# Optimisation of histocompatibility testing in living-donor renal transplantation.

By

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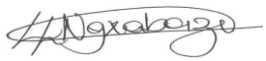
Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy (PhD) in Medical Immunology
At the University of Pretoria

2018

# Declaration

I declare that the thesis, which I hereby submit for the degree of PhD in Medical Immunology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.



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# Publications

Evaluation of three different laboratory methods to detect preformed human leukocyte antigen antibodies in a South African kidney transplant population

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# Presentation:

Some of this work has been presented in 32nd European Immunogenetics and Histocompatibility conference, in Venice May 9-12, 2018.

# Summary

Notwithstanding ABO erythrocyte typing, pre-transplantation analysis of human leukocyte antigen (HLA) class I and II antigens carried out on 15 donor and 15 recipient lymphocytes, as well as detection of pre-existing cytotoxic antibodies reactive with donor lymphocytes, remain the cornerstones of donor-recipient matching in clinical organ transplantation. The laboratory procedures used in this setting have undergone considerable refinement in the past 5-10 years with traditional complement-based cytotoxocity methods having been largely superseded by polymerase chain reaction (PCR)-based molecular and fluoroanalytical procedures for HLA-typing and detection of alloreactive cytotoxic antibodies respectively. Although these innovations in HLA cross-matching technologies will contribute significantly to improved graft survival, several limitations remain, most importantly realisation of the potential of assays based on T-lymphocyte alloreactivity to complement molecular/humoral histocompatibility testing procedures. This issue was addressed in this thesis in the setting of living-donor clinical renal transplantation. Recipient T-cell alloreactivity results were assessed using:

i) a novel, 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE), flow cytometric two-way mixed lymphocyte reaction (MLR); ii) flow cytometric detection of expression of early markers (CD25, CD69, HLA class II) of T-cell activation in the two-way mixed leucocyte culture (MLC); and iii) fluoro-analysis of T-cell-derived cytokines in plasma and the supernatant fluids generated in the two-way MLC. The potential of these assays of T-cell alloreactivity was assessed pre- and post-transplantation in combination with histocompatibility and standard cross-matching procedures to monitor the development of recipient immune responsiveness to donor antigens. With respect to standard histocompatibility testing, HLA class I and II antigen genotyping was performed using a high-resolution PCR-based procedure, while 3 procedures were compared for the detection of donor-alloreactive HLA class I and II cytotoxic antibodies *viz*: i) Luminex®-based fluoro-analysis using HLA antigen-coated beads and recipient serum; ii) flow cytometry using donor lymphocytes and recipient serum; and iii) conventional antibody-mediated, complement-dependent cytotoxicity (CDC) using donor lymphocytes and recipient serum.
The key findings of the study, as presented sequentially in the thesis, as follows:

In **Chapter 3**, three cross-match procedures were compared. All 3 methods identified positive HLA Class I cross-match in two (13%) of the 15 patients. The complement-dependent cytotoxicity cross-match (CDCXM) assay showed that 6 (40%) out of the 15 patients tested positive for HLA class II antibodies compared to the donor-specific antibody cross-match (DSAXM) and flow cytometry cross-match (FCXM) assays. When confirming the results using the Luminex® based single antigen assay, HLA class II antibodies were not detected in 4 (67%) out of the 6 patients, indicating that false-positive results were obtained with the CDCXM assay. The FCXM and DSAXM methods performed equally well.

In **Chapter 4**, a panel of cytokines was assayed in plasma of patients who were divided into 2 groups, those who rejected the renal transplant (G1) and those with a successful graft (G0). Increased median cytokine levels were observed in plasma of those patients that rejected the renal grafts, although the eosinophil chemotactic protein, eotaxin, was the only cytokine out of the 27 tested that increased significantly ($p=0.03$) in the G1 group compared to the G0 group. In the second part of the study, the levels of the same pre-transplant growth factors, pro- and anti-inflammatory cytokines were determined in MLC supernatant fluids of both the patients and donors. However, little or no cytokine secretion was detected when compared to the levels observed in plasma for the same cytokines, with the exception of IL8. A general decrease in the level of cytokines in plasma, with the exception of one patient, was observed post-transplantation. In this part of the study, the involvement of eosinophil and increase of anti-inflammatory cytokines were identified as possibly contributing to the initiation of kidney transplant rejection.

In **Chapter 5**, the CFSE-based two-way MLC was used to measure T-cell proliferation of the recipient/donor cells. In these experiments, one of the participating sets of lymphocytes (recipients or donors) was stained with CFSE, and percentage proliferation in these populations was tracked using flow cytometry. The proliferating cells in this CFSE-based MLC were counterstained with CD25, CD69 and HLA-DR. An increase in the expression of these early surface markers of T-cell activation is an indication of cell activation and proliferation, which can also be monitored by the progressive loss of CFSE fluorescence as the cells divide. The mean percentage proliferation of the recipients that rejected ($\geq 15\%$) was higher than those individuals with successful renal grafts. In addition, significant increases between the autologous MLC and the allogeneic MLC in T-lymphocyte activation marker expression of

CD25, CD69 and HLA-DR was observed. Accordingly, loading lymphocytes of one of the participants, either donor or recipient, with CFSE, CFSE-based 2-way MLC can be used to predict GVHD and may be able to predict those patients that are at increased risk of transplant rejection.

In conclusion, the findings described in this thesis may contribute to optimising histocompatibility testing in renal transplantation by improving HLA matching criteria as well as identifying potential strategies to monitor and prevent GVHD.

# Acknowledgements

Through my life there have been people or events that have shaped who I am today and this process has been very humbling and has reinforced just how much I still have to learn, due to these factors, I would like to acknowledge the following people for their contribution:

I would like to thank God for giving me strength and vision to pursue this project.

I have had the privilege to be supervised by Dr Pieter WA Meyer, I would like to thank him for his guidance, patience and support during this project- I know I am not the easiest person to work with, but he had his way to keep me on course.

I am so honoured and grateful to Professor R Anderson for sharing his knowledge and insight throughout this project. He assisted me in the drafting of this project and writing of grant applications. His guidance provided knowledge that will assist me to survive as a research scientist.

To my co-supervisor, Prof VOL Karusseit, I would like to thank him for his contribution to this project, for giving us clinical access and facilitating the study at SBAH.

I would like to acknowledge the research grant provided by the NHLS Research Trust that made this project possible and also extend my thanks to my colleagues in the Department of Immunology for their support.

A special appreciation to Professor Annette Theron for her motivational chats and sense of humour that always helped me to progress with my thesis when the willpower waned, thank you for your unconditional love and support.

With greatest respect and gratitude to Prof Theresa Rossouw for making time and assisting me with clinical knowledge and statistical analysis.

The contribution of Dr Helen Steel cannot be understated. I would like to express my sincere gratitude for her support in reviewing and editing this thesis. I am immensely grateful for her willingness to share her expertise, support and advice.

Dr Chrisna Durandt and Ms Voula Stivaktas for their immeasurable contribution in data and technical analysis. I really appreciate them taking time out of their busy schedules to assist me. My thanks to the Netcare Transplant Coordinator, Lydia Botes, the participating hospitals and the kidney transplant patients, as well as the donors who have donated blood. Without your professional, accurate, timely support, and participation, this project will not have been a success.

I am so fortunate to have a family that supports and believes in my capabilities, even when I doubt myself. To my siblings, Ziyanda, Nathi, Nomtha and Phumelela, I have to thank them for their unwavering support throughout the past few years, whether it was looking after my son when I needed time to concentrate or through assisting me with typing or proofreading the thesis. If I can be given an opportunity to choose my siblings, I will definitely have you as my siblings. YOU are the best!

Last, but definitely not least, I would like to express my eternal gratitude to the two most important men in my life, my husband, Bernard (Wuzzie) and son, Kwabena (Koby), for their unwavering love, support and patience. Instead of complaining about the long hours I have devoted to this work, they provided me with plenty of encouragement to keep me from getting stale. Above all, I would like to thank them for believing in my dream, I love them very much.

I dedicate this thesis to my Mother, Nomathemba (Lovey), whom I miss so much. She always encouraged us to try our best while enjoying life to the fullest. I know she would have been proud of me. I want to take this opportunity to thank her for loving us unconditionally and for teaching us to love each other; they really helped me during the writing stage of my thesis; her teachings paid off. I love her so much and may her soul rest in perfect peace.

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# List of abbreviations

| | |
|---|---|
| α | Alpha |
| ACD | Acid-citrate-dextrose |
| ACR | Acute T-cell rejection |
| AMR | Antibody-mediated rejection |
| AO | Acridine orange |
| AP-1 | Activator protein-1 |
| APCs | Antigen presenting cells |
| β | Beta |
| BAF | Backgound adjustment factor |
| β2M | Beta-2-microglobulin |
| $Ca^{2+}$ | Calcium |
| cAMR | Chronic Antibody-Mediated Rejection |
| CD | Cluster of differentiation |
| CDC | Complement-dependent lymphocyte cytotoxicity |
| CDCXM | Complement-dependent cytotoxicity cross-match |
| CFSE | 5,6 carboxyfluorescein diacetate succinimidyl ester |
| CKD | Chronic kidney disease |
| $CO_2$ | Carbon dioxide |
| CON | Control |
| CSIF | Cytokine synthesis inhibitory factor |
| CTLA4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DAG | Diacylglycerol |
| DC | Dendritic cells |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleic triphosphate |
| DSA | Donor-specific antibody |
| DSAXM | Donor-specific antibodies cross-match |
| CY5 | Cyanine-5 |
| EB | Ethidium bromide |
| ELISA | Enzyme-linked immunosorbent assays |

| | |
|---|---|
| ESKD | end-stage kidney disease |
| Fab | Fragment antigen-binding |
| FBS | foetal bovine serum |
| Fc | Fragment crystallizable |
| FCXM | Flow Cytometry cross-match |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| FOXP3 | Factor forkhead box P3 |
| FS | Forward Scatter |
| G-CFS | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GVHD | Graft versus host disease |
| HD | Haemodialysis |
| HLA | Human leukocyte antigen |
| IgG | Immunoglobulin G |
| IP-10 | Interferon gamma inducible protein 10 |
| IP3 | Inositol 1, 4, 5-trisphosphate |
| IP3R | Inositol trisphosphate receptor |
| kDa | Kilodalton |
| LAT | Linker for activated T cells |
| LFA-1 | Leukocyte function antigen 1 |
| LMX | LABScreen mixed assay |
| LSA | LABScreen single antigen assay |
| Mb | Mega base pair |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MFI | Mean fluorescence intensity |
| MHC | Major histocompatibility complex |
| MICA | Major histocompatibility complex class I- related chain A |
| min | Minutes |
| MIP-1α | Macrophage inflammatory protein 1-alpha |
| MIP-1β | Macrophage inflammatory protein 1-beta |
| µl | Microlitre |

| | |
|---|---|
| MLC | Mixed lymphocyte culture |
| MLR | Mixed lymphocyte reaction |
| MNL | Mononuclear leukocytes |
| ng | Nanogram |
| NIH | National Institutes of Health |
| NK | Natural Killer |
| NFAT | Nuclear factor of activated T cells |
| $NF_KB$ | Nuclear factor kappa B |
| OX40L | OX40 ligand |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PD | Peritoneal dialysis |
| PDGF | Platelet-derived growth factor |
| PE | Phycoerythrin |
| pg/ml | Picograms per mililitre |
| PHA | Phytohaemagglutinin |
| $PIP_2$ | Phosphatidylinositol bisphosphate |
| PLCγ1 | Phosphorylation of phospholipase Cγ1 |
| PMPs | Paramagnetic particles |
| PF | Precursor Frequency |
| PI | Proliferation Index |
| RANTES | Regulated on activation, normal T cell expressed and secreted |
| Rh | Rhesus |
| RPMI | Roswell Park Memorial Institute Medium |
| RRT | Renal replacement therapies |
| RT | Room temperature |
| SD | Stained donor cells |
| SAPE | R-phycoerythrin-conjugated strepavidin |
| SOT | Solid organ transplant |
| SR | Stained recipient cells |
| SS | Side Scatter |
| SSP | Sequence-specific primer |

| | |
|---|---|
| SSO | Sequence-specific olignucleotide |
| SSOP | Sequence-specific olignucleotide probes |
| Tc2 | Type 2 cytotoxic T cell |
| TCR | T cell Receptor |
| Th | T helper cell |
| TNF-α | Tumor necrosis factor-alpha |
| Tregs | Regulatory T cells |
| VEGF | Vascular endothelial growth factor |
| ZAP-70 | Zeta chain of T-cell receptor associated protein kinase 70 |

# Chapter 1

# Introduction

# 1. Introduction

## 1.1 *History of kidney transplantation*

End-stage kidney disease (ESKD) can be treated with organ transplantation. This treatment enhances the quality and duration of the life of patients suffering from kidney failure (Batabyal et al., 2012; Shrestha and Haylor, 2014). The history of kidney transplantation is believed to have begun in the early twentieth century, with experimental xenografting (Watson and Dark, 2012). The first successful experimental kidney transplantation was performed by Emerich Ullmann (Vienna, Austria, 1902) who successfully completed the procedure by using the kidney of a dog (Hoffart, 2014)
.

The next milestone in the history of transplantation was made by surgeon Alexis Carrel, whose work led to the improvements of the surgical procedures required for clinical transplantation. In 1912 he received a Nobel prize for his work on blood vessel surgery and organ transplantation. His flawless techniques revealed the limitations of post-transplant rejection (Carrel and Guthrie, 1905; Carrel and Guthrie, 1906; Toledo-Pereyra and Palma-Vargas, 1999; Langer and Kahan, 2002; Sade, 2005).

The first allotransplantation of an organ from a living person was performed in 1952 by the French physician, Jean Hamburger, at the Hospital Necker in Paris. Despite the use of cortisone, a glucocorticoid, released by the adrenal gland, which suppresses the immune system and was administered to prevent transplant rejection, the kidney was rejected after three weeks, and the first biopsy of a rejected kidney was recorded (Legendre and Kreis, 2010; Vanikar, 2014).

After several attempts, the first long-term successful kidney transplant was performed by Joseph E. Murray on December 23, 1954 between genetically identical twins. The transplanted kidney functioned for eight years, until the recipient died, without any apparent immunological problems. In 1990, Murray received the Nobel prize in Physiology and Medicine (Toledo-Pereyra and Palma-Vargas, 1999; Legendre and Kreis, 2010; Vanikar, 2014).

## 1.2 *Underlying diseases leading to end-stage kidney disease*

Kidney failure, or ESKD, is a worldwide pandemic and a major concern to public health. Patients with ESKD can be treated with hemo- or peritoneal dialysis, but renal transplantation continues to be the preferred treatment for ESKD with regard to patient survival (Hariharan, 2002). ESKD is caused by primary underlying kidney diseases, developmental abnormalities or trauma. Several groups of primary kidney diseases, such as arteriopathic renal diseases, glomerulonephritis, diabetes, infective obstructive nephropathy, congenital diseases, hereditary renal diseases, toxic nephropathy, neoplasms, miscellaneous conditions and other diseases can also lead to ESKD (Maisonneuve et al., 2000; Hariharan et al., 2002). See Table1.1 page 3.

**Table 1.1: Underlying diseases that can lead to end-stage kidney disease**

| |
|---|
| Glomerular diseases: |
| • IgA - glomerulonephritis<br>• Membranous glomerulonephritis<br>• Focal segmental glomerulosclerosis<br>• Diabetic nephropathy<br>• Amyloidosis |
| Vasculitis: |
| • Wegener's granulomatosis<br>• Vasculitis and its derivatives<br>• Polyarthritis<br>• Goodpasture's syndrome |
| Urologic diseases: |
| • Renal and urinary tract tumours<br>• Lymphoma of kidney<br>• Congenital obstructive uropathy<br>• Neurogenic/neuropathic bladder |
| Interstitial diseases: |
| • Multiple myeloma<br>• Acute interstitial nephritis<br>• Analgesic nephropathy |

| Hereditary kidney diseases: |
| --- |
| • Alport syndrome<br>• Cystic kidney disease<br>• Hereditary/familial nephropathy, nephrotic syndrome<br>• Steroid-resistant nephrotic syndrome |
| Collagenosis: |
| • Scleroderma<br>• Systemic lupus erythematosus (SLE nephritis) |

Adapted with permission (Maisonneuve et al., 2000).

## 1.3 *Transplantation immunology*

Transplantation immunology refers to a series of events that occurs after an allograft from a donor is transplanted to a recipient. An inflammatory reaction follows resulting in the damage of tissue at the transplantation and graft site.

Sir Peter Medawar's (1915-1987) pioneering work in tissue transplantation brought together surgery and immunology. In 1960, he won a Nobel prize in medicine for his work in organ rejection the findings of which were documented earlier in 1940s, demonstrated the central role of lymphocytes in the rejection process (Gibson and Medawar, 1943; Medawar, 1944; Kahan, 1991).

As already mentioned, the first successful kidney transplantation was performed at the Peter Bent Brigham Hospital in Boston by Joseph E. Murray. Murray performed a kidney transplant between the identical Herrick twins without any immunosuppressive (IS) medication. Both recipient and donor survived the surgery. The recipient survived eight years and the donor died at the age of seventy five, proving Murray's theory that renal transplantation could be safely performed without any IS agents in living patients (Murray, 2011).

After the first successful transplantation between identical monozygotic twins, surgical techniques developed rapidly, but the problem of immunological rejection of tissue was not resolved. The causes and mechanisms of graft rejection were unknown. The next step was the development of immune biology and immunosuppressive medication (Groth et al., 2000).

Kidney transplantation outcomes are generally measured by patients' survival, and the graft survival rates. In general, the steadily-improving graft survival time in clinical renal transplantation and clinical organ transplantation, is attributable to two major factors. Firstly, to the acquisition of more effective and selective chemotherapeutic and immunotherapeutic agents (Merion et al., 1984; Sollinger, 1995; Pirsch et al., 1997; Shapiro et al., 2005; Stucker et al., 2012). Due to use of newer immunosuppressive drugs such as mycophenolate mofetil and tacrolimus, there has been a reduction in cases of acute rejection episodes (Thiel et al., 1994; Sollinger, 1995; Pirsch et al., 1997; Tönshoff and Höcker, 2006). However, no significant improvements have been found to prolong long-term graft survival (Thiel et al., 1994; Lodhi and Meier-Kriesche, 2011).

Secondly, the development of histocompatibility testing procedures with improved sensitivity and precision, including molecular analysis of Human Leukocyte Antigen (HLA) class I and class II antigens, as well as the detection of donor-generated cytotoxic antibodies reactive with recipient alloantigens, which initiate acute rejection, has contributed significantly to prolonged graft survival (Takemoto et al., 2000; Terasaki, 2000; Sheldon and Poulton, 2006; Gritsch et al., 2008; Cecka, 2010). Detection of cytotoxic antibodies is not only a prerequisite for donor-recipient matching, but also critical in the post-transplantation monitoring of allograft sensitisation (Patel and Terasaki, 1969; Terasaki and Ozawa, 2004; Iacomini and Sayegh, 2006; Kimball et al., 2011). However, improvements in histocompatibility procedures based on T cell alloreactivity, an index of cell-mediated chronic rejection (Sayegh and Turka, 1998; Sayegh and Carpenter, 2004; Issa et al., 2008), have been less impressive. Despite the improvements, it cannot be claimed that all problems have been resolved in kidney transplantation. Prevention of antibody-mediated rejection (AMR) still remains a major challenge.

The current study is focused primarily on the development and evaluation of novel procedures for the rapid, reproducible and efficient measurement of T cell alloreactivity both pre- and post-transplantation. As a secondary objective, the 3 currently-available major procedures for the detection of cytotoxic antibodies have been compared to identify which is best suited to optimising pre-transplantation donor-recipient matching (used in conjunction with HLA class I and II typing by high-resolution polymerase

chain reaction (PCR)-based molecular procedures), as well as in the post-transplantation detection of sensitisation to donor alloantigens. The clinical setting for the proposed study is that of living-donor renal transplantation, with the potential for extrapolation to other types of organ transplantation and possibly cadaver transplantation.

## 1.4 *Antibody-mediated rejection (AMR)*

### 1.4.1 Definition

AMR is an allograft rejection that involves donor-specific antibodies (DSA) i.e. antibodies against donor specific (HLA), ABO isoagglutinins and endothelial cell antigens. AMR is a central cause of acute and chronic allograft dysfunction and graft loss. The diagnosis of AMR depends on typical histological lesions, $CD4^+$ T cell staining and serum DSA detection (Montgomery et al., 2004; Singh et al., 2009; Ahmed and Senzel, 2012; Sun and Yang, 2013).

### 1.4.2 Types of antibody-mediated rejection

Renal allograft rejection can be divided into acute T cell-mediated rejection (ACR) and acute AMR based on the biopsy morphology and presence of donor-specific antibody. Antibodies targeting donor antigen can cause various types of symptoms ranging in severity. For histological characterization, the Banff criteria are used (Davis, 2004; Solez, 2010; Solez and Racusen, 2013; Djamali et al., 2014):

- Hyperacute AMR
- Acute AMR
- Chronic AMR

#### 1.4.2.1 Hyperacute AMR

Hyperacute AMR occurs within minutes of revascularization of a transplanted organ. It occurs due to the DSA present in high titers and appears as graft failure after transplantation. It is uncommon for this type of rejection to occur, due to the universal use of histocompatibility procedures (Davis, 2004; Bharat and Mohanakumar, 2017)

In hyperacute rejection, pre-existing antibodies in the recipient's serum bind to donor antigens on endothelial cells and activate the complement and clotting cascades. Preformed alloantibodies recognize vascular endothelial cells and ABO blood type antigens, but they are mainly directed against HLA antigens (Patel and Terasaki, 1969; Terasaki, 2003; Singh et al., 2009; Bartel et al., 2011; Kim et al., 2014; Bharat and Mohanakumar, 2017). See Figure1.1A page 7.

#### 1.4.2.2 **Acute AMR**

Acute AMR is caused by DSA against HLA molecules and other antigens such as non-HLA molecules expressed on endothelial cells. It can be categorized into acute cellular (interstitial and vascular) (Figure1.1B page 8) and acute humoral rejection, as well as their mixed form. Acute AMR usually begins seven days after transplantation and occurs in about 5-7% of all kidney transplants, with about 40% of recipients experiencing graft loss (Davis, 2004; Terasaki and Mizutani, 2006; Morath et al., 2012; Puttarajappa et al., 2012; Wang et al., 2017).

Histopathology in kidney transplant patients is related to endothelial injury mediated by antibodies, which is less severe than that of hyperacute rejection. With the use of newer immunosuppression strategies, graft damage caused by cellular rejection has almost disappeared. AMR rejection has become the major problem for long-term graft survival (Mauiyyedi and Colvin, 2002; Davis, 2004; Terasaki and Mizutani, 2006; Morath et al., 2012; Puttarajappa et al., 2012; Wang et al., 2017). See Figure 1.1B page 7.

#### 1.4.2.3 **Chronic AMR (cAMR)**

Chronic antibody-mediated rejection (cAMR) is one of the main causes of late renal allograft loss. Although, chronic renal failure is caused by various non-immunological and immunological factors, donor-specific anti-human leukocyte antibodies are considered the most detrimental to graft survival. In 2001, chronic rejection was suggested as a new disease entity. It can occur from one month, to years after kidney transplantation. In the early phase there are no symptoms, but later in the course proteinuria and oedema can develop. In cAMR, it is well recognized that antibodies can

mediate chronic allograft injury and chronic rejection. Such mediation is characterized by fibrosis and vascular abnormalities with loss of graft function occurring over a prolonged period (Figure1.1C page 7) (Tanabe and Inui, 2013; Chung et al., 2014; Shrestha and Haylor, 2014).

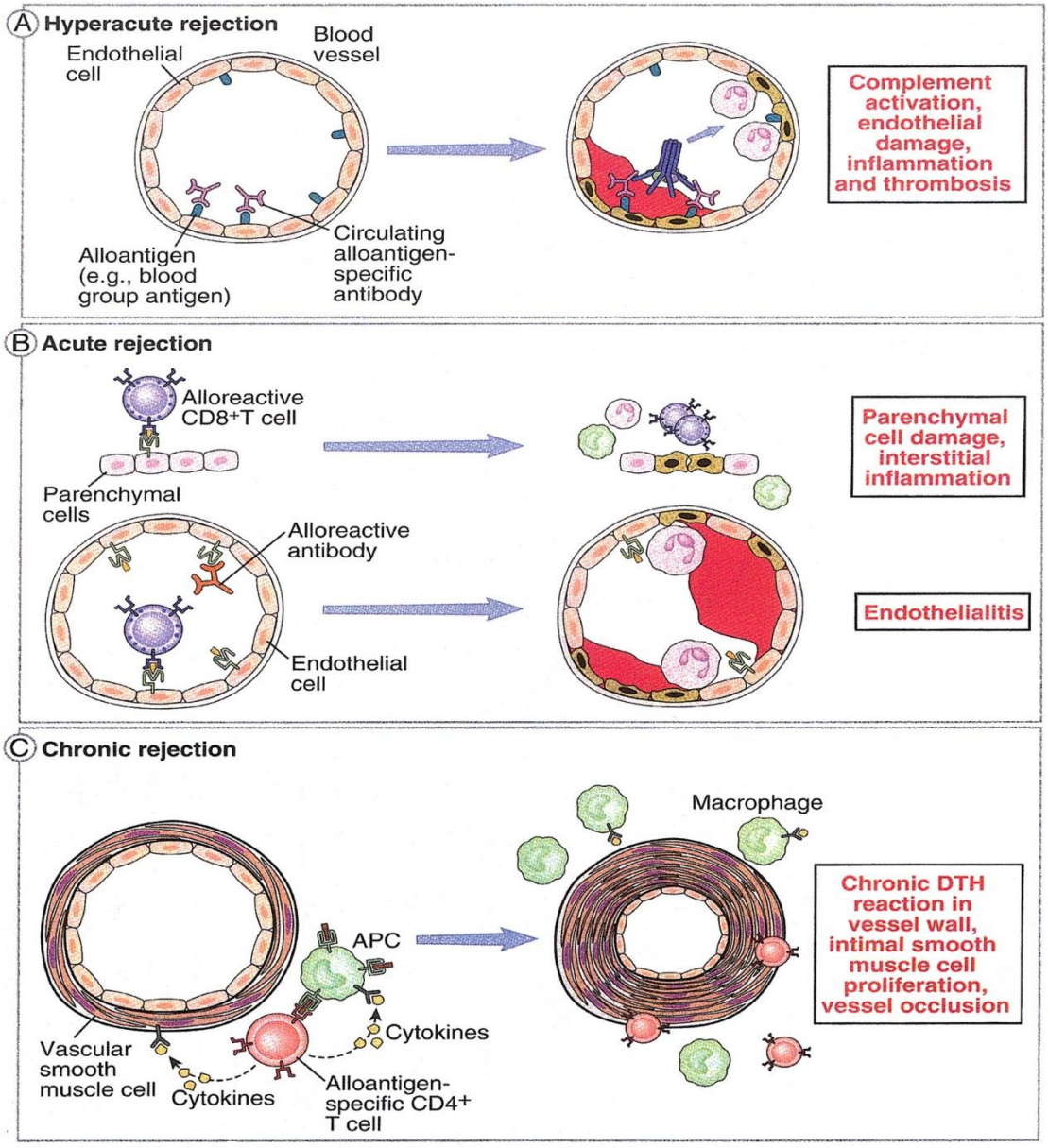


**Figure 1.1A: Hyperacute allograft rejection.** Preformed donor-specific antibodies react with alloantigens, such as blood group or HLA which activates complement causing inflammation.

**Figure 1.1B: Acute allograft rejection.** Cellular form of acute rejection recognizes alloantigen on the graft and causes damage. In the humoral acute rejection, alloreactive antibodies induce vascular injury.

**Figure 1.1C: Chronic allograft rejection.** T-lymphocytes reacting with graft alloantigens may produce cytokines that induce proliferation of endothelial cells, resulting in arteriosclerosis of the graft. Based on reference: (Davis, 2004): with permission.

## 1.5 *Alloantigens*

Alloantigens are histocompatibility antigens present in different form in individuals of the same species. The alloimmune response to a transplant is different from the adaptive response against conventional antigens, posing a unique challenge to the recipient's immune system. Two populations of antigen-presenting cells (APCs) (those of donor origin carried with the allograft and those of recipient origin) can be recognised by recipient T cells (Lombardi et al., 1991; Suchin et al., 2001; Nadazdin et al., 2011; Ayala García et al., 2012; Wood and Goto, 2012; Marino et al., 2016). .

In the context of transplantation, T cell allorecognition is suggested to occur through two different pathways, see Figure 1.2 page 9.

