AALBORG UNIVERSITY

Evaluation of an automated method for viability testing of lymphocytes for crossmatching before kidney transplantation and a retrospective evaluation of transplantation outcome

Master's Thesis | Translational Medicine

Anette Sand Svendsen and Marie Weinreich Petersen

29-05-2015

Medicine with Industrial Specialization

Title:

Evaluation of an automated method for viability testing of lymphocytes for crossmatching before kidney transplantation and a retrospective evaluation of transplantation outcome

Department:

Department of Health Science and Technology

Project period:

1st September 2014 to 29th May 2015

Group members:

Anette Sand Svendsen and Marie Weinreich Petersen

Internal supervisor:

Linda Pilgaard, Associate Professor

External supervisors:

Bjarne Kuno Møller, Head Consultant at Department of Clinical Immunology

My Hanna Sofia Svensson, Staff Specialist, Department of Renal Medicine

External partner:

Aarhus University Hospital

Number of pages:

60

Number of appendices:

6

Acknowledgements:

We would like to express our gratitude to Linda, Bjarne, and My for supervision, and the staff at Department of Clinical Immunology at Aarhus University Hospital for valuable guidance.

English summary

In order to improve transplantation outcome a new method for evaluation of crossmatch would be valuable.

Aim: The present study had three aims. The first aim was to evaluate kidney transplantation outcome in relation to results from tests performed before transplantation. The second aim was to test the magnetic activated cell sorting (MACS) for separating T-and B-cells from isolated peripheral blood mononuclear cells (PBMC) and defrosted spleen. The third aim was to evaluate the Vi-CELL XR Cell Viability Analyzer (Vi-CELL) for analyzing lymphocyte concentration and viability.

Material and methods: Clinical data was gathered from 147 recipients who had been kidney transplanted with deceased donor in the years 2011-2013 at Aarhus University Hospital, Skejby. MACS was used to separate T- and B-cells. Lymphocyte concentration and viability before and after separation were evaluated using flow cytometry. Samples with lymphocytes from defrosted spleen and PBMC were analyzed for lymphocyte concentration and viability with the Vi-CELL and flow cytometry, and the results were compared.

Results: No significant results were obtained when evaluating different tests performed before transplantation and their relation to transplantation outcome ($P > 0.05$). However, the results indicated that presence of human leukocyte antigen-antibodies and donor specific antibodies in the recipient contributes to poor estimated glomerular filtration rate (eGFR) and higher risk of rejections. A difference in lymphocyte viability ≤ 5 PP between before and after separation with MACS was acceptable and seen for two out of three. However, it was not possible to reach an acceptable purification yield (> 90 %) and lymphocyte loss (< 10 %). It was not possible to set the Vi-CELL parameters to measure a lymphocyte concentration ≤ 15 % difference compared to flow cytometry. However, the difference in lymphocyte viability between the Vi-CELL and flow cytometer was acceptable for the majority of the samples (≤ 5 PP).

Conclusion: It was found that kidney transplantation outcome could not be predicted by pre-transplantation tests. When testing the utility of MACS acceptable values were obtained for lymphocyte death, but not for lymphocyte loss and purification yield. Evaluation of the Vi-CELL for analyzing lymphocyte concentration and viability showed that the Vi-CELL was able to measure viability correct, but not lymphocyte concentration compared to flow cytometry.

Dansk resumé

For at forbedre udfaldet af transplantation, vil en ny metode til evaluering af crossmatch være værdifuld.

Mål: Dette studie havde tre mål. Det første var at evaluere udfaldet af nyretransplantation i forhold til resultater fra tests foretaget før transplantationen. Det andet mål var at teste magnetisk aktiveret celle sortering (MACS) til separation af T- og B-celler fra isolerede mononukleare celler fra perifert blod (PBMC) og optøede miltceller. Det tredje mål var at evaluere Vi-CELL XR Cell Viability Analyzerens (Vi-CELL) evne til at analysere lymfocytkoncentration og –viabilitet.

Materialer og metoder: Klinisk data blev indhentet fra 147 recipienter, der havde fået en nyretransplantation med afdød donor i 2011-2013 på Aarhus Universitetshospital, Skejby. T- og B-celler blev separeret med MACS, og lymfocytkoncentration og –viabilitet før og efter separation blev evalueret med flowcytometri. Prøver med lymfocytter fra milt og PBMC blev analyseret for lymfocytkoncentration og –viabilitet med Vi-CELL og resultaterne blev sammenlignet med dem fra flowcytometri.

Resultater: Ingen signifikante resultater blev fundet under evalueringen af tests udført forud for transplantation og deres relation til transplantationsoutcome ($P > 0,05$). Dog indikerede resultaterne, at tilstedeværelsen af humane leukocyt antigen-antistoffer og donorspecifikke antistoffer i recipienten førte til lavere estimeret glomerulær filtrationsrate (eGFR) og højere risiko for afstødning. En forskel i lymfocytviabilitet ≤ 5 PP mellem før og efter separation med MACS var acceptabel og blev fundet i to ud af tre prøver. Dog var det ikke muligt at opnå en acceptabel oprensningskvalitet (> 90 %) og tab af lymfocytter (< 10 %). Det var ikke muligt at indstille Vi-CELL parametrene til at måle ≤ 15 % forskel i lymfocytkoncentration i forhold til flowcytometri. Dog var forskellen i lymfocytviabilitet mellem Vi-CELL og flowcytometri acceptabel for størstedelen af prøverne (≤ 5 PP).

Konklusion: Det blev fastslået, at udfaldet af nyretransplantationer ikke kunne forudsiges af test foretaget før transplantation. Ved test af MACS's anvendelighed blev acceptable værdier opnået for lymfocytdød, men ikke for lymfocyttab og oprensningskvalitet. Evaluering af Vi-CELLen for analyse af lymfocytkoncentration og –viabilitet viste, at Vi-CELLen kunne måle viabilitet korrekt, men ikke lymfocytkoncentration sammenlignet med flowcytometeret.

Table of contents

Introduction

The first kidney transplantation in human was performed in 1954 in Boston, U.S. 10 years later the transplantation was performed in Aarhus, Denmark. Every year around 225 kidney transplantations are being performed in Denmark. In 2013, 2,420 people were living with a donated kidney, and around 500 people were waiting for a new kidney (1).

Although transplantation can be an effective treatment for kidney diseases, the outcome of the kidney transplantation is varying. However, the graft is usually functioning a few days after the transplantation. After one year 90-95 % of the grafts are still functioning, but the degree of function decreases over time, which can result in the recipients being obligated to return to dialysis. The decrease results in total loss of kidney function after 10-15 years for about 50 % of the recipients. Eventually, second kidney transplantation can be the only treatment, but some complications are related to repeated transplantation: The immune system can develop defences against the grafts, which can lead to higher risk of rejection of the second graft, caused by donor specific antibodies (DSA), or cellular rejection initiated by T-cells. In general, several complications are related to kidney transplantations, and especially prevention of rejection is critical for graft function. Therefore it is important to match donor and recipient on different parameters before transplantation (1).

Kidney transplantation

Epidemiology

In Denmark 212[1] kidney transplantations were performed in 2013. Of the 212 transplantations in 2013, 107 were with deceased donor and 105 were with living donor (Table 1) (2).

The percentage of living donor versus deceased donor has been registered by Danish Nephrology Register (DNR) since 2000, and shows an increase in transplantation with living donor. About 50 % of the living donors had a family relation to the recipient in 2013 (2).

[1] 9 transplantations have been excluded for analysis in the Danish Nephrology Register's (DNR) year report from 2013

Table 1: Flow-chart of donor status for kidney transplantation in Denmark year 2013 (2)

2013		
Kidney transplantations (N = 212)		
Deceased donor (N = 107)	**Living donor (N = 105)**	
	Unrelated donor (N = 49)	Related donor (N = 56)
		Parent (N = 28)
		Sibling (N = 19)
		Other (N = 9)

Population

Patients with chronic kidney failure can be considered for kidney transplantation. To be able to be considered for kidney transplantation, the patient has to be thoroughly examined. E.g. the cardiovascular system will be examined, and it will be determined if it is possible to connect the transplanted kidney to the patient's artery. All patients in Denmark who fulfil the criteria for receiving a kidney will be enrolled in the Nordic organisation, Scandiatransplant, which administers waiting lists and possible exchanges of organs between the participating countries (1). In Denmark 2515 patients were in dialysis for end-stage renal disease (ESRD) in 2013, with 634 new ESRD patients registered in that year. The leading causes of ESRD are diabetes and hypertensive diagnoses, but a majority of ESRD are unknown (2). Around 80 % of the patients in chronic dialysis cannot get kidney transplantation, because of comorbidities: Often cardiovascular diseases. Also the patients have to be otherwise physically strong enough to survive the operation and the immunosuppressive treatment (1). There is currently no age-limitation for receiving a new kidney in Denmark, and in 2013 2.8 % of the recipients were 70 years or older (2).

Complications and prognosis

Several complications are associated with kidney transplantation. One of the complications associated with kidney transplantations is the side-effects of the life-long use of immunosuppressive treatment to prevent graft rejection. Unfortunately, the immunosuppressive treatment can be toxic to the recipient and cause nephrotoxicity, hypertension and diabetes among others (3). Another side-effect of the immunosuppressive treatment is immunodeficiency. This leads to an increased risk of infections, especially cytomegalo virus, and a higher risk of cancer (4,5).

One of the more severe complications to kidney transplantation is graft rejection. In Denmark acute rejection is seen for 10-15 % of the recipients within the first months after the transplantation. This is treated with intense immunosuppressive treatment (1). The timeline from the transplantation until the eventual graft rejection occurs influences the overall survival rate of the graft. The longer time before the rejection occurs, the longer the graft will survive (6). The function of the graft will decrease with time, and after one year 5-10 % of the grafts will have lost function entirely, while the function will be lost for around 50 % after 10-15 years. However, it seems that the graft survival has increased over the years, since 1990, especially with deceased donor (Figure 1 and 2) (1,2).

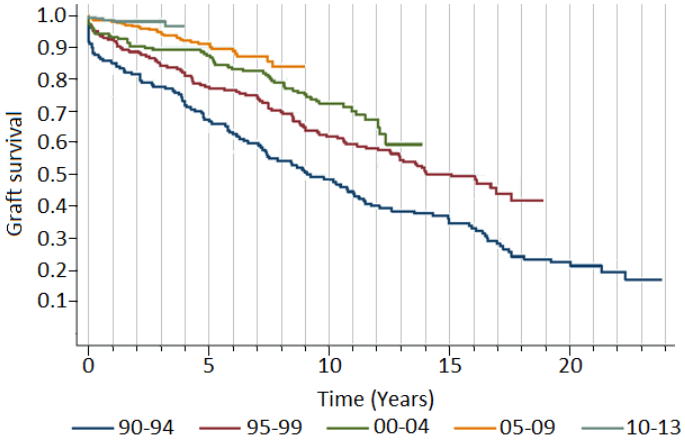

Figure 1: Graft survival in Denmark after kidney transplantations performed in 1990-2013 (living donor). Dark blue line: Graft survival after transplantation performed in 1990-1994. Red line: Graft survival after transplantation performed in 1995-1999. Green line: Graft survival after transplantation performed in 2000-2004. Yellow line: Graft survival after transplantation performed in 2005-2009. Light blue line: Graft survival after transplantation performed in 2010-2013. Data on graft survival where recipient is deceased has been excluded (2)

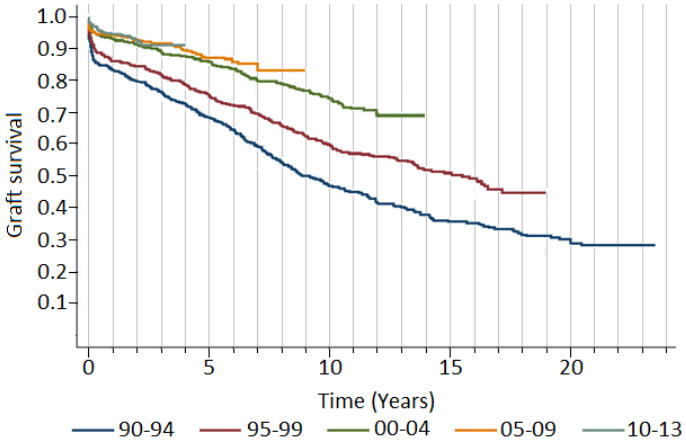

Figure 2: Graft survival in Denmark after kidney transplantations performed in 1990-2013 (deceased donor). Dark blue line: Graft survival after transplantation performed in 1990-1994. Red line: Graft survival after transplantation performed in 1995-1999. Green line: Graft survival after transplantation performed in 2000-2004. Yellow line: Graft survival after transplantation performed in 2005-2009. Light blue line: Graft survival after transplantation performed in 2010-2013. Data on graft survival where recipient is deceased has been excluded (2)

In addition to increasing graft survival it also seems like the patient survival has increased over the years (Figure 3 and 4). For transplantations performed in 1990-94 the patient survival was around 70 % after 10-15 years, and has increased to around 80 % for transplantations performed in 1995-2004 with a living donor. However, the numbers are lower for a deceased donor; around 40-50 % patient survival for transplantation performed in 1990-94 after 10-15 years, and around 60-70 % patient survival for transplantations performed in 1995-04 (2).

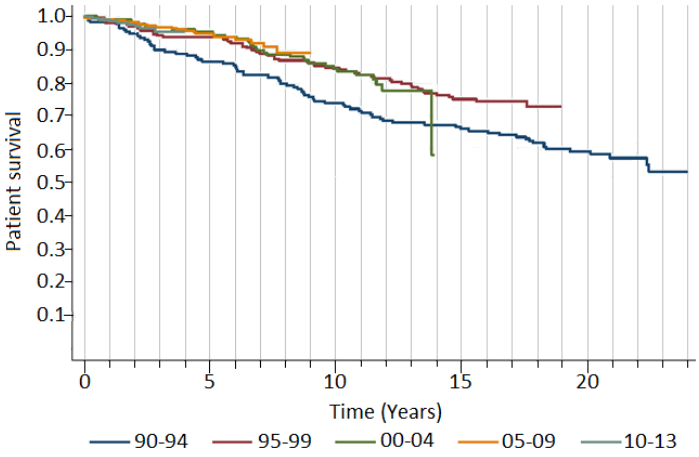

Figure 3: Recipient survival in Denmark after kidney transplantation performed in 1990-2013 (living donor). Dark blue line: Recipient survival after transplantation performed in 1990-1994. Red line: Recipient survival after transplantation performed in 1995-1999. Green line: Recipient survival after transplantation performed in 2000-2004. Yellow line: Recipient survival after transplantation performed in 2005-2009. Light blue line: Recipient survival after transplantation performed in 2010-2013 (2)

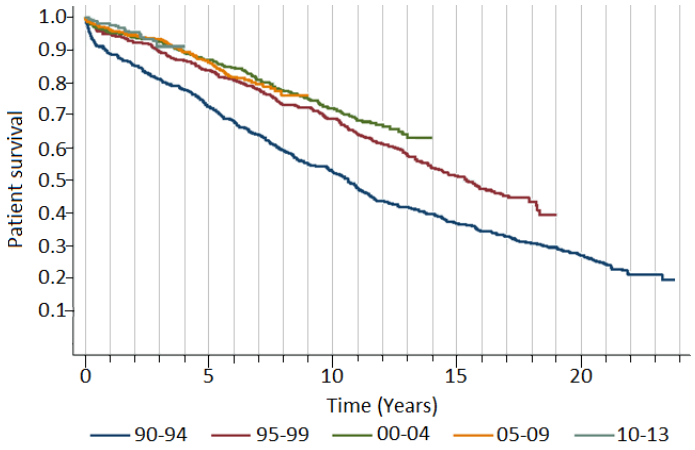

Figure 4: Recipient survival in Denmark after kidney transplantation performed in 1990-2013 (deceased donor). Dark blue line: Recipient survival after transplantation performed in 1990-1994. Red line: Recipient survival after transplantation performed in 1995-1999. Green line: Recipient survival after transplantation performed in 2000-2004. Yellow line: Recipient survival after transplantation performed in 2005-2009. Light blue line: Recipient survival after transplantation performed in 2010-2013 (2)

Lymphocytes – The basis of the adaptive immune system

In the human body the immune system protects against external microorganisms. The immune system can be divided into two parts: The innate and the adaptive immune system. The innate immune system is represented by fagocytes and cells that release inflammatory mediators (7,8). The cellular basis of the human adaptive immune system is lymphocytes. These cells can be divided into two groups: T-cells and B-cells. The cells are divided according to their surface molecules (Cluster of differentiation (CD)) (9).

