanations can be envisioned. First, it is well known that the CDC assay, in particular the B cell CDC, have a tendency to exhibit a high degree of background. We found that in 31% of the CDC+ B cell XMs no C3d deposition or IgG binding was observed. Importantly, the majority of those sera were negative for HLA IgM which may indicate that a large number of false positive B cell CDC crossmatch tests were observed. As discussed above, the signal-to-noise ratio for complement deposition on B cells are low which may cause insufficient levels of sensitivity, particularly evident with low avidity antibodies.

The result from paper I show that both T and B cells co-purified during the isolation of Tie-2+ EPCs can be used as targets for determination of complement deposition on HLA class I and HLA class I+II expressing cells, respectively. More importantly we show that the XM-ONE® kit can potentially be used as an assay for determination of both HLA and non-HLA antibody-induced complement factor deposition. Interestingly, in 30% of CDC T-/B-– crossmatch tests using sera without HLA class I or II antibodies resulted in positive C3d staining on EPC. This finding suggests that

non-HLA antibodies present in kidney patients are able to induce deposition of complement on EPC. However, it would be of interest to formerly verify the presence of non-HLA antibodies in those sera by other techniques. Optimally, one would select a panel of HLA antibody negative sera with EPC reactivity (IgG or/and IgM) and optimize this assay further. In particular it would be of interest to investigate the efficacy of various IgG subclasses (IgG1 to 4) on complement deposition [137]. Jackson *et al* has shown that anti-endothelial antibodies consisted predominately of complement non-fixing IgG2 and IgG4 subclasses [126].

Paper II

As described in previous sections, the EPCXM assay (XM-ONE®) enables the detection of donor reactive HLA and non-HLA antibodies in patients undergoing evaluation for living donor (LD) transplantation. Data from a multicenter clinical trial published in 2011 showed that EPC antibodies as defined by the XM-ONE® pre-transplantation are associated with and increased incidence of early rejections and increased serum creatinine levels at 3 and 6 months post-Tx [108]. The multicenter study was conducted between June 2005 and October 2006 on patients negative for lymphocyte crossmatch tests, both CDC and FCXM (when performed). The Tx-center at Karolinska, Huddinge and Gothenburg (data presented in paper III) participated in that study. Here in paper II we report data from Karolinska Tx-center on the EPCXM performed between February 2007 and December 2009. The data presented in this paper include all patients irrespective of immunization status, both lymphocyte crossmatch negative and positive ones. The patient (n=99) demographics is summarized in Table 1 (paper II) and show that the EPCXM+ and EPCXM– groups were comparable. A few exceptions can be noted though; i) higher frequencies of females were found to be EPCXM+, ii) a higher frequency of EPCXM+ patients received treatments pre-Tx compared to EPCXM– patients, and iii) there were differences in the immunosuppression protocol between the EPCXM groups. All in all 199 EPCXM were performed on 177 patients. Sixty-four out of 199 crossmatches (32%) were found to be EPCXM positive (16/64 IgG+, 35/64 IgM+ and 13/64 IgG+/IgM+). In our study we observed a high frequency (25%) of EPCXM IgM+ crossmatches. In the majority of cases the IgM antibodies were not reactive to HLA. The significance of IgM antibodies in transplantation is not clear. There are reports suggesting that AECA IgM is associated with increased risk of rejection [108].

However, there are also reports unable to find any correlation between presence of IgM and increased incidence of rejection [126]. Notably, autoantibodies, predominantely IgM, have been suggested to play a role in graft rejection [138]. In our study we found that 27% of allo-EPCXM IgM+ were positive also for auto-EPCXM IgM.

