THE EFFECT OF CYTOKINE GENE POLYMORPHISMS ON RENAL TRANSPLANTATION AND ATHEROSCLEROSIS

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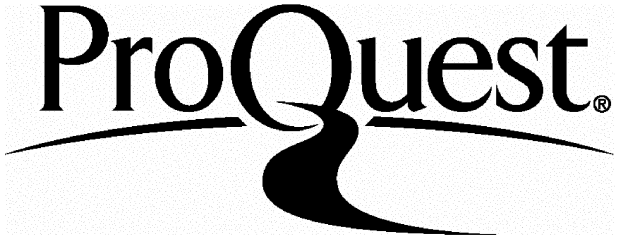

ABSTRACT

BACKGROUND: Genetic variability in cytokine production could influence the outcome of immune and inflammatory responses. Renal transplantation, which is considered to be the most efficient form of renal replacement therapy for patients with end stage renal disease, is adversely affected by acute rejection, chronic allograft dysfunction, and accelerated atherosclerosis. The aim of this thesis was to explore the influence of cytokine gene polymorphism on acute rejection, chronic allograft dysfunction, and atherosclerosis.

METHODS: 113 kidney allograft recipients, 66 atherosclerotic renal artery stenosis patients and 100 normal (control) individuals were genotyped for single nucleotide polymorphisms of TNF-alpha, IL-2, IL-6, IL-10, and TGF-beta genes. Microsatellite polymorphisms of IFN-gamma, IL-10G, IL-10R, TNF-a and TNF-d were also investigated. Each cytokine gene polymorphism was studied for its influence on secretory status *in vitro*.

RESULTS: The frequencies of IL-10 (-1082) AA, GA (low producer) genotypes and TNF-a9 microsatellite allele were high, independently, in individuals with more than one episode of acute allograft rejection when compared to rejection free patients after renal transplantation. The frequencies of IL-10 (-1082) AA, GA genotypes were also high in individuals with chronic allograft dysfunction within five years post transplant. Similarly, the frequencies of IL-10 (-1082) AA, GA genotypes were high in renal artery stenosis patients when compared to normal healthy controls. There was increased production of IL-10 mRNA and protein in individuals with the IL-10 (-1082GG) genotype compared to IL-10 (GA and AA) genotypes. Other cytokine gene polymorphism did not show any association with either renal transplant outcome or atherosclerosis.

CONCLUSION: These results suggest that IL-10 (-1082) promoter and TNF-a microsatellite polymorphisms independently may predict the development of acute rejection after renal transplantation. The results also suggest that IL-10 may have an important role in regulating inflammatory signals in the pathogenesis of both chronic allograft rejection and atherosclerosis.

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List of Abbreviations:

ADCC	: Antibody Dependent Cell-mediated Cytotoxicity
AP	: Activating Protein
APC	: Antigen Presenting Cell
ARE	: AU- Rich Element
BSA	: Bovine Serum Albumin
CAD	: Chronic Allograft Dysfunction
CD	: Cluster of Designation
CRE	: cAMP Responsive Element
CRP	: C- Reactive Protein
CSIF	: Cytokine Synthesis Inhibitory Factor
CTL	: Cytotoxic T Lymphocyte
DC	: Dendritic Cell
DEPC	: Diethyl Pyrocarbonate
DISC	: Death Inducing Signal Complex
DNA	: Deoxyribonucleic Acid
DTH	: Delayed Type Hypersensitivity
EBV	: Epstein Barr Virus
ECL	: Enhanced Chemiluminescence
ECP	: Eosinophil Cationic Protein
EGF	: Epidermal Growth Factor
ELISA	: Enzyme Linked Immunosorbent Assay
EPO	: Eosinophil Peroxidase
GM-CSF	: Granulocyte/Macrophage Colony Stimulating Factor
GRE	: Glucocorticoid Responsive Element

HIV : Human Immunodeficiency Virus

HLA : Human Leukocyte Antigen

HRP : Horseradish Peroxidase

ICAM : Inter Cellular Adhesion Molecule

IFN : Interferon

Ig : Immunoglobulin

IL : Interleukin

LAF : Lymphocyte Activation Factor

LDL : Low Density Lipoprotein

LPS : Lipopolysaccharide

LRD : Live Related Donor

LT : Lymphotoxin

MCP : Monocyte Chemoattractant Protein

MDC : Monocyte Derived Chemokine

MHC : Major Histocompatibility Complex

MIF : Migration Inhibitory Factor

MLC : Mixed Lymphocyte Culture

MLR : Mixed Lymphocyte Reaction

MRE : Multiple Responsive Element

mRNA : Messenger RNA

MS : Multiple Sclerosis

NF-AT : Nuclear Factor of Activated T cell

NF-κB : Nuclear Factor-kappa B

NK : Natural Killer

NLB : Nuclear Lysis Buffer

PBMC : Peripheral Blood Mononuclear Cell

PBS : Phosphate Buffered Saline

PCR : Polymerase Chain Reaction

PHA : Phytohaemagglutinin

PMA : Phorbol 12-Myristate 13-Acetate

PNK : Polynucleotide Kinase

RA : Rheumatoid Arthritis

RAS : Renal Artery Stenosis

RCLB : Red Cell Lysis Buffer

RFLP : Restriction Fragment Length Polymorphism

RNA : Ribonucleic Acid

RT : Reverse Transcription

SDS : Sodium Dodecyl Sulphate

SLE : Systemic Lupus Erythematosus

SNP : Single Nucleotide Polymorphism

SSP : Sequence Specific Primer

SSOP : Sequence Specific Oligonucleotide Probing

STAT : Signal Transducers and Activators of Transcription

STR : Short Tandem Repeat

TAP : Transporter associated with Antigen Processing

TARC : Thymus and Activation Regulated Chemokine

TCGF : T cell Growth Factor

TCR : T cell Receptor

TGF : Transforming Growth Factor

Th : T helper

TLR	: Toll Like Receptor
TNF	: Tumour Necrosis Factor
TRAF	: TNF Receptor Associated Factor
UTR	: Untranslated Region
VCAM	: Vascular Cell Adhesion Molecule
VEGF	: Vascular Endothelial Growth Factor
VNTR	: Variable Number Tandem Repeats
YY-1	: Yin-Yang-1

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1. George S, Turner D, Reynard M, Navarrete C, Rizvi I, Fernando ON, Powis SH, Moorhead JF, Varghese Z. 'Significance of cytokine gene polymorphism in renal transplantation.' Oral presentation at the 17th International Society of Transplantation, Rome, 2000 (August 27th -Sept 1st).

2. George S, Turner D, Reynard M, Navarrete C, Rizvi I, Fernando ON, Powis SH, Moorhead JF, Varghese Z. 'Cytokine gene polymorphism may predict rejection following renal transplantation.' Poster presentation at the American Society of Transplantation meeting, Chicago, 2000 (May 13th – 17th).

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4. George S, Turner D, Reynard M, Navarrete C, Rizvi I, Fernando ON, Powis SH, Moorhead JF, Varghese Z. 'Significance of cytokine gene polymorphism in renal transplantation.' Transplantation Proceedings, 2001 Feb-Mar; 33 (1-2): 483-484.

5. George S, Ruan XZ, Navarrete C, Sweny P, Wheeler D, Powis SH, Moorhead JF, Varghese Z. 'Influence of Cytokine gene polymorphism on atherosclerotic renal artery stenosis.' Poster presentation at the World Congress of Nephrology in San Francisco, 2001 (October 13th-17th).

6. George S, Ruan XZ, Navarrete C, Sweny P, Wheeler D, Powis SH, Moorhead JF, Varghese Z. 'The effect of cytokine gene polymorphism on atherosclerotic renal artery stenosis. 'Poster presentation at Renal Association meeting (Spring-2001), London.

7. George S, Navarrete, Sweny P, Powis SH, Varghese Z. 'Impact of Cytokine gene Polymorphism on the outcome of renal transplantation.' Oral presentation at the European School of Haematology, 4th Euro Conference, Paris, France, 2002 (December 13th –15th).

8. George S, Navarrete, Sweny P, Powis SH, Varghese Z. 'The influence of cytokine gene polymorphism on acute and chronic rejection after renal transplantation.' Poster presentation at the sixth British Transplantation Society Annual Congress, London, 2003 (April 8th-10th).

CHAPTER 1

INTRODUCTION

The human genome project, one of the major scientific achievements of all time has generated a vast database of genomic information that has been of great benefit to the field of immunogenetics. One of the major goals of this area of scientific enquiry has been to understand the myriad associations of genetics to immune related phenomena. This process involved identification of genetic variation within the genomic regions of interest that might affect gene expression and function, and therefore phenotype - encompassing variations within the promoter regions, exons and introns as well as the surrounding regions. Identification of the polymorphisms was followed by attempts to evaluate changes in phenotype and the final step has been to correlate these changes with changes in clinical outcome.

The work described in this thesis has been an attempt to identify cytokine gene polymorphisms which could have an influence on the development of acute and chronic allograft rejection as well as atherosclerotic renal artery stenosis.

1.1. CYTOKINES

Cytokines are small glycoprotein molecules that are important in immune and inflammatory responses. They are produced by numerous cell types in response to diverse stimuli and can act in an autocrine, paracrine or in an endocrine fashion. Many of the cytokines have overlapping functions conferring a degree of redundancy on the cytokine network. Some of the cytokines exhibit unique as well as many redundant activities. This is believed to be the result of sharing of receptors or receptor chains between these m olecules (Oppenheim 1993).

1.1.1 Historical Overview

The identification of lymphocytes as the principal immunocompetent cells (Gowans 1959) and the development of tissue culture techniques for studies of *in vitro* lymphoproliferative reactions to phytohaemagglutinin (Nowell 1960) laid the groundwork for the detection of lymphocyte derived soluble mediators initially called lymphokines and, later, cytokines.

The demonstration of macrophage migration inhibitory factor (MIF) released from tuberculin sensitized lymphocytes stimulated with specific antigen in 1966 by Bloom and Bennett was a cardinal development (Bloom and Bennett 1966). In 1968 Ruddle and Waksman, discovered lymphotoxin activity in the supernatant from activated lymphocyte cultures (Ruddle and Waksman 1968). These cell-free soluble factors were called lymphokines, because they were released from sensitized lymphocytes on interaction with specific antigen (Dumonde et al. 1969). It has been known since 1970s that lymphocyte can produce one or more mitogenic factors for other lymphocytes.

Studies of lymphotoxin were amplified by the subsequent discovery by Carswell in 1975 (Carswell et al. 1975) of serum factors with *in vitro* cytotoxic effects and which induced *in vivo* tumour necrosis; this first monocyte/macrophage derived cytokine was called tumour necrosis factor (TNF). TNF production was induced by endotoxin and, in contrast to lymphocyte derived lymphotoxin, was predominantly a macrophage-derived product. Both of these factors were considered to contribute to host defence against infectious and neoplastic diseases.

Lymphocyte activation factor (LAF) was originally detected in supernatants of adherent cells isolated from human peripheral blood by Gery *et*

al in 1971 (Gery et al. 1971). This monocyte-derived cytokine is now known as interleukin-1 (IL-1).

Issacs and Lindenmann first described interferon in 1957, as a factor secreted by virus-infected cells capable of inducing cellular resistance. In 1965, Wheelock described a functionally related virus-inhibitory protein produced by mitogen activated T-lymphocytes later called interferon-gamma (Isaacs and Lindenmann 1987).

The "molecular" era of cytokine research began in the early 1980s with the development of techniques such as high performance liquid chromatography, microsequencing and the production of monoclonal antibodies to cytokines. These developments permitted the purification and amino acid sequencing of the very small quantities of cytokines secreted into culture supernatants. Application of molecular biological techniques made it possible to produce larger quantities of cloned and expressed recombinant cytokines. It also resulted in the identification of more cytokines by direct expression gene cloning. Using the recombinant form, IL-2 was shown to be a major lymphoproliferative cytokine for T cells, B cells and NK cells (Taniguchi et al. 1983). In 1984 the two cytotoxic factors lymphotoxin (LT) and tumour necrosis factor (TNF) were cloned and expressed (Gray et al. 1984; Pennica et al. 1984). Generation of mice with targeted deletion of the TNF gene revealed it to be a key regulator of inflammation in host defence rather than a cytotoxic anti-tumour factor (Candeias et al. 1997).

De Larco and Todaro in 1978 described a growth factor, originally termed sarcoma growth factor that promoted the growth of normal rat fibroblasts in soft agar (de Larco and Todaro 1978). Since then two families of

transforming growth factors (TGF) have been identified- TGF-β1 and TGF-β2. These are distinct peptides with different biological activity. TGF-β is closely related to epidermal growth factor (EGF) The first of the cytokines which proved subsequently to have potent immunosuppressive and anti-inflammatory effects, namely transforming growth factor-β (TGF-β) was cloned in 1985 by Derynck (Derynck et al. 1985). In addition, a cytokine synthesis inhibitory factor (CSIF), initially discovered by Mosmann (Moore et al. 1990), and now known as IL-10, was found to have immunoenhancing effects on humoral immunity and immunosuppressive effects on cell mediated immune responses.

1.1.2 Cytokines and their gene polymorphisms

Cytokine genes are generally polymorphic. The majority of polymorphisms in these genes are either located in the non-translated regions of the gene or involve silent mutations of exons. Conservative mutations, where protein structure is not altered, may still influence levels of protein expression in a number of ways. Polymorphism within 5' and 3' regulatory sequences may affect transcription by altering the structure of transcription factor binding sites. Intronic polymorphism may affect mRNA splicing or the structure of enhancers or silencers. Finally, polymorphisms may alter the structure of binding sites for other transcription factors that are known to modulate promoter activity. The effects of conservative polymorphism often have less impact in terms of cytokine production levels than those that alter the structure of the protein. This makes the study of the relationship between cytokine polymorphism and altered cytokine levels difficult.

The majority of the cytokine gene polymorphisms that have been identified are either single nucleotide polymorphisms (SNPs) or microsatellites.

Most of the polymorphism has been found in the promoter region of genes, which are important regulators of their expression. Therefore, it has been speculated that these polymorphisms may have a role in controlling gene expression. Microsatellites are short tandem repeat sequences in the DNA that consists of one to five repeat base pair units and polymorphism arises from the variation in the number of repeats from allele to allele. Microsatellites are distributed more or less evenly across the genome (Bennett 2000) and their biological function is not clear. However, their abundance, conservation and frequent location closer to the genes suggest that a few may have some biological effects. Advances in mutation detection techniques and increased availability of sequence based databases have resulted in increased recognition of gene polymorphism and their *in vivo* effects as well as their disease associations (Bidwell et al. 2001; Haukim et al. 2002).

There have been several studies showing that certain cytokine gene polymorphisms are associated with predisposition to and severity of certain diseases (Bidwell et al. 2001; Bidwell et al. 1999). Many studies looking at the same disease have given conflicting results. There may be several reasons for this including ethnic differences in allele frequency, small study cohorts, and limitations in the analytical procedures applied. The majority of disease association studies involve analysis of individual SNPs or a series of SNPs in a number of different cytokine genes.

Recent studies of cytokine polymorphism in genetically diverse populations have highlighted inherent ethnic genetic variation (Hoffmann et al. 2002; Meenagh et al. 2002; Padyukov et al. 2001; Reynard et al. 2000). Inter-population discrepancies in allele frequencies, particularly between Caucasian

and non-Caucasian sample cohorts are often huge. This was particularly apparent for IL-10 (-1082) polymorphism and TNF (-308) polymorphism. Many of these established cytokine allele frequencies are Caucasian allele frequencies as much of the work was done in European and North American laboratories. Moreover, these are the loci that are most frequently analysed in disease association studies and have been positively associated with a number of immune diseases. However, their relevance to other ethnic populations is at best limited and in some cases non-existent.

1.1.3. Tumour Necrosis Factor (TNF)

1.1.3.1. TNF gene and gene regulation

The gene is located on human chromosome 6p23-q12, consisting of a single gene of 3.6 kb, split by three introns (Nedwin et al. 1985). The 5' flanking region of the TNF gene contains a TATA box, GC box, consensus binding sites for transcription factors such as NF-κB, PU.1 (purine rich box), a cyclic AMP response element (CRE), ATF-2, c-jun/AP-1, AP-2, SP-1, Krox-24, and NF-AT (Tsai et al. 1996a). The CRE binding site in the human TNF gene promoter binds to ATF2/jun proteins and this CRE site is critical to the regulation of the gene in multiple cell types (Tsai et al. 1996a; Tsai et al. 1996b). In macrophages TNF production has been shown to be dependent on NF-κB activation (Foxwell et al. 1998). The 3' untranslated region (UTR) contains AU rich elements (ARE), which are known to control post transcriptional regulation of TNF gene expression by destabilising mRNA and interfering with translation (Carballo et al. 1998). Agents such as tristetraprolin bind to ARE and promote the deadenylation and destabilisation of TNF-alpha mRNA (Lai et al. 1999). Certain agents such as lipopolysaccharide (LPS) have been shown to induce

TNF expression by inducing factors that bind to other regions in the TNF promoter (Myokai et al. 1999). The regulation of the TNF gene is cell specific and different response elements in the TNF promoter are activated by different stimuli (Foxwell et al. 1998).

1.1.3.2. TNF protein

Mature human TNF is a 17 kDa, 157 amino acid long protein containing one disulphide bridge and no carbohydrates. The first 5-10 residues of the N-terminus of the protein appear to be critical for its biological activity (Aggarwal 1992). The active form of TNF appears to be homotrimer. The crystal structure of TNF revealed that each monomer consists of two anti-parallel beta pleated sheets (Eck and Sprang 1989; Jones et al. 1989).

Although monocytes and macrophages are the major sources of TNF, it is also produced by T cells, NK cells, dendritic cells, endothelial cells, osteoblasts, mast cells, Kupffer cells and smooth muscle cells (Aggarwal et al. 2001).

1.1.3.3. Functions

TNF-α is a pleiotropic cytokine that produces varying immunological and inflammatory host defence responses. It is chemotactic to monocytes and neutrophils. Stimulation of these cells with TNF-α induces phagocytosis, adherence of these cells to endothelial cells, and generation of free radicals.
On activated endothelial cells, TNF-α induces expression of ELAM-1 and ICAM-1 (leading to neutrophil adhesion) and VCAM-1 (thereby promoting lymphocyte and monocyte activation) (Pober and Cotran 1990). It also increases the secretion of the chemokines IL-8 (Strieter et al. 1989) and monocyte chemotactic protein-1 (Matsushima and Oppenheim 1989) from activated

endothelial cells causing an increase in the migration of leukocytes to the site of inflammation. TNF-α also increases both vascular permeability and vasodilatation via the modulation of nitric oxide and endothelin production by endothelial cells (Marsden and Brenner 1992), (Lamas et al. 1991). TNF-α induced endothelial activation leads to the structural reorganisation of the endothelium, resulting in vascular leakiness which is partly due to its capacity to upregulate vascular endothelial growth factor (VEGF), also known as vascular permeability factor (Giraudo et al. 1998).