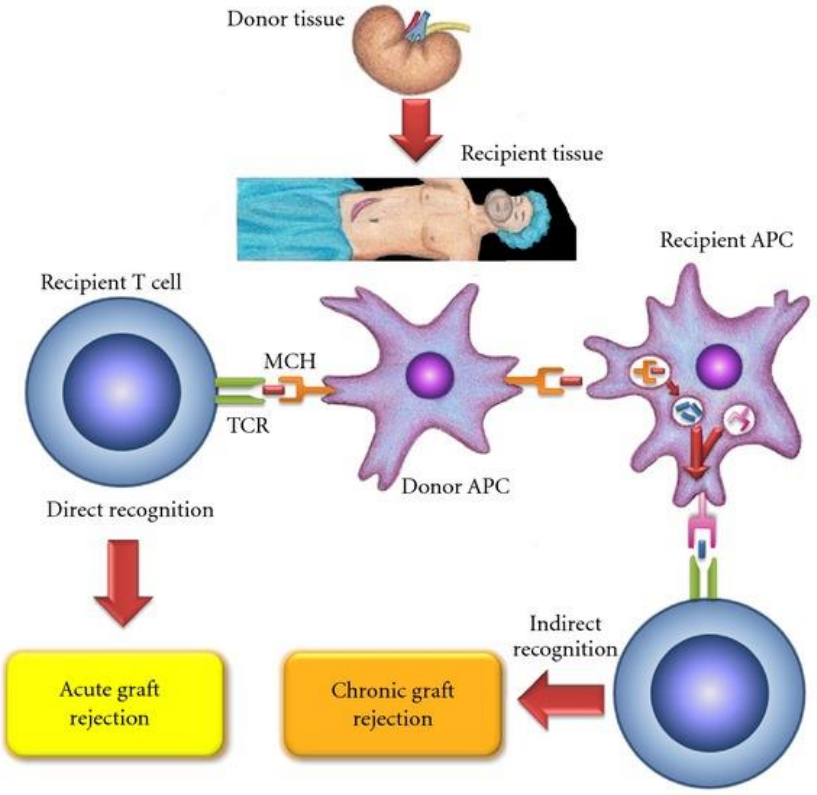


**Figure 1.2: Direct and indirect allorecognition of alloantigens during a mixed lymphocyte culture (MLC) reaction.** Indirect, direct and semi-direct recognition are the three mechanisms recognised to cause an allogenic immune response. Reproduced from (Ayala García et al., 2012): with permission.

### 1.5.1 Direct allorecognition

Direct allorecognition involves the recognition of an intact donor major histocompatibility complex (MHC) molecule on donor APCs by the T cells of the recipient. These molecules may be loaded with peptides produced within the donor's cells before transplantation. The amino acid differences in the α helices of the donor MHC molecule or peptide may, in turn, be recognised by the recipient's T cells (Lombardi et al., 1991; Suchin et al., 2001; Nadazdin et al., 2011; Ayala García et al., 2012; Wood and Goto, 2012; Marino et al., 2016).

### 1.5.2 Indirect allorecognition

The indirect pathway of allorecognition involves recognition of donor MHC or non-MHC peptides, loaded on class II molecules of the recipient's APC's by the recipient's T cells (Brennan et al., 2009; Ayala García et al., 2012).

Several research teams have provided evidence that supports the important role that the indirect-pathway plays in allograft rejection (Cosgrove et al., 1991; Bradley et al., 1992; Fangmann et al., 1992; Priestley et al., 1992; Auchincloss et al., 1993; Sayegh and Carpenter, 1996; Lantz et al., 2000; Honjo et al., 2004; Brennan et al., 2009; Harper et al., 2015)**.** The indirect pathway is known to have a dominant role in chronic rejection and there is also growing evidence that the indirect-pathway of allorecognition may be important early after transplantation. This contention is supported by Auchincloss' demonstration of acute skin allograft rejection in the absence of direct pathway allorecognition. Brennan *et al.* also describes an elegant T cell receptor (TCR)-transgenic model in which direct and indirect-pathway cluster of differentiation (CD)$4^+$ T cells could be simultaneously tracked (Brennan et al., 2009; Ayala García et al., 2012). Graft rejection-mediated via the indirect pathway is believed to be slower and more relevant for chronic allograft rejection (Ali et al., 2016).

## 1.6 *T cell activation*

The TCR provides the primary stimulus for T cell activation. The T cell is held in contact with the APC by interactions between adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) with leukocyte function antigen-1 (LFA-1) that allows the TCR to survey MHC grooves for foreign peptides. The TCR is composed of two chains, *viz.* the α and the β chains, which are complexed with the chains of CD3 on T cells. Binding of alloantigens by the TCR transduces a signal of activation to the T cell via the CD3 complex. In addition to the primary stimulus received via the TCR, naïve T cells require a secondary signal for activation to be completed. This is provided by co-stimulatory molecules on the APCs (Smith-Garvin et al., 2009; Valujskikh et al., 2010; Priyadharshini et al., 2012; Kinnear et al., 2013).

### 1.6.1 Co-stimulation

Three signals are required for the activation of T cells. Firstly, an antigen-specific signal is provided by the interaction of the antigenic peptide-MHC complex with the TCR. This is followed by an antigen-independent co-stimulatory or co-inhibitory signal. This signal is delivered to the T cells by APCs to either promote or inhibit T cell clonal expansion, cytokine production, and effector function. A third signal, mediated by cytokines such as interleukin (IL)-2, further enhances T cell proliferation (Ingulli, 2010; Murakami and Riella, 2014) .

Host T cells can directly recognize donor HLA molecules on graft cells (direct pathway of antigen presentation). The TCR on $CD4^+$ and $CD8^+$ T cells bind peptides presented by HLA class II and HLA class I antigens respectively. Co-stimulatory molecules on the T cell need to be engaged by their cognate ligands, e.g. CD28 / cytotoxic T-lymphocyte-associated protein 4 (CTLA-4):CD80/CD86, CD40:CD154, inducible co-stimulator (ICOS): inducible T cell co-stimulator ligand (ICOSL), OX40:OX40 ligand (OX40L), and CD27:CD70, in order for T cells to be activated (Lechler and Batchelor, 1982; Croft, 2003; Priyadharshini et al., 2012; Ford, 2016). CD28 is located on resting T cells. The CTLA-4/CD28 molecules on the T cell bind to B7 (CD80/CD86) molecules on the APC. Interaction between these molecules triggers a signalling pathway distinct from the pathway initiated via the TCR. The CD40L molecule on the T cell, which binds to CD40 on the APC triggers, an immune response (Lenschow et al., 1992; Baliga et al., 1994; Linsley et al., 1996; Glysing-Jensen et al., 1997; Greenwald et al., 2001; Wells et al., 2001; Ingulli, 2010; Gardner et al., 2014; Murakami and Riella, 2014; Bertelli et al., 2016).

When expressed, it binds to ligands B7.1 (CD80) and B7.2 (CD86) which are expressed on APC. Interaction between these molecules triggers a signalling pathway distinct from the pathway initiated via the TCR. The threshold of TCR signalling to promote T cell proliferation, cytokine production, and differentiation can be reduced by signalling through CD28. The blocking of CD28 signalling on T cells shown in animal models prevents both acute and chronic allograft rejection.(Gardner et al., 2015; Ford, 2016).

CD40, belonging to the gene superfamily of tumour necrosis factor (TNF), is one of the molecules involved in co-stimulation in the adaptive immune response. It is expressed preferentially on B lymphocytes, monocytes/macrophages and dendritic cells. The

CD40L molecule on the T cell, which binds to CD40 on the APC triggers an immune response (Lenschow et al., 1992; Baliga et al., 1994; Linsley et al., 1996; Glysing-Jensen et al., 1997; Greenwald et al., 2001; Wells et al., 2001; Ingulli, 2010; Gardner et al., 2014; Murakami and Riella, 2014; Bertelli et al., 2016).

$CD4^+$ T cells can differentiate into different types of T lymphocytes, that include the T helper (Th)1, Th2, and Th17 cells. Membrane-bound forms of cytokines and soluble cytokines are also involved in co-stimulation. Soluble cytokines include IL-1, IL-6, IL-12 and TNF-alpha (α) which binds to surface receptors on the T cell surface. Th1 cells produce cytokines such as IL-2, and interferon-gamma (IFN-γ), which drive a cell-mediated immune response. In contrast, Th2 cells produce cytokines such as IL-4 and IL-10, which drive antibody-mediated immune responses (Stivaktas, 2008; Chen and Flies, 2013; Liu et al., 2016). Th17 cells play a key role in mucosal immunity, mainly through the production of IL-17, which is highly inflammatory and has been demonstrated as a causative agent in human disease as well as graft rejection (Miossec et al., 2009; Noack and Miossec, 2014; Beringer et al., 2016; Miossec, 2017; Chamoun et al., 2018).

### 1.6.2 Intracellular events following TCR ligation

TCR ligation triggers a cascade of kinase activity. The earliest step in intracellular signalling begins with the activation of the Src family protein tyrosine kinases Lck, and recruitment of the Syk family kinase. The following step is the activation of the cytosolic tyrosine kinase zeta chain of T cell receptor associated protein kinase 70 (ZAP-70) which phosphorylates the trans-membrane protein Linker for activation T cells (LAT) and SLP-76. Together they facilitate activation by tyrosine phosphorylation of phospholipase Cγ1 (PLCγ1). PLCγ1 hydrolyzes the membrane lipid phosphatidylinositol bisphosphate ($PIP_2$), releasing inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG) (Stivaktas, 2008; Smith-Garvin et al., 2009; Chen and Flies, 2013; Perley et al., 2014; Balagopalan et al., 2015; Bilal and Houtman, 2015; Chapman et al., 2015; Su et al., 2016).

The IP3 then stimulates, calcium ($Ca^{2+}$)-permeable ion channel receptors, IP3 receptor (IP3R) on the endoplasmic reticulum (ER) membrane, leading to the release of ER $Ca^{2+}$ into the cytoplasm (Conley et al., 2016; Liu et al., 2016; Porciello and Tuosto, 2016; Schmiedeberg et al., 2016).

$Ca^{2+}$ released, together with membrane events which result in the influx of additional $Ca^{2+}$, allows sustained high $Ca^{2+}$ concentrations in the cytosol. Calcineurin dephosphorylates the transcription regulatory protein, nuclear factor of activated T cells (NFAT), leading to its translocation from the cytoplasm to the nucleus. NFAT moves to the nucleus to its deoxyribonucleic acid (DNA) binding site in the regulatory region of the IL-2 gene, resulting in IL-2 gene transcription. NFAT also induces transcription of genes encoding IFN-γ, IL-4, TNF-α, some of which also require the binding of transcription factor activator protein-1 (AP-1). The diacylglycerol released by PLC-γ1 activates protein kinase C which is involved in the chain of cytosolic signalling events leading to the activation of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells ($NF_KB$) (Stivaktas, 2008; Smith-Garvin et al., 2009; Chen and Flies, 2013; Perley et al., 2014; Balagopalan et al., 2015; Bilal and Houtman, 2015; Chapman et al., 2015; Su et al., 2016). All of these events are summarized in Figure 1.3 page 14.

Ultimately, T cell activation leads to the induction of gene transcription, resulting in cytokine production, blast formation, mitosis and differentiation into functional effector T cells.

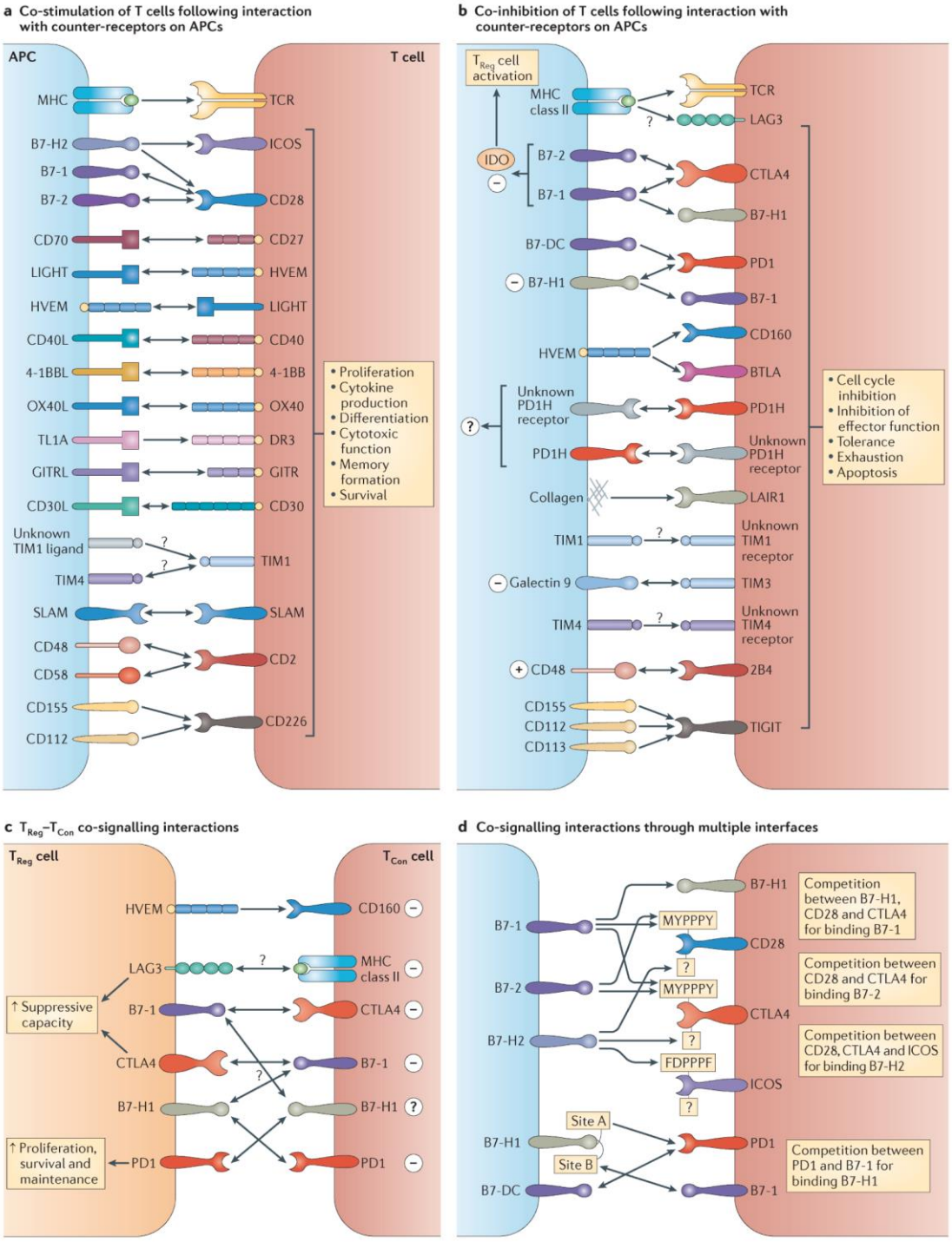


**Figure 1.3: Co-signalling interactions in T cell activation**. Surface molecules involved in co-stimulation, co-inhibition and co-signalling of T -cells (Chen and Flies, 2013): with permission**.**

## 1.7 *Regulatory T cells*

Regulatory T cells (Tregs) are recognized as a major mechanism of T cell suppression and prevention of autoimmunity (Bluestone et al., 2015; Grant et al., 2015; Zhang et al., 2016). The concept of 'suppressed T cells' which are immune cells with suppressor properties were first described in the early 1970s by Gershon and Kondo who suggested that thymocytes in mice are able to suppress the antigen-induced response of other thymocytes without affecting B cells (Gershon and Kondo, 1971).
Subsequently, Hall et al. proved that CD25+ CD4+ T cells were responsible for mediating tolerance in a rat cardiac allograft model (Hall et al., 1985; Hall et al., 1990). Follow-up work showed these cells could control autoimmunity and graft versus host disease (GVHD) (Juvet et al., 2014).

The study by Sakaguchi et al. showed that the interleukin-2 receptor alpha (IL-2Rα),CD25 chain is critical for the control of autoreactive T cells in *vivo*. It acts as a marker for an immune-suppressive T cell population that is derived from the thymus and is important for the prevention of autoimmunity (Sakaguchi et al., 1995; Asano et al., 1996).

The next major breakthrough was the discovery of the X-linked transcription factor forkhead box P3 (FOXP3) in 2003, which was identified as a master regulator of Tregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003).

Two major populations of Tregs with multiple subsets have been characterized (Sharma and Rudra, 2018):

- Natural Tregs, develop in the thymus as a specific T cell lineage to maintain peripheral tolerance against self-antigens and have a role in regulating against autoimmunity (Zhu, 2015; Inomata et al., 2016).
- Adaptive Tregs are differentiated in the periphery from naïve T cells. They are selected to have higher affinity for non-self-antigens and have a role in suppressing immune responses against foreign antigens (Kendal et al., 2011; Zhu, 2015).

## 1.8 *Immunological strategies to assess donor/ recipient compatibility in clinical organ transplantation.*

End-stage renal disease is a global problem. Renal transplantation continues to be the treatment of choice with organ transplantation becoming a routine therapy, despite improvements in dialysis techniques. Such procedures are however, very expensive, necessitating on-going development of immunological strategies to assess donor/recipient compatibility in order to improve the chances of graft survival. The strategies include the following:

### 1.8.1 HLA typing

The pioneers of transplant immunology were the first to recognize the importance of MHC, although its physiological function was discovered much later. In 1937, Peter Gorer discovered a strain-specific antigen in inbred mice which affected the survival of an allogeneic tumour transplant. This discovery played a major role in the development of human organ transplantation (Klein, 1986). For almost forty years thereafter, MHC products were only known for their ability to induce graft rejection. It was only in 1974 when their role as cell surface markers was discovered. This enabled infected cells to signal cytotoxic and T helper cells (Zinkernagel and Doherty, 1974; Mellet et al., 2016).

MHC genes encompass a 7.6 mega base pair (Mb) region on the short arm of chromosome six (6p21) in humans as depicted in Figure 1.4 page 18. The HLA region can be divided into three different sub-regions (McCarty et al., 2010; Ayala García et al., 2012; Mahdi, 2013; Ozaki et al., 2015; Callus et al., 2017). The HLA class I molecules (A,B,C) are found on all nucleated cells, while HLA class II molecules (DR, DP, DQ) have a much more limited distribution being located on APCs including B-lymphocytes, and a few other cell types (T cells, mast cells, basophils, microvascular endothelial cells, β-islet cells), following exposure to antigens and/or pro-inflammatory cytokines (Muczynski et al., 2003; Arnold et al., 2005; Tinckam and Chandraker, 2006; Callus et al., 2017). HLA class I and II regions contain the most polymorphic genes in the human genome. They are made up of a heavy chain (α-chain) encoded within the HLA region, which binds non-covalently to the non-polymorphic light beta-2-microglobulin (β2M) (HLA class I). HLA class II genes encode a heavy α-chain and a

light β-chain as shown in Figure 1.4 page 18 (Davis, 2004; Horton et al., 2004; Vang and Xie, 2017).

The primary functions of HLA class I and II antigens involve the activation of T cells in the processes of immune surveillance (CD8+ cytotoxic T cells) and antigen presentation (CD4+ helper T cells) respectively (Scholl and Geha, 1994; DeBenedette et al., 1999; von Andrian and Mackay, 2000; Ono et al., 2003; Callus et al., 2017). HLA molecules are also potent alloantigens, triggering non-self T-cells, with resultant alloantigen-specific antibody- and cell-mediated immune responses, which promote allograft rejection. HLA incompatibility is generally considered to be the major cause of allograft rejection. Immune responses to non-HLA antigens such as ABO blood group antigens, which are located in humans on chromosome nine, and polymorphic major histocompatibility complex class I- related chain A (MICA) antigens, which are expressed on microvascular endothelial cells, but not on peripheral blood lymphocytes, also result in immune/inflammation-mediated endothelial dysfunction, ischaemia, and graft rejection (Patel and Terasaki, 1969; Kristensen et al., 1976; Sumitran-Holgersson, 2008; Bharat and Mohanakumar, 2017).

Serological typing of HLA antigens was introduced in the 1960s, originally using unfractioned blood mononuclear leukocytes (MNL) to detect HLA class I antigens. The procedure is based on using a panel of antibodies with different HLA specificities contained in the wells of micro-HLA typing trays which are commercially prepared and contain anti-sera to all the common, as well as many HLA alleles which only occur occasionally. Typing is performed by adding test MNL to the wells of the micro-tray, followed by incubation and addition of rabbit complement. Recognition of a specific HLA antigen on the surface of the test MNL by antibody results in complement-mediated cytotoxicity which can be detected using fluorescence microscopy with non-permeant fluorescent dyes (do not penetrate viable cells, but stain dead cells). This procedure was later refined using immunomagnetic bead strategies to isolate pure T and B cell populations from MNL preparations, which can then be used to type HLA class I and II antigens respectively. Although relatively rapid and useful, HLA serological typing lacks the precision inherent in PCR-based molecular typing procedures, with resolution occurring at the amino acid level (Robinson et al., 2003; Claas et al., 2004; Nakatsugawa et al., 2011). In this context, Tinckam and others, reported that more sensitive technologies can be employed to assay HLA such as sequence-specific primer

PCR (SSP), sequence-specific oligonucleotide probes (SSOP), and direct DNA sequencing, which are all molecular-based methods (Tinckam and Chandraker, 2006; Tinckam, 2009; Sun and Xi, 2014; Mellet et al., 2016).

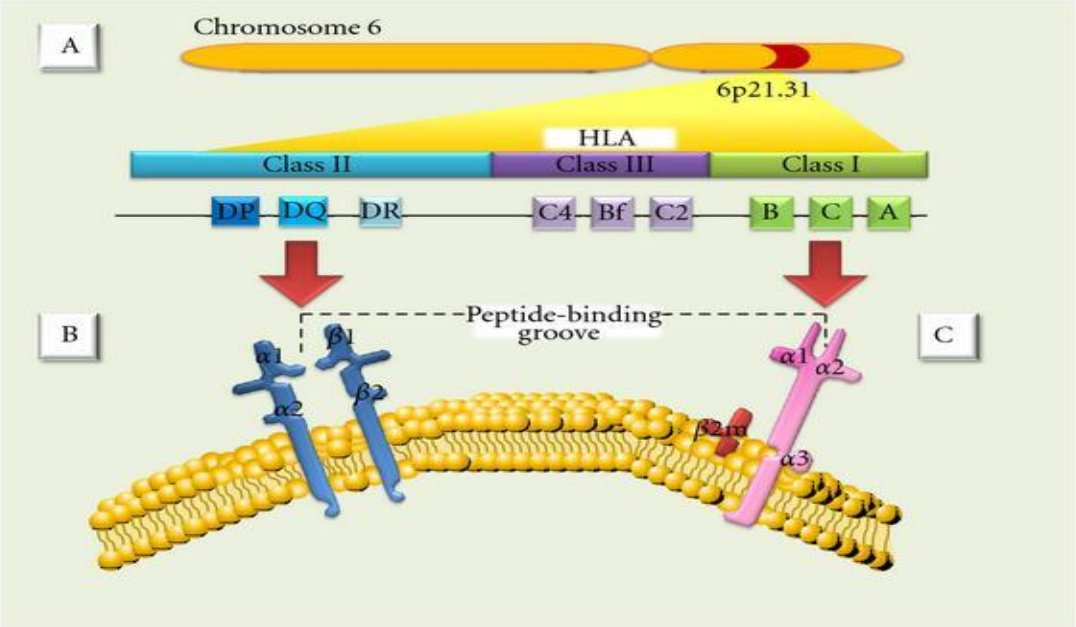


**Figure 1.4: major histocompatibility complex (MHC)** found on the short-arm of chromosome 6. Reproduced from (Ayala García et al., 2012): with permission.

### 1.8.2 Detection of cytotoxic antibodies

Pre-existing, persistent cytotoxic antibodies to donor HLA alloantigens represent a barrier to renal transplantation as these antibodies precipitate acute rejection. Notwithstanding pre-transplantation detection of cytotoxic antibodies, post-transplantation monitoring is of considerable value in detecting recipient sensitization to donor alloantigens. Three procedures are generally used for the detection of anti-recipient cytotoxic antibodies. These include: i) complement-dependent lymphocyte cytotoxicity assays (CDC) using donor lymphocytes and recipient serum; ii) flow cytometry-based procedures also use donor lymphocytes and recipient serum; and iii) solid phase assays using known alloantigens bound to a solid phase, recipient serum, and an appropriate detection system (Tait, 2016).

#### 1.8.2.1 **Cytotoxicity assays**

CDC cross-match was the first method to be developed around 50 years ago (Pei et al., 2003).

In this procedure, donor lymphocytes (isolated T cells and B cells) are incubated with recipient serum and rabbit complement, and stained with a non-permeant dye. The presence or absence of cytotoxic antibodies is then evaluated microscopically. The alloantigen specificity of the cytotoxic antibodies can be established using a panel of lymphocytes, usually from a large number of healthy donors, with different HLA phenotypes. Although it is a useful screening procedure for the detection of cytotoxic antibodies and is still considered the 'gold standard' by many transplant centres, this method does have a number of limitations. These include: i) relatively low sensitivity; ii) antibodies to MICA antigens are not detected; iii) false-positive reactions due to autoantibodies occur; and iv) the antigen spectrum is limited by the test panel (Gebel and Bray, 2000; Heinemann, 2009; Tait, 2016; Callus et al., 2017).

#### 1.8.2.2 **Flow cytometry**

Flow cytometry is a rapid screening procedure for the detection of anti-HLA class I and II antibodies, uncomplicated by the requirement for CDC. Using this procedure, donor MNL are incubated with recipient serum followed by the addition of fluorochrome-labelled, anti-human monoclonal antibodies. Using flow cytometric analysis, the T and B cell populations can be distinguished by using monoclonal antibodies labelled with different fluorochromes to cell-specific CD markers (usually CD3 and CD19 respectively) as can the presence or absence of alloantibodies bound to these distinct lymphocyte sub-populations. The exclusion of false-positive reactions due to autoantibodies is done by including a control system known as an "autocrossmatch", consisting of recipient cells and recipient serum. The major limitation of flow cytometry in allocrossmatching is the lack of availability of the complete spectrum of fluorochrome-labelled HLA-alloantibodies. However, it is a useful screening test in the pre-transplantation setting, and in post-transplantation detection of alloantigen sensitisation (Scornik et al., 1997; Lee and Won, 2011; Tait, 2016; Callus et al., 2017).

#### 1.8.2.3 **Solid phase detection of alloantibodies**

Solid phase assays for detection of alloantibodies in clinical organ transplantation do not utilise donor lymphocytes unlike the aforementioned assays. Instead HLA class I or II alloantigens (or MICA antigens) are coupled to the surfaces of the wells of microtitre plates in enzyme-linked immunosorbent assays (ELISA) or to microbeads in multiplex platforms (Kao et al., 1993; Pei et al., 1999; Zachary et al., 2001; Pei et al., 2003; Tinckam and Chandraker, 2006). The systems can be adapted for the detection of class-specific (HLA class I, HLA class II, MICA) alloantibodies in screening procedures or alloantibodies of single specificity, while microbead-based multiplexing systems such as the Luminex® platform enable the simultaneous detection of multiple specificities (Gebel and Bray, 2000; Heinemann, 2009; Callus et al., 2017). This system uses various microbeads in liquid suspension, each conjugated with a specific alloantigen. The beads contain different ratios of two spectrally distinct fluorophores, thereby assigning a unique spectral identity to each alloantigen-specific bead. The alloantigen-coupled, colour-coded microbeads are then incubated with recipient serum followed by washing and addition of an anti-human immunoglobulin G (IgG) biotinylated antibody, incubation, washing and addition of streptavidin-phycoerythrin (PE) (streptavidin binds to the biotinylated anti-IgG, while phycoerythrin is a fluorochrome). The beads are then analysed using an automated fluorimeter. This highly sensitive semi-automated procedure enables the relatively rapid, simultaneous detection of multiple different alloantibody specificities in a single specimen, and is becoming the method of choice for detection of alloantibodies in both the pre- and post-transplantation settings.

### 1.8.3 **Assays of T cell alloreactivity**

Advances in the methodology for the detection of T cell alloreactivity have been remarkably unimpressive unlike HLA typing and the procedures used for the detection of donor-targeted alloantibodies. Bach and Voynow's original description of the one-way mixed lymphocyte reaction (MLR) in 1966 has undergone little or no improvement or innovation since then (Bach and Voynow, 1966).

### 1.8.3.1 Types of MLC/ MLR

There are two types of mixed lymphocyte culture (MLC), the one-way MLC and the two-way MLC.

#### 1.8.3.1.1 The one-way MLC

The one-way MLC was introduced by Bach and Voynow in 1966 (Bach and Voynow, 1966). It is used for the detection of histocompatibility *in vitro* and has been used to predict and evaluate individual responses to transplanted organs. In this procedure, the response of one cell population is measured. This method involves co-culturing MNL from two individuals, a potential recipient (responder cells), with the inactivated MNL from the potential donor (stimulator cells). For the prediction of GVHD the reverse strategy is used, using stimulator cells from the recipient and responder cells from the donor. The proliferation of the stimulator cells is blocked by irradiation or treatment with mitomycin C. Proliferation is then measured by the incorporation of $^{3}$H-thymidine into the DNA of proliferating responder cells (Kabelitz et al., 1985; Jooss et al., 1988; Sato et al., 1999; Popma et al., 2000).

The major problem of the one-way MLC is that a transplanted organ contains antigen-presenting cells and immunocompetent cells such as T, B and natural killer (NK) lymphocytes, which leave the graft and persist in host tissue for long periods of time. These cells are preactivated (HLA-DR+), CD8+ and CD45RO+ T lymphocytes, which recognize the tissue of the recipient as foreign, resulting in GVHD. Following organ transplantation, the immune reaction is bilateral and therefore it cannot be qualitatively represented by the standard one-way MLC (Schlitt et al., 1993; Richter et al., 1994).