T-cells

The T-cells arise in the lymphoid tissue in the bone marrow and travel to the thymus, where they mature. During the maturation, they develop T-cell receptors (TCR). This receptor can bind specific antigens in a pocket. Furthermore, the T-cells differentiate into two sub-classes during maturation: T-helper-cells (Th-cells) and T-cytotoxic-cells (Tc-cells). The Th-cells are characterized by expressing CD4 on their surface, and recognize antigens bound and presented by human leukocyte antigen (HLA)-II molecules. The Tc-cells express CD8 on their surface and can only recognize antigens presented by HLA-I molecules. The TCR cannot recognize antigens alone, only when the antigen is bound to HLA molecules (9).

After maturation and differentiation, the T-cells start circulating in the blood as naive T-cells, as they have not met their respective antigen yet. When the T-cells encounter their antigens, they will be activated into mature T-cells (Figure 5). The CD4 on the Th-cells makes a complex with the antigen presented by HLA-II on an antigen presenting cells (APC). Further, the protein called B7 on the surface of the APC binds to the CD28 on the Th-cells, which leads to complete activation of the Th-cells. The Th-cells are then auto-stimulated by secreted IL-2, and develops into lymphoblasts and further into memory- or effector Th-cells (Figure 6). The effector-Th-cells are then developed into two types (Th_1 and Th_2). The effector-Th_1-cells activate Tc-cells at cellular immune response, and activate non-specific cells, e.g. macrophages and NK-cells. The effector-Th_2-cells activate B-cells at humoral immune response (9).

The Tc-cells are activated when their CD8 surface molecule makes a complex with the antigen presented by HLA-I on a target cell. The Tc-cells then express an IL-2 receptor and secretes IL-2, which activates the Tc-cell along with IL-2 secreted from the Th-cells. This leads to complete

activation, and then differentiation to lymphoblasts, which develops into memory-Tc-cells and effector-Tc-cells. The effector-Tc-cells kill cells by inducing apoptosis, in cells that present an antigen through HLA-I (9).

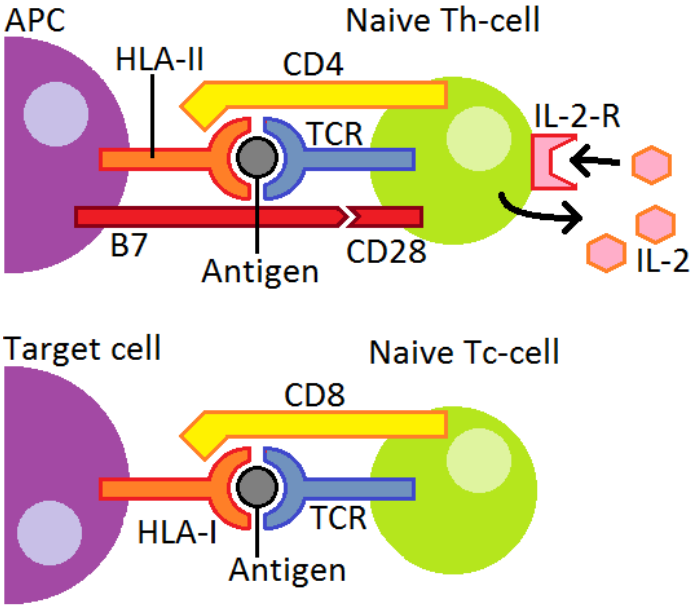

Figure 5: Activation of T-cells. The naive Th-cell is activated through interaction with antigen presenting cells (APC). The HLA-II from the APC binds antigen, which is presented to the T-cell receptor (TCR) on the Th-cell. The CD4 on the Th-cell binds to the HLA-antigen-TCR complex, and the CD28 on the Th-cell binds to the B7 on the APC. This leads to autostimulation with IL-2, which leads to full activation of the Th-cell. The naive Tc-cell is activated through interaction with target cells (any cell that presents the antigen through HLA-I). The HLA-I from the target cell binds antigen, which is presented to the TCR on the Tc-cell. The CD8 on the Tc-cell binds to the HLA-antigen-TCR complex. This leads to full activation of the Tc-cell (9)

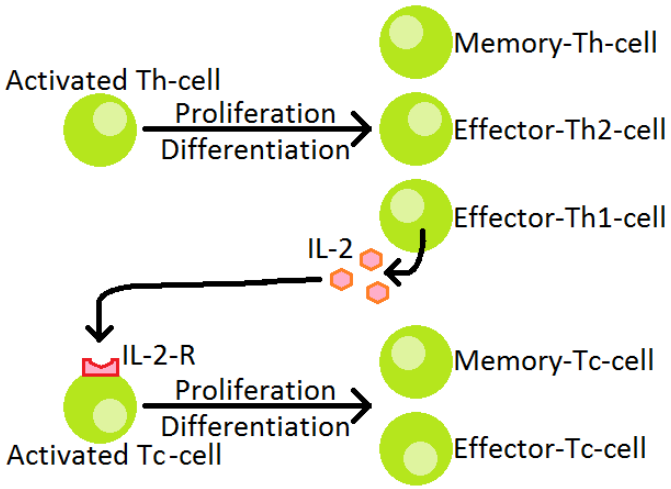

Figure 6: Proliferation and differentiation of T-cells. The activated Th-cell proliferates and differentiates to memory-Th-cell and the two types of effector-Th-cells (Th1 and Th2). The effector-Th1-cell secretes IL-2, which stimulates the activated Tc-cell through the IL-2-receptor (IL-2-R). The Tc-cell then proliferates and differentiates to memory-Tc-cell and effector-Tc-cell (9)

B-cells

The B-cells arise from the bone marrow, just as for the T-cells. However, they remain in the bone marrow, where their maturation takes place. The B-cells develop antigen specific B-cell receptors (BCR). The BCR can react with an antigen without presentation through an HLA molecule. The B-cells function as APC, as they express HLA-II on their surface and can present antigens to e.g. T-cells. The immature B-cells in the bone marrow have to undergo selection before entering the blood. The immature B-cells express IgM, but if the cells survive the selection, they leave the bone marrow and express both IgM and IgD (9).

The B-cells get activated when they interact with an antigen (Figure 7). The B-cells encounter an antigen, which leads to two IgG molecules cross-binding the antigen. Then two reactions happen in the B-cells. First, the antigen will be consumed by the B-cells by endocytosis, and then it will be presented through the HLA-II-molecules. This leads to the second reaction where the B-cells express B7 (which is necessary for activation of Th-cells). To achieve full activation, the B-cells need to be activated by activated Th_2-cells. Hereafter, the B-cells will differentiate into memory-B-cells or plasmablasts (9).

The memory-B-cells express all classes of immunoglobulins and several adhesion molecules. The plasmablasts differentiate into plasmacells, which secretes antibodies (9).

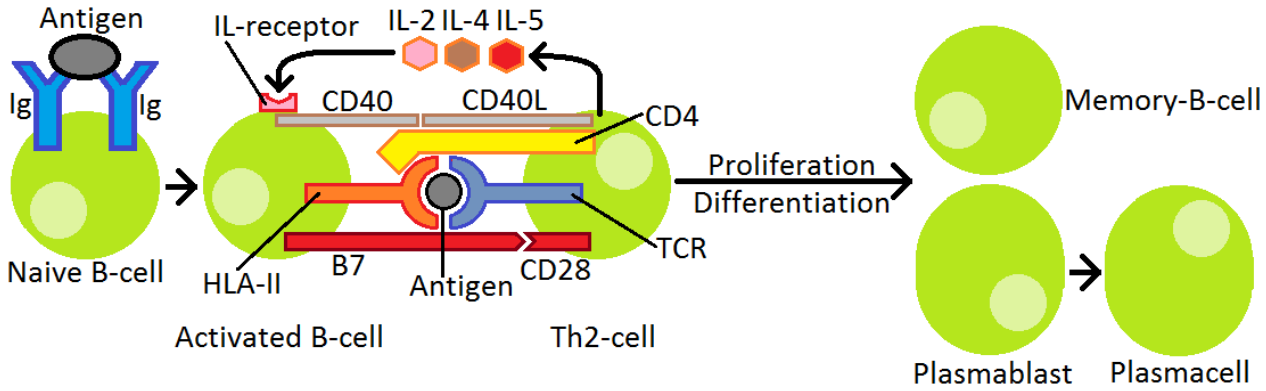

Figure 7: Activation, proliferation and differentiation of B-cells. The immunoglobulin (Ig) molecules on the naïve B-cell cross-bind an antigen. The antigen is then presented through an HLA-II on the activated B-cell to a T-cell receptor (TCR) on the Th_2-cell. The CD4 on the Th-cell binds to the HLA-antigen-TCR complex, and the B7 on the B-cell binds to the CD28 on the Th_2-cell. The CD40 on the B-cell binds to the CD40L on the Th_2-cell. This leads to secretion of IL-2, IL-4, and IL-5 from the Th_2-cell, which binds to the IL-receptor on the B-cell. This leads to proliferation and differentiation of the B-cell into memory-B-cell and plasmablast, which further differentiates into plasma cells (9)

Transplant immunology

As previously mentioned it is the immune system that causes rejection of the graft after transplantation. Even though both the innate and the adaptive immune system participate in graft rejection, it is especially the adaptive immune system that initiates rejection and therefore especially B- and T-cells contribute to rejection (7,8,10,11).

Allorecognition

A graft transplanted between individuals of the same species is called allograft and is the most common type of transplantation (8). Allorecognition is the recognition of donor-derived antigens by the recipient's immune system, after allograft transplantation. The antigens that can activate an immune response are often HLA. The allorecognition can be both humoral (antibody mediated) and cellular (T-cell mediated). Both kinds of allorecognition can potentially cause rejection of the graft and they often act together, e.g. by initiating each other (8,11,12).

Cellular rejection

As already mentioned, cellular rejection is caused by differences in HLA between donor and recipient. If the HLA between donor and recipient are mismatched, the recipient's T-cells will acknowledge the donor-HLA-I and -HLA-II molecules on the graft cells as foreign, and this will initiate an immune response against the graft. It is often the tubules and arterial endothelium in the graft that will be infiltrated by the T-cells (7,8,13).

Cellular rejection can occur through different pathways: The direct pathway where the T-cells recognize donor-HLA molecules present on the surface of the donor cells, or the indirect pathway where the T-cells recognize donor-HLA molecules presented as peptides by self-HLA molecules (11,12,14).

The direct pathway of cellular rejection

In order for T-cells to recognize HLA directly on the donor cells, the donor cells have to migrate from the graft to connect directly with T-cells in the lymph nodes. One theory which explains how this happens is the passenger leukocyte theory (Figure 8). Here, the immature donor dendritic cells (DC) migrate to the blood of the recipient. They express donor-HLA and migrate to the lymph

nodes where the recipient T-cells are located. Here the DCs activate the T-cells, which now can recognize the donor-HLA. The activated T-cells migrate to and infiltrate the graft, and then recognize the donor-HLA directly on the graft parenchyma. However, over time the APC on the surface of the graft deplete, and the allorecognition continue by the indirect pathway (11,12). The direct pathway has been shown to be important in acute rejection (14).

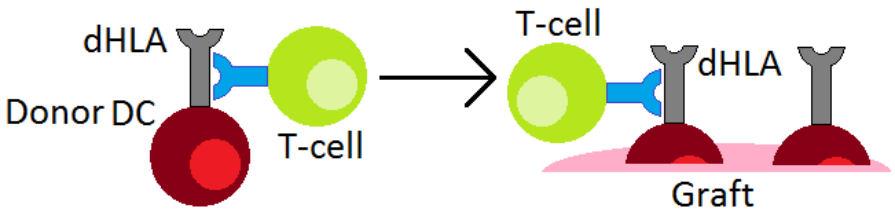

Figure 8: Allorecognition – direct pathway. Donor dendritic cell (DC) encounters the T-cell in the blood, and the T-cell can now recognize dHLA on the graft and initiate rejection (11,12)

The indirect pathway of cellular rejection

The indirect pathway represents the way most foreign antigens activates the T-cells, and has been shown to be important in chronic rejection (11,14). The indirect pathway has three different mechanisms of antigen presentation to T-cells (Figure 9). The first mechanism is that donor-HLA from the graft are released into the blood and absorbed by recipient-DC. The second mechanism is that donor cells migrate to secondary lymphoid tissues and are absorbed by recipient-DC. The third mechanism is that recipient-DC migrate into the graft where they collect donor-HLA and then migrate to secondary lymphoid tissues (11).