TNF- α may also be involved directly in parenchymal cell damage. Although it is believed that the majority of cell death during rejection is due to specific mechanisms such as the release of granzyme and perforins from cytotoxic lymphocytes or the binding of specific alloantibodies, TNF-α, released by macrophages, may mediate cell killing through its p55 receptor, via apoptosis (Tartaglia et al. 1993).

1.1.3.4. TNF polymorphisms

The TNF- α gene has one of the most extensively studied cytokine gene polymorphisms. At least 20 SNPs have been described within this gene. The most widely studied TNF- α SNP is a guanine to adenine substitution located at position –308, first described by Wilson *et al.* in 1992 (Wilson et al. 1992). Using reporter gene assays, several groups have found that an adenine at this position (also known as TNF2 allele) increased transcriptional activity and is therefore associated with increased TNF secretion (Braun et al. 1996; Kroeger et al. 1997; Wilson et al. 1997; Wu and McClain 1997). In contrast, there are also other studies that found no increase in gene transcription with TNF-308A allele (Brinkman et al. 1996; Stuber et al. 1996). However, it was speculated by

Kroeger *et al.* in 1997 that the differential effect seen between the two TNF alleles is only apparent when the reporter gene constructs contained the TNF 3'untranslated region (UTR) (Kroeger et al. 1997).

There are other *in vitro* studies to support the functional relevance of the TNF-308A allele. Bouma *et al* (1996) showed increased TNF secretion in peripheral blood mononuclear cell (PBMCs) cultures of individuals carrying TNF –308A allele, when stimulated with CD3/CD28 (Bouma et al. 1996). Lipopolysaccharide (LPS) stimulated whole blood has been reported to have higher levels of TNF in individuals with TNF-308A allele (Louis et al. 1998).

Other polymorphisms within the TNF gene may also have functional importance. A cytosine to adenine substitution at position –863 was described (Skoog et al. 1999) and concavalin-A activated PBMCs secreted increased amounts of TNF when carrying the TNF-863A allele (Higuchi et al. 1998). Another study showed that TNF-863 polymorphism was situated in the NF-κB binding site (Hohjoh and Tokunaga 2001). The TNF-863C allele is able to bind both forms of NF-κB, but the TNF-863A allele is unable to bind the p50-p50 form of NF-κB, which normally acts as a transcriptional repressor (Udalova et al. 2000). It has also been observed there is allele specific binding of the transcription factor OCT-1, to the –863 polymorphism (Hohjoh and Tokunaga 2001).

In addition to the SNPs within the TNF gene, the TNF locus contained several microsatellite polymorphisms. Several microsatellites have been mapped and characterised in the TNF locus, namely TNF-a, TNF-b, TNF-c, TNF-d and TNF-e (Jongeneel et al. 1991; Nedospasov et al. 1991). The TNF-a and TNF-b microsatellites are located 3.5 kilobases upstream of the TNF-β gene.

TNF-c is located in the first intron of TNF-β. TNF-d and TNF-e microsatellite loci are located 8-10 kilobases downstream of the TNF-α gene.

The a2 allele of TNF-a microsatellite, which is composed of AC dinucleotide repeats has been associated with differential production of TNF (Obayashi et al. 1999; Pociot et al. 1993). The TNFd3 allele of TNF-d microsatellite is composed of TC repeats and has been shown to be associated with higher TNF production in heart transplant recipients (Turner et al. 1995).

1.1.3.5. Disease associations

Polymorphisms in the TNF gene have been linked to a variety of infectious diseases. The TNF-308 polymorphism was studied in malaria, as increased levels of the cytokine have been associated with disease severity (McGuire et al. 1994). They have reported that homozygotes for the TNF-308A allele had a relative risk of seven for death or severe neurological sequelae due to cerebral malaria. The TNF-308A allele has also been associated with other infectious diseases such as mucocutaneous leishmaniasis (Cabrera et al. 1995) and brucellosis (Caballero et al. 2000). Another polymorphism at position –238 has been associated with severe malarial anaemia characterised by low TNF levels (McGuire et al. 1999). An association between the TNF-238 single nucleotide polymorphism and the development of chronic hepatitis B infection has been reported (Hohler et al. 1998). It has also been reported that TNF-238A and –308A conferred a higher risk of cirrhosis from hepatitis C infection (Yee et al. 2000). Results from HLA studies in HIV have suggested that the HLA-DR3, B8, A1 haplotype is associated with faster progression and this haplotype is in linkage disequilibrium with the TNF-c1 microsatellite allele (Hajeer et al. 1996).

The TNF-308A allele was originally shown to be associated with development of systemic lupus erythematosus (SLE) in Caucasians. This allele forms part of the SLE haplotype, HLA-DR3, B8, A1 (Wilson et al. 1994). More recently Rood *et al* (Rood et al. 2000) found that –308A allele association with SLE is independent of HLA-DR*0301.

In rheumatoid arthritis (RA), TNF-α is the driving force for the inflammatory response in the joints (Brennan et al. 1992). While TNF-308 alleles were not found to be associated with RA (Wilson et al. 1995), the TNF-a6 microsatellite allele was associated with RA (Hajeer et al. 1996). A later study of familial RA in Spanish patients showed that the TNF-a6, TNF-b5 haplotype was preferentially transmitted to affected offspring independent of HLA-DR (Martinez et al. 2000). These results suggest that the polymorphic loci of the TNF region confer susceptibility to RA independently of HLA-DR.

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system associated with HLA-DR2 in North Europeans and North American Caucasians. The TNF-c1, TNF-a11, TNFb4 microsatellites have been associated with MS, possibly because of their association with HLA-DR*1501 haplotypes (Roth et al. 1994). However TNF-308 polymorphism investigated by several groups did not show any association with MS (Epplen et al. 1997; Kirk et al. 1997; Mycko et al. 1998).

1.1.4. Interferon-γ (IFN-γ)

1.1.4.1. IFN-γ gene and gene regulation

The human IFN-γ gene is located on chromosome 12 at 12q14. It contains three introns and four exons. The current evidence suggests a complex mechanism of IFN-γ gene transcription that is regulated through methylation,

binding of activating transcription factors, and binding of repressors (Ye and Young 1997). Hypomethylation of CG islands in the proximal promoter region and in the first intron correlates with expression of the gene. Two distinct activation specific regulatory elements are present in the human promoter (position –108 to-40bp), which are essential for induction by PMA (Phorbol 12-Myristate 13-Acetate) and ionomycin (Phenix et al. 1993). The first intron contains several important sites for control of transcriptional regulation. It contains a c-Rel and an NFκB p65/p50 binding element (Sica et al. 1992) that enhance promoter activity and also consensus sites for STAT-1, STAT-4, STAT-5 and STAT-6 (Xu et al. 1996). There appears to be an important role for repressor nuclear factors in silencing IFN-γ gene transcription. A silencer element at position –251 to -215bp has been identified to which both the nuclear repressor yin-yang-1 (YY-1) and an AP-2 like repressor can bind (Ye et al. 1994). This co-operative binding results in inhibition of IFN-γ expression (Ye et al. 1994). Thus both enhancing and repressing transcription factors appear to regulate expression of the IFN-γ gene through interaction with promoter and intronic cis-elements in a concerted manner.

1.1.4.2. IFN-γ protein

The mature form of IFN-γ protein consists of 143 amino acids. Its natural conformation is a V shaped globular dimer of 34 kDa formed by association of two identical subunits (Ealick et al. 1991). IFN-γ is produced almost exclusively by NK cells and a certain sub populations of T lymphocytes.

1.1.4.3. Functions

One of the important functions of IFN-γ is its ability to upregulate expression of MHC class II molecule (Billiau 1996). It can upregulate class II molecules on antigen presenting cells and induces *de novo* synthesis of class II molecules in other cell types. IFN-γ also affects antigen processing by enhancing the expression of various transporter proteins such as TAP-1 and TAP-2 (Epperson et al. 1992). MHC class I antigen expression can also be enhanced under the influence of IFN-γ (Billiau 1996).

IFN-γ also has anti-viral activity. It can induce several endogenous enzymes that protect against viral infection by inhibiting protein synthesis. It can also upregulate proteins that inhibit the enzymes that are involved in driving the cell cycle (Kusari and Sen 1986).

IFN-γ can augment or inhibit immunoglobulin secretion and inhibit or promote the differentiation and proliferation of B cells. It also has an important role in regulation of class switching of the immunoglobulin heavy chain (Boehm et al. 1997). IFN-γ can activate macrophages, enhance their capacity for phagocytosis and increase their bactericidal activity through induction of reactive oxygen species and nitric oxide generation (Kaplan and Schreiber 1999).

1.1.4.4. IFN-γ polymorphism

In the first intron of the IFN-γ gene a microsatellite consisting of variable number of CA repeats has been described (Gray and Goeddel 1982). Pravica *et al.* showed a significant increase in IFN-γ production in PBMCs stimulated with concavalin A in individuals who had allele 2 of this microsatellite (Pravica et al. 1999). Furthermore, this microsatellite has been associated with a SNP at the 5'

end of CA repeat region in the first intron of the IFN-γ gene (+874 T/A) that lies in a putative NFκB binding site. There was complete correlation between the presence of the T allele, allele 2 of the microsatellite (Pravica et al. 2000), although Cartwright *et al* reported the contrary, with no association between the presence of this microsatellite and IFN-γ levels (Cartwright et al. 1999).

1.1.5. Interleukin-2 (IL-2)

1.1.5.1. IL-2 gene and gene regulation

The human IL-2 gene is located on chromosome 4, band q26-28. Approximately 350bp upstream from the transcription start site lie the binding sites for transcription factors, AP-1, NFκB, and NF-AT. These three transcription factors synergise to promote maximal transcriptional activation of the IL-2 gene (Garrity et al. 1994). Exclusion of any one of these factors results in marked attenuation of IL-2 gene expression. T cells are the only cells that have been found to express the IL-2 gene and IL-2 gene expression is strictly controlled by the T cell antigen receptor (TCR) (Meuer et al. 1984; Smith et al. 1980).

1.1.5.2. IL-2 protein

IL-2, originally termed T cell growth factor (TCGF), was the first protein to be isolated, purified and characterised at the molecular level. Human IL-2 is a small (15.5kDa) globular glycoprotein of 133 amino acids (Robb and Smith 1981; Taniguchi et al. 1983).

1.1.5.3. Functions

The physiological role of IL-2 is believed to be in promoting the proliferation and augmenting the differentiation of T cells and NK cells (Smith 2001). Its involvement in the expansion of cytotoxic T lymphocytes has been

shown by experiments using IL-2 knockout mice, where antigen induced CD8+ T cell expansion was reduced by 90% (Cousens et al. 1995). IL-2 has also been shown to promote cytolytic activity by activating the expression of cytolytic molecules such as perforin and the serine esterase found in the cytolytic granules of cytototoxic T lymphocyte (Liu et al. 1992). IL-2 is also a growth factor for CD4+ T cells and required for their differentiation into Th1 and Th2 cells (Smith 2001).

1.1.5.4. IL-2 polymorphisms

Two single nucleotide polymorphisms at positions –330 and +166 have been described in the IL-2 gene (John et al. 1998). But the functional relevance of these polymorphisms is not yet known.

1.1.6. Interleukin-6 (IL-6)

1.1.6.1. IL-6 gene and gene regulation

The human IL-6 gene is located on chromosome 7 (Sehgal et al. 1986), is approximately 5kb in size, and has 5 exons separated by 4 introns. Potential binding sites for transcription factors GRE, AP-1 site, CRE and NFκB (Matsuda and Hirano 2001), NF-IL-6 binding sites, a multiple responsive element (MRE) and an IL-1 responsive element have been identified in the promoter region (Akira et al. 1994; Isshiki et al. 1990).

1.1.6.2. IL-6 protein

The mature form of IL-6 consists of 186 amino acids (Heinrich et al. 1990). At least five different forms of IL-6 have been described, arising from post-translational modifications such as glycosylation and phosphorylation (Matsuda and Hirano 2001). IL-6 is produced by a variety of cell types such as

T cells, B cells, macrophages, fibroblasts, endothelial cells, and vascular smooth muscle cells and may be involved in their growth (Nabata et al. 1990).

1.1.6.3. Functions

IL-6 has been shown to be involved in the regulation of the immune system by stimulating both the humoral and cellular arms of the immune response. It acts on B cells activated by IL-4 and IL-5 to induce the production of IgM, IgG, and IgA and causes their terminal differentiation into plasma cells (Muraguchi et al. 1988). IL-6 acts synergistically with IL-1 to promote the proliferation of activated T cells and is necessary for the development of antigen-specific cytotoxic T cells (Renauld et al. 1989). It has also been shown to be a major inducer of acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (Castell et al. 1988).

1.1.6.4. IL-6 polymorphisms

A single nucleotide polymorphism at position –174 (guanine to cytosine substitution) studied using a luciferase reporter gene assay in HeLa cells, revealed that IL-6-174C construct had lower expression than –174G construct when stimulated with IL-1 or LPS (Fishman et al. 1998). IL-6 plasma levels were also found to be lower in healthy individuals having the –174C allele (Fishman et al. 1998).

Functional differences have been noted between the –597 G to A, -572 G to C, -373 A_nT_n , -174 G to C haplotype polymorphisms (Terry et al. 2000). Other SNPs at –634 and +4391 have also been described in this gene (Nakajima et al. 1999).

1.1.6.5. Disease associations

Studies from Fishman *et al* demonstrated that an SNP at –174 was associated with systemic onset juvenile chronic arthritis. The CC genotype was significantly less common in juvenile chronic arthritis patients than in Caucasian control subjects (Fishman et al. 1998). There is also evidence that the IL-6 gene is involved in determining susceptibility and disease phenotype in Crohn's disease. Genotype and allele frequencies of IL-6 4470G/A biallelic polymorphism in the fourth intron and the variable number tandem repeats (VNTR) polymorphism in the 3' flanking region of the IL-6 gene were associated with Crohn's disease. An increased frequency of less common IL-6 G allele was found in patients with Crohn's disease compared to controls (Koss et al. 2000b).

1.1.7. Interleukin-10 (IL-10)

1.1.7.1. IL-10 gene and gene regulation

The IL-10 gene is located on chromosome 1q, spans 4.7 kb and consists of five exons separated by four introns (Eskdale et al. 1997a). Regulatory consensus transcription factor binding sequences have been identified in the TATA box is located 84bp 5' of the first methionine codon, CCAAT motifs at -148,-363,-864, AP-1 binding sites at -29 and -695, IL-6 consensus binding sites, glucocorticoid response element (GRE) and cAMP response element (CRE) (De Waal Malefyt 2001). The CRE appears to be functional, as agents that raise intracellular cAMP levels enhance the expression of IL-10 in monocytes. Transcription of IL-10 is known to be influenced by other cytokines such as TNF, IFN-γ, and IL-12 (Kube et al. 2001). Expression of the IL-10 gene in monocytes is not dependent on NFκB activation (Bondeson et al. 1999).

1.1.7.2. IL-10 protein

Mature human IL-10 is a 17kDa protein consisting of 160 amino acids. The molecule is biologically active as a non-covalently linked homodimer (De Waal Malefyt 2001). IL-10 is expressed by naïve and memory T cells, T cell clones belonging to Th1, Th2, Th0 and Tr1 subsets, NK cells, activated monocytes, mast cells, eosinophils, keratinocytes, trophoblasts, B cells and various tumour cells (Del Prete et al. 1993; Pretolani 1999) .

1.1.7.3. Functions

IL-10 was originally described as cytokine synthesis inhibitory factor (CSIF), from the observation that supernatants from activated Th2 cells could inhibit cytokine production from activated Th1 cells (Fiorentino et al. 1989). Subsequent studies on IL-10 have shown that it has a number of properties that can be grouped as "anti-inflammatory" effects which repress the progression of inflammation.

IL-10 strongly inhibits the production of other cytokines including IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α, GM-CSF and IL-10 itself by activated monocytes/macrophages (Berkman et al. 1995; D'Andrea et al. 1993; de Waal Malefyt et al. 1991a; de Waal Malefyt et al. 1993; Fiorentino et al. 1991; Gruber et al. 1994; Marfaing-Koka et al. 1996). The mechanism for the inhibition of IL-12 occurs at the transcriptional level and is dependent on new protein synthesis (Aste-Amezaga et al. 1998) and inhibition of TNF-α production occurs by transcriptional and post transcriptional mechanisms that are independent of the ability of IL-10 to inhibit NFκB activation (Clarke et al. 1998).

IL-10 has been shown to induce production of IL-1Ra (Cassatella et al. 1993; de Waal Malefyt et al. 1991a; Jenkins et al. 1994) and expression of

soluble p55 and p75 TNFR (Dickensheets et al. 1997; Hart et al. 1996; Joyce and Steer 1996; Linderholm et al. 1996) indicating that IL-10 induces a shift from production of pro-inflammatory to anti-inflammatory mediators. IL-10 also down regulates the expression of MHC class II antigens, ICAM-1, B7.1 and B7.2 (de Waal Malefyt et al. 1991b; Ding et al. 1993; Kubin et al. 1994; Willems et al. 1994) and therefore down regulates the antigen presenting capacity of professional APCs. IL-10 inhibits MHC class II expression by monocytes through a post transcriptional mechanism involving inhibition of transport of mature, peptide-loaded MHC class II molecules to the plasma membrane (Koppelman et al. 1997).

In contrast, IL-10 upregulates expression of FcR molecules on monocytes, including CD16 and CD64 (Calzada-Wack et al. 1996; de Waal Malefyt et al. 1993; te Velde et al. 1992) and these molecules bind the Fc region of antibodies onto monocytes/macrophages and large granular lymphocytes (Hutchinson et al. 1995), promoting antibody dependent cell mediated cytotoxicity (ADCC). Although the exact role of the humoral arm of the immune response during acute rejection is unclear, it is obvious that IL-10 can not be thought of solely as a beneficial cytokine in transplantation.

1.1.7.4. IL-10 polymorphism

Several polymorphisms have been observed in the human IL-10 gene in the 5'flanking sequence. They include two areas of 6-11 (CA)n repeat microsatellite polymorphisms - IL-10G and IL-10R (Eskdale et al. 1997a; Hurme et al. 1998) . IL-10G lies upstream of the gene between –1193 and –1151 and is highly polymorphic having up to sixteen alleles (Eskdale and Gallagher 1995). The IL-10R lies between –4004 and –3978 in the 5'flanking region and

this CA repeat is less polymorphic. These microsatellites have been shown to be associated with differential cytokine production. The IL-10R2/IL-10G7 haplotype has been associated with high IL-10 secretion and IL-10R3/IL-10G7 haplotype has been associated with low IL-10 secretion (Eskdale et al. 1998).

