GENE POLYMORPHISMS IN RENAL TRANSPLANTATION

by

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ABSTRACT

Despite optimal HLA matching and advances in immunosuppressive therapy, acute rejection remains a leading cause of transplant failure. Increasing evidence suggests that T-cell activation and response to immunosuppression vary widely between individuals, and that these functional differences may be related at least in part to polymorphisms within critical immune response genes. Testing for these polymorphisms may therefore provide a simple and clinically important method for predicting rejection risk and guiding therapy in renal transplantation.

Genetic polymorphisms of two groups of genes (T-cell signaling molecules and cytokine genes) were studied in renal transplant patients who did (AR group) or did not (NR group) experience acute rejection episodes during the first year after transplant. CTLA-4, CD45, and CD40L signaling molecules and TGF-β1, IL-10, TNF-α, and IFN-γ cytokine gene polymorphisms were examined. PCR-RFLP, SNP, and fragment length analysis of dinucleotide repeats were used to study the relationship between genetic polymorphisms and the frequency of acute rejection. The allele frequencies at each gene locus were determined for the study groups and compared to the control population by Fisher’s exact test. Multiple logistic regression analysis was performed using statistical language R, version 1.8.0 (2004), software.

There was no significant difference in the frequency of the CTLA-4 (-318 C/T and +49 A/G), and TGF-β1 (+600 T/C and +650 G/C) genes RFLP in patients with acute renal transplant rejection in a comparison with patients with no rejection. The variant (G^77)
allele of the CD45 gene was not detected in the study population. Univariate analysis of SNP polymorphisms at the IL-10 (-1082 A/G) and TNF-α (-308 A/G) genes, and IFN-γ and CD40L dinucleotide repeat polymorphisms did not reach a level of statistical significance. However, multiple logistic regression analysis which incorporated demographic and clinical data, demonstrated a significant difference for the CD40L 157 1* and 2* alleles between the AR and NR groups (OR=0.58 and OR=0, respectively; p value=0.005), with the proportion of patients carrying the CD40L 157 allele being higher in the NR than in the AR group. This finding suggests that the CD40L 157 allele may have a protective effect in renal transplantation and requires further investigation.
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<tr>
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<td>APC</td>
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<td>B cell receptor</td>
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<td>CDR</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ESRD</td>
<td>end-stage renal disease</td>
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<tr>
<td>Fas</td>
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<tr>
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<td>FK-binding proteins</td>
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<td>G</td>
<td>guanine</td>
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<td>GFR</td>
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<td>human leukocyte antigen</td>
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<td>ICAM-1</td>
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CHAPTER ONE

INTRODUCTION

1.0 Background

1.1 Chronic Renal Failure

Chronic renal failure is a pathophysiologic process leading to loss of function of nephrons and resulting eventually in end-stage renal disease (ESRD). ESRD is caused by a diverse group of kidney disorders including diabetic nephropathy (28.2%), hypertension (22.2%), and others (United Network for Organ Sharing, 2003). The incidence and prevalence of end-stage renal disease in developed countries have increased up to 10% per year during the last decade particularly in patients over 65 for whom hypertension is the most common cause of chronic renal failure (1). The diagnosis of early renal failure is normally reflected by an elevation in serum urea and creatinine concentration analyses that estimate glomerular filtration rate (GFR). As glomerular filtration rate continues to decline, clinical evidence of deteriorating kidney function known as “uremia” becomes obvious. This may include symptoms of nocturia, mild anemia, and abnormalities of calcium and phosphorus metabolism. Decline in GFR below 5-10% of normal is defined as end-stage renal disease when survival without renal replacement therapy becomes impossible.

There are three principal treatment options for the patients diagnosed with end-stage renal failure: hemodialysis, peritoneal dialysis, and a renal transplant (2). Every year growing numbers of patients who are diagnosed with chronic renal failure commence dialysis...
treatment and are put on a renal transplant waiting list. In the US in 2003 there were 58,709 registered patients on waiting lists for a kidney transplant (The United States Renal Data System, 2003) and in Canada there were 3,956 patients awaiting a renal transplant (The Canadian Organ Replacement Registry, 2003). The societal economic burden related to dialysis and management of patients with ESRD is substantial and continuous. The direct economic impact of renal disease care is estimated to approach $700 million per year in Canada and exceed $17.9 billion in the United States (The Canadian Organ Replacement Registry, 2000; The United States Renal Data System, 2001). Although the initial cost of the first year after a kidney transplant approximates hemodialysis costs and is around $55,000 (in Canadian dollars) (3), this cost decreases by the 2nd year to $28,000 (4). Transplantation becomes even more cost-effective by the 5th year of transplant, approaching $10,000 (US dollars) per life-year saved (5). In addition to economic advantages, for the majority of patients with end-stage renal failure, renal transplant facilitates considerable rehabilitation, improvement of quality of life and return to normal social life (6, 7).

One of the serious concerns associated with a renal transplant is acute rejection, which still occurs despite advances in immunosuppressive therapy. This is closely associated with the occurrence of subsequent chronic rejection with progressive transplant dysfunction and an ultimate return to dialysis (8).
1.2 Clinical Aspects of Renal Transplantation

Renal transplant provides an opportunity for the patients diagnosed with end-stage renal failure to avoid the inconveniences and complication associated with a hemodialysis treatment. Renal transplant therefore the preferred treatment option for the majority of patients with ESRD.

Short- and long- term outcomes of renal transplant depend on a number of variables, including:
1. factors related to the donor, such as the donor’s age and the organ cold preservation time
2. factors related to the recipient, such as the recipient’s age, previous sensitization (panel-reactive antibodies), and co-morbid conditions
3. factors related to the donor-recipient relationships, such as the number of HLA mismatches, and antigen independent factors (body size index and age)
4. external factors, such as an immunosuppressive treatment

These prognostic parameters and their relationships offer only a partial guide to outcome, as there are complex interactions among these prognostic variables and predisposing genetic factors.

1.2.1 Transplant Donor Factors

The success of renal transplantation is conditional on donor and recipient parameters that determine renal allograft function and survival, with donor factors being responsible for
35-45% of the variability in early allograft function (9). Kidneys for transplant can be obtained from a cadaveric donor (CAD), a living related donor (LRD), or a living unrelated donor (LUD). Donor-dependent variables that include donor age and organ preservation time (cold ischemia time) influence the quality and function of the donor organ and are important considerations in renal transplantation.

Donor age determines residual function of the transplanted organ and is an important factor for transplant outcome. The glomerular filtration rate is known to decrease with age in association with acquired glomerulosclerosis with incremental loss of both glomerular number and size (10, 11). The relative number of sclerotic, obsolescent glomeruli reach values of about 30% in persons more than 80 years old (12). Therefore the supply of viable donor nephrons and the physiologic demands of the transplant recipient are important determinants of long-term graft survival (13).

Cold ischemia of the donor organ occurs during preservation of the donor organ after retrieval, and is an important limiting factor in graft survival. Longer cold preservation time is associated with poor prognosis, including delayed graft function, acute rejection, and early graft loss due to ischemia/reperfusion injury (14, 15). Experimental evidence has demonstrated that grafts with a cold preservation time greater than six hours developed pronounced vascular endothelial injury and worse tubular necrosis compared to grafts with shorter preservation times (16). Moreover, the glomerular filtration rate is diminished and kidney function is further compromised with cold ischemia time greater than 24 h (17). Noteworthy is the fact that recipients of fully HLA matched kidneys with
cold ischemia time longer than 36 hours do not have superior outcomes compared with recipients of kidneys with one or more mismatches (18).

Living donor grafts are generally superior to CAD grafts (19-21). One-year graft and patient survival in 2001 for the CAD transplant was 89.2% and 96.1%, and for the LD transplant was 94.3% and 98.3%, respectively (United Network for Organ Sharing, 2003). The superior results of LD versus CAD transplants can be partly explained by the potential for better HLA matching in LRD, reduction in the cold ischemia period and the absence of other factors pertinent to the transplanted organ itself, for instance, brain death and cardiovascular instability of the donor before nephrectomy.

1.2.2 Transplant Recipient Factors

The serious shortage of donor organs restricts access to renal transplantation patients with end-stage renal failure. Discrete criteria are therefore used to select an optimal recipient on the bases of age, sensitization, co-morbid conditions (cancer, HIV, advanced diabetes), weight and other factors.

Recipient age at transplant is an important predictor of outcome with higher rate of acute rejection in pediatric and young adult transplants. In adult renal transplantation, 5-year graft survival declines with an increase of a patient’s age. In contrast, in pediatric renal transplantation the age most commonly associated with increased risk is infancy (highest patient death rate in early post-transplant period and lowest graft survival) and
adolescence (increased risk of graft loss, late acute rejection, and incomplete rejection reversal) with poor long-term graft survival (22).

The presence of high titers of pre-formed anti-HLA class I specific immunoglobulin G (IgG) antibodies (panel-reactive antibodies, PRA), induced by previous failed grafts, blood transfusions, or pregnancy, may contribute to hyperacute or acute rejection and is often a barrier to allotransplantation. Highly sensitized patients with PRA against more than 85% of the donors may have difficulty in identifying a crossmatch negative kidney transplant and there have been alternative strategies developed that are aimed at defining acceptable mismatches (23) or a shared antigens based algorithm, *HLA-Matchmaker* (24).

Diabetes accounts for one-third of the patients with end-stage renal failure and therefore comprises a significant proportion of primary disease in the renal transplant population. Diabetes is one of the most important predictive variables for poor outcome after kidney transplantation, resulting in reduced graft and patient survival. (25, 26). Patients with diabetes have an increased incidence of postoperative infection following renal transplantation associated with poor perioperative hyperglycemic control. Moreover, it has been shown that histological features of diabetic nephropathy recur in some patients following renal transplantation (27).

Obesity has also been implicated as an important co-morbid factor associated with short- and long-term transplant outcome. A recent study clarified that it is not the obesity itself,
but the cardiovascular disease associated with it, that is responsible for inferior graft and patient survival in obese renal transplant recipients (28).

1.2.3 The Donor-Recipient Relationship

A combination of donor and recipient antigen-dependent and antigen-independent factors are frequently used as surrogate predictive markers for kidney transplant outcome. Antigen-dependent factors include the number of HLA A, B, and DR mismatches between a donor and a recipient. Antigen-independent factors include a ratio of donor to recipient body size, and age and gender of both.

It has been shown that the best HLA-matched transplants have a lower incidence of acute rejection episodes and higher rates of survival in renal transplant patients when adjusted for other risk factors (29). Introduction of molecular DNA typing techniques that replaced previously employed serological typing enabled precise definition of HLA class I and II alleles contributing to better HLA matching (30). However, genetic diversity in the population creates difficulties in finding a perfect HLA match between a donor and a recipient. Therefore more potent immunosuppression is usually employed to prevent acute rejection in HLA-mismatched patients.

It has been suggested that antigen independent factors that are associated with lower donor kidney mass or increased recipient size play a significant role in both cadaver and living donor kidney transplant loss (31). However when body surface area (BSA) was used as a surrogate for nephron mass in a study assessing the impact of matching donor
and recipient BSA on long-term renal transplant outcome in CAD transplants, graft survival and kidney function over 5 years did not differ among groups. It was concluded therefore that donor-recipient BSA, independent of other risk factors, does not affect the risk of allograft loss (32).

Donor age is considered an important factor limiting renal transplant outcome, and giving older kidneys to older recipients reduces renal allograft and patient survival (33, 34). As discussed earlier, donor and recipient gender may also play a role in kidney transplant outcome. Although some studies analysed large database sets, they neither reached a clear conclusion as to the magnitude of the effect that gender plays in kidney transplantation nor provided an explanation as to the physiological background of such a phenomenon.

1.2.4 Acute Rejection Risk Factors

Acute rejection is a major complication after renal transplantation and an important risk factor for chronic rejection. HLA-DR mismatch, delayed graft function, and prolonged cold ischemia time have a profound effect on both short- and long-term graft survival. The frequency of acute rejection episodes is significantly associated with the number of HLA-DR mismatches, and the lowest frequency of acute rejection episodes is seen in fully HLA matched grafts. The incidence of acute rejection is also significantly associated with prolonged cold ischemia time (14), and delayed graft function and HLA mismatch, are independent risk factors for early acute rejection. With development of novel immunosuppressive therapy and advances in new diagnostic techniques, the
incidence of acute rejection was reduced from 47-49.7% in 1982-1993 to around 22.0% in 1995-2002 (35). Real-time ultrasound-guided allograft biopsy and adoption of the Banff criteria for standardizing histologic evaluation have became the gold standard for the diagnosis and monitoring of acute rejection (36). It has been demonstrated that graft survival correlates closely with Banff grade I (interstitial rejection: i2-3 and t2-3), grade II (intimal arteritis: v1-2), and grade III rejection (transmural arteritis or fibrinoid necrosis within the vessel wall: v3). Grafts have a poorer prognosis if an intimal arteritis or fibrinoid arterial necrosis is present in contrast to interstitial or borderline rejection according to the Banff 97 criteria (37, 38).

Despite progress in diagnosis of acute rejection, modern diagnostic procedures can only confirm, not predict this event. New explorative analysis of the entire human genome, however, has identified several predictive clusters of genes, which can be functionally grouped into categories. This may allow construction of predictive models that incorporate polymorphic gene haplotypes to predict rejection risk.

Advances in immunosuppressive therapy have decreased acute rejection rates, and 1-year renal graft survival is now 90% or more. However, long-term results of kidney transplantation are still inadequate. According to the National Organ Procurement and Transplantation Network 2003 report, the 5-year graft survival rate is 65.7% for CAD transplant and 78.6% for LD graft transplants. When all other risk factors are stratified, the single most important factor in short- and long-term graft loss is a history of an acute rejection episode (39).
1.2.5 Immunosuppressive Therapy

The primary aim of immunosuppression in solid organ transplantation is to inhibit the recipient immune response to donor alloantigens without precluding the normal development of immunity to infection and immunosurveillance of abnormal or cancerous cells. The protocols employed for immunosuppression have changed dramatically over the last two decades. They typically include three components: induction, maintenance and, if necessary, acute rejection treatment.

Induction immunosuppressive therapy consists of a short-term high-dose immunosuppression when the recipient's immune system is initially exposed to the foreign allograft. It can be administered before the transplantation procedure and is continued for a short time after surgery. Drugs used during this period may include potent antilymphocyte antibody preparation [antithymocyte globulin (Thymoglobulin®), muromonab-CD3, daclizumab (Zenapax®), or basiliximab (Simulect®)] (40). During the past decade the proportion of patients that received antibody induction therapy increased and reached 65% by 2002 (The United States National Organ Procurement and Transplantation Network /Scientific Registry of Transplant Recipients, 2003).

Maintenance immunosuppressive therapy follows the induction period and is intended to prevent acute and chronic allograft rejection. Lower doses are typically administered and adjustments are made based on efficacy, side effects and patient tolerance, and drug-interactions (41). Agents used for maintenance therapy are steroids (methylprednisone), a
calcineurin inhibitor (cyclosporin or tacrolimus), and an antimetabolite (azathioprine or mycophenolate mofetil).

If rejection is suspected, high-dose pulse steroid therapy or a course of antilymphocyte antibody therapy may be necessary, depending on the clinical situation. The principal groups of drugs used in clinical transplantation are outlined below.

1.2.5.1 Corticosteroids

For many years corticosteroids (prednisone, methylprednisone) were the backbone of post-transplant immunosuppression and the main treatment for acute rejection episodes due to their potent anti-inflammatory properties. Corticosteroids bind to intracellular receptors and block lymphocyte proliferation and activation of several pro-inflammatory cytokine genes. Subsequently, synthesis of interleukins (IL-1, IL-2, IL-3, and IL-6; interferon gamma (IFN-γ); and tumor necrosis factor alpha (TNF-α) is inhibited (42, 43).

Steroids also inhibit antigen presentation by monocytes and migration of lymphocytes and macrophages to the site of inflammation via down-regulation of the synthesis and release of chemokines (44). Chronic use of corticosteroids is associated with a number of undesirable side-effects, including growth retardation, Cushingoid changes, weight gain, osteoporosis and increased susceptibility to infections (45). Therefore, alternate day administration of prednisone is often used with the aim of reducing adrenal-pituitary axis suppression and other steroid side-effects (46). In addition, low-dose steroids, late steroid withdrawal or steroid-sparing protocols have been implemented in renal transplantation.
In 2001 91% of renal transplant patients were on maintenance therapy with corticosteroids during the first year after transplant in comparison to 99.5% in 1992 (The United States National Organ Procurement and Transplantation Network /Scientific Registry of Transplant Recipients, 2003).

1.2.5.2 Cytotoxic drugs

Cytotoxic drugs, or antimetabolites that include azathioprine and mycophenolate mofetil inhibit both DNA and RNA synthesis by interfering with the synthesis of precursors of the de novo purine synthesis.

Azathioprine (AZA, Imuran), a derivative of 6-mercaptopurine, is a non-selective inhibitor of both DNA and RNA synthesis, which acts by suppression of de novo purine synthesis in all hematopoietic cells. It affects both B and T lymphocyte proliferation, suppresses primary T–cell and secondary antibody synthesis; however, it has no effect on cytokine production. It was used initially in combination with corticosteroids to prevent acute renal allograft rejection (51). Later azathioprine became a part of the triple therapy protocol together with cyclosporin and prednisone until the introduction of the more potent inhibitor MMF in 1995. Common side-effects of AZA treatment include bone marrow suppression resulting in leukopenia, thrombocytopenia, and anemia, hepatotoxicity, and increased risk of infections (52, 53).

Mycophenolate mofetil (MMF, Cell Cept) is an ester pro-drug of mycophenolic acid, a byproduct of a penicillin mold. It is a selective inhibitor of purine nucleotide synthesis in
T and B lymphocytes. MMF inhibits B and T cell proliferation, cell-mediated and humoral immunity and it is hypothesized that MMF interferes with adhesion molecule biosynthesis and thus suppresses entry of effector cells into inflammatory regions. It was found to be superior to AZA in its immunosuppressive properties with fewer side effects (54). In kidney transplant recipients, several large randomized trials have confirmed the superiority of MMF to AZA as part of triple drug maintenance therapy (55, 56). In these trials, patients receiving MMF in a maintenance regimen had fewer episodes of acute rejection and required less antibody treatment for rejection episodes than those on AZA, although short-term allograft and patient survival rates were similar between groups. MMF gradually replaced AZA in clinical practice, thus administration in kidney transplant patients increased from 1% in 1992 to 80% in 2002 (The United States National Organ Procurement and Transplantation Network /Scientific Registry of Transplant Recipients, 2003). The major adverse effects of MMF are gastrointestinal (abdominal pain, nausea, diarrhea, gastroenteritis and gastritis), hematologic (leukopenia and thrombocytopenia), and opportunistic infections (CMV and herpes simplex) (53).