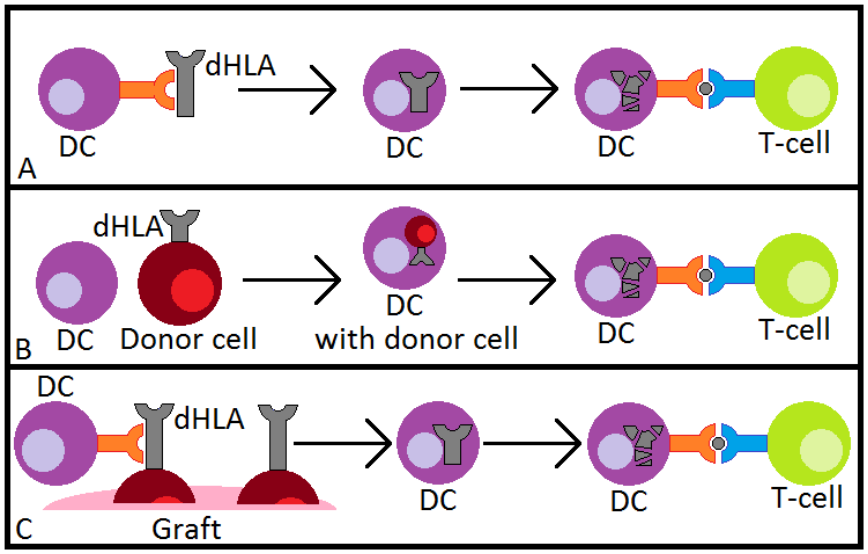

Figure 9: Allorecognition – indirect pathways. A: The recipient dendritic cell (DC) encounters donor-HLA (dHLA) in the blood, where it engulfs the dHLA. The recipient-DC presents the dHLA as peptides in the lymph node, where the T-cells are. B: Donor cells migrate to secondary lymphoid tissue and are engulfed by recipient-DC. The recipient-DC presents the dHLA as peptides in the lymph node, where the T-cell is. C: The recipient-DC migrates into the graft and engulfs dHLA. The recipient-DC presents the dHLA as peptides in the lymph node, where the T-cells are (11,12)

Humoral rejection

Many studies have focused on the cellular rejection but it is clear that humoral rejection also plays a part in all types of graft rejection (10,13). These types are classified according to the clinical symptoms (13). As earlier mentioned, the biggest risk factor for humoral rejection is immunization of the recipient to donor-HLA. The source of this immunization can be previous transplantations and pregnancy. Preformed DSAs in the recipient play a major role in this immunization (7,10).

Hyperacute humoral rejection is caused by preformed DSA. It is clinically characterized by vascular thrombosis and immediate graftectomy is required in order to secure the survival of the patient. This type of rejection is histological characterized by hemorrhagic necrosis, tubular injury, fibrin deposition, and thrombosis. Also immunoglobulin G (IgG) antibodies have been shown to appear in the glomerular and peritubular capillaries (10,13).

Acute humoral rejection is characterized by necrosis, microangiopathy, and arterial inflammation. This type also presents with a deposition of C4d in the peritubular capillaries (13).

DSA can also cause chronic humoral rejection which has several characteristics: Arterial intimal fibrosis, duplication of the glomerular basement membranes in glomeruli, and lamination of the peritubular basement membrane (10,15). Patients suffering from the chronic form of humoral

rejection appear with circulating HLA-I and -HLA-II antibodies which causes glomerulopathy and arteriopathy in the graft. However, the mechanisms of chronic humoral rejection are still unclear (13).

Recipient-donor compatibility

Clinical relevance of HLA-matching

Matching of HLA between donor and recipient is an important factor in preventing graft rejection after transplantation. Especially mismatch between A, B, and DR loci should be tested before considering transplantation. If there are a total match in all HLA-I and HLA-II loci (not just A, B, and DR) between a recipient and a donor, the recipient cannot develop DSA. If there are HLA mismatches between the recipient and donor, there are risk of a worse transplantation outcome (14,16).

When there are HLA mismatches between recipient and donor, there is a risk of the recipient developing DSA. Furthermore, more HLA mismatches in a recipient, lead to more possibilities of developing DSA (17). If the recipient has DSA the risk of rejection is greater. The recipient can have pre-formed DSA, which are developed before transplantation, and can be caused by e.g. previously transplantations, blood transfusions, or pregnancy. The recipient can also develop DSA after the transplantation, which is caused by antigens from the graft (18). HLA matching is often done by determining DNA and amino acid sequences, e.g. with polymerase chain reaction (16). The presence of DSA in the recipient can be tested with several crossmatch methods as described below (19).

Crossmatch tests

Crossmatches make it possible to identify the presence of DSA in a potential recipient.

In the complement dependent cytotoxicity crossmatch (CDC-XM) donor lymphocytes are divided into T- and B-cells (10). These are mixed with serum from the recipient and complement (Figure 10). If DSA are present in the recipient's serum, they will bind to HLA on the donor-lymphocytes: HLA-I present on both B-and T-cells and HLA-II present on B-cells. If the DSA are complement dependent, the donor cells undergo complement-mediated death (8,19).

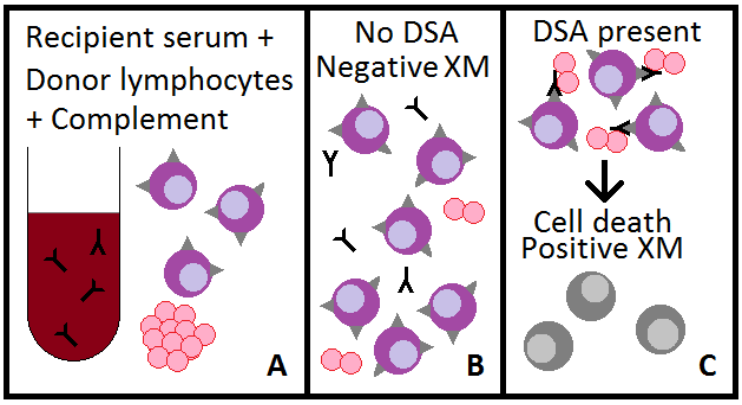

Figure 10: Complement-dependent cytotoxicity crossmatch (CDC-XM). A: Recipient serum, donor lymphocytes and complement is mixed. B: If there are no donor specific antibodies (DSA) in the recipient serum the crossmatch (XM) is negative. C: If there are DSA present in the recipient serum, the DSA will initiate a complement-dependent cell death and the XM is positive (19)

After the crossmatch, the cells are stained, e.g. with trypan blue, and the dead cells will stand out (8,19). The result of the test is determined by manual cell counting through microscopy where the percentage of dead cells is calculated. The bigger percentage of dead cells, the more positive the test is (19).

A positive T-cell crossmatch always contradicts transplantation, but a positive B-cell crossmatch does not (19). To determine whether the cytotoxic effects in the test really are from the binding of DSA in the recipient's serum, positive and negative controls should be included in the test (8). Also a test with dithiothreitol (DTT) should be made. The DTT test investigates if the donor cells are dead from IgM antibodies and not by DSA, since the IgM is considered not having any important role in rejection after transplantation. If the result with DTT is negative, then the crossmatch is considered negative (19).

To achieve a more precise crossmatch result flow cytometry can be used for evaluation. This is called a flow cytometric crossmatch (FC-XM) (11). The FC-XM is performed in the same manner as CDC-XM by incubating recipient serum with donor lymphocytes. Fluorescence labelled antibodies are added after incubation (Figure 11). Fluorescence labelled antibodies are targeted against DSA. If DSA is present in the recipient serum, the DSA will bind to the donor lymphocytes, which allows for the fluorescence labelled antibodies to bind indirectly to the lymphocytes. When the sample is analyzed with flow cytometry, the fluorescence will be measured and the amount of fluorescence is a direct measure of the amount of DSA bound to donor lymphocytes (8,19).

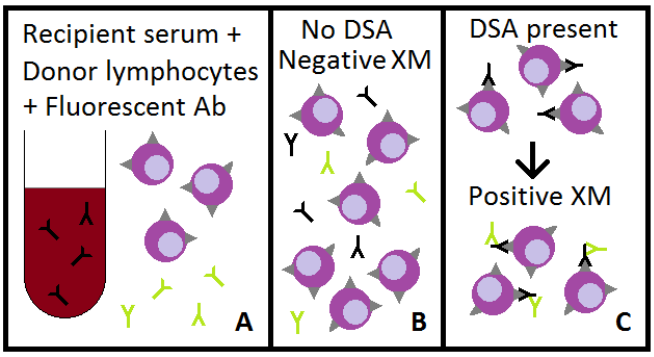

Figure 11: Flow cytometric crossmatch (FC-XM). A: Recipient serum, donor lymphocytes and fluorescent antibodies (Ab) are mixed. B: If there are no donor specific antibodies (DSA) in the recipient serum the crossmatch (XM) is negative. C: If there are DSA present in the recipient serum, the DSA will bind to the donor lymphocytes and the fluorescent Ab bind to DSA: The XM is positive (19)

Antibody screening with use of Luminex

The Luminex method investigates the presence of HLA-antibodies in the recipient serum using synthetic microbeads coated with specific HLA. The microbeads are incubated with serum from the recipient (Figure 12). Reporter dye is added along with a detector antibody. If the recipient serum contains HLA-antibodies, they will bind to the antigens on the microbeads. Then the detection antibody can bind to the HLA-antibodies and the reporter dye will emit fluorenscence. Hence, the result can be read by flow cytometry. The more fluorescent the sample is, the more DSA are present in the sample. If the test is positive for HLA-antibodies, they can be compared to the HLA identity from a potential donor and by virtual crossmatching determine the presence of DSA (19).

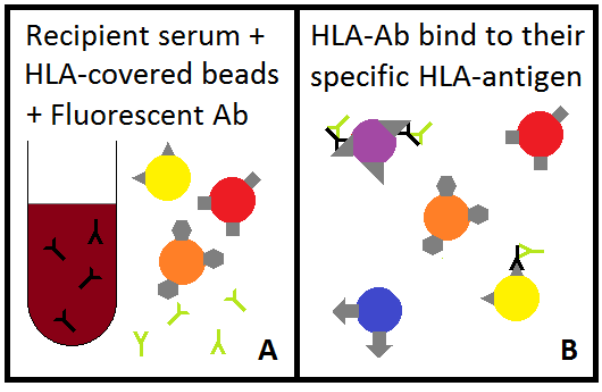

Figure 12: Detection of HLA-antibodies by Luminex. A: Recipient serum, HLA-covered microbeads and fluorescent antibodies (Ab) are mixed. B: If there are HLA-antibodies in the recipient serum they will bind to the specific HLA-antigen on the beads, and the fluorescent antibodies will bind to the HLA-antibodies. the HLA-antibodies in the recipient serum can be identified with flow cytometry (19)

The standard crossmatch is the CDC-XM, which is performed before every transplantation. If the T-cell CDC-XM is positive, the transplantation will not be performed with that recipient-donor match, because this greatly reduces graft survival, function, and maybe recipient survival. Therefore a crossmatch with high predictive value is important, as a false negative crossmatch can lead to transplantation with very poor outcome, and furthermore repeated transplantation. There are several factors to be considered with repeated transplantation: Resources; both organs and financial and the risk of developing HLA-antibodies, including DSA. The change in immune status may lead to a repeated transplantation not being possible, as the immune system will attack the graft (20). Another factor that can be improved from a highly predictive crossmatch is the need for immunosuppressive treatment. If the crossmatch strongly indicates that the graft and recipient are highly compatible, it may lower the need for treatment, which will ultimately lead to fewer side-effects. Therefore a highly predictive crossmatch giving as few false results as possible will be of great value (8).

Methodological theory

Preparation of lymphocytes

Isolation of human peripheral blood mononuclear cells

Using lymphoprep for isolation of peripheral blood mononuclear cells (PBMC) is a widely used method. The method takes advantage of the fact that monocytes and lymphocytes have a lower density than erythrocytes and granulocytes. Most of PBMC have a density below 1077 g/mL and therefore they can be isolated by centrifugation on a medium with a density of 1077 g/mL, e.g. lymphoprep. Lymphoprep consists of sodium diatrizoate, an ionidated density gradient media, and polysaccharide. The polysaccharide contributes to the density of the medium and it increases the sedimentation of the erythrocytes (21).

When using lymphoprep, blood is diluted 1:1 in saline before placing it on the lymphoprep. This is done in order to obtain the maximum yield. During centrifugation the erythrocytes and granulocytes travel through the lymphoprep medium, displacing this upwards. Hence, the plasma and the PBMC will lie on top of the lymphoprep (Figure 13). In this way the PBMC can easily be aspirated from the lymphoprep surface (21).

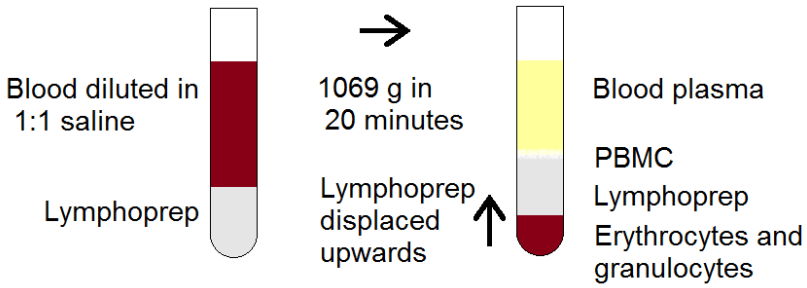

Figure 13: Isolation of peripheral blood mononuclear cells (PBMC). Before centrifugation the diluted blood is placed on the lymphoprep. After centrifugation with 1069 g in 20 minutes the erythrocytes and granulocytes are aggregated to the bottom, and the PBMC are between the lymphoprep and the blood plasma (22)

Magnetic activated cell sorting

Magnetic activated cell sorting (MACS) can separate cells using magnetic microbeads that consist of ferritin coated with specific antibodies. The cells are incubated with the microbeads, and are ready for separation after this one step (Figure 14) (23). The cells will be separated in a MACS-column, which is a tube containing a matrix with magnetic spheres. The MACS-column will be placed in a MACS-separator, which acts as the magnet. The magnetic force from the separator is

amplified by the magnetic spheres in the column. When the labelled (target) and unlabelled cells flow through the column the target cells will remain in the column, due to the magnetic forces, whereas the unlabelled cells will flow through the column. Afterwards the column can be removed from the separator, and the target cells can be eluted from the column, as the magnetic force is now much smaller (23,24).

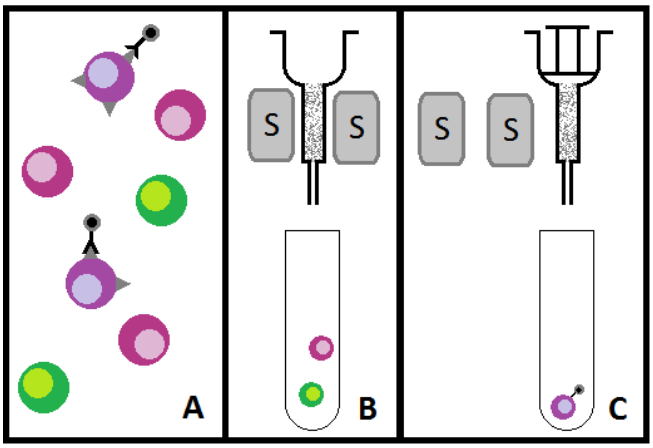

Figure 14: Magnetic activated cell sorting (MACS). A: Magnetic labelling of target cells with microbeads (black and grey) bound to antibodies which bind to target antigens. B: The column is placed in the separator (S) and the unlabelled cells are washed through the column and target cells remain due to magnetic force from the separator. C: Target cells are eluted after removal of the column from the separator (23)

Assessment of cell viability

Cell viability analysis with Vi-CELL XR Cell Viability Analyzer

The Vi-CELL XR Cell Viability Analyzer (Vi-CELL) can be used to determine cell concentration and viability. The cells are loaded into the Vi-CELL, where they are stained with trypan blue. The trypan blue and the cells are mixed several times, a parameter that can be determined before loading cells into the Vi-CELL. The trypan blue dye penetrates the membrane of dead cells, which makes the dead cells dark compared to viable cells. This gives basis for a distinction between dead and viable cells (25). After staining with trypan blue, the Vi-CELL uses a video imaging system to record the flow through of cells. The number of images can be set beforehand. The Vi-CELL then determines the cell concentration and viability depending on different parameters, which can be adjusted after flow through of the cells (25).