Three linked SNPs at positions –1082 (G to A), -819 (C to T), -592 (C to A) have been described. The –1082 polymorphism is associated with differential IL-10 production. Lymphocyte cultures of individuals who lacked the –1082A allele were shown to produce significantly increased amounts of IL-10 when stimulated with concavalin A (Turner et al. 1997b). Similar findings have obtained from LPS stimulated whole blood (Koss et al. 2000a). Recently other SNPs have been described at positions –627 and –1117 (Grove et al. 2000). However their functional relevance is not yet known.

1.1.7.5. Disease association

IL-10 probably has a role in systemic lupus erythematosus (SLE) as the production of IL-10 was shown to be increased in these patients (Eskdale et al. 1997b). The differences found in the frequencies of microsatellite alleles of the gene also suggest a role for IL-10 in SLE (Eskdale et al. 1997b).

In rheumatoid arthritis, although there were no differences in the frequencies of the promoter region SNPs (Hajeer et al. 1998), at position –1082, allele A was found more frequently in patients with an Ig-A type rheumatoid factor than in those with Ig-G type rheumatoid factor (Hajeer et al. 1998), which might indicate that the severity of rheumatoid arthritis is influenced by IL-10 gene polymorphism.

Another study has shown almost all EBV seronegative adults had allele G at position –1082 suggesting that a high IL-10 producer status somehow

protects from EBV infection (Helminen et al. 1999). However Westendrop *et al.* (Westendorp et al. 1997) have demonstrated that a genetically determined high capacity to produce IL-10 increases mortality in a meningococcal disease. Together these reports suggest that defence mechanisms against different microbes vary greatly, and changes at the level of a given cytokine do not necessarily exert the same effect in all infections.

1.1.8. Transforming growth factor-beta (TGF-β)

1.1.8.1. TGF-β gene and gene regulation

Humans express three distinct isoforms of TGF-β, TGF-β1, TGF-β2, and TGF-β3 encoded by unique genes located on different chromosomes. All three genes share a similar intron/exon structure. The TGF-β1 gene has been mapped to the long arm of chromosome 19 (Fujii et al. 1986) and contains seven exons. It has extensive 5' and 3' UTRs resulting in mRNA transcripts of 2.5kb, 1.9kb and 1.5kb. The most efficiently transcribed is the one with 1.9kb length (Flanders et al. 2001). The TGF-β promoter lacks a TATA box, but has several GC boxes proximal to the transcriptional start site, which mediate its regulation by both SP-1 and Zf9/CPBP, a core promoter binding protein (Kim et al. 1998). Other transcriptional regulatory sites include AP-1 site, early growth response-1 (Egr-1) and retinoblastoma response element (Flanders et al. 2001).

1.1.8.2. TGF-β Protein

TGF-β exists as a 112 amino acid long protein. Natural TGF-β exists as a 25kDa homo or heterodimer. Nearly every cell can express TGF-β receptors and their ligand. In most cells and tissues TGF-β1 is the predominant isoform.

1.1.8.3. Functions

The TGF-β protein family appears to have major roles in cell growth, neoplasia, inflammation and immunoregulation. Roberts and Sporn in 1990 described their potent immunosuppressive action on numerous functions of T lymphocytes (Roberts et al. 1990).

TGF-β is a prototype of a multifunctional growth factor. It may have one or several biological actions in a given cell or tissue and these are regulated by the cellular environment. It has variable effects on proliferation of mesenchymal cells which range from inhibitory to stimulatory depending on cell type and growth conditions (Fynan and Reiss 1993). It also increases the synthesis of matrix proteins and protease inhibitors, as well as cell adhesion receptors (Sporn and Roberts 1990). TGF-β can block antibody production by B cells and it depresses activity of natural killer cells. It also inhibits generation of cytotoxic T cells and induces expression of MHC class II molecules (Letterio and Roberts 1998).

1.1.8.4. TGF-β polymorphism

Many SNPs have been described within the TGF-β1 gene both in the 5' non-coding region and within the exons, causing amino acid changes. Three SNPs at –988, -800 and –509 have been described (Cambien et al. 1996). In addition three SNPs within the exons were found and they were in codon 10 T to C (Leu to Pro), in codon 25 G to C (Arg to Pro) and codon 263 C to T (Thr to Ile). The Leu 10 Pro and Arg 25 Pro polymorphisms are located in the signal peptide sequence that is cleaved from the TGF-β1 precursor at the level of codon 29 (Cambien et al. 1996). Stimulated lymphocytes from individuals with the

genotype homozygous for GG at codon 25 secreted increased amounts of TGF-β1 (Awad et al. 1998).

1.1.9. Mechanisms of cytokine action through their receptors

Cytokines mediate their biological activities through binding to their surface receptors on target cells. Receptors for most cytokines have now been characterised. They consist of more than one chain, components of which may be shared by different cytokines that suggests that some of these cytokines exhibit unique as well as many redundant activities (Oppenheim 1993).

On the basis of shared structural and sequence similarities, receptors have been classified into different families. The largest family is the cytokine or haemopoietin receptor family or Type I (or Class I) family which include chains of many cytokines receptors such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, GM-CSF, and erythropoietin. The class II cytokine receptor family includes receptors for interferons and IL-10 and has structural similarities with the class I family. The class III or nerve growth factor receptor family includes TNF receptors. The class IV receptor family, whose extracellular part contains immunoglobulin – like domains, includes IL-1 receptors.

For most of the cytokine receptors, signal transduction is due to the association of cytoplasmic domain of the receptor with one of the Janus kinases (Jak) or other kinases (Ihle 1995; Karnitz and Abraham 1995). Recently it has emerged that signal transducers and activators of transcription (STATs), first identified as transcription factors in interferon signalling (Darnell et al. 1994), are also involved in regulating the expression of genes induced by other members of the cytokine receptor family (Ihle et al. 1994). The STATs become activated in the cytoplasm following phosphorylation by Jaks, are transported to

the nucleus where they controll the expression of cytokine induced genes (Shuai 1994; Shuai et al. 1993).

The cytoplasmic parts of the TNF receptor family have no known enzymatic activities like class I and class II receptors. TNF receptor associated factors (TRAF) are major intracellular signalling proteins involved in TNF actions and TRAF 2 appears to play a role in TNF induced activation of NFκB transcription factor by both TNF receptor I and II (Wallach et al. 1997). These receptors also possess an intracytoplasmic death domain which enables interaction with other elements involved in apoptosis (Tewari and Dixit 1996; Wallach 1997).

1.1.10 Role of cytokine gene polymorphism in transplantation

There has been great interest in the role of cytokines in solid organ transplantation and a wide variety of genotypic polymorphisms have been examined for their influence on transplant outcome.

Early studies by Turner *et al* showed a higher risk of rejection in heart transplant recipients carrying TNF-308A allele in combination with IL-10 -1082GG genotype (Turner et al. 1997a). Similarly, renal transplant recipients who had TNF-308A allele and IL-10-1082GG genotype showed higher risk of multiple rejection episodes in HLA-DR mismatched transplants (Sankaran et al. 1999). A recent study has also shown that the presence of TNF-308A allele alone was associated with increased risk for acute rejection after renal transplantation (Hahn et al. 2001). In addition TNF-308AA genotype was also associated with acute rejection after liver transplantation (Bathgate et al. 2000).

Microsatellite alleles have also been shown to influence the outcome of solid organ transplantation. The frequency of TNF-a9 allele was found to be

higher in recipients who had acute rejection following renal transplantation (Asano et al. 1997). Awad *et al* showed increased frequency of allele-2 of IFN-γ microsatellites in patients developing lung allograft fibrosis (Awad et al. 1999). Lu *et al* reported an association between the development of bronchiolitis obliterans syndrome, a form of chronic rejection in the transplanted lung and the presence of IL-6 –174 and IFN-γ +874 polymophism (Lu et al. 2002).

In contrast, a study by Marshall *et al* in renal transplant recipients reported that there was no association between cytokine genotypes and acute rejection three months post transplant in spite of testing eleven cytokine and their receptor polymorphisms (Marshall et al. 2000). However, genotyping these polymorphisms in the corresponding donors suggested that donor IL-6-174 polymophism had a role in the development of acute rejection (Marshall et al. 2001).

TNF and IL-10 gene polymorphisms have been reported to be associated with differential outcomes in haemopoietic stem cell transplantation. Middleton *et al* showed that transplant recipients homozygous for TNF-d3 allele had increased incidence of graft verse host disease (GVHD) (Middleton et al. 1998). Similarly, they reported presence of IL-10G microsatellite alleles 12 to 16 was associated with GVHD (Middleton et al. 1998). Nordlander *et al* have also reported a positive correlation between TNF-d4 allele and IL-10-1064 allele-13 with acute GVHD after allogeneic stem cell transplantation (Nordlander et al. 2002).

The bewildering array of associations reported between various cytokine gene polymorphisms and transplant outcomes highlights the complex role of cytokines in organ transplantation.

1.2. RENAL TRANSPLANTATION

1.2.1. Current status

Despite improvements in HLA matching, cross-match techniques and the advent of more powerful immunosuppressive agents, long-term allograft survival has not improved significantly. Approximately 15% of the cadaveric kidney grafts fail during the first year and there is a steady attrition rate thereafter due to chronic graft dysfunction, such that 50% (half-life) of the functioning graft at the end of the first year are predicted to survive for 10-15 years. Acute rejection has an impact on the development of chronic allograft dysfunction (Meier-Kriesche et al. 2000).

Graft failure creates problem of alloantibody formation and sensitisation, making future donor selection a more difficult task and requiring more sensitive immunosuppression. Immunosuppression, on the other hand, is not without problems. All immunosuppressive drugs have unwanted side effects, with opportunistic infections and increased incidence of malignancies being the main concerns. Nephrotoxicity is a particular concern with calcineurin inhibiting Cyclosporin (Cecka 1997; Hariharan et al. 2000; Varghese 1999) (Olyaei et al. 2001)

Most of the currently available immunosuppressive agents are selected their ability to reduce acute rejection and with appropriate protocols it is possible to reduce acute rejection to less than 10%. Unfortunately, these measures have failed to reduce the incidence of chronic rejection and transplant half-life of a kidney has remained static around 8 years. However, an improvement is beginning to occur because of the range of measures taken, which include: reduction in cold ischemia time, reduction in ischemic reperfusion injury, age

and size matching of the kidney, adequate immunosuppression, control of hypertension and treatment for hyperlipidaemia (Cecka 1997; Hariharan et al. 2000; Varghese 1999).

The success of renal transplantation has produced its own problems, however in that there are far more patients on dialysis waiting for a transplant than there are kidneys available for transplantation, and this discrepancy becomes more marked each year. In the United Kingdom, there are nearly 6000 patients waiting for a kidney transplant, but the transplant rate remains relatively stable at around 1500 transplants per year while over 2000 new patients are added to the waiting list each year. Living renal transplants have slowly increased (3%) each year (UKTSSA-www.uktransplant.org.uk).

1.2.2. ACUTE ALLOGRAFT REJECTION

Acute rejection episodes may occur at any time after the first few days following transplantation but with decreasing frequency after the first 3 months. The process is T cell mediated and, therefore, the treatment is usually with increased doses of standard immunosuppressive drugs or with antilymphocyte antibodies. Current immunosupression involving calcineurin inhibitors, MMF, and mono and polyclonal antibodies targeted against T cells have reduced the incidence of acute rejection to below 20% (Group. 1996). Recent studies have shown that as many as 80% of kidney transplant recipients never experience an episode of acute rejection (Group. 1996) and it is now rare to lose a transplanted organ to cell mediated rejection during the first year after transplantation. The experimental study of cell mediated rejection is important for several reasons. Firstly, acute rejection may contribute to the process of chronic rejection. Secondly, a better understanding of the mechanisms may help to design assays

that could identify rejection and monitor its response to immunosppressive protocols. More importantly, a better understanding of how T cells cause graft rejection may help in designing strategies to eliminate deleterious pathways and at the same time protect pathways involving regulatory cells for which can preserve the transplant tolerance.

1.2.2.1. Mechanisms of acute allograft rejection

Acute rejection is an immunological process resulting from the recognition of alloantigens by recipient T cells. The alloreactive response can be divided into three successive stages.

1. Recognition of alloantigens by naïve host T cells.
2. Activation and expansion of alloreactive T cells.
3. The effector phase, which involves the destruction process itself.

1.2.2.1.1 Recognition of alloantigens by naïve host T cells.

The first step in the initiation of an immune response against a graft is recognition of the graft as 'non-self'. This is believed to occur by two mechanisms - the 'direct' pathway and the 'indirect' pathway of antigen recognition.

2.2.1.1.a. Direct pathway

The direct pathway is dependent on the recognition of allo-MHC and peptide on professional antigen presenting cells of donor origin from within the graft (Lechler and Batchelor 1982). Evidence suggests that this is the major route of allorecognition and is responsible for acute cellular rejection of the graft. It is believed to arise from the high reactivity of T cells to alloMHC molecules with a precursor frequency that is 10 -100 fold higher than for other antigens. The reason for this high precursor frequency is unclear (Lechler et al.

1990). It could be due to recognition of the allo MHC molecule itself independent of the peptide or due to MHC binding a wide variety of peptides of exogenous or endogenous origin.

Renal allograft experiments on a rat model provide firm evidence for the role of the 'passenger leukocyte' and the direct pathway (Braun et al. 1993a). Allogeneic rat kidneys transplanted into irradiated hosts are normally not rejected. 'Parking' the kidney would allow donor antigen presenting cells (APCs) to migrate out and slowly, host antigen presenting cells would infiltrate the graft. Placing a second kidney allograft and simultaneously infusing a costimulation dependant T cell clone recognising donor MHC class II caused rejection of the second unmanipulated graft while the first graft was unaffected. The explanation offered is that only passenger leukocytes expressing donor MHC class II can activate the direct pathway and cause acute rejection. Evidence that these passenger leukocytes are dendritic cells comes from Lechler and Batchelor who showed that infusion of donor dendritic cells at the time of retransplantation of the 'parked' kidney caused rejection (Lechler RI and Batchelor 1982b).

Looking at the sequence of events involved in direct allorecognition, in skin grafts, maturation and migration of Langerhaan's cells have been documented as early as 4 hours after skin grafting (Larsen et al. 1990b). Mature dendritic cells capable of activating host T cells can be demonstrated after 18 hours (Kripke et al. 1990) in the draining lymph nodes. In vascularised allografts (cardiac), dendritic cells migrate to the spleen where they associate with $CD4^+$ T lymphocytes (Larsen et al. 1990a) and to the regional lymph nodes (Sriwatanawongsa V et al. 1993). Approximately 90% of dendritic cells leave

the graft within 4 days (Larsen et al. 1990a), but some persist for long periods (Demetris AJ et al. 1992). The mature immunocompetent dendritic cell interacts with naive T cells in the spleen and lymph nodes and current evidence points to these sites as the places where allosensitisation occurs through the direct pathway (Austyn JM and Larsen 1990). Dendritic cell traffic appears to be a one way phenomenon from graft to lymph node, as mature dendritic cells do not migrate from blood into graft tissue (Larsen CP et al. 1990). Naive recipient T cells traffic through the graft as part of the inflammatory infiltrate, but fail to become activated - probably because the dendritic cells are immature (Larsen CP et al. 1990). In the spleen and lymph nodes, dendritic cells directly activate $CD8^+$ T cells to differentiate into cytotoxic lymphocytes (Young JW and Steinman 1990). $CD4^+$ T helper cell activation can initiate alloantibody production and after trafficking back to the graft, and these cells can initiate delayed hypersensitivity reactions by recruiting monocytes.

Strategies of targeting the direct pathway for tolerance induction would aim to deplete the target of immunostimulatory cells to prevent interaction between these cells and host T cells. Facilitating interaction between the remaining graft cells and host T cells would facilitate graft survival as it induces a state of anergy. Numerous studies have shown the efficacy of passenger leukocyte depletion in experimental systems. Strategies such as 95% oxygen culture (Hullett DA et al. 1989), prolonged low temperature culture (Lacy PE et al. 1979), treatment with an MHC class II antibody (Faustman D et al. 1981), antidendritic cell antibody (Faustman DL et al. 1984), and irradiation of the graft (Lau H et al. 1984) in experimental pancreatic islet transplantation have all targeted this route.

Several intracellular signals follow the engagement of co-stimulatory molecules (providing signal 2) (Bretscher and Cohn 1970). The binding of CD28 receptor triggers the production IL-2 in naïve CD4+ T cells by inducing the activation of several transcription factors. Furthermore, it protects T cells from apoptosis (Constant and Bottomly 1997; Watts and DeBenedette 1999) and these actions together favour the proliferation alloreactive T cells. Constitutive expression of co-stimulatory molecules (providing signal 2) (Bretscher and Cohn 1970) is crucial to the role of these cells. The presence of costimulatory molecules on the surface of the APC makes them a professional antigen presenting cells (APC), as MHC class I and class II (Braun et al. 1993b) expression alone on the surface tends to anergise rather than activate T cells. Co-stimulatory blockade is also of proven benefit and may work by inhibiting interaction between donor APC and host responder cells. CD3 (Cosimi AB et al. 1981), CD4 (Lehmann M et al. 1997), B7-1 and B7-2 (Lenschow et al. 1995), CTLA-4 (Kirk AD et al. 1997), CD40 ligand and ICAM-1 (Isobe M et al. 1992) have all been targeted with positive results in both experimental and clinical settings. The important strategy in limiting the rapid polyclonal expansion of effector cells - as seen in a direct pathway response - with cyclosporin A, tacrolimus and azathioprine - has been effective in bringing about some of the current success of clinical transplantation.

1.2.2.1.1.b. Indirect pathway

The normal physiological route through which T cells are stimulated is through T cell receptor (TCR) recognition of foreign peptides on MHC molecules of recipient APCs. In transplantation immunology, this route is referred to as the indirect pathway of antigen presentation. The term is

misleading as it fails to imply that it is the normal pathway of antigen presentation (Shoskes and Wood 1994).

The results of early studies on transplantation suggested the existence of a second pathway through which graft rejection could occur (Lechler RI and Batchelor 1982a). Initially, this route was termed 'cross priming' (Bevan 1976). There was early evidence in a rat model, 'parked' kidneys depleted of passenger leukocytes, were still acutely rejected in some strains after retransplantation (Lechler RI and Batchelor 1982a). Skin grafts from MHC Class I and Class II deficient mice could still be rejected by CD4 T cells of normal allogeneic recipients (Dierich A et al. 1993; Grusby MJ et al. 1993) suggesting that proteins from the graft are processed and these peptides are able to initiate rejection. The best piece of evidence for the role of the indirect pathway so far is that T cells reactive to a single immunodominant self restricted allo-peptide could induce skin graft rejection - i.e. completely through the indirect pathway without any direct pathway involvement (Valujskikh A et al. 1998).