1.2.5.3 Inhibitors of signaling

Inhibitors of signaling (cyclosporin, tacrolimus, and rapamycin) belong to a group of immunosuppressants that interfere with T cell proliferation. Cyclosporine (CsA, Neoral®), a cyclic peptide of fungal origin, is a selective inhibitor of lymphocyte signal transduction, which inhibits cytokine synthesis by binding to cytoplasmic proteins (cyclophyllins) in T lymphocytes. The immunophillin-drug complex inhibits calcineurin, a calcium-calmodulin-dependent protein phosphatase. This complex prevents
dephosphorylation and nuclear translocation of Nuclear Factor of Activated T cells (NFAT), which is an important transcription factor in interleukin-2 (IL-2) production. Cyclosporine therefore reduces the transcription of IL-2 and other cytokines and regulatory proteins necessary for T-cell activation and proliferation. The major adverse effects of cyclosporine are nephrotoxicity, neurotoxicity, hepatotoxicity, cardiovascular (hypertension), metabolic (electrolyte abnormalities, hyperlipidemia, impaired glucose tolerance), hirsutism, gingival hyperplasia, and facial dysmorphism in children. The major long-term side-effects are infections and malignancies (57, 58).

Tacrolimus (FK506), structurally related to macrolides antibiotics, is a potent immunosuppressive agent similar to CsA in its pharmacodynamic action. Tacrolimus functions in a similar fashion to CsA, by binding to FK-binding proteins (FKBP) in the cytoplasm. The events that follow are similar to those with CsA and result in inhibition of nuclear regulatory proteins and impaired synthesis of cytokines (IL-2) necessary for T-lymphocyte proliferation (59). Initially tacrolimus was used as a rescue therapy in CsA-treated patients. However, for the past decade tacrolimus has become the more frequently used calcineurin inhibitor as initial therapy in combination with MMF and steroids. In 2002 tacrolimus was used in 63% of kidney transplant recipients in comparison to 2% in 1993 (The United States National Organ Procurement and Transplantation Network /Scientific Registry of Transplant Recipients, 2003). Common adverse events with tacrolimus are nephrotoxicity (with less hypertension than with CsA), neurologic, gastrointestinal (anorexia, diarrhea, dyspepsia, nausea), and metabolic (hyperkalemia, hyperglycemia, and diabetes) (60).
Rapamycin (sirolimus) is a macrocyclic triene antibiotic, structurally similar to tacrolimus. It binds to FKBP 12, but, it does not affect calcineurin activity. Rapamycin inhibits mTOR (FRAP), and alters p70S6 kinase and PHAS-1 activity. Rapamycin does not inhibit cytokine synthesis, but rather inhibits the response of T cells to IL-2, IL-4 and other cytokines acting at a later stage in the cell cycle. Rapamycin has been shown to have an efficacy similar to CsA in preventing acute graft rejection but a different side-effect profile: Rapamycin is associated with hyperlipidemia and thromboleukopenia, anemia, arthralgia, and rash (67). Rapamycin was used in only in 15% of renal transplant patients in 2002 (The United States National Organ Procurement and Transplantation Network /Scientific Registry of Transplant Recipients, 2003).

None of the four drugs described above is able to prevent acute or chronic rejection in solid organ transplants when used alone. Treatment protocols therefore employ a combination of immunosuppressive agents which typically includes an inhibitor of signaling (CsA or Tacrolimus), a cytotoxic drug (AZA or MMF) and prednisone, with or without induction therapy by ALG, OCT 3, anti-IL2 receptor monoclonal antibody, or thymoglobulin, and prednisone free maintenance immunosuppression.

1.2.5.4 Blocking antibodies

Blocking antibodies are now commonly used for induction therapy in kidney recipients reflecting a shift from the predominant use as an acute rejection treatment a decade ago. Among them are the polyclonal antithymocyte globulin (Atgam®), monoclonal antibody
muromonab-CD3 (OKT3®) directed at the CD3 receptor on human T lymphocytes, and inhibitors of IL-2 receptor: basiliximab (Simulect®) and daclizumab (Zenapax®). The therapeutic effects of polyclonal antithymocyte globulin antibodies and OKT3 are thought to result from a depletion of T lymphocytes through opsonization and sequestration into the reticuloendothelial system and complement-mediated T cell lysis or induction of T-cell anergy (62, 63). Interleukin-2 receptor antagonists bind directly to the CD25 (alpha-chain) of the IL-2 receptor expressed on activated T lymphocytes, thereby inhibiting IL-2-mediated activation and proliferation.

Adverse effects of antibody therapy range from mild flu-like symptoms to severe anaphylactic reactions, encephalopathy, nephrotoxicity, and posttransplantation lymphoproliferative disease (64).

1.2.6 Immunosuppressive therapy monitoring

Adequate dosing of immunosuppressive drugs is a crucial factor for the prevention of acute rejection or drug toxicity. Because of variable pharmacokinetics and narrow therapeutic indices of immunosuppressive drugs, pharmacokinetic and pharmacodynamic (therapeutic) drug monitoring has been introduced into clinical practice.

Pharmacokinetics describes the absorption, distribution, metabolism, and excretion of drugs in the body. Pharmacokinetics measurements in renal transplantation are used for inhibitors of signaling cyclosporine, tacrolimus and rapamycin.
For measurement of the pharmacokinetics of cyclosporine (Neoral), a trough level (at 0 time) and a single-point concentration at 2 hours post-dose (C2) measuring are used currently. It has been shown that C2 measuring optimises the cyclosporine dosing and minimises the risk of acute rejection. Reducing the C2 level to the maintenance dose (approximately 800 mcg/l) may prevent adverse events linked to cyclosporine toxicity, and improve general well-being of renal transplant patients (65).

Tacrolimus has a 10- to 100-fold greater in vitro immunosuppressive activity compared with cyclosporine, therefore therapeutic whole blood trough concentrations for tacrolimus are around 20-fold lower than the corresponding cyclosporine concentrations. Although tacrolimus trough concentrations correlate with toxicity they do not strongly correlate with acute rejection risk (66). Nevertheless, sampling at other time-points offers no advantage over trough level monitoring. Pharmacokinetics measurements of trough levels of Rapamycin are also used in renal transplantation.

Pharmacodynamic monitoring measures biologic response to a drug by determining activity of enzymes involved in T cell signaling or expression level of the T cell surface molecules. To date, the most detailed studies have involved pharmacodynamic monitoring of CsA and MMF, by measurement of the activity of the enzymes calcineurin and inosine monophosphate dehydrogenase, respectively (67). OKT3 therapeutic drug monitoring has been done by measuring CD3 levels (68). However, reliance on blood counts to monitor azathioprine therapy can be misleading, and they do not provide information on immunosuppressive efficacy. Azathioprine efficacy is measured by
thiopurine S-methyltransferase enzyme activity and the quantification of intracellular 6-thioguanine nucleotides concentrations in red blood cells. There are no standard protocols currently available for the monitoring of other groups of immunosuppressive drugs used in renal transplant patients and dose adjustments commonly based on clinical outcomes. This approach is not optimal because of individual differences in the effects of immunosuppressive drugs. Various immune function assays were proposed lately to optimize the effects of drug monitoring by measurement of lymphocyte proliferation, expression of T-cell surface activation antigens (CD25, CD71, CD11a, CD95, CD154), production of intracellular cytokines (IL-2, INF-γ, TNF-α), and detection of lymphocyte subsets (CD4, CD8, CD16, CD20) by flow cytometry (69). Recently MRI was introduced as an effective, non-invasive method of renal graft monitoring (70). Another method of transplant monitoring is a measurement of post-transplant donor-specific anti-HLA antibodies (DS-HLA Abs) utilizing the ELISA technique (71). A recently reported microarray assay for the detection and differentiation of acute renal rejection (72) offers promising approach for better understanding the molecular mechanisms underlying renal acute rejection, its early detection and the assignment of individualized treatment protocols.

1.3 Immunology of Transplantation

In kidney transplantation there is always a conflict present between donor and recipient immune system elicited by genetic dissimilarity in allogeneic transplants. This conflict frequently leads to acute allograft rejection that presents an ongoing problem in renal transplantation because it may precede acute or chronic graft loss. It is an immunological
process that involves professional and non-professional antigen-presenting cells (APC), T and B cells, and macrophages and is triggered by the recognition of alloantigens by recipient T cells.

The acute rejection process consists of three major events:

1. Alloantigen recognition by naïve host T cells
2. T cells activation, differentiation and proliferation
3. Infiltration of the allograft by the effector T cells

These events lead to a graft destruction or, alternatively, to a development of immunological tolerance.

The first event occurs through the direct or indirect HLA class I or II recognition of alloantigens presented by professional APCs, dendritic cells and macrophages, in the graft or recipient lymph nodes or spleen. The second phase is the activation T cells by the T cell receptor (TCR) and co-stimulatory signals, differentiation into alloantigen-specific cytotoxic T cells (CTL), T-helper cells (Th1, Th2, and T-regulatory phenotypes) and proliferation. The third phase begins when activated T cells move into the allograft, attracted by chemokines released by the graft, where they can cause reversible or irreversible graft destruction, or tolerance.

1.3.1 Antigen Recognition

Antigenic differences between a donor and a recipient are encoded by HLA (human leukocyte antigen) class I and II genes and minor histocompatibility (mH) antigens. Their
products are displayed on the surface of all nucleated cells and therefore create a powerful barrier to allotransplantation. HLA class I molecules express self-proteins in the form of peptides and HLA class II express digested and processed allo-proteins both of which serve for the T cell direct, or indirect recognition of alloantigens. Direct recognition involves the donor’s HLA presented on the surface of donor-derived antigen-presenting cells (APC) that are recognised by the recipient’s T cell receptor (TCR). CD8+ T cells recognize allo-HLA class I, while CD4+ T cells recognize allo-HLA class II molecules. In contrast, indirect recognition involves the recipient’s antigen presenting cells processing the donor’s HLA class I and II and a presentation in the form of peptide in a context of HLA class II to the recipient’s CD4+ T cells (73-75). Both HLA class I and II also present minor histocompatibility antigens which are peptides derived from allelically polymorphic host proteins distinct from HLA molecules, and they may cause a strong immune response even in HLA-identical transplants (76). The recognition of allograft HLA and mH antigens is the crucial initial event that leads to acute rejection and graft destruction. It has been suggested that the initial response of the recipient T cells is limited to a few dominant allogenic determinants of the donor HLA with increasing response at the onset to infrequently presented cryptic allogeneic HLA peptides (77). This phenomenon may play an important role in the amplification and the continuation of the rejection process.

1.3.2 T Cell Activation, Differentiation and Proliferation

T cells that recognize alloantigen presented by self- or donor-derived antigen-presenting cells become activated and begin rapid proliferation. Differentiation and proliferation of
the T cells depends upon affinity and avidity of the alloantigen presented, timing of an antigen encounter, co-stimulation, and the cytokine environment produced by T-helper (Th) cells, dendritic cells, and macrophages (78-80).

The strength of the T cell proliferative response within the direct pathway of allore cognition depends largely upon the concentration of foreign HLA complexes presented to alloreactive T cells during the first few weeks after transplantation, when donor-derived dendritic cells are available within the recipient lymphoid tissue (81). Also it has been suggested that indirect pathway allore cognition plays an important role in late-onset acute and chronic rejection, even in the absence of direct allore cognition (82).

T cell activation is mediated by an intricate network of signaling pathways that determines the efficacy of the immune response and initially depends upon signals delivered through the TCR and co-stimulatory molecules including CD28, CTLA-4 (Cytotoxic T-Lymphocyte-associated Antigen-4, CD152), CD40 ligand (CD40L), and the transmembrane protein tyrosine phosphatase CD45. CD28 and CTLA-4 are expressed on the T cell and both utilize the same, CD80 (B7-1) ligand for stimulation with opposing effect on T cells activation leading to stimulation or downregulation of T cell activity (83). Antigen-driven proliferation and activation of naive CD4+ T cells in vivo is dependent on CD28-derived signals (84), and is inhibited by CTLA-4 (85, 86), which is not expressed on naive T cells but up-regulated upon T cell activation (87). CTLA-4 signaling blocks interleukin- two (IL-2) production, IL-2 receptor alpha chain (IL-2Rα) and CD69 (an early marker of T cell activation) expression, and cell cycle progression.
and is important in the induction of T cells tolerance *in vivo* (89). CD28 blocking by CTLA-4 fused to immunoglobulin (CTLA-4Ig fusion protein) resulted in a long-lasting survival of organ transplants in several models (90, 91).

CD40/CD40L interaction is also among the first critical events in immune activation. The CD40 molecule is expressed on APCs and B lymphocytes, and CD40 ligand (CD40L) is expressed on T lymphocytes. Their engagement up-regulates B 7-1 and B 7-2 expression on dendritic cells (92) and induces interleukin-12 (IL-12) production followed by priming of CD8+ T cells into cytotoxic T lymphocytes (CTLs) (93).

CD40 ligation on B lymphocytes is essential for the regulation of B cell proliferation, production of immunoglobulins (Ig), and Ig class switching. CD40 stimulation on monocytes and macrophages augments their pro-inflammatory activities and CD40 engagement on endothelial cells up-regulates cell adhesion molecules that are essential for the migration of leukocytes into sites of inflammation (94). The blockade of CD40 ligand-CD40 interaction impairs CD4+ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and effector function, by inhibiting the induction of B 7-1 on APC (95). In animal models, blocking anti-CD40L antibodies were found to be beneficial in renal (96) and cardiac allotransplantation (97), where prolongation of a graft survival was accompanied by immune deviation toward Th2 cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10). Moreover, the CD154 blockade on CD4+CD25+ regulatory T cells enhanced their immunosuppressive activities *in vivo* in a model of allograft tolerance.
which could be a contributing factor to the long-lived therapeutic effects of anti-CD154 treatment (98).

CD45 (PTPRC) is a transmembrane protein tyrosine phosphatase that is expressed on all nucleated haematopoietic cells and is a key regulator of T cell receptor (TCR) and B cell receptor (BCR) signaling required for the activation and development of lymphocytes (99, 100). CD45 can function as a positive regulator of antigen-receptor signaling via dephosphorylation of Src kinases (101) or as a negative modulator of cytokine-receptor signaling via dephosphorylation of JAK kinases (102). CD45 is expressed in different isoforms (RA, RB, RC, and RO) as a result of alternative splicing of variable CD45 exons coding for the extracellular domain (103, 104). The manipulation of various CD45 splice variants with antibodies prevented transplant rejection in mouse model of renal transplantation (105). It has been suggested that anti-CD45RB antibodies up-regulate CTLA-4 expression and this therefore demonstrates a link between CD45 and CTLA-4 that depends on calcineurin-mediated signaling (106). Moreover, the combination of anti-CD45RB and anti-CD40L antibodies also demonstrated significant improvement in the induction of tolerance to islet allografts by inhibition of CD8\(^+\) cells, B cells, and monocytes islet infiltration and the inhibition of Th1 cytokines and an increase of Th2 cytokine expression within the graft (107).

T cells activated through the TCR and co-stimulatory signals described above, require T cell growth factors for further proliferation. Cytokines are key factors driving Th1, Th2, Th3 or Tr expansion during the initial phase of CD4\(^+\) T cell activation. There are two
main subgroups of cytokines produced by differentiated T cells. One of them is represented by pro-inflammatory tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ); another is represented by interleukin-10 (IL-10) and tumor growth factor-beta (TGF-β), cytokines that exhibit anti-inflammatory or tolerogenic properties. Th1 cells secrete IL-2 and IFN-γ, Th2 cells secrete IL-4, IL-10, and IL-13, and Tr cells secrete IL-10, and TGF-β (108, 109, 110). The role of cytokines in the acute rejection or induction of graft tolerance has been largely studied in the context of the Th1/Th2 paradigm. This paradigm proposes that Th2 lymphocytes support tolerance induction by producing IL-4 and IL-10, and Th1 lymphocytes inhibit tolerance induction by secreting IL-2, TNFα/β and IFN-γ (111). This hypothesis is based upon assessment of intragraft cytokine expression by RT-PCR or by immunohistochemistry techniques. It has been reported that serum levels of TNF-α, IL-1α and IL-6 are increased during acute rejection episodes, and that these cytokines are overexpressed within the graft (112-115). Another group reported intragraft gene expression of granzyme B, IL-10, and IL-2 in acute rejection and of TGF-β1 in chronic rejection, findings that correlated well with histological diagnoses using the Banff criteria for acute and chronic rejection, respectively (116). It has also been reported that mRNA for IFN-γ is consistently expressed in human renal allografts prior to or during rejection (117) and could contribute to the degree of renal graft infiltration and to the severity of the rejection episode. However, it was also suggested that IFN-γ may be required for the induction of long-term transplant tolerance by the CTLA-4 Ig blockade in the MHC-mismatched donor-recipient pairs (118) and that IL-2 also triggers negative feedback mechanisms in T lymphocytes, which limits alloimmune responses (119). Although studies of cytokine
gene expression may provide information on immune events underlying acute rejection or acceptance of the graft, it is not clear whether pro-inflammatory cytokine infiltration is a result, or consequence, of acute rejection. Moreover cytokine function may not be unidirectional as it was previously thought.

1.3.3 Effector Phase: Destruction of the Graft or Induction of Tolerance

Following activation, alloreactive T cells become capable of crossing the vascular endothelial barrier and entering the graft. The inflammatory response within the graft elicited by transplant and ischemia-reperfusion injury, causes up-regulation of cytokine and chemokine production, and recruitment of monocytes and macrophages that can provide in situ antigen presentation and co-stimulation to the T cells. Allograft destruction by activated cytotoxic T cells occurs via two major cytotoxic mechanisms: perforin/granzyme (CD8+ and NK cells) and Fas/Fas-ligand system (CD4+ cytotoxic lymphocytes) (120). Additionally, activated macrophages, neutrophils, and eosinophils may contribute to the acute rejection process with the ultimate endpoint: destruction of the graft and loss of the transplant.

The induction of antigen-specific tolerance is an important goal in de-novo transplant. Peripheral transplantation tolerance can be divided into two phases: induction phase immediately post-transplant and a maintenance phase (121).

The induction phase is characterized by a large number of alloreactive T cells stimulated by graft antigens that ultimately result in acute rejection unless the inflammatory process
is well controlled by various mechanisms including T cell depletion, anergy and production of inhibitory cytokines.

The maintenance phase of allograft tolerance requires active immunoregulatory control of antigen-specific alloreactive T cells by a specific subset of T regulatory (Tr) cells (122). Several subsets of Tr cells with distinct mechanisms of action are now identified, including Tr1, Th3, and CD4+$\text{CD}25^+$ T cells. Tr1 cells secrete high levels of IL-10 and moderate to low levels of TGF-beta with the immunosuppressive effect attributed to the downregulatory properties of secreted IL-10 (123). Tr1 cells display an increased ability to migrate into inflamed tissues and are present in increased numbers within the tolerated grafts (124, 125). Th3 cells produce high levels of TGF-$\beta$ and moderate amounts of IL-10 and IL4 (126). CD4+$\text{CD}25^+$ T cells comprise approximately 10% of all T cells and constitutively express CTLA-4 on their surface (127). They are powerful suppressors of the activation of both CD4$^+$ and CD8$^+$ T cells. Suppression is mediated by cell-cell interaction (128) and IL-10 and CTLA-4 dependent mechanism (129). CD4$^+$CD25$^+$ regulatory cells were shown to suppress rejection of skin grafts (129) and adoptively transfer transplantation tolerance in the mouse model (130). CD4$^+$CD25$^+$ cells were found in stable renal transplant patients, and their action was implicated in downregulation of alloreactive donor-specific T cells activated through the indirect pathway of allore cognition (131).