The parameters are: Diameter (minimum and maximum), cell brightness, cell sharpness, viable cell spot brightness, viable cell spot area, minimum circularity, and decluster degree. The diameter

determines which events on the images that should be considered cells. Cell brightness describes when an event is considered a cell according to how dark or how light the event is at the boundary compared to the background of the image (Figure 15). Cell sharpness determines how blurry a cell boundary can be, to be counted a cell. Viable cell spot brightness describes how bright the cell has to be inside. The viable cell spot area describes the size of the bright area in the cell. Both parameters affects when a cell is considered viable. Minimum circularity only affects the considered non-viable cells, and the events will only be counted as cells if they have certain circularity. The decluster degree is set to determine how well a cluster of cells can be counted as individual cells. All these parameters allows for re-analysis after the run-through of the sample (25,26).

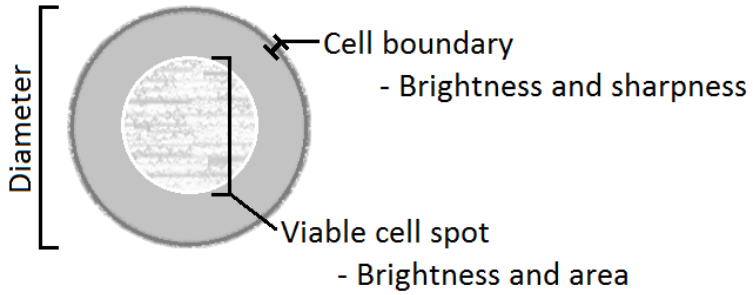

Figure 15: Vi-CELL XR Cell Viability Analyzer parameters. Diameter defines cell size limits. Cell boundary brightness and sharpness determines when an event is defined as a cell. Viable cell spot brightness and area determines when an event is defined as a viable or dead cell (25)

Flow cytometry

Flow cytometry is a sensitive and widely used method for analyzing cells. The cells will run through the cytometer, which can detect the cells, based on their size, granularity and eventual labelling (27).

The cells have to be in a suspension before entering the cytometer. The cell suspension enters the cytometer in a buffer-solution through a tube, which ends inside a flow chamber and releases the cell suspension into a stream of fluid (sheath fluid). This centres the cell suspension and allows for a small diameter of cell suspension to pass by the laser. If the cell suspension has a low sample pressure, the diameter will be smaller which will allow for only one cell to pass the laser at a time. If the pressure is high, more cells can pass the laser. This gives a faster reading time, but also a risk of an imprecise result (27,28).

When a cell passes the laser it will scatter the light. If the cells are labelled the fluorophore will absorb light of a specific wavelength from the laser and emit light at a different wavelength. The light scatter and the emitted light can then be detected, and this data can then be used to analyze the cell population (27). The scattered light will be detected by two sensors. One is placed at the opposite site of the laser and detects forward scattered light (FSC). The other sensor is placed at a 90˚ angle from the laser and detects side scattered light (SSC). The FSC describes the cell size, whereas the SSC describes granularity. The detectors used for FSC and SSC detect light at the same wavelength as the wavelength of the laser (27,28). The data from the sensors are shown on a plot, where each data point represents one event, which is usually one cell, depending on sample pressure. The difference in colour means that several events are plotted in the same coordinates: the warmer the colour the more events (Figure 16) (27).

The target cells can be labelled with a fluorescent molecule (fluorophore) bound to a specific antibody, e.g. anti-CD45, which binds to CD45 present on the surface of lymphocytes (Figure 16). Different lasers have to be used to excite different fluorophores and different fluorophores emit light at different wavelengths (Table 2) (27,28).

Table 2: Summary of flurophores, where excitation and emission is given as maximum wavelength

Flurophore	Excitation (nm)	Emission (nm)	Colour emission
Flourescein isothiocyanat	494	519	Green
Phycoerythrin	546	578	Green-yellow
Allophycocyanin	650	660	Red
7- aminoactinmyocin	482	678	Red

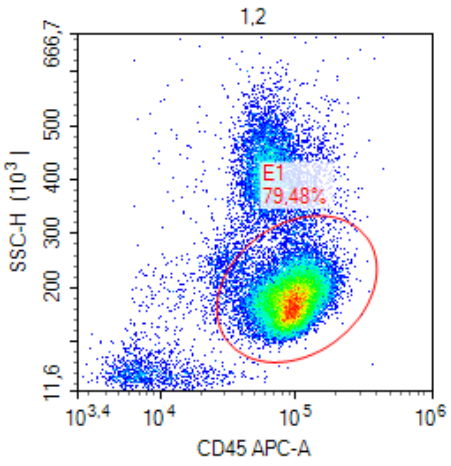

Figure 16: Plot from the flow cytometer. The horizontal axis shows intensity of allophycocyanin (APC) anti-CD45 bound to CD45. The vertical axis shows intensity of side scattered light (SSC). The red circle is gated around lymphocytes, indicating that 79.48 % of the cells in the cell suspension are lymphocytes

After a plot is made, a specific area can be analyzed further, by making a gate; e.g. a circle around a group of cells. The gate can now be made into different plots, which can show different parameters, than the first plot. E.g. the possible lymphocytes on a CD45-SSC-plot can be gated, and the next plot can show CD3 and CD19 to determine which of the lymphocytes are T-cells, and which are B-cells (Figure 17) (27).

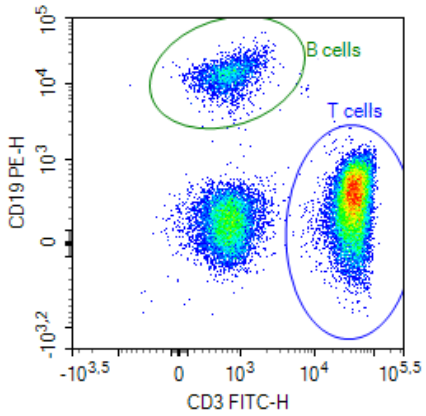

Figure 17: Plot from the flow cytometer made from gate in figure 16. The horizontal axis shows intensity of fluorescein isothiocyanat (FITC) anti-CD3 bound to CD3. The vertical axis shows intensity of phycoerythrin (PE) anti-CD19 bound to CD19. The green circle is gated around B-cells and the blue circle is gated around T-cells

Data obtained from the fluorophores can be presented in several ways, but is often presented graphically with the marker intensity on the horizontal axis and either cell count (single-colour flow analysis) or intensity of another marker on the vertical axis (Figure 18) (27). A commonly used viability marker is 7-aminoactinmyocin D (7-AAD) which penetrates the membrane of dead cells and binds to the guanine-cytosine region of its DNA (29).

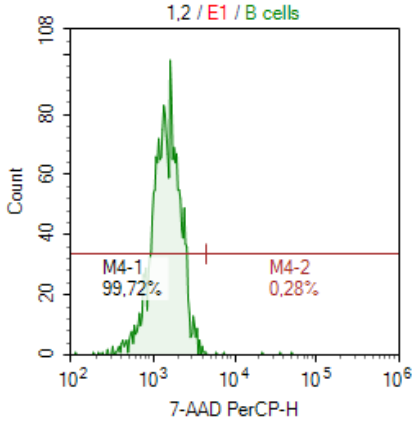

Figure 18: Plot from the flow cytometer made from gate in figure 17. The horizontal axis shows intensity of 7-aminoactinmyocin D. The vertical axis shows cell count. The limit between viable and dead cells are defined by 7-AAD intensity and indicates 99.72 % viable B-cells

Aim and objectives

Aim

The first aim of the study was to evaluate kidney transplantation outcome to corresponding tests performed before transplantation with deceased donor.

The second aim of the study was to test the utility of MACS for separating T- and B-cells from isolated PMBC and defrosted spleen.

The third aim was to evaluate the Vi-CELL for analyzing lymphocyte concentration and viability.

Objectives

The first objective was to investigate results from tests performed before transplantation (CDC-XM, FC-

XM, HLA-mismatch, HLA-antibodies, and DSA) in relation to transplantation outcome measured in number of rejections, eGRF 3 months (eGFR 3M) post transplantation, and eGRF one year (eGFR 1Y) post transplantation.

The second objective was to test the utility of MACS by testing lymphocyte concentration and viability before and after separation and calculating lymphocyte loss and purification yield. A decrease in viability from before to after separation in the positive target lymphocyte fraction should be ≤ 5 percentage point. The lymphocyte loss from separation should be ≤ 10 %. The purification yield should be > 90 % after separation. The lymphocyte concentration and viability was evaluated with flow cytometry before and after separation.

The third objective was to test whether the Vi-CELL was able to read the T- and B-cells when attached to the microbeads used in MACS. The same homogenous sample was tested with both Vi-CELL and flow cytometry.

The fourth objective was to test the capability of the Vi-CELL to measure the correct lymphocyte concentration and viability in a solution, by changing the Vi-CELL parameters. A difference in lymphocyte concentration ≤ 15 % between flow cytometer and Vi-CELL was acceptable. A difference in viability ≤ 5 percentage points was acceptable. The same homogenous sample was tested with both Vi-CELL and flow cytometry.

Methodological considerations

Isolation of donor lymphocytes by MACS

At Aarhus University Hospital, Skejby, lymphocyte isolation has been done with Dynal beads, which are bigger than the microbeads. It has been attempted to evaluate the lymphocyte concentration and viability after separation with the Vi-CELL. These attempts have not been successful, since the Vi-CELL could not distinguish Dynal beads from dead cells. Therefore a new method for cell separation is necessary, if the Vi-CELL should be used to evaluate the CDC-XM. With the new method, the donor lymphocytes will be isolated with magnetic microbeads and a MACS-column. After isolation of the T- and B-cells with MACS, an investigation has to be made in order to conclude that the relevant lymphocytes are present. Furthermore, it has to be evaluated if MACS isolates a high percentage of the T- and B-cells. The lymphocyte suspension before and after (negative and positive fractions) separation will be analyzed by flow cytometry. The loss of lymphocytes should be ≤ 10 %.

The lymphocyte viability is suspected to be affected by the movement through the MACS-column. The lymphocyte viability will be analyzed by flow cytometry before and after the separation. This will determine a possible decrease in viability caused solely by the separation. The decrease in viability should be ≤ 5 PP.

The purification yield in the positive fraction should be high (> 90 %) in order to ensure that the target lymphocytes are isolated to be used in later crossmatching.

Vi-CELL

The Vi-CELL could be a new method for determining crossmatch results. The Vi-CELL uses the same method for evaluation as is standard today (CDC-XM). However, the Vi-CELL provides an objective method for distinguishing dead and viable cells, based on trypan blue staining. The CDC-XM is cytotoxic but not quantitative, whereas the Vi-CELL is non-cytotoxic but quantitative. Therefore a new procedure which combines these two methods and provides an objective result would be valuable.

The Vi-CELL has different parameters which decide lymphocyte concentration and viability in a sample. To ensure that the results obtained from the Vi-CELL are valid they will be compared to the results from the flow cytometer. The parameters described in the theory section have to be

set to fit the lymphocyte characteristics. The results from the flow cytometer will be used as a guideline to match the lymphocyte concentration and viability in order to achieve the most optimal setting for each parameter. Previous investigations performed on location have shown that magnetic beads in a cell suspension can influence the Vi-CELL analysis, if the beads are the same size as dead cells. As small microbeads (< 1 µm) are used in this study, it is believed that they do not influence the Vi-CELL analysis. Since magnetic beads do not affect the flow cytometry analysis, the results from the two analyses can be compared and determine if the microbeads influence the Vi-CELL analysis.

To allow for methodological and technological variations between flow cytometry and the Vi-CELL, a difference in lymphocyte concentration ≤ 15 % is acceptable. Since the viability determines the result of the crossmatch it is the most important result from the Vi-CELL analysis. Therefore a difference in viability has to be minimal, and is set for ≤ 5 PP between the two methods.

Materials and methods

The project was approved by the Local Ethics Committee (Central Denmark Region) and the Danish Data Protection Agency, journal number: 1-16-02-689-14.

For list of materials confer Appendix 1.

Clinical data

All recipients that were kidney transplanted with deceased donor in 2011 to 2013 at Aarhus University Hospital, Skejby, were included in this study. Data was gathered on location through the Electronic Patient Journal (EPJ), Hospital System software, and laboratory journals. Basic recipient information, results from tests performed before transplantation, and transplantation outcome was collected (Table 3).

Table 3: Clinical data obtained from recipients that were kidney transplanted with deceased donor in 2011 to 2013 at Aarhus University Hospital, Skejby. TX = Transplantation, CDC-XM = Complement dependent cytotoxicity crossmatch, HLA = Human leukocyte antigen, FC-XM = Flow cytometry crossmatch, DSA = Donor specific antibodies, eGFR 3M and 1Y = estimated glomerular filtration rate obtained 3 months and one year after transplantation, Banff = Classification for assessment of kidney allograft biopsies

Clinical data	Specification
Age at TX	Years
Sex	Male or female
Previous TX	Number of TX and organ
Diabetes at TX	Present or not
Original disease	Type of disease
CDC-XM	Positive or negative for T-cells and/or B-cells
HLA-mismatch	Number of HLA-I mismatches:number of HLA-II mismatches
HLA-antibodies	Positive or negative for HLA-I and/or HLA-II antibodies
FC-XM	Positive or negative for T-cells and/or B-cells
DSA at TX	Name of DSA present
Basis biopsy	Histology of the TX kidney
Standard immunosuppressant	Tacrolimus, non-steroid, or cyclosporine
eGFR 3M and 1Y	eGFR in ml/min
Biopsies without rejection	Given in days after rejection
Biopsies with rejection	Given in days after rejection, classified according to Banff (or described otherwise), treatment

Preparation of lymphocytes

In the present study lymphocytes from spleen and whole blood were used.

Lymphocyte defrost

The lymphocytes were from a spleen from a deceased kidney donor in the autumn 2014. The lymphocytes were stored in a –80 ˚C freezer. The lymphocytes were defrosted in a 37 ˚C bath, centrifuged and resuspended in balanced salt solution (BSS) twice (Appendix 2).

Isolation of PBMC

The blood was diluted 1:1 with a mixture of BSS and heparin (Appendix 3). Diluted blood was then placed carefully on top of the lymphoprep (Blood-lymphoprep ratio 2:1). The samples were centrifuged at 1069 g for 20 minutes. After centrifugation the interphase was aspirated and resuspended in BSS/heparin-mix. The samples were centrifuged at 500 g for 10 minutes. The pellet was resuspended in MACS-buffer, which consisted of 0.5 % inactivated human serum, 2 mM ethylenediaminetetraacetic acid (EDTA) and BSS.