The first step in the indirect pathway would be uptake of alloantigen by host dendritic cells that are known to infiltrate grafts after a variable period of residency (Armstrong HE et al. 1987). These infiltrating cells are immature dendritic cells which are well equipped for antigen capture and presentation through the presence of abundant Fc receptors, high phagocytic and macropinocytic activity and abundant intracellular MHC class II molecules (Thomson AW and Lu 1999). There is evidence that dendritic cells process apoptotic cells through expression of $\alpha_v\beta_5$ integrin and CD36 (Albert ML et al. 1998a; Albert ML et al. 1998b) and present these processed antigens to T cells (Adler AJ et al. 1998). Dendritic cells will then migrate out of the graft, mature

on their way to the regional lymph node or spleen and then activate effector cells - $CD8^+$ T cells (Lee RS et al. 1994), $CD4^+$ T cells (Grusby MJ et al. 1993), B cells (Steele DJ et al. 1996) for alloantibody production and macrophages (Waaga AM et al. 1997) for inducing delayed type hypersensitivity responses. The importance of lymphoid organs in this process is proved by the fact that mice lacking lymph nodes and a spleen ignore the alloantigens and accept vascularised cardiac allografts indefinitely (Lakkis et al. 2000).

An important feature of T cell responses in the indirect pathway response is that these cells are primed against a single or few immunodominant epitopes, and are, therefore oligoclonal (Benichou G et al. 1998; Benichou G et al. 1999). However, with the passage of time and persistence of the graft, clones against cryptic epitopes are generated - a factor which may be responsible for features of chronic rejection.

The frequency of these self restricted T cells recognising processed allo-MHC in the indirect pathway is approximately 100 fold lower than that for T cells recognising intact allo-MHC (i.e. the direct pathway) (Liu Z et al. 1993). Although this may suggest that the indirect pathway plays a minor role in allograft rejection, there is evidence to suggest that rejection can occur through this route just as rapidly as through the direct pathway (Lee RS et al. 1997) and rejection can occur utilising the indirect pathway alone (Valujskikh A et al. 1998). Although direct antigen recognition tends to overshadow the indirect pathway, careful analysis of the contribution of each to allograft rejection showed that indirect pathway accounted for 10% of the response (Benichou G et al. 1999).

Once activated by TCR and costimulatory signals, T lymphocyte expansion further requires T cell growth factors such as IL-2, IL-4 and IL-15. Mice lacking one or even two of these growth factors are still able to reject fully mismatched allografts (Li et al. 1999b; Smith et al. 2000). However, blockade of the gamma –c chain shared by the IL-2, IL-4 and IL-15 receptors will lead to allograft acceptance and profound immunosuppression (Li et al. 2000).

1.2.2.1.2 Differentiation of alloreactive CD4 cells into Th1 and Th2 phenotype

CD4+ T cells play an important role in rejection, as demonstrated by the inability of CD4 deficient mice to reject organ allografts (Krieger et al. 1996). In the course of an immune response, CD4+ T cells can differentiate into two different subsets whose functional properties are characterised by the cytokines they secrete. The Th1 phenotype is characterised by production of cytokines such as IL-2, IFN-γ and lymphotoxin and TNF-alpha in the context of graft rejection and will result in the activation of CD8+ cell mediated cytotoxicity, macrophage dependent delayed type hypersensitivity and the synthesis of complement fixing IgG2a antibody by B cells. Th1 cells also may become cytotoxic by the expression of Fas-ligand on their surface. In contrast, the Th2 phenotype is characterised by the production of IL-4, IL-5, IL-6, IL-9 and IL-10 and IL-13. These will mainly trigger eosinophil activation, a process that can itself mediate graft rejection. Alloreactive Th2 cells do not express Fas-ligand and therefore do not mediate direct cytotoxicity. CD4+ cells appear to go through a stage prior to their differentiation into Th1 or Th2 cells, designated Th0, in which their pattern of cytokine secretion is broader (IL-2, IL-4, IFN-γ, lymphotoxin).

Cytokines are the major factor driving Th1 or Th2 expansion during the initial steps of CD4+ T cell activation. IL-12 and IFN-γ promote Th1 differentiation. IL-12 directly acts on CD4+ precursors where it stimulates IFN-γ synthesis and inhibits IL-4 production (Constant and Bottomly 1997). IFN-γ has anti-proliferative effects on emerging Th2 cells, but not on Th1 cells because functional IFN-γ receptors are expressed only on Th2 cells (Tau et al. 2000). In addition, IFN-γ upregulates the expression of IL-12 receptor β2 chain on naïve T cells and inhibits their IL-4 production (Zhang et al. 2001). IL-4 favours a Th2 response by directly down regulating the transcription factors promoting IFN-γ synthesis (Goenka et al. 1999; Ohmori and Hamilton 1997). *In vitro* studies show the presence of IL-4 in culture promotes the Th2 phenotype (Seder et al. 1992) whereas the presence of IL-12 favours Th1 cell differentiation (Manetti et al. 1993).

The $CD4^+$ Th mediated response is certainly more than just Th1 and Th2, but these represent two extremely polarised forms that play important roles in immune responses. Other cytokine patterns also exist. Th3 cells are TGF-beta secreting $CD4^+$ cells that do not secrete IL-2, IL-4, IFN-γ, IL-6, IL-10 or IL-13 appear to be a unique T cell subset that can down regulate Th1 and other immune cells.

The phenotypes of DC as well as the organs from which they originate are other factors influencing Th cell differentiation. DC can be functionally separated into high (DC1) or low IL-12 producing DC (DC2) leading to Th1 or Th2 differentiation respectively (Liu et al. 2001).

Recent evidence indicates that activation of CD8+ T cells by donor MHC class I molecules will prevent the production of Th2 cells and skew the immune

response toward the Th1 type, whereas in the absence of alloreactive CD8+ T cells, CD4 cells will produce a mixture of Th1 and Th2 phenotypes (Braun et al. 2000; Chan et al. 1995). One of the mechanisms involved in this pathway is that alloreactive CD8+ precursors are able to rapidly produce IFN-γ, which will stimulate IL-12 production by donor DC (Kopf et al. 1995; Srikiatkhachorn and Braciale 1997). This will promote a Th1 type alloreactive response and inhibit the differentiation into Th2 type cells. Few animal studies support this concept. In a mouse model of cardiac transplantation across combined MHC class I and Class II disparities a Th1 response occurs in unmodified recipients. However following CD8 depletion or IL-2 neutralisation the response is shifted towards a Th2 type (Chan et al. 1995; Piccotti et al. 1996; Piccotti et al. 1997).

1.2.2.1.3. Role of chemokines in transplant rejection

Alloreactive cells primed in lymph nodes or spleen and circulating blood leukocytes such as monocytes and eosinophils are guided to the allograft by a chemoattractant gradient of chemokines released by the allograft itself. Although the initial burst of chemokines is associated with ischemia/reperfusion injury, it is likely that those graft infiltrating leukocytes then further contribute to chemokine production (Hancock et al. 2000a; Lu et al. 1999; Nelson and Krensky 2001).

Several recent experiments indicate that chemokines play a major role in the allograft rejection process (Hancock et al. 2000a; Nelson and Krensky 2001). The neutralisation of monokine induced IFN-γ (Mig) by rabbit antiserum prevents graft T cell infiltration and significantly prolongs the survival of MHC class II as well as minor histocompatibility antigen- disparate skin allografts (Koga et al. 1999; Watarai et al. 2000). Similarly CXCR3 knockout mice, which

lack receptor for three CXC chemokines, Mig, IP-10, and I-TAC, showed a substantial prolongation of vascularised heart allograft survival (Hancock et al. 2000b). Among these three chemokines IP-10 is the first to be produced after transplantation (Hancock et al. 2001). Early donor derived IP-10 plays a critical role in triggering rejection since cardiac allografts from IP-10+/+ donor mice are normally rejected by IP-10 knockout recipients, whereas cardiac allografts from IP-10 knockout donors survive long term in IP-10+/+ recipients (Hancock et al. 2001). These experiments focus on chemokines that recruit mainly Th1 cells. Whether Th2 –recruiting chemokines such as thymus and activation regulated chemokine (TARC), monocyte derived chemokine (MDC) or eotaxin affect the rejection process mediated by Th2 cells remains to be investigated.

1.2.2.1.4. The effector phase

1.2.2.1.4.a. Alloreactive T cell cytotoxicity

Once T cells are activated, they acquire cytotoxic properties that enable them to kill the targets. The two main cytotoxic mechanisms are the perforin/granzyme and Fas/Fas-ligand systems. Whichever mechanism is considered, the end point is the death of the target cell by apoptosis (Berke 1995; Graubert and Ley 1996).

The perforin/granzyme pathway is used by CD8+ T cells and NK cells. The acquisition of cytotoxic properties by CD8+ T cell precursors requires Th1 cytokines, mainly IL-2. During the 48 hours after TCR engagement, activated CD8+ T cells synthesise perforin and granzymes, which are then targeted to intracellular cytotoxic granules (Shresta et al. 1998). When a CTL recognizes the allo-MHC molecule, it forms a tight junction with the allogeneic cell, allowing CTL granules to fuse with the target cell membrane. Perforin

molecules insert within the allogeneic cell membrane and form polymers that create channels, through which granzyme A and B penetrate into the cytoplasm. From the cytoplasm, granzymes can either directly enter the nucleus or can cleave cytoplasic procaspases into caspases, which will then move into the nucleus (Graubert and Ley 1996). Caspases are a family of cysteine proteases that cleave aspartate residue from many substrates including the caspases themselves. Granzyme B acts on the mitochondria to release cytochrome C, which can also trigger the caspase system (Heibein et al. 1999). Caspase activation is responsible for the appearance of functional nuclease activity that finally triggers DNA fragmentation and leads to apoptosis (Graubert and Ley 1996; Heibein et al. 1999).

In vitro experiments have shown that CD8 cells need both perforin and granzymes to mediate maximal cytotoxicity. *In vitro* target cell lysis by CD8+ T cells from perforin deficient mice is impaired. Similarly, CD8+ T cells from mice equipped with a normal perforin system but knockout for both granzyme A and B are still able to make cell membrane pores but do not induce target cell apoptosis (Simon et al. 1997). The *in vivo* role of the perforin/granzyme pathway in allograft rejection has been demonstrated by the transplantation into perforin deficient mice (Graubert et al. 1997; Schulz et al. 1995). Rejection of skin and heart allografts has significantly delayed when perforin deficient mice received organs from donors showing a single MHC class I antigen disparity (Schulz et al. 1995). In contrast MHC classes I and II mismatched skin and heart allografts are normally rejected by perforin deficient mice. These observations might be explained by the fact that CD8+ T cells play a dominant role in the rejection of organs having only MHC class I antigen mismatch,

whereas other effector mechanisms can mediate rejection of combined MHC class I and class II antigen mismatched allografts.

Fas/Fas-ligand (FasL) interaction is the most important mechanism for CD4+ CTL mediated cytotoxicity (Kagi et al. 1996). Fas is a member of the tumour necrosis factor family of death receptors, is constitutively expressed on most cell surfaces (Peter and Krammer 1998) and Fas-ligand is essentially inducible. Its expression becomes apparent 4-5 hours after T cell activation. The Fas-ligand expression is restricted to Th1 and not Th2 alloreactive T cells (Kagi et al. 1996; Matesic et al. 1998). On the cell surface, FasL is rapidly cleaved by metalloproteinases and then binds to Fas on the target cell surface. Fas engagement results in death inducing signal complex (DISC) formation and the activation of the caspase cascade, which will in turn induce target cell apoptosis similar to that involved induced by the perforin/granzyme system. This apoptotic process can be interrupted by several anti-apoptotic proteins such as Bcl-2 and their homologues, which interfere with caspase activation (Tschopp et al. 1998).

There is evidence supporting the role for Fas/FasL interactions in allograft rejection. Experiments have shown that mice reject MHC class II disparate skins by two distinct effector pathways, one mediated by eosinophils and the other by Fas/FasL interactions (Le Moine et al. 1999).

1.2.2.1.4.b Alloantibodies

Patients who have been in contact with HLA alloantigens through transfusions, pregnancy or transplantation may develop anti-HLA class I or class II antibodies. In this scenario, a B cell that has bound an HLA molecule by its surface immunoglobulin will internalise the HLA-Ig complex, cleave the HLA

molecules into allo-peptides, load the suitable ones within the groove of its own MHC class II and return these allo-peptides/MHC class II complex back to the B cell surface. The alloreactive B cell then requires T cell help to divide and produce antibodies. Only CD4+ cells able to recognise complexes of allo-peptides /self MHC class II molecules will be able to establish cognate interactions with alloreactive B cells. These T cells belong exclusively to the subset involved in the indirect recognition of MHC alloantigens (Pettigrew et al. 1998).

1.2.2.1.4.c. Macrophage activation and delayed type hypersensitivity reaction

Delayed type hypersensitivity (DTH) reactions are characterised by tissue swelling and induration due to increased vascular permeability and the presence of an inflammatory infiltrate rich in T cells, macrophages and neutrophils. This reaction is delayed, because unlike immediate hypersensitivity mediated by preformed antibodies, some days are required to prime antigen specific Th1 cells. Activated Th1 cells are crucial for DTH through the release of IFN-γ and TNF-α. This in turn will trigger macrophages to produce toxic molecules such as nitric oxide (NO), oxygen intermediates, and TNF-α. NO, a highly reactive nitrogen metabolite, produced by the inducible form of NO synthase, is cytotoxic at high concentrations. It also elicits the vasodilatation and oedema characteristic of DTH. TNF-α or TNF-β bind to TNF receptors and induce target cell apoptosis or necrosis through caspase activation, as described for Fas/FasL and perforin/granzyme systems. Activated neutrophils release myeloperoxidase, which will then generate toxic metabolites as oxygen species and hydrogen peroxide.

Clinical, as well as experimental, evidence supports the view that DTH reactions are able to mediate allograft rejection. Macrophages are often present among leukocytes infiltrating rejected allografts (Grimm et al. 1999; Valujskikh A et al. 1998).

1.2.2.1.4.d. Eosinophils and Th2 type alloreactive response

Eosinophils are recruited and activated within the allograft through the combined action of IL-4, IL-5, and IL-13 produced by alloreactive Th2 cells. IL-5 plays an essential role in the differentiation and proliferation of eosinophils in the bone marrow (Foster et al. 1996; Kopf et al. 1996) IL-4 and IL-13 upregulate the expression of VCAM-1 on endothelial cells, a critical adhesion molecule for eosinophils, which express the counter receptor VLA-4 on their membrane (Doucet et al. 1998; Fukushi et al. 2000; Pretolani et al. 1994). Furthermore IL-4 and IL-13 stimulate the production of eotaxin, a CC chemokine, by several cell types including endothelial cells (Li et al. 1999a; Matsukura et al. 1999). IL-5 and eotaxin together recruit and activate eosinophils within inflamed tissues (Collins et al. 1995). Activated eosinophils release granules that contain toxic molecules such as eosinophil peroxidase (EPO) and eosiniphil cationic protein (ECP) (Assa'ad et al. 2000), which leading to the formation of brominating species responsible for oxidative damage of tissue proteins (Wu et al. 2000).

The first clue that a Th2 eosinophil pathway could represent an effector mechanism of allograft rejection came from the work of Chan *et al* (Chan et al. 1995). They observed that fully mismatched hearts rejected by unmodified mice contained the Th1 cytokines IL-2 and IFN-γ, together with anti-donor CD8+ CTL. The Th2 cytokines IL-4, IL-5 and IL-10 could not be detected. When

recipients were depleted of CD8+ cells or had IL-12 blocked by various means, rejection still occurred but was characterised by an aggressive eosinophil cellular infiltrate and the presence of IL-4, IL-5 and IL-10 mRNA within rejected grafts (Piccotti et al. 1996; Piccotti et al. 1997).

1.2.3 CHRONIC ALLOGRAFT DYSFUNCTION (CHRONIC REJECTION)

Chronic allograft dysfunction (nephropathy or rejection) has been defined as the progressive deterioration of structure and function of an organ allograft in the months or years after transplantation. This is believed to arise as a result of alloantigen dependent immune responses, alloantigen independent inflammatory processes and non immune processes (Kreis and Ponticelli 2001). Alloantigen dependent immunological factors include HLA incompatibility; number, timing and severity of acute rejection episodes; panel reactive antibodies and modulation of ongoing alloresponsiveness by immunosuppression (Paul 2001). Alloantigen independent factors include cold and warm ischemic-reperfusion injury, donor age, size of the kidney, hyperlipidaemia and hypertension (Fellstrom 2001). The non-immune processes include calcineurin inhibitor toxicity and acute tubular necrosis. It is difficult to separate the individual entities in the final picture, as there is complex interplay among the different processes and the similar pathologic changes they induce.

The effects of non-immune and immune interactions in chronic rejection have been hypothesised to accelerate the process of senescence in vascular endothelial and renal tubular epithelial cells (Halloran et al. 1999). As a consequence, the repair capacities of vascular and renal cells have been postulated to become exhausted, resulting in inadequate tissue repair and

contributing to graft vascular disease, tubular atrophy, interstitial fibrosis, and glomerular sclerosis. It has also been postulated that inadequate nephron dosing due to size and age mismatch between donor and recipient, together with the rest of the donor antigen dependant and independent factors can promote chronic allograft nephropathy by lowering nephron mass and causing hyperfiltration related injury in the remaining nephrons (Brenner and Mackenzie 1997).