Experimental data suggests that maintenance of established T cell tolerance may be related to the immunoregulatory action of transforming growth factor beta (TGF-$\beta$), a
cytokine known to inhibit the Th1 cells responses. In \textit{in vitro} experiments TGF-\(\beta\) production increased upon the addition of IL-4 to CD4\(^+\) T cell cultures and significantly increased after addition of exogenous TGF-\(\beta\) (132). Resting \textit{naïve} T cells constitutively produce TGF-\(\beta\), but when stimulated with peptide and APC under conditions that allow clonal expansion, TGF-\(\beta\) production is abrogated. However, anergic T cells secrete TGF-\(\beta\) and this secretion can not be down-regulated by re-stimulation under immunogenic conditions (antigen and APC) (133).

1.4 Genetics of Transplantation

T cell activation and regulation are central in the generation and expression of the alloimmune responses that lead to allograft rejection. It is now clear that immune activity varies markedly between individuals, and has been suggested that this difference may be determined by polymorphisms within the genes for certain immunoregulatory molecules or their receptors (134, 135). Various genetic factors contribute to an immunological response, including diversity of human leukocyte antigen genes, signaling molecules and cytokine genes.

1.4.1 Human Leukocyte Antigen Genes

Human leukocyte antigen (HLA) genes are the most polymorphic genes known in humans. There are more than 1300 alleles defined by molecular typing that represent a major barrier to successful solid organ and hematopoietic stem cell transplantation (136). The HLA genes that are involved in the allograft immune response are located on the
short arm of chromosome 6 and encode two structurally and functionally different classes of HLA class I (HLA-A, B, C) and class II (HLA-DR, DQ, DP) antigens that are highly polymorphic cell surface glycoproteins (137, 138).

The HLA class I genes (A, B, and C) encode the α-chain of the HLA class I molecule that is associated with β2 microglobulin on the cell surface. The α chain consists of α1, α2, and α3 domains with variable regions located in the α1- and α2-domains that form a peptide binding site. HLA class II genes (DR, DQ, and DP) encode the α-chain and the β-chain of the functional molecule (139). All of the major polymorphisms of the HLA DR gene are located in the β1-domain of the β-chain genes (140).

The peptide-binding groove of the HLA molecule consists of two parts, the two α helixes that form walls of the groove and a floor formed by the β-pleated sheet. Pockets formed by amino acids in the floor and sides of the groove determine which peptides an HLA molecule binds, because the side chains that fit into the grooves serve as the peptide's anchors. The residues lining the pockets are encoded in genetic segments defined by the individual alleles of the HLA genes and can bind to peptides distinguished by two or three amino acid residues (motifs) that fit into the anchoring pockets. It has been shown that each of the peptide-binding pockets plays a significant and distinct role in binding of self-peptides required for recognition of the HLA molecule by alloreactive cytotoxic T lymphocytes (CTLs) (141).
In the HLA class I peptide-binding groove, the ends of the α helixes of the α1 and α2 domains join together to close the groove accommodating short 7 to 15 residue peptides, whereas in the HLA class II molecules, the groove is open-ended, harbouring longer peptides. The orientation of the peptides is fixed, their amino terminal ends positioned at the same end of the groove as defined by the orientation of the HLA polypeptide chains and with the carboxy terminal at the opposite end.

Allelic variations among the HLA class I and class II gene products are the basis for the differential peptide binding, and this constitutes a major problem for transplantation as the HLA molecules function as ligands for both T cells and NK cells implicated as mediators in allograft rejection. Several studies suggested that the HLA phenotype of the recipient, especially the HLA-DR antigens, may determine solid organ transplant outcome. Two restriction fragment length polymorphisms, Bam HI of the HLA DR and Eco RV of the HLA DQ regions were suggested as molecular markers of acute rejection (142), however, this finding was not confirmed later. The HLA-DR6 positive phenotype in recipients was found to be associated with acute rejection of HLA mismatched kidney grafts (143) whereas the HLA-DR1 phenotype was reported to be associated with the a lower incidence of acute rejection in studied groups of patients (144). In another study, kidney grafts from donors mismatched for HLA-DR6 were shown to be less immunogenic than grafts with other HLA-DR mismatches (145, 146). Despite earlier reports, data on the association of particular HLA-DR antigens or particular HLA mismatches with acute allograft rejection were not confirmed in other studies.
1.4.2 The T Cell Receptor

Generation and maintenance of an effective repertoire of T cell antigen receptors are essential to the immune system. Conventional T cells express αβ T cell receptors (TCRs), which engage processed antigen presented on the HLA molecules. T cells can also express γδ TCRs, which are not well characterized and are linked to innate immunity in part due to their ability to recognize unprocessed ligands directly, without a requirement for presentation by HLA.

The αβ TCR, the most common heterodimer, consists of two disulphide-linked alpha and beta chains, each of which has a membrane-distal variable (Vα or Vβ) domain that forms three complementarity-determining regions (CDRs) or loops. The less variable CDR1 and CDR2 loops interact primarily with the relatively conserved amino acid residues of the HLA alpha helixes, whereas the more variable CDR3 loops touch the most variable part of the peptide, determining the specificity of the interaction (147). Several attempts were made to define the genetic TCR differences on the T cells infiltrating allografts in acute rejection and under tolerogenic conditions. These studies produced conflicting and inconclusive results. The studies focused on the Vβ length polymorphism in the hypervariable CDR3 region. Alloreactive T lymphocytes derived from acutely rejected human kidney allografts were shown to exhibit restricted repertoires including Vβ2, Vβ3, Vβ7, Vβ13, and Vβ14 (148, 149), or with predominant involvement of Vβ8 (150, 151). The same increase in Vβ8 expression was observed in rejected heart allografts (152). In another study (153) Vβ8 and Vβ23 were also overexpressed in chronically rejected kidneys with superimposed acute lesions. However, in experimental studies that
involved animal models of allotransplants, Vβ transcript regulation and CDR3 length distribution during acute rejection exhibited increased diversity of the TCR repertoire without any clone predominance of the Vβ family. In contrast, tolerated grafts harbored T cells with a highly altered repertoire attributed to regulatory cells (154, 155). Discrepancy in the published results may be explained by a difference in the transplant models studied (human vs. inbred animal). Moreover, it has been suggested by Lennon et al (156), that the increase in transcription of the specific Vβ chain is not dependent on a specific antigen, but determined by the TCR’s upstream promoter. This may explain why studies of the TCR Vβ repertoire analysis inconsistently identified the TCR Vβ expression in the rejected kidney transplants.

The role of γδ T cells in acute allograft rejection remains undefined. One reported study found infiltration by γδ T cells in 30% to 40% of rejected kidney allografts (up to 10% of the total T cell population in a contrast to < 1% in non-rejected kidneys) (157), but this finding has not been confirmed in subsequent studies.

1.4.3 Signaling Molecules Genes

In addition to the variability in HLA and the TCR that affects allorecognition, polymorphisms in co-stimulatory molecule genes may also contribute to a differential immune response in the recipient. Recent studies showed a differential expression of co-stimulatory molecules in renal biopsies of allograft recipients undergoing acute or chronic rejection (158). An intense focal infiltration of the CTLA-4 positive T lymphocytes (mainly CD8+) was present in acute rejection biopsies, whereas CD40L positive T cells were present in chronic rejection. At the same time, increased CD40L
gene expression was observed in peripheral CD4+ T cells during acute rejection and in chronic allograft nephropathy (159).

1.4.3.1 The CTLA-4 gene

The human CTLA-4 gene, located on the long arm of chromosome 2 at 2q 33-34, is composed of four exons that encode a leader sequence, extracellular domain, transmembrane domain, and cytoplasmic domain (160, 161). It has been shown that CTLA-4-deficiency results in spontaneous lymphocyte activation and development of lymphoproliferative disorders and early lethality in mice suggesting an important role of this molecule in lymphocyte homeostasis (162). Recently, synergy of the CTLA-4 gene with a particular HLA susceptibility haplotype (DR4-DQA1*0301-DQB1*0302) has been shown in autoimmune diseases that include type I diabetes, Graves’ disease, and Hashimoto thyroiditis (163). The CTLA-4 gene is known to contain several polymorphisms including restriction fragment length polymorphisms (RFLP) at a position −318 (C to T substitution) of the promoter region (Genebank # M74363), and in the exon 1, at a position 49 (A to G substitution) coding for threonine or alanine, respectively (Genebank # M74363). Two polymorphic repeat sequences located 13.9 cM centromeric of CTLA-4 gene (164), and dinucleotide (AT)n repeat sequence in the 3’-untranslated region, at a position 642 (Genebank # M37243) have also been described. Certain CTLA-4 gene polymorphisms were found to be associated with a number of autoimmune disorders. An increased frequency of the G49 allele was reported in multiple sclerosis (165), Hashimoto thyroiditis and Addison’s disease in patients, carrying the human leukocyte antigen DQA1*0501 (166). Autoimmune hepatitis (167), insulin-
dependent diabetes mellitus (IDDM) (168), and systemic lupus erythematosus (169) were also associated with G<sup>49</sup> allele. The CTLA-4 gene 106 bp (AT)<sub>n</sub> repeat allele was also associated with autoimmune disorders, including Addison disease (170), and Graves disease (171), and was found to be in strong linkage disequilibrium with G<sup>49</sup> allele (172).

A study of liver and kidney transplant recipients of mixed ethnic origin (Caucasian, African American, Hispanic, and Asian) reported an increased frequency of 92, 94 and 100 bp (AT)<sub>n</sub> repeat alleles in patients that experienced at least one episode of acute rejection (173). However, the most recent report from the same group could confirm an association between 92, 94, and 100 bp (AT)<sub>n</sub> alleles and graft survival for African-American liver transplant recipients only (174). This group also found a significant association of the CTLA-4 G<sup>49</sup> allele with reduced 5 and 10-year graft survival (39.7% and 9.9%, respectively) independent of the frequency of acute rejection episodes in the cohort of liver transplant recipients studied.

Several studies attempted to demonstrate an association between CTLA-4 gene polymorphisms and CTLA-4 molecule expression or T cell proliferative responses and cytokine production. A study of myasthenia gravis (MG) patients found a positive correlation between the CTLA-4 (AT)<sub>n</sub> length polymorphism and serum IL-2 receptor alpha chain (IL-2Rs<sub>α</sub>) concentration, a marker for the T cell activation (175). Longer CTLA-4 (AT)<sub>n</sub> alleles were associated with an increased concentration of IL-2Rs<sub>α</sub> in patients that did not receive an immunosuppressive therapy. Ligers et al. (176) have reported a relationship between thymine at position -318 of the CTLA4 promoter (T<sup>-318</sup>)
and adenine at position 49 (A<sup>49</sup>) in exon 1 and increased cell-surface CTLA-4 expression in stimulated CD3+ T cells. The presence of T<sup>318</sup> allele was also associated with higher promoter activity in a study by Wang et al. (177). This finding was supported by a recent report that linked the G<sup>49</sup> allele to reduced CTLA-4 surface expression and increased IL-2 secretion by activated T cells in GG homozygous subjects (178).

1.4.3.2 The CD40L Gene

The human CD40 ligand (CD40L) gene has been mapped to the long arm of the X chromosome at Xq26. The CD40L gene spans over 12 kb and consists of five exons (179). Mutations in CD40L cause a severe form of immunodeficiency, hyper IgM syndrome, resulting in recurrent bacterial infections (180). The CD40L gene has been implicated as a risk factor for increased susceptibility to tuberculosis (181), though this finding was not confirmed in later studies (182).

Nine single nucleotide polymorphisms (SNPs) have been identified in the CD40L gene to date, including the promoter region SNP at a position −726 (C<sup>−726</sup>), associated with the lower risk for severe malaria in African males (183). Although, this association has not yet been confirmed. Moreover, none of the identified SNPs could be linked to an obvious function.

A dinucleotide (CA)<sub>n</sub> repeat polymorphism in the 3’ untranslated region of the CD40L gene has heterozygosity suitable for the further genetic study (184). In a study examining the influence of the CD40L (CA)<sub>n</sub> dinucleotide-repeat polymorphism on susceptibility to
and severity of multiple sclerosis (MS) in Nordic patients, allele frequencies for the CD40L (CA)n repeat did not differ significantly between MS patients and controls, or between subgroups of MS patients (185). In contrast, an association of the longer (CA)n alleles of the CD154 gene in patients with systemic lupus erythematosus in comparison to normal controls has been reported recently (186). There have not been any reports on association of dinucleotide-repeat alleles in the CD40L gene in renal transplant patients to date, which should be explored given the data that the longer alleles were associated with more prolonged CD154 protein expression in activated lymphocytes of the healthy controls in the aforementioned study (186).

1.4.3.3 CD45 Gene

The CD45, transmembrane protein-tyrosine phosphatase receptor type C (PTPRC), is a signaling molecule indispensable for the T cell receptor activation that modulates the strength of signals upon interaction with an antigen-presenting cell (187). CD45-deficiency in humans leads to combined immunodeficiency disease (188); conversely, constitutive expression of active form of CD45 is associated with development of autoimmune disorders in mice (189). The human CD45 gene, located in the long arm of the chromosome 1 at a position 1q31-q32, consists of 33 exons (190). Three of these exons [exons 4 (A), 5 (B), and 6 (C)] are alternatively spliced producing at least five different isoforms distinct in their extracellular domain (191). The pattern of CD45 splicing is highly regulated and depends upon the cell type and state of activation. Resting or naïve T cell express high molecular weight CD45 isoforms produced by different combinations of exons 4, 5, and 6, whereas activated T cell change to
expression of low molecular weight isoforms lacking exons 4 and 6. It has been found that a variation in exon 4 (C to G transition at a position 77) of the CD45 gene causes variant splicing in humans (192-194). Activated or memory lymphocytes of the individuals with the G\textsuperscript{77} continue to express both high and low molecular weight isoforms in contrast to the usual pattern of low molecular weight isoform expression. It has been suggested that the CD45 gene could serve as a modifier of human autoimmunity, generating a number of conflicting reports related to the association of the CD45 alleles with various autoimmune disorders. In support of this hypothesis, several reports on association of the CD45 G\textsuperscript{77} allele and autoimmune hepatitis (195), multiple sclerosis (196), and systemic sclerosis (197) have emerged recently. However, the association of multiple sclerosis and the CD45 G\textsuperscript{77} allele has not been confirmed in larger studies (198, 199). An attempt to find an association of G\textsuperscript{77} allele and autoimmune myasthenia gravis has also been unsuccessful (200).

1.4.4 Cytokine Genes

Cytokines play an important role in the regulation of normal immune function including T and B cell priming in allotransplantation. In recent years, cytokines and their receptors have been shown to be highly polymorphic. Numerous studies indicate a direct association between some polymorphisms in cytokine gene promoter sequences and the levels of mRNA expressed or protein produced. This has been shown for the IL-10 gene, where GG\textsuperscript{1082} polymorphism in the promoter region was associated with lower production of IL-10 cytokine \textit{in vitro} in comparison to AG or AA genotypes (201) and for IL-6 gene, where a G to C substitution at position −174 in the promoter of IL-6 also
increased in vitro transcription of IL-6 (202). It has also been shown that a polymorphism at a site other than the promoter (for example, Taq I polymorphism within exon 5 of the IL-1β gene) may influence gene transcription (203). Although single nucleotide polymorphisms of cytokine genes were linked to in vitro cytokine production this fact could not always be confirmed in vivo studies. This may be due to a complexity of cytokine interaction in vivo and marked diversity of cytokine polymorphism frequencies in different ethnic groups as it has been shown (204). Polymorphisms in cytokine genes have been associated with number of autoimmune diseases as well as organ transplant outcomes in heart, liver, and renal transplantation. IL-10 gene promoter polymorphism has been implicated in rheumatoid arthritis (205); IFN-γ and IL-6 (206), and TGF-β1 and TGF-β1 receptor type II (207) gene polymorphisms were found to associate with graft-versus-host disease in HLA-matched sibling bone marrow transplantation. Polymorphisms of IL-10 (allele G-1082) and TGF-β1 +600 T/C (codon 10) were reported to be associated with acute liver graft rejection (208). Recently TNF-α and IL-10 polymorphisms linked to TNF-α high / IL-10 high production have been associated with acute graft rejection in renal transplantation (135), while a TNF-α high / IL-10 low producer phenotype was found to be associated with heart transplant rejection (209). However, there is little agreement between different research groups as to the functional significance of each polymorphism studied and its role in transplant outcome. Despite optimism in earlier reports, current renal transplant association data is confusing and often contradictory and requires careful interpretation and clarification.
1.4.4.1 IFN-\(\gamma\) Gene

Interferon gamma (IFN-\(\gamma\)) belongs to a family of interferon proteins and glycoproteins that are produced in response to infection. Interferon-gamma induces an increase of antibody production by B cells, activates natural killer cells, macrophages and neutrophils. Interferon-gamma induces expression of major histocompatibility complex (MHC) class II antigens on macrophages, therefore augmenting antigen presentation in direct- and indirect- allorecognition in transplantation. Interferon-gamma also modulates macrophage and CD4\(^+\) T cell function by up-regulating TNF-\(\alpha\) production and down-regulating interleukin-4, interleukin-5, and interleukin-10 production.

IFN-\(\gamma\) is encoded by a single gene, mapped to a long arm of chromosome 12 at q24.12 in humans. The gene consists of four exons with three intervening sequences. A variable dinucleotide polymorphism has been described in humans within the first intron of this gene between positions 1349-1373 \(210\) and a single nucleotide polymorphism has also been detected at position +874 \(211\). Certain alleles of dinucleotide repeat polymorphism in IFN-\(\gamma\) gene have been found to be associated with IgA nephropathy and lupus nephritis in Japanese patients \(212, 213\), development of sepsis after trauma \(214\), and graft-versus-host disease in bone-marrow transplantation \(206\).

A correlation was found between high levels of secreted IFN-\(\gamma\) and an increased incidence of acute rejection after renal transplantation \(215\). More recent evidence suggests that a particular allele, corresponding to 12 bp dinucleotide repeat, is associated with higher level of IFN-\(\gamma\) secretion, and hence may be of biological importance in the
process of acute rejection, particularly in patients receiving cyclosporin monotherapy (216, 217). In contrast, there was no significant relationship found between the IFN-γ dinucleotide repeat polymorphism and the incidence of acute rejection in heart and liver transplant recipients (218, 219).

1.4.4.2 TNF-α Gene

Tumor necrosis factor-alpha (TNF-α) is a 17kd polypeptide, that is structurally related to several other cytokines including TNF-β. Among this family of cytokines, TNF-α is the most potent mediator of inflammation. The role of TNF-α includes activation and chemotaxis of leukocytes; induction of the expression of adhesion molecules on neutrophils and endothelial cells, and regulation of the secretion of other pro-inflammatory cytokines.