Standard lymphocyte crossmatch tests (CDC and FCXM) were performed for the majority of patients that were crossmatch tested with XM-ONE®. Of patients with CDC T−/B− 24% were found to be EPCXM+ (5% IgG+; 15% IgM+; 4% IgG+/IgM+) and of patients with negative T and B cell FCXM, 20% were EPCXM+ (0% IgG+; 14% IgM+; 6% IgG+/IgM+). In the CDC and FCXM negative group of patients, 18% had IgM Abs and 7% both IgG and IgM Abs binding donor EPC. No patient with IgG Ab alone was detected. These results show that in 20-25% of the living donor (LD) patients with negative in LXM had detectable EPC antibodies. In the multicenter study, AECA were found in 24% of patients [108]. Although there may exist differences in the patient inclusion criteria and study design comparable results (22-40%) have been reported by others [126, 139, 140]. Xavier *et al* found that EPCXM+ patients had increased risk of graft rejection. Notably, this study was performed retrospectively on third-party EPC which suggests that EC antigens are broadly expressed and less donor-specific than HLA. However, data from Zitzner et al show that sera reacting against one donor may not necessarily bind to another donor [139].

Panel reactive HLA class I and II antibodies were determined in 125 of the sera used for EPCXM. As summarized in Table 3, paper II, around one-third of the EPCXM+ was found in each sensitization group (non-sensitized, sensitized and highly

sensitized). For sera without EPCXM IgG reactivity the frequencies for these three groups were 76%, 16% and 8%, respectively. There was more sensitized and highly sensitized patients in the EPCXM+ than in the EPCXM– group (Fisher exact test: p=0.0002). This finding was expected since the Tie-2+ EPC isolated with XM-ONE® express HLA class I and II. Notably, the expression levels on Tie-2+ EPC are lower, particularly for HLA class II, than on B cells [125]. EPCXM positive reactions may be due to HLA and/or non-HLA antibodies. Determination of donor specific anti-HLA antibodies in patient sera will obviously give valuable information. Another strategy to discriminate between HLA and non-HLA antibodies using XM-ONE® assay is to include anti-CD3 and CD19 antibodies in the staining protocol as described above in paper I. In case of an EPCXM+ IgM, gating on the co-isolated lymphocyte population may provide some guidance for interpretation of results. If the lymphocyte population proves to be negative it strongly indicates the presence of IgM+ non-HLA antibodies.

In the multicenter clinical trial in which only patients with negative pre-transplant lymphocyte crossmatch tests were included, it was found that EPCXM+ (IgG or IgM) patients experienced an increased risk of acute rejections and increased serum creatinine [108]. In our study described here we did not find any difference in the frequencies of rejections in the EPCXM+ and EPCXM– group of patients. Nor did we observe any significantly differences in serum creatinine levels at 1 or 3 months post-Tx. The reason for this discrepancy is not known. However, a large percentage (58%) of the EPCXM+ patients received pre-treatment that may have affected the incidence of acute rejections. Interestingly, there were more patients with EPCXM+ and pre-treated that experienced delayed graft function as compared to the EPCXM– group. Another important observation was that the EPCXM+ patients with DSA had higher

levels of serum creatinine 1 and 3 month compared to the EPCXM– patients with DSA. This finding did not reach statistical significance and should be interpreted with caution. One interpretation is that presence of non-HLA antibodies together with donor specific HLA antibodies may influence transplantation outcome [141, 142].

Paper III

The transplantation center at Sahlgrenska University Hospital, Gothenburg, Sweden was one of six centers that participated in the multicenter prospective XM-ONE® clinical trial in 2005-2006 [108]. Here in paper III we present 4-year follow-up data from those fifty-three patients (n=53) that were recruited at Sahlgrenska. Twenty-eight of the patients received a kidney from a live donor. The majority of patients (83%) in the multicenter study were transplanted with a living donor kidney. At Sahlgrenska a large part of the patients (47%) were transplanted with a deceased donor. The patients included were all negative in the CDC XM which was the inclusion criteria for all participating centers in the multicenter study. Here we also performed FCXM on 32 out of 53 patients. Twelve percent (12%) were positive in the T cell FCXM and 9% in B cell FCXM.