The pathological changes observed in chronic allograft nephropathy are usually not specific to a given disease. They are the result of immune and non-immune mediated injury to the vessel wall with a local inflammatory response; the changes often combine patchy fibrosis of the interstitium with or without inflammation, tubular atrophy, glomerular sclerosis, and vascular endarteritis. The exact mechanism by which the initial injury leads to chronic allograft nephropathy is not clearly defined. Injury to the endothelial cells of the arterial wall is thought to initiate the pathogenic process. This may be accompanied by infiltration of the site by inflammatory cells which, along with the endothelial cells, secrete a variety of growth factors and increase expression of MHC class II molecules and cell adhesion molecules (Fuggle and Koo 1998; Hayry 1998). In addition, production of nitric oxide, which has vasodilatory and antiproliferative properties, is reduced (Fellstrom and Larsson 1993). The growth factors induce proliferation of smooth muscle cells that contribute to the eventual narrowing of renal arteries. This resultant ischemia could lead to proliferation of mesangial cells and other changes characteristic of allograft nephropathy (Sahai et al. 1997). The inflammatory mediators generated by endothelial injury could result in mesangial cell proliferation and fibrotic scarring leading to glomerulosclerosis (Valdes and Sonora 1999). Within the interstitium, injury results in the influx of

mononuclear cells, proliferation of fibroblasts and deposition of extracellular matrix leading to scar formation and fibrosis (Waller and Nicholson 2001). Macrophage infiltrates are commonly present in chronic allograft nephropathy and provide a wealth of profibrotic factors and extracellular matrix proteins (Nathan 1987). Resident fibroblasts are also particularly important in fibrogenesis (Desmouliere and Gabbiani 1995) and fibroblasts from diseased kidneys deposit more collagen than normal fibroblasts (Stetler-Stevenson 1996). Under the influence of cytokines and growth factors, fibroblasts differentiate into myofibroblasts (Grinnell 1994), the main cellular element of the interstitium (Border and Noble 1994) and this phenotypic switch from a quiescent state to a secretory state is a key event in the accumulation of extracellular matrix. The role of the tubular epithelial cell in the production of extracellular matrix in allograft fibrosis is unclear, although upregulation of tubular collagen IV has been demonstrated in diabetic rats (Ceol et al. 1996) and TGF-β stimulated rabbit proximal tubular cells increase synthesis of proteoglycan and fibronectin (Humes et al. 1993). Eventually the fibrotic processes dominate the vascular, glomerular and interstitial compartments leading to loss of graft function.

As mentioned before, numerous profibrotic cytokines and growth factors are generated in response to injury at the time of transplantation. Over the course of time, the balance between synthesis and degradation of extracellular matrix is tilted in favour of accumulation, leading to fibrosis and subsequent graft failure. TGF-β and angiotensin-II appear to play prominent roles in this process.

IL-1 induces fibrosis through upregulation of adhesion molecules on the endothelial cells (di Giovine and Duff 1990) and by inducing smooth muscle cell

and fibroblast proliferation (Nikolic-Paterson et al. 1996; Raines et al. 1989). IL-6 is also a profibrotic cytokine which acts as a mitogen for smooth musle and mesangial cells (Ruef et al. 1990). TNF-α can stimulate leukocyte and endothelial cell production of adhesion molecules and other cytokines (Paulnock 1992). It increases extra cellular matrix (ECM) formation by down regulation of ECM proteases and upregulation of tissue inhibitors of metalloproteinases. TGF-β is a potent profibrotic cytokine signalling molecule that is essential for normal wound healing (Sporn and Roberts 1992). However, sustained or increased synthesis of TGF-β results in a shift towards accumulation of extra-cellular matrix by increasing matrix synthesis and inhibiting its degradation (Ignotz et al. 1987; Sharma et al. 1996). These properties suggest TGF-β has a role in allograft fibrosis. Angiotensin II can also induce profibrotic signals through TGF-β (Kagami et al. 1994) by inducing ECM deposition.

Chronic allograft dysfunction remains the leading cause of graft failure after the first year following transplantation. The two major causes associated with long-term graft loss continue to be cardiovascular death with a functioning graft and chronic allograft dysfunction. Both conditions are associated with progressive atherosclerosis and vasculopathy resulting in macrovascular and microvascular disease, one systemic, and the other within the graft. At present, no effective drug is available to reverse or inhibit this process. However a better understanding of the molecular mechanisms involved in atherosclerosis offers a number of potential new strategies.

1.3. ATHEROSCLEROSIS

Atherosclerosis appears to be a highly characteristic response of arteries (Ross 1981) arising from a chronic inflammatory reaction between modified lipoproteins, monocyte derived macrophages, T cells, and the normal cellular elements of the arterial wall (Glass and Witztum 2001). This inflammatory process can eventually lead to the development of complex lesions, or plaques that protrude into the arterial lumen. Plaque rupture and thrombosis would lead to vascular occlusion with catastrophic consequences. Occlusion of coronary arteries would lead to myocardial infarction and cerebrovascular occlusion to stroke, the major causes of death (Ross 1999).

Atherosclerosis occurs mainly in large and medium sized elastic and muscular arteries. It was initially believed that atherosclerosis was the response to injury and that endothelial denudation was the first step in progression of this disease (Ross and Glomset 1976a; Ross and Glomset 1976b). Current evidence points to endothelial dysfunction rather than denudation. Possible causes of endothelial dysfunction include elevated and modified LDL, free radicals generated by cigarette smoking, hypertension, diabetes mellitus, elevated plasma homocysteine concentrations, and infectious microorganisms (Libby et al. 2002). It must be pointed out that much of the new evidence regarding the causative agents is derived from *in vitro* studies or from studies in mouse models of atherosclerosis derived in the setting of very high blood cholesterol levels. The quantitative contribution of each of the other pathogenic agents is unclear, but could make a crucial difference between disease progression and a stable plaque *in vivo*.

There is general consensus regarding the sequence of events that lead to the development of the earliest visible lesion of atherosclerosis, the fatty streak (Steinberg 2002). Most of the work relating to this comes from studies in the hypercholesterolemic rabbit and mouse models. Hypercholesterolemia has been shown to increase expression of vascular cell adhesion molecule-1 (VCAM-1), a key adhesion molecule for monocytes and T cells, on the surface of endothelial cells of large arteries (Cybulsky et al. 2001). Knockout studies in mice also suggest involvement of P and E selectins (Dong et al. 1998) and monocyte chemoattractant protein-1 (MCP-1) (Gosling et al. 1999). This results in the recruitment of monocytes and, later, T lymphocytes to these sites where they penetrate through the endothelium and take up residence in the subendothelial space. There, the monocytes undergo phenotypic modification, imbibe LDL to become foam cells, loaded with multiple cytoplasmic droplets containing cholesterol esters.

The mechanisms relating to foam cell formation are still a subject of intense research, as the lipid accumulations within the cells are derived from plasma lipoproteins and under physiological conditions, lipid uptake through the LDL receptor is subject to feedback inhibition. Uptake of chemically modified LDL through the scavenger receptor-A and CD36 has been shown to result in foam cell formation (Kodama et al. 1988), (Brown and Goldstein 1983), (Endemann et al. 1993). The best studied modification that might account for foam cell formation is oxidative modification (Steinberg et al. 1989). The observation of increased LDL concentrations in the susceptible sites of rabbit aortic wall (Schwenke and Carew 1989) along with tight adherence of LDL to arterial wall proteoglycans (Camejo et al. 1998) and increased susceptibility of

proteoglycan bound LDL to oxidation (Hurt-Camejo et al. 1992) has led to the proposition that oxidation of LDL trapped at susceptible sites may be the initiating factor in atherosclerosis – the oxidative modification hypothesis (Berliner and Heinecke 1996). Furthermore, oxidised LDL has been shown to have other proatherogenic properties such as being directly chemoattractant for monocytes and T cells (Quinn et al. 1987), cytotoxic to endothelial cells(Hessler et al. 1983), mitogenic for macrophage (Yui et al. 1993) and smooth muscle cells (Chatterjee and Ghosh 1996) and stimulation of release of monocyte chemoattractant protein-1 and monocyte colony stimulating factor from endothelial cell (Yui et al. 1993). These observations add support to the oxidative modification hypothesis.

The fatty streak itself is clinically benign, but acts as the precursor to the clinically relevant lesions, which develop later. Formation of a complex lesion from a simple fatty streak is characterised by the migration of smooth muscle cells from the medial layer of artery wall past the intimal elastic lamina and into the intimal or sub endothelial space. These smooth muscle cells may proliferate and take up modified lipoproteins and form foam cells. The foam cells synthesise extracellular matrix proteins and lead to the formation of a fibrous cap (Paulsson et al. 2000; Ross 1999). Unfortunately, there is, as yet, no neat linear pathogenic hypothesis to connect the development of the fatty streak to the later plaques. All the main cell types in a lesion are capable of secreting a variety of chemokines, cytokines and growth factors which interact in a complex way (Glass and Witztum 2001; Libby et al. 2002). Over time, macrophages become engorged with lipids, undergo mitosis and die with the residual lipids developing into a necrotic, debris laden core. Smooth muscle cells change their

phenotype, replicate, change into foam cells, and eventually die. In later stages of the evolution of the plaque, the endothelial cells are damaged and slough off, forming a nidus for a thrombus. Ultimately, the fibrous cap thins down and ruptures, exposing the tissue factor rich interior – precipitating a thrombotic event. These processes are not necessarily sequential, they may occur concurrently. Moreover, they may not progress linearly with time (Bruschke et al. 1989; Yokoya et al. 1999).

1.3.1. Immunological responses in atherosclerosis.

It is now widely accepted that inflammation is an important component of the early events and progression of atherosclerosis. The appreciation that the inflammatory processes also participate in events leading to plaque rupture, which causes the thrombotic complications and clinical events, has fundamentally changed the view on the pathogenesis of atherosclerosis. Both adaptive and innate immune responses modulate the rate of progression as well as the composition of the lesions. But it has to be mentioned that immune mechanisms by themselves do not appear to be the primary cause of atherosclerosis (Binder et al. 2002).

Macrophages are fundamental innate immune cells through production of reactive oxygen species, proteases, and cytokines as well as their scavenging function mediated by pattern recognition receptors such as scavenger receptors (SR-A and SR-B), CD 36 and toll like receptors (TLRs). The recruitment of monocytes appears to be essential formation as hypercholesterolemic mice that are deficient in macrophage chemoattractant protein-1 (MCP-1) or its cognate ligand on monocytes CCR2, have greatly reduced incidence of atherosclerosis (Boring et al. 1998; Gosling et al. 1999). In the initiating stages of

atherosclerosis, modified LDL activates endothelial cells to express vascular cell adhesion molecule-1 (VCAM-1) which preferentially recruits monocytes and T cells through their interaction with the integrin very late antigen 4 (VLA-4) (Shih et al. 1999). Cytokines such as IL-1 and TNF-α are also involved. Oxidised LDL, cytokines and activated complement stimulate arterial cells to secrete macrophage chemoattractant protein –1 (MCP-1) which recruits monocytes and T cells to the subendothelial space (Witztum and Berliner 1998). Hypercholesterolemic *Op/Op* (*Csf1*$^{-/-}$) mice which lack monocyte colony stimulating factor and therefore lack differentiated macrophages in their tissues also show minimal atherosclerosis (Smith et al. 1995). Ingestion of oxidised LDL by macrophages through SR-A and SR-B has been shown to lead to foam cell formation, a rate limiting step in atherosclerosis (Febbraio et al. 2000; Suzuki et al. 1997). TLRs are expressed in atherosclerotic lesions as well. Although TLR4 deficiency does not reduce atherosclerosis in ApoE knockout mice (Wright et al. 2000), a TLR-4 polymorphism that attenuates receptor signalling is associated with reduced atherosclerosis in humans (Kiechl et al. 2002). CD14, the lipopolysaccharide receptor, initiates inflammatory responses through interactions with TLRs. A polymorphism in the CD14 promoter region which increases its phenotypic expression has been identified as a risk factor for myocardial infarction (Hubacek et al. 1999). A variety of stimuli can activate macrophages to secrete cytokines which regulate scavenger receptor expression and inflammatory responses in general (Binder et al. 2002).

The complement system and C-reactive protein (CRP) are also involved in atherogenesis. C4, C3 and C5b-9 deposition was documented in atherosclerotic lesions (Vlaicu et al. 1985). CRP has been identified in

atherosclerotic lesions and is known to co-localize with oxidised and other forms of modified LDL (Chang et al. 2002), raising the possibility that it is also involved in the pathogenesis of atherosclerosis. Natural antibody deposition has also been documented in atherosclerotic plaques and some of these have been shown to be specific for epitopes of oxidised LDL (Shaw et al. 2000).

The best evidence for the role of adaptive immunity in the genesis of atherosclerosis comes from experiments using Apoprotein E knockout and lipoprotein receptor knockout mice crossed on to a recombination activation gene (Rag) knockout background, generating hypercholesterolaemic mice lacking T and B cells. When these mice were fed high cholesterol diets, resulting in plasma cholesterol levels of 600-800 mg/dl, a 40-80% reduction in the extent of atherosclerotic lesions was noted (Reardon et al. 2001; Song et al. 2001). When $CD4^+$ T cells from the immunocompetent parental strains were transferred into these crossbred mice, there was no difference in the extent of atherosclerotic plaque development (Zhou et al. 2000).

T cells are a component of both early and late lesions. Most T cells in lesions express CD3, CD4 and αβ–TCRs (Hansson 2001). In contrast there are relatively few B cells are found in the adventitia surrounding lesions (Sohma et al. 1995; Zhou and Hansson 1999). All lesion cells express MHC class II molecules, indicative of IFN-γ mediated activation. In turn MHC class II molecules can interact with the TCRs of CD4+ Th cells (Hansson 2001). Interestingly, CD40 and CD40L have been reported to be widely expressed on most cells within the lesion, not limited to T and B cells (Mach et al. 1997). These interactions appear to be important as genetic disruption of CD40L in apoprotein E gene knockout animals (Lutgens et al. 1999), as well as using anti

CD40L monoclonal antibody in LDL receptor knockout mice (Mach et al. 1998) reduced atherosclerotic plaque development by 60%. Furthermore, it was reported that treatment with anti CD40L monoclonal antibody stabilised and retarded plaque development (Lutgens et al. 2000; Schonbeck et al. 2000). IFN-γ induces CD40 and binding to its cognate ligand CD40L induces matrix metalloproteinase expression by macrophages, which can destabilise plaques. Th1 cells are dominant in atherosclerotic lesions, especially early lesions, as the cytokines IFN-γ IL-2 and TNF-α are highly expressed, where as only low amounts of the Th2 cytokines IL-4, IL-5 and IL-10 can be detected. In addition, IgG2a antibodies against oxLDL epitopes, formed by Th1 help, predominate in plasma during early stages in LDL receptor knockout and apo E knockout mice. IL-4 expression was detected only in the advanced stages of atherosclerotic lesions of apo E knockout mice in the presence of hypercholestrolemia. IgG1 antibodies formed by Th2 help found more prominently at the later stages of atherosclerosis (Hansson 2001).

In the context of atherogenesis, the Th1 cytokine IFN-γ is pro-inflammatory and pro-atherogenic. In addition to activating macrophages, it inhibits smooth muscle cell proliferation and collagen synthesis and therefore promotes destabilisation of the plaque. IL-1 and TNF-α have similar functions in promoting inflammatory responses. *In vitro* experiments using human mesangial cells, which are modified macrophages, demonstrated that the pro inflammatory cytokines IL-1 and TNF-α can modify cholesterol homeostasis through the dysregulation of LDL receptor, suggesting that they are important risk factors for atherogenesis (Ruan et al. 1998; Ruan et al. 2001). They have shown that these cytokines could overcome sterol induced suppression of LDL

receptor expression and induce scavenger receptor A expression (Ruan et al. 1999) and mesangial foam cell formation (Ruan et al. 2001). IL-12 and IL-18 are also highly expressed in atherosclerotic lesions and can further augment IFN-γ secretion (Gerdes et al. 2002; Uyemura et al. 1996). Apo E knockout mice deficient in the IFN-γ receptor have significantly reduced lesions and increased collagen content (Gupta et al. 1997). Another study showed daily administration of IFN-γ promoted atherosclerosis in Apo E knockout mice (Whitman et al. 2000). Daily administation of IL-12 (Lee et al. 1999) or IL-18 (Whitman et al. 2000), both of which promotes Th1 effects increased atherosclerosis in Apo E knockout mice, suggesting that the pro-atherogenic effect of IL-12 and IL-18 is mediated through IFN-γ. Thus, Th1 cells secrete IFN-γ, which activates macrophages and that leads to their release of IL-12, which in turn augments IFN-γ secretion by T cells. IFN-γ also inhibits the production of the Th2 cytokines IL-4 and IL-10.

In contrast, Th2 responses seem to antagonise pro-atherogenic Th1 effects and thereby confer atheroprotection. IL-10 can potently suppress IL-12 and IFN-γ secretion. *In vitro* studies have shown that recombinant IL-10 inhibited the oxidised LDL induced production of IL-12 by human monocytes indicating a protective function for IL-10 (Uyemura et al. 1996). IL-10, which cross regulates Th1 cells, has potent deactivating properties in macrophages and modulates several other cellular processes that may interfere with the development and stability of the atheroslerotic plaque (Uyemura et al. 1996). Mildly hypercholesterolaemic, genetically modified C57bl/6 mice demonstrated a protective function for Th2 biased responses in fatty streak formation (Huber et al. 2001). In addition, IL-10 deficient mice exhibited a three fold increase in

lipid accumulation, indicating an antiatherogenic role for IL-10 (Mallat et al. 1999). Consistent with this, transgenic overexpression of IL-10 in T cells using the human IL-2 promoter resulted in significant inhibition of lesion development in C57BL/6J mice fed an atherogenic diet (Pinderski et al. 2002; Pinderski Oslund et al. 1999). These same mice also had decreased circulating IFN-γ secreting CD4+ cells in peripheral blood and in the spleen and found an increased IgG1 antibodies than IgG2a antibodies against malonedialdehyde-LDL. Furthermore, treatment of Apo E knockout mice with pentoxifylline, which inhibits Th1 differentiation, Th2 derived cytokine IL-4 appear to have complex effects on lesion development. IL-4 exerts a number of effects that are predicted to be antiatherogenic, including antagonistic effects on IFN-γ activity in macrophages and inhibition of Th1 cell function. However, IL-4 is also proven to be pro-atherogenic in mice (King et al. 2002). In summary, Th2 responses seem to have a protective effect. T cells and macrophages engage in an interaction and the local dominance of one subset of Th cells could well influence the course of lesion progression and stability.

The anti-inflammatory cytokine TGF-β is secreted by macrophages, smooth muscle cells, and the subsets of Th cells, Th3 that exerts regulatory functions. TGF-β could be involved in plaque stabilisation, because in contrast to IFN-γ, it stimulates collagen synthesis and is fibrogenic (Lutgens et al. 2002). There is now much evidence that both innate and adaptive immune mechanisms are involved in atherogenesis, as might be anticipated for a disease that is chronic inflammatory process. Elucidation of pathways involved could lead to insights into pathogenic events that could explain in part the diversity in the expression of this disease in individuals apparently equal in regard to risk

factors, such as plasma LDL. Study of the action of the immune system in atherogenesis is in its infancy, and much remains to be learned. Most of the work so far has been done on experimental animals, mainly mice, and the relevance of these observations to human disease remains to be determined. The challenge will be to translate what has been learned already, and what will be learned through future experimental studies, to human populations.