Magnetic separation of lymphocytes

The magnetic separation was done according to manufacture protocol (Appendix 4). In brief anti-human-CD3 microbeads and anti-human-CD19 microbeads were used to isolate T- and B-cells respectively. The separation of lymphocytes was tested with three samples to investigate lymphocyte viability, loss, and purification yield. Lymphocyte concentration and viability was tested with flow cytometry before and after separation. The lymphocytes used were both defrosted spleen lymphocytes and PBMC.

Assessment of cell concentration and cell viability

Flow cytometry

Each sample was stained with 3 µL anti-CD3 [FITC], 3 µL anti-CD19 [PE], 1.5 µL anti-CD45 [APC], and 0.5 µL7-AAD and incubated in darkness at room temperature for minimum 30 minutes.

Fluorescence-activated cell sorting (FACS) fluid was added to each sample and run through the NovoCyte Flow cytometer (Acea Biosciences, Inc., USA).

The parameters on the flow cytometer were set as follows: The stop condition was set at 50 µL, the flow rate was set at Medium, the FSC-H threshold was set at 100,000, and the APC threshold was set at 4,000.

Vi-CELL

A lymphocyte suspension of 500 - 2000 µL was loaded into a sample cup which was placed in the Vi-CELL carousel. To start analysis the button "Start queque" was pressed. The Vi-CELL was tested with nine samples to determine the parameter settings for which the Vi-CELL results for lymphocyte concentration and viability matched the results from flow cytometry. Furthermore, it was noted if the Vi-CELL could distinguish between dead lymphocytes and microbeads.

Dilution series: Spleen lymphocytes and PMBC

A dilution series was made by mixing the spleen lymphocytes with the PBMC (Figure 19). This gave a dilution series with 100 %, 75 %, 50 %, 25 %, and 0 % PBMC diluted in 0 %, 25 %, 50 %, 75 %, and 100 % spleen lymphocytes respectively. This was done to investigate the Vi-CELL's ability to measure viability, as the defrosted spleen cells had lower viability than the fresh PBMC.

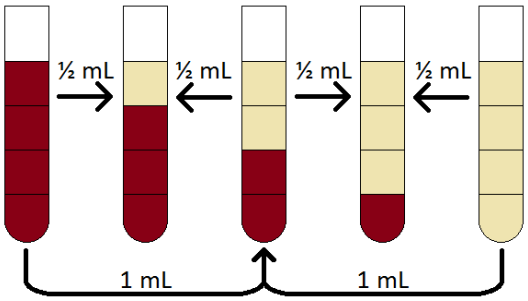

Figure 19: Dilution series with peripheral blood mononuclear cells (PBMC) (dark red) and defrosted lymphocytes from spleen (beige). 1 mL PBMC and 1 mL spleen lymphocytes were mixed (50/50 %). 0.5 mL 50/50 % solution was mixed with 0.5 mL PBMC (75/25 %) and 0.5 mL spleen lymphocyte (25/75 %)

Dilution series: CH_2O-treated cells and non-CH_2O-treated cells

One part of a PBMC sample was resuspended in 100 µL 4 % formaldehyde (CH_2O) in phosphate buffered saline (PBS) and 2.4 mL MACS-buffer and incubated for 30 minutes at room temperature. A dilution series was made by mixing the CH_2O-treated cells with the non-CH_2O-treated cells (0 %, 20 %, 40 %, 60 %, 80 % and 100 %). In a second dilution series the same set up was used, however, the CH_2O-treated cells were incubated in pure 4 % CH_2O in PBS (500 µL) for 10 minutes.

Data analysis

For data analysis IBM SPSS Statistics 22 was used. All metric data was tested for normality. When data was normal distributed two-sample T-test or two-way ANOVA were performed. When data was not normal distributed Mann-Whitney U-test or Kruskal-Wallis test were performed. Graphs and figures were made in Microsoft Excel 2007 – 2010.

Graphs in section "Clinical results, Number of rejections in comparison to tests performed before transplantation" were made from the mean number of rejections occurring for each group, and was calculated from the following equation:

$$Mean_{No.rejections} = \frac{\sum(x\ rejections\ \cdot n_{x\ rejections})}{N}$$

Number of lymphocytes in cell suspensions was calculated as

$$No.of\ lymphocytes = Lymphocyte\ concentration\ \cdot Sample\ volumen$$

Loss of lymphocytes (lymph) during preparation and separation was calculated as

$$Loss\ of\ lymph = \frac{No.of\ lymph_{Pre-sep} - No.of\ lymph_{Positive} - No.of\ lymph_{Negative}}{No.of\ lymph._{Pre-sep}} \cdot 100\ \%$$

Pre-separation (pre-sep), positive, and negative indicates separation fraction.

Purification yield of sample was calculated as

$$Purification\ yield = \frac{No.of\ target\ lymph_{Positive}}{No.of\ target\ lymph_{Positive} + No.of\ nontarget\ lymph_{Positive}} \cdot 100\ \%$$

Positive indicates the positive separation fraction. Target lymphocytes (lymph) are e.g. B-cells in the B-cell fraction and non-target cells are e.g. T-cells in the B-cell fraction.

Clinical results

Study population

In this study 147 recipients were included (Appendix 5). The recipients had been transplanted at Aarhus University Hospital, Skejby, in 2011 to 2013 with deceased donor. 57 of the recipients were female (≈ 40 %) and 90 of the recipients were male (≈ 60%). Mean age at time of transplantation was 52.7 years for females and 52.3 years for males. Age at time of transplantation ranged from 16 to 74 years (Figure 20). 50 % of the patients were between 45 and 62 years at time of transplantation.

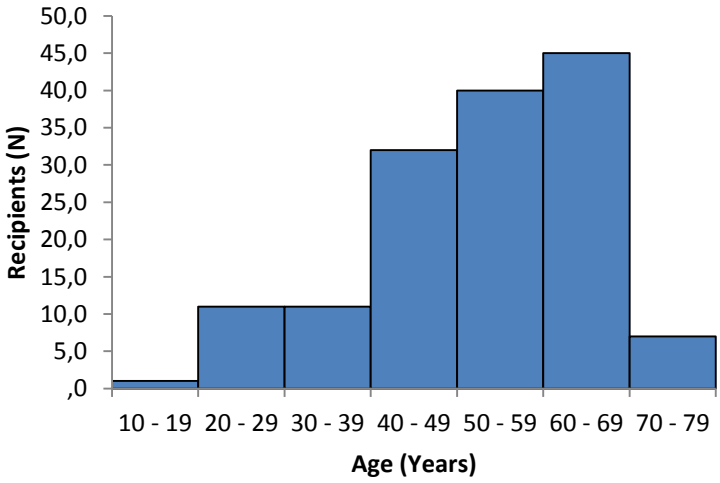

Figure 20: Age frequency of recipients at time of transplantation

Of the 147 recipients 26 (17.7 %) had diabetes at time of transplantation.

The recipients' basis diseases were divided into six categories: Unknown, cystic, other, glomerulonephritis, diabetes, and hypertension. The most frequent basis diseases are unknown and cystic kidney diseases (Figure 21).

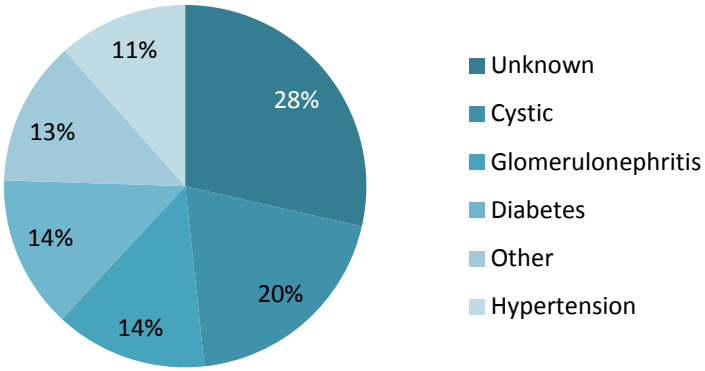

Figure 21: Basis disease frequency of recipients. Unknown: Renal failure, nephropathy, nephrotic syndrome, and unknown; Cystic: Polycystic kidney disease, medullary cystic kidney, and cystic kidney; Glomerulonephritis: Glomerulonephritis, anti-glomerular basement membrane antibody-disease, IgA-nephritis, and Schönlein-Henochs purpura; Other: Contracted kidney, nephrectomy, urinary tract infection, congenital small kidney, lithium nephropathy, congenital myelomeningocele, congenital valvulae urethrae, kidney stones, vesicoureteral reflux, AA amyloidose, damage from calcineurin inhibitor, sepsis, reflux nephropathy, acute tubule-interstitial nephropathy, and obstructive nephropathy; Diabetes: Diabetic nephropathy; Hypertension : Hypertension and glomerulosclerosis

In total 120 (81.6 %) of the recipients had not been transplanted previously, 25 (16.9 %) recipients had one to three kidney transplantations previously, and 2 (1.4 %) recipients had transplantations with other organs.

To investigate the immunization status of the recipients, several tests can be performed prior to transplantation: CDC-XM, FC-XM, HLA-mismatch, and detection of HLA-antibodies and DSA. The CDC-XM has been performed for all 147 recipients and all recipients except one had a negative B-cell-CDC-XM. FC-XM was performed for 19 recipients (Table 4).

Table 4: Results from flow cytometric complement-dependent cytotoxicity crossmatch (FC-XM) according to number of recipients. T and B indicates FC-XM results from T- and B-cells, which can be positive (+) or negative (-)

FC-XM	Recipients (N)
$T^- B^-$	7
$T^- B^+$	5
$T^+ B^-$	1
$T^+ B^+$	6

HLA-mismatch was gathered for 114 recipients, where 110 had a HLA-A or -B mismatch, 93 had a HLA-DR mismatch, and 4 had a zero-HLA-mismatch.

HLA-antibodies were investigated for 111 recipients (Table 5). DSA was present in 26 of them.

Table 5: Results from human leukocyte antigen (HLA)-antibody detection according to number of recipients. HLA-I and HLA-II indicates HLA-I antibodies and HLA-II antibodies, which can be present (+) or absent (-) in recipient serum

HLA-antibodies	Recipients (N)
HLA-I$^-$ HLA-II$^-$	75
HLA-I$^+$ HLA-II$^-$	14
HLA-I$^-$ HLA-II$^+$	8
HLA-I$^+$ HLA-II$^+$	14

Rejection of the graft was seen for 42 (28.6 %) of the 147 recipients and 21 had rejection more than once. The maximum number of rejections observed in one recipient was six.

Transplantation outcome across tests performed before transplantation

To investigate the outcome of the transplantation two parameters were used: eGFR and number of rejections.

eGFR in comparison to pre-transplantation tests

eGFR values were obtained from 121 patients for both eGFR 3M and eGFR 1Y. Data was checked for normality ($P > 0.05$). It was investigated if eGFR varied between different results from pre-transplantation tests: CDC-XM, FC-XM, HLA-mismatch, presence of HLA-antibodies, and presence of DSA. No significant difference was found for any of the tests between groups for both eGFR 3M and 1Y ($P > 0.05$) which indicated that the outcomes of the pre-transplantation tests were not a prediction for graft function. However, a tendency was seen for HLA-antibodies, indicating that eGFR decreases with increasing number of HLA-antibodies (Figure 22).

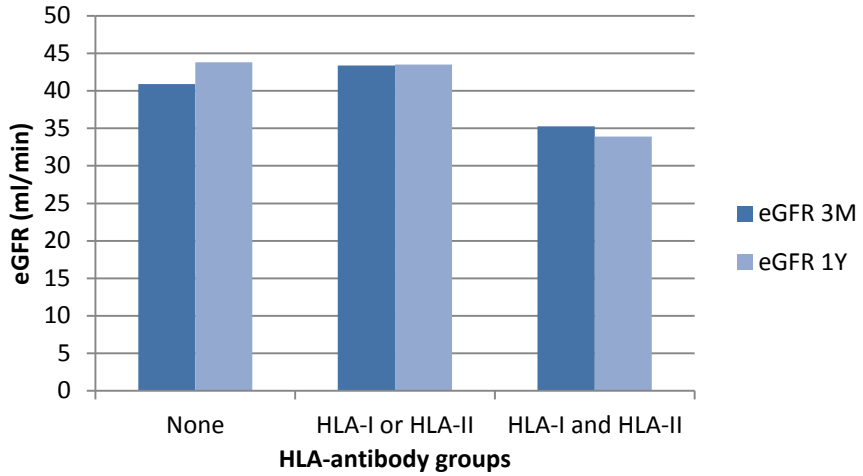

Figure 22: Mean estimated glomerular filtration rate (eGFR) measured in ml/min across HLA-antibody groups. eGFR was measured 3 months (eGFR 3M) and 1 year (eGFR 1Y) after kidney transplantation. None = No HLA-antibodies present in recipient serum; HLA-I or HLA-II = Either HLA-I or -II antibodies present in recipient serum; HLA-I and HLA-II = Both HLA-I and -II antibodies present in recipient serum

The same tendency was seen for DSA, hence, increasing number of DSA lead to lower eGFR (Figure 23).

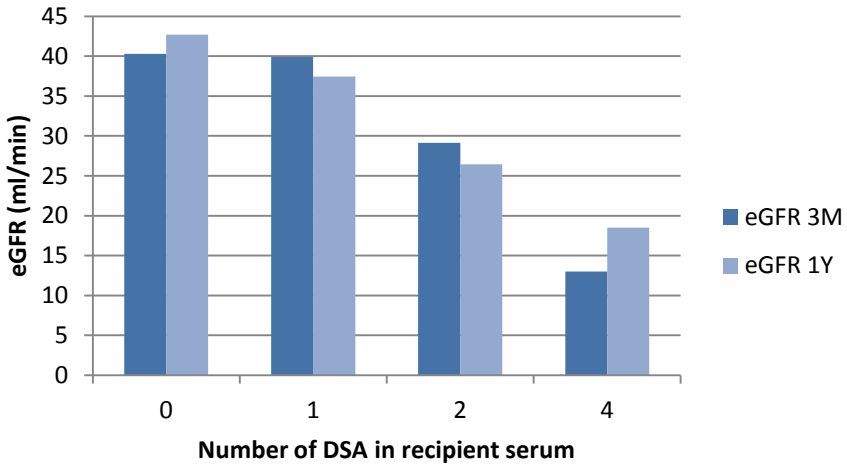

Figure 23: Mean estimated glomerular filtration rate (eGFR) measured in ml/min across number of donor specific antibodies (DSA) in recipient serum. eGFR was measured 3 months (eGFR 3M) and 1 year (eGFR 1Y) after kidney transplantation.

It was further investigated if eGFR was different between the two crossmatch groups, CDC-XM and FC-XM. The groups were defined as following: The result from the CDC-XM was the same as the FC-XM, or the CDC-XM results were better than the FC-XM results. The best result for a crossmatch was a negative crossmatch for both T- and B-cells, and the worst result was a positive crossmatch for both T- and B-cells. The results were ranked as following: T-, B- → T-, B+ → T+, B-

→ T+, B+. No significant difference was found (P > 0.05), indicating that the graft function did not worsen with a poorer crossmatch result.