#### 1.8.3.1.2 The two-way MLC

The two-way MLC was introduced by Bain in 1964. In this method, MNL from two individuals are co-cultured for up to seven days. If alloreactive T cells are present, they will be activated and proliferate. Proliferation is measured by incorporation of $^{3}$H-thymidine into the DNA of the dividing cells (Bain et al., 1964). Alternatively, the DNA-binding fluorescent dye 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE) can be used to track cell division flow cytometrically according to loss of

fluorescence. Although useful in assessing compatibility, the limitation associated with the two-way MLC is the inability to distinguish between GVH rejection and host versus graft rejection in the observed allogeneic response (Danzer and Rink, 1996).

#### 1.8.3.2 **CFSE measurement of T cell alloreactivity**

Stivaktas, in her dissertation titled "Comparison of cyclosporin A with mitomycin C and gamma irradiation as inactivators of stimulator cells in the one-way mixed lymphocyte reaction" underscored the limitations of the conventional one-way MLC, advocating instead an alternative two-way MLC based on the flow cytometric detection of the DNA-binding CFSE, to track cell proliferation in the MLC (Stivaktas, 2008). The CFSE-based two-way MLC clearly demonstrated a significant increase in accumulation of daughter cells as early as day 2 during the time-course of the MLC. Importantly, CFSE-based proliferation is amenable to counter-staining with fluorochrome-labelled monoclonal antibodies to phenotype the proliferating cell population (CD3+ T cells). It is also able to detect up-regulation of expression of CD25 (the IL-2 receptor), an early marker of immune activation on these cells, which is correlated with the number of daughter cells. This modified procedure for the detection of T cell alloreactivity has, however, not been evaluated in the setting of clinical organ transplantation. A primary focus of the proposed study was therefore to evaluate the potential of the two-way MLC as an adjunct to molecular HLA typing and detection of cytotoxic alloantibodies using conventional and more recently introduced, sophisticated technologies in optimising pre-transplantation donor/recipient matching and post-transplantation allosensitization in the setting of living-donor renal transplantation. CFSE-based flow cytometric detection of T cell alloreactivity, has been complemented by measurement of expression of early surface markers of T cell activation (CD25, CD69, HLA class II), as well as production of cytokines associated with activation of Th1, Th2, Th17 and cytotoxic T cells.

#### 1.8.3.2.1 Advantages of a CFSE MLC assay

- CFSE labelling enables the detection of proliferating cells within the heterogeneous MLC because CFSE-labelled cells show distinct peaks for each generation of dividing cells (Popma et al., 2000; Wallace et al., 2008; Carollo et al., 2012).
- It is able to detect a T cell response to a lower antigen concentration (Mannering et al., 2003; Wallace et al., 2008).
- CFSE labelling eliminates the use and disposal of radioisototes (Popma et al., 2000; Wallace et al., 2008; Carollo et al., 2012).
- CFSE produces a bright fluorescence signal which can be detected easily by table-top flow cytometers (Popma et al., 2000; Wallace et al., 2008; Carollo et al., 2012).

### 1.8.4 Cytokine analysis

Measurement of cytokines associated with T cell activation in serum or in the supernatant fluids of donor/ recipient MNL co-culture represents an additional strategy to detect activation. The fate of a transplanted organ depends on a variety of factors, but primarily on the cytokine environment. Cytokines can be destructive immune-modulators in graft rejection (Mosmann and Coffman, 1989; Mosmann and Sad, 1996; Holdsworth and Gan, 2015). They may act locally, either on other cell types (paracrine), on the same cell type (autocrine), or systemically (endocrine). They can also be classified as either pro-inflammatory or anti-inflammatory cytokines (Dinarello, 2000; Opal and Depalo, 2000; Wadia and Tambur, 2008; Su et al., 2012). Cytokines can be grouped as lymphokines, interleukins, interferons, chemokines and growth factors (Wallace et al., 2008).

The Th1 cytokines such as IL-2, IFN-γ, and TNF α, promote cell-mediated immune responses, activating cytotoxic T lymphocytes, NK cells and monocytes (Stechova et al., 2007). These cells play a role in graft destruction, directly or by acting on effector cells (Antin and Ferrara, 1992; Nickerson et al., 1997; Alakulppi et al., 2004; Wramner et al., 2004; Wadia and Tambur, 2008; Dhaouadi et al., 2013; Rashad et al., 2016).

Th2-type cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which promote humoral immune responses and may contribute to tolerance and stable graft survival (Boehler et al., 1999; Wadia and Tambur, 2008; Bertelli et al., 2016; Rashad et al., 2016). Circulating IFN-γ was found to be elevated in the circulation of recipients of heart and kidney transplants during rejection (Saiura et al., 2001; Obata et al., 2005). However, it is reported that rejection was aggravated in animal models of heart and kidney transplantation when the IFN-γ gene was knocked out (Halloran et al., 2001; Miura et al., 2003). Therefore, these studies indicated that rejection might not be mediated by Th1-mediated immune mechanisms alone. Recent studies show that Th1 cytokines such as IL-2 and IFN-γ correlate with proliferation in the MLC and as such predict graft rejection (Zhou et al., 2014).

Proinflammatory cytokines such as TNF-α, IL-2 and IFN-γ and IL-6 have been identified as the main factors involved in allograft rejection (Müller-Steinhardt et al., 2002; Alakulppi et al., 2004; Wramner et al., 2004; Dhaouadi et al., 2013; Rashad et al., 2016).

Genetic polymorphisms occur, which result in high levels of the anti-inflammatory cytokine IL-10, which has been shown to inhibit the development and function of Th1 cells, thereby suppressing inflammation, has been associated with allograft tolerance (Asderakis et al., 2001; Xiong et al., 2015; Rashad et al., 2016).

Another subset of T helper cells, Th17 cells, distinct from Th1 or Th2, has been characterized by the production of IL-17A (Ouyang et al., 2008; Atalar et al., 2009; Heidt et al., 2010). These cells orchestrate inflammatory responses which are characterized by the activation of neutrophils and monocytes/macrophages (Miura et al., 2003; Korn et al., 2009). Th17 cells have also been associated with allograft rejection (Afzali et al., 2007; Atalar et al., 2009), with elevated IL-17A levels having been associated with renal and lung graft rejection in humans (Loong et al., 2002; Vanaudenaerde et al., 2006; Vanaudenaerde et al., 2008; Deteix et al., 2010).

Although the mid-1990s saw a decrease of acute renal allograft rejection occurrences, an estimated 10-20% of patients still suffer an episode of acute rejection (Magee and Pascual, 2004). Mechanisms fundamental to this process are complex and are not completely understood. Very few studies have investigated the role of IL-17A in

allograft rejection, including clinical renal transplantation (Van Kooten et al., 1998; Loong et al., 2002; Yapici et al., 2011). The contribution of Th17-derived cytokines to allograft rejection pathology therefore, remains to be completely elucidated and may be of potential importance in developing new therapies in transplantation (Normanton and Marti, 2013).

## 1.9 *Hypothesis, Aims, Objectives*

### 1.9.1 Hypothesis

The CFSE-based 2-way MLC procedure, complemented by detection of early markers of T cell activation and cytokine production, is a useful index of T cell alloreactivity, and will optimise pre-transplantation histocompatibility testing and post-transplantation monitoring of allosensitisation in living-donor renal transplantation. Alternatively, this procedure will prove to be of little value as an adjunct to molecular HLA typing and antibody-based procedures for the detection of allosensitisation.

### 1.9.2 Aims

The ultimate aim of transplantation diagnostics is to identify optional donor/recipient compatibility in the shortest time. This thesis addresses the following:

- Development and evaluation of novel procedures for the rapid, reproducible and efficient measurement of T cell alloreactivity both pre- and post-transplantation, with the aim of optimizing donor/ recipient compatibility in renal transplantation.
- Comparison of the three currently-available procedures for the detection of cytotoxic antibodies to identify which of these is best suited to optimizing pre-transplantation donor-recipient matching, as well as in post-transplantation detection of sensitization to donor alloantigens.

### 1.9.3 Objectives

- Measurement of donor and recipient HLA genotype and ABO, Rhesus (Rh) phenotypes (once-off), as well as circulating alloantibodies (HLA class I, HLA class II, MICA) in all potential recipients.
- Measurement of pre-transplantation T cell alloantigen reactivity to donor lymphocytes in all recipients.
- Cross-matching of T cell alloreactivity using flow cytometry.
- Measurement of post-transplantation recipient sensitization to donor alloantigens using solid phase alloantibody procedures.
- Assessment of time taken to allosensitization according to immunological (T cell alloreactivity/cytotoxic antibody) and renal function criteria, as well as time taken to initiation of step-up/step-down immunosuppressive therapy.
- Evaluation of two-way CFSE MLC markers counterstained with CD25, CD69, HLA class II for the measurement of expression of early surface markers of T cell activation.
- Measurement of circulating cytokines associated with activation of Th1, Th2, Th17 and cytotoxic T cells on the recipient serum and in supernatant fluid of MLC.

The following chapters of this thesis consist of a description of methodology followed by three chapters of results each with a separate abstract, results, discussion and reference sections and, finally, an overall general conclusions and limitations section.

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# Chapter 2

# Evaluation donor/ recipient compatibility in clinical organ transplantation: Methodology and Study design

# 2. Evaluation donor/ recipient compatibility in clinical organ transplantation

## 2.1 *Introduction*

Various obstacles of genetic and immunological nature are encountered with solid organ transplants (SOT). The rejection response to grafted tissue is caused by cell surface molecules that induce an immune stimulus. These cell surface molecules include MHC/ HLA, minor histocompatibility antigens and ABO blood grouping antigens (Ayala García et al., 2012; Ferrari-Lacraz et al., 2012). The impact of HLA antibodies on transplanted organs during the early years of clinical renal transplantation has already been discussed in Chapter 1. Hence, the detection of cytotoxic antibody status is one of the most important investigations to be undertaken in potential organ transplant recipients (Bittencourt et al., 1998; Eng et al., 2008; Kupatawintu et al., 2016; Baranwal et al., 2017).

## 2.2 *Patients and study design*

Patient demographic and clinical data is discussed in detail in Chapters 3 and 4 of the dissertation. The present study is a prospective investigation with the primary objective of evaluating the measurement of donor and recipient HLA genotype and ABO, Rh phenotypes (once-off), as well as circulating alloantibodies (HLA class I, HLA class II,) in all potential recipients. In addition, the novel carboxyfluorescein succinimidyl ester (CFSE)–based two-way MLC procedure, complemented by detection of early markers of T-cell activation and cytokine production, will be tested as an index of T-cell alloreactivity, which will optimise pretransplantation histocompatibility of allosensitisation in living-donor renal transplantation.

The study was approved by the Research Ethics Committee of the Faculty of Health Science, University of Pretoria and conformed to good laboratory practice and with the 1964 Helsinki declaration and its later amendments (2013) or comparable ethical standards. The purpose and objective of the study was explained in detail to both patients and donors and all participants were given the opportunity to read the consent document (Appendix B). Upon agreement to participate, they were requested to sign

the consent document. Written informed consent was obtained from each individual, prior to enrolment into the study**.** Ethics certificate reference number: 242/2013.

The blood was drawn by the phlebotomy services of Steve Biko Academic hospital, the National Health Laboratory Service of South Africa and the Netcare coordinators from patients who were being evaluated for renal transplantation, as well as from potential related donors. The results are reported anonymously (no identifiers were used that would enable results to be tracked to individual participants).

Fifteen patients who were candidates for renal transplantation from Steve Biko Academic Hospital and Jakaranda Private Hospital, Pretoria and their potential donors were enrolled in the study from September 2014 through April 2015. Blood samples were drawn pre-transplant from both recipients and donors and 8 weeks post-transplant from recipients only. The blood was collected in acid-citrate-dextrose (ACD) and clotted blood collection tubes. The respective samples were used for the following analyses.

***Acid-citrate-dextrose (ACD)*** (blood) was used for the following assays:

- ABO and Rh blood typing, which was performed within 72 hours post collection.
- Complement dependent microlymphocytotoxicity CDC cross-match and flow cytometry crossmatch: For these assays, samples were processed and analysed within 72 hours ensuring maximal cell viability.
- DNA extraction for HLA genotying. DNA was isolated and if not processed immediately, was stored at -70ºC until use.

***Clotted blood*** (serum) was used for the following assays

- Cross-match
- Antibody detection

An additional ethylenediaminetetraacetic (EDTA)-containing tube was requested for isolation of MNL for MLR and cytokine assays.

## 2.3 *Laboratory Methods*

The patients were cross-matched with their potential donors by three different methods. Firstly, by means of the NIH Terasaki complement-dependent microlymphocytotoxicity cross-match (CDCXM); secondly, by flow cytometry cross-match (FCXM) and thirdly, by Luminex® based Donor Specific Antibody cross-match (DSAXM).

### 2.3.1 **ABO-Rh red blood cell grouping**

One of the limitations of renal transplantation has been the ABO blood group system. ABO incompatibility can result in hyperacute humoral rejection of primarily vascularised grafts (Ayala García et al., 2012; Ferrari-Lacraz et al., 2012). Hence ABO-compatibility, between the recipient and donor, remains a defining criteria for transplantation in our centre.

ABO-Rh red blood grouping was performed using a standard agglutination procedure. All reagents (monoclonal antibodies) were supplied by BioTec Laboratories, (Bridport, Dorset, United Kingdom).
The participants (donor and recipient) blood groups were determined by using forward RBC typing, mixing one drop of the patients- or donors whole blood, which contains antigen, with one drop of known anti-A, anti-B and anti-Rh reagent, which contains antibody on a slide. The slide was tilted back and forth and observed for agglutination. The presence of antigen-antibody reactions resulted in visible agglutination and the blood group was determined to be A, B, or AB. No agglutination with anti-A or anti-B was observed for group O and samples showing no agglutination with Rh were considered Rh- negative.

### 2.3.2 **Complement-dependent microlymphocytotoxicity cross-match (CDCXM)**

The CDCXM assay was performed using the standard two-stage National Institutes of Health (NIH) technique (Patel and Terasaki, 1969; Peña et al., 2013). Briefly, a drop of liquid paraffin was added to each Terasaki tray. Donor T- and B-cells were isolated using CD2 and CD19 monoclonal antibodies (One Lambda, Inc. Hannover, Germany)

attached to beads. One microlitre (µl) of donor cells was incubated with 1µl of recipient serum for 30 minutes (min) at room temperature (RT). After incubation, 5µl rabbit complement was added and incubated for a further 60 min at RT. The cells were then stained with 5µl of fluoroquench$^{TM}$ AO/EB (One Lambda, Inc. Hannover, Germany), a fluorescent dye cocktail, containing acridine orange (AO) and ethidium bromide (EB), and incubated for 10 to 15 min at RT. The cells were observed for cytotoxicity using an immunofluorescence microscope. Dead cells stained red with EB, while viable cells stained green with AO as shown in Figure 2.1 A and B. The presence of donor-specific antibodies (DSA) bound to the donor cells is indicative of the complement cascade having been activated via the classical pathway resulting in lysis of lymphocytes. CDC results for T- and B-cells respectively were considered positive when cell death exceeded that of the negative control by 20%.

A B

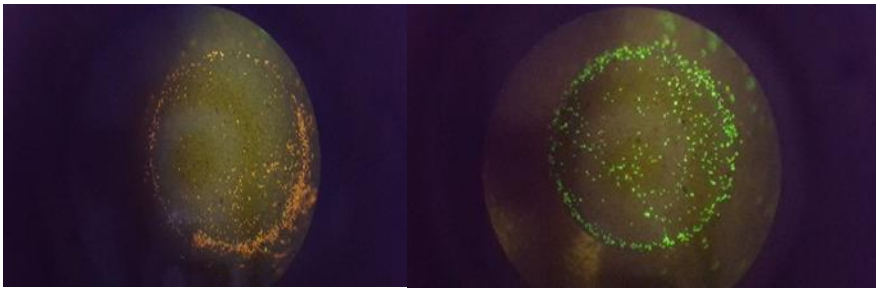

**Figure 2.1: Images of cells stained with fluoroquench$^{TM}$ dye:** A. Immunofluorescent microscopic results of dead cells stained red with EB.
B. Immunofluorescent microscopic results of viable cells stained green with AO.

### 2.3.3 Flowcytometric cross-match (FCXM)

FCXM was performed using serum samples from patients and peripheral blood MNL isolated from donor blood. In short, 100µl of donor lymphocytes ($1x10^6$ cells/ml) were mixed with 20µl of patient sera and incubated for 60 min at RT. Following three washes with phosphate-buffered saline (PBS: 5min at 600xg) the samples were incubated for further 30 min with fluorochrome-labelled antibodies, 5µl of phycoerythrin-cyanine-5 (CY5) labelled anti-CD3 (Beckman Coulter, USA), 5µl of phycoerythrin-labelled anti-CD19 monoclonal antibody (Beckman Coulter, USA), and 10µl of goat $F(ab)_2$ anti-human IgG-fluorescein isothiocyanate (FITC) antibody (Beckman Coulter, USA) at RT in the dark to identify alloantibodies and differentiate between T and B cells. Finally,

two wash steps were performed and cells were re-suspended in 200µl of PBS prior to analysis.

Multicolour flow cytometric analysis was performed using the Cytomics FC 500 instrument (Beckman Coulter, USA). Viable lymphocytes were gated on the basis of their forward and side-scatter characteristics. Positive flow cytometry cross-match results were expressed as a mean channel shift to the right of either the T-peak and/or the B-peak values.

### 2.3.4 **Luminex based donor-specific antibody cross-match (DSAXM)**

DSAXM was performed as per the manufacturer's instructions (Gen-Probe, LIFECODES, Stamford, CT, USA). Briefly, 30ml of donor lymphocytes were isolated by layering whole blood on 15ml of Histopaque®-1077, a lymphocyte density separation medium (Sigma-Aldrich, St Louis, MO, USA.) Following centrifugation (1000g with break off for 20 min at RT), the layer of cells at the density separation media interface was collected. The cells were then pelleted by centrifugation (1000g for 10 min, at RT) and washed three times in cell culture media by gentle centrifugation (1000g, 10 min, RT) to pellet the cells. The final wash supernatant was discarded and the pellet stored at -70ºC for future use.

Donor lysate was prepared by adding diluted lysis buffer (1:10 dilution) to the cell pellet. The cell suspension was then mixed by vortexing to completely lyse the cells. The mixture was centrifuged at 1000g for 5 min to sediment the cell membranes and the supernatant (lymphocyte lysate) was transferred into a clean labelled tube (Huh et al., 2012).

Donor lysate and control lysate (8µl) were incubated with 5µl of capture beads for 30 min at RT in the dark with mixing at 10 min intervals to enable binding of the solubilized donor HLA molecules with the immunofluorescent beads. A volume of 42µl of wash buffer was added to the bead/lysate mixture, and 55µl of this mixture was then transferred to the wells of a 96-well filter plate. The plate was then washed three times using a vacuum pump. A volume of 38µl of specimen diluent and 12µl of patient's serum were added, and the plate was incubated on an orbital shaker (Thermo Fisher Scientific, MA USA) for 30 min at RT in the dark. Following another three washes,

50µl of diluted IgG PE conjugate (SAPE) was added to all the wells containing donor lysate and lysate control and 50 µl diluted conjugate concentrate to all wells containing serum sample and serum control. Following a final 30 min incubation on an orbital shaker, at RT in the dark, the plate was washed three times and 150µl of wash buffer was added to each well. The plate was then analysed using a Bio-Plex suspension array system, Luminex 100 analyser (Bio-Rad Laboratories, USA).

A captured bead is determined to be positive or negative for DSA by comparing the mean fluorescence intensity (MFI) value of each bead with three control (CON) beads in each well. The cut off values (background adjustment factors) calculated for a CON bead was subtracted from the MFI value of the capture bead. The process was repeated for each of the remaining two CON beads to obtain three results (Adjusted MFI Values). A sample is considered positive if two or more Adjusted MFI Values are positive and a sample is considered to be negative if two or more Adjusted MFI Values are negative.

### 2.3.5 **HLA antibody analysis**

HLA-DSA detection was determined using solid-phase immunoassay (SPI). Serum samples from the recipients were analysed for Class I and Class II IgG HLA antibodies using the commercially available LABScreen SAB assay kit (One Lambda, Inc., Canoga Park, CA, USA) on a Luminex® 100 IS platform (Bio-Rad Laboratories, USA). The procedure was performed according to the manufacturer's instructions. Specificity of HLA class I and II IgG antibodies in recipient sera was determined using the LABScreen mixed (LMX) and single antigen assay (LSA) (One Lambda, Canoga Park, CA). Twenty µl of serum was first incubated with 5µl of LABScreen® beads (Mixed, class I or class II), followed by three washes using wash buffer to remove unbound antibodies. Alloantibodies present in the test serum bind to the antigen-coated beads which are labelled with 100µl of R-Phycoerythrin (R-PE)-conjugated goat anti-human IgG.

All incubations were performed on a gently rotating platform (Thermo Fisher Scientific, MA USA) in the dark at RT for 30 min. After the final wash step, 80µl of PBS buffer was added to each well and the samples were analyzed using Luminex® 100 IS v 2.3 software (Bio-Rad Laboratories, USA) for data acquisition. Data analysis was done with HLA Fusion software (One Lambda, Inc.). Results with MFI values over

1000 were regarded as positive. This procedure enables detection of the entire spectrum of alloantibodies in the recipient's serum.

### 2.3.6 HLA tissue typing

HLA Class I and Class II play a critical role in both cellular- and antibody-mediated alloresponses, which in turn, play a critical role in the longevity of a transplanted organ. The major impact of loss of graft comes from the effects of HLA-B and HLA-DR antigens. Few months after transplantation, the effects of HLA-DR are the most prominent HLA-B related-rejection occurs within the first two years while HLA-A mismatches have a harmful effect on long-term graft survival (Mahdi, 2013; Callus et al., 2017).

Various HLA test methodologies are performed in our centre to support the transplant program. Routinely HLA Class I typing is conducted by serologic testing while Class II is done using sequence-specific primer (SSP) molecular typing methodology. For this study a third DNA-based sequence-specific oligonucleotide (SSO) high-definition typing method was used in order to ascertain whether it had higher sensitivity, accuracy and more resolving power than the serologic and SSP typing methods.

#### 2.3.6.1 Serological typing

This procedure was based on using a panel of antibodies with different HLA specificities pre-coated in the wells of commercially prepared micro-HLA typing plates (BAG Health Care GmbH, Amtsgerichtsstrβe, Germany).

Recognition of a specific HLA antigen on the surface of the test MNL by antibody results in complement-mediated cytotoxicity which can be detected using fluorescence microscopy with the aid of Fluoroquench® a non-permeate fluorescent dye (does not penetrate viable cells, but stains dead cells). Non-lysed cells fluoresce green, while lysed (positive) cells fluoresce red.

The assay was performed by adding 1µl MNL T cells separated using immunomagnetic beads (One Lambda, USA) to the wells of the micro-tray, followed by 30 min incubation at RT. A further 5µl rabbit complement was added to each well and incubated for 1 hour at RT. Those antisera which reacted positively were reported as the HLA types of the patient.

### 2.3.6.2 **Molecular typing**

DNA-based tissue typing techniques (SSP and SSO) require purified DNA.

#### **2.3.6.2.1 DNA sample preparation**

Genomic DNA from the MNL was extracted from whole blood (350µl) using the Maxwell®16 Instrument (Promega Corporation, USA), which is an efficient automated purification process. The instrument was designed for use with predispensed reagent cartridges, maximizing simplicity and convenience. Following DNA extraction, the purity of the DNA was determined and the concentration adjusted to approximately 20 nanograms (ng)/µl for SSO and 25-200 ng/µl for the SSP technique.

#### **2.3.6.2.2 Micro SSP™ HLA DNA typing**

The Micro SSP™ DNA typing test (One Lambda, Inc., USA) distinguishes between the different alleles during the polymerase chain reaction (PCR) process. The Micro SSP™ DNA typing trays provide sequence-specific oligonuleotide primers for amplification of HLA alleles and the human β-globin gene by PCR. Pre-optimised primers are present in different wells of a 96-well tube tray for PCR. The method was followed as described by the manufacturer. Thirty nine microliters (µl) of DNA sample and 2 µl recombinant Taq polymerase were added to 360µl deoxyribonucleic triphosphate (dNTP)-buffer mix (D-mix) and mixed well. Ten µl of D-mix/DNA mixture were then added to each well. Matched primer pairs result in the amplification of target sequences. Separation of amplified DNA fragments by 2% agarose gel electrophoresis is then performed followed by visualisation by nucleic acid gel stain, gel red (20µl) (Biotium, CA, USA) and exposure to ultraviolet light. HLA Fussion software from One Lambda, Inc. was used to interpret the PCR results based on the presence or absence of a specific amplified DNA fragment.

#### 2.3.6.2.3 PCR-SSO-Luminex method

The LABType®SSO (One Lambda, Inc., USA) method uses SSO probes bound to fluorescently coded microspheres to identify alleles encoded by the DNA. LABType® applies Luminex® technology, to the reverse SSO DNA typing method. The method was followed as outlined by the manufacturer.

Firstly, target DNA was PCR-amplified using a group-specific primer. The PCR product was then denatured and allowed to rehybridize to complementary DNA probes conjugated to fluorescently coded microspheres. The microsphere mixture consisted of a set of fluorescently labelled microspheres that bore unique sequence-specific oligonucleotide probes for HLA alleles. Each microsphere mixture included negative and positive control microspheres for subtraction of non-specific background signal and normalization of raw data in order to adjust for possible variation in sample quantity and reaction efficiency.

The PCR product was biotinylated, allowing for detection using R-phycoerythrin-conjugated strepavidin (SA-PE). Analysis was done using a Luminex® 100 which identifies the fluorescence intensity of PE on each microsphere. The assignment is done by software (Fusion 2, One Lambda, USA) and the HLA type is based on the reaction patterns compared to known patterns associated with published HLA gene sequences.

### 2.3.7 CFSE-based two-way MLC

$^{3}$H-thymidine uptake has been a standard method for measuring proliferation in the MLC. However, due to the hazards and costs associated in working with radioactivity, alternative non-radioactive methods have been investigated. CFSE is recognized as being a favourable alternative. It provides the cheapest alternative and has been shown to correlate well with $^{3}$H-thymidine uptake (Stivaktas, 2008; Quah and Parish, 2012; Lašťovička et al., 2016).

The two-way MLC procedure is used to measure histocompatibility between two different individuals. In this method, the mononuclear layers (MNL) from two unrelated or related individuals are cultured together for 1-5 days in the presence of CSFE, a nontoxic green fluorescent dye, which is taken up by the cells, and is trapped irreversibly within the cytosol of the cell. On division of the cells, the CFSE-labelled macromolecules of the cell segregate into two daughter cells allowing each daughter

cell to contain half the CFSE content of the parent cell. The fluorescence intensity in each generation of daughter cells is therefore half that of the previous generation. Proliferating cells can then be tracked using flow cytometry. CFSE-labelled cells can also be counterstained with other fluorescent dye-labelled monoclonal antibodies and viability markers to allow for the phenotyping of the proliferating population, and to determine the viability of these cells. The CFSE-based MLC assay is divided into two stages: i) CFSE labelling of cells and ii) flow cytometric CFSE-based two-way MLC.

#### 2.3.7.1 **CFSE-labelling of cells**

Both donor and recipient MNL were labelled with CFSE (Molecular Probes, Eugene, OR, USA) as follows: Recipient MNL at 1 x $10^6$ cells/ml, were suspended in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich, St Louis, MO, USA) (5ml), were incubated with 1µl of 0.5µM CFSE (concentration predetermined in preliminary experiments) at 37ºC for 7 min. The staining was terminated by washing with equal volume of cold culture medium containing 10% foetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) at 200rpm for 10 min at 4°C. After washing twice with cold culture medium, both recipient and donor CFSE-labelled cells were resuspended in RPMI to 5ml and adjusted to a concentration of 1 x $10^6$ cells/ml in RPMI 1640 culture medium.

#### 2.3.7.2 **Flow cytometric CFSE-based two-way MLC**

Each sterile, non-pyrogenic 15ml test tube received 900µl of a 1 x $10^6$ cells/CSFE-labelled MNL suspension and 900µl of a 1 x $10^6$ cells/CSFE-unlabelled MNL in RPMI 1640 culture medium and 200µl FBS. Autologous control systems of each donor received 1.8ml of 1 x $10^6$ cells/ml CSFE-labelled MNL in RPMI 1640 culture medium. The tubes were incubated for 5 days at 37ºC in a humidified 5% carbon dioxide ($CO_2$) incubator. followed by flow cytometric analysis of: i) loss of CSFE fluorescence and ii) expression of T cell activation markers; CD25, CD69 and HLA class II (antibodies specification refer to appendix C) as indices of donor-recipient alloantigen responsiveness, which were then analysed on days 1, 3 and 4 of culture using a Beckman Coulter Cytomics FC 500 instrument (Beckman Coulter, Miami, Florida, USA). Cells (200µl) were added to 10µl of monoclonal antibodies (CD25, CD69, and

HLA-DR) and incubated for 10 min at RT in the dark. For accurate counting, 200µl of Flow-Count™ Fluorospheres (Beckman Coulter, Miami, FL, US), were added prior to the samples being analysed. Lymphocytes and blast populations were identified and gated using a forward scatter (FS) vs. side scatter (SS) scattergram (explained in details section 5.3.2).

Fluorescence data for CFSE and CD25, CFSE and CD69, as well as CFSE and HLA DR were collected from gated regions. The fluorescence measurements were then displayed on dual parameter plots of CFSE vs. CD25 PE, CFSE vs. CD69 PC5 and CFSE vs. HLA DR FITC. The percentage positive CD25, CD69 and HLA DR cells and counts of accumulated daughter cells were derived from gated regions.