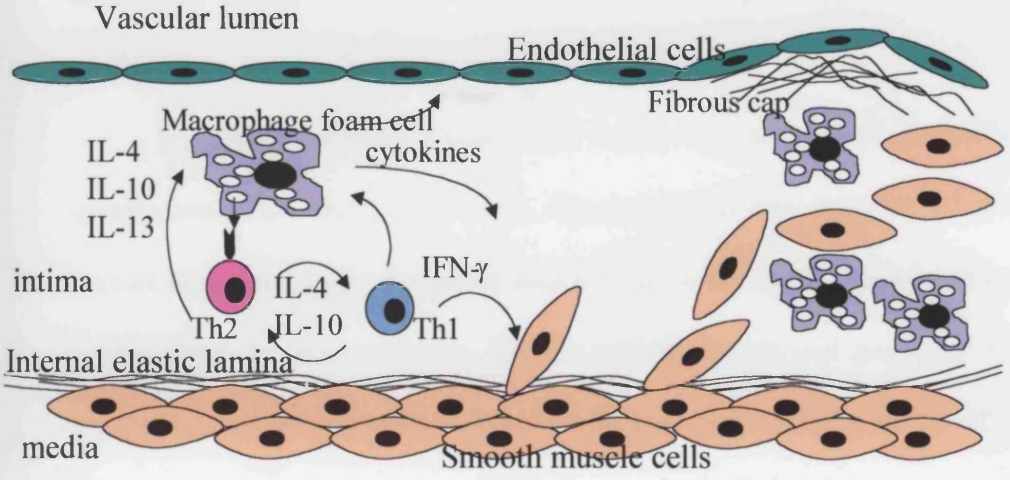


Figure:1.1 Lesion progression: Interaction between macrophage foam cells, Th1 and Th2 cells establishes a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages influence both pro- and anti-atherogenic effects on each of the cellular elements of the vessel wall.

1.3.2. Analogy between chronic rejection and atherosclerosis

It is interesting to note that the histological pattern of luminal narrowing consequent to smooth muscle cell and fibroblast proliferation, as well as extra cellular matrix deposition in blood vessels in chronic allograft dysfunction is similar to the early pathological lesions described in atherosclerosis (Billingham 1987). In atherosclerosis, endothelial injury is believed to be secondary to lipoproteins, hypertension, viral infections, and other agents which activate the cell. This activated phenotype could recruit macrophages and T cells through the expression of adhesion molecules and secretion of chemokines with consequent changes in the intima and media leading to smooth muscle cell proliferation, foam cells formation and other changes.

In chronic rejection, endothelial injury can occur through antibody, complement, CD4 and CD8 T cells. Consequent to this, a variety of growth factors are secreted into the vessel wall leading to the same sequence of events as described for atherosclerosis, namely smooth muscle cell proliferation and extracellular matrix deposition followed by luminal narrowing of the affected blood vessel (Foegh 1990; Ross 1993). Thus, once endothelial injury occurs, the response to the injury seems to be the same in both situations. The major difference appears to be the nature of the initial insult to the endothelial cell in these two conditions.

Since the final histological appearance is similar in these two conditions, it is possible that the response to injury (inflammatory response) is mediated by the same factors. Cytokines have been shown to be key factors in the inflammatory response in both these situations and it is a well-recognised fact that individuals vary in their propensity to mount an inflammatory response.

Renal artery stenosis (RAS) could be due to fibromuscular dysplasia as well as atherosclerosis. In contrast to atherosclerotic RAS, patients with fibromuscular RAS of dysplastic origin seldom develop decline in renal function even with progression of the stenosis (Schreiber et al. 1984). Progressive nephrosclerosis in association with renal dysfunction is a common consequence of atherosclerotic RAS regardless of the adequacy of blood pressure control (Rimmer and Gennari 1993; Schreiber et al. 1984). Therefore, it is likely that the atherosclerotic process may extend to involve the small vessels in the kidney. Atherosclerotic RAS has become recognised as a major cause of renal disease in the elderly leading to progressive renal failure. In one study 39 out of 106 (38%) patients presenting with hypertension and renal impairment and in another study 14% of the patients over the age of 50 presenting with advanced renal failure had atherosclerotic renal artery disease as an underlying cause (Scoble et al. 1989; Ying et al. 1984). In a study of 1302 individuals undergoing coronary angiography, 30% were found to have atherosclerotic RAS (Harding et al. 1992). Atherosclerotic RAS is also a marker for atheroembolic disease which has recently been recognised as an important cause of renal disease in the elderly (Robson and Scoble 1996; Scoble et al. 1989). Patients with atherosclerotic RAS on haemodialysis have a very poor prognosis and over 50% of such patients die within the first two years of starting dialysis (Mailloux et al. 1988)

In 1982 Moorhead *et al* hypothesised that chronic progressive kidney disease could be mediated by abnormalities of lipid metabolism. This original hypothesis is supported by numerous animal studies that implicate an involvement of hyperlipidaemia in the pathogenesis of progressive renal disease. Clinical examples of focal glomerulosclerosis and diabetic glomerular disease

reinforce the idea of an analogy between atherosclerosis and glomerulosclerosis. One of the significant features of focal segmental glomerulosclerosis is the occurrence of lipid within the mesangium as small droplets, within large vacuoles, or in lipid-laden macrophages. These histological features have many analogies to atherosclerotic lesions. Foam cells are also a key feature in the formation of the fatty streak in atherosclerosis, along with smooth muscle cell proliferation. It was these characteristics, together with coincident hyperlipidaemia, hypertension, inflammation, risk factors for atherosclerosis, which led Moorhead *et al* (1997) to put forward the idea of ' glomerular atherosclerosis'.

It is becoming apparent that both alloantigen-dependent and independent factors may co-ordinate their impact through activation of the same cellular and molecular mediators of tissue injury, remodelling and repair. The vascular lesions of chronic graft rejection are thought to progress through cyclical episodes of endothelial injury followed by inflammation and repair. This process involves vascular smooth muscle cell and fibroblast proliferation and extra cellular matrix deposition, all of which are similar to naturally occurring atherosclerosis (Varghese 1999). Therefore, we sought to study the influence of cytokine polymorphism in a clearly defined model of atherosclerotic renal disease such as atherosclerotic RAS. This, we hope, would reinforce the influence of inflammation in the process of chronic graft dysfunction and atherosclerosis.

1.4. HYPOTHESIS & AIMS

Cytokines have been shown to influence immune and inflammatory responses. Single nucleotide and microsatellite polymorphisms have been described within the genes. Polymorphisms in the 5' promoter and regulatory region can alter transcription of these genes and the polymorphisms may also be in linkage disequilibrium with other regulatory sites. There are several reports describing associations between these polymorphisms and differential cytokine production, disease susceptibility or severity and transplantation outcome.

HYPOTHESIS:

Polymorphisms within the cytokine genes may alter the production of cytokines and influence the outcome of transplantation and development of atherosclerosis.

AIMS:

This study was undertaken to investigate the influence of polymorphisms in cytokine (TNF, IL-2, IL-6, IL-10, TGF-β1, and IFN-γ) genes on acute allograft rejection and chronic allograft dysfunction after renal transplantation. Due to the analogy between atherosclerosis and chronic rejection, a separate study was carried out to assess the influence of cytokine (TNF, IL-2, IL-6, IL-10, and IFN-γ) gene polymorphisms on atherosclerotic renal artery stenosis. These cytokines were chosen on the basis of previous studies showing their influence on the inflammatory processes. The effect of these cytokine gene polymorphisms on cytokine production was also assessed.

CHAPTER- 2

MATERIALS AND METHODS

2.1. DNA EXTRACTION FROM WHOLE BLOOD:

2.1.1. General reagents:

1M Tris-HCl (1L)

Tris: 121.1g

dH_2O: 800ml

adjusted to pH 8.2 with concentrated HCl

made up to 1000ml with dH_2O

200mM EDTA(disodium ethylene diamine tetraacetate) (1L)

Na_2EDTA: 74.4g

dH_2O: 800ml

adjusted to pH 8.0 with 5M NaOH

made up to 1000ml with dH_2O

20XSSC (salt sodium citrate) (1L)

sodium chloride: 175.2g(3M)

sodium citrate: 88.2g(0.3M)

made up to 1000ml with dH_2O

10% SDS (sodium dodecyl sulphate) (1L)

SDS: 100g

made up to 1000ml with dH_2O

2.1.2. Reagents used for DNA extraction

10X Lysis buffer (1L)

ammonium chloride: 82g(1.55 M)

1M potassium hydrogen carbonate: 100ml(1M)

EDTA: 50ml 200mM EDTA (0.5M)

adjusted to pH 7.4

made up to 1000ml with dH_2O

NLB (nuclear lysis buffer) (1L)

sodium chloride: 23.37g(0.4M)

dH_2O: 900ml

1M Tris-HCl (pH8.2): 10ml(10mM)

200mM Na_2EDTA (pH 8.0):10ml(2mM)

made up to 1000ml with dH_2O

NLB/SDS

NLB: 300ml

10% SDS: 20ml

mixed and stored at room temperature

6M NaCl(1L)

sodium chloride: 350.55g

made up to 1000ml with dH_2O.

2.1.3. Procedure

2 ml of whole blood was transferred into a labelled PS/PE 15ml tube and centrifuged for 10 minutes at 1430g. The plasma was removed and 10ml of red cell lysis buffer (RCLB) was added to the cell layer and mixed for 10 minutes. Nuclei were collected by centrifugation at 1430g in a PE 'Ficoll'tube. Pellets were resuspended in 1.5 ml of NLB/SDS and 400 µl 6M NaCl and mixed well. 2 ml chloroform was added to the samples. After mixing for 5 mins and centrifugation at 1430g for 10 minutes, the upper aqueous layer was removed in to clean PS tube and 8 ml 100% ethanol added to precipitate the DNA. The DNA was washed with 70% ethanol and allowed to air dry for 5 to 10 minutes.

The DNA was redissolved in 250µl of dH_2O by mixing on a tube rotator overnight.

2.2. POLYMERASE CHAIN REACTION (PCR)

2.2.1. Reagents used for PCR

Dinucleotide triphosphates (dNTPs) (MBI Fermentas)

For (2mM) stock

20µl dATP(100mmol/l)

20µl dCTP(100mmol/l)

20µl dGTP(100mmol/l)

20µl dTTP(100mmol/l)

10xPCR buffer (MBI Fermentas, Lithuania)

TDMH

25mM $MgCl_2$:	11.04 ml
10 X ammonium sulphate buffer:	13.36 ml
100mM dATP :	336 µl
100mM dCTP :	336 µl
100mM dGTP :	336 µl
100mM dTTP :	336 µl
8.33% sucrose solution:	26.48 ml

2.2.2. Reagents used for agarose gel electrophoresis

10XTBE (Tris borate electrophoresis) buffer (5L)

Tris: 540g (0.89M)

Boric acid: 75g (0.89M)

EDTA: 500ml 200mM EDTA (0.02M)

made up to 5000ml with dH_2O

Agarose gel

2% agarose gel: 2g agarose dissolved in 100ml 1XTBE buffer

5µl ethidium bromide

Orange G

Glycerol: 500ml

Orange G: 1.25g

Made up to 1litre with 1X TBE buffer

2.2.3. Procedure

Polymerase Chain Reaction and conditions

All PCRs were carried out on a Perkin Elmer 9600 thermal cycler as follows unless specifically stated otherwise. 30µl reaction mixtures were prepared containing 3µl test DNA (at a concentration of 200ng/ml), 3µl 10x PCR buffer, 3µl 25mM $MgCl_2$, 3µl 2mM dNTPs, 3µl 5µM of each primer,1U Taq Polymerase enzyme and 12µl dH_2O.

The cyclic conditions for each PCR were as follows: 95°C for 5minutes, 30 cycles of 95°C for 30 seconds, annealing temperature (56°C) for 30 seconds, 72°C for 1 minute and 72°C for 5minutes.

The detection of PCR Product

The presence of amplified product was assessed following each set of PCR reactions by monitoring on a 2% agarose gel, 10µl of PCR product was mixed with 5µl orange G buffer prior to loading in to gel.

2.3. DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISM IN THE TNF-α GENE PROMOTER USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP).

2.3.1. Method

Polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP).

Restriction endonuclease (*Nco-I*) enzyme was used to cut the polymorphic site of TNF-α promoter (-330) region.

PCR product, 10µl was digested with *Nco-I* restiction endonuclease for 17 hours at 37°C (optimal temperature of the enzyme), and the digested product confirmed two alleles. Allele1 gives two fragments of 87 bp and 20 bp, allele 2 a single 107-bp fragment. These were separated by polyacrylamide gel electrophoresis, the bands were visualised by silver staining the gel, and the position of bands was used to determine the genotype.

2.3.2. Reagents used for polyacrylamide gel electrophoresis

8% 49:1 acrylamide:bisacrylamide gel (75ml)

40% 49:1 acrylamide: bisacrylamide (BDH) stock:	15ml
dH_2O:	52ml
10XTBE:	7.5ml
10% ammonium persulphate (APS):	500µl
TEMED:	60µl

5Xloading buffer(10ml)

10XTBE:	5ml
glycerol:	4.9ml
10%SDS:	0.1ml

bromophenol blue added to colour

2.3.3. Reagents used for $AgNO_3$ staining

Fixing solution(1L)

ethanol: 100ml

glacial acetic acid: 5ml

made up to 1000ml with dH_2O

$AgNO_3$ staining solution (10X stock, 500ml)

silver nitrate: 10g

dH_2O: 500ml

Working solution prepared by 1/10 dilution in dH_2O

Developing solution (300ml)

sodium hydroxide: 4.5g

formaldehyde: 1.2ml of 37% stock

made up to 300mlwith dH_2O

Stop solution (10X stock, 1L)

sodium carbonate: 75g(7.5%)

made up to 1000ml with dH_2O

Working solution made up by 1/10 dilution in dH_2O

2.3.4. Procedure

PCR reagents and conditions

DNA was extracted as described previously in section 2.1.3. PCRs were carried out on a Perkin Elmer 9600 thermal cycler using the following reagents. 30µl reaction mixtures were prepared containing 3µl test DNA (at a concentation of 200ng/ml), 3µl 10x PCR buffer, 2µl 25mM $MgCl_2$, 3µl 2mM

dNTPs, 3µl 5µM of each primer (originally decribed by Wilson *et al* in 1992) (table 2.1), 1U Taq polymerase enzyme and 13µl dH_2O.

The cycling conditions for each PCR were as follows: 95°C for 5minutes, 30 cycles of 95°C for 30 seconds, annealing temperature (59°C) for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes.

Primers and probes

Primer pairs were designed to minimise primer dimer formation and 3' looping and to ensure identical annealing temperature (Tm). These were estimated using the formula Tm= 4 (G+C)+2(A+T).

Table.2.1 Nucleotide sequences of primers used in TNF-α (-330) PCR-RFLP.

Primer Pairs	Sequence	*T*(°C)	Region amplified
TNF-α sense	5'-AGGCAATAGGTTTTGAGGGC-3'	59	-224 to -331
TNF-α antisense	5'-TCCTCCCTGCTCCGATTCCG-3'		

Restiction enzyme digestion of PCR product

10 µl of specific amplified products were digested with 1 unit of restriction endonuclease enzyme (*NcoI*) for 17 hours at 37°C.

Polyacrylamide gel electrophoresis

The polyacrylamide gel was prepared and injected without any bubbles between two 32cm X 17cm glass plates separated with spacers and an 18 well comb was placed on the top end. The gel was left to set for 2 hours.

The digested products were mixed with 2 µl of 5xTris-borate electrophoresis loading buffer and loaded on to the wells on the polyacrylamide gel. A ladder was loaded to the last well to identify the size of the fragment. The loaded samples were run using 1xTris-borate electrophoresis loading buffer at 800 volts, 40 mA, 27 watts for 2 hours.

Fixing

Following electrophoresis, the gel was removed from the plates and fixed in 500 ml of fixing solution for 10 minutes.

Silver nitrate staining

After fixing, the gel was stained by placing in 500 ml of 0.2 % silver nitrate solution with constant shaking for 15 minutes.

Developing

Following staining, the gel was washed with dH_2O twice to remove excess silver nitrate. Then the gel was placed in 500 ml of developing solution until the bands were seen clearly. An 87bp band corresponds to the allele TNF1, whilst a band of 107bp corresponds to the TNF2.

Stopping

Following developing the bands, the reaction was stopped by adding stop solution.

Drying

The gel was carefully placed between wet cellophane films and allowed to dry.

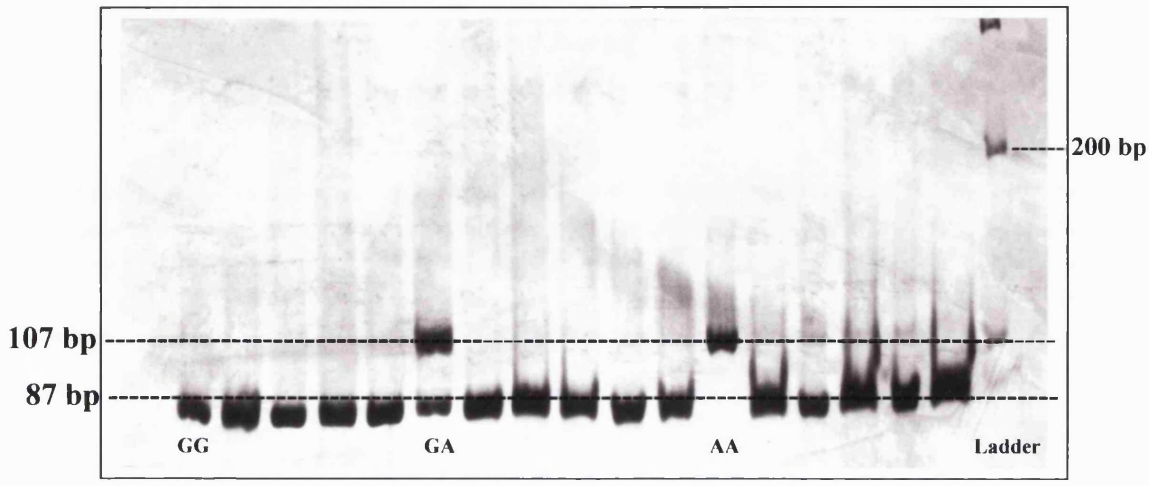


Figure.2.1 Silver nitrate stained polyacrylamide gel showing the digestion products for the TNF-α(-308) PCR-RFLP. The restriction enzyme NcoI produced an 87bp product when a guanine is present at position –308. The presence of adenine prevents the cleavage by the enzyme, leaving the 107bp PCR product intact. Therefore, a lane with an 87bp band corresponds to the GG genotype; a lane with an 107bp band corresponds to the AA genotype; and, heterozygosity (GA) was identified by the presence of both bands.