Number of rejections in comparison to tests performed before transplantation

Number of rejections was obtained for all 147 patients. Data was checked for normality (P < 0.05). It was investigated if number of rejections varied between different results from pre-transplantation tests: CDC-XM, FC-XM, HLA-mismatch, presence of HLA-antibodies, and presence of DSA. No significant difference was found for any of the tests between groups (P > 0.05), which indicated that the outcome of the pre-transplantation tests were not a prediction for graft survival. However, as for the eGFR a tendency was seen for HLA-antibodies and DSA, indicating that the increasing number of HLA-antibodies and DSA lead to higher risk of rejection (Figure 24 and 25). As for the eGFR it was investigated if number of rejections varied between CDC-XM compared to FC-XM groups, and no significant difference was found (P > 0.05). This indicated that number of rejections did not increase with poorer crossmatch result.

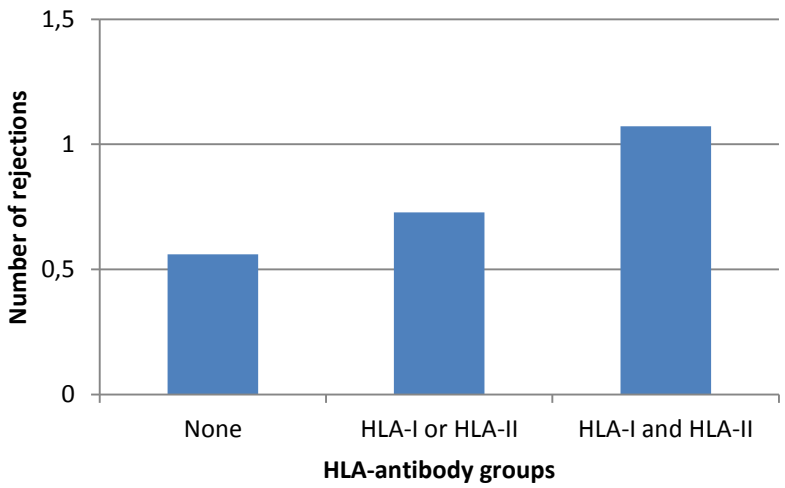

Figure 24: Mean number of rejections across HLA-antibody groups. None = No HLA-antibodies present in recipient serum; HLA-I or HLA-II = Either HLA-I or -II antibodies present in recipient serum; HLA-I and HLA-II = Both HLA-I and -II antibodies present in recipient serum

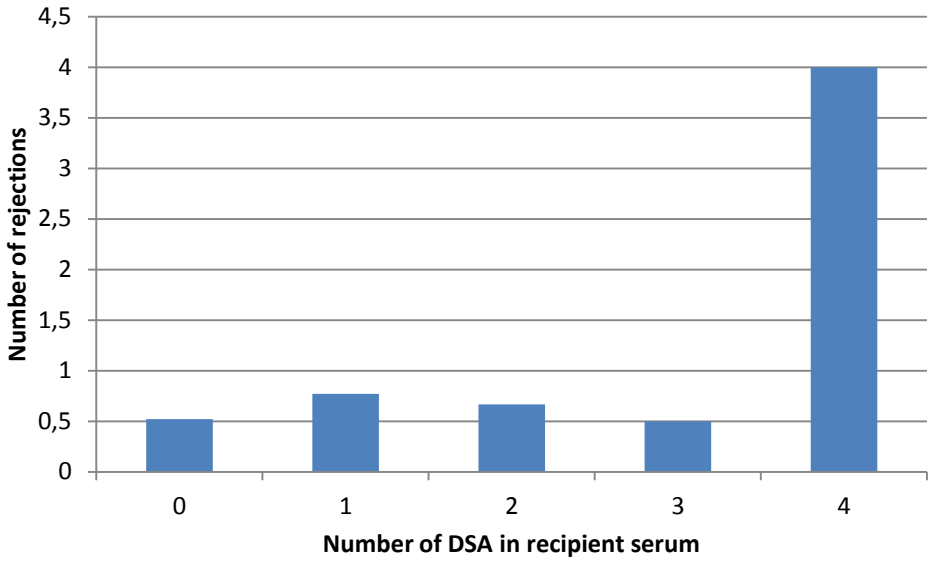

Figure 25: Mean number of rejections across number of donor specific antibodies (DSA) in recipient serum

Experimental results

Lymphocyte viability, loss, and purification yield from MACS

Lymphocyte viability was measured before and after separation (Table 6). A decrease in viability before and after separation smaller or equal to 5 PP or an increase was acceptable, which was seen for the T-cell fraction in two samples and B-cell fractions in all three samples.

Table 6: Results from MACS separations. Results are obtained from three independent samples (A, B, and C) divided into target B- and T-cell fractions. Viability was measured before separation, and after separation in both the negative and positive fraction, for both B- and T-cells in all samples and target cell type fractions. ΔViability target cells = Difference in viability for the target cell type before separation and the positive fraction; PP = Percentage points; + = Increase in viability; - = Decrease in viability; PB = Peripheral blood

Target cell type fraction	T-cell A	B-cell A	T-cell B	B-cell B	T-cell C	B-cell C
Cell source	Spleen	Spleen	PB	PB	PB	PB
ΔViability target cells (PP)	- 18.07	+ 14.23	- 0.31	+ 8.11	- 0.11	+ 8.17
Target cell loss (%)	88.1	92.5	49.79	41.29	23.41	40.88
Purification yield for target cells (%)	49.3	99.3	99.42	86.75	99.38	89.26

Lymphocyte loss decreased for both T-cells and B-cells across samples, but did not reach an acceptable level (< 10 %). A purification yield of > 90 % was obtained for 50 % of the sample fractions. In conclusion, the results from MACS, besides difference in viability, did not fulfill the objectives.

Vi-CELL parameter setting

It was noted that the microbeads did not appear on the Vi-CELL software images and therefore it was concluded that the microbeads did not influence on the Vi-CELL results. Two samples were used to set the Vi-CELL parameters. The Vi-CELL parameters were changed during several reanalyses (Table 8 and 9). A few parameters were changed for each reanalysis. Lymphocyte concentration and viability obtained from the Vi-CELL were compared to those given by the flow cytometer and images from the Vi-CELL video imaging system were observed and evaluated for each reanalysis. The starting point was the Chinese Hamster Ovary (CHO) cell type. With this setting the Vi-CELL results differed from the flow cytometer with 50.72 % for lymphocyte concentration (Flow: 1700 cells/µL) and 0.2 PP for lymphocyte viability (Flow: 98.80 %) (Table 7).

Table 7: Parameter settings with the Chinese Hamster Ovary (CHO) cell type from the Vi-CELL XR Cell Viability Analyzer

Parameter	Value
Minimum diameter (µm)	6
Maximum diameter (µm)	50
Dilution factor	1
Cell brightness (%)	85
Cell sharpness	100
Viable cell spot brightness (%)	75
Viable cell spot area (%)	5
Minimum circularity	0,6999
Decluster degree	Medium

Table 8: Change of Vi-CELL XR Cell Viability Analyzer parameters during the first test. The parameter settings for the Chinese Hamster Ovary cell type was the starting point and is given in reanalysis 1. Yellow highlights indicate which parameter was changed for each reanalysis. The results for each reanalysis are given as cell concentration and viability

Reanalysis	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Minimum diameter (µm)	6	6	6	6	6	6	6	6	6	6	5	5,5	6	6
Maximum diameter (µm)	50	10	10	10	10	10	10	15	12	12	12	12	12	12
Dilution factor	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cell brightness (%)	85	85	75	75	75	75	75	75	75	75	75	75	75	75
Cell sharpness	100	100	100	200	200	200	200	200	200	200	200	200	200	200
Viable cell spot brightness (%)	75	75	75	75	80	80	80	80	80	85	80	80	80	80
Viable cell spot area (%)	5	5	5	5	5	4	4	4	4	4	4	4	4	4
Minimum circularity	0.7	0.7	0.7	0.7	0.7	0.7	0.4	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Decluster degree	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	High	Low
Cell concentration (cells/µL)	3.45	3.04	1.30	1.3	1.29	1.3	1.3	1.58	1.55	1.54	2.19	1.86	1.47	1.44
Viability (%)	99	99.1	99.1	99.1	99.1	99.1	98.7	99.1	99.2	99.2	99.2	99.3	99.2	99.2

Table 9: Change of Vi-CELL XR Cell Viability Analyzer parameters during the second test. The parameter settings from the first test were the starting point, and the parameters changed from the last reanalysis in the first test to the first reanalysis in the second test is highlighted in yellow, and given in reanalysis 1. Yellow highlights indicate which parameter was changed for each reanalysis

Reanalysis	1	2	3	4	5	6	7	8	9	10	11	12	13
Minimum diameter (µm)	6	6	6	6	6	6	6	6	6	6	6	6	6
Maximum diameter (µm)	13	10	10	10	10	10	10	10	10	10	10	10	10
Dilution factor	1	1	1	1	1	1	1	1	1	1	1	1	1
Cell brightness (%)	85	85	70	75	76	77	78	78	78	78	78	78	78
Cell sharpness	200	200	200	200	200	200	200	200	200	200	200	200	200
Viable cell spot brightness (%)	80	80	80	80	80	80	80	85	90	95	95	95	95
Viable cell spot area (%)	4	4	4	4	4	4	4	4	4	4	5	6	5,9
Minimum circularity	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0,7
Decluster degree	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
Cell concentration (Cells/µL)	0.80	0.69	0.36	0.39	0.42	0.44	0.46	0.46	0.46	0.45	0.46	0.46	0.46
Viability (%)	93.60	92.70	99.70	97.80	97.20	97.00	96.10	96.10	96.10	95.80	95.80	95.80	95.80

The starting point for the second setting was the parameters from the first setting, but slightly modified. The parameters that were changed from reanalysis 14 in the first test to reanalysis 1 in the second test were maximum diameter, cell brightness and decluster degree. The procedure for the second setting was the same as for the first setting and new parameters that were closer to the flow cytometry results were found. In general the parameters that influenced most on the lymphocyte concentration were maximum and minimum diameter and cell brightness, while the other parameters only influenced slightly on the viability.

The final setting were named HumLymph and it differed from the flow cytometer results with 9.68 % for lymphocyte concentration (Flow: 404 cells/µL) and 0.2 PP for viability (Flow: 92.00 %) (Table 10). Hence, the HumLymph setting fulfilled the objectives for lymphocyte concentration and viability and this setting was applied for further research.

Table 10: Parameter settings with the HumLymph setting from the Vi-CELL XR Cell Viability Analyzer

Parameter	Value
Minimum diameter (µm)	6
Maximum diameter (µm)	10
Dilution factor	1
Cell brightness (%)	78
Cell sharpness	200
Viable cell spot brightness (%)	95
Viable cell spot area (%)	5,9
Minimum circularity	0,7
Decluster degree	Medium

Lymphocyte concentration and viability measured with Vi-CELL

The difference in lymphocyte concentration between the Vi-CELL and flow cytometer results should be ≤ 15 %. Measuring cell concentration with the HumLymph setting gave a smaller difference in lymphocyte concentration compared to the CHO settings. However, a difference in lymphocyte concentration ≤ 15 % was still not consistent: Only 27 % of the tests using HumLymph setting showed a difference in lymphocyte concentration ≤ 15 %, indicating that the Vi-CELL was unable to measure accurate lymphocyte concentration.

In order to illustrate the difference in lymphocyte concentration for all samples measured with flow cytometer and Vi-CELL (HumLymph setting), a Bland-Altman plot was made (Figure 26). This plot showed that most of the data points lay within ± 1.96 standard deviations (SD) from mean

which generally means that two methods are in agreement. However, the difference between the two methods increased with higher mean lymphocyte concentration which lead to the belief that the two methods did not provide agreeable results.

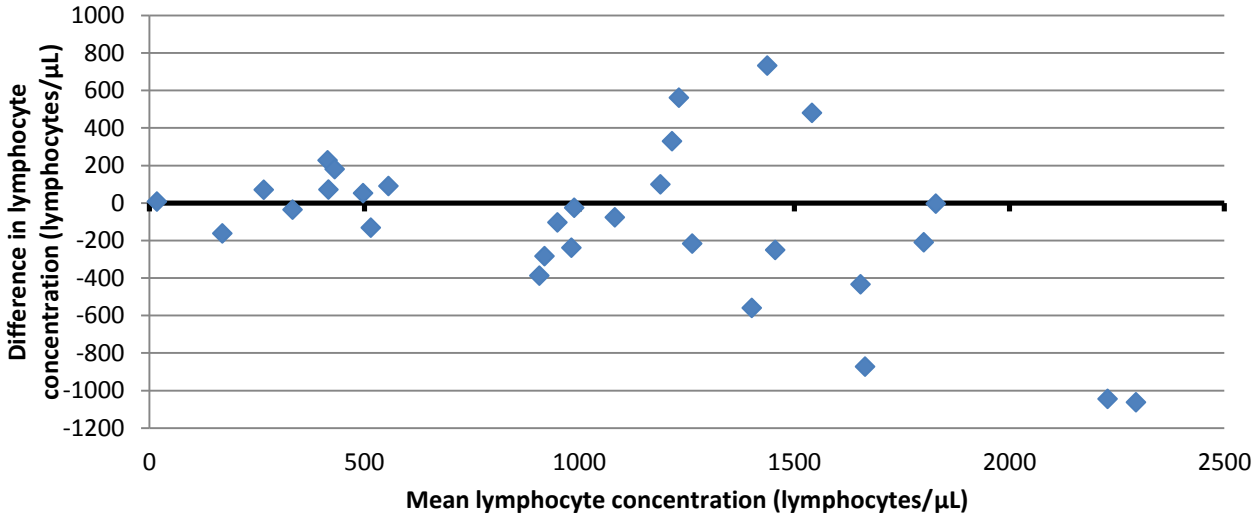

Figure 26: Bland-Altman plot showing the difference in lymphocyte concentration obtained for all samples using flow cytometer and Vi-CELL XR Cell Viability Analyzer (HumLymph). 1.96 standard deviations = 809.92, indicating a 95 % limit of agreement

The lymphocyte viability was tested with the HumLymph setting. In general, the difference in viability between the Vi-CELL and the flow cytometer was low: 93 % of the tests using HumLymph had a viability difference < 5 PP. This indicated that the Vi-CELL was able to measure lymphocyte viability accurately.

As for the lymphocyte concentration, a Bland-Altman plot was made for the lymphocyte viability (Figure 27). This plot showed that all data points lay within ± 1.96 SD from mean, indicating agreement between the two methods.