### 2.3.8 **Cytokines**

Circulating cytokine profiles, as well as those present in MLR supernatants, representative of Th1, Th2 and Th17 cells were determined using the Bio-Plex suspension bead array system (Bio-Rad Laboratories, Hercules, CA, USA). This system utilizes the Luminex xMAP® multiplex technology which permits multiplexing of up to 100 different analytes within a single sample. The system uses a liquid suspension array consisting of microspheres. The beads are filled with different ratios of two different fluorescent dyes, resulting in transmission of a unique spectral identity.

Analysis of the sample was performed using a Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA, USA. Precision fluidics aligns the beads in a single file through a flow cell where two lasers excite the beads individually. The red classification laser excites the dye in each bead, identifying the specific bead address. The green reporter laser excites the reporter molecule associated with the bead, allowing quantitation of the captured analyte. High-speed digital signal processors and Bio-Plex Manager software record the fluorescent signals simultaneously for each bead, translating the signal into data for each bead-based assay. The cytokine analysis kits were obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

The following analytes were measured simultaneously using a 27-plex kit: interleukin (IL)-1β, IL-1ra, IL-5, IL-7, IL8, IL-9, IL-12p70, IL-13, IL-15, eotaxin, basic fibroblast growth factor (FGF), interferon gamma-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-

1α), macrophage inflammatory protein 1- beta (MIP-1β), platelet-derived growth factor (PDGF) BB, regulated on activation, normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF). The method was followed as described by the manufacturer. Briefly, the cytokine levels were determined in either plasma (1:4 dilution) or MLR (undiluted) samples. Fifty microliters of pre-mixed antibody covered magnetic beads were added to each of the wells of 96 well plate. Appropriate standards provided with the kits, were added to the wells to facilitate quantification of the selected cytokines. An additional internal control from pooled plasma from volunteer donors was added onto each of the plates for internal quality control. The samples were then added to the designated wells. The plates were sealed and incubated with agitation on an orbital shaker for 2 hours at room temperature. The sample fluid was removed, and the plate washed three times using an automated plate washer (Bio-Rad Laboratories Hercules, CA, USA) after which 60µl of streptavidin-PE was added, washed for three times and incubated as described above for 30 minutes. After the streptavidin-PE incubation step, the fluid was removed followed by a further three washes and resuspension of the beads in assay buffer, prior to being assayed on a Bio-Plex Suspension Array platform (Bio-Rad Laboratories Hercules, CA, USA). Bio-Plex Manager Software 6.0 was used for beads acquisition and analysis of median fluorescence intensity. Results are reported as pg/ml.

## 2.4 *Statistical analyses*

Data was summarized using descriptive methods (median, range, standard deviation, frequency and percentage). The experiments were done three times over three days. Difference testing employed the Fisher's exact and the Mann-Whitney tests (Wilcoxon rank-sum) for discrete and continuous variables respectively where applicable. Spearman's rank correlation coefficients, with 95% confidence intervals (CI), were used to determine correlations between the various parameters, with values of 0.75-0.99, 0.5-0.74, and 0.25-0.49 indicating high, moderate and low degrees of correlations. Comparisons were considered to be statistically significant if the two-tailed P-values were less than 0.05.

## 2.5 *References*

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# Chapter 3

# Detecting preformed human leukocyte antigen antibodies in a South African kidney transplant population: evaluating three laboratory methods

# 3. Detecting preformed human leukocyte antigen antibodies in a South African kidney transplant population: evaluating three laboratory methods.

## 3.0 *Abstract*

**Background**

Transplant guidelines advocate the screening of kidney transplant patients' serum for donor-specific anti-human leukocyte antibodies (anti-HLA). If present, these autoantibodies may lead to graft rejection and loss.

**Objectives**

This is a prospective study; three methods were compared to detect cytotoxic anti-HLA antibodies, to establish which of these would optimise pre-transplant donor-recipient matching.

**Methods**

Serum samples from 15 pre-operative renal transplant recipients were tested for the presence of anti-HLA antibodies. Three methods used to analyse donor lymphocytes and recipient sera were: i) cytotoxic-dependent cross-match (CDCXM), ii) flow cytometric cross-match (FCXM) and iii) Luminex-based donor-specific antibody cross-match (DSAXM). Luminex was used to confirm the presence of preformed HLA antibodies.

**Results**

All three methods identified positive HLA Class I cross-match in two (13%) of the 15 patients. The CDCXM and FCXM identified positive HLA Class I in the same two patients. The DSAXM identified positive HLA Class I in two (13%) patients, one who tested positive using CDCXM and FCXM. The CDCXM identified positive HLA Class II in six (40%) of the 15 patients. The DSAXM and FCXM identified positive HLA Class II in 2 (13%) and 1 (7%) of patients, respectively. When the 4 non-congruent CDCXM HLA Class II positive samples were analysed with the lymphocyte single

antigen (LSA) assay, no HLA Class II antibodies were identified, indicating that these 4 (67%) samples tested false-positive.

**Conclusions**

The DSAXM method delivered the most accurate results for identifying anti-HLA antibodies in prospective, pre-sensitised kidney transplant patients.

## 3.1 *Introduction*

Chronic kidney disease (CKD) is a worldwide public health problem that can be managed using renal replacement therapy which requires either dialysis or organ transplantation. However, dialysis is life-long and is associated with reduced quality of life and increased risk of mortality. Compared to dialysis, kidney transplantation offers better survival and quality of life for patients with end-stage kidney disease (Alheim et al., 2015; Tait, 2016; Baranwal et al., 2017; Callus et al., 2017).

According to the South African Renal Registry Annual Report of 2015, 10 360 patients were on renal replacement therapies (RRT). In South Africa, Gauteng province had the highest number of patients at 3238; of whom 958 were from the public sector and 2280 from the private sector. Of the 10 360 patients, 1 440 (13.9%) were on peritoneal dialysis and 7 529 (72.2%) were on haemodialysis. Of all these patients, only 1 391 (13.4%) underwent renal transplantation (Davids et al., 2017), underscoring that South Africa has one of the lowest organ donation rates in the world. The total organ donation rate in South Africa is approximately two-million donations per year, compared to 13 million in the UK and over 30 million in Spain (Davids et al., 2017; Sparaco, 2017; Venter, 2017). In South Africa, the potential of kidney transplantation has not been realised, resulting in transplant waiting lists of between two and seven years (Montgomery et al., 2011; Steyn, 2011; Pauly, 2012; van Heerden and du Plessis, 2016; Bhatt and Das, 2017).

To ensure the optimal use of available organs, best-practice methods should be applied when screening for potential rejection risk. In clinical renal transplantation, rejection risk can be predicted by detecting pre-formed human leukocyte antigen (HLA)

antibodies reactive with transplanted organs (Worsley et al., 2012; Ramparsad, 2014). Presently, complement dependent cytotoxicity cross-match (CDCXM) is the predominant pre-transplant cross-match technique used in South African settings (Worsley et al., 2012; Ramparsad, 2014). This technique is limited by the low viability of cells in both cadaver and living-related donor screening. Therefore, in this study we evaluate alternative methods for detecting recipient serum antibodies directed against donor antigens (Bittencourt et al., 1998; Eng et al., 2008; Kupatawintu et al., 2016; Baranwal et al., 2017).

## 3.2 *Methods*

The participants were cross-matched with their corresponding donors using three different methods. The 3 procedures detect donor alloreactive antibodies and consisted of: (i) the NHI / Terasaki microlymphocytotoxicity technique (CDCXM), (ii) the flow cytometric-based cross-match (FCXM) and; (iii) the Luminex-based donor specific antibodies cross-match (DSAXM). The Luminex-based single antigen fluoro-analysis was performed to confirm the presence of preformed HLA antibodies. All methodologies are comprehensively described in Chapter 2.

## 3.3 *Results*

The recipient group comprised 9 (60%) females and 6 (40%) males, and the living-related donor group consisted of 6 (40%) females and 9 (60%) males. The mean age of the recipients was 39.3 (range 21-61 years) and the mean donor age was 41.7 (range 23-58 years), with Caucasians accounting for 73% and Africans accounting for 27% from each group. Recipients and their potential donors were all blood group compatible. The types of renal replacement therapy received by the participants were haemodialysis (HD) ($n$=9) and peritoneal dialysis (PD) ($n$=6) and the average duration of dialysis before transplantation was 23.5 months (ranging from 6 months to 46 months). Nine (60%) patients were diagnosed with hypertensive nephropathy, 2 (13%) with SLE, 1 (7%) with glomerulonephritis, 1 (7%) chronic allograph nephropathy, 1 (7%) chronic glomerulonephritis and 1 (7%) for which the diagnosis was not stated. Donor and recipient profiles are shown in Table 3.1.

| **Table 3.1: Recipient-donor profile for 15 allograft recipients awaiting kidney transplants in South Africa (September 2014- April 2015)** | | |
|---|---|---|
| **Variables** | **Donors (n=15)** | **Recipients (n=15)** |
| **Age in Years - Mean (SD)** | 47.7 (11) | 39.3 (13.4) |
| | | |
| **Gender - n (%)** | | |
| Male | 9 (60) | 6 (40) |
| Female | 6 (40) | 9 (60) |
| | | |
| **Ethnicity- n (%)** | | |
| Caucasian | 11 (73) | 11 (73) |
| African | 4 (27) | 4 (27) |
| | | |
| **Blood Type- n (%)** | | |
| A | 5 (33) | 7 (47) |
| B | 1 (7) | 4 (27) |
| O | 9 (60) | 3 (20) |
| AB | 0 (0) | 1 (6) |
| | | |
| **Medical history (recipients) - n (%)** | | |
| Diabetic mellitus | | 1(7) |
| Hypertension | | 12(80) |
| Hyperuricaemia | | 2(13) |
| Systemic lupus erythematosus (SLE) | | 2(13) |
| Hypothyroidism | | 2(13) |
| Hypercholesterolaemia | | 5(33) |
| Unknown | | 1(7) |
| Dialysis (HD/PD) | | 9/6 |
| Average duration (mon) | | 23.5(10.5) |

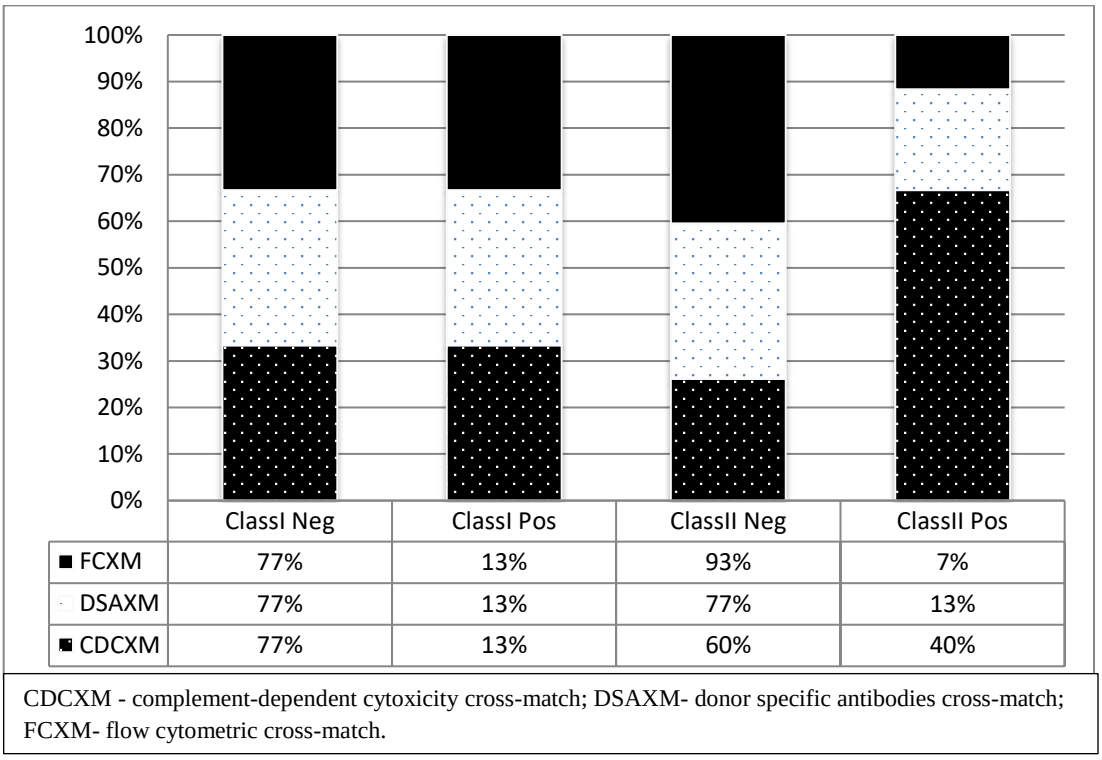
As shown in Table 3.2, the CDCXM identified seven (47%) of the fifteen patients as class I- or class II-positive. Two (13%) patients were class I-positive, 13 (87%) were class I-negative, while 6 (40%) patients were class II-positive and 9 (60%) were class II-negative. One (7%) patient was class I- and class II-positive.

**Table 3.2: Results comparing the three cross-match methods for patients awaiting kidney transplants (September 2014 – April 2015)**

| | **NIH** | | **Luminex** | | **Flow** | |
|---|---|---|---|---|---|---|
| | CDCXM | | DSAXM | | FCXM | |
| **Patient** | Class I | Class II | Class I | Class II | Class I | Class II |
| **1** | N | P | N | N | N | N |
| **2** | N | N | N | N | N | N |
| **3** | N | N | N | N | N | N |
| **4** | N | P | P | P | N | N |
| **5** | N | N | N | N | N | N |
| **6** | N | N | N | N | N | N |
| **7** | P | N | P | P | P | N |
| **8** | N | N | N | N | N | N |
| **9** | N | N | N | N | N | N |
| **10** | P | P | N | N | P | P |
| **11** | N | P | N | N | N | N |
| **12** | N | P | N | N | N | N |
| **13** | N | P | N | N | N | N |
| **14** | N | N | N | N | N | N |
| **15** | N | N | N | N | N | N |
| Total Positive - n (%) | 2 (13) | 6 (40) | 2 (13) | 2 (13) | 2 (13) | 1 (7) |
| Total Negative - n (%) | 13 (87) | 9 (60) | 13 (87) | 13 (87) | 13 (87) | 14 (93) |
| N = Negative; P= Positive | | | | | | |

The results of the CDCXM, FCXM and DSAXM assays are presented collectively in Figure 3.1. For all three methods, two (13%) patients were true HLA class I positives. Both the CDCXM and FCXM assays identified the same patients as having antibodies

to HLA class I, whereas DSAXM only identified one of these patients, as well as another unrelated one. CDCXM presented a high percentage of positive HLA class II (40%), while DSAXM identified only two (13%) patients and FCXM identified one (7%). HLA antibody screening using HLA class I and class II single antigens (LSA) was performed for all samples to detect possible antibodies against the donor antigens.




| | ClassI Neg | ClassI Pos | ClassII Neg | ClassII Pos |
|---|---|---|---|---|
| FCXM | 77% | 13% | 93% | 7% |
| DSAXM | 77% | 13% | 77% | 13% |
| CDCXM | 77% | 13% | 60% | 40% |

CDCXM - complement-dependent cytoxicity cross-match; DSAXM- donor specific antibodies cross-match; FCXM- flow cytometric cross-match.

**Figure 3.1: Comparison between cross-match testing methods to identify HLA antibodies in recipients awaiting kidney transplants.**

Table 3.3 shows results of 4 patients who were CDCXM class II positive, however LSA class II results did not detect any antibodies reactive against donor antigens.

**Table 3.3: LSA results for the four patients transplanted with positive HLA CDC cross-match**

| Patient | HLA Class I PRA | Alleles | HLA Class II PRA | Alleles |
|---|---|---|---|---|
| **1** | 5% | A66, B45, B60, B76. | 2% | DPQ1, DPQ5 |
| **11** | 14% | A2, B18, B41, B45, B48, B60, B61, B71, B72, B76. | 0% | |
| **12** | 1% | B73 | 0% | |
| **13** | 0% | | 0% | |

## 3.4 *Discussion*

Renal transplantation is a RRT option of choice in patients with end-stage kidney disease (Dieplinger et al., 2014; Haque and Rahim, 2017). In South Africa, the CDCXM method is still widely used in the pre-renal transplantation work-up to match recipients and donors (Worsley et al., 2012; Ramparsad, 2014), and is used routinely in our setting for both deceased and living donor transplantation in order to evaluate histocompatibility between recipients and donors. A negative CDCXM assay before transplantation is known to significantly reduce the risk of hyperacute rejection (Lee et al., 2017). A positive CDCXM, on the other hand, indicates the presence of HLA antibodies; these antibodies, especially DSA, are associated with graft rejection and eventual graft loss (Gloor et al., 2010; Bentall et al., 2013; Chung et al., 2014; Chung et al., 2018). However, the CDCXM is known for its lack of sensitivity, resulting in both false negative and -positive results. False-negative results may occur if there are low levels of DSA or if HLA antibodies belonging to the immunoglobulin (Ig)G2 and IgG4 subclasses are unable to activate the complement cascade and remain undetected (Mulley and Kanellis, 2011; Roelen et al., 2012; Haque and Rahim, 2017). Additionally, CDCXM can also react with non-specific antibodies (i.e. IgM antibodies), leading to false-positive reactions. Accordingly, more specific and sensitive techniques are required to match recipients to potential donors (Bose et al., 2013; Tait, 2016; Haque and Rahim, 2017). In this study, HLA antibody testing was used to confirm the CDCXM results and found a 40% false-positive rate for HLA class II, confirming the

lack of sensitivity of the CDCXM. These results suggest that the CDCXM HLA class II method has no diagnostic application. On the other hand, CDCXM HLA class I testing, which has limited application in cadaver donor transplant, may be beneficial in living-related donor transplantation when used in conjunction with a more sensitive cross-match method.

Luminex DSAXM is a new procedure in our local setting. This procedure is not widely used for routine diagnoses in tissue immunology centres in South Africa, increasing the local relevance of this study. Although DSAXM class II false results have occasionally been reported (Billen et al., 2009; Lal and Mishra, 2015; Mishra and Lal, 2016; Vimal et al., 2017). In this study, the DSAXM results for HLA class II correlated well with FCXM results. Although the sample size was small, DSAXM gave better results than the CDCXM assay eliminating the reliance on viable cells. DSAXM uses donor lysate, which can also be stored for future analyses. In addition, DSAXM detects only IgG DSA and is therefore more sensitive (Eng et al., 2008; Huh et al., 2012; Chacko et al., 2016).

The DSAXM and FCXM methods performed equally well. Four patients with positive CDCXM HLA class II reactions were negative with DSAXM and FCXM HLA class II. In addition, the LSA results did not detect any HLA class II antibodies (Table 3). However, one patient with a positive FCXM reaction was negative with DSAXM. This patient was pre-sensitised, as LSA detected, but did not have specific antibodies to the donor's HLA antigens. It is therefore concluded that, despite the small size of the study group, which is a limitation for this type of study, DSAXM could help to identify pre-sensitised kidney transplant patients who may not reject the donor graft.
In conclusion, these findings suggest that CDCXM should be replaced with more sensitive methodologies such as FCXM, DSAXM or LSA.

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# Chapter 4

# Association of pretransplant cytokine levels with graft outcome among renal transplant recipients.

# 4. Association of pretransplant cytokine levels with graft outcome among renal transplant recipients.

## *4.0 Abstract*

The success of renal transplantation relies, in most part on the prevention of graft rejection by the host's immune system, which recognizes alloantigens present in the transplanted organ. Cytokines play a critical role in the development of the alloimmune response leading to rejection.

**Aim**

To evaluate pretransplant cytokine levels in plasma and supernatant of MLC compared to graft outcome in renal transplant recipients and to ascertain whether cytokines could be used as early predictors of graft rejection.

**Patients and Methods**

Patients awaiting renal transplant together with their living related donors were enrolled in the study. A panel of 15 patients and plasma from 4 healthy, volunteer control donors were analysed. A multiplex cytokine detection kit was used that is able to detect 27 different cytokines per sample. The average age of the patients was 39.9 ± 13.4 years (range 21-61). The total average duration of renal replacement therapy before the renal transplant was 23.5 ± 10.5 months.

**Results**

Patients were distributed into two groups *viz.* those who rejected the renal transplant (G1) and those with a successful graft (G0). Median concentrations of the 27 cytokines tested in both plasma and MLC supernatant were higher in the G1 group as compared to G0, however, different cytokine secretion patterns were observed. Increased cytokine median concentrations were observed in plasma. Of the 27 cytokines analysed, twenty-six cytokines did not show any statistically significant difference between the groups, however, the eosinophil chemotactic protein, eotaxin ($p=0.03$) was higher in G1 compared to G0. Higher median concentrations, albeit not attaining statistical significance, of anti-inflammatory cytokines were observed for G1, in both the plasma and supernatants of the MLC.

**Conclusion**

The results of this study suggest that the higher median concentration of anti-inflammatory cytokines in those patients that rejected their organ grafts may be indicative of the initiation of kidney rejection episodes.

## 4.1 *Introduction*

Despite significant advances in immunosuppressive therapy, employing more sensitive HLA matching methods and improvement in transplant care, acute and chronic rejection still remain major problems with organs received from both deceased and living donors (Karczewski et al., 2008; Karczewski et al., 2009; Karczewski et al., 2010; Nazari et al., 2013; Alves et al., 2015).

Success of renal transplantation relies on preventing graft rejection by the recipients' immune system. The key inflammatory, soluble peptide mediators involved in development of an alloimmune response leading to acute rejection are called cytokines. T cells are a crucial component of the immune response and they are important immunoregulatory cells involved in the activation of B cells, dendritic cells and macrophages, with the many functions of these cells mediated by cytokines (Priyadharshini et al., 2012). Cytokines play an important role in directing the magnitude and type of immune response mounted against the organ transplant. T-helper (Th)1-type cytokines mediate the cellular response,Th2-type cytokines are known to mediate the humoral immune response, while Th17-type cytokines are known to be involved in inflammatory processes during infection and organ-specific auto immune diseases (Harrington et al., 2005; Park et al., 2005; Abadja et al., 2012; Jin and Dong, 2013; Kuwabara et al., 2017). There is evidence that Th1-associated cytokines are involved in allograft rejection while Th2 cytokines are associated with tolerance. However, other studies have shown that Th2-related cytokines can also be involved in acute kidney rejection. In addition, Th17-associated cytokines are also known to contribute to graft rejection (Amirzargar et al., 2005; Karczewski et al., 2008; Karczewski et al., 2009; Nazari et al., 2013; Kamińska et al., 2016; Cortvrindt et al., 2017; Erol et al., 2017).

The aim of the present study was to evaluate whether an association exists between pretransplant cytokine levels and graft outcome in renal transplant recipients.

## 4.2 *Methods*

Circulating cytokine profiles, as well as those present in mixed lymphocyte reaction (MLR) supernatants, representative of Th1, Th2 and Th17 cells as well as Growth Factors were determined using the Bio-Plex suspension bead array system as described in Chapter 2. The Bio-Plex system utilizes the Luminex xMAP® Multiplex technology. The analytes measured in the Multiplex system include:

| **Pro-inflammatory** | **Anti-inflammatory** | **Chemokines** | **Growth Factors** |
|---|---|---|---|
| G-CSF | IL-1RA | IL-8 | VEGF |
| GM-CSF | IL-4 | Eotaxin | PDGF-BB |
| IFN-γ | IL-9 | MCP-1 (MCAF) | FGF |
| IL-1β | IL-10 | MIP-1α | |
| IL-2 | IL-13 | MIP-1β | |
| IL-5 | | RANTES | |
| IL-6 | | | |
| IL-7 | | | |
| IL-12 (p70) | | | |
| IL-15 | | | |
| IL-17 | | | |
| IP-10 | | | |
| TNF-α | | | |
| The abovementioned panel constitutes known cytokines, chemokines and growth factors associated with rejection episodes. | | | |

## 4.3 *Results*

Demographic data of patients are shown in Table 4.1. The average age of the total number of patients was 39.9 ± 13.4 years (range 21-61). The group that rejected the kidney transplant had an average age of 30.7 ± 1 years while the group with successful grafts had an average age of 41.5 ± 14.5 years. Nine (60%) of the kidney transplant patients included in this study were females and six (40%) were males. The average duration of dialysis before transplantation was 23.5 months (ranges from 6 months to 46 months). The dialysis duration was longer in the group that rejected the renal transplant (31.7 months) as compared to the successfully grafted group, which was 21.4 months. The types of renal replacement therapy received by the participants were haemodialysis (HD) (*n*=9) and peritoneal dialysis (PD) (*n*=6).

A total of 15 renal transplant patients and 4 healthy, volunteer controls were recruited. The patients were grouped according to those who underwent successful kidney transplant (G0, *n*=12), those who rejected the graft (G1, *n*=3) and a control group (G2, *n*=4).

**Table 4.1: Demographic data of patients**

| Variables | Total (n=15) | Rejected (n=3) | Successful graft (n=12) |
|---|---|---|---|
| Age (years ± SD) | 39.3 ±13.4 | 30.7±0.9 | 41.5±14.5 |
| Sex (F/M) | 9/6 | 1/2 | 8/4 |
| Dialysis (HD/PD) | 9/6 | 2/1 | 7/5 |
| Duration of dialysis (months) | 23.5 ±10.5 | 31.7±10.1 | 21.4±9.6 |
| Deceased | 1 | 1 | 0 |

### 4.3.1 **Comparison of pre-transplant cytokine concentrations between groups**

Prior to statistical analysis, the normality of the data distribution was evaluated by the Shapiro-Wilk test. Statistical significance was determined using the distribution of data with Kruskall-Wallis test being used for non-normal distributed data and the unpaired t-test for normally distributed data. The results were expressed as median concentration between the 25th and the 75th inter-quartile rage (Table 4.2). The median concentrations of the pro-inflammatory cytokines, including the chemokines (IL-1β, IL-6, IL-7, IL-8, IL-12, IL-15, IL-17, INF-γ, TNF-α, eotaxin, MCP-1, MIP-1α, MIP-1β and RANTES), as well as those of the growth factors

(FGF, G-CSF, GM-CSF, VEGF and PDGF-bb) were higher in the transplant rejection group (G1) compared to the successfully grafted group (G0), while IL-2 and IP-10 levels were slightly higher in G0 (Table 4.2).

**Table 4.2: Plasma cytokine levels in renal transplant patients and healthy controls.** Results are presented as pg/ml for the 25, 50 and 75 percentiles.

| Cytokine Concentration (pg/ml) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Group 0 (*n*=12) | | | Group 1 (*n*=3) | | | | Group 2 (*n*=4) | | |
| | Percentiles | | | Percentiles | | | | Percentiles | | |
| | 25 | 50 | 75 | 25 | 50 | 75 | p value | 25 | 50 | 75 |
| **Pro-inflammatory** | | | | | | | | | | |
| **IL-1β** | 3 | 15 | 34 | 5 | 45 | 62 | | 5 | 5 | 10 |
| **IL-2** | 11 | 32 | 54 | 0 | 29 | 371 | | 7 | 23 | 67 |
| **IL-6** | 31 | 65 | 147 | 16 | 248 | 968 | | 19 | 22 | 37 |
| **IL-7** | 9 | 12 | 15 | 11 | 17 | 106 | | 25 | 41 | 58 |
| **IL-8** | 109 | 1742 | 4709 | 1038 | 2113 | 12192 | | 47 | 51 | 84 |
| **IP-10** | 970 | 1860 | 2276 | 1423 | 1581 | 1993 | | 678 | 746 | 985 |
| **IL-12** | 54 | 64 | 92 | 71 | 95 | 481 | | 51 | 103 | 200 |
| **IL-15** | 0 | 7 | 20 | 0 | 58 | 113 | | 0 | 0 | 8 |
| **IL-17** | 53 | 114 | 168 | 46 | 131 | 542 | | 61 | 81 | 314 |
| **INF-γ** | 49 | 70 | 103 | 71 | 108 | 1705 | | 140 | 177 | 296 |
| **TNF-α** | 49 | 66 | 92 | 45 | 132 | 717 | | 100 | 115 | 185 |
| **Eotaxin** | 101 | 163 | 208 | 236 | 333 | 563 | 0.030 | 81 | 90 | 110 |
| **MPC-1** | 183 | 322 | 684 | 379 | 498 | 3023 | | 43 | 55 | 62 |
| **MIP-1α** | 6 | 11 | 94 | 19 | 48 | 145 | | 7 | 7 | 13 |
| **MIP-1β** | 787 | 1538 | 4539 | 1812 | 2161 | 2772 | | 151 | 176 | 305 |
| **RANTES** | 20825 | 52001 | 72642 | 24737 | 110604 | 148428 | | 13528 | 13689 | 19607 |
| **Anti-inflammatory** | | | | | | | | | | |
| **IL-1Rα** | 145 | 230 | 367 | 132 | 264 | 3700 | | 257 | 457 | 688 |
| **IL-4** | 8 | 11 | 15 | 11 | 14 | 49 | | 12 | 14 | 24 |
| **IL-5** | 0 | 1 | 2 | 1 | 1 | 42 | | 6 | 9 | 17 |
| **IL-9** | 68 | 119 | 145 | 74 | 110 | 1061 | | 31 | 34 | 66 |
| **IL-10** | 11 | 20 | 41 | 21 | 33 | 282 | | 18 | 49 | 216 |
| **IL-13** | 5 | 10 | 15 | 5 | 15 | 109 | | 17 | 26 | 57 |
| **Growth factors** | | | | | | | | | | |
| **FGF** | 68 | 88 | 101 | 71 | 111 | 361 | | 89 | 102 | 173 |
| **G-CSF** | 171 | 248 | 275 | 218 | 389 | 1397 | | 616 | 666 | 840 |
| **GM-CSF** | 133 | 214 | 266 | 200 | 250 | 520 | | 0 | 1 | 2 |
| **VEGF** | 82 | 213 | 404 | 460 | 600 | 774 | | 0 | 0 | 7 |
| **PDGF-bb** | 2841 | 4671 | 8107 | 3699 | 8595 | 8787 | | 653 | 778 | 1654 |

The median concentrations of the anti-inflammatory cytokines (IL-1Rα, IL-5, IL-10, IL-13, the pro-inflammatory cytokines IL-12 and INF-γ) and the growth factor G-CFS were all higher in the control group (G2) when compared to G0 and G1. On the other hand, IL-1RA, IL-4, IL-10 and IL-13 were higher in G1 when compared to G0. The levels of IL-4 did not differ significantly between groups G1 and G2 and IL-9 levels were highest in G0. Due to the very small number of participants in the study group, no statistically significant differences were observed in the 2 groups with the exception of eotaxin (p=0.03) and VEGF that almost reached statistical significance (p=0.08); consequently, the results should be regarded as preliminary, and conclusions drawn as tentative. Nevertheless, definite trends in respect of increased systemic levels of pro- and anti-inflammatory cytokines were evident in those patients experiencing rejection episodes.