2.4. SEQUENCE SPECIFIC OLIGONUCLEOTIDE PROBING (SSOP) ANALYSIS OF THE THREE SINGLE BASE PAIR POLYMORPHISMS IN IL-10 PROMOTER

2.4.1. Method

Polymerised chain reaction (PCR) followed by Sequence specific oligonucleotide probing (SSOP). Two 5' biotinylated oligonucleotide probes were designed to detect each polymorphism by a dot blot technique

1a- IL-10 1*G 1b- IL-10 1*A

2a- IL-10 2*C 2b- IL-10 2*T

3a- IL-10 3*C 3b- IL-10 3*A

PCR product, 2µl was blotted on to Hybond N^+ nylon transfer membrane, and probes were hybridised at 42.5°C. Probes 1a and 1b were washed at 52 °C and probes 2a, 2b, 3a and 3b were washed at 53.5°C. A streptavidin-horseradish peroxidase conjugate was used for the detection of bound probe.

2.4.2. Reagents:

Denaturing solution (1L)

sodium hydroxide: 20g(0.5M)

sodium chloride: 87.7g(1.5M)

made up to 1000ml with dH_2O

Neutralising solution (1L)

sodium chloride: 87.7g(1.5M)

Tris: 60.6g(0.5M)

made up to 1000ml with dH_2O

Hybridisation buffer (250ml)

SSC: 62.5ml 20XSSC (5XSSC)

blocking agent (milk powder): 1.25g(0.5%)

N-laurylsarcosine: 0.25g(0.1%)

SDS: 0.5ml 10%SDS (0.02%)

Wash buffer (1L)

SSC: 250ml 20XSSC (5XSSC)

SDS: 10ml 10% SDS (0.1%)

made up to 1000ml with dH_2O

Stringency wash buffer (1L)

SSC: 50ml 20XSSC (1XSSC)

SDS: 10ml 10%SDS (0.1%)

made up to 1000ml with dH_2O

Buffer 1 (1L)

sodium chloride: 8.77g (0.15M)

Tris: 12.11g (0.1M)

dH_2O: 800ml

adjusted to pH 7.5

made up to 1000ml with dH_2O

Buffer 2 (1L)

sodium chloride: 23.38g (0.4M)

Tris: 12.11g (0.1M)

dH_2O: 800ml

adjusted to pH 7.5

made up to 1000ml with dH_2O

Blocking solution (200ml)

blocking agent (milk powder): 1g

buffer 1: 200ml

2.4.3. Procedure

PCR reagents and conditions

DNA was extracted as described previously in section 2.1.3. PCRs were carried out on a Perkin Elmer 9600 thermal cycler using the following reagents. 30µl reaction mixtures were prepared containing 3µl test DNA (at a

concentration. of 200ng/ml), 3µl 10x PCR buffer, 3µl 25mM $MgCl_2$, 3µl 2mM dNTPs, 3µl of each primer (5µM), 1U Taq Polymerase enzyme and 12µl dH_2O. The cycling conditions for each PCR were as follows: 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, annealing temperature (56°C) for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes.

Dot blotting of PCR products

2µl of PCR product was dotted on to Hybond N^+ nylon membrane and denatured in denaturing solution for 5 minutes at room temperature. Following 1 minute in neutralizing solution, the membrane filters were baked at 70° C for 10 minutes and crosslinked in a UV-Stratalinker for 30 seconds.

Probe hybridization

Membranes were pre-hybridized at 42.5°C for 30 minutes by continual mixing in 10ml hybridization buffer.

Washing filters

Following hybridization, filters were washed twice for 5 minutes at room temperature in wash solution. 30 minutes stringent wash was then performed at the specific temperature of the probes.

Enhanced chemiluminescence (ECL) detection

Membranes were washed in buffer 1, followed by incubation for 30 minutes in blocking solution, followed by incubation with buffer 1 containing 6µl streptavidin/horseradish peroxidase conjugate (Amersham). Following two washes in buffer 2, membranes were drained and placed on Saran wrap. Equal volumes of the two detection solutions (Amersham) were then mixed and this solution was applied to the membrane. Following incubation for 1 minute, the excess detection solution was removed and the membranes were wrapped in

cellophane. An autoradiography film was placed over the filters and exposed for 1 minute. When the film was removed for developing a second film was placed in the cassette, which was subsequently removed after a suitable exposure time.

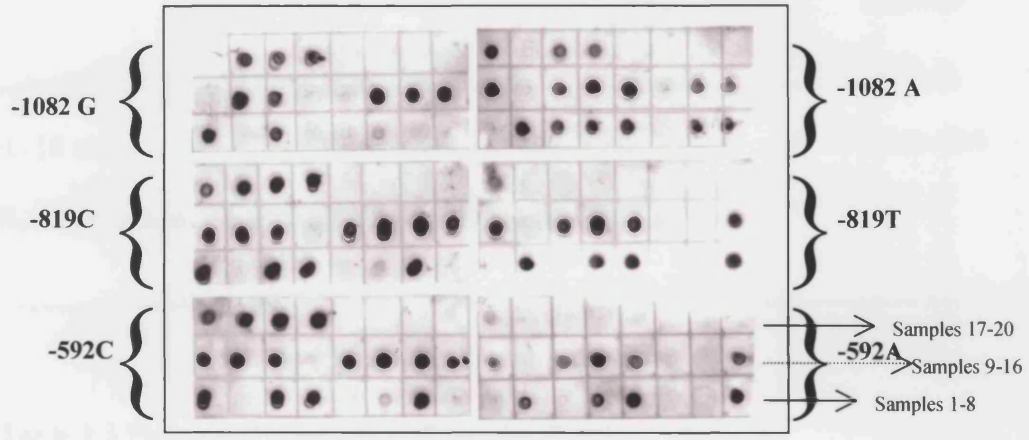


Figure.2.2 Results from PCR-SSOP photographic film for the detection of the IL-10 (-1082, -819, -592) promoter polymorphisms. Six Hybond N^+ nylon membranes were blotted with 20 different PCR amplified test samples, each membrane having a single nucleotide probe specific for –1082G, -1082A, -819C, -818T, -592C or –592A. The presence of the allele with binding to the probe appeared as dark spots.

Primers and probes

Primer pairs were designed to minimise primer dimer formation and 3' looping and to ensure identical annealing temperatures (Tm) which were estimated by the formula Tm= 4 (G+C)+2(A+T).

Table.2.2 Nucleotide sequences of primers used in IL-10 (-1082,-819,-592) PCR-SSOP.

Primer Pairs	Sequence	*T*(°C)	Regions amplified
IL-10 sense	5'-ATCCAAGACAACACTACTA	56	-1115 to-528
IL-10 antisense	5'-TAAATATCCTCAAAGTTCC-3'		

Table.2.3 Probes and stringent wash temperatures

Probe	Sequence	*T*(°C)
Probe 1.-1082*G (coding)-	5'-TCTTTGGGAGGGGGAAGTAG-3'	52
Probe 2.-1082*A (noncoding)-	5'-CTACTTCCCCTTCCCAAAGAA-3'	52
Probe 3.-819*C (coding)-	'-GGTGATCTAACATCTCTGTGC-3'	53.5
Probe 4.-819*T (noncoding)-	5'-GCACAGAGATATTACATCACCT-3'	53.5
Probe 5.-592*C (coding)	-5'-CCCGCCTGTCCTGTAGGAA-3'	53.5
Probe 6.-592*A (noncoding)-	5'-TTCCTACAGTACAGGCGGG-3'	53.5

2.5. ANALYSIS OF MICROSATELLITE POLYMORPHISM IN THE FIRST INTRON OF IFN-γ.

2.5.1. Method

Polymerase chain reaction (PCR) was carried out, followed by capillary electrophoresis on ABI Prism^{TM} 310 genetic analyser using GeneScan analysis. Microsatellites are short tandem repeat sequences (STR) in the DNA. The size of the microsatellite varies depending on the number of nucleotide repeats and this can be detected by electrophoresis (figure 2.3).

In the first intron of IFN-γ, (CA) nucleotide repeats occur 11 to 16 times. Therefore each allele was defined on the basis of number of repeats.

2.5.2. Materials

TAMRA (dye) 500 size standard (PE Applied Biosystems)

Performance Optimised polymer 4(POP4) (PE Applied Biosystems)

310 Genetic Analyzer Capillaries (47cm x 50μm)

1X buffer with EDTA (PE Applied Biosystems)

Deionized formamide

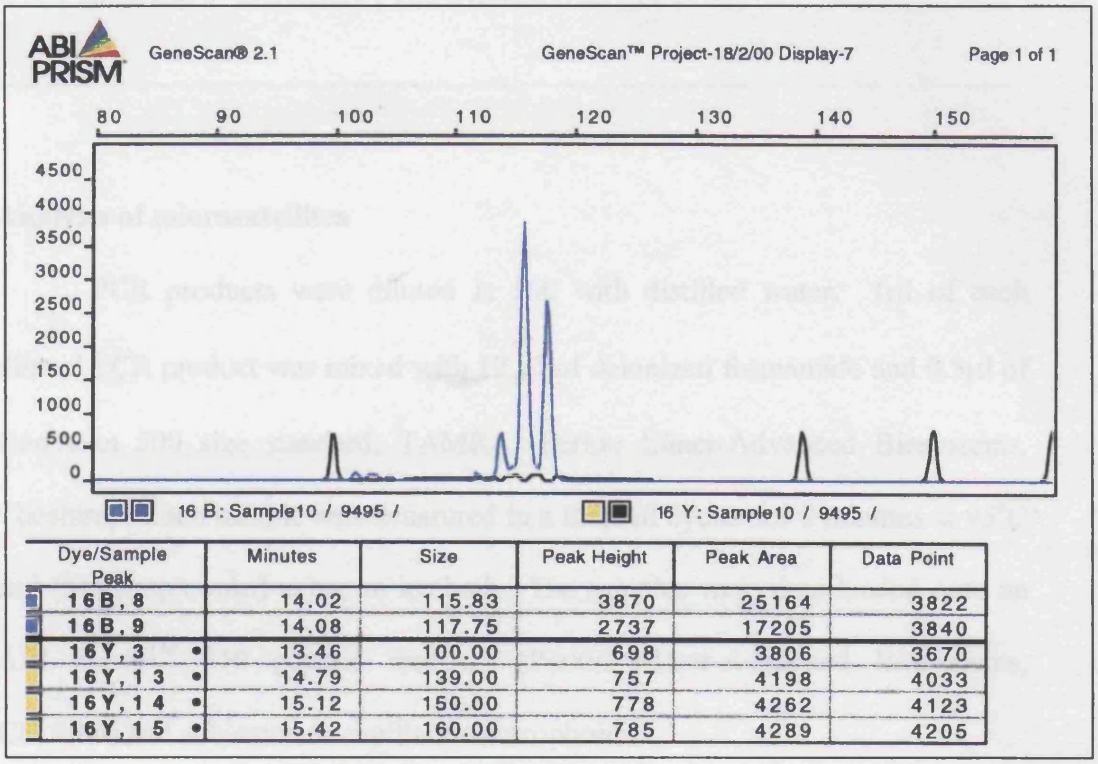
GeneScan analysis software.

2.5.3. Procedure

PCR reagents and conditions

30μl reaction mixtures were prepared containing 3μl test DNA (at a concentration of 200ng/ml), 3μl 10x PCR buffer, 3μl 25mM $MgCl_2$, 3μl 2mM dNTPs , 3μl of IFN-γ (sense) primer (5μM) (fluorescently labelled with FAM), 3μl 5μM of IFN-γ (antisense) primer(Pravica *et al* 1999; table 2.4), 1U Taq polymerase enzyme and 12μl dH_2O.

The cycling conditions for each PCR were as follows: 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, annealing temperature (56°C) for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
16B, 8	14.02	115.83	3870	25164	3822
16B, 9	14.08	117.75	2737	17205	3840
16Y, 3 •	13.46	100.00	698	3806	3670
16Y, 13 •	14.79	139.00	757	4198	4033
16Y, 14 •	15.12	150.00	778	4262	4123
16Y, 15 •	15.42	160.00	785	4289	4205

Figure.2.3. Results from the ABI PRISM^{TM}310 machine for IFN-γ microsatellite analysis. The black peaks show standard sizes from 100bp to 160bp and blue peaks show sample alleles sizes of 116bp and 118bp, which correspond to allele 2 and allele 3.

Table.2.4. Primers and annealing temperature for PCR to amplify the first intronic region of IFN-γ.

Primers	Sequence	*T*(°C)	Region amplified
IFN-γ sense	5'-XGCTGTCATAATAATATTCAGA-3'	56	+812 to+932
IFN-γ antisense	5'-CGAGCTTTAAAAGATAGTTCC-3'		

Analysis of microsatellites

PCR products were diluted 1: 100 with distilled water. 1μl of each diluted PCR product was mixed with 12 μl of deionized formamide and 0.5μl of Genescan 500 size standard, TAMRA (Perkin Elmer-Advanced Biosystems, Cheshire). Each sample was denatured in a thermal cycler for 3 minutes at 95°C and then snap cooled using an ice bath. The samples were then loaded onto an ABI Prism™ 310 genetic analyser (Perkin Elmer-Advanced Biosystems, Cheshire) and subjected to capillary electrophoresis.

2.6. ANALYSIS OF TNF-a AND TNF-d MICROSATELLITE POLYMORPHISMS

The TNF locus is located within the MHC on chromosome 6, which is centromeric to HLA-B and telomeric to the class III region. Several microsatellites have been mapped and characterised in the TNF locus, namely TNF-a, TNF-b, TNF-c, TNF-d and TNF-e. The TNF-a and TNF-b microsatellites are located 3.5 kilobases upstream of the TNF-β gene. TNF-c is

located in the first intron of TNF-β̃. TNF-d and TNF-e microsatellite loci are located 8-10 kilobases downstream of the TNF-α gene (figure 2.4)

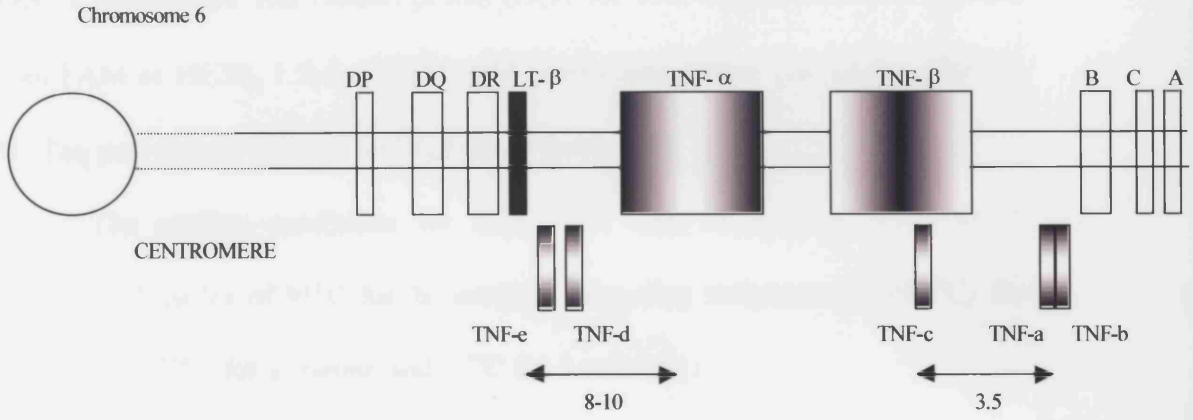


Figure.2.4 Location of TNF microsatellites.

2.6.1. Method

Polymerase chain reaction (PCR) followed by capillary electrophoresis on ABI Prism™ 310 genetic analyser in the same way as those for the IFN-γ microsatellites. TNF-a region, 13 alleles were identified in which (AC) dinucleotide repeats seveal times, ie- $(AC)_6$- $(AC)_{18}$. Alleles were numbered relative to cell line DNAs of known TNF-a type (Udalova et al. 1993).

In the TNF-d region, six alleles were identified with (TC) dinucleotide repeats. Alleles were numbered relative to cell line DNAs of known TNF-d type (Udalova et al. 1993).

2.6.2. PCR reagents and conditions

13μl reaction mixtures were prepared containing 3μl test DNA (at a concentration of 200ng/ml), 150 μM each of dATP, dCTP, dGTP, dTTP (MBI Fermentas, Lithuania), 1.22 μM $MgCl_2$, 9.8 μM ammonium sulphate, 39.6 μM Tris , 1.5μl of 5μM IR2 (sense) primer (IR11 for TNF-d) (fluorescently labelled with FAM or HEX), 1.5μl of 5μM IR4 (antisense) primer (IR 12 for TNF-d), 1U Taq polymerase enzyme and 4μl dH_2O (table 2.5).

The cycling conditions for each PCR were as follows: 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, annealing temperature (59.5°C) for 45 seconds, 72°C for 1 minute and 72°C for 5 minutes.

Primers and annealing temperature for PCR

TNF-a and TNF-d microsatellites were amplified by PCR using primers described by Udalova *et al*, 1993.

Table.2.5 Nucleotide sequences of primers used in TNF-microsatellite PCR.

Primers		Sequence	*T*(°C)
TNF-a	IR2:	5'-**X**GCCTCTAGATTTCATCCAGCCACA-3'	59.5
TNF-a	IR4:	5'- CCTCTCTCCCCTGCAACACACA-3'	
TNF-d	IR11:	5'-**X**AGATCCTTCCCTGTGAGTTCTGCT-3'	59.5
TNF-d	IR12:	5'-CATAGTGGGACTCTGTCTCCAAAG-3'	

Analysis of microsatellites

PCR products were treated in the same way as those for the IFN-γ microsatellites before commencing capillary electrophoresis on the ABI PRISM™ 310 machine.

2.7. ANALYSIS OF IL-10 MICROSATELLITE POLYMORPHISMS

To identify IL-10 G and IL-10 R microsatellite polymorphisms in the upstream of the gene. IL-10 G is a CA repeat that lies upstream of the gene between –1193 and –1151 and is very polymorphic, having up to 16 alleles (Eskdale and Gallagher 1995). IL-10 R lies between –4004 and –3978 in the 5' flanking region (Eskdale et al. 1996).

2.7.1. Method

Polymerase chain reaction (PCR) followed by capillary electrophoresis on ABI Prism™ 310 genetic analyser in the same way as those for the IFN-γ microsatellites.