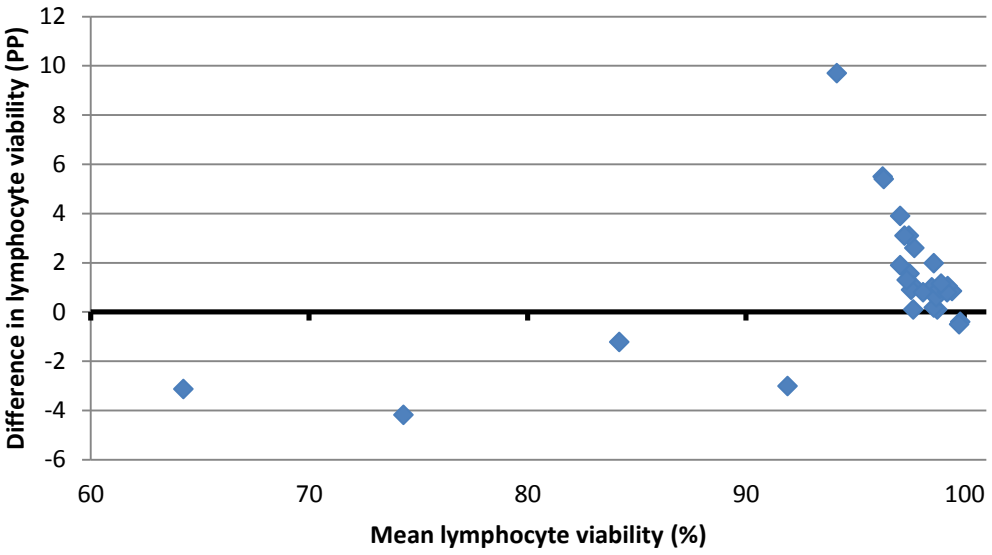

Figure 27: Bland-Altman plot showing the difference in lymphocyte viability obtained for all samples using flow cytometer and Vi Vi-CELL XR Cell Viability Analyzer (HumLymph). 1.96 standard deviations = 15.29, indicating a 95 % limit of agreement

Discussion

In the present study it was investigated if the CDC-XM could be evaluated objectively with an automated video imaging system, the Vi-CELL.

Only recipients that had received a kidney from a deceased donor were included. This inclusion criterion was chosen, as recipient and graft survival is generally lower for transplantations with deceased donor compared to living donor (2). Therefore outcomes from deceased donor transplantation are more likely to benefit from a higher predictive crossmatch result. Lower recipient and graft survival can be caused by several factors. One of them is an increased risk of rejection, as the possibilities for performing tests prior to transplantation are limited. A reason for this is the cold ischemia time, which is 24 hours for a kidney. When the transplantation is with a living donor more results from pre-transplantation tests can be evaluated as the transplantation is planned ahead. Therefore a better donor-recipient match can be found, leading to a better transplantation outcome (19). A way of making the same quality, when crossmatching with a deceased donor, is to have a more precise and predictive crossmatch evaluation. E.g. by making the evaluation of the CDC-XM test more objective.

Results

During this study it was investigated if transplantation outcome varied across results from tests performed before kidney transplantation with deceased donor. Furthermore, the ability of MACS for separating T- and B-cells was investigated, and the Vi-CELL's ability to analyze lymphocyte concentration and viability was tested.

Clinical results

Two parameters were chosen as a measure of transplantation outcome: eGFR and number of rejections. eGFR values were obtained for three months and one year after transplantation. The timelines were chosen to account for graft adaption. eGFR is a biomarker for kidney function, and indicates how well the graft is working (30). Number of rejections was chosen as a biomarker for graft survival. This parameter is closely related to the tests performed before transplantation, where the results of these should give an idea of compatibility between the graft and the

recipient. eGFR and number of rejections are related as the graft function (eGFR) decreases with increasing number of rejections, as the graft tissue is damaged every time a rejection occurs (6).

The present study showed no significant differences between any of the tests performed before kidney transplantation for eGFR 3M, eGFR 1Y, or number of rejections. However, tendencies were seen for both time points for eGFRs and number of rejections across presence of HLA-antibodies and number of DSA. As DSA are donor-specific HLA-antibodies those two parameters are related. Therefore it was expected, that the same tendency was seen for these two parameters for transplantation outcome. The results from this study are in line with previous studies showing that the presence of HLA-antibodies and especially DSA are predisposing factors for poor graft survival (31–35).

The lack of significance can be caused by the skewed distribution of clinical data. Presence of HLA-antibodies and eGFR 3M values were obtained from 103 recipients and presence of HLA-antibodies and eGFR 1Y values were obtained for 95 recipients. This indicates that the sample sizes should be sufficient to ensure a valid result, but the distribution of HLA-antibodies was unequal as most of the recipients did not have any HLA-antibodies (N = 70 for eGFR 3M and N = 65 for eGFR 1Y). The unequal distribution was also seen for the number of DSA, where eGFR 3M and 1Y values were obtained for 92 recipients and a large part of the recipients did not have any DSA (N = 68 for eGFR 3M and N = 63 for eGFR 1Y).

Presence of HLA-antibodies and number of rejections was obtained for 111 recipients. This indicated a sufficient sample size, but the distribution of data was skewed since 76 recipients did not have any rejections. Number of DSA and number of rejections were obtained for 99 recipients. Again there was an unequal distribution with 69 recipients having no rejections.

Other studies have stated that the formation of HLA and especially DSA in a recipient can have a great influence on graft function and survival. Even though no statistical significant results were found, this study showed the same tendencies. The sensitization of the recipient can lead to a poor transplantation outcome since the recipient's adaptive immune system attacks the graft faster than if the recipient was not present with DSA (31–35). Furthermore, it is much more complicated to account for this sensitization medically since humoral rejection respond poorly to immune suppressive treatment (10). In the clinic the recipients are tested for the presence of HLA-antibodies and DSA. However, the presence of non-HLA-antibodies and antibodies against minor

histocompatibility molecules are not investigated, since these antibodies are not believed to have a major influence on graft rejection (8,36,37).

Even though no tendency was seen for the other pre-transplantation tests, previous studies have shown that crossmatch results and HLA-mismatch degree do affect transplantation outcome. However, it seems that DSA are the most relevant as predictor of transplantation, even though all the tests can have a predictive value regarding transplantation outcome. The CDC-XM and the FC-XM indicate if DSA are present in the recipient and the HLA-mismatch degree predicts if there is a basis for developing DSA. If there is a HLA-mismatch, donor specific HLA-antibodies can be developed (16,17,38).

Experimental results

In this study lymphocytes were isolated from whole blood using lymphoprep. This is a widely used method and it should be adequate for isolation of T- and B-cells (22). However, several steps in the protocol make the method somehow unreliable when performed by unexperienced personal. After centrifugation and dividing of cells, the layer of lymphocytes has to be aspirated from the tube. In this step it is important to aspirate the whole layer of lymphocytes without stirring the erythrocytes and granulocytes in the bottom. If this happens it is possible that some of the unwanted cells could be mixed with the lymphocytes. This could give an unreliable result for the obtained lymphocyte concentration and viability, when analyzed with the Vi-CELL as it only distinguish cells according to morphology. However, when this stirring occurred, further aspiration was postponed until the layers had re-established. Therefore the results are considered reliable.

MACS

To ensure that T- and B-cell crossmatches could be performed the lymphocytes had to be separated. The chosen method was MACS, which was evaluated with flow cytometry.

A lymphocyte loss ≤ 10 % due to the separation was acceptable but even though the lymphocyte loss decreased during the tests it was still > 10 %. The reason for this decrease was assumed to be the choice of lymphocyte source and procedure for the separation. In the first test defrosted lymphocytes from spleen were used and they had a lower viability than PBMC. In the later tests the cell type used was PBMC which had a higher viability (almost 100 %). Furthermore the procedure was changed so the pre-separation lymphocyte concentration was obtained *after* wash

and centrifugation instead of before. This secured that the only difference between the pre-separation sample and the post-separation sample was the separation. This lowered the difference in lymphocyte concentration, and thereby reduced the lymphocyte loss. For one of the samples an increase in lymphocyte number was seen from pre-separation to post-separation. As this is not possible, an error has occurred somewhere: E.g. measuring of the wrong sample or a miscalculation. An acceptable level of lymphocyte loss (< 10 %) was not reached for any of the samples. A study by Willasch et al. reached a low cell loss after MACS (39). However, another study by Woodside et al. have obtained cell loss results similar to findings in the present study (40).

The purification yield was also investigated, and was acceptable at > 90 %. In the first test the lymphocytes were from spleen, where the concentration of B-cells is higher than T-cells (55 % B-cells and 31 % T-cells) (41). This may lead to a very high purification yield for B-cells, as the B-cell concentration was very high before separation. It could also lead to a lower T-cell purification, as all the B-cells might not be cleared from the MACS Column until they are flushed through with the force of the plunger. In later tests, when PBMC was used instead of spleen lymphocytes, the concentration relation was reversed, as T-cells are present in a higher concentration compared to B-cells in the peripheral blood (73 % T-cells and 12 % B-cells) (42). This could lead to the same purification yield issues as mentioned for the spleen lymphocytes, but reversed. The spleen lymphocytes had a lower viability than PBMC which may influence their binding to the microbeads: dead lymphocytes bind unspecific to the microbeads (43). This would lead to e.g. dead T-cells binding the anti-CD19 microbeads, and ending in the positive B-cell fraction, and the B-cells not binding the anti-CD19 microbeads: The B-cells would end in the negative B-cell fraction. A high purification yield was obtained for some of the samples, however, even with PBMC, which is assumed not to bind unspecific, the purification yield did not reach > 90 % for all the samples. This is in correlation with previous studies (39,44).

It was also investigated if lymphocytes died from the separation. A decrease in viability ≤ 5 PP from the pre-separation sample to the positive fraction was accepted. As only the positive fraction will be used in a crossmatch in a clinical setting, the change in viability was only relevant for this fraction. A decrease in viability ≤ 5 PP was seen for all three samples with PBMC, but not for the spleen lymphocytes in the T-cell fraction. This is assumed to be caused by the low viability for the

spleen lymphocytes, and the low concentration of T-cells. However, an increase in viability was seen for all three B-cell fractions. This cannot be explained, as no other studies have mentioned this increase. The T-cell viability in the T-cell fractions was slightly decreased after separation (< 1 PP), so in general the difference in viability was highly acceptable.

Vi-CELL

The lymphocyte concentration and viability measured with the Vi-CELL was compared to the corresponding values obtained by flow cytometer for several samples. A difference in lymphocyte concentration ≤ 15 % and a difference in viability ≤ 5 PP were acceptable.

As the microbeads did not appear on the Vi-CELL software images, it was concluded that the Vi-CELL did not read and count them as lymphocytes. This is due to the small size of microbeads that makes the Vi-CELL unable to read them.

To make the Vi-CELL results as close to the flow cytometer results as possible, two samples were used to decide the parameter settings for the Vi-CELL and the result was the HumLymph setting. Several parameters were changed during the procedure, but it was noted, that only the parameters minimum and maximum diameter and cell brightness had a major influence on the results. The chosen diameter resembled the known size of lymphocytes. Cell brightness was found to change the lymphocyte concentration, and therefore several values for this parameter were tested. Through the procedure of the parameter settings, it was discovered that the other parameters only influenced the viability. Changing these parameters did not influence major on the results, as the samples run through the Vi-CELL were all from PBMC, which generally had a high viability (> 90 %). The HumLymph setting had a very low cell sharpness of 200, which was the maximal value for this setting. This was chosen, because the dead cells had blurry cell lines, and therefore a false high viability could be avoided, as the dead cells now would be included in the cell count. The other parameters were set to values, where the viability was closest to the viability obtained by flow cytometry.

The parameter settings were made from only two samples, and therefore an acceptable difference between Vi-CELL and flow cytometer results could be by chance. The lymphocyte viability difference between the two methods was small even with the CHO cell type. Therefore several samples were reanalyzed and new tests were performed with the HumLymp setting. Further tests

with the HumLymph showed that the difference in lymphocyte concentration was acceptable for 27 % of the tests. The difference in viability between the Vi-CELL and the flow cytometer was acceptable in almost all the tests. This could indicate that the HumLymph setting was not the most appropriate cell type setting for measuring lymphocyte concentration. If more tests had been done in order to get more suitable settings, maybe the Vi-CELL would be able to obtain results closer to the flow cytometer results. However, several attempts to make the HumLymph parameters better were done but they did not succeed. It was attempted to make the big difference in lymphocyte concentration between flow cytometry and Vi-CELL smaller, without increasing the difference in viability. Unfortunately, this was not possible, as the lymphocyte viability changed along with the lymphocyte concentration.

The lymphocyte concentration was considered incorrect, and therefore it was assumed that the lymphocyte viability was questionable. To test the viability a dilution series was made from spleen lymphocytes and PBMC, as previous results had shown that spleen lymphocytes had a low viability (≈ 60 %). In this dilution series the differences in viability were ≤ 5 PP, which indicates that the HumLymph setting can be used to measure viability for lymphocytes.

Two other dilution series were made with CH_2O-treated cells, in order to test if the Vi-CELL was able to measure viability, when this was close to zero. However, it did not succeed to reach viability that low. CH_2O is a solution used to fixate cells, where the cells die but keep their morphological appearance (45). In the first series, the lymphocytes were resuspended in buffer and then CH_2O was added. The viability did not decrease from this, and it is believed that the dilution of the CH_2O in buffer made it ineffective. In the second series the lymphocytes were resuspended in pure CH_2O, and the lymphocytes were expected to die from this. However, when viability was tested, it was still very high. It was assumed that the CH_2O killed the lymphocytes, but also made them impermeable, and therefore the staining used to measure viability did not work. To overcome this problem, ethanol could be added after fixation to make the cells permeable (45). The fluorophore used for the flow cytometer was 7-AAD which binds DNA in dead cells, and the Vi-CELL uses trypan blue, which also needs to enter the cells to stain them. Therefore, the dead lymphocytes would not be stained; hence, the obtained viability remained high.

To investigate agreement between flow cytometry and Vi-CELL methods, Bland-Altman plots were made. These are often used to illustrate the difference between two methods, to show if the

methods are in agreement (46). For lymphocyte concentration it was seen that the methods appeared to be in agreement, as almost all differences in concentration were within 1.96 SD from the mean concentration. However, as the difference in lymphocyte concentration increased with increasing mean concentration, it cannot be concluded that the two methods are in agreement. There is a risk that the difference in lymphocyte concentration might increase even more if the mean concentration increases further and the difference in concentration might exceed the 1.96 SD. For viability it also seemed as if the two methods were in agreement even though the difference in viability increased with mean viability. However, it is still believed that the agreement between the two methods is reliable since the viability in the samples was around 100 %, and the viability can never exceed 100 %. In conclusion the methods were in agreement for lymphocyte viability, but not for lymphocyte concentration.

It seemed that the Vi-CELL measured a correct viability of lymphocytes both in samples with PBMC and spleen lymphocytes. Therefore, it was still considered if the Vi-CELL could be used to evaluate crossmatches since lymphocyte viability and not lymphocyte concentration is relevant to crossmatch results. However, as the difference in lymphocyte concentration between the Vi-CELL and the flow cytometer was > 15 %, it was not possible to determine if the Vi-CELL only read the lymphocytes in the samples. Hence, it was assumed that it was not possible to make the right parameter settings that made the Vi-CELL able to measure both lymphocyte concentration and viability accurately.