The human TNF locus is situated on the short arm of chromosome 6 at p21.3 in close proximity to the HLA-B locus. Variations in levels of TNF-α production have been linked to differences in the TNF gene specific polymorphisms, providing conflicting results (220, 221). The TNF-α gene promoter polymorphism at position –308 has been associated with susceptibility to systemic lupus erythematosus (222), and microsatellite polymorphism was associated with rheumatoid arthritis (223). It has also been suggested that the combination of the high TNF-α (AA\textsuperscript{-308}) and low IL-10 (AA\textsuperscript{-1082}) gene polymorphisms is associated with heart transplant rejection (209). In renal transplant the frequency and number of acute rejection episodes was reported to be increased in patients with low TNF-α (GG\textsuperscript{-308}) and high IL-10 (GG\textsuperscript{-1082}) combination phenotypes.
and in TNF-α high / IL-10 high producers in HLA-DR mismatched transplants according to the report of Sankaran et al (135). That was contrary to the finding in heart transplant recipients with history of acute rejection.

1.4.4.3 IL-10 Gene

Interleukin-10 (IL-10) is a 36 kDa homodimeric cytokine that is produced by T cells, B cells and macrophages. IL-10 has a different effect in different cell types: it increases major histocompatibility complex (MHC) class II gene expression, proliferation and differentiation of human B cells, whereas it blocks MHC class II expression and B7 expression on the surface of monocytes, and reduces MHC class I expression on the surface of cytotoxic T-lymphocytes. IL-10 inhibits secretion of certain cytokines: IL-1, TNF-α, IL-6, IL-8, and IL-12 from monocytes/macrophages, and IFN-γ and IL-2 from T cells therefore limiting an inflammatory response. Differential expression of IL-10 has been implicated in rheumatoid arthritis (225, 226), systemic lupus erythematosus (227), and in induction of long-term transplantation tolerance (228).

The human IL-10 gene is located on the long arm of chromosome 1 at a position 1q 31-32. Family studies of first-degree relatives, twins and unrelated individuals have indicated that around 75% of inter-individual variability in IL-10 production is associated with genetic variation (229). Twenty-eight single nucleotide polymorphisms (SNPs) and two (AC) dinucleotide repeats, termed IL-10G and IL-10R in IL-10 gene have been identified to date. None of the SNPs causes amino acid change, although a SNP at the promoter region at a position −1082 (G to A substitution) has been shown to correlate
with IL-10 production after stimulation of T cells *in vitro*. The G\textsuperscript{1082} allele has been associated with a high producer phenotype compared to A\textsuperscript{1082} allele (230). A number of other SNP alleles of the IL-10 gene, including T\textsuperscript{−819} and A\textsuperscript{−592}, constitute extended IL-10 haplotypes and may correlate with IL-10 production (231). Nonetheless, the influence of these allelic variations on transcriptional activity remains to be defined. Several studies have reported an association between IL-10 polymorphism and the risk of several autoimmune disorders, including asthma (232), systemic lupus erythematosus in African-Americans (233), and reactive arthritis in Finnish patients (234). However, there have been discrepancies in association studies related to IL-10 promoter polymorphisms in hematopoietic stem cell, liver and kidney transplantation. It has been reported that a SNP in the promoter region of IL-10 (AA\textsuperscript{−592}) was associated with a decreased risk of graft-versus-host disease (GVHD) in hematopoietic stem cell recipients (235), whilst in a different study the IL-10 (GG\textsuperscript{1082}) polymorphism was significantly associated with susceptibility to GVHD (236). An association between the polymorphism in a promoter region of IL-10 (GG\textsuperscript{−1082}) and acute rejection after liver transplantation has been reported by Warle et al (208), a finding that was not confirmed by a different group (237). In renal transplantation, the combination of recipient IL-10 G\textsuperscript{−1082} and donor IL-10 A\textsuperscript{−1082} (recipient high/donor low producer phenotypes) was significantly decreased in multiple rejectors (238). In contrast, another group reported an association of the GG\textsuperscript{−1082} genotype with increased incidence of multiple rejections (135). This finding was not confirmed by later study that reported an association of the AA\textsuperscript{−1082} genotype with acute rejection (239).
1.4.4.4 TGF-β Gene

Transforming growth factor-β1 (TGF-β1) is produced by variety of cell types, including cells of the immune system. TGF-β1 is secreted primarily in a latent 105 kDa form, which is subsequently cleaved to the mature active 25-kDa cytokine. TGF-β1 has been shown to be a potent immunomodulatory molecule, with both immunosuppressive and pro-inflammatory abilities. TGF-β1 can, under certain conditions, stimulate proliferation and differentiation of T cells (240, 241), but most commonly has been shown to inhibit lymphocyte proliferation, downregulate receptor and cytokine expression and inhibit generation of cytotoxic T lymphocytes (242, 243). In association with its immunosuppressive properties, TGF-β1 has been demonstrated to inhibit the onset or severity of autoimmune disease (244-246), and to mediate the induction of oral tolerance (247). Over-expression of TGF-β1 was reported to correlate with chronic allograft nephropathy (57), the rate of decline in renal function, and cyclosporine toxicity in human allografts (248, 249).

Human TGF-β is located on long arm of chromosome 19 at q13. Eleven single nucleotide polymorphisms of TGF-β1 have been reported in the promoter region, in non-translated regions, and in the signal sequence of the TGF-β1 gene (250). There are two SNPs in the first exon of the TGF-β1 gene encoding the leader sequence of the TGF-β1 protein. These polymorphisms located at +600 (T to C substitution) and +650 (G to C substitution), as reported by Hutchinson (251), relative to the transcription start site result in a change in the amino acid sequences at codon 10 (Leucine to Proline) and codon 25 (Arginine to Proline), respectively. Importantly, the TGF-β1 polymorphism linked to
codon 25 Arginine to Proline substitution was found to be associated with inter-individual variation in levels of TGF-β1 production (252). Homozygosity for the Arg$^{25}$ allele, correlated with higher TGF-β1 production in vitro, was found to be associated with allograft fibrosis in lung transplantation (252). TGF-β1 polymorphism linked to codon 10 Leucine to Proline substitution in donors was associated with the development of acute graft-versus-host disease in children with HLA-matched bone marrow transplantation (207), and with chronic allograft nephropathy in renal transplant recipients (253). In heart transplantation, patients with certain TGF-β1 allele combinations [TT (Leu-Leu)$^{10}$ and GG (Arg-Arg$^{25}$) or TC (Leu-Pro)$^{10}$ and GG (Arg-Arg$^{25}$)] had a longer time to first rejection than those with the other TGF-β1 genotypes [CC (Pro-Pro)$^{10}$ and GC (Arg-Pro)$^{25}$ or CC(Pro-Pro)$^{10}$ and CC (Pro-Pro)$^{25}$] (254).

1.4.5 Other Genes and Their Effects

A number of other cytokine, cytokine receptor and adhesion molecule genes have been implicated in a short- and long-term renal transplant outcome. For example, a promoter region polymorphism allele C$^{-174}$ of the donor IL-6 gene was reported to be associated with an increased incidence and severity of acute rejection (255) whilst the same polymorphism in recipient IL-6 gene was associated with decreased long-term allograft survival in CAD kidney transplant recipients (256). Although the IL-2 gene promoter region polymorphism allele T$^{-330}$ has been shown to be associated with acute kidney rejection during the first 3 months after transplantation (257), this study has not yet been confirmed. A monocyte chemoattractant protein-1 (MCP-1) promoter region polymorphism (allele G$^{-2518}$) was found to be significantly associated with a higher
MCP-1 secretion and premature graft failure in renal transplant recipients (258). An adhesion molecule ICAM-1 R 241 polymorphism in exon 4 was reported to be associated with chronic renal allograft failure in a study by McLaren et al (259). The risk of acute rejection in renal transplantation was associated with a genetic variation in the chemokine receptors CCR2 (V641) and CCR5 (allele A<sup>-59029</sup>) (260), while vascular endothelial growth factor (VEGF) alleles G<sup>-1154</sup> and C<sup>-2578</sup> in the promoter, encoding higher VEGF production, were reported to be strongly associated with acute rejection (261). Despite large number of reports claiming significant associations of single nucleotide or dinucleotide repeat polymorphism in various genes with renal transplant outcome, none has been confirmed as a reliable predictive factor. The studies reported above were generally conducted in single centres on a limited number of patients, and it require conformation in larger or multi-center studies, with careful patient selection and unbiased interpretation of the results.
1.5 Premise and Null Hypothesis

The ability to identify factors influencing the risk of acute rejection prior to solid organ transplant is important in establishing the optimal approach to immunosuppression. Defining the polymorphisms of immune regulatory genes for each patient may serve as a tool for this purpose, enabling the individualization of the immunosuppressive therapy according to genetic risk.

The null hypothesis for this study therefore assumes that there is no relationship between polymorphisms in the immune response genes and the risk of acute rejection in renal transplant patients.
CHAPTER TWO

METHODS

2.1 Rationale
It was hypothesized that the identification of genetic polymorphisms predicting immunological responsiveness to the graft would enable physicians to:

a. identify patients with high or low risk for a graft rejection prior to the transplant using genetic polymorphism-based predictive algorithms

b. avoid overimmunosuppression by tailoring the immunosuppressive therapy for each patient according to the risk of graft rejection

2.2 Sample Size Calculations
Sample size calculation was based upon the detection of a minimum absolute difference in target allele prevalence of 25% between patient groups, predicated upon the observed differential frequency of comparable markers in the reported literature. Power analysis based on a Chi-square test indicated that a sample size of 50 patients per group was sufficient to provide power of over 80% for detection of such a difference, assuming a prevalence in the control population from 5% to 70%, which encompasses the reported range of allele frequencies for most polymorphic loci investigated. Power was expected to be enhanced through the use of logistic regression models allowing for adjustment for other relevant clinical variables.
2.3 Study Subjects Selection Criteria

The study design was based upon comparison of two groups of renal transplant patients with (AR group) or without diagnosed episode(s) of acute rejection (NR group) during the first year after transplant. A group of normal subjects (C group) served as an indicator of polymorphic gene frequency in the population.

The study subjects were selected from the renal transplant patients at Vancouver General Hospital and St. Paul Hospital transplant clinics, who consented to participate and provided their blood samples for the DNA extraction. In order to ensure a homogeneous population, the selection criteria were established for the study:

a. male or female patients of Caucasian origin
b. age from 18 to 65 years
c. did not receive any induction therapy with antilymphocyte agents
d. maintenance immunosuppressive therapy was a triple combination of CsA or Tac, AZA or MMF, and prednisone

Patients had no history of previous transplants and were followed for at least 1 year after transplant. All participants’ charts were reviewed and relevant information was entered into the study database. Data collected included age at the time of transplant; gender; donor source; frequency and timing of acute rejection episode; histology of renal biopsy using Banff 93 and 97 criteria where available; cold ischemia time, number of HLA A, B, DR mismatches, maintenance immunosuppression and acute rejection treatment, if applicable. A clinical acute rejection episode was defined by increase in creatinine level
by at least 20% from post-operative baseline and the administration of anti-rejection treatment. Each documented acute rejection episode was confirmed by renal biopsy. For the patients in group NR, the serum creatinine level did not exceed 10% of post-operative baseline throughout the period of follow up. Control subjects answered a health-related questionnaire that allowed selection of participants with no history of autoimmune, kidney and cardiovascular disorders.

2.4 Polymorphic Genes
Genetic polymorphisms of two groups of genes (signaling molecules and cytokine genes) were studied in renal transplant patients with and without acute rejection episodes during the first year after transplant. We examined CTLA-4, CD45, and CD40L signaling molecules and TGF-β1, IL-10, TNF-α, and IFN-γ cytokine gene polymorphisms. These genetic markers were selected because of their critical role in the T cell activation and priming and their putative relationship to acute rejection or induction of transplant tolerance.

2.5 Materials and Solutions

Red Blood Cells Lysis Buffer

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<th>Concentration</th>
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<tr>
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<td>15.2 g</td>
</tr>
<tr>
<td>0.001M NaHCO₃</td>
<td></td>
<td>0.17 g</td>
</tr>
</tbody>
</table>

-added dH₂O to 2.0L
-stored at 4°C
Nuclei Lysis Buffer

10.0 mM Tris base 0.61 g
400.0 mM NaCl 11.7 g
2.0 mM Na$_2$EDTA 0.37 g or 2.0 ml of 0.5M Na$_2$EDTA stock

-added dH$_2$O to 500 ml
-stored at 4°C

Proteinase K Stock Buffer

2.0 mM Na$_2$EDTA 2.0 ml (0.5M stock)
1.0% SDS (10% SDS stock) 50.0 ml
dH$_2$O 448.0 ml

-stored frozen in 50 ml aliquots

Proteinase K Solution

Protease K 2.0 mg
Protease K buffer 1 ml

-mixed well
-stored frozen in 600 µl aliquots

1x TE

4.7 mM Tris HCl 0.37 g
1.0 mM Na$_2$EDTA 1.0 ml (0.5M stock)

-added dH$_2$O to 400 ml
-adjusted pH to 8.0 with 10N NaOH
-added dH$_2$O to 500 ml
-autoclaved or filter sterilized
-stored at room temperature

Saturated Ammonium Acetate

19.2M NH$_4$ acetate* 740.0 g

-added dH$_2$O to 500 ml
-stored at room temperature

*saturated solution-added additional dH2O with a dropper until it went into solution without heating
### 10 x Loading Buffer

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<tr>
<td>25.0% Ficoll type 400</td>
<td>25.0 g</td>
</tr>
<tr>
<td>0.1% SDS (10% SDS stock)</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

- QS to 100 ml with dH2O
- adjusted pH to 7.5
- stored at room temperature

### 5 x Loading Buffer

<table>
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</thead>
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<tr>
<td>SDS</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40.0 g</td>
</tr>
</tbody>
</table>

- dissolved Tris and EDTA in 50 ml dH₂O
- adjusted pH to 7.6
- dissolved SDS, Bromphenol Blue, sucrose in Tris-EDTA
- adjusted final volume to 100 ml with dH₂O
- stored at room temperature

### 1% Ethidium Bromide

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium Bromide</td>
<td>10.0 mg</td>
</tr>
</tbody>
</table>

- added dH₂O to 1.0 ml
- stored at 4°C

### 10 x TBE

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.89M Tris base</td>
<td>215.6 g</td>
</tr>
<tr>
<td>0.89M Boric acid</td>
<td>110.0 g</td>
</tr>
<tr>
<td>0.02 Na₂EDTA</td>
<td>80.0 ml  (from 0.5M stock)</td>
</tr>
</tbody>
</table>

- added dH₂O to 2.0 L
- stored at room temperature
5 x TBE

Tris base 54.0/108.0 g
Boric acid 27.0/54.0 g
Stock Na₂EDTA (0.5M) 20.0/40.0 ml

-added dH₂O to 1.0/2.0 L
-adjusted pH to 8.3
-stored at room temperature

0.1M NaOH

NaOH 4.0 g

-added 1.0 L dH₂O and dissolved
-stored at room temperature

0.1M HCl

concentrated HCl 8.3 ml

-added 800 dH₂O, mixed and QS to 1.0L with dH₂O

1.5% Agarose Gel

Agarose 1.2 g
0.5x TBE 80 ml

-boiled, constantly stirring
-cooled, stirring to 50C and added 1.5µl of ethidium bromide
-poured into a casting form, inserted 1mm slot former and cooled for 30 min
-placed into electroforetic chamber with 0.5x TBE, pipetted samples into the slots and ran
12.5% Polyacrilamide Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/Bis (29:1)</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>5 x TBE</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>4.0 ml</td>
</tr>
</tbody>
</table>

-mixed together and de-aerated for 5 min

-then added: 10% ammonium persulfate 70 µl

-then added: TEMED 7 µl

-mixed by swirling gently

-loaded the gel

-let set for at least 1 hour

-assembled electroforetic chambers with the gel, added 1 x TBE, pipetted samples and ran

10% Ammonium Persulfate

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Persulfate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>dH2O</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

-mixed together

-stored at room temperature

30% Acrylamide/Bis

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis (29:1)</td>
<td>30 g</td>
</tr>
<tr>
<td>dH2O</td>
<td>73 ml</td>
</tr>
</tbody>
</table>

-mixed together

-stored protected from light (aluminum foil cover) at room temperature

Standard Size Markers

-100 bp DNA ladder (Pharmacia)

-80 bp DNA size markers were use for Beckman Coulter Sequencer
PCR (Polymerase Chain Reaction) Reagents

5nM primers
- primers were received in a freeze-dried form and diluted to the 5nM working solution with ddH₂O, aliquoted to 200 µl and stored at −20°C

10x dNTP’s
- supplied in a kit form Pharmacia as 100 mM of dATP, dCTP, dGTP, and dTTP
- prepared working mix using 10µl of each nucleotide and 960µl of dH₂O (total volume = 1000µl) and stored at −20°C

Taq DNA Polymerase
- supplied from Roche and stored at −20°C

10x PCR Buffer and 10x Mg
- supplied from Promega and stored at −20°C. 10x Mg was supplied as 25 mM MgCl₂. A 1000µl 15 mM working solution was prepared by diluting 600 µl of stock MgCl₂ and 400 µl of dH₂O and stored at 4°C

PCR Mixes
- all PCR reaction mixes were prepared in a restricted access C Level Laboratory that limited a potential risk of DNA contamination.
- prepared in volumes sufficient for 33 reactions, labeled and stored at -20°C:
-10x PCR buffer 100 μl
-10x dNTP’s mix 100 μl
-5’ (forward) Primer* 100 μl
-3’ (reverse) Primer* 100 μl
-dH₂O 470 μl

*specific primers were use for each gene locus

**SNP and Sequencing Reagents** (were supplied from Beckman Coulter)

**Separation gel**

CEQ™ Separation Gel- LPA I

**Sequencing separation buffer**

CEQ™ Separation Buffer

**SNP primer extension kit**

-Prepared working mix using:

-10x reaction buffer 210μl
-ddUTP dye terminator 210μl
-ddGTP dye terminator 210μl
-ddCTP dye terminator 210μl
-ddATP dye terminator 210μl
-polymerase enzyme 105μl

Total 1155μl

-mixed and aliquoted 190μl into sterile 0.5ml microfuge tubes, each aliquot was enough for 16 samples
-stored the aliquots in a –20°C non-frost free freezer
Exonuclease I / Shrimp Alkaline Phosphatase (Exo/SAP)

- received from Amersham Biosciences
- used for PCR product purification from single-stranded DNA and residual dNTPs

Shrimp Alkaline Phosphatase

- received from Amersham Biosciences
- added 1 unit to each sample tube and mixed thoroughly
- incubated samples for 1 hour at 37°C to treat unincorporated dye terminators
- incubated samples 15 minutes at 75°C to deactivate the enzyme
- stored samples on ice before loading into CEQ Beckman Coulter sequencer

Microsatellite Analysis Reagents (were supplied by Beckman Coulter)

- CEQ™ DNA Size Standard Kit-600
- CEQ™ Separation Gel
- CEQ™ Separation Buffer
- CEQ™ DNA Separation Capillary Array
- CEQ™ Sample Loading Solution

RFLP Reagents

- restriction endonucleases specific for each polymorphic site were received from New England Biolabs and stored at -20°C

2.6 Deoxyribonucleic Acid (DNA) Extraction and Quantification Methods

Approximately 10ml of whole blood was collected into ACD tube from each research subject. All DNA extractions were done in the laminar flow hood using sterile techniques. Tubes were spun for 20 minutes at 2000 rpm and the buffy coat (white blood cells) with approximately 1 ml of underlying red blood cells were transferred to labeled 15 ml conical polypropylene centrifuge tubes. Cold red blood cell (RBC) buffer was
added to 9 ml mark on the tube and mixed well by inverting the tube. Tubes were then left at room temperature for 15 minutes and were inverted several times during this incubation to ensure even lysis. The tubes were spun again for 20 minutes at 2000 rpm and the supernatant was discarded. A small amount of cold RBC lysis buffer was added to the pellet to remove leftover RBC and ghost cells without disturbing the pellet. This procedure was repeated 2-3 times after which 3 ml of cold nuclei lysis buffer was added to the white cell button and mixed well to totally resuspend the cells. 200 µl of 10% SDS (a detergent that lyses nuclear and white blood cell membranes by degrading their lipid content) and 600 µl of Proteinase K solution (used to digest endonucleases and histoproteins) was added to each tube. Samples were mixed by gently inverting the tubes and incubated for 2 hours at 65°C. 1 ml of saturated ammonium acetate was added to each tube and shaken vigorously for 30 seconds. Then samples were spun for 15 minutes at 2500 rpm and supernatant, containing DNA, was poured into another labeled 15ml polypropylene tube. Exactly two volumes of room temperature absolute ethanol was added to each tube and mixed gently by inverting the tube until the DNA precipitated. Precipitated DNA was gently spooled from the tube with 1 µl inoculation loop and air-dried for one minute. Then the DNA was transferred to a sterile screw cap Sarstedt 2ml tube containing 300-500µl of 1xTE buffer (amount of 1xTE was dependent on amount of precipitated DNA), incubated for at least 1 hour before quantification, cooled to room temperature and stored at 4°C.
2.6.1 DNA Quantification

DNA quantifications were done on spectrophotometer Gene Quant (Pharmacia). 10 μl of each well-vortexed sample was transferred to another EZ tube with 990 μl of deionized water (total volume=1 ml) and vortexed thoroughly. The spectrophotometre reference reading was set at 260 nm with deionized water to “0” prior to each quantification procedure. The absorbance for each sample was measured at 260 nm and 280 nm. The A260/A280 ratio was also measured and expected to be greater than 1.6. DNA concentration for each sample was then determined using the DNA/RNA measurement x 100=μg/μl. The DNA sample in the original EZ tube was then diluted to a concentration of 100 ng/μl for a PCR set up.