Seven patients were EPCXM positive (7/53; 13%). Notably, no IgM+ EPCXM were observed. This observation is different from the experience of other centers participating in the multicenter study. As discussed above in paper II, relatively large frequencies (25%) of the patients at Karolinska, Huddinge were EPCXM IgM+. Furthermore, 20% of the patients at Northwestern University in Chicago (also a participant in the multicenter study) were EPCXM IgM+ [139]. Similar data have been reported by Annette Jackson at John Hopkins in Baltimore. The reason for these inter-center differences could be the disease history (e.g. autoimmune disorders) or ethnicity of the patients. The patient group studied here in paper III is small and a bigger patient cohort may clarify this issue.

Although the EPCXM enable detection of donor specific anti-endothelial antibodies the assay design do not allow discrimination between HLA antibodies and non-HLA

as discussed above. Patients recruited to this study were negative in CDC but 3 of 7 EPCXM+ patients (43%) were positive in T cell FCXM suggesting the presence of donor-specific HLA antibodies (for details see Table 1 in Paper III). This interpretation is supported by data showing that a high proportion (57%) of EPCXM+ patients was sensitized. The role of complement non-fixing HLA antibodies in graft survival is still debated [143-145]. Many Tx-centers do not consider those antibodies as an immunological risk factor. Nevertheless, the presence of HLA antibodies in EPCXM+ patients complicates the interpretation of data on graft rejection and function in our study.

In this study 5 out 7 EPCXM+ patients experienced rejection within 3 months. Apart from one "borderline" they were all classified as acute T cell mediated Type IIA rejections. The rejections were diagnosed within one week after Tx. The EPCXM– patients (5/46) rejected after approximately 3 weeks. No rejection occurred in the EPCXM+ patient group beyond 3 months post-Tx. Concordant results have been reported by others [126, 139]. EPCXM+ patients in our study had lower glomerular filtration rate (GF) at 6 months but this statistically significant difference was lost after 1 year and beyond. Serum creatinine levels were increased in the EPCXM+ group at 3 and 6 month suggestive of perturbed kidney function. However, the serum creatinine differences between EPCXM+ and EPCXM– groups were not observed at later time points. This is in line with patient data presented by Jackson et al [126]. The reason for improved survival and kidney function late after Tx is not clear. One reason could be extended or beneficial immunosuppression given to EPCXM+ patients.

Concluding remarks

- Complement factors C3c and C3d, but not C1q nor C4d, were easily detected on EPCs and co-purified lymphocytes following incubation with serum containing HLA antibodies
- The amount of C3c deposition and IgG binding on EPCs and T cells, but not on B cells, correlated
- The specificity and sensitivity for C3d deposition on co-purified T cells *vs* the T CDC assay were 69% and 72%, while for B cells the sensitivity was considerably lower
- In our cohort of patients evaluated for living donor kidney transplantation, 32% had IgG and/or IgM-binding donor EPCs in their pre-Tx sera
- Of the patients with negative lymphocyte crossmatch tests, 25% had EPC Abs mainly of IgM class not reactive with HLA
- The pre-Tx EPCXM positive group had significantly more patients with delayed graft function
- It appears as if differences in serum creatinine and glomerular filtration rates observed between EPCXM positive and negative groups early on after transplantation disappears beyond 6 months post-Tx

Future perspectives

- To validate the clinical significance of the EPCXM adopted for detection of complement-fixing anti-EPC antibodies as a risk assessment tool in kidney transplantation
- To assess the ability of the EPCXM to detect non-HLA antibodies induced post-Tx and the clinical significance of such antibodies
- To further develop the EPCXM such that it allows detection of non-HLA antibodies in the presence of HLA antibodies
- To assess whether there is an overlap in terms of antigens recognized by the EPCXM and known non-HLA, like MICA/B, AT_1R and others
- To identify the antigens recognized by antibodies giving rise to a positive EPCXM
- To develop a solid phase immunoassay with antigens identified as targets for anti-EPC antibodies

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