Cytokines were measured as continuous variables. Aiming to further categorise the cytokine profile, the results were grouped into categories of 4 groups from the highest group to the lowest group. The analysis among the subgroups were performed by means of testing differences between categories with the Fisher's exact method. Patients in the G0 and G1 groups were compared according to their distribution: highest (quantile 4) versus lowest (quantile 1) as shown in Table 4.3.

From the results obtained, it was observed that one of the patients that rejected the graft was in the highest quantile for the pro-inflammatory cytokines: IL-2, IL-6, IL-7, IL-12, IL-15, IL-17, INF-γ, TNF-α and eotaxin, the growth factors: FGF, G-CSF, GM-CSF, VEGF, PDGF-bb and the anti-inflammatory cytokines: IL-1RA, IL-4, IL-5, IL-9, IL-10 and IL-13. Two more scores were then created: firstly, the highest two quantiles (3 and 4) versus the lowest two quantiles (1 and 2). These results indicate that for 24(89%) of the 27 cytokines, two of the rejection category patients fell in to the high category. Proportionally, there were more patients that rejected kidney grafts in the high category than in the low category. Lastly, the highest quartile (4) was compared with the three lower quartiles, (1, 2, 3 *vs* 4). At least one patient that rejected was in the highest quartile for 25 (92%) of the 27 cytokines tested. One patient who underwent graft rejection, in particular, appeared in the highest quantile for 20 (75%) of the 27 cytokines measured. Proportionally, patients who underwent successful graft fell in the low category rather than the high category (Table 4.4).

**Table 4.3: binary representation of cytokine levels in renal transplant group and rejection.** The 0 in the cytokine level column represents quantiles 1, 2 and 3 combined, while a score of 1 represents quantile 4 of the cytokine levels detected. Group 0 represents the successful graft group and Group 1 represents those individuals with rejected kidney transplants in the rejection columns.

**Pro-inflammatory Cytokines**

| IL-1β | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 10 | 2 | 12 |
| 1 | 2 | 1 | 3 |
| Total | 12 | 3 | 15 |

| IL-2 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 11 | 2 | 13 |
| 1 | 1 | 1 | 2 |
| Total | 12 | 3 | 15 |

| IL-6 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 11 | 2 | 13 |
| 1 | 1 | 1 | 2 |
| Total | 12 | 3 | 15 |

| IL-7 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 9 | 1 | 10 |
| 1 | 3 | 2 | 5 |
| Total | 12 | 3 | 15 |

| IL-8 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 12 | 3 | 15 |
| 1 | 0 | 0 | 0 |
| Total | 12 | 3 | 15 |

| IL-12 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 9 | 1 | 10 |
| 1 | 3 | 2 | 5 |
| Total | 12 | 3 | 15 |

| IL-15 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 11 | 2 | 13 |
| 1 | 1 | 1 | 2 |
| Total | 12 | 3 | 15 |

| IL-17 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 9 | 2 | 11 |
| 1 | 3 | 1 | 4 |
| Total | 12 | 3 | 15 |

| INF-γ | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 9 | 2 | 11 |
| 1 | 3 | 1 | 4 |
| Total | 12 | 3 | 15 |

| TNF-α | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 9 | 1 | 10 |
| 1 | 3 | 2 | 5 |
| Total | 12 | 3 | 15 |

| Eotaxin | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 4 | 0 | 4 |
| 1 | 8 | 3 | 11 |
| Total | 12 | 3 | 15 |

| IP-10 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 12 | 3 | 15 |
| 1 | 0 | 0 | 0 |
| Total | 12 | 3 | 15 |

| MCP-1 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 11 | 2 | 13 |
| 1 | 1 | 1 | 2 |
| Total | 12 | 3 | 15 |

| MIP-1α | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 9 | 2 | 11 |
| 1 | 3 | 1 | 4 |
| Total | 12 | 3 | 15 |

| MIP-1β | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 7 | 3 | 10 |
| 1 | 5 | 0 | 5 |
| Total | 12 | 3 | 15 |

| RANTES | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 3 | 1 | 4 |
| 1 | 9 | 2 | 11 |
| Total | 12 | 3 | 15 |

**Anti-inflammatory Cytokines**

| IL-1Ra | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 8 | 2 | 10 |
| 1 | 4 | 1 | 5 |
| Total | 12 | 3 | 15 |

| IL-4 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 8 | 1 | 9 |
| 1 | 4 | 2 | 6 |
| Total | 12 | 3 | 15 |

| IL-5 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 10 | 2 | 12 |
| 1 | 2 | 1 | 3 |
| Total | 12 | 3 | 15 |

| IL-9 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 4 | 1 | 5 |
| 1 | 8 | 2 | 10 |
| Total | 12 | 3 | 15 |

| IL-10 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 8 | 2 | 10 |
| 1 | 4 | 1 | 5 |
| Total | 12 | 3 | 15 |

| IL-13 | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 10 | 2 | 12 |
| 1 | 2 | 1 | 3 |
| Total | 12 | 3 | 15 |

**Growth Factors**

| FGF | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 6 | 1 | 7 |
| 1 | 6 | 2 | 8 |
| Total | 12 | 3 | 15 |

| G-CSF | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 12 | 2 | 14 |
| 1 | 0 | 1 | 1 |
| Total | 12 | 3 | 15 |

| GM-CSF | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 4 | 0 | 4 |
| 1 | 8 | 3 | 11 |
| Total | 12 | 3 | 15 |

| PDGF | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 4 | 0 | 4 |
| 1 | 8 | 3 | 11 |
| Total | 12 | 3 | 15 |

| VEGF | Rejection | | |
|---|---|---|---|
| | 0 | 1 | Total |
| 0 | 10 | 1 | 11 |
| 1 | 2 | 2 | 4 |
| Total | 12 | 3 | 15 |

### 4.3.2 Comparison of cytokine profile of MLC co-cultures over three days

The harvested supernatants were analysed for the same cytokines. The cytokine profiles for the MLC on day 1, 2 and 3 following transplant were analysed in 15 renal transplant recipients and the results are shown in Table 4.4 to Table 4.6.

On day 1, the pro-inflammatory cytokines IL-1β, IL-8, eotaxin and MIP-1β, the anti-inflammatory cytokines IL-10 and IL-13 and growth factor PDGF-bb, were higher in those individuals who rejected the kidney graft as compared to those patients who underwent successful transplant with no significant statistical difference (Table 4.4).

**Table 4.4: Cytokine profile of MLC on day one in patients following renal transplant.** Results are presented as pg/ml for the 25, 50 and 75 percentiles.

| **TIMEPOINT 1** | | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (pg/ml) | | | | | |
| | **Group 0 (*n*=12)** | | | **Group 1 (*n*=3)** | | |
| | **Percentiles** | | | **Percentiles** | | |
| | **25** | **50** | **75** | **25** | **50** | **75** |
| **Pro-inflammatory Cytokines** | | | | | | |
| **IL-1β** | 13 | 27 | 137 | 17 | 38 | 978 |
| **IL-2** | 13 | 22 | 45 | 15 | 15 | 84 |
| **IL-6** | 65 | 208 | 1273 | 94 | 151 | 40477 |
| **IL-7** | 7 | 10 | 12 | 7 | 9 | 16 |
| **IL-8** | 6275 | 8819 | 16768 | 5312 | 11005 | 34714 |
| **IP-10** | 261 | 581 | 6047 | 1202 | 2135 | 3218 |
| **IL-12** | 36 | 53 | 72 | 38 | 50 | 145 |
| **IL-15** | 26 | 60 | 63 | 24 | 46 | 63 |
| **IL-17** | 65 | 87 | 132 | 71 | 74 | 196 |
| **INF-γ** | 32 | 50 | 77 | 46 | 47 | 236 |
| **TNF-α** | 28 | 53 | 79 | 38 | 39 | 542 |
| **Eotaxin** | 13 | 14 | 19 | 13 | 17 | 46 |
| **MCP-1** | 1249 | 1888 | 208842 | 1309 | 1836 | 2096 |
| **MIP-1α** | 60 | 110 | 13250 | 36 | 162 | 91756 |
| **MIP-1β** | 1350 | 2266 | 4789 | 1139 | 2364 | 7204 |
| **RANTES** | 5986 | 7476 | 9766 | 6752 | 7530 | 7768 |
| **Anti-inflammatory Cytokines** | | | | | | |
| **IL-1Ra** | 92 | 156 | 261 | 113 | 122 | 795 |
| **IL-4** | 4 | 5 | 6 | 4 | 5 | 12 |
| **IL-5** | 0 | 0 | 1 | 0 | 0 | 0 |
| **IL-9** | 32 | 42 | 62 | 32 | 41 | 70 |
| **IL-10** | 15 | 18 | 28 | 14 | 26 | 136 |
| **IL-13** | 3 | 4 | 10 | 2 | 6 | 9 |
| **Growth Factors** | | | | | | |

| TIMEPOINT 1 | | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (pg/ml) | | | | | |
| | Group 0 (*n*=12) | | | Group 1 (*n*=3) | | |
| | Percentiles | | | Percentiles | | |
| | 25 | 50 | 75 | 25 | 50 | 75 |
| **FGF** | 29 | 40 | 55 | 29 | 29 | 67 |
| **G-CSF** | 118 | 174 | 395 | 133 | 173 | 3953 |
| **GM-CSF** | 60 | 105 | 120 | 44 | 80 | 168 |
| **VEGF** | 202 | 285 | 398 | 185 | 209 | 829 |
| **PDGF-bb** | 1716 | 2216 | 2811 | 19076 | 2430 | 3055 |

On day 2 following transplant, the pro-inflammatory cytokines: IL-7, IP-10, MCP-1, MIP-1α; the anti-inflammatory cytokines: IL-10 and IL-13 and the growth factors: G-CSF, GM-CSF, VEGF, PDGF-bb were shown, albeit not significantly, to be slightly higher in those patients with rejected transplants as compared to patients with successful grafts (Table 4.5). It was also observed that cytokine levels were higher on day 2 following transplant as compared to day1 with the exception of IL-1β, IL-7 and MIP-1β in successfully grafted patients and IL-1β, IL-8, eotaxin, MIP-1α, MIP-1β, IL-9 and PDGF-bb in those patients who rejected the donor kidney.

**Table 4.5: Cytokine profile of MLC co-culture on day two in patients following renal transplant.** Results are presented as pg/ml for the 25, 50 and 75 percentiles.

| TIMEPOINT 2 | | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (pg/ml) | | | | | |
| | Group 0 (*n*=12) | | | Group 1 (*n*=3) | | |
| | Percentiles | | | Percentiles | | |
| | 25 | 50 | 75 | 25 | 50 | 75 |
| **Pro-inflammatory Cytokines** | | | | | | |
| **IL-1β** | 13 | 23 | 64 | 10 | 19 | 580 |
| **IL-2** | 39 | 53 | 75 | 18 | 48 | 192 |
| **IL-6** | 91 | 266 | 1455 | 105 | 262 | 43691 |
| **IL-7** | 6 | 9 | 12 | 6 | 10 | 10 |
| **IL-8** | 9157 | 15679 | 30807 | 8271 | 9604 | 10304 |
| **IP-10** | 1285 | 3731 | 9536 | 2047 | 4731 | 9166 |
| **IL-12** | 50 | 59 | 88 | 42 | 56 | 183 |
| **IL-15** | 57 | 60 | 65 | 57 | 57 | 66 |
| **IL-17** | 71 | 88 | 114 | 68 | 77 | 261 |
| **INF-γ** | 43 | 60 | 91 | 46 | 54 | 815 |
| **TNF-α** | 39 | 54 | 73 | 41 | 47 | 472 |
| **Eotaxin** | 15 | 17 | 19 | 12 | 16 | 44 |
| **MCP-1** | 1911 | 1972 | 2039 | 1610 | 2026 | 2135 |
| **MIP-1α** | 64 | 80 | 192 | 17 | 99 | 91756 |

| TIMEPOINT 2 | | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (pg/ml) | | | | | |
| | Group 0 (*n*=12) | | | Group 1 (*n*=3) | | |
| | Percentiles | | | Percentiles | | |
| | 25 | 50 | 75 | 25 | 50 | 75 |
| **MIP-1β** | 1306 | 2054 | 3028 | 703 | 2049 | 6854 |
| **RANTES** | 7354 | 8079 | 10675 | 6870 | 7090 | 8984 |
| **Anti-inflammatory Cytokines** | | | | | | |
| **IL-1Ra** | 124 | 205 | 266 | 138 | 153 | 902 |
| **IL-4** | 4 | 5 | 6 | 4 | 5 | 13 |
| **IL-5** | 0 | 0 | 1 | 0 | 1 | 3 |
| **IL-9** | 29 | 42 | 58 | 25 | 37 | 106 |
| **IL-10** | 16 | 23 | 29 | 15 | 36 | 149 |
| **IL-13** | 4 | 5 | 11 | 4 | 6 | 64 |
| **Growth Factors** | | | | | | |
| **FGF** | 28 | 32 | 39 | 28 | 30 | 60 |
| **G-CSF** | 132 | 207 | 469 | 136 | 237 | 7398 |
| **GM-CSF** | 76 | 85 | 109 | 56 | 96 | 200 |
| **VEGF** | 237 | 317 | 478 | 218 | 256 | 1189 |
| **PDGF-bb** | 2004 | 2273 | 2575 | 2054 | 2278 | 3514 |

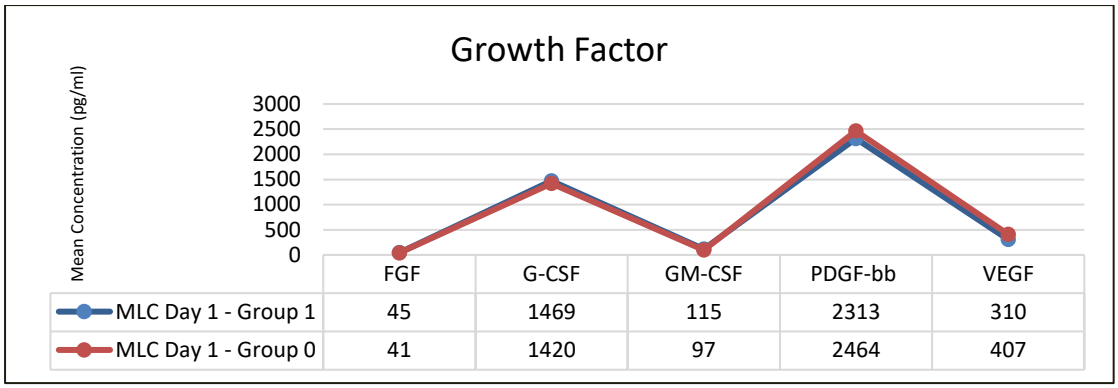
**Table 4.7: Cytokine profile of MLC on day three in patients following renal transplant.** Results are presented as pg/ml for the 25, 50 and 75 percentiles.

| TIMEPOINT 3 | | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (pg/ml) | | | | | |
| | Group 0 (*n*=12) | | | Group 1 (*n*=3) | | |
| | Percentiles | | | Percentiles | | |
| | 25 | 50 | 75 | 25 | 50 | 75 |
| **Pro-inflammatory Cytokines** | | | | | | |
| **IL-1β** | 9 | 16 | 65 | 9 | 19 | 1926 |
| **IL-2** | 39 | 61 | 150 | 17 | 59 | 181 |
| **IL-6** | 72 | 205 | 2209 | 0 | 129 | 45548 |
| **IL-7** | 6 | 8 | 11 | 3 | 9 | 19 |
| **IL-8** | 6869 | 9955 | 19663 | 7568 | 13726 | 17050 |
| **IP-10** | 2465 | 7856 | 20026 | 56 | 2490 | 21237 |
| **IL-12** | 45 | 91 | 93 | 51 | 75 | 169 |
| **IL-15** | 60 | 64 | 66 | 50 | 57 | 80 |
| **IL-17** | 70 | 96 | 115 | 60 | 82 | 212 |
| **INF-γ** | 56 | 60 | 107 | 51 | 58 | 1916 |
| **TNF-α** | 47 | 54 | 88 | 51 | 58 | 1916 |
| **Eotaxin** | 14 | 16 | 21 | 14 | 15 | 44 |
| **MCP-1** | 1747 | 1968 | 2083 | 0 | 1729 | 20013 |
| **MIP-1α** | 35 | 52 | 63 | 16 | 76 | 23977 |
| **MIP-1β** | 1067 | 1471 | 1681 | 595 | 2233 | 5744 |

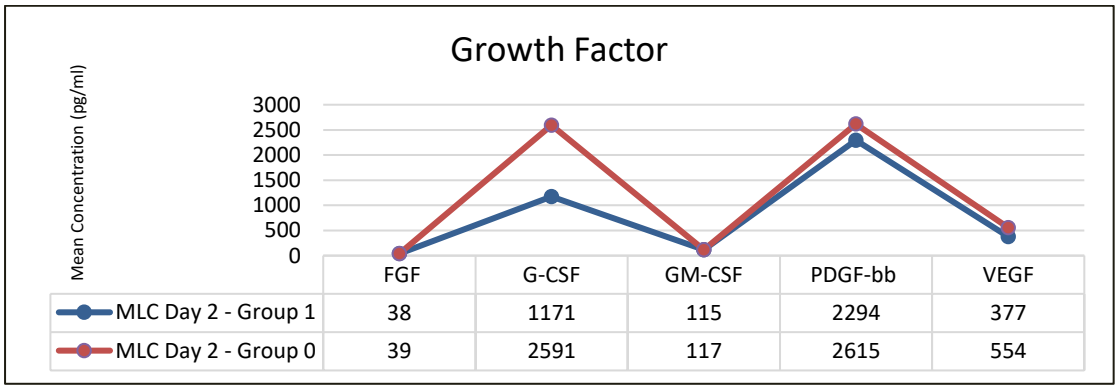
| **TIMEPOINT 3** | | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (pg/ml) | | | | | |
| | **Group 0 (*n*=12)** | | | **Group 1 (*n*=3)** | | |
| | **Percentiles** | | | **Percentiles** | | |
| | **25** | **50** | **75** | **25** | **50** | **75** |
| **RANTES** | 6333 | 7330 | 11034 | 0 | 5563 | 6404 |
| **Anti-inflammatory Cytokines** | | | | | | |
| **IL-1Ra** | 155 | 171 | 321 | 140 | 184 | 1222 |
| **IL-4** | 4 | 5 | 7 | 4 | 5 | 17 |
| **IL-5** | 0 | 1 | 2 | 0 | 6 | 7 |
| **IL-9** | 31 | 41 | 54 | 26 | 73 | 210 |
| **IL-10** | 16 | 24 | 42 | 25 | 32 | 152 |
| **IL-13** | 5 | 9 | 21 | 4 | 32 | 92 |
| **Growth Factors** | | | | | | |
| **FGF** | 30 | 34 | 50 | 27 | 33 | 75 |
| **G-CSF** | 124 | 179 | 581 | 154 | 199 | 33152 |
| **GM-CSF** | 72 | 94 | 123 | 51 | 96 | 620 |
| **VEGF** | 204 | 323 | 518 | 336 | 438 | 838 |
| **PDGF-bb** | 2007 | 2524 | 2752 | 2307 | 2784 | 3435 |

On day three of the MLC, the median concentrations of pro-inflammatory cytokines: IL-1β, IL-8, TNF-α, MIP-1α, MIP-1β; growth factors: G-CSF, GM-CSF, VEGF and PDGF-bb and median concentrations of anti-inflammatory cytokines: IL-1RA, IL-5, IL-9, IL-10, IL-13, were higher in the group with rejected transplants (G1) compared to the successfully grafted group (G0), The concentration of IL-2 and IL-7 did not differ between the groups. The pro-inflammatory cytokine concentrations for IL-2, IP-10, IL-12, IL-17, TNF-α and INF-γ were found to be higher on day 3 of the MLC, as compared to day 2 and day 1 for G1 as shown in Table 4.5 to Table 4.7. Almost all anti-inflammatory cytokine levels were found to be higher in G1 compared to G0 with the exception of IL-1RA (Figure 4.1).

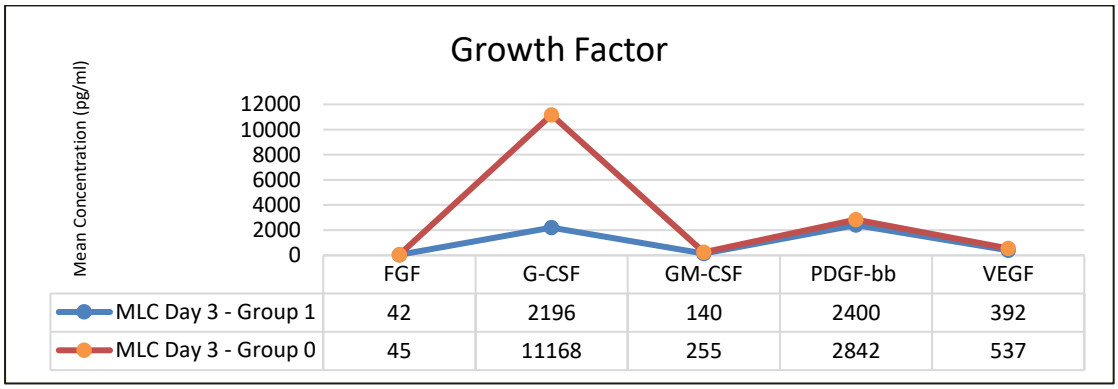
**Figure 4.1: Mean cytokine concentrations by subgroups of MLC, Day 1 – 3, between those patients who rejected (Group 1) the renal transplant and the successfully grafted patients (Group 0).**



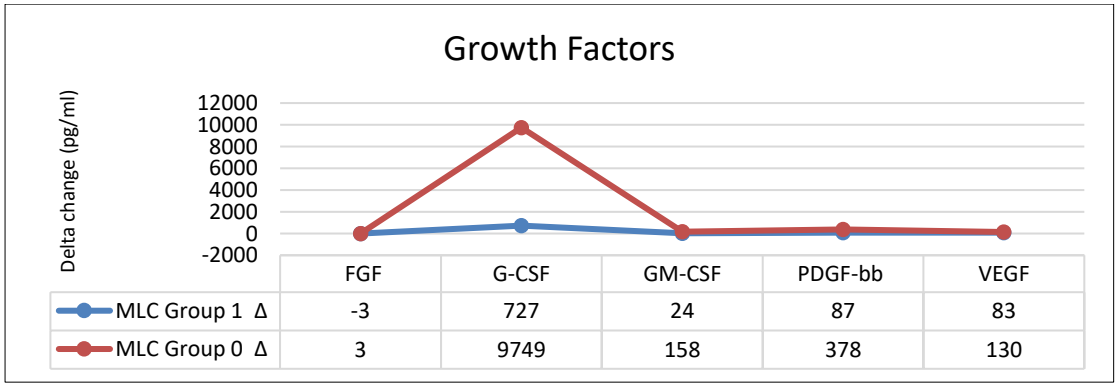

| | FGF | G-CSF | GM-CSF | PDGF-bb | VEGF |
|---|---|---|---|---|---|
| MLC Day 1 - Group 1 | 45 | 1469 | 115 | 2313 | 310 |
| MLC Day 1 - Group 0 | 41 | 1420 | 97 | 2464 | 407 |



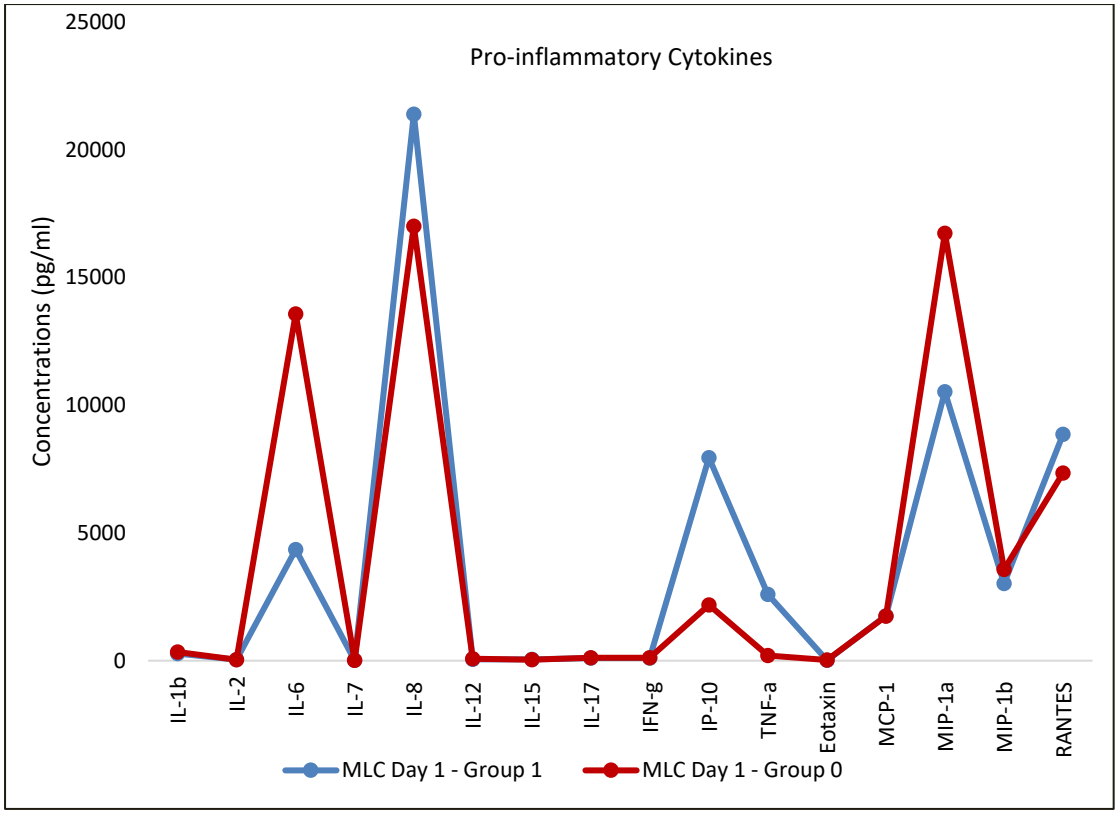

| | FGF | G-CSF | GM-CSF | PDGF-bb | VEGF |
|---|---|---|---|---|---|
| MLC Day 2 - Group 1 | 38 | 1171 | 115 | 2294 | 377 |
| MLC Day 2 - Group 0 | 39 | 2591 | 117 | 2615 | 554 |