2.7 Polymerase Chain Reaction (PCR)

2.7.1 Primers Design

The primers used in this study were designed using available gene sequences from the Gene Bank in order to produce more sensitive and specific primers and often with higher melting points than the published primers. Where it was necessary, primers were modified according to previously published studies to produce recognition sites for restriction endonucleases.

2.7.1.1 CTLA-4 primers:

-5’ Primer (CTLA-4 F1) 5’-GGG ATT TAG GAG GAC CCT TGT AC
-3’ Primer (CTLA-4 R1) 5’-AGT CTC ACT CAC CTT TGC AGA AG

(Gene Bank: M74363)
2.7.1.2 CD 45 primers:

- 5' Primer (CD45 F) 5'-GAT TGA CTA CAG CAA AGA TGC
- 3' Primer (CD45 R) 5'-TTG TGG TCT CTG AGA AGT CA

(Gene Bank: NM_002838)

2.7.1.3 CD40 Ligand (CD40L) primers

5'-Primer (CD40LF) 5'-CAA TCT CTC TGT TTC CCT TTG TC*
3'-Primer (CD40LR) 5'-AAG AGA ACT GAC TAG CAA CGG C

*Labeled primer (Beckman dye D3-PA)

(Gene Bank: D31797.1)

2.7.1.4 TGF-beta 1 (TGF-β1) primer sets:

- TGF-β1 +600 T/C (Arg^{25}-Pro)

- 5 Primer (TGF-β1F) 5'-ACC ACA CCA GCC CTG TTC GC
- 3' Primer (TGF-β1R) 5'-AGC TTG GAC AGG ATC TGG CC

- TGF-β1 +650 G/C (Leu^{10}-Pro)

- 5 Primer (TGF-β1F) 5'-ACC ACA CCA GCC CTG TTC GC
- 3' Primer (TGF-β1RM) 5'-AGT AGC CAC AGC AGC GGT AGC AGC TGC*

*Underlined nucleotide denotes a mutation introduced in order to create a recognition site for the restriction endonuclease (262).

(Gene Bank: X05839.1)
2.7.1.5 Interleukin-10 (IL-10) primers

5'-Primer (IL-10F) 5'-'AAT CCA AGA CAA CAC TAC TAA GGC
3'-Primer (IL-10R) 5'-CAA GGA AAA GAA GTC AGG ATT CC
5'-Primer (IL-10F2)* 5'-AAT CCA AGA CAA CAC TAC TAA GGC TTC TTT GGGA
* SNP interrogation primer
(Gene Bank: AF295024.1)

2.7.1.6 TNF alpha (TNF-α) primers

5'-Primer (TNF-αF) 5'-'ATC CTG TCT GGA AGT TAG AAG G
3'-Primer (TNF-αR) 5'-GCC ACT GAC TGA TTT GTG TGT AG
5'-Primer (TNF-αF2)* 5'-G AAA TGG AGG CAA TAG GTT TTG AGG GGC ATG
*SNP interrogation primer
(Gene Bank: AB048822.1)

2.7.1.7 Interferon gamma (IFN-γ) primers:

-5’ Primer (IFN-γ F) 5'-AG ACA TTC ACA ATT GAT TTT ATT CTT AC*
-3’ Primer (IFN-γ R) 5'-CCT TCC TGT AGG GTA TTA TTA TAC G
* Labeled primer (Beckman dye D3-PA)
(Gene Bank: J00219.1)
2.7.2 Polymerase Chain Reaction (PCR) Reagents and Conditions

All PCRs were carried in a total volume of 30μl per sample and consisted of:

- PCR mix 26μl
- MgCl₂ (15mM) 3μl
- Taq DNA Polymerase 0.15μl
- Genomic DNA (100 ng/μl) 1μl

A master mix that consisted of all reagents except DNA was prepared and aliquoted in a volume of 29 μl per vial, than one microliter of DNA was added to each vial and one drop of mineral oil to prevent an evaporation during the PCR cycles. A positive (DNA with known amplification under the same conditions) and negative (without DNA) controls were added to each sample set. Prepared samples were quickly transferred to the pre-heated to 95°C Perkin Elmer Thermal Cycler where after 5 minutes at 95°C (hot start) an appropriate combination of cycling parameters was applied accordingly to Tm (melting temperature) and specificity of primers design.

2.7.3 Cycling File Parameters

- CTLA-4 amplifications:

Hot start (95°C) for 5 minutes followed by 30 cycles of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>60°C</td>
<td>45 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>60 seconds</td>
</tr>
</tbody>
</table>
• **CD40L amplifications:**

  Hot start (95°C) for 5 minutes followed by 30 cycles of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>59°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>60 seconds</td>
</tr>
</tbody>
</table>

  with 15 minutes final extension at 72°C

• **CD45 amplifications:**

  Hot start (95°C) for 5 minutes followed by 30 cycles of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>60°C</td>
<td>45 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>60 seconds</td>
</tr>
</tbody>
</table>

• **IFN-γ amplifications:**

  Hot start (95°C) for 5 minutes followed by 30 cycles of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>45 seconds</td>
</tr>
<tr>
<td>62°C</td>
<td>60 seconds</td>
</tr>
</tbody>
</table>

  with 15 minutes final extension at 72°C

• **IL-10 and TNF-α amplifications:**

  Hot start (95°C) for 5 minutes followed by 25 cycles of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>59°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>60 seconds</td>
</tr>
</tbody>
</table>

  with 15 minutes final extension at 72°C
• TGF-β1 amplifications:

Hot start (95°C) for 5 minutes followed by 30 cycles of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>64°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>60 seconds</td>
</tr>
</tbody>
</table>

with 15 minutes final extension at 72°C

After the completion of the PCR cycles all samples underwent an amplification control. Five microliters of each sample was added to 1μl of loading buffer and applied to 1.5% agarose gel. The gel was then ran for 15 minutes at 150 volts and photographed under UV light.

2.8 Restriction Fragment Length Polymorphism (RFLP)

Restriction endonucleases purchased from New England Biolabs were used to digest previously amplified DNA at specific polymorphic sites that differed by one nucleotide base pair. This produced distinct DNA fragments that migrated with different rate through 12.5% polyacrilamide gel during electrophoresis. When stained with ethidium bromide, DNA fragments became visible under UV light and were photographed using a Polaroid camera. Generally 1 μl of enzyme was added to 9 μl of reaction mixture (specific enzyme buffer, de-ionized water, with or without BSA) and incubated at 37°C or 65°C according to manufacturer's instructions. Comparison of DNA fragments between samples permitted discrimination between samples with or without polymorphic sites based upon cut versus non-cut results. As an internal quality control DNA samples
known to have recognition site for the tested enzyme, and mixed batches of samples from both study groups and controls, were used throughout experiments.

2.8.1 CTLA-4 PCR-RFLP

Polymerase-chain reaction was used to amplify a 504 bp fragment of DNA that included a non-translated region of the CTLA-4 gene promoter and part of the first exon. It contained two known polymorphic sites: at a position −318 in the promoter (C to T) and a position 49 in the first exon (A to G). The polymorphic site at a position −318 contained a recognition site for the restriction enzyme Mse I (5’-TTAA-3’) that produced a cut when the variant type (thymine instead of cytosine) was present. In a person with the variant allele (T) this would produce 65 bp and 439 bp fragments as depicted in Table 2.1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DNA Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>504 bp</td>
</tr>
<tr>
<td>CT</td>
<td>504 bp, 439 bp, and 65 bp</td>
</tr>
<tr>
<td>TT</td>
<td>439 bp and 65 bp</td>
</tr>
</tbody>
</table>

The second polymorphic site of the amplified region at a position 49 in the first exon contained a recognition site for the restriction enzyme Bbv I (5’- GCTGC-3’) that cut when the variant type (guanine instead of adenine) was present. In a person with the
variant (G) allele this would produce 88 bp and 416 bp fragments as presented in Table 2.2.

**Table 2.2: Bbv I enzyme digest patterns**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DNA Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>504 bp</td>
</tr>
<tr>
<td>AG</td>
<td>504 bp, 416 bp, and 88 bp</td>
</tr>
<tr>
<td>GG</td>
<td>416 bp and 88 bp</td>
</tr>
</tbody>
</table>

Combination of both polymorphisms was revealed by simultaneous presence of 65 bp and 88 bp fragments as shown in Table 2.3.

**Table 2.3: Bbv I and Mse I enzymes digest patterns**

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>DNA Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bbv I</td>
<td>416 bp and 88 bp</td>
</tr>
<tr>
<td>Mse I</td>
<td>439 bp and 65 bp</td>
</tr>
<tr>
<td>Bbv I/Mse I</td>
<td>351 bp, 88 bp, and 65 bp</td>
</tr>
</tbody>
</table>

To detect *cis*- versus *trans*- configuration of the polymorphisms, double digestion by both enzymes was used. This was achieved by sequential digestion with Bbv I enzyme for 1
hour at 37°C, than brief (30 minutes) incubation at 65°C to stop activity of the enzyme after which second, Mse I enzyme, was added and samples were incubated for 1 more hour at 37°C. Upon completion of the incubation, samples were stained with ethidium bromide (1.5μl of 10x buffer) and run on 12.5% polyacrilamide gel and photographed under UV light. A standard DNA size marker, DR52 allele 0301, Msp I digest (129 bp, 78 bp, and 63 bp size fragments) was used.

### 2.8.2 CD 45 RFLP

Polymerase-chain reaction with CD45 F/R primers was used to amplify a 120 bp fragment of DNA that included exon 4 of the CD 45 gene. It contained a previously described RFLP site at a position 77 (C/G). A restriction endonuclease Msp I that recognizes the 5’...CCGG...3’ (variant form) of the gene was used. In a homozygous person (GG genotype) this would produce 75 bp and 45 bp fragments as presented in Table 2.4.

**Table 2.4: Msp I enzyme digest patterns**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DNA Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>120 bp</td>
</tr>
<tr>
<td>CG</td>
<td>120 bp, 75 bp, and 45 bp</td>
</tr>
<tr>
<td>GG</td>
<td>75 bp and 45 bp</td>
</tr>
</tbody>
</table>
2.8.3 TGF-beta (TFG-β1) RFLP

PCR with two different sets of primers was used to study TGF-β1 Leu$^{10}$-Pro and TGF-β1 Arg$^{25}$-Pro polymorphisms located at the position +600 (T to C substitution) and +650 (G to C substitution) relative to the transcription site of the TGF-β1 gene.

First set of primers (TGF-β1 F/TGF-β1 RM) produced 110 bp fragments that included a T/C$^{600}$ (Leu$^{10}$-Pro) polymorphism. A restriction endonuclease PstI that recognizes the sequence (5’...CTGCA^\*G...3’) was used to cut the allele containing T at the +600 (translated as Leu$^{10}$) generating two 24 bp and 86 bp fragments as presented in Table 2.5.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DNA Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>110 bp</td>
</tr>
<tr>
<td>TC</td>
<td>110 bp, 24 bp, and 86 bp</td>
</tr>
<tr>
<td>TT</td>
<td>24 bp, 86 bp</td>
</tr>
</tbody>
</table>

A second set of primers (TGF-β1 F/TGF-β1 R) produced a 224 bp fragment that contained +650 G/C (Arg$^{25}$-Pro) polymorphism. A distinct restriction endonuclease Sau 96 I that recognizes the sequence (5’...G’GNCC...3’) was applied to cut a variant (cytosine instead of guanine) sequence yielding two 100 bp and 124 bp fragments as presented in Table 2.6.
Table 2.6: Sau 96I enzyme digest patterns

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DNA Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>224 bp</td>
</tr>
<tr>
<td>GC</td>
<td>224 bp, 100 bp, and 124 bp</td>
</tr>
<tr>
<td>CC</td>
<td>100 bp, 124 bp</td>
</tr>
</tbody>
</table>

2.9  Electrophoresis

Electrophoresis was used to separate DNA fragments of different length. DNA was visualised with ethidium bromide. DNA size markers, PhiX174 DNA/Hae III (Promega) or 100 bp DNA ladder (Pharmacia), were applied to determine the size of the fragments. For enzyme digested DNA, 12.5% polyacrilamide gel was used.

2.9.1  Polyacrilamide Gel Electrophoresis

Gel Electrophoresis System (GIBCO BRL) with a 0.75 mm spacer was used for electrophoresis of enzyme digested DNA. Ten milliliters (for two symmetrical gels) of 12.5% polyacrilamide gel with one time TBE buffer was used to run the gels. 1.5µl of 10x loading buffer was added to the enzyme digested amplicon, spun briefly and loaded into the slots. Gels were run at 200 volts until the bromphenol blue dye ran of the bottom of the gel (approximately 1 hour). Gels were then stained separately in 10 ml of 0.1µg/ml ethidium bromide for 5 minutes on top of a gentle shaker, rinsed for 10 minutes with dH2O and photographed under UV light.
2.9.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis (1.5%) was used to perform amplification control after the PCR reaction.

2.10 Single Nucleotide Polymorphism (SNP) Analysis

A Beckman Coulter DNA sequencer (CEQ™ 8000 Genetic Analysis System) has been employed to analyse single nucleotide polymorphisms for which restriction endonucleases were unavailable. In order to perform this kind of analysis, first a PCR reaction was performed to produce DNA fragments with a polymorphic site of interest. Second, a cleaning step (to remove residual PCR primers and residual dNTPs) with sequential use of Exo/SAP enzyme and Shrimp Alkaline Phosphatase was applied. SNP products were created by the hybridization of an unlabeled locus interrogation primer to a complementary template followed by single-base extension by fluorescent dye labeled terminators and DNA polymerase. The resulting labeled SNP fragments were combined with an aliquot of the CEQ™ DNA Size Standard 80 and loaded into the CEQ™ 8000 Genetic Analysis System for automated separation, detection, and genotyping. As a quality control, ten randomly selected samples were sequenced and the results were compared with the SNP data.

2.10.1 SNP Cleaning Procedure

Approximately 2μl of each amplified DNA sample was mixed with 0.8μl of Exo/SAP enzyme, quickly spun and incubated in a Thermocycler for 15 min at 37°C, then at 80°C for an additional 15 min for the pre-cycle-sequencing clean-up. For the cycle sequencing,
2.8μl of a DNA product was mixed with 13.2 dH2O, 1μl of internal (interrogation) primer at a concentration 0.2μM, and 3 μl of SNP pre-mix (all samples were kept on ice during the entire procedure), quickly spun and transferred to a cycle sequencer. For the post-cycle sequencing clean-up, samples were mixed with 2μl of 10x RX buffer and 10μl of SAP enzyme and transferred to a cycle-sequencer for the final incubation. After the incubation samples were transferred to the Beckman Coulter sequencer for the SNP analysis with 13 and 80 bp size standards.

2.10.2 IL-10 SNP Analysis

As a first step for the IL-10 SNP analysis, PCR with a first set of primers (IL-10F/IL-10R) was used to amplify a part of the IL-10 gene promoter, including a potential polymorphic site at a position −1082, with a resulting 112 bp fragment. The quality of the product obtained was determined by 1.5% agarose gel electrophoresis. For the second step an additional, PAGE purified, locus interrogation primer, IL-10F2, was applied that would allow a single-base extension by fluorescent dye labeled terminators, A or G nucleotides. This produced a distinct peak on electropherogram (red colour with A and green with G) and allowed easy discriminate homo- and heterozygous samples.

2.10.3 TNF-α SNP Analysis

The primary amplification step for the TNF-α gene promoter that included a polymorphic site at position −308 was initiated using a TNF-α F/TNF-α R set of primers. This produced a 129 bp fragment that was used for the next step of the SNP analysis. The second part necessitated a design of an interrogation, PAGE purified, primer TNF-α F2,
that permitted a discrimination between a wild-type (A) and a mutated (C) form when run on a Beckman Coulter sequencer.

2.11 Microsatellite Polymorphism Analysis

For the dinucleotide repeats (microsatellites), a Beckman Coulter sequencer with appropriate supportive software was used that enabled high precision analysis of variable length polymorphisms. For the purpose of this analysis, fluorescent-labeled forward primers were generally employed with unlabeled reverse primers. Electropherograms demonstrated peaks of a different size so that were homo- and heterozygosity was easily interpreted.