Other studies have used the Vi-CELL for assessing human lymphocyte concentration and viability, but no studies, that investigated the Vi-CELL's ability to measure lymphocyte concentration correct, were found (47–49).

Vi-CELL compared to flow cytometry

Throughout the study, the flow cytometer results have been the guideline for parameter setting and evaluation of the Vi-CELL. It was expected that it was possible to compare the two methods and get a valid and true result. However, this might not be the case. For viability testing, the two methods use the same principle: Dead cells have a permeable cell membrane that allows for staining with a viability marker, 7-AAD and trypan blue (29). This was a possible explanation of the small difference in viability between the two methods. The principle for measuring lymphocyte

concentration, however, is very different between the two methods. The flow cytometer uses fluorophores that bind to surface molecules to distinguish lymphocytes from other cells and to quantify T- and B-cells (50). The Vi-CELL uses a video imaging system to distinguish between target and not-target cells in a sample. The cells are therefore classified according to their morphology, and a method for separating T- and B-cells has to be applied before Vi-CELL analysis (25). The different principles for obtaining cell concentration might be the reason why the difference in lymphocyte concentration between the two methods was so large. To overcome this problem another method could be used as a guideline for determining Vi-CELL parameters. This method has to be similar in the way of obtaining cell concentration, e.g. manual cell counting with haemocytometer. A laboratory technician that is highly experienced in this might provide a better guideline for lymphocyte concentration. However, if manual cell counting is used as guideline the Vi-CELL might not provide a better result compared to the standard procedure currently used in the clinic.

Flow cytometry is a comprehensive method and requires knowledge of a certain level to analyze the events with gates and to interpret the results. However, if this is done exact, the results are very reliable (50). As time is an important factor in regard to transplantations, it is a small drawback that the cells and fluorescents have to be incubated for 30 minutes. The fluorescent dyes needed for the flow cytometry are also more expensive than the reagents used for the Vi-CELL. The flow cytometer uses a more complicated method for measuring, as several dyes, lasers etc. are used for each individual sample, whereas only a camera and trypan blue is used for the Vi-CELL. Regarding sample size, the flow cytometer only requires a very small volume (40 µL), whereas the Vi-CELL needs a volume of minimal 0.5 mL with a certain cell concentration, as it cannot give a valid result, if the cell concentration is too small. The flow cytometer does not need a certain concentration to give a valid result. Homogenization is very important for a reliable result obtained with the Vi-CELL, but this is the only factor that can vary between analyses (intra-examiner and inter-examiner reliability). Otherwise the machine is fully automated: Mixing of trypan blue with sample, assessment of cell concentration and viability. The staining procedure is manually performed when using the flow cytometer, and there can be several differences between analyses. Another thing to consider is safety regarding the personnel performing the analyses. With the Vi-CELL there is no contact with the reagents, as everything is within the

machine. As the mix of dyes and cells has to be performed manually for the flow cytometer, the personnel will be in contact with the dyes, which can be carcinogenic.

In general the Vi-CELL is a more simple method for analyzing lymphocyte concentration and viability, and it is a cheaper and faster method compared to flow cytometry. However, if the Vi-CELL is to be used for crossmatch it should be considered that a lymphocyte separation has to be applied before analysis – in this study MACS was used, which costs extra time and money.

Conclusion

In the present study it was found that kidney transplantation outcome (eGFR and number of rejections) could not be predicted by pre-transplantation tests. The utility of MACS was tested and acceptable values were obtained for lymphocyte death from separation, but not for lymphocyte loss and purification yield. Evaluation of the Vi-CELL for analyzing lymphocyte concentration and viability showed that the Vi-CELL was able to measure viability correct, but not lymphocyte concentration compared to flow cytometry.

In conclusion the MACS separation and Vi-CELL cannot be used for crossmatching.

Future perspectives

In this study MACS was used to separate T- and B-cells. To optimize this method in future studies it should be considered that the separation can be improved if performed in a cold environment (51). Furthermore, a dead cell removal-kit could be used before separation to avoid unspecific binding of the microbeads to dead cells. The best separation results were obtained when the T- and B-cells were isolated from PBMC. Since donor lymphocytes used for crossmatching today are from spleen future studies should involve the optimization of the spleen lymphocyte separation. Otherwise the clinical procedure could be changed to use PBMC lymphocytes instead of spleen lymphocytes in the future. This could increase the time available for matching donor and recipient before transplantation with deceased donor as a blood sample can be obtained faster than a spleen.

The Vi-CELL was tested for assessment of lymphocyte concentration and viability. To further test this method a large sample size should be used and these should be run in triplicates as a minimum. To determine the parameter settings for the Vi-CELL a different guideline than flow cytometry could be used in the future. E.g. by using a hemocytometer which could give the Vi-CELL the same properties as manual cell counting, but the objective element would be added.

Bibliography

1. Strandgaard S, Klamer F Kronisk nyresygdom og kronisk nyresvigt [Internet]. sundhed.dk [cited 2014 Sep 18]. Available from: https://www.sundhed.dk/sundhedsfaglig/laegehaandbogen/nyrer-og-urinveje/tilstande-og-sygdomme/nyresygdomme/kronisk-nyresygdom-og-kronisk-nyresvigt/

2. Danish nephrology registry. Annual report 2013. 2013.

3. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med. 2004 Dec 23;351(26):2715–29.

4. Birkeland SA, Storm HH. Cancer risk in patients on dialysis and after renal transplantation Maternal and fetal hepatitis C virus exposure by intrauterine transfusion. The Lancet. 2000;355:1886–7.

5. Strandgaard S, Klamer F, Sahl Andersen J. Nyretransplantation [Internet]. sundhed.dk. [cited 2014 Sep 18]. Available from: https://www.sundhed.dk/sundhedsfaglig/laegehaandbogen/nyrer-og-urinveje/tilstande-og-sygdomme/behandlinger/nyretransplantation/

6. Opelz G, Döhler B. Influence of time of rejection on long-term graft survival in renal transplantation. Transplantation. 2008 Mar 15;85(5):661–6.

7. Lillevang S, Møller B. Immunologi - En kortfattet lærebog. 2nd ed. København: FADL's forlag; 2009.

8. Zachary AA, Leffell MS, Walker JM. Transplantation Immunology. Methods and protocols. 2nd ed. Humana Press; 2013.

9. Geneser F. Histologi - på molekylærbiologisk grundlag. Munksgaard Danmark; 2010.

10. Michaels PJ, Fishbein MC, Colvin RB. Humoral rejection of human organ transplants. Springer Semin Immunopathol. 2003 Sep;25(2):119–40.

11. Ingulli E. Mechanism of cellular rejection in transplantation. Pediatr Nephrol. 2010 Jan;25(1):61–74.

12. Game DS, Lechler RI. Pathways of allorecognition: Implications for transplantation tolerance. 2002;10:101–8.

13. Lucas JG, Co JP, Nwaogwugwu UT, Dosani I, Sureshkumar KK. Antibody-mediated rejection in kidney transplantation: an update. Expert Opin Pharmacother. 2011 Mar;12(4):579–92.

14. Nguyen H Do, Williams RL, Wong G, Lim WH. The Evolution of HLA-Matching in Kidney Transplantation. 2013.

15. Mauiyyedi S, Colvin RB. Humoral rejection in kidney transplantation: new concepts in diagnosis and treatment. Curr Opin Nephrol Hypertens. 2002 Nov;11(6):609–18.

16. Takemoto S, Port FK, Claas FHJ, Duquesnoy RJ. HLA matching for kidney transplantation. Hum Immunol. 2004;65(12):1489–505.

17. Kosmoliaptsis V, Gjorgjimajkoska O, Sharples LD, Chaudhry AN, Chatzizacharias N, Peacock S, et al. Impact of donor mismatches at individual HLA-A, -B, -C, -DR, and -DQ loci on the development of HLA-specific antibodies in patients listed for repeat renal transplantation. Kidney Int. Nature Publishing Group; 2014 Nov;86(5):1039–48.

18. Malheiro J, Tafulo S, Dias L, Martins LS, Fonseca I, Beirão I, et al. Analysis of preformed donor-specific anti-HLA antibodies characteristics for prediction of antibody-mediated rejection in kidney transplantation. Transpl Immunol. Elsevier B.V.; 2015 Feb 7;1–6.

19. Mulley WR, Kanellis J. Understanding crossmatch testing in organ transplantation: A case-based guide for the general nephrologist. Nephrology (Carlton). 2011 Feb;16(2):125–33.

20. Magee CC. Transplantation across previously incompatible immunological barriers. Transpl Int. 2006 Feb;19(2):87–97.

21. Axis-Shield. Purification of mononuclear cells, monocytes and polymorphonuclear cells. 2011;(4):1–16.

22. Axis-Shield. Lymphoprep. Isolation of human mononuclear cells. p. 5–6.

23. MS Columns - Miltenyi Biotec [Internet]. [cited 2014 Oct 24]. Available from: http://www.miltenyibiotec.com/en/products-and-services/macs-cell-separation/manual-cell-separation/columns/ms-columns.aspx

24. Miltenyi Biotec. MACS Technology. The gold standard. Now and forever. 2013.

25. Beckman Coulter Inc. Vi-CELL XR Cell Viability Analyzer. Reference manual.

26. The Vi-CELL Series Cell Viability Analyzers - Beckman Coulter, Inc. [Internet]. [cited 2014 Oct 24]. Available from: https://www.beckmancoulter.com/wsrportal/wsrportal.portal?_nfpb=true&_windowLabel=UCM_RENDERER&_urlType=render&wlpUCM_RENDERER_path=/wsr/research-and-discovery/products-and-services/flow-cytometry/cell-counters/vi-cell-series/index.htm#2/10//0/25/1/0/asc/2/383080///0/1//0/

27. Jahan-Tigh RR, Ryan C, Obermoser G, Schwarzenberger K. Flow cytometry. J Invest Dermatol. Nature Publishing Group; 2012 Oct;132(10):e1.

28. Leach RM, Drummond M, Doig A. Practical Flow Cytometry in Haematology Diagnosis. John Wiley & Sons; 2013.

29. Zembruski NCL, Stache V, Haefeli WE, Weiss J. 7-Aminoactinomycin D for apoptosis staining in flow cytometry. Anal Biochem. Elsevier Inc.; 2012;429(1):79–81.

30. McCance KL, Huether SE. Pathophysiology - The biologic basis for diseases in adults and children. 6th ed. Brashers VL, Rote NS, editors. 2010.

31. Eng HS, Bennett G, Tsiopelas E, Lake M, Humphreys I, Chang SH, et al. Anti-HLA donor-specific antibodies detected in positive B-cell crossmatches by Luminex predict late graft loss. Am J Transplant. 2008 Nov;8(11):2335–42.

32. Baxter-Lowe L a, Cecka M, Kamoun M, Sinacore J, Melcher ML. Center-defined unacceptable HLA antigens facilitate transplants for sensitized patients in a multi-center kidney exchange program. Am J Transplant. 2014 Jul;14(7):1592–8.

33. Caro-Oleas JL, González-Escribano MF, Gentil-Govantes M a, Acevedo MJ, González-Roncero FM, Bernal-Blanco G, et al. Influence of donor specific HLA antibodies detected by Luminex in kidney graft survival: a multivariate analysis. Hum Immunol. American Society for Histocompatibility and Immunogenetics; 2013 May;74(5):545–9.

34. Fidler SJ, Irish AB, Lim W, Ferrari P, Witt CS, Christiansen FT. Pre-transplant donor specific anti-HLA antibody is associated with antibody-mediated rejection, progressive graft dysfunction and patient death. Transpl Immunol. Elsevier B.V.; 2013 Jun;28(4):148–53.

35. Iniotaki-Theodoraki A. The role of HLA class I and class II antibodies in renal transplantation. 2001;150–2.

36. Luo L, Li Z, Wu W, Luo G, Mei H, Sun Z, et al. The effect of MICA antigens on kidney transplantation outcomes. Immunol Lett. Elsevier B.V.; 2013;156(1-2):54–8.

37. Sapák M, Chreňová S, Tirpáková J, Žilinská Z, Ďurmanová V, Shawkatová I, et al. Donor non-specific MICA antibodies in renal transplant recipients. Immunobiology. 2014 Feb;219(2):109–12.

38. Lim WH, Gray N a, Chadban SJ, Pilmore H, Wong G. Graft and patient outcomes of zero-human leucocyte-antigen-mismatched deceased and live donor kidney transplant recipients. Transpl Int. 2015 Feb 16.

39. Willasch a, Eing S, Weber G, Kuçi S, Schneider G, Soerensen J, et al. Enrichment of cell subpopulations applying automated MACS technique: purity, recovery and applicability for PCR-based chimerism analysis. Bone Marrow Transplant. 2010 Jan;45(1):181–9.

40. Woodside SM, Makowichuk RC, Eaves AC, Thomas TE. Isolation of Highly Purified Lymphocyte Subsets Directly from Whole Blood. StemCell Technologies Inc.;

41. Colovai AI, Giatzikis C, Ho EK, Farooqi M, Suciu-Foca N, Cattoretti G, et al. Flow cytometric analysis of normal and reactive spleen. Mod Pathol. 2004;17(8):918–27.

42. Agger R, Andersen V, Leslie G, Aasted B. Immunologi. 4th ed. København: Biofolia; 2007.

43. Miltenyi Biotec. CD19 Antibodies Human. Protocol. 2007.

44. Lyons P a, Koukoulaki M, Hatton A, Doggett K, Woffendin HB, Chaudhry AN, et al. Microarray analysis of human leucocyte subsets: the advantages of positive selection and rapid purification. BMC Genomics. 2007 Jan;8:64.

45. Yang H, Yorke E, Hancock G, Clutton G, Sande N, Angus B, et al. Improved quantification of HIV-1-infected CD4+ T cells using an optimised method of intracellular HIV-1 gag p24 antigen detection. J Immunol Methods. Elsevier B.V.; 2013;391(1-2):174–8.

46. Bowers D. Medical Statistics from Scratch. An Introduction for Health Professionals. 2nd ed. Chichester: John Wiley & Sons; 2008.

47. Ramsay AG, Evans R, Kiaii S, Svensson L, Hogg N, Gribben JG. Chronic lymphocytic leukemia cells induce defective LFA-1-directed T-cell motility by altering Rho GTPase signaling that is reversible with lenalidomide. Blood. 2013 Apr 4;121(14):2704–14.

48. Ramsay AG, Clear AJ, Fatah R, Gribben JG. Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide : establishing a reversible immune evasion mechanism in human cancer. 2015;120(7):1412–22.

49. Boyce C, Lane C, Hingorani R, Mcintyre C. Human Regulatory T-Cell Isolation and Measurement of Function. BD Biosci. 2010;

50. Weaver JL. Introduction to flow cytometry. Methods (San Diego, Calif.). 2000.

51. Alipoor FJ, Gilani MAS, Eftekhari-Yazdi P, Hampa AD, Hosseinifar H, Alipour H, et al. Achieving high survival rate following cryopreservation after isolation of prepubertal mouse spermatogonial cells. J Assist Reprod Genet. 2009 Mar;26(2-3):143–9.