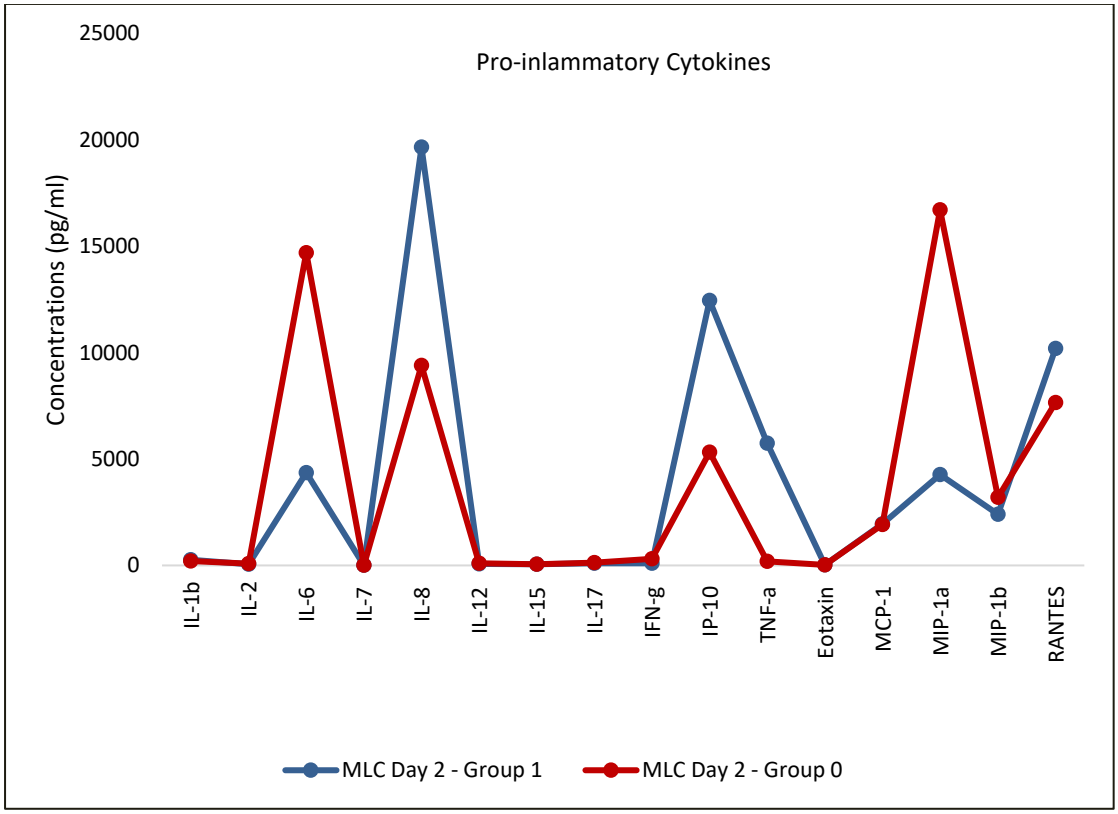


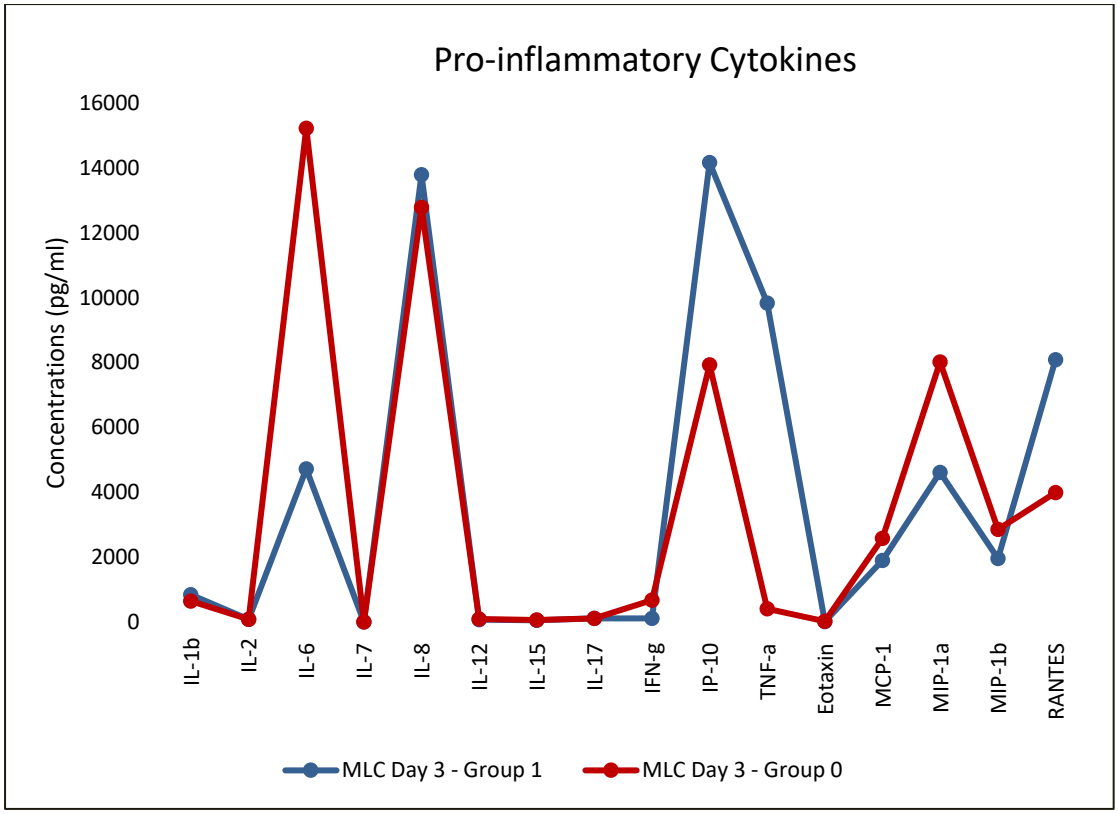

| | FGF | G-CSF | GM-CSF | PDGF-bb | VEGF |
|---|---|---|---|---|---|
| MLC Day 3 - Group 1 | 42 | 2196 | 140 | 2400 | 392 |
| MLC Day 3 - Group 0 | 45 | 11168 | 255 | 2842 | 537 |

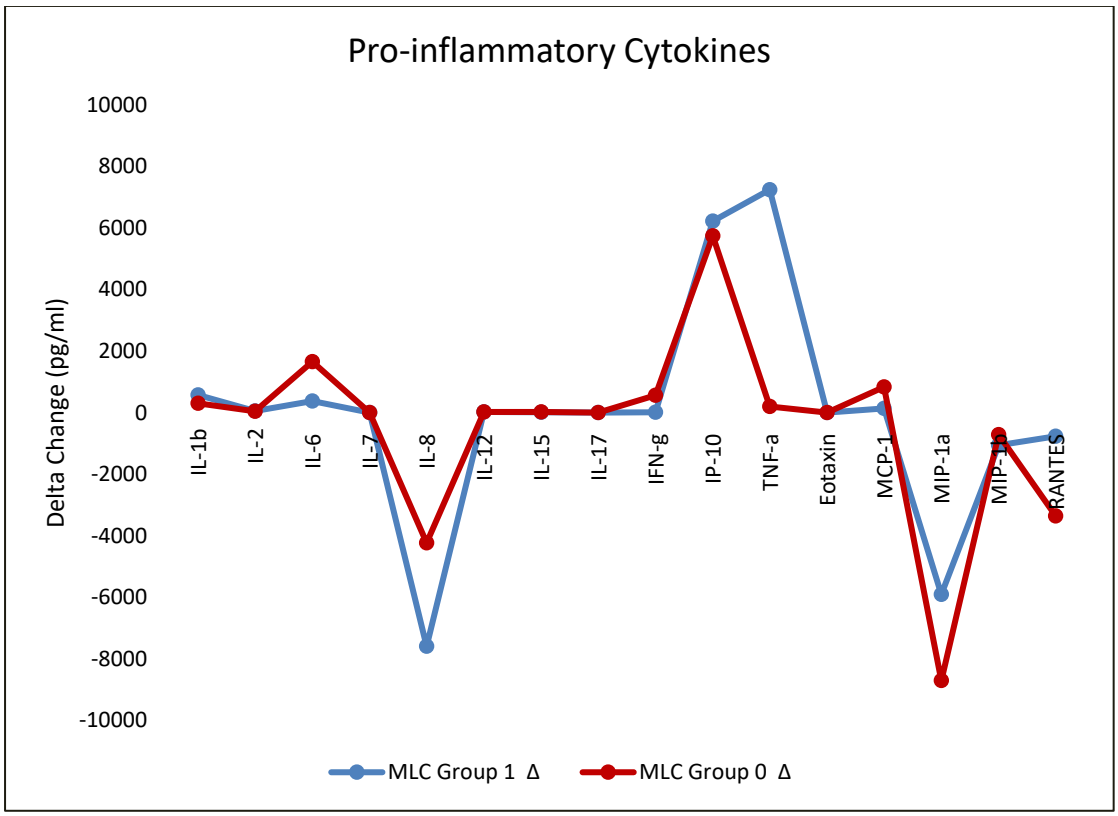


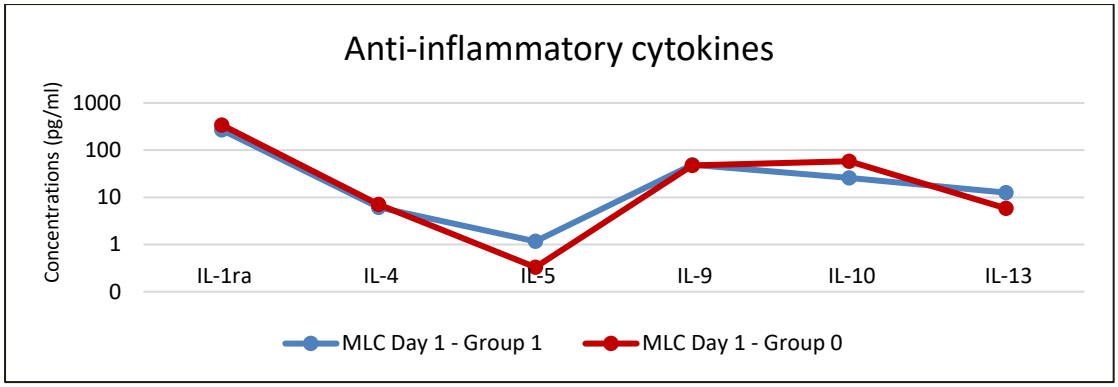

| | FGF | G-CSF | GM-CSF | PDGF-bb | VEGF |
|---|---|---|---|---|---|
| MLC Group 1 Δ | -3 | 727 | 24 | 87 | 83 |
| MLC Group 0 Δ | 3 | 9749 | 158 | 378 | 130 |

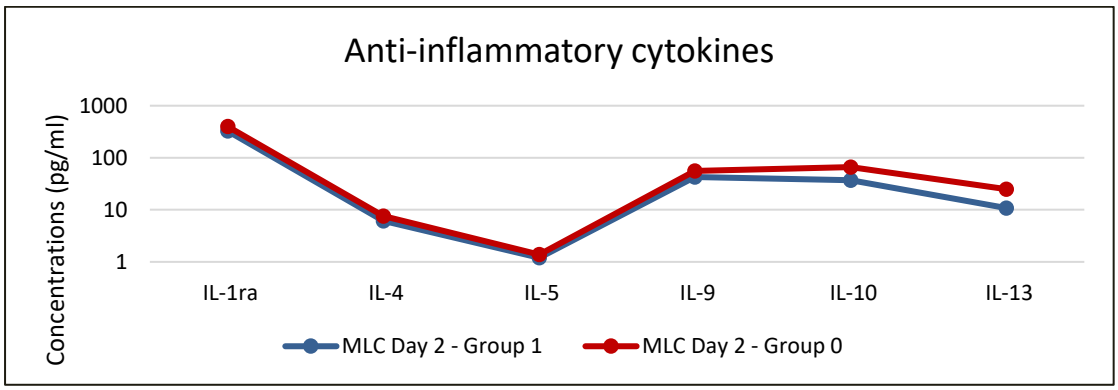

Pro-inflammatory Cytokines
Concentrations (pg/ml)
25000
20000
15000
10000
5000
0
IL-1b
IL-2
IL-6
IL-7
IL-8
IL-12
IL-15
IL-17
IFN-g
IP-10
TNF-a
Eotaxin
MCP-1
MIP-1a
MIP-1b
RANTES
MLC Day 1 - Group 1
MLC Day 1 - Group 0

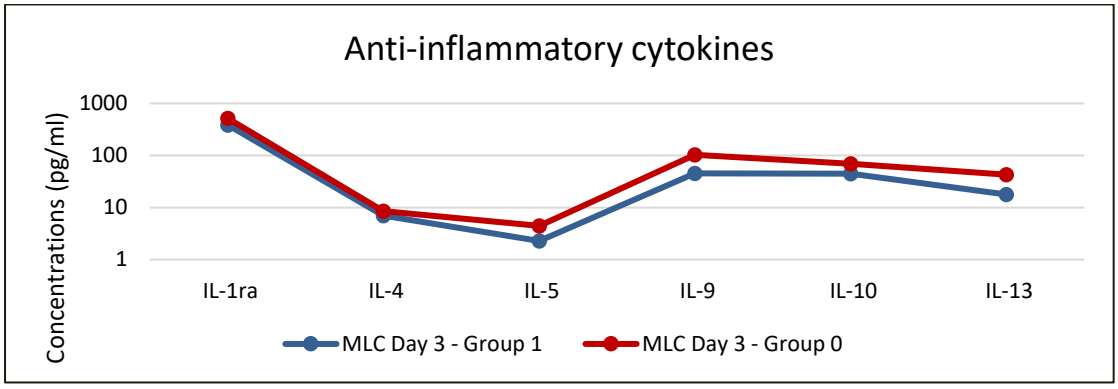

Pro-inlammatory Cytokines
Concentrations (pg/ml)
25000
20000
15000
10000
5000
0
IL-1b
IL-2
IL-6
IL-7
IL-8
IL-12
IL-15
IL-17
IFN-g
IP-10
TNF-a
Eotaxin
MCP-1
MIP-1a
MIP-1b
RANTES
MLC Day 2 - Group 1
MLC Day 2 - Group 0

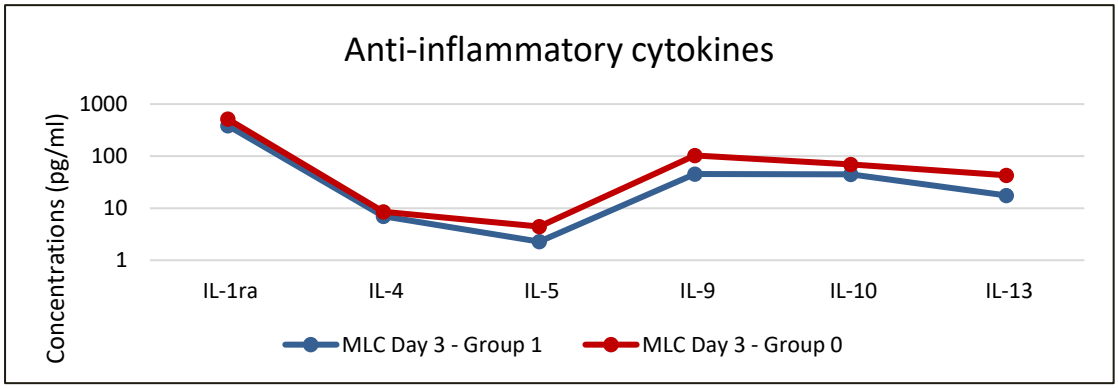

Pro-inflammatory Cytokines
Concentrations (pg/ml)
16000
14000
12000
10000
8000
6000
4000
2000
0
IL-1b
IL-2
IL-6
IL-7
IL-8
IL-12
IL-15
IL-17
IFN-g
IP-10
TNF-a
Eotaxin
MCP-1
MIP-1a
MIP-1b
RANTES
MLC Day 3 - Group 1
MLC Day 3 - Group 0


Pro-inflammatory Cytokines
Delta Change (pg/ml)
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
IL-1b
IL-2
IL-6
IL-7
IL-8
IL-12
IL-15
IL-17
IFN-g
IP-10
TNF-a
Eotaxin
MCP-1
MIP-1a
MIP-1b
RANTES
MLC Group 1 Δ
MLC Group 0 Δ




The cytokine levels released by the MLC over the 3 days from those patients with rejected transplants were then compared. Significant differences were found for three cytokines *viz.* IL-2 (p=0.011), IL-5 (p=0.032) and IL-13 (p=0.029) when comparing day three with day one (Figure 4.2).

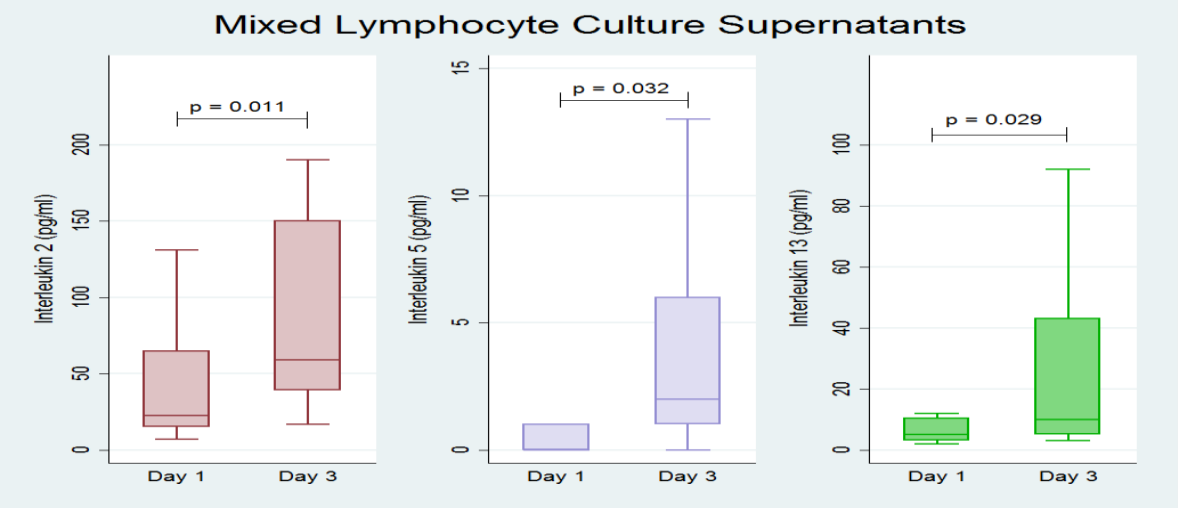
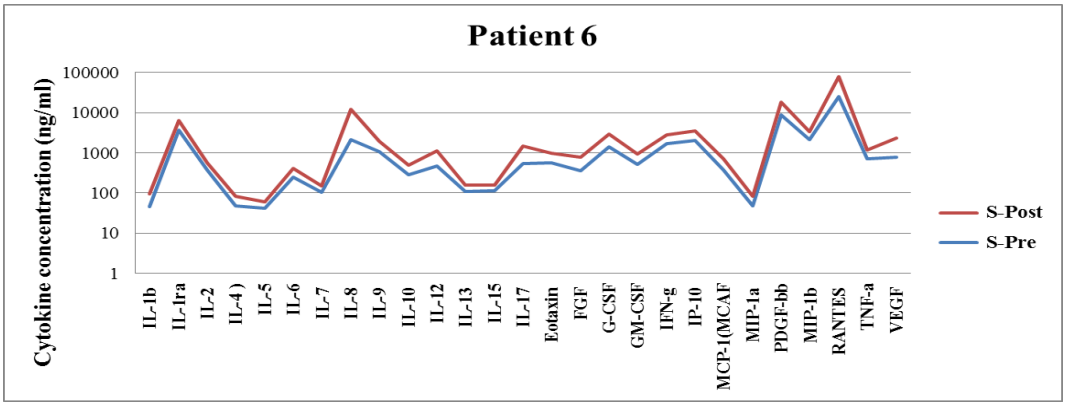



**Figure 4.2: The release of IL-2, IL-5 and IL-13 by the MLC of the patients who rejected the kidney transplants over a 3-day period**

### 4.3.3 Comparison of pre- and post-transplant cytokine profiles

Finally, pre- and post-transplant cytokine profiles were compared in representatives of both those patients that rejected the kidney transplant (Patient 6 and Patient 10) and those with successful grafts (Patient's 11, 13 and 14). Of the 27 cytokines compared, a general decrease in the level of cytokines was observed following transplant with the exception of Patient 11 (Figure 4.3). There were no marked differences in cytokine levels between the two groups. Only one patient (Patient 11) showed a slight increase in most of the cytokine levels post-transplant.



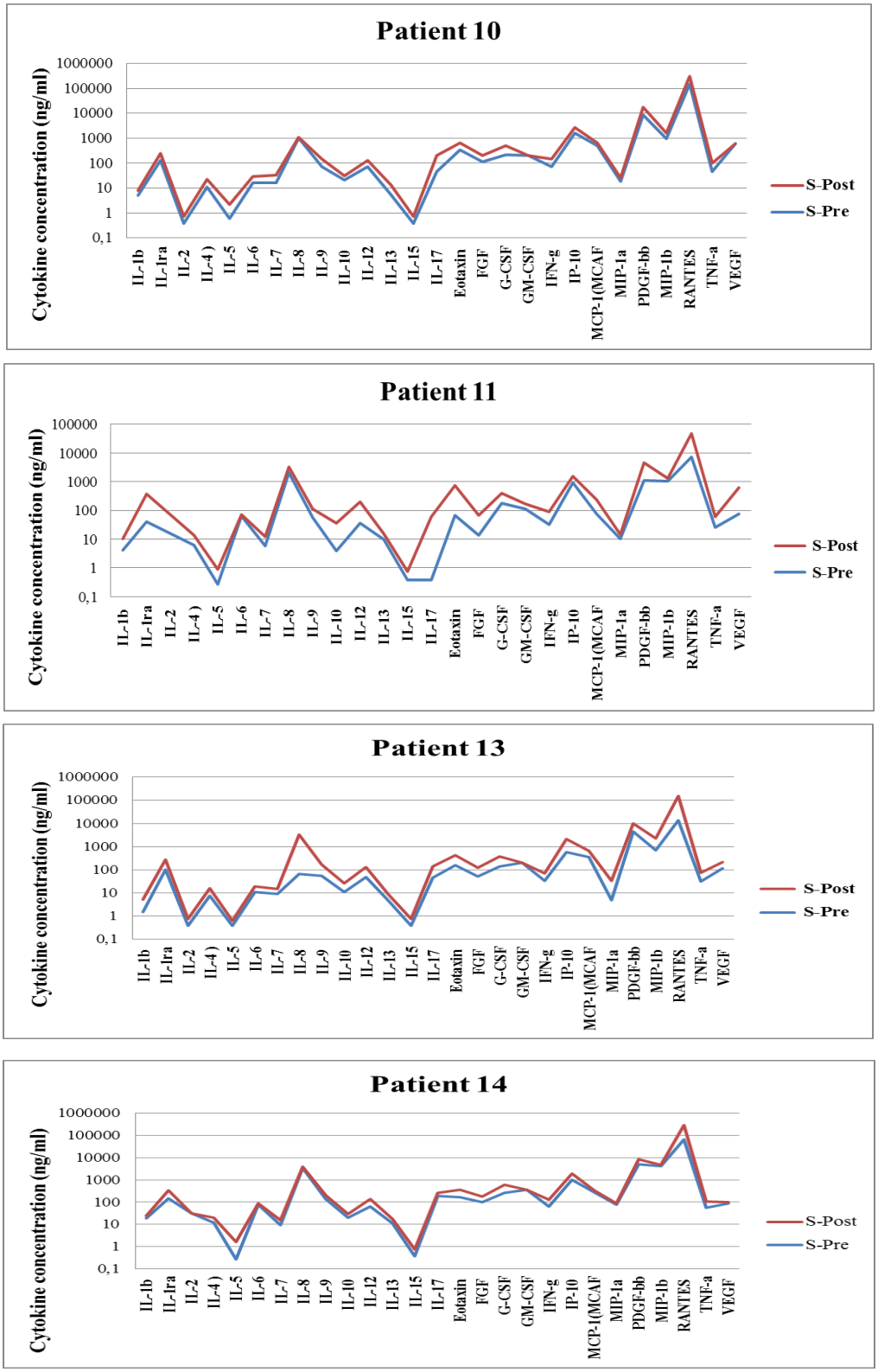



**Figure 4.3: Pre- and post-transplant cytokine profiles of five representative patients**

## 4.4 *Discussion*

This study determined levels of cytokines, chemokines and certain growth factors in peripheral blood, as well as MLC of patients undergoing kidney transplantation. In organ transplantation, donor-recipient histo-incompatibility may stimulate an inflammatory immune response that might result in rejection episodes. Loss of graft function due to acute and chronic rejection remains one of the main complications leading to end-stage organ failure today. Renal allograft involves a network of cytokines, growth factors and co-stimulatory molecules released from the leukocyte infiltration associated with the transplanted kidney (Borel et al., 2003). The main types of infiltrating cells are subsets of T cells and macrophages at different stages of activation (GRIMM et al., 1999). It has been reported that a dominant Th1 cytokine pattern is associated with allograft rejection (Bumgardner and Orosz, 1999; Gudmundsdottir and Turka, 1999; Amirzargar et al., 2005; Mota et al., 2017) while, on the other hand, the presence of a Th2-associated cytokine pattern may lead to transplant tolerance and stable graft survival (Mota et al., 2017). Pre-transplant cytokine profiles of the recipients' blood and supernatant of the MLC may be of assistance in predicting transplant outcome.

Cytokines and chemokines play an important role in the activation of the immune response and play a key role in controlling leukocyte mobility and migration. However, the role of cytokines in graft versus host disease (GVHD) can only be understood when the full cytokine profile is present (Bennett et al., 2011; Thiant et al., 2016).

The Luminex multiplex immune-assay was employed to detect multiple cytokines in a single 96- well plate. This technique requires only a small amount of plasma and produces a good correlation among different cytokines. The findings from this study showed higher plasma levels of the pro-inflammatory cytokines and chemokines *viz.* IL-1β, IL-6, IL-7, IL-8, IL-12, IL-15, IL-17, INF-γ, TNF-α, eotaxin, MCP-1, MIP-1α, MIP-1β and RANTES and the growth factors *viz*. FGF, G-CSF, GM-CSF, VEGF and PDGF-bb in the group that rejected the transplant (G1) compared to the successfully grafted group (G0) and healthy controls (G2). In contrast, IL-2 and IP-10 levels were slightly higher in the successfully grafted group compared to the healthy controls and rejected groups. The predominance of a pro-inflammatory cytokine pattern in G1 is consistent with the findings of other published reports (Sadeghi et al., 2003; Amirzargar et al., 2005; Karczewski et al., 2009; Mota et al., 2017).

Many studies have investigated the involvement of pro-inflammatory cytokines in graft rejection, especially the role of IL-6 (Booth et al., 2011; Wang et al., 2013; Kamińska et al., 2016). In the current study, the median concentration of IL-6 was higher in the group that rejected the kidney transplant than the group with successful grafts. This was consistent with previous studies, which showed the pro-inflammatory property of IL-6 with high levels of this cytokine being expressed in the transplant rejected group (Diller et al., 2003; Pasare and Medzhitov, 2003; Booth et al., 2011; Wang et al., 2013). Therefore, an increase in levels of IL-6 has been associated with risk of transplant rejection episodes and graft loss (Berber et al., 2008; Kamińska et al., 2016).

IL-2 is essential for the regulation of immune processes that induces IL-2 dependent antigen-specific helper and cytotoxic T cell proliferation and differentiation. Although IL-2 levels were found to be higher in the successfully grafted group, others have found that post-transplantation, the expression of IL-2 has been associated with organ rejection (Kishimoto, 2006; Bennett et al., 2011; Su et al., 2017). The concentrations of IL-4 did not differ between the G1 and G2 groups. Interestingly, the only statistically significant differences between the two groups of patients was found for eoxatin (Table 4.2), demonstrating a possible predictive role for this chemokine. Eotaxin has been reported previously to be significantly secreted in chronic allograft rejection animal models (Dosanjh, 2014). Eosinophils have also been shown to play a crucial role in the mechanism of injury during adverse prognosis on graft rejection episodes (Almirall et al., 1993; Jezior et al., 2003; Bush et al., 2016; Yuvaraj et al., 2017) and eotaxin plays an important role in the recruitment of eosinophils. Unfortunately, circulating eosinophils counts were not monitored throughout this study.

The second part of this study involved determining the pre-transplant cytokine profile (growth factors, pro- and anti-inflammatory cytokines) in 15 patients and donors using MLC. In general, there appeared to be a lack of cytokine secretion by the MLC co-culture as compared to the levels observed in plasma for the same cytokines, with the exception of the chemokine, IL-8. IL-8 is an inflammatory chemokine which is known to induce recruitment of neutrophils to sites of infection (Bennett et al., 2011).

The secretion of several cytokines was first detected at twenty-four hours, with a marked increase of some of the other cytokines thereafter. The secretion of IL-5 was not detected until

after seventy-two hours and only in the rejection group. Notably, IL-5 is also associated with the differentiation, proliferation and survival of eosinophils (Takatsu and Nakajima, 2008).

The elevation of the anti-inflammatory cytokines in pre-transplant plasma, as well as in the MLC was observed in patients that underwent graft rejection, which may be consistent with an on-going counteracting anti-inflammatory response with adverse prognostic potential. Strehlau *et al* found high levels of IL-10 associated with acute transplant rejection (Strehlau et al., 1997). Hueso *et al*, also reported that in chronic rejection, the poor graft survival was accompanied by the up-regulation of IL-10 gene expression (Hueso et al., 2010). The involvement of IL-10 is surprising, as it would be expected that the high production of IL-10 would counteract the production of pro-inflammatory cytokines and suppress acute rejection. Furthermore, IL-10 has been shown to be a potent stimulator of the immune system, leading the immune response towards the humoral pathway (Th2) and enhancing antibody responses against the graft, and therefore, the acute rejection response may, in part, be antibody mediated (Rousset et al., 1992; Hueso et al., 2010). Most likely, however, elevated levels of IL-10 are simply indicative of a compensatory co-existent anti-inflammatory response with adverse prognostic potential.

In the present study, pre- and post-transplant cytokine profiles were investigated. Unfortunately, follow-up clinical samples were only collected from five patients. From these post-transplant samples, it was observed that there was a general decrease in cytokine secretion in all patients with the exception of one. The reason for the decrease in cytokine production post-transplant may be as a result of the immunosuppressive regimen that these patients undergo. It remains unclear why one patient showed a slight increase in the majority of the cytokines secreted post-transplantation.

Despite the large cytokine profile evaluated, a significant difference was only observed for eotaxin when the conventional statistical analysis was used. A new approach in analysing the cytokine levels in relation to outcome was therefore used in renal transplant. The analysis included the determination of ‘high’ and ‘low’ cytokine producers, as well as the construction of the frequency diagram of ‘high’ and ‘low’ procedures, with this procedure 2 of the 3 rejected patients appear on the high group for 24 of the 27 cytokines, which revealed that the rejected group had higher cytokine concentration compared to the group that was successfully grafted.