2.11.1 Microsatellite Fragment Analysis Procedures

The PCR reactions were prepared with a pair of fragments consisting of fluorescent-labeled forward primers and unlabeled reverse primers. All reagents were kept on ice while preparing the PCR reactions. After an amplification control was completed, 8μl of each sample was mixed with 200μl of SLS, and 1μl of Size Standard-600. Each well of the sample plate was filled with 25μl of the prepared sample/size standards mix. One drop of mineral oil was added to each of the wells and loaded into the CEQ™ 8000 for the analysis.
2.12 Statistical Analysis Methods

Allele frequencies and the influence of categorical allele groups for each of the candidate genes studied were compared in both study groups and controls using the Fisher’s exact test. The Odds Ratio (OR) was calculated and p value determined between the acute rejection (AR) and no rejection (NR) groups of patients. For analysis of the influence of cytokine gene polymorphisms on the frequency of acute rejection in HLA DR mismatched recipients, the patients were grouped according to the presence or absence of at least one HLA DR mismatched allele.

A forward stepwise multiple logistic regression model employing the likelihood ratio was used to examine the influence of each polymorphic allele and important clinical covariates on the occurrence of acute rejection in the AR and NR groups of patients. Covariates that were included in the analysis were: age at the time of transplant; cold ischemia time; year of transplant; primary renal diagnosis; donor source; number of HLA A, B and DR mismatches; and immunosuppressive therapy. P values for the variable of interest were calculated using the Likelihood ratio test, which is preferable for small sample sizes.

Statistical analysis was performed using the statistical language R, version 1.8.0 (2004), software. The significance level for the univariate and multiple logistic regression was set at 0.05. Because the research was designed to confirm previously reported significant associations, adjustment was not made for multiple testing. Where new findings were
reported, they were regarded as preliminary results that require confirmation in larger studies.
CHAPTER THREE

RESULTS

3.1 Introduction

In the period from 1989 to 1999 for the Greater Vancouver Area a total number of 1092 first kidney transplants were performed, including 281 that received their graft from living donors. Among the transplanted patients there were 585 Caucasians in the age group between 18 and 65, who were still alive at the beginning of the study and resided in the Greater Vancouver Area. Of these, 327 patients had at least one episode of acute rejection during the first six months post-transplant or lost their graft due to acute rejection, and 132 patients did not have an acute rejection in the first year after transplant. Out of 201 patients identified who met study selection criteria, 100 sequential patients were selected who consented to participate in the study and were allocated into two groups of 50, each either with acute rejection (AR group) or without acute rejection episode (NR group) during the first year after renal transplant. Patients selected for the study were Caucasians aged from 18 to 65 years. All patients were transplanted in the same program between 1989 and 1999 at one of the two centers: St. Paul Hospital or Vancouver General Hospital. They received their first kidney transplant from either cadaveric (CAD) or living (LD) donor, and were treated with conventional triple therapy (CsA/Tac, ASA/MMF, Prednisone). Patients who were treated for an acute rejection episode received Solumedrol and OKT3, or ALG, if the rejection was steroid resistant, and a rejection episode was confirmed by a renal biopsy. A third, control C group,
consisted of healthy individuals who filled out a health questionnaire and consented to participate in the study.

The demographic and laboratory data of the study patients are presented in Table 3.7 and Table 3.7.1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Acute Rejection (AR)</th>
<th>No Rejection (NR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45 ± 10</td>
<td>43 ± 11</td>
</tr>
<tr>
<td>Male/Female ratio</td>
<td>34/16</td>
<td>23/27</td>
</tr>
<tr>
<td>CAD/LD</td>
<td>33/17</td>
<td>26/23 (1-n/a)*</td>
</tr>
<tr>
<td>HLA mismatch (number)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>57</td>
<td>36</td>
</tr>
<tr>
<td>B</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>DR</td>
<td>47</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>101</td>
</tr>
<tr>
<td>Mean</td>
<td>3.22 ± 1.13</td>
<td>2.2 ± 1.62</td>
</tr>
<tr>
<td>Cold ischemia time (mean hours)</td>
<td>11.4 ± 7.8</td>
<td>9.4 ± 7.7</td>
</tr>
<tr>
<td>Treatment: Neoral/AZA</td>
<td>32 (64%)</td>
<td>31 (62%)</td>
</tr>
<tr>
<td>Neoral/MMF</td>
<td>14 (28%)</td>
<td>17 (34%)</td>
</tr>
<tr>
<td>Tac/MMF</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Neo</td>
<td>2 (4%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Tac</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Primary diagnosis:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>8 (16%)</td>
<td>9 (18%)</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>5 (10%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Lupus erythematosus</td>
<td>4 (8%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>9 (18%)</td>
<td>9 (18%)</td>
</tr>
<tr>
<td>Polycystic kidneys</td>
<td>5 (10%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>Renal vascular hypertension</td>
<td>3 (6%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Focal glomerulosclerosis</td>
<td>2 (4%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Pyelonephritis/Interstitial</td>
<td>4 (8%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Other renal disorders</td>
<td>10 (20%)</td>
<td>7 (14%)</td>
</tr>
</tbody>
</table>

Values represent mean +/- Standard Deviation or number of subjects (%)

*n/a denotes information that was unavailable for the analysis

All differences between two study groups are insignificant except for the number of male patients (p-value<0.05) and HLA mismatches (p-value<0.05)
Table 3.7.1 Number of patients with 0, 1, and 2 HLA mismatches in AR and NR groups

<table>
<thead>
<tr>
<th>Number of mismatches</th>
<th>AR group (number of patients)</th>
<th>NR group (number of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA A</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>HLA B</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>HLA DR</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

*Four patients in the NR group were unavailable for the analysis

Patients for the study groups were carefully selected for the following criteria: transplant number, type of immunosuppression, original disease, age category, donor source and gender. There was no significant difference in the key parameters for each study group, except for the male/female ratio (34/16 in acute rejection group vs. 23/27 in non-rejected group) and number of HLA mismatches (mean value 3.22 in acute rejection group vs. 2.2 in non-rejected group). Univariate analysis revealed that the influence of recipient gender on outcome of renal transplant was significant (OR=2.47; p value=0.04). The same was observed for number of HLA mismatches with p values for HLA A, B, and DR mismatch 0.011, 0.005, and 0.066, respectively. The combined effect of all HLA mismatches was also significant (p value=0.003). However, sub-analysis revealed, that the effect observed was due principally to the influence of HLA mismatch in the LD group (p value=0.004) and was not significant in CAD group (p value=0.326).
3.2 Signaling Molecules Genes

3.2.1 Cytotoxic T Lymphocyte Antigen - 4 (CTLA-4) Gene

3.2.1.1 CTLA-4 (-318) Polymorphism

CTLA-4 promoter region (-318) polymorphism frequencies in the study groups and controls are shown in Table 3.8.

Distribution of the genotypes followed the Hardy-Weinberg equilibrium (p value=0.88; Chi-square=0.14). Comparison of genotype frequencies by univariate analysis between AR and NR groups revealed that eighty-two percent of patients (41/50) from the AR group were homozygous for the C allele in comparison to ninety-two percent (46/50) from the NR group (OR=0.39; p value=0.23). The difference was non-significant. Eighteen percent (9/50) of patients from the AR group were heterozygous (TC genotype) compared to eight (4/50) patients from the NR group. This difference also did not reach statistical significance. Although there was a trend towards association (OR=2.52; p value=0.23). None of the subjects in the AR or NR groups was homozygous for the T (variant) allele (OR not defined).

Comparison of genotype frequencies in the AR group and the control group revealed that 82% (41/50) of the AR group had the CC genotype versus 78% (39/50) of the control group subjects (OR= 1.28; p value=0.81). Eighteen percent of the AR group subjects (9/50) had TC genotype, versus 20% (10/50) in the control group (OR= 0.88; p value=1.0). None of the AR group subjects was homozygous for the variant, T allele, in
contrast to a 2% frequency (1/50) in the control group, although this difference was not significant either (OR=0.32; p value=0.49).

Comparison of the NR group gene frequencies to the control group revealed that the CC genotype was present in ninety-two percent (46/50) of the NR group subjects versus seventy-eight percent (39/50) of the control group patients (OR=3.24; p value=0.09). Eight percent (4/50) of patients in the NR group carried the heterozygous TC genotype in comparison to twenty percent (10/50) in the control group (OR=0.35; p value=0.14). None of the NR group subjects was homozygous for the variant T allele in comparison to 2% (1/50) of patients in the control group (OR=0.32; p value=0.49). The overall p value from the Fisher’s exact test for the presence or absence of the variant T allele was 0.23 (not significant).

Table 3.8. CTLA-4 (-318) polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
<th>ORb</th>
<th>p b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%) ORa</td>
<td>p a</td>
<td>n (%) ORa</td>
<td>p a</td>
</tr>
<tr>
<td>CC</td>
<td>39 (78%)</td>
<td>41 (82%) 1.28 ns</td>
<td></td>
<td>46 (92%) 3.24 ns</td>
<td>0.39 ns</td>
</tr>
<tr>
<td>TC</td>
<td>10 (20%)</td>
<td>9 (18%) 0.88 ns</td>
<td></td>
<td>4 (8%) 0.35 ns</td>
<td>2.52 ns</td>
</tr>
<tr>
<td>TT</td>
<td>1 (2%)</td>
<td>0 (0%) 0.32 ns</td>
<td></td>
<td>0 (0%) 0.32 ns</td>
<td></td>
</tr>
</tbody>
</table>

* overall p value from Fisher’s exact test 0.23

T-318 - variant allele
a compared with controls
b AR group compared with NR group
3.2.1.2 CTLA-4 (+49) Polymorphism

CTLA-4 first exon region (+49) polymorphism frequencies in study groups and controls are shown in the Table 3.9.

The distribution of the genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (p value= 0.71; Chi square=8.34). Analysis of frequencies of the first exon CTLA-4 gene polymorphism at a position +49 (A/G) revealed that thirty-six percent (18/50) of patients in the AR group were homozygous for the A allele in comparison to forty-six percent (23/50) of patients in the NR group (OR= 0.27; p value=0.42). The difference was not significant. Fifty-eight percent (29/50) of the AR group patients and forty-eight percent (24/50) of the NR group were heterozygous (AG genotype) but the difference was not significant (OR=1.49; p value=0.31). The homozygous G (variant) allele was present equally (6%) in both AR (3/50) and NR group (3/50), and the difference in the allele frequency was not significant (OR= 1; p value=0.99). The overall p value for the comparison of frequencies of the variant form between AR and NR groups was 0.58.

Comparison of genotype frequencies in the AR group and the control group revealed that 36% (18/50) of the AR group had the AA genotype versus 42% (21/50) of the control group subjects (OR= 0.77; p value=0.68). Fifty-eight percent of the AR group subjects (29/50) were heterozygous (AG genotype), identical to the 58% (29/50) in the control group (OR= 1.0; p value=1.0). None of the control group subjects was homozygous for the variant G allele, in a contrast to 6% frequency (3/50) in the AR group, although this
difference was not significant despite a trend towards association (OR=7.45; p value=0.11).

Comparison of the NR group gene frequencies to the control group revealed that the AA genotype was present in thirty-six percent (18/50) of the control group subjects versus forty-six percent (23/50) of the NR patients (OR=1.17; p value=0.84). Forty-eight percent (24/50) of patients in the NR group carried the heterozygous AG genotype in comparison to fifty-eight percent (29/50) in the control group (OR=0.60; p value=0.42). None of the control group subjects was homozygous for the variant G allele in comparison to 6% (3/50) of patients in the NR group. This difference did not reach statistical significance with a trend towards association (OR=7.45; p value=0.11). The overall p value from the Fisher’s exact test for the presence or absence of the variant G allele was 0.58 (not significant).

Table 3.9. CTLA-4 (+49) polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>OR&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AA</td>
<td>21 (42%)</td>
<td>18 (36%)</td>
<td>0.77 ns</td>
</tr>
<tr>
<td>AG</td>
<td>29 (58%)</td>
<td>29 (58%)</td>
<td>1.00 ns</td>
</tr>
<tr>
<td>GG</td>
<td>0 (0%)</td>
<td>3 (6%)</td>
<td>7.45 ns</td>
</tr>
</tbody>
</table>

* overall p value from Fisher’s exact test 0.58

G<sup>49</sup>- variant allele
<sup>a</sup> compared with controls
<sup>b</sup> AR group compared with NR group
3.2.2 CD45 Gene

The polymerase-chain reaction with CD45 forward and reverse primers was used to amplify a 120 bp fragment of DNA that included exon 4 of the CD 45 gene. This contains an RFLP site at position 77 (C to G replacement) with a reported frequency ranging from 1 to 8%. A restriction endonuclease Msp I that recognizes the variant form of the gene was used. However, in the population studied, none of the patients had a variant (guanosine instead of cytosine) allele detected. This rendered further analysis impossible.

3.2.3 CD40 Ligand Gene

3.2.3.1 CD40L Microsatellite Polymorphism

The frequencies of the CD40L gene microsatellite polymorphism are shown in Table 3.10.

There were 10 alleles typed ranging from 143 bp (23 repeats) to 161 bp (32 repeats). When the AR group was compared to the NR, there was no significant difference between microsatellite fragment frequencies. None of the AR group patients carried the shortest, 143 bp or the longest, 161 bp allele while 2% (1/50) of the NR group patients had short 143 bp allele and 2% (1/50) had long 161 bp allele (OR=0.32; p value=0.49). Ten percent (5/50) of the AR group patients had the 145 bp allele vs. 4% (2/50) of the NR group (OR=2.30; P value=0.43). The 147 bp allele was also present in 10% (5/50) of the AR group patients vs. 14% (7/50) of the NR group (OR=0.43; p value=0.75). The same 10% (5/50) frequency was observed for 149 bp allele in the AR group in a contrast to 24% (12/50) of the NR group (OR=0.20; p value=0.10).
Table 3.10. CD40L microsatellite polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Controls</th>
<th>AR</th>
<th>OR$^a$</th>
<th>p$^a$</th>
<th>NR</th>
<th>OR$^a$</th>
<th>p$^a$</th>
<th>OR$^b$</th>
<th>p$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>143 bp</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
<td>1 (2%)</td>
<td>3.06</td>
<td>ns</td>
<td>0.32</td>
<td>ns</td>
</tr>
<tr>
<td>145 bp</td>
<td>5 (10%)</td>
<td>5 (10%)</td>
<td>1.00</td>
<td>ns</td>
<td>2 (4%)</td>
<td>0.37</td>
<td>ns</td>
<td>2.30</td>
<td>ns</td>
</tr>
<tr>
<td>147 bp</td>
<td>4 (8%)</td>
<td>5 (10%)</td>
<td>1.27</td>
<td>ns</td>
<td>7 (14%)</td>
<td>1.87</td>
<td>ns</td>
<td>0.43</td>
<td>ns</td>
</tr>
<tr>
<td>149 bp</td>
<td>4 (8%)</td>
<td>5 (10%)</td>
<td>1.27</td>
<td>ns</td>
<td>12 (24%)</td>
<td>3.63</td>
<td>0.05</td>
<td>0.20</td>
<td>ns</td>
</tr>
<tr>
<td>151 bp</td>
<td>27 (54%)</td>
<td>26 (52%)</td>
<td>0.92</td>
<td>ns</td>
<td>22 (44%)</td>
<td>0.66</td>
<td>ns</td>
<td>1.37</td>
<td>ns</td>
</tr>
<tr>
<td>153 bp</td>
<td>14 (28%)</td>
<td>14 (28%)</td>
<td>1.00</td>
<td>ns</td>
<td>11 (22%)</td>
<td>1.37</td>
<td>ns</td>
<td>1.37</td>
<td>ns</td>
</tr>
<tr>
<td>155 bp</td>
<td>5 (10%)</td>
<td>3 (6%)</td>
<td>1.74</td>
<td>ns</td>
<td>5 (10%)</td>
<td>1.00</td>
<td>ns</td>
<td>0.57</td>
<td>ns</td>
</tr>
<tr>
<td>157 bp</td>
<td>12 (24%)</td>
<td>3 (6%)</td>
<td>4.94</td>
<td>0.02</td>
<td>9 (18%)</td>
<td>1.43</td>
<td>ns</td>
<td>0.20</td>
<td>ns</td>
</tr>
<tr>
<td>159 bp</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>0.32</td>
<td>ns</td>
<td>0 (0%)</td>
<td>3.06</td>
<td>ns</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>161 bp</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
<td>1 (2%)</td>
<td>0.32</td>
<td>ns</td>
<td>0.32</td>
<td>ns</td>
</tr>
</tbody>
</table>

$^a$ compared with controls  
$^b$ AR group compared with NR group

The most frequent allele found in the groups studied was the 151 bp allele that was present in 52% (26/50) of the AR group patients and 44% (22/50) of the NR group patients (OR=1.37; p value=0.54). The second most frequent was the 153 bp allele that was found in 28% (14/50) of the AR group patients and in 22% (11/50) of the NR group patients (OR=1.37; p value=0.64). The two largest alleles found in the AR group were
155 bp and 157 bp, each with frequency 6% (3/50) in contrast to 10% (5/50) for the 155 allele and 18% (9/50) for the 157 bp allele in the NR group (OR=0.57; p value=0.71) and (OR=0.20; p value=0.12), respectively.

Comparison of microsatellite polymorphism frequencies between the AR and the control group revealed that none of the differences was significant, except for the 157 bp allele which was found in 6% (3/50) of the AR group patients and in 24% (12/50) of the control subjects (OR=4.94; p value=0.02). Comparison of microsatellite polymorphism frequencies between the NR and the control group also did not reveal differences except for the 149 bp allele which was found in 24% (12/50) of the NR group patients and in 8% (4/50) of the control group subjects (OR=0.27; p value 0.05).

Figure 3.1 illustrates the distribution of CD40L dinucleotide repeat polymorphisms in the study groups.
Although univariate analysis of the frequencies of dinucleotide repeat polymorphism in the CD40L gene did not reveal any statistically significant difference between the AR and the NR groups of patients, stepwise multiple logistic regression analysis, adjusted for demographic and clinical variables, demonstrated a significant difference in the frequency of the CD40L 147 and 157 bp alleles between patients with or without acute rejection, (p values 0.059 and 0.005, respectively).

Detailed results are presented in Table 3.11 and Table 3.12.