In conclusion, the results of this study suggest that the interaction of pro- and anti-inflammatory cytokines may contribute to the initiation of kidney transplant rejection. The elevation of IL-5 and IL-10 serving as growth and differentiation factors for B cells may be indicative of an

increase in antibody production. Furthermore, the IL-1β / IL-1 Ra ratio, although not statistical meaningful is shown to feature throughout the study and needs further elucidation in follow-up cohorts with increased participants, as IL-1β and its antagonist IL-1 Ra is of importance in inflammation and fibrosis as previously shown (Arend, 2002; Borthwick, 2016). In addition, eotaxin and IL-5 may be indicative of eosinophil involvement at the site of the graft and should be monitored as a predictor of graft rejection. Importantly, the cytokines released during the immune response following renal transplant should not be interpreted individually, but rather as a part of an interrelated network. The cytokines identified here may be promising targets for further investigation as mediators of kidney transplant rejection, and/or serve as potential clinical biomarkers for prediction of the possibility of graft loss. The ultimate goal would be to identify potential means for therapeutic intervention in order to improve the success of kidney grafts.

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# CHAPTER 5

# CFSE-based two-way mixed lymphocyte culture as predictor of renal allograft rejection.

# 5. CFSE-based two-way mixed lymphocyte culture as predictor of renal allograft rejection.

## *5.0 Abstract*

**Introduction**

Mixed lymphocyte culture (MLC) is one of the oldest and most significant *in vitro* assays for determining histocompatibility. MLC Incompatibility is known to induce a T cell proliferation response which may lead to transplant rejection. The purpose of this study was to evaluate the two-way MLC as a predictive assay of acute and chronic graft *vs* host disease (GVHD) and to determine the relationship between MLC assays and graft survival in living-donor kidney transplantation.

**Method**

The study included 15 patients who underwent a living-donor kidney transplant. They were divided according to those who grafted successfully (12 patients) and those who rejected the kidney (3 patients). Mixed lymphocyte culture utilising CFSE based two-way flow cytometry method was used to measure the proliferation percentage, precursor frequency and phenotype of alloreactive T cells *in vitro* using living related donor-specific cells for allostimulation. The proliferating cells in this CFSE-based MLC were counterstained with CD25, CD69 and HLA-DR.

**Results**

There was a significant increase between the autologous control and the MLC in T-lymphocyte activation marker expression (CD25 $p<0.001$, CD69 $p=0.004$ and HLA-DR $p=0.002$). The mean proliferation percentage of the 3 recipients that rejected the transplant (proliferation $\geq$ 15%) was higher than those with successful kidney grafts. One donor (responder cells) of the patient that was successfully grafted had a mean proliferation of 19%.

**Conclusion**

The CFSE-based two-way MLC may be able to predict patients that are at risk of transplant rejection. Furthermore, the flow cytometric MLC method may possibly be the tool of choice to indicate GVHD occurrences.

## 5.1 *Introduction*

MLC has been used historically to predict adverse graft versus host immune stimulation. The assay was developed to measure T- lymphocyte proliferation, which provides information on the alloreactivity (Bain et al., 1964; DeWolf et al., 2016). It offers an exact method by which an immunological response To the graft can be measured *in vitro*. In renal transplant, there is justification for, and against the use of MLC to predict rejection and clinical outcome (Cullen et al., 1977; Harmon et al., 1982; Langhoff et al., 1985; Burke et al., 1994; Steinmann et al., 1994). This test has an advantage over the serological typing strategy, in that, it may possibly give a degree of successful outcome of graft survival. Proliferation can be detected and monitored *in vivo* which simulates the probable graft vs. host interaction, which is not possible with serotyping. However, viable cells are mandatory to carry out the essay. The standard MLC is fraught with numerous specialized technical difficulties, such as the prerequisite for a large volume of blood of both donor and recipient, use of radioactive isotopes and extended incubation periods (3-7 days), which are not conducive for routine diagnostic applications. Therefore, an alternative method which is non-radioactive, with a shorter incubation and simplified analysis has been suggested (Bach et al., 1970; Chen et al., 2003).

CFSE is one of the preferred indicators of proliferation. One of the redeeming features of using CSFE is that it has been shown to correlate well with $^{3}$H-thymidine uptake (Fulcher and Wong, 1999; Popma et al., 2000; Nitta et al., 2001). The reason for this is that CFSE binds covalently to the intracellular amine groups of macromolecules. It is therefore possible to accurately determine how many times the cell has divided as the dye is distributed equally between the daughter cells with the fluorescence intensity of the daughter cell being half that of the parent cell (Lyons and Parish, 1994; Parish, 1999; DeWolf et al., 2016). Furthermore, the CFSE-based 2-way MLC method can demonstrate an increase in daughter cells as early as day 2 of culture thereby shortening the time-course of the MLC dramatically. Importantly, CFSE-based proliferation is amenable to counter-staining with fluorochrome-labelled monoclonal antibodies. This enables the determination of phenotypes of the proliferating cell population (i.e. CD3+ T-cells), as well as detection of up-regulation of CD25 (interleukin-2 receptor) expression, an early marker of immune activation of T-cells. This modified procedure for the detection of T-cell alloreactivity has not been extensively explored in the setting of clinical organ transplantation. Therefore, in the present study, the 2-way CFSE MLC analysis was complemented by counterstaining with monoclonal antibodies directed against CD25, CD69 and HLA class II (HLA-DR) that allow for the measurement of expression of early surface

markers of T-cell activation. The expression of activation markers combined with the two-way MLC was evaluated as a predictive assay of acute rejection and/or chronic GVHD.

## 5.2 *Methods*

### 5.2.1 *In vitro* MLC set-up

The detailed method is described in Chapter 2 Page 52. Briefly, all MLC where performed in 15 ml sterile, non-pyrogenic tubes. Two different experiments were set up:

1. Allogeneic MLC: 900µl of 1 x$10^6$ recipient cells/ml CFSE-labelled mononuclear leukocytes (MNL) were cultured with 900µl of 1 x$10^6$ cells/ml unlabelled lymphocytes isolated from donor blood.
2. The reciprocal allogeneic MLC: Lymphocytes from the donor were stained with CFSE and tracked when cultured with recipient's unstained lymphocytes.
3. Two controls were included with each experiment.
    - **Autologous MLC**: Acts as a negative control. It includes either 1.8 ml of a 1 x $10^6$ recipient's cells/ml CFSE labelled MNL in 1640 RPMI alone or 1.8 ml CFSE-labelled donor cells alone in 1640 RPMI.
    - **Positive control**: 2.5mg/ml Phytohemagglutinin (PHA) (CALBIOCHEM, USA) was used as a positive control in order to test whether the cells could proliferate.

All samples were incubated for 3 days at 37ºC in a humidified $CO_2$ incubator (5% $CO_2$). MLCs were sampled and analysed at 3 different time points, on day 1, 2 and 3 of culture, utilising the Beckman Coulter FC500 flow-cytometer. The intracellular fluorescence label, CFSE, was used to tag proliferating cells and responding T cells were reported as mean proliferation %.

For immunophenotyping, the cells were counterstained with anti-CD25 PE, anti-CD69 PC7, and anti-HLA-DR PC5.

## 5.3 *Results*

### 5.3.1 CFSE based 2-way MLC

There was an increase in proliferating responder T cells (CFSE-labelled MNL; both donor and recipient cells present) as compared to autologous control systems in which only CFSE-labelled MNL of either donor or recipient was present. The results for day 3 are presented in Figure 5.1.

- In Figure 5.1, the CFSE based 2-way MLC (recipient was labelled (SR) with CFSE cultured with unlabelled donor cells D) and its autologous control (only recipient (SR) cells were stained with CFSE), there was an increase in the percentage of the proliferating responder T-cells (SR) compared to the autologous control systems.
- It was observed that the 3 patients that rejected the kidney transplant had the highest percentage of proliferating responder T-cells. The responder T-cells of Recipient 3 increased from 4% (baseline) to 17% on day 3, Recipient 6 increased from 2% (baseline) to 15% and Recipient 10 from 2% to 15%.
- The average percentage of the proliferating responder T-cells (SR) of the patients that did not reject varied from 4% to 10% as shown on the scatter graph in Figure 5.1.

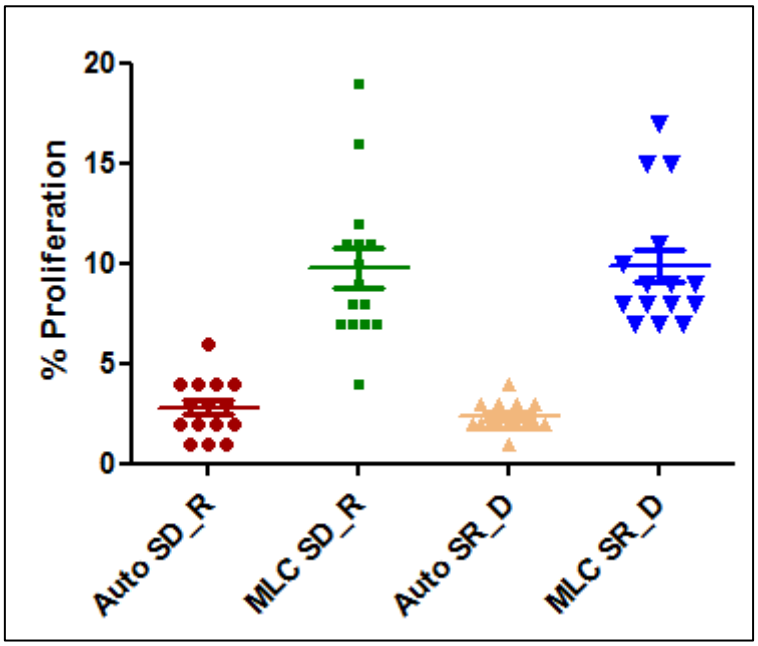



**Figure 5.1 Comparison of the proliferation percentage of accumulation of daughter cells of 15 CFSE-based two-way MLCs.**
**Auto SD:** negative control - included donor cells only.

**Auto SR:** negative control - included recipient cells only.

**Allogeneic MLC SR_D**: CFSE-stained recipient cells (responding cells-SR) were cultured with unlabelled donor c (D).

**Reciprocal allogeneic MLC SD_R:** CFSE-stained donor cells (responding cells-SD) were cultured with unlabelled recipient cells (R).

#### 5.3.1.1 Reciprocal CFSE-labelling in the two-way MLC

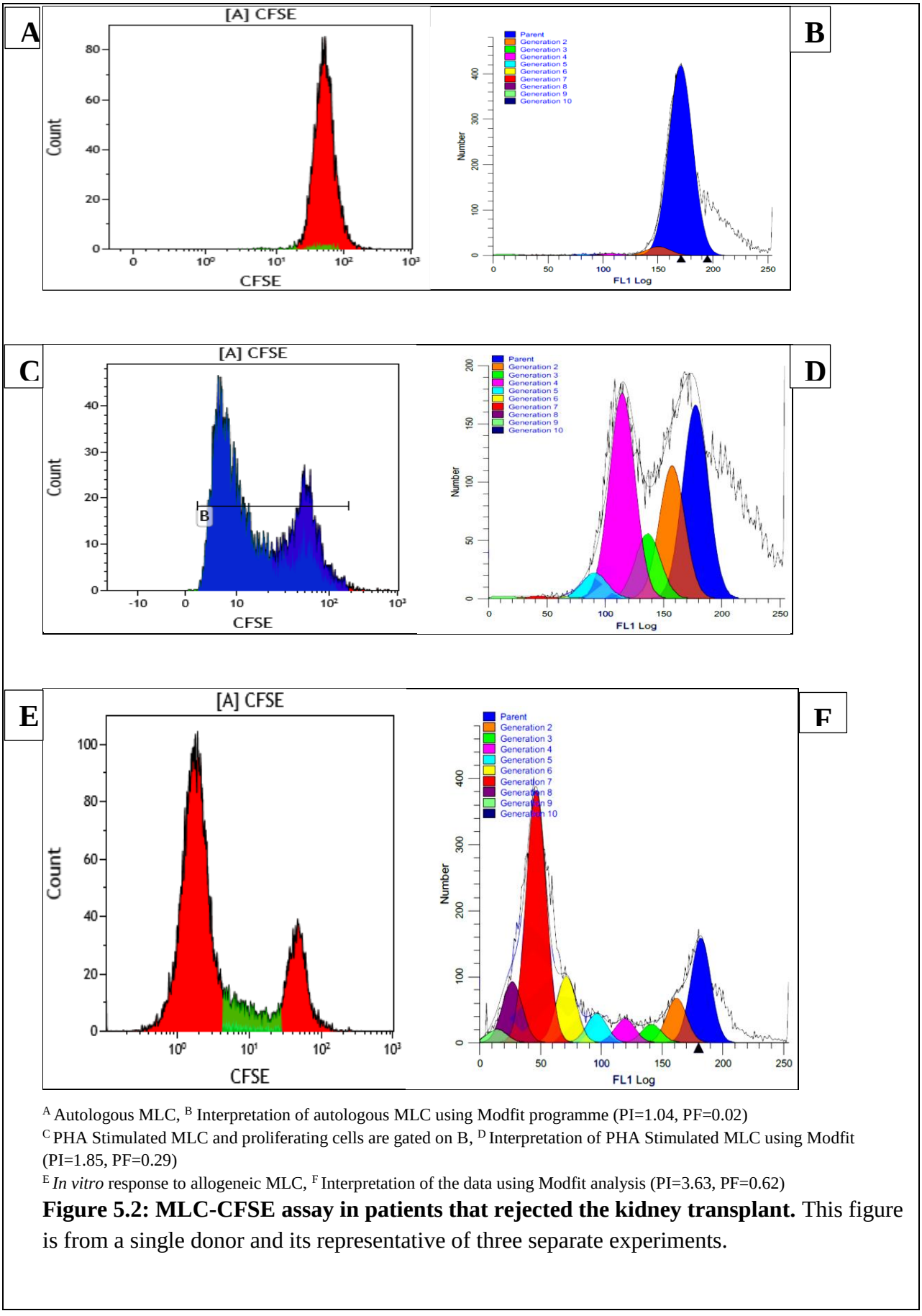
The CFSE-labelled lymphocytes of donors (SD) were cultured with unlabelled lymphocytes of recipients, allowing for the tracking of the response and proliferation of donor's lymphocytes. In the corresponding reciprocal experiment, the lymphocytes of the recipient were labelled with CFSE and cultured with unlabelled lymphocytes of donor cells, allowing tracking of the response of proliferation of donors' lymphocytes. Thus, the relative intensities of the responses of participating donors in the MLC can be tracked individually and compared.

- Following a 3-day culture period, the percentage of proliferating T-cells varied from 3% to 19%.

- It was observed that 2 donors had a high percentage of accumulated daughter cells. The first one was a donor to the patient that rejected (Recipient 10) with proliferating percentages ranging from 2% (baseline) to 16%.
- The second donor was donor to one of the patients that did not reject (Recipient 12) with proliferating percentages ranging from 2% (baseline) to 19% as shown in Figure 5.1. It was also observed that this donor\recipient pair had one of the highest pro-inflammatory cytokine profiles in the supernatant of the MLC.

In addition to proliferation of cells, two-way CFSE-based MLC can also be used for tracking cell division *in vitro*. The ModFit programme was used to correlate data, as well as to calculate precursor frequencies and proliferation indices from the list mode data. Figure 5.2 shows an experiment following a day3 culture period of a patient that rejected (autologous controls, cells stimulated with PHA and allogeneic MLC).

- Figure 5.2A. (Autologous MLC/control): No cell division was detected in the autologous MLC/control. On the ModFit programme negligible background proliferation was observed with Proliferation Index (PI) = 1.04 and Precursor Frequency (PF) = 0.02 (Figure 5.2.B).
- Figure 5.2C. (Stimulated cells): The PHA stimulated cells were found to proliferate with PI =1.85 and PF = 0.29 (Figure 5.2D).
- Figure 5.2E. Allogeneic MLC: This MLC shows the *in vitro* response to alloantigen stimulation of MNL (CFSE-labeled recipient cells and unlabelled donor cells). The histogram representative of the proliferation assay, by sorting the negative donor cells (left pick), proliferating ($CFSE^{low}$) and non-proliferating/ resting recipient cells ($CFSE^{high)}$ is shown in E. Figure 5.2F shows the cell tracking and number of generations after a 3-day culture period. PI =3.63 and PF = 0.62.




[A] Autologous MLC, [B] Interpretation of autologous MLC using Modfit programme (PI=1.04, PF=0.02)
[C] PHA Stimulated MLC and proliferating cells are gated on B, [D] Interpretation of PHA Stimulated MLC using Modfit (PI=1.85, PF=0.29)
[E] *In vitro* response to allogeneic MLC, [F] Interpretation of the data using Modfit analysis (PI=3.63, PF=0.62)

**Figure 5.2: MLC-CFSE assay in patients that rejected the kidney transplant.** This figure is from a single donor and its representative of three separate experiments.

### 5.3.2 Immunophenotyping

To further characterise the MLC responder cells, the difference in the proportion of cells that expressed the early (CD25, CD69) and late T-cell activation markers (HLA-DR) cell surface expression was assessed in the two-way based CFSE-MLC of living donor renal transplant patients.

Peripheral blood mononuclear cells (not depleted for monocytes) were gated on FS vs SS to identify lymphocytes and proliferating cells and exclude debris and dead cells (Region A). Region A was gated for CFSE vs CD25 to identify proliferating T cells by increasing CD25 expression and decreasing CFSE expression (Region B). Region B excluded monocytes, which may lie in the same region as proliferating T cells by excluding $CFSE^{+++}$ events. Based on backgating, monocytes express CFSE brighter than lymphocytes and can be excluded from the analysis. Region B was also used to generate CD69 and HLADR positive cells as a percentage of $CD25^{+}$ CFSE positive cells from CD69 vs CD25 and HLADR vs CD25 bivariate plot (Figure 5.4B).

#### 5.3.2.1 CD25 expression

CFSE-labelled lymphocytes isolated from the transplant recipient (SR) were cultured with unlabelled lymphocytes isolated from the corresponding living-related donor (D) allowing for the tracking of the response and proliferation of recipient's lymphocytes.

A corresponding, reciprocal CFSE labelled two-way MLC experiment was also done. In this experiment CFSE-labelled lymphocytes isolated from the donor (SD) were cultured with unlabelled lymphocytes isolated from the transplant recipient (R), allowing tracking of the response and proliferation of the labelled donor's lymphocytes in the presence of unlabelled recipient lymphocytes (SD_R). This enabled tracking and comparing of the relative intensity of both the labelled donor and labelled recipient responses separately in the MLC.

The results shown in Figure 5.3 are as follows:

- There was a significant increase in CD25 expression on proliferating responding T cells ($P<0.05$) in relation to the control for the two-way CFSE-MLC following day 3 of the culture period.
- In this MLC SR_D, there was an increase in the mean percentage of CD25 proliferating responder T cells (Recipient) from 9.26 ±1.33% to 35.28 ±3.27% ($p<0.0001$).

- For MLC SD_R, there was an increase in the mean percentage of CD25 on proliferating responder T-celsl (donor) from 11.68 ±2.76% to 20.57 ±2.58% (p=0.030).

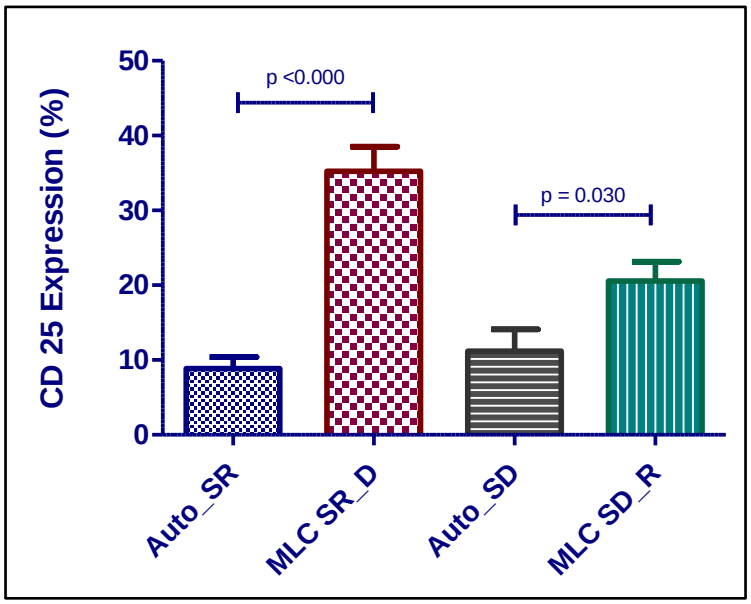



**Figure 5.3: CD25 expression of CFSE-based two-way MLC.** The results are representative of the mean of CD25 expression ± SEM for CFSE-based two-way MLC with the corresponding background (autologous controls – Auto_SR or _SD).

It should be noted that monocytes also demonstrate a significant increase in CD25 expression (Figure 5.4A) and lie in the same region as the T-lymphocytes. Monocytes were found to express very bright CFSE fluorescent peaks. Thus, it is important to exclude these monocytes by gating out the $CFSE^{+++}$ (bright) events. CD25 was found to be up-regulated over the 3-day MLC incubation period (Figure 5.5), with significant differences between day 1 and day 3 being observed (P=0.03).

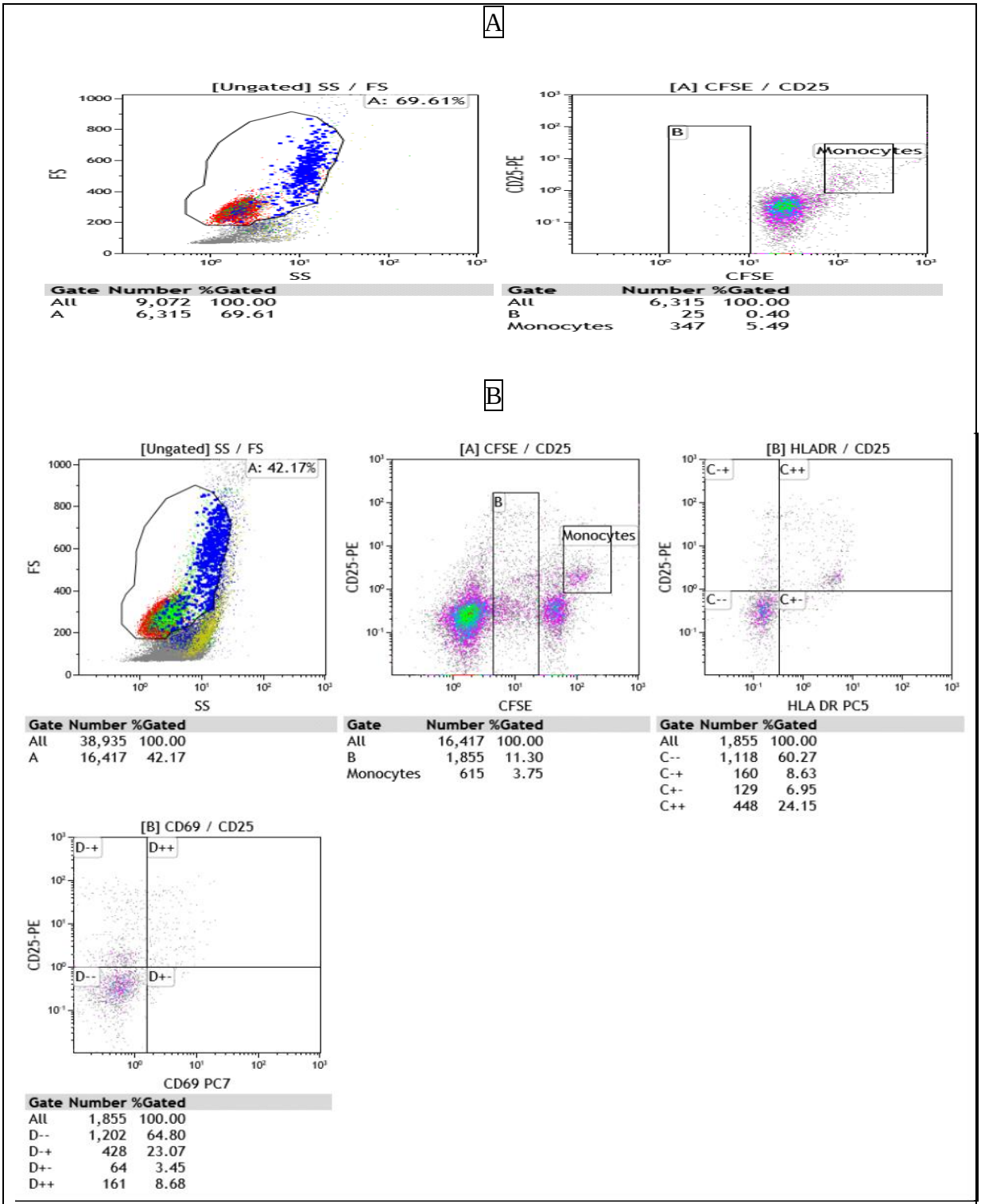



**Figure 5.4: A. CD25 expression on monocytes.** Monocytes demonstrate a brighter CFSE fluorescent peak than lymphocytes.

**: B**. Flow cytometric profile (stained recipient and unstained donor).

Histogram 1 (SS/FS): light scatter profile gated on lymphocytes and blast (RegionA)

Histogram 2 (CFSE/CD25): gated on events in Region A: CFSE vs. CD25 MLC to identify proliferating T cells (Region B) and region B excludes monocytes.

Histogram 3 (HLADR/CD25): Region C shows positive HLADR cells

Histogram 4 (CD69/CD25): Region D shows positive CD69 cells. Both CD69 and HLADR positive cells were generated from Region B.

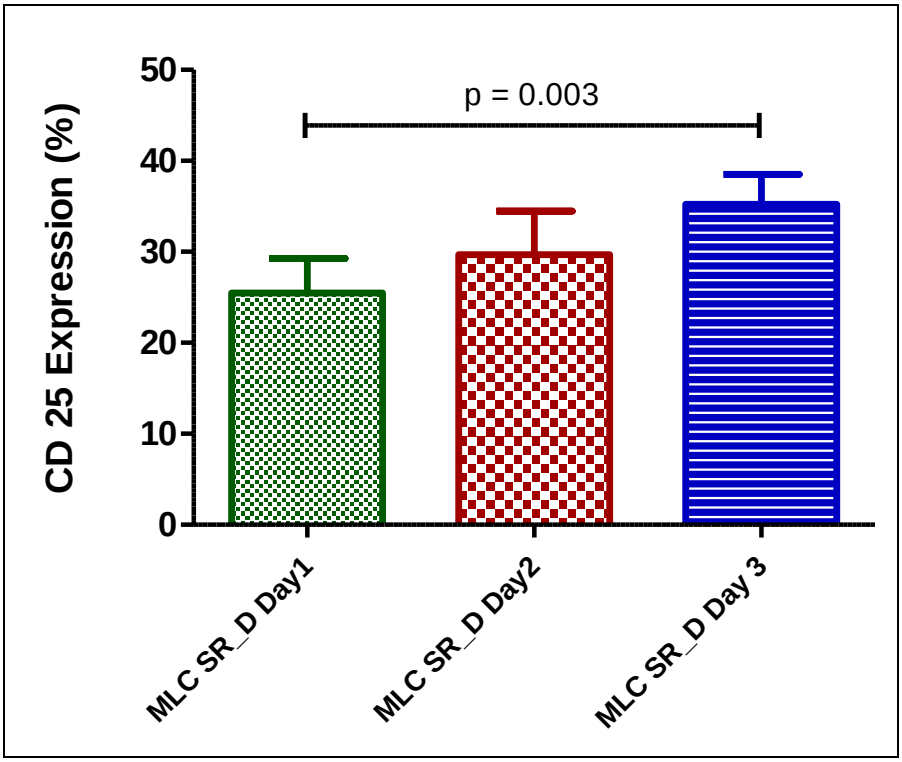



**Figure 5.5: Up-regulation of CD25 over a three-day MLC period.**

### 5.3.2.2 **CD69 expression**

The CFSE-labelled lymphocytes isolated from recipients (SR) were cultured with unlabelled lymphocytes isolated from the corresponding donor (D) allowing tracking of the response and proliferation of the recipients' lymphocytes.

In the corresponding reciprocal CFSE labelling two-way MLC experiment, CSFE-labelled lymphocytes isolated from donors (SD) were also cultured with unlabelled lymphocytes of recipients (R), allowing the response and proliferation of the labelled donor in the presence of unlabelled recipient (SD_R) to be followed.

- A significant difference in the mean percentage of CD69 expression on proliferating SR_D [where SR-recipient is a responder (CFSE-loaded)] T-cells (P=0.004) from 3.25 ±0.78 (baseline) to 13.93 ±3.83 in relation to the autologous MLC for the two-way CFSE-MLC following a 3-day culture period was observed (Figure 5.6).
- In the reciprocal experiment, a significant difference in CD69 expression on T-cell (SD_R) proliferation in relation to the autologous control for the two-way CFSE-MLC

following a3 day culture period from 3.49 ±0.79 to 17.04 ±5.88 (p=0.002) was noted (Figure 5.7)

- CD69 was found to be down regulated over the 3 days (Figure 5.7) with a significant decrease between day 1 and day 2 (p=0.01) as well as day1 and day3 (p=0.011) being observed. There was no statistically significant difference between day 2 and day 3.