### Table 3.11 CD40L 147 bp allele microsatellite polymorphism multiple logistic regression analysis

<table>
<thead>
<tr>
<th>Factors</th>
<th>Df</th>
<th>Deviance</th>
<th>LRT</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at transplant</td>
<td>1</td>
<td>104.999</td>
<td>0.343</td>
<td>0.55820</td>
</tr>
<tr>
<td>Cold ischemia time</td>
<td>1</td>
<td>104.881</td>
<td>0.225</td>
<td>0.63536</td>
</tr>
<tr>
<td>Year of transplant</td>
<td>1</td>
<td>104.737</td>
<td>0.081</td>
<td>0.77596</td>
</tr>
<tr>
<td>Principal diagnosis</td>
<td>2</td>
<td>107.203</td>
<td>2.547</td>
<td>0.27987</td>
</tr>
<tr>
<td>Donor source</td>
<td>1</td>
<td>104.680</td>
<td>0.023</td>
<td>0.87848</td>
</tr>
<tr>
<td>HLA mismatches:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA A</td>
<td>1</td>
<td>106.344</td>
<td>1.688</td>
<td>0.19392</td>
</tr>
<tr>
<td>HLA B</td>
<td>1</td>
<td>108.249</td>
<td>3.592</td>
<td>0.05805'</td>
</tr>
<tr>
<td>HLA DR</td>
<td>1</td>
<td>105.789</td>
<td>1.133</td>
<td>0.28717</td>
</tr>
<tr>
<td>MMF</td>
<td>1</td>
<td>104.867</td>
<td>0.210</td>
<td>0.64642</td>
</tr>
<tr>
<td>CD40L147</td>
<td>2</td>
<td>110.287</td>
<td>5.631</td>
<td>0.05989'</td>
</tr>
</tbody>
</table>

Significance codes: 0 *** 0.001 ** 0.01 * 0.05 ‘ 0.1 ‘‘ 1
LRT- Likelihood Ratio
### Table 3.12 CD40L 157 bp allele microsatellite polymorphism multiple logistic regression analysis

<table>
<thead>
<tr>
<th>Factors</th>
<th>Df</th>
<th>Deviance</th>
<th>LRT</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at transplant</td>
<td>1</td>
<td>99.806</td>
<td>0.029</td>
<td>0.863686</td>
</tr>
<tr>
<td>Cold ischemia time</td>
<td>1</td>
<td>100.016</td>
<td>0.239</td>
<td>0.624995</td>
</tr>
<tr>
<td>Year of transplant</td>
<td>1</td>
<td>99.822</td>
<td>0.045</td>
<td>0.831299</td>
</tr>
<tr>
<td>Primary Diagnosis</td>
<td>2</td>
<td>102.990</td>
<td>3.213</td>
<td>0.200570</td>
</tr>
<tr>
<td>Donor Source</td>
<td>1</td>
<td>99.812</td>
<td>0.036</td>
<td>0.850334</td>
</tr>
<tr>
<td>HLA mismatches:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA A</td>
<td>1</td>
<td>103.489</td>
<td>3.712</td>
<td>0.054015 '</td>
</tr>
<tr>
<td>HLA B</td>
<td>1</td>
<td>104.092</td>
<td>4.315</td>
<td>0.037777 *</td>
</tr>
<tr>
<td>HLA DR</td>
<td>1</td>
<td>100.493</td>
<td>0.717</td>
<td>0.397292</td>
</tr>
<tr>
<td>MMF</td>
<td>1</td>
<td>100.130</td>
<td>0.353</td>
<td>0.552366</td>
</tr>
<tr>
<td>CD40L157</td>
<td>2</td>
<td>110.287</td>
<td>10.510</td>
<td>0.005221 **</td>
</tr>
</tbody>
</table>

Significance codes: 0 *** 0.001 ** 0.01 * 0.05 ' 0.1 `` 1

Df- degree of freedom
LRT- Likelihood ratio

- Stepwise multiple logistic regression analysis performed with or without gender as an independent co-variate had no effect on the significance level for the CD40L157 allele (p value=0.0037 for the analysis with gender included). A table without gender as a co-variate is presented.

- The estimated odds ratio (OR) calculated for CD40L157 1* (1 allele) was 0.587 and for the CD40L157 2* (2 alleles) was 4.1x10⁻⁹.
3.3  Cytokine Genes

3.3.1  Tumor Growth Factor Beta 1 (TGF-β1) Gene

3.3.1.1  TGF-β1 +600 T/C (Leu¹⁰-Pro) Polymorphism

TGF-β1 +600 T/C (codon 10) polymorphism frequencies in the study groups and controls are shown in Table 3.13.

Distribution of the genotypes did not deviate from the Hardy-Weinberg equilibrium (p value=0.57; Chi-square=0.24). Analysis of frequencies of the TGF-β1 +600 T/C (codon 10) polymorphism revealed that thirty-two percent (16/50) of the AR group as well as the NR group patients were homozygous for T (Leu/Leu) allele (OR=1.00; p value=0.83). The distribution of the heterozygous TC (Leu/Pro) genotype was also identical between the groups studied with 48% (24/50) of patients positive in each (OR=1.00; p value=0.84). Twenty percent (10/50) of AR and NR group patients were homozygous carriers of the variant C (Pro/Pro) allele (OR=1.00; p value=0.80). Therefore, there was no difference detected in the TGFβ1 gene +600 T/C (codon 10) polymorphism frequencies between AR and NR groups (overall p value=1). Comparison between both AR and NR groups and the control group displayed that 32% (16/50) of the AR and NR group patients were carriers of the homozygous T (Leu/Leu) allele vs. 34% (17/50) of the control group subjects (OR= 0.91; p value=0.82). Forty-eight percent (24/50) of AR and NR groups patients had the TC (Leu/Pro) genotype vs. 56% (28/50) of control group subjects (OR= 0.72; p value= 0.54). The variant CC (Pro/Pro) genotype was found in 20% (10/50) of the AR and NR groups in contrast to 10% (5/50) of the control group subjects (OR=2.25; p value= 0.26). The overall p value from the Fisher's
exact test for the presence or absence of the variant C (Pro\textsuperscript{10}) allele was 1 (not significant).

Table 3.13. TGF\textbeta 1 +600 T/C (Leu\textsuperscript{10}-Pro) polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>OR\textsuperscript{a}</td>
</tr>
<tr>
<td>TT (Leu/Leu)</td>
<td>17 (34%)</td>
<td>16 (32%)</td>
<td>0.91 ns</td>
</tr>
<tr>
<td>TC (Leu/Pro)</td>
<td>28 (56%)</td>
<td>24 (48%)</td>
<td>0.72 ns</td>
</tr>
<tr>
<td>CC (Pro/Pro)</td>
<td>5 (10%)</td>
<td>10 (20%)</td>
<td>2.25 ns</td>
</tr>
</tbody>
</table>

* overall p value from Fisher’s exact test 1

C (Pro\textsuperscript{10}) – variant allele
\textsuperscript{a} compared with controls
\textsuperscript{b} AR group compared with NR group

3.3.1.2 TGF-\textbeta 1 +650 G/C (Arg\textsuperscript{25}-Pro) Polymorphism

TGF-\textbeta 1 +650 G/C (codon 25) polymorphism frequencies in the study groups and controls are shown in Table 3.14.

All the genotypes were in the Hardy-Weinberg equilibrium (p value=0.91; Chi-square=4.38). Comparison of the TGF\textbeta 1 gene +650 G/C (codon 25) polymorphism frequency between the AR and NR groups demonstrated that 80% (40/50) of the AR group patients were homozygous for the G (Arg/Arg) allele vs. 86% (43/50) of the NR group patients (OR= 0.65; p value= 0.59). Sixteen percent (8/50) of AR group patients
had heterozygous GC (Arg/Pro) genotype vs. 12% (6/50) of the NR group (OR=1.39; p value=0.77). There were 4% (2/50) of the AR group patients who carried the variant CC (Pro/Pro) genotype in comparison to 2% (1/50) of the NR group (OR=2.04; p value=0.99).

Table 3.14. TGFβ1 +650 G/C (Arg^{25}-Pro) polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
<th>OR\textsuperscript{a}</th>
<th>p\textsuperscript{a}</th>
<th>OR\textsuperscript{b}</th>
<th>p\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (Arg/Arg)</td>
<td>45 (90%)</td>
<td>40 (80%)</td>
<td>43 (86%)</td>
<td>0.44 ns</td>
<td></td>
<td>0.65 ns</td>
<td></td>
</tr>
<tr>
<td>GC (Arg/Pro)</td>
<td>5 (10%)</td>
<td>8 (16%)</td>
<td>6 (12%)</td>
<td>1.71 ns</td>
<td></td>
<td>1.39 ns</td>
<td></td>
</tr>
<tr>
<td>CC (Pro/Pro)</td>
<td>0 (0%)</td>
<td>2 (4%)</td>
<td>1 (2%)</td>
<td>5.29 ns</td>
<td></td>
<td>2.04 ns</td>
<td></td>
</tr>
</tbody>
</table>

* overall p value from the Fisher’s exact test 0.67

C (Pro^{25}) – variant allele
\textsuperscript{a} compared with controls
\textsuperscript{b} AR group compared with NR group

When the AR and NR groups were compared to the control group subjects, none of the control group patients was homozygous for the variant C (Pro/Pro) allele in contrast to the AR (OR=5.2; p value=0.24) or the NR group (OR=3.06; p value=0.49). The GG (Arg/Arg) genotype was less frequent in the AR (OR=0.44; p value=0.26) and the NR (OR=0.68; p value=0.75) groups when compared to the control group were it was carried by 90% (45/50) of subjects. In contrast, heterozygocity was more frequent in the AR (OR= 1.71; p value=0.55) and the NR (OR=1.22; p value=0.99) groups compared to the
only 10% (5/50) of the control group subjects. None of the differences was significant (overall p value=0.67).

3.3.1.3 TGF-β1 T<sup>600</sup>-G<sup>650</sup> (Leu<sup>10</sup>-Arg<sup>25</sup>) Polymorphism

TGF-β1 T<sup>600</sup>-G<sup>650</sup> (codon 10 and 25) combination polymorphism frequencies in study groups and controls are shown in Table 3.15.

Analysis of the frequency of the combination of the +600 TT (Leu<sup>10</sup>/Leu<sup>10</sup>) and +650 GG (Arg<sup>25</sup>/Arg<sup>25</sup>) in the groups studied revealed that 32% (16/50) of the AR group and the NR group patient expressed this polymorphism (OR=1.00; p value=0.82). Thirty-four percent (17/50) of the control group patients carried these alleles combination. The difference was not significant when compared to either the AR or NR group (OR=0.91; p value=0.83).

<table>
<thead>
<tr>
<th>Genotype (Leu&lt;sup&gt;10&lt;/sup&gt;/Arg&lt;sup&gt;25&lt;/sup&gt;)</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
<th>OR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sup&gt;600&lt;/sup&gt;/G&lt;sup&gt;650&lt;/sup&gt;</td>
<td>n (%)</td>
<td>n (%)</td>
<td>OR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>p&lt;sup&gt;a&lt;/sup&gt;</td>
<td>OR&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>17 (34%)</td>
<td>16 (32%)</td>
<td>0.91 ns</td>
<td>ns</td>
<td>16 (32%)</td>
</tr>
</tbody>
</table>

Table 3.15. TGFβ1 +600 TT / +650 GG (Leu<sup>10</sup>-Arg<sup>25</sup>) combination polymorphism frequencies in the AR and NR groups of patients and controls

T<sup>600</sup> (Leu<sup>10</sup>)-non-variant allele
G<sup>650</sup> (Arg<sup>25</sup>)-non-variant allele
<sup>a</sup> compared with controls
<sup>b</sup> AR group compared with NR group
3.3.2 Interleukin -10 (IL-10) Gene

3.3.2.1 IL-10 (-1082) Polymorphism

IL-10 promoter region (-1082) polymorphism frequencies in study groups and controls are shown in Table 3.16.

All the genotypes were in the Hardy-Weinberg equilibrium (p value=0.49; Chi-square=8.61). Frequencies of the IL-10 gene (-1082) polymorphism when compared between the AR and the NR groups revealed that 32% (16/50) of the AR group were homozygous for the variant A allele, which was in contrast to 22% (11/50) of the NR group. However, this difference did not reach statistical significance (OR=1.66; p value=0.37). The frequency of the heterozygous AG genotype was 40% (20/50) for the both AR and NR group (OR=1.00; p value=0.84). Twenty-eight percent (14/50) of the AR group were homozygous for the G allele in comparison to 38% (19/50) of the NR group (OR=0.63; p value=0.39).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
<th>OR</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>OR</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>15 (30%)</td>
<td>14 (28%)</td>
<td>1.10</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>17 (34%)</td>
<td>20 (40%)</td>
<td>0.77</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>18 (36%)</td>
<td>16 (32%)</td>
<td>1.19</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

*: overall p value from the Fisher’s exact test 0.45

A<sup>-1082</sup> - variant allele

*compared with controls

AR group compared with NR group
Overall comparison for the frequency of the G allele between the AR and NR groups revealed no significant difference (p value=0.45).

When the frequency of the AA genotype in the AR and NR groups was compared to the frequency of 36% (18/50) in the control group, the difference was not significant with an OR=0.83; p value=0.83 and OR=0.50; p value=0.18, respectively. The heterozygocity (AG genotype) was found in 34% (17/50) of the control group subjects which was not significant difference when compared to AR and NR groups (OR=1.29; p value=0.67). Thirty percent (15/50) of the control group subjects were homozygous for the G allele which did not differ significantly from the AR (OR=0.90; p value=1) and NR groups (OR=1.43; p value=0.52).
3.3.3 Tumor Necrosis Factor Alpha (TNF-α) Gene

3.3.3.1 TNF-α (-308) Polymorphism

TNF-α promoter region (-308) polymorphism frequencies in study groups and controls are shown in Table 3.17.

Table 3.17. TNF-α (-308) polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>OR&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GG</td>
<td>40 (80%)</td>
<td>36 (72%)</td>
<td>0.64 ns</td>
</tr>
<tr>
<td>GA</td>
<td>7 (14%)</td>
<td>11 (22%)</td>
<td>1.73 ns</td>
</tr>
<tr>
<td>AA</td>
<td>3 (6%)</td>
<td>3 (6%)</td>
<td>1.00 ns</td>
</tr>
</tbody>
</table>

* overall p value from the Fisher’s exact test 0.51

A<sup>-308</sup> variant allele

<sup>a</sup> compared with controls

<sup>b</sup> AR group compared with NR group

The Hardy-Weinberg analysis of the genotype data indicated random distribution (p=0.83; Chi-squire = 21.2). Comparison of the TNF-α (-308) polymorphism frequency between the AR and NR groups demonstrated no difference for the GG genotype which was found in 72% (36/50) of both AR and NR group patients (OR=1.00; p value=0.82). Twenty-two percent (11/50) of the AR group had a heterozygous GA genotype vs. 16% (8/50) of the NR group patients (OR=1.48; p value=0.61). There was a relatively high frequency of 12% (6/50) of the variant AA genotype in the NR group in contrast to 6% (3/50) of the AR group. This difference did not reach statistical significance.
(OR= 0.46; p value=0.48). Analysis for the overall presence of the variant A allele in the AR and NR groups did not reveal statistically significant difference (p value=0.51).

The frequency of the GG genotype was lower in the AR and NR groups when compared to the frequency of 80% (40/50) in the control group subjects, but this difference was not significant (OR=0.64; p value=0.48). Heterozygous GA genotype frequency also did not differ significantly between the AR or NR groups when compared to 14% (7/50) in the control group subjects with OR=1.73; p value=0.43 and OR=1.17; p value=1, respectively. Six percent (3/50) of the control group subjects were homozygous for the variant A allele, which did not differ from the AR (OR=1; p value=0.99) or NR group (OR=2.13; p value=0.48).

Analysis of the HLA DR mismatched subgroup of the AR and NR patients did not show significant difference in the variant A allele distribution between the groups studied.

Details of the sub-analysis are presented in Table 3.18

Table 3.18

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AR group</th>
<th>NR group</th>
<th>*p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>27 (40%)</td>
<td>23 (34%)</td>
<td>ns</td>
</tr>
<tr>
<td>GA</td>
<td>9 (13%)</td>
<td>3 (4%)</td>
<td>ns</td>
</tr>
<tr>
<td>AA</td>
<td>3 (4%)</td>
<td>2 (3%)</td>
<td>ns</td>
</tr>
</tbody>
</table>

*overall p value from the Fisher’s exact test 0.39
A⁻³⁰⁸ variant allele
3.3.4 Interferon Gamma (IFN-γ) Gene

3.3.4.1 IFN-γ Microsatellite Polymorphism

The frequencies of the IFN-γ gene microsatellite polymorphisms are shown in Table 3.19. There were 5 alleles typed ranging from 126 bp (12 repeats) to 134 bp (16 repeats) in the three groups studied. Allele distribution analysis revealed that the two shortest alleles were relatively more frequent in the AR group in comparison to the NR group, but this difference did not reach statistical significance. The most frequent allele was the shortest, 126 bp allele, found in 72% (36/50) of the AR group patients in comparison to 60% (30/50) of the NR group patients (OR=1.71; p value=0.29). Seventy percent (35/50) of the AR group patients and 66% (33/50) of the NR group patients had 128 bp allele (OR=1.20; p value=0.83). The 130 bp allele was also more frequent in the AR group found in 12% (6/50) of patients vs. 8% (4/50) of the NR group (OR=1.56; p value=0.74). The 132 bp allele, displayed a reverse pattern of distribution being less frequent (6%; 3/50) in the AR group in contrast to the NR group (14%; 7/50). The difference was not significant (OR=0.39; p value=0.31).

Comparison of the IFN-γ allele frequencies between the AR or NR groups and the control group did not reveal any statistically significant differences in. Moreover, the 126 bp allele was found with the same frequency in the AR and the control groups (72% of subjects) and the 128 bp allele was found in 33% of the control subjects and the NR group patients. The longest, 134 bp allele was found in 2% (1/50) of the control group but was not present neither in the AR nor in the NR group patients (OR=3.06; p value=0.49).
Table 3.19. IFN-γ microsatellite polymorphism frequencies in the AR and NR groups of patients and controls

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Controls</th>
<th>AR group</th>
<th>NR group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>OR¹</td>
</tr>
<tr>
<td>126 bp</td>
<td>36 (72%)</td>
<td>36 (72%)</td>
<td>1.00</td>
</tr>
<tr>
<td>128 bp</td>
<td>33 (66%)</td>
<td>35 (70%)</td>
<td>0.83</td>
</tr>
<tr>
<td>130 bp</td>
<td>7 (14%)</td>
<td>6 (12%)</td>
<td>1.19</td>
</tr>
<tr>
<td>132 bp</td>
<td>2 (4%)</td>
<td>3 (6%)</td>
<td>0.65</td>
</tr>
<tr>
<td>134 bp</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>3.06</td>
</tr>
</tbody>
</table>

¹ compared with controls
² AR group compared with NR group

Figure 3.2: IFN-γ dinucleotide repeat polymorphism allele frequencies in the AR, NR, and control group of subjects

Study subjects are indicated by lines and icons: AR (n=50) [●]  NR group (n=50) [□]  Control group (n=50) [○]
Analysis of the HLA DR mismatched subgroup (n=67) also did not reveal any significant
differences in the distribution of the IFN-γ microsatellite polymorphism between the AR
and NR groups of patients as shown in Table 3.20.

Table 3.20. IFN-γ microsatellite polymorphism frequencies in the HLA DR
mismatched subgroup (n=67) of the AR and NR groups of patients

<table>
<thead>
<tr>
<th>Alleles</th>
<th>AR group</th>
<th>NR group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>126 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>18 (27%)</td>
<td>12 (18%)</td>
<td>0.62</td>
</tr>
<tr>
<td>2*</td>
<td>10 (15%)</td>
<td>5 (7%)</td>
<td></td>
</tr>
<tr>
<td>128 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>24 (36%)</td>
<td>13 (19%)</td>
<td>0.30</td>
</tr>
<tr>
<td>2*</td>
<td>4 (6%)</td>
<td>7 (10%)</td>
<td></td>
</tr>
<tr>
<td>130 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>4 (6%)</td>
<td>0 (0%)</td>
<td>0.13</td>
</tr>
<tr>
<td>2*</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>132 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>2 (3%)</td>
<td>3 (4%)</td>
<td>0.64</td>
</tr>
<tr>
<td>2*</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

* numbers 1 and 2 denote number of alleles
CHAPTER FOUR

DISCUSSION

In this study we attempted to discern an association between polymorphisms of signaling molecules and cytokine genes and the development of acute rejection in renal transplant patients. The signaling molecule and cytokine gene polymorphisms studied were selected on the basis of prior laboratory or clinical studies suggesting that they were important for acute rejection or tolerance induction. Genetic typing was employed to examine single nucleotide and dinucleotide repeat polymorphisms within the target genes in renal transplant patients with (AR group) or without (NR group) at least one episode of acute rejection during the first year after transplant. A control group (C group) was used for comparison of the distribution of the polymorphic genes studied in the general population.

This case-control study looked specifically at the signaling molecule and cytokine gene polymorphisms in renal transplant patients. Previous studies included all consecutive kidney transplants (135, 238, 263), a mixed group of the kidney and liver (173), kidney and pancreas (224) transplant patients, or patients from mixed ethnic and age groups (264). By contrast, this investigation selected only renal transplant recipients, and contemporaneous controls identified according to stringent inclusion criteria. To achieve homogeneity of the study population, all individuals selected for the study were adults (18-65 years old), of the same ethnic origin (Caucasians), treated with standard triple therapy, and all diagnoses of acute rejection were confirmed by a biopsy. This approach
limited the number of participating patients, but permitted comparison between well-matched groups in terms of the organ transplanted, recipient age, cold ischemia time, initial diagnosis and treatment. Studies previously done on large groups of renal transplant patients did not define sex as acute rejection risk factor. Nevertheless, having significant difference in the male/female ratio between AR and NR groups, the data was analysed with and without gender as a co-variate and there was no difference between the analyses. The diagnosis of renal acute rejection in all cases was defined by an increase in creatinine level over 20% above the post-transplant baseline, anti-rejection treatment, and was confirmed by histology.

The candidate genes for this study included signaling molecules CTLA-4 (chromosome 2), CD40L (X chromosome) and CD45 (chromosome 1), and cytokines IL-10 (chromosome 1), TNF-α (chromosome 6), TGF-β (chromosome 19), and IFN-γ (chromosome 12). With the exception of the CD45 and IL-10 genes that shared the same chromosome, the other candidate genes were more broadly distributed throughout the genome residing on different chromosomes, therefore increasing the probability of detecting a possible effect of the other gene loci that were in linkage disequilibrium with the polymorphisms studied, on the frequency of acute rejection.

The first polymorphisms examined occur in the promoter region (position -318) and first exon (position +49) of the CTLA-4 molecule. These have been reported to be capable of altering the function or the expression of the CTLA-4 molecule. It has been reported that the presence of the T-318 allele correlates with higher promoter activity (177) and the G+49
allele with reduced inhibitory function of the molecule (265). Analysis revealed that the frequency of the SNP (cytosine to thymine) at the position –318 in the CTLA-4 promoter in our control population did not differ significantly from those reported previously (266, 267). Despite earlier reports on the associations of the CTLA-4 SNP C\textsuperscript{–318} allele with autoimmune diseases, including Graves' disease and Hashimoto thyroiditis (268), and the T\textsuperscript{–318} allele with peripheral neuropathy (269), this polymorphism has not been studied extensively in renal transplant patients. A recent study of the CTLA-4 –318 and +49 polymorphisms did not find an expected association between the T\textsuperscript{–318} polymorphism and steroid-resistant rejection in kidney transplant patients. Instead, these investigators reported a significant increase in the frequencies of the wild-type G\textsuperscript{+49} polymorphism in steroid-sensitive compared to steroid-resistant patients (270). The effect observed has not been confirmed yet.

In the study reported here, none of the patients from the AR or NR groups was homozygous for the variant T\textsuperscript{–318} allele, thus rendering comparison for this impossible. Nevertheless, there was a trend towards association when the frequencies of heterozygotes were compared between groups. Eighteen percent (9/50) of patients from the AR group were heterozygous for the TC allele compared to eight percent (4/50) of patients from the NR group (OR=2.52; p value=ns).

The single nucleotide polymorphism (adenine to guanine substitution at position +49) in the first exon of the CTLA-4 gene has been studied in a combined group of kidney and liver transplant patients (173). The conclusion from this study supports our own finding
of a lack of association between the G$^{+49}$ polymorphism and the incidence of acute rejection in kidney transplant recipients. This group also reported a significant association between dinucleotide (AT)n repeat alleles 3 and 4 (92 and 94 bp) and acute rejection in a combined group of renal and liver transplant recipients. We could not confirm this finding since we did not include the dinucleotide repeat polymorphism analysis in our study design. It has been reported that (AT)n polymorphism, located in the 3' untranslated region of the last exon, is in linkage disequilibrium with the SNP +49 in the first exon (172). Therefore we did not perform the microsatellite analysis having not found any significant correlation between the G$^{+49}$ SNP and the frequency of acute rejection in our study groups.

A dinucleotide repeat polymorphism located in the 3'untranslated region (UTR) of the CD40L gene includes five dispersed AT-rich motifs, transcribed as AU to mRNA, with a similar structure to identified cis-acting instability elements, or AU-rich elements (ARE), and a highly pyrimidine-rich region important for mRNA stabilisation (271). It has been suggested that the differential stability of the CD40L mRNA may be dependent on the binding of the activation-dependent ribonucleoprotein complexes to cis-acting elements in the 3'UTR, controlling CD40L expression.

Analysis of the dinucleotide repeat polymorphism detected all 6 alleles reported to date in Caucasians (184) in our study groups. The most frequent was the 151bp allele (presented in 54% of controls), which is similar to the previously reported frequency of 52.5% in the
control group reported in the European population (185). However, univariate analysis of the frequencies of the alleles in the AR and NR groups did not reveal any significant difference between study groups, despite previous reports of an association of longer alleles with autoimmune disorders (186). There was, however, a marginally significant difference in frequency of the CD40L 147 bp allele (p value = 0.059) and highly significant difference in the frequency of the CD40L 157 allele, the longest allele detected in our study group, (p value = 0.005) when multiple logistic analysis was employed. The estimated odds ratio (OR) calculated for CD40L157 1* (1 allele) was 0.587 and for the CD40L157 2* (2 alleles) was 4.1x10^-9, i.e. close to 0, therefore considering all other clinical factors, CD40L 157 2* allele is much less likely to be associated with acute rejection then 1* allele or no alleles. Considering the fact that CD40L gene is located to X chromosome, we performed separate analysis for the distribution of the CD40L 157 allele between male and female patients in the AR and NR groups. The difference was not significant thus the effect observed was not due to unequal numbers of male and female patients in the AR and NR groups. Taking into account the exploratory nature of this study, we cannot therefore conclude with confidence that these polymorphisms serve as reliable markers for the prediction of acute rejection in renal transplant patients. However, this is the first report of CD40L gene polymorphisms in acute rejection in transplantation, and the data suggests further investigations are wanted.

We also examined the correlation between acute rejection and the CD45 protein tyrosine phosphatase SNP at exon 4, position 77, that has been implicated in T cell functional response. The frequency of the G^77 allele has been reported to range from 1.8% in healthy
controls to 6-7% in patients with MS and systemic sclerosis (196, 197). In our study of renal transplant patients we did not detect the $G^{77}$ allele in either patients or controls, and further comparative analysis could not therefore be performed.

The relationship between cytokine gene polymorphisms and the outcome of solid organ transplantation has been studied extensively for the past decade, but has produced conflicting results. A marked inter-and intra-individual variation in the production of TNF-α, IL-2, IL-4, IL-10, and IFN-γ and other cytokines production has been reported (237, 272, 273), and is seen in our own unpublished observations. While certain authors denote single-nucleotide polymorphisms in various cytokine genes by their presumed association with high or low in vitro cytokine production, attempts to define the effect of particular genotypes on cytokine production both in vitro and in vivo has resulted in a number of inconsistent reports. Studies of transfected B-cell line indicated that the IL-10 A$^{-1082}$ allele confers a two fold increase in transcriptional activity of the promoter compared to the G$^{-1082}$ allele (272), but the G$^{-1082}$ allele has also been associated with a high production of IL-10 compared to A$^{-1082}$ allele in vitro (230, 274). Despite certain studies demonstrating correlation between the TNF-α$^{-308}$, TNF-d3, IL-10$^{-1082}$, and IFN-γ gene polymorphisms and increased production of the relevant peptides (201, 216, 237), other reports have shown no relationship between these polymorphisms and cytokine production (263, 273, 275).

Regardless of the controversy surrounding the relationship between genotype and cytokine production, certain cytokine gene polymorphisms have been reported to
correlate with the incidence of acute rejection or long-term graft survival in renal, heart and liver transplantation. Functional analysis demonstrated that the polymorphic -1082 region of the IL-10 gene lies within a putative ETS-like transcription factor-binding site, which is an important site for a protein interaction. Therefore, polymorphisms in the promoter region could influence the affinity of transcription factors binding and the subsequent level of gene transcription and cytokine production. Genotyping of the IL-10 promoter region polymorphism in our study revealed that the frequency of the variant, A<sup>-1082</sup> allele, did not differ significantly from the previously published frequencies in the similar studies (135, 238). Univariate statistical analysis of the frequencies of the IL-10<sup>-1082</sup> SNP in AR and NR patients did not reveal any differences in the allele distribution between groups. This result is in contrast to the previously reported association of the IL-10 (-1082) polymorphism with the risk of acute rejection in renal (239, 238, 276), heart (209, 277), and liver (208) transplantation. It is in accord, nevertheless, with the recently reported lack of association between this polymorphism and acute renal rejection (263, 264, 278, 279). We also could not confirm an association reported between the IL-10 (GG) genotype and multiple acute rejection episodes in HLA DR mismatched grafts (135) due to the low frequency of multiple rejection episodes in our AR group. The frequency of acute rejection episodes did not differ significantly between the HLA DR mismatched study subgroups (p value=0.16).

The TNF-α (-308) polymorphism (guanine to adenine substitution) is situated in the 5' flanking region of the TNF-α gene, which contains multiple regulatory sites, including consensus sequences for the transcription factors AP-1, and AP-2, cAMP-response
element, and sequences similar to those found in the immunoglobulin and cytokine regulatory elements (280). It has been shown that the TNF-α -308 polymorphism, associated with HLA A1, B8, and DR3 haplotypes, affects the affinity of the transcription factors binding in the promoter (281). Several reports have linked TNF-α promoter region polymorphism at a position -308 to TNF-α cytokine production and outcome in solid organ transplantation.

Genotyping of the TNF-α (-308) SNP revealed a non-significant difference of the A allele distribution in the control (20%) and both AR and NR groups (28%) of patients studied, similar to the frequency reported in Caucasian population (229, 264, 282, 283). Contrary to the studies that reported a significant association between the TNF-α (-308) polymorphism and incidence of acute rejection in heart (209, 277), liver (237, 284), and kidney (135, 285, 282) transplants, we did not observe such an effect in our group of renal transplant patients. Our failure to confirm the association in renal transplant recipients is in accord with the recent reports from other groups (238, 263, 278). Analysis of the sub-group of HLA DR mismatched patients did not reveal an association previously reported between the TNF-α (-308) SNP and acute rejection episodes in HLA DR-mismatched kidney recipients (135, 264). In another report on kidney transplants, the TNF-α GA-308 and AA-308 alleles correlated with frequency of recurrent acute rejection episodes, however there was no relationship between the TNF-α genotype and the incidence of acute rejection (224). We did not include the frequency of recurrent rejection episodes in our analysis due to a low incidence of recurrent episodes in the acute
rejection group, perhaps explained by the efficient treatment strategies for first rejection episodes.

The IFN-\(\gamma\) gene first intron (CA)n dinucleotide repeat polymorphism has been described in association with IFN-\(\gamma\) production (216). Individuals homozygous for the allele 2 (12 bp) were shown to produce significantly more IFN-\(\gamma\) than individuals with the other combination of alleles, an effect explained by a proximity of the dinucleotide repeat polymorphism to a putative NF-\(\kappa\)B transcription factor binding site (211). As an important immunoregulatory cytokine, IFN-\(\gamma\) attracted attention as a possible genetic marker for a number of autoimmune diseases and in transplantation. IFN-\(\gamma\) polymorphism has been associated with type I diabetes (286, 287), rheumatoid arthritis (288), lung allograft fibrosis and chronic rejection in lung transplants (289), acute rejection in kidney transplantation, (217, 276), and recurrence of hepatitis C in liver transplant recipients (219).

The frequencies of the two most common alleles, allele 2 (126 bp) and allele 3 (128 bp) of the IFN-\(\gamma\) dinucleotide (CA)n repeat polymorphism in our control population were 72\% and 66\%, respectively. These frequencies were similar to the previously reported in the UK population (217) and a US study (224) and higher than reported by Cartwright, et al (263). Comparative analysis of the frequencies of the IFN-\(\gamma\) dinucleotide repeats did not reveal significant differences in the distribution of the alleles between AR and NR groups that support the previous report (263). There was a tendency prevalence of allele 2 (126 bp repeats), which corresponds to the high IFN-\(\gamma\) production reported phenotype,
detected in the AR group (OR=1.71). This finding was in accordance with the report from the UK group (217). This difference, nevertheless, did not reach the level of statistical significance. Analysis of the HLA DR mismatched subgroup (n=67) also did not reveal any significant differences in the distribution of the IFN-γ microsatellite polymorphism between the AR and NR groups of patients in contrast to previously reported finding by Asderakis et al (217).

The TGF-β1 gene single nucleotide polymorphisms, associated with the inter-individual variations of the TGF-β1 cytokine production, are located in the gene region that encodes the leader sequence of the TGF-β1 protein and is responsible for the amino acid changes in codon 10 (leucine to proline) and codon 25 (arginine to proline). Codon 10 is a part of a structure which directs transport of the TGF-β1 protein through the cell, and codon 25 polymorphism is close to the enzyme cleavage site. Both polymorphisms have been reported to alter the transport and processing of the active protein. Therefore, the highest production of the TGF-β1 cytokine is associated with the leucine at codon 10 and arginine at codon 25 (251). Individual variations in TGF-β1 production associated with the codon 10 and codon 25 polymorphisms have been implicated in lung fibrosis (252), acute rejection in kidney transplantation (251) and chronic rejection in lung (290), heart (291, 292), and renal transplantation (253, 276).

Genotyping of the TGF-β1 codon 10 and 25 SNPs revealed that the frequencies of the polymorphisms in our control population corresponded to the frequencies for the healthy
controls previously reported (290). TGF-β1 gene polymorphism analysis was performed for all 100 patients included in the study. Despite reports showing increased TGF-β1 expression during acute and chronic rejection episodes in renal (293) and heart (294) transplants, the role of a genetic predisposition remains unclear. In our study we hypothesised that TGF-β1 may have a protective effect against acute rejection. Nonetheless, we could not confirm a correlation previously reported between TGF-β1 polymorphisms and the incidence of acute rejection in kidney transplantation (257). We did not find significant differences in the codon 10 and codon 25 polymorphisms distribution between the AR and NR groups. The results of this study are similar to those observed earlier (278). It is likely that increased TGF-β1 expression observed in rejected kidneys relates not only to the polymorphic variations in the TGF-β1 gene, but also to the complex interactions with other cytokines.

Our findings may contrast with some of the work published earlier for several possible reasons. In this study we addressed the role of recipient genotype in renal transplantation, while most of the work that has been done previously was focused on heart (209), lung (295), liver (237), or combined group of kidney and liver transplants (173). Noteworthy is that in a large study of renal transplant patients that reported a positive association of TNF-α and IL-10 polymorphisms with recurrent acute rejection (135), the majority of the patients were initially on cyclosporine monotherapy, whereas in our study all patients were on the triple therapy, including CsA or tacrolimus, AZA or MMF, and prednisone. It is plausible that potent immunosuppressive therapy may mask or counteract the effect of a cytokine genotype. Nevertheless, the differences in patient selection and the
definition of acute rejection episodes may explain the conflicting results reported in different transplant cohorts. In our study all the acute rejection diagnoses were confirmed by a renal biopsy, whereas, in the study by Sankaran et al (135), discussed above, the biopsies were obtained in fewer than 50% of cases. Moreover, study groups were analysed according to the presence or absence of two or more rejection episodes. In our study the individuals with no acute rejection episode and a stable renal function during the first year after the transplant were compared to the individuals with a rejection episode during the first six months after the transplant. This post-transplant period allows the detection of the majority of patients with acute rejection during the first year after the transplant, in contrast to a study with only 30 days of follow up (278). Acute rejection was included in our study design as an objective short-term outcome of kidney transplant, which is closely linked to long-term outcome and a good predictor of a graft survival.

A strength of this study design was that the presence of acute rejection was defined by stringent clinical and histological criteria. Despite the relatively small sample size, the study subjects included in both groups represented clear categorical phenotypes of the immunological response to a transplanted organ. If a genetic difference exists in the signaling molecules and the cytokine genes chosen for the analysis, the design of this study should have been sensitive enough to detect significant associations. Indeed, the results of the multiple logistic regression of the CD40L gene dinucleotide repeat polymorphism confirm that the sample size of the cohort selected was sufficient to detect statistically significant differences between the study groups, if present. Our failure to identify a significant association between the signaling molecules and cytokine gene
polymorphisms studied and the incidence of acute rejection in renal transplantation does not necessarily exclude the possibility that these polymorphisms may play a lesser role in renal transplantation. It is important to conduct larger, multi-center studies with rigorous selection criteria and inclusion of both short- and long-term outcomes to explore this further. For such studies, polymorphic gene selection for the analysis should be based on the gene expression profile with an option of analysis of extended haplotypes. This may serve as a better method for defining the importance of the signaling molecules and the cytokine gene polymorphisms in discrete patterns of the clinical and immunological response.
CHAPTER FIVE

SUMMARY

In this study we addressed the important question of whether genetic markers can be identified that predict acute rejection risk in de-novo renal transplant patients. The study design employed rigorous patient selection criteria to minimize differences between the study groups for known risk factors influencing the frequency of acute rejection. Patients were allocated to two groups based upon the presence or absence of acute rejection during the first year after transplantation.

Two groups of genes were examined in this study: signaling molecules, and cytokine genes implicated in acute rejection or induction of transplant tolerance. Selection of the polymorphic markers was based upon available data on their functional role in cell signaling and/or protein production. Univariate analysis did not detect any significant differences in the distribution of the polymorphisms of the cytokine genes studied between the AR (acute rejection) and NR (no rejection) groups. However, analysis of signaling molecule genes revealed that the CD40L dinucleotide repeat polymorphism, allele 157, was more frequent in the NR than in AR group. This difference achieved significance when the data was compared using a multiple logistic regression model, which allowed stratification between the study groups for the major clinical and demographic factors implicated in acute rejection.
Despite encouraging initial results, we cannot conclude with confidence that this genetic marker can be included in the acute rejection predictive algorithm. The stringent selection criteria employed limited number of participants and impacted the sensitivity of the study. This result should therefore be confirmed in a larger study before any conclusions as drawn regarding the usefulness of the CD40L polymorphism for the prognosis of short-term and long-term renal transplant outcome.
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