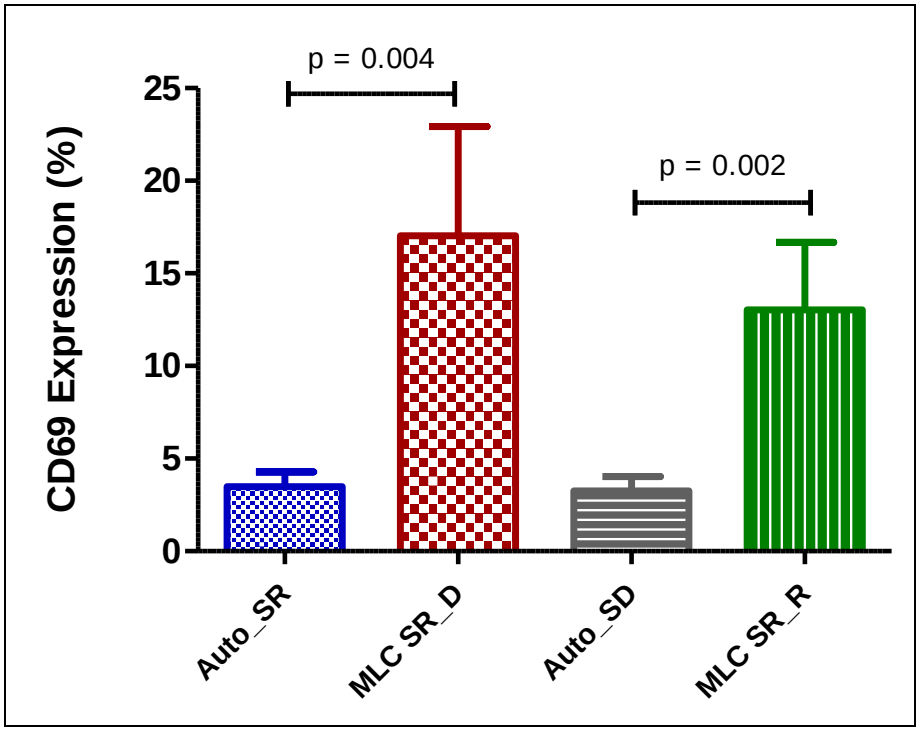



**Figure 5.6: CD69 expression of CFSE-based two-way MLC.** The results are representative of the mean of CD69 expression ± SEM for CFSE-based two-way MLC. With SR (stained recipient: CFSE-loaded), with the corresponding reciprocal MLC with SD (stained donor: CFSE-loaded).

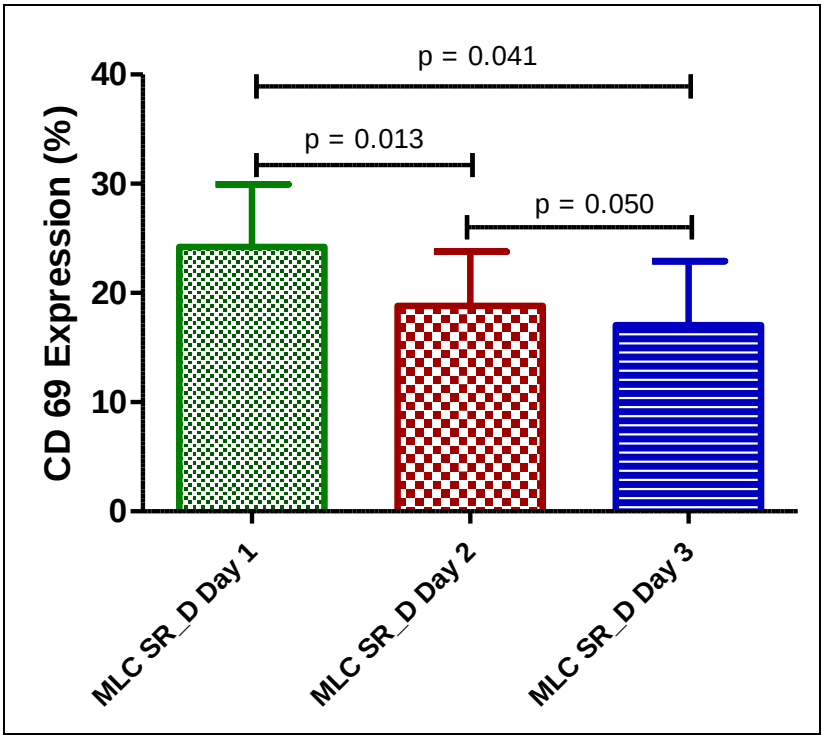



**Figure 5.7: Down-regulation of CD69 over the three-day MLC.**

### 5.3.2.3 HLA-DR expression

HLA-DR expression was performed as described above for CD25 as well as CD69. The results are as follows:

- A significant difference in the mean percentage of HLA-DR expression on proliferating SR_D T-cells (P=0.002) from 6.09 ±0.60 to 40.5 1±3.98 in relation to the autologous control for the two-way CFSE-MLC following day 3 culture period was observed (Figure 5.8).
- In the reciprocal experiment, a significant difference in HLA-DR expression on proliferating T-cell (SD_R) in relation to the autologous control for the two-way CFSE-MLC following day 3 culture period from 5.42 ±0.72 to 35.16 ±4.16 (P=0.002) was found (Figure 5.8).
- HLA-DR was found to be up regulated over the 3 days of incubation (Figure 5.9.) with significant increases noted between day 1 and day 3 (p=0.006), as well as day 2 and day 3 (p=0.047). There was no statistically significant difference between day 1 and day 2 (p=0.055) Figure 5.9.

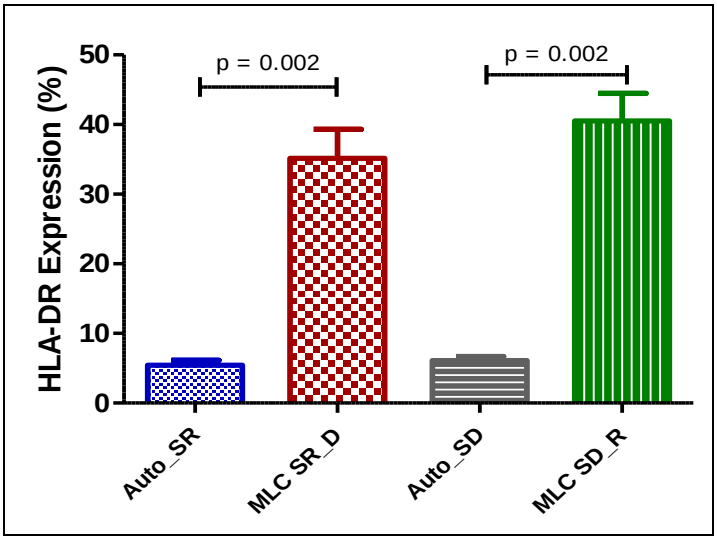



**Figure 5.8: HLA-DR expression of CFSE-based two-way MLC.** The results are representative of the mean of HLA-DR expression ± SEM for CFSE-based two-way MLC. With SR_ (stained recipient: CFSE-loaded), with the corresponding reciprocal MLC with SD (stained donor: CFSE-loaded).

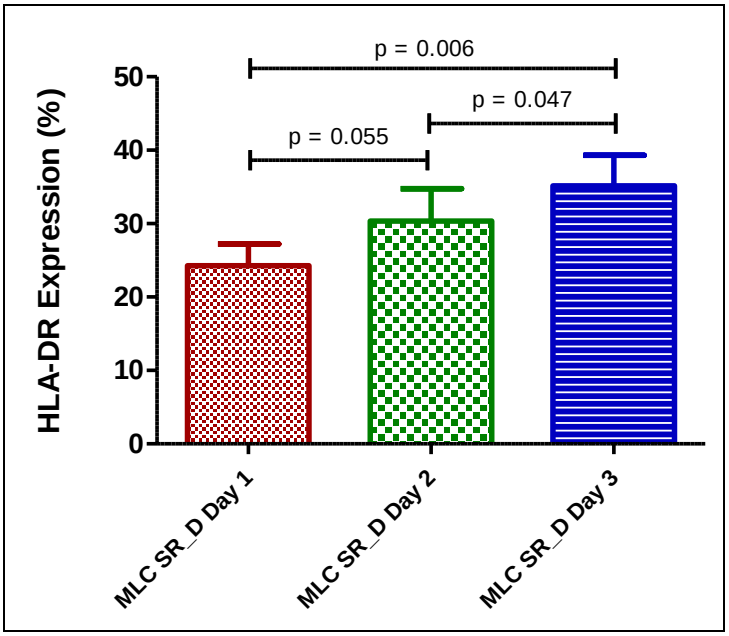



**Figure 5.9: Up-regulation of HLA-DR over the three-day MLC.**

### 5.3.3 Comparison between patients who rejected transplants and those with successfully grafted kidneys.

Patients who rejected the transplants were found to have observably lower CD69 expression levels on day 3 when compared to those individuals with successful grafts albeit not statistically significant. However, the expression of CD25 (p=0.038) and HLA-DR (p=0.030) T-lymphocytes were observed to be significantly higher in patients that rejected the transplanted kidney compared to those with successful grafts (Figure 5.10).

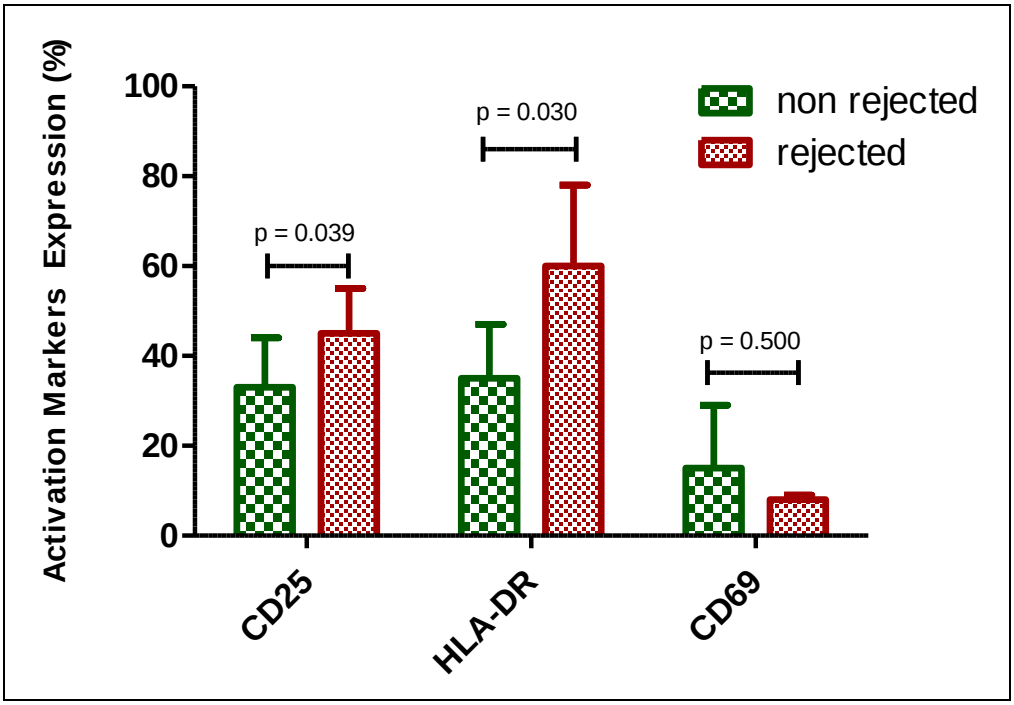



**Figure 5.10: Expression of T-cell activation markers (CD25, CD69, HLA-DR) in the MLC supernatant** in all patients that rejected the transplant *vs* patients with successful grafts.

## 5.4 *Discussion*

MLC is one of the most widely used *in vitro* assays to study the donor specific cellular and humoral host immune response (Harmon et al., 1982; DeWolf et al., 2016). One-way MLC has been the standard method for measuring cell proliferation in the past by measuring the uptake and incorporation of $^{3}$H-thymidine into the DNA of dividing cells. However, this assay is limited in its utility, not giving any further information as to the type of proliferation, relative number of reactive T-cell precursors, the nature of the immune response, etc. (Chen et al.,

2003). Published reports have shown the correlation between MLC outcome and acute rejection and graft survival (Merion et al., 1984; Francis et al., 1988; Nishigaki et al., 1990; Massy et al., 1996; Kerman et al., 1997; El-Agroudy et al., 2004). However, the available literature on the use of CFSE-based two-way MLC assays is sparse, especially in conjunction with the added ability to detect alterations in of CD25, CD69, and HLA-DR. These lymphocyte activation markers also correspond with the number of daughter cells. This modified procedure for the detection of T-cell alloreactivity appears to be relatively unexplored in the setting of clinical organ transplantation.

In this study, the use of CFSE-based two-way MLC and flow cytometry was found to enable simultaneous tracking and phenotyping of proliferation. The use of lymphocyte activation markers in human solid organ transplantation has been reported to be limited mostly to cell surface expression on resting lymphocytes (Schowengerdt et al., 2000; Posselt et al., 2003). An increase in expression of these T-lymphocyte activation markers is a positive indicator that the cells are undergoing mitosis and serves as an indication of cell proliferation. Early expression CD69 and CD25 T-lymphocyte activation markers were observed, followed by a reduced CD69 expression and elevation of HLA-DR expression, secondary to CD25 and CD69. Of note, patients that rejected the kidney transplants showed significantly elevated expression of these activation markers compared to those patients with successful grafts. The conventional method of quantifying cell proliferation in a CFSE MLC is by measuring the accumulation of daughter cells. In the present study, the ModFit statistical software was used to calculate the PF and PI. It was observed that the PF and PI of patients with rejected transplants were higher than those with successful grafts, as well as higher that the autologous control as shown in Figure 5.2, and enabled differentiation between those patients that rejected and those that did not reject by checking the proliferation percentage. The proliferation percentages of the three patients that rejected were greater than 15%. Interestingly, looking at the reciprocal MLC (where the donor was the responder), a donor to one of the patients that did not reject (Recipient 12) had a proliferation percentage of 19%. This pair also had the highest concentration of pro-inflammatory cytokines in the supernatant of the MLC. Unfortunately, no post-transplant follow-up samples were received on this patient, hence the presence of *de novo* antibodies post-transplant to indicate possible GVHD could not be evaluated.

Conclusion: The CFSE-based two-way MLC assay enabled demonstration of proliferation in response to non-self and to differentiate which cell population proliferated. The results of this pilot study show that, using this assay in combination with the ModFit-cell tracking statistical

analysis, has the potential to be a novel way of detecting cell proliferation, as well as possible adverse reactions in the MLR. From the results obtained, it can be concluded that the CFSE-based two-way MLC may predict those patients that are at greater risk of rejecting renal transplants, as well as the possibility of detecting GVHD.

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# CHAPTER 6

# General Conclusions

# And Limitations

# 6. Conclusions and limitations

## 6.1 *Conclusion*

Organ transplantation is one of the greatest therapeutic achievements of the twentieth century. It is the ultimate form of renal replacement therapy because it provides an improved quality of life for patients with end-stage renal failure (Fiebiger et al., 2004; Gentile et al., 2013; Gautam et al., 2018). The field of organ and tissue transplantation has improved remarkably since the discovery of the MHC (Bach and Amos, 1967; Van Rood et al., 1968). Organ rejection is the main obstacle to overcome to achive successful transplantation and the success of the kidney transplant depends on patient selection, advances in surgical techniques, pre-operative management, and immunosuppressive regimens (Millán et al., 2013; Alves et al., 2015; Erol et al., 2017). The allocation of suitable donors is becoming a possibility and priority, because of improved crossmatch techniques along with the identification of HLA antibodies and their specificity.

Cross-match and tissue-typing testing are vital tools in assessing the immune compatibility of a particular donor/recipient pairing (Ansari et al., 2013; Walton et al., 2016; Jayant et al., 2018). The presence of DSA has been considered a contraindication for renal transplantation which may lead to hyper-acute rejection and graft loss (Patel and Terasaki, 1969; Erol et al., 2017; Lee et al., 2017; Jayant et al., 2018), hence cross matching is a mandatory component of the transplant work-up. antibodies to HLA class I (HLA-A, HLA-B) and HLA class II (HLA-DR and HLA-DQ) play a crucial role in acute and chronic rejection (Jin et al., 2005; Campos et al., 2006; Gerbase-DeLima et al., 2006; Jindra et al., 2008; Amirzargar et al., 2014). It has also been reported that anti-HLA-C and anti-HLA-DQ play a major role in renal graft loss (Gloor et al., 2007; Pollinger et al., 2007; Duquesnoy et al., 2008; Tran et al., 2011; Ling et al., 2012). Few case reports have demonstrated anti-HLA DP as having detrimental outcomes for renal graft survival (Jolly et al., 2012; Cippà et al., 2014). As methods improve to identify HLA antigen specificities, the allocation of organs to recipients will become more onerous. Pre-transplant immunological risks remain a major stumbling block and more research still needs to be conducted to improve donor-recipient matching.

The evidence presented in the preceding chapters describes comparative techniques to determine a "best practice" approach to HLA-antibody determination against the donors' antigens, HLA tissue typing of recipients and donors, as well as a novel MLC approach.
In essence, however, a "one size fits all" approach encompassing both LRD as well as cadaver donor transplant remains elusive.
In the current study, a 40% false positive rate for HLA Class II of CDCXM was detected, which confirms the lack of sensitivity reported by others (Mulley and Kanellis, 2011; Roelen et al., 2012; Haque and Rahim, 2017; Jayant et al., 2018). The results from this study suggest that the CDC HLA Class II method has limited diagnostic application because of the high false-positivity rate. However, CDCXM HLA Class I showed good correlation with other cross-match testing (DSAXM and FCXM) and may be beneficial in living-related donor transplants when used in conjunction with another more sensitive cross-match method. At present, no other centre in South Africa has evaluated the Luminex based DSAXM making this study novel in our setting.
There have been reports by others of false-positive DSAXM Class II results (Billen et al., 2009; Lal and Mishra, 2015; Mishra and Lal, 2016; Vimal et al., 2017). However, the DSAXM Class II results recorded in the current study correlated well with FCXM, as well as with the Luminex DSA method. DSAXM was found to be more advantageous than CDCXM. It can therefore be proposed that, DSAXM could add potential value in pre-sensitised kidney transplant patient screening in order to identify those pre-sensitised patients that may not reject the donor graft due to the absence of donor-specific antibodies.

Only 2-3% of kidney transplants are lost due to acute rejection, while a larger percentage is lost due to chronic rejection. Thus, it is critical to monitor the recipient's immune response and the nature of the responding cells in order to improve and refine immunosuppressive therapy. This will lead to customized treatment for the individual, helping to balance suppression of the allogeneic response, while maintaining an adequate immune response to pathogens.
To achieve this, the recipients' T-cell response needs to be characterized following transplantation, by identifying the responding cells and targeting them specifically. For example, CD8+ T-cell activation is independent of the CD40L-CD40 pathway, and therefore immunosuppressive therapy based on blocking CD40L interactions will not suppress CD8+ T-cell activation, proliferation, differentiation, cytokine production and homing to the target allograft. Thus, CD4+ assistance is not always necessary for the production of CD8+ CTL.

Cytokines are the key mediators involved in development of alloimmune responses leading to acute rejection (Kleiner et al., 2013; Yasmeen et al., 2013). Median concentrations of the 27 cytokines in both blood and MLR supernatants were shown to be elevated, as well as having different expression levels, in the group of patients that had rejection episodes (G1) compared to the non-rejection patients (G0). The predominance of a pro-inflammatory cytokine pattern in G1 group was consistent with other published reports (Sadeghi et al., 2003; Amirzargar et al., 2005; Karczewski et al., 2009; Mota et al., 2017).

The counteracting anti-inflammation response charecterised by elevated levels of IL-10 and IL-1Rα also appear to be indicative of a poor outcome. In this context, an elevation of anti-inflammatory cytokine in pre-transplant serum samples and in the MLC supernatant was observed in patients that rejected the renal transplant. Previous findings indicated that poor graft survival or loss was accompanied by the up-regulation of IL-10 gene expression (Strehlau et al., 1997; Hueso et al., 2010; Elnokeety et al., 2017).
Despite the large cytokine profile evaluated, no significant differences were found by conventional statistical analysis; this is probably due to the relatively small sample size, a potential limitation of the study. Our results suggest that elevated anti-inflammatory cytokines may be predictive of the initiation of kidney rejection, but needs confirmation with a larger cohort. The plasticity and interplay of cytokines released during an immune response cannot be interpreted individually, but rather as a part of an interrelated network, but the findings do provide justification for more extensive study.

Another important objective was addressed in Chapter 5, focused on the evaluation of a flow cytometric-based two-way CFSE MLC assay counterstained with CD25, CD69 and HLA-DR for the measurement of expression of early surface markers of T-cell activation. The CFSE-based two-way MLC demonstrated an increase in accumulation of daughter cells, with proliferation detected as early as 24 hours during the time-course of the MLC. CFSE-based proliferation was also successfully combined with counter-staining with monoclonal antibodies to phenotype proliferating cells. Increases in activation markers observed via phenotyping correlated with the growth in the number of daughter cells as determent by CFSE measurement. It could therefore be demonstrated that the proliferation index could utilised to distinguish between those patients that rejected the kidney transplants and those that did not. Additional studies with larger sample groups will be required to confirm and support this contention.

The multiparameter nature of the CFSE assay and flow cytometry afford the opportunity to characterize the long-term immune response of the recipient. A further proposed extension, is the addition of phenotyping of the proliferating cells to characterise the responding cells, *i.e.* whether they are CD4 T-cell- or CD8 T-cell-mediated. In addition, the responding cells can be further characterized to determine if they will develop into effector memory T-cells by counterstaining with CD44, CD62L and CD45RB. It would also be important to determine if regulatory T cells (Tregs) are induced, in light of their essential role in the maintenance of immunological tolerance. FoxP3 expression is considered the distinguishing marker for Tregs, together with co-expression of CD4 and CD25. This remains untested, however, but will be earmarked in further studies.

The two-way MLC may provide a platform for detecting alloreactive immune responses, while simultaneously tracking lymphocyte proliferation/activation flow-cytometrically using CFSE-labelling and immunophenotyping with early surface markers of T-cell activation of the responder cells. CFSE-based two-way MLC may predict the patients that are at high risk of rejecting transplants. In addition, by reciprocal loading of cells with CFSE, it may be able to predict GVHD.

## 6.2 *Limitation of the study*

The leading limitation of this study was the low numbers of subjects recruited overall, particularly for the post-transplant study. One of the reasons being that most recipients were not residing in Gauteng in close proximity to the hospitals. Hence, they were unavailable for follow-up study. In the present study, the rate of rejection was low (only 3 patients) making any significant correlation very difficult. Overall, the time period for this study was also limited being a post-graduate study set within a timeframe of 2-3 years and the fact that the Greater Tshwane transplantation institutions do limited LRD transplantations. Nevertheless, potential interesting findings with translational potential resulted from the study.

Because of the low numbers of patients who were available for the post-transplant study, the cytokines and anti-HLA antibodies post-transplant could not be evaluated. In this context, development of *de novo* anti-HLA antibodies might be a sign of non-optimal immunosuppressive therapy and may give an indication to the clinician to revise the patient's immunosuppressive regimen.

Lack of published reports on the application of the CFSE-based two-way MLC assay in this setting also made it difficult to corroborate the findings of the current study with those of our peers. This is, therefore, the only known study to demonstrate the use of CFSE-based two-way MLC in living-related donor renal transplants in South Africa.

Cellular rejection is not a major problem anymore, due to the use of newer immunosuppressive drugs. Chronic rejection will also be better controlled in the future, with numerous studies involving the development of B-cells activation suppressants, together with the enhanced understanding of co-receptor expression in transplantation immunology. Further innovation in enhanced phenotyping of proliferation of T-cell activation markers, as well improved knowledge of cytokine production in the GVHD, will also assist in enhanced characterisation and regulation of the alloimmune responses.

Finally, this study included living-related donors only, a decision which was taken based on the low frequency of cadaver and living non-related donors in the planned time period, as well as avoiding additional variables introduced in cadaver transplants. This may also represent a selective bias since there is a greater graft success in surgery with living-related donors than in living non-related and deceased donors. Hence, inclusion of living non-related and deceased donors should be considered in similar studies in the future.

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# Appendix A

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

**1/08/2013**

**Approval Notice**
**New Application**

**Ethics Reference No.:** 242/2013

**Title:** Optimising histocompatibility testing in living-donor renal transplantation

Dear Ms LLI Kwofie

The **New Application** for your research received on the 24/05/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 31/07/2013.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years.
- Please remember to use your protocol number (242/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

**Ethics approval is subject to the following:**
**Standard Conditions:**
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

**Additional Conditions:**
- PhD Committee approval must be submitted to our Research Ethics Committee.

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

We wish you the best with your research.

**Yours sincerely**

pp. **Professor Werdie (CW) Van Staden**
MBChB MMed(Psych) MD FCPsych FTCL UPLM
*Chairperson: Faculty of Health Sciences Research Ethics Committee*

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# Appendix B

**INFORMATION LEAFLET AND INFORMED CONSENT**

**TITLE OF STUDY:**

Optimising histocompatibility testing in living-donor renal transplantation

Dear Patient

**1. INTRODUCTION**
We invite you to participate in a research study. This information leaflet will help you to decide if you want to participate. Before you agree to take part you should fully understand what is involved. If you have any questions that this leaflet does not fully explain, please do not hesitate to ask Prof V.O.L Karusseit.

**2. NATURE AND PURPOSE OF THE STUDY**
The aim of the study is to improve donor-recipient renal transplantation histotocompatibility. Previous studies have shown that histocompatibility testing plays an important role in organ acceptance.

**3. EXPLANATION OF PROCEDURE TO BE FOLLOWED**
In addition to the normal blood that the doctor will be taking an extra 10 ml (2 teaspoons) of blood will be required.

**4. POSSIBLE BENEFITS OF THE STUDY**
The results of this study may improve donor-recipient renal transplantation histocompatibility.

**5. WHAT ARE YOUR RIGHTS AS A PARTICIPANT?**
Your participation in this study is entirely voluntary. You can refuse to participate or stop at any time without giving any reason. Your doctor will still treat you to the best of his or her ability even if you do not participate in the study.

**6. HAS THE STUDY RECEIVED ETHICAL APPROVAL?**
This study has received written approval from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria. A copy of the approval letter is available if you wish to have one.

**7. INFORMATION AND CONTACT PERSON**
The contact person for the study is Dr P.W.A Meyer. If you have any questions about the study contact him on his office line (0123192916) or email:
pieter.meyer@nhls.ac.za

**8. COMPENSATION**
Your participation is voluntary. No compensation will be given for your participation.

**9. CONFIDENTIALITY**
All information that you give will be kept strictly confidential. No personal identification information like your name will be used. We will only use a number to

identify you. Research reports and articles in scientific journals will not include any information that may identify you.

**CONSENT TO PARTICIPATE IN THIS STUDY**

- I confirm that the person asking my consent to take part in this study has told me about the nature, process and benefits of the study.
- I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study.
- I am aware that the results of study, including personal details, will be anonymously processed into research reports.
- I am participating voluntarily.
- I have had time to ask questions and have no objection to participate in the study.
- I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect any treatment or care in any way.
- I will receive a signed copy of this informed consent agreement

Participant's name (Please print): _____

Participant's signature: _____Date:_____

Investigator's name (Please print): _____

Investigator's signature: _____Date:_____

Witness' name (Please print): _____

Witness' signature: _____Date:_____

**VERBAL INFORMED CONSENT**

- I, the undersigned have read and have fully explained to the participant the contents of the information leaflet, which explains the nature, process, and benefits of the study to the participant whom I have asked to participate in the study.
- The participant indicates that he/she understand the results of the study, including personal details regarding the interview will be anonymously processed into a research report.
- The participant indicates that he/she had time to ask questions and has no objection to participate in the interview.
- He/she understands that there is no penalty should he/she wish to discontinue with the study and his/her withdrawal will not affect any treatment and care in any way.

I hereby certify that the patient has agreed to participate in this study.

Participant's name (Please print): _____

Participant's signature: _____Date:_____

Investigator's name (Please print): _____

Investigator's signature: _____Date:_____

Witness' name (Please print): _____

Witness' signature: _____Date:_____

# Appendix C

Specifications of antibodies used

| Specificity | **HLA-DR** |
|---|---|
| Clone | Immu357 |
| Fluorochrome | PC5 |
| Species | Mouse |
| isotype | IgG1 |

| Specificity | **CD3** |
|---|---|
| Clone | UCHT1 |
| Fluorochrome | PC5 |
| Species | Mouse |
| isotype | IgG1 kappa |

| Specificity | **CD69** |
|---|---|
| Clone | TP1.55.3 |
| Fluorochrome | PC7 |
| Species | Mouse |
| isotype | IgG2b |

| Specificity | **CD25** |
|---|---|
| Clone | B1.49.9 |
| Fluorochrome | PE |
| Species | Mouse |
| isotype | IgG2a |

| Specificity | **CD19** |
|---|---|
| Clone | J3-119 |
| Fluorochrome | PE |
| Species | Mouse |
| isotype | IgG1 |

| Specificity | **Goat F(ab')2 Anti-Human IgG** |
|---|---|
| Clone | Polyclonal |
| Fluorochrome | FITC |
| Species | Goat |
| isotype | IgG |