Alleles of the IL-10 microsatellites were detected by electrophoresis of fluorescently labelled PCR products through an ABI Prism™ 310 genetic analyser (Applied Biosystems). DNA samples were set up in 24μl PCR reactions containing 1.25μM of a forward and a reverse primer and were originally described by Eskdale *et al* in 1995 (see table.2.6), 3.125μM dNTPs, 3μl of 10x PCR buffer (MBI Fermentas) and 1U Taq polymerase. The reaction conditions were 95°C for 5 minutes, 30 cycles of 95°C for 15 seconds, annealing temperature (65°C) for 1 minute, 72°C for 1 minute and 72°C for 5 minutes. Following PCR the samples were set up to run on the ABI Prism™ 310 using the same procedure as described for the IFN-γ.

Table.2.6 Nucleotide sequences of primers used in IL-10 microsatellite PCR

Primers	Sequence
IL-10-1G:	d(XGTCCTTCCCCAGGTAGAGCAACACTCC)
IL-10-2G:	d(CTCCCAAAGAAGCCTTAGTAGTGTTG)
IL-10-3R:	d(XCCCTCCAAAATCTATTTGCATAAG)
IL-10-4R:	d(CTCCGCCCAGTAAGTTTCATCAC)

2.8. CYTOKINE GENOTYPING USING PCR-SSP

Single nucleotide polymorphisms at -1082, -819 and -592 in the IL-10 promoter; at -308 in the TNF-α promoter; at -330 in the IL-2 promoter; at -174 in the IL-6 and codon 10, codon 25 in the first exon of the TGF-β1 gene were analysed using sequence specific primers in the PCR. DNA was amplified in a 10 μl reaction, containing 3μl of DNA (50- 200ng), 3 μl of TDMH buffer, 2 μl of specific primer mix (1μl each of 5 μM generic primer and one of the two allele specific primers), 2 μl of internal control primer mix (1μl each of 5 μM internal control primer 1 and primer 2) and 0.25 units of Taq polymerase enzyme. Internal control primers were used to check for successful amplification and these primers amplify a human growth hormone sequence. All these primers were previously decribed by Perrey *et al* (Perrey C et al. 1999) (see table 2.7).

The cycling conditions for each PCR were as follows: 95°C for 1minute followed by 10 cycles of 95°C for 15 seconds, 65°C for 50 seconds 72°C for 40

seconds followed by 30 cycles of 95°C for 20 seconds, annealing temperature (59°C) for 50 seconds, 72°C for 50 seconds.

Table.2.7 Nucleotide sequences of primers used in cytokine PCR-SSP.

Primer	**sequence**
IL-10 (-1082 generic antisense)	5'-CAGTGCCAACTGAGAATTTGG-3'
IL-10 (-1082G specific sense)	5'-CTACTAAGGCTTCTTTGGGAG-3'
IL-10 (-1082A specific sense)	5'-ACTACTAAGGCTTCTTTGGGAA-3'
Amplified product size = 258bp	
IL-10 (-819/-592* generic antisense)	5'-AGGATGTGTTCCAGGCTCCT-3'
IL-10 (-819C/-592C* specific sense)	5'-CCCTTGTACAGGTGATGTAAC-3'
IL-10 (-819T/-592A* specific sense)	5'-ACCCTTTGACAGGTGATGTAAT-3'
Amplified product size = 233bp	
TNF-α (-308 generic antisense)	5'-TCTCGGTTTCTTCTCCATCG-3'
TNF-α (-308G specific sense)	5'-ATAGGTTTTGAGGGGCATGG-3'
TNF-α (-308A specific sense)	5'-AATAGGTTTTGAGGGGCATGA-3'
Amplified product size = 184bp	
TGF-β1 (C10 generic sense)	5'-TCCGTGGGATACTGAGACAC-3'
TGF-β1 (C10C specific antisense)	5'-GCAGCGGTAGCAGCAGCG-3'
TGF-β1 (C10T specific antisense)	5'-AGCAGCGGTAGCAGCAGCA-3'
Amplified product size = 241bp	
TGF-β1 (C25 generic antisense)	5'-GGCTCCGGTTCTGCACTC-3'
TGF-β1 (C25G specific sense)	5'-GTGCTGACGCCTGGCCG-3'
TGF-β1 (C25C specific sense)	5'-GTGCTGACGCCTGGCCC-3'
Amplified product size =233bp	
IL-6 (generic antisense)	5'-TCGTGCATGACTTCAGCTTTA-3'
IL-6 (G specific)	5'-AATGTGACGTCCTTTAGCATC-3'
IL-6 (C specific)	5'-AATGTGACGTCCTTTAGCATG-3'
Amplified product size = 176bp	
IL-2 (generic sense)	5'-CCAAAGACTGACTGAATGGATG-3'
IL-2 (Tspecific)	5'-ATTCACATGTTCAGTGTAGTATTAT-3'
IL-2 (G specific)	5'-ATTCACATGTTCAGTGTAGTATTAG-3'
Amplified product size = 176bp	
Internal control primer 1	5'-GCCTTCCCAACCATTCCCTTA-3'
Internal control primer 2	5'-TCACGGATTTCTGTTGTGTTTC-3'
Amplified product size = 429bp	

* IL-10 –592 polymorphism is in linkage disequilibrium with –819 polymorphism. Allele C at –592 is always present when at position –819 is allele C and allele A at –592 is always present when at position –819 is allele T.

2.9. INTERNATIONAL CYTOKINE WORKSHOP STUDY.

Fifty control DNA samples supplied from 13th International Workshop Cell and Gene Bank, Seattle were genotyped for IL-2 (-330), IL-6 (-174), IL-10 (-1082, -819, -592), TGF-β1 (codon 10 and codon 25), and TNF-α (-308) using PCR-SSP cytokine typing tray kit (University of Heidelberg, Germany) as a part of the international cytokine workshop-phase I study. These DNA samples were also typed using previously described PCR-SSP methods (section 2.8) and the results were compared to validate the typing methods used in this thesis.

In the phase II study, 15 Quality Control DNAs from the 13th International Workshop Cell and Gene Bank, Seattle were genotyped for IL-2 (-330), IL-6 (-174), IL-10 (-1082, -819, -592), TGF-β1 (codon 10 and codon 25), and TNF-α. (-308). using a PCR-SSP cytokine typing kit (University of Heidelberg, Germany). These DNAs were also genotyped using previously described PCR-SSP methods as well as other typing methods described in this thesis to confirm the results. Each of the above cytokine polymorphisms were analysed and compared with the methods used in this thesis for the reliability of the genotyping results.

2.10. MEASUREMENT OF CYTOKINE PRODUCTION

Culture medium with 10% human (blood group AB) serum (100ml)

RPMI 1640:	88ml
Heat inactivated human AB serum:	10ml
L-glutamine (2mM):	1ml
Antibiotics (100 units/ml penicillin + 100μg/ml streptomycin):	1ml

Isolation of PBMCs

Mononuclear cells were separated from peripheral blood samples using density gradient centrifugation (Lymphoprep, Nycomed Pharma As, Oslo, Norway). Blood from normal healthy volunteers was double diluted with sterile Dulbecco's phosphate buffered saline (PBS). Two volumes of diluted blood was layered slowly on one volume of lymphoprep and centrifuged at 514g for 20 minutes without braking. The interface, which contains PBMCs, was carefully collected and washed once with sterile PBS at 228g for 15 minutes. Two further washes were carried out with the culture medium at 200g for 10 minutes. The PBMCs were resuspended in culture medium and the differential viable lymphocyte counts were quantified after staining of this preparation with 0.2% trypan blue. The cells were used immediately for experiments.

Measurement of IL-2, TGF-β, IL-6, IL-10 and TNF-α by *in vitro* stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from normal healthy blood donors. From each individual, $1x10^6$ cells/ml of cell culture medium containing 10% heat inactivated human AB serum in RPMI 1640 supplemented with L-glutamine (2mM), 100 units/ml penicillin and 100μg/ml streptamycin were cultured at 37°C in 5% CO_2 for 24 hours with and without various stimuli. Dose response and time course experiments were carried out in order to assess the optimal doses and time point for various stimuli such as LPS and PHA. The controls were treated in the same way without any addition of stimuli. Supernatants were collected after 24 hours and measured the cytokine production was measured using enzyme linked immunosorbent assays (ELISA).

Measurement of IL-2, IFN-γ and TGF-β1 by *in vitro* stimulation of PBMCs

PBMCs were cultured in cell culture medium containing 10% heat inactivated human AB serum in RPMI 1640 supplemented with L-glutamine (2mM), 100 units/ml penicillin and 100μg/ml streptamycin. Dose response and time course experiments were carried out in order to assess the optimal doses of phorbol 12- myristate 13-acetate (PMA) and ionomycin (both Sigma-Aldrich Company Ltd.) and an appropriate time point for supernatant collection. Cultures containing $1x10^6$ cells/ml culture medium were stimulated with 5ng/ml PMA and 1μg/ml of ionomycin and incubated at 37°C in 5% CO_2. Culture supernatants were collected at 48 hours and cytokine levels were measured using ELISA.

Standardisation of one way-mixed lymphocyte culture (MLC)

It was necessary to standardise MLC for the reliability of results. For this reason, the critical steps were evaluated. Firstly, we tested that the irradiated stimulator cells were not able to proliferate or respond to any mitogen stimuli. Experiments were carried out to find the ratio of responder cells to stimulator cells, the best incubation period and appropriate cell density in order to assess the optimal condition. Each experiment was repeated with five individuals.

Measurement of cytokines by *in vitro* mixed lymphocyte culture

PBMCs were isolated as described above. 2.5 $x10^6$cells/ml: $2.5x10^6$cells/ml of responders and stimulators in cell culture medium containing 10% heat inactivated human AB serum in RPMI 1640 supplemented with L-glutamine (2mM), 100 units/ml penicillin and 100μg/ml streptamycin, were

incubated at 37°C in 5% CO_2 for 48 hours. 2.5 x10^6cells/ml of responder cells alone were incubated as controls at 37°C in 5% CO_2 for 48 hours. The supernatants were collected and each cytokine was measured using ELISA.

2.11. ENZYME LINKED IMMUNOSORBENT ASSAYS

2.11.1. Reagents:

Wash buffer: PBS containing 0.05 % Tween 20

PBS: 500ml

Tween 20: 250µl

pH : 7.2-7.4

Block buffer: PBS containing 1 % BSA (5 % Tween 20 for TGF-β), 5 % sucrose and 0.05 % NaN_3

BSA: 5g (for TGF-β assay, 25 ml of Tween 20 instead of BSA was used)

Sucrose: 25g

NaN_3: 0.25g

Made up to 500ml with PBS

General reagent diluent: Tris buffered saline containing 0.05 % Tween 20 and 0.1 % BSA

BSA: 0.5g

Tween 20: 0.25ml

TRIZMA: 1.211g

NaCl: 4.383g

Made up to 500 ml with distilled H_20, 0.2 µm filtered and pH adjusted to 7.2-7.4.

Reagent Diluent for IL-10 assay: (1 % BSA in PBS)

BSA: 5g

PBS: 500ml

pH : adjusted to 7.3, filtered through 0.2 μm filter

Reagent Diluent for TGF-βassay (500ml): 1.4% delipidised bovine serum and 0.05% Tween 20 in PBS

Delipidised bovine serum: 7g

Tween 20: 250μl

Made up to 500 ml, filtered through 0.2 μm filter and pH is adjusted to 7.3

1.2 M NaOH/0.5 M HEPES (100ml):

10M NaOH: 12ml

HEPES: 11.9g

Made up to 100ml

Substrate solution:

1:1mixture of colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine) (R&D systems)

Stop solution:

2 N H_2SO_4

2.11.2. General ELISA Protocol

Plate preparation (DuoSet ELISA development system, R&D systems)

The capture antibody (monoclonal mouse anti human cytokine) was diluted in PBS without carrier protein to the working concentration (table 2.8). 100μl of diluted capture antibody was added to a 96 well microplate and coated by incubating the plate at room temperature overnight. Each well was aspirated and washed three times with 300μl wash buffer using an automatic washer. The

plate was blocked by adding 300µl of block buffer to each well and incubated at room temperature for minimum of one hour. Then the plate was aspirated and washed three times with the automatic washer and dried under vacuum and stored at 4 ^{0}C until used.

Assay procedure

100µl of sample or standard (recombinant cytokine) in reagent diluent was added to each well and incubated for 2 hours at room temperature after sealing with an adhesive strip. Then the plate was aspirated and washed three times using an automatic washer. 100µl of diluted detection antibody (table 2.8) in reagent diluent was added and covered with a new adhesive strip and incubated for another 2 hours at room temperature. The plate was aspirated and washed as before. 100µl of working solution of streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature in the dark followed by three washes. Then 100µl of substrate solution was added to each well and incubated for another 20 minutes avoiding direct light. The reaction was stopped by adding 50µl of stop solution to each well and the optical density of each well was measured using a microplate reader (Bio-rad model 3550) set at 450nm with a reference wavelength of 540nm.

Activation of samples for the detection of TGF-β1

In order to measure TGF-β1, it must firstly be converted from its inactive form to its active form so that the anti- TGF-β1 antibody used in the ELISA is able to bind to its site. To activate latent TGF-β1 to immunoreactive TGF-β1,100µl culture supernatants were treated with 20µl of IM HCl, mixed well and incubated for 10 minutes at room temperature. Then the samples were neutralised by adding 30µl of 1.2M NaOH/0.5 M HEPES.

Detection of IL-2, IL-6, IL-10 TNF-α, IFN-γ, and TGF-β by ELISA

Levels of cytokines (IL-2, IL-6, IL-10 TNF-α, IFN-γ, and TGF-β) in the culture supernatant were measured using a sandwich ELISA kit called DuoSet ELISA Development system (R&D systems, Abingdon, Oxon,UK).

Table:2.8. Concentrations of cytokine standards and antibodies used in ELISA.

Cytokine	Capture Ab	Detection Ab	Standard serial dilution
IL-2	4μg/ml	50 ng/ml	15.625pg/ml to 1000pg/ml
IL-6	2μg/ml	200ng/ml	4.6875pg/ml to 300pg/ml
IL-10	4μg/ml	500ng/ml	62.5pg/ml to 4000pg/ml
TNF-α	4μg/ml	300ng/ml	15.625pg/ml to 1000pg/ml
IFN-γ	4μg/ml	100ng/ml	15.625pg/ml to 1000pg/ml
TGF-β1	2μg/ml	300ng/ml	31.25pg/ml to 2000pg/ml

2.12. RNA ISOLATION

2.12.1. Reagents

RNase-Free water:

ddH_2O was treated by 0.1% diethyl pyrocarbonate (DEPC) (Sigma, Dorset, UK) at 37^{0}C overnight and then autoclaved.

0.75M sodium citrate (pH 7.0):

citrate-3 Na (Sigma, Dorset, UK): 11.029g

Dissolved in dH_2O and made up to 50ml.

Add 0.2 ml of DEPC and stir for 2 hours and then autoclaved.

10% Sarcosyl:

N-lauroylsarcosine sodium salt (Sigma, Dorset, UK): 10g

Dissolved in dH_2O and made up to 100ml.

Add 0.2 ml of DEPC and stir for 2 hours and then autoclaved.

Denaturation solution:

Guanidium thiocyanate (GTC): 250g

0.75M sodium citrate (pH 7.0): 17.6ml

10% sarcosyl: 26.4ml

RNase-free water: 293ml

The mixture was stirred at 65°C until GTC was mixed.

Before use, add 2-mercaptoethanol (Sigma, Dorset, UK) 0.36 ml per 50 ml solution.

2M sodium acetate (pH 4.0):

Sodium acetate (Sigma, Dorset, UK): 27.216g

Dissolved in dH_2O and made up to 100ml, and the pH was adjusted to 4.0 using glacial acetic acid and then filtered.

0.5M EDTA:

Disodium ethylene diamine tetra acetate.$2H_2O$ (Sigma, Dorset, UK): 186.1g

Dissolved by adding 800ml dH_2O and the pH was adjusted to 8.0 with NaOH (about 20g NaOH pellets). Then sterilised by autoclaving.

3M sodium citrate (pH 5.2)

Sodium acetate.$3H_2O$: 408.1g

Dissolved in dH_2O and made up to 1 litre. pH was adjusted to 5.2 using glacial acetic acid and then autoclaved.

Phenol:Chloroform:Isoamylalcohol (25:24:1, pH 4.7) (Sigma, Dorset, UK)

Isopropanol (Sigma, Dorset, UK)

75% ice-cold ethanol RNase free (Sigma, Dorset, UK)

Creating a ribonuclease free environment

Ribonucleases are difficult to inactivate. Therefore, care should be taken to avoid inadvertently introducing RNase activity in to the isolation procedure. The following precautions will help to prevent accidental contamination of samples. The more common sources of RNase contamination are the user's hands, bacteria and moulds that may be present on airborne dust particles. Therefore, gloves were worn at all times and proper microbiological sterile techniques used when handling the reagents. Sterile plastic disposable plasticware was used for handling RNA. These materials are generally RNase free and thus do not require pre-treatment to inactivate RNase. Non disposable glassware and plasticware was treated before use to ensure that it is RNase free. Glassware was baked at 200°C overnight. Plasticware was thoroughly rinsed with 0.1M NaOH, 1mM EDTA and then with nuclease free water. RNase free materials were used for weighing chemicals. Solutions were treated with addition of diethyl pyrocarbonate (DEPC) to 0.1% overnight at room temperature and then autoclaved for 30 minutes to remove any traces of DEPC. Tris buffers were prepared by using a container of Tris designated only for RNA isolations and DEPC treated water that had been autoclaved.

Total RNA isolation and purification

Cultured PBMCs from tissue cuture plates were collected and pelleted in to each Eppendorfs and then washed once in ice cold phosphate buffered saline. The cell pellet (about 1x 10^6) was lysed in 600µl of ice cold denaturation

solution. Cell lysates were sheared six times using a 21-gauge needle and 1ml syringe. 60µl of 2M sodium acetate (pH 4.0) was added to the lysate and mixed thoroughly by inverting the tube four to five times. 1 volume of phenol: chloroform: isoamylalcohol was added. The mixture was vortexed for 10 seconds and then centrifuged at 12,000rpm for 10 minutes at 4°C. The aqueous phase was carefully transferred to a fresh eppendorf tube, taking care not to touch the interface. An equal volume of isopropanol was added to this and precipitated at -20 ° C for at least 2 hours.

The crude RNA pellet was recovered by centrifugation at 12,000 rpm for 20 minutes at 4 ° C and the pellet was washed with 1ml of 75% ice cold ethanol. The RNA was recovered by centrifugation at 12,000 rpm for 10 minutes at 4 ° C. The pellet was dried in air and resuspended in 150µl RNase free water. 10µl of 3M sodium acetate (pH5.2) was added and mixed thoroughly by inverting the tube four to five times. 150µl (1 volume) of phenol: chloroform: isoamylalcohol was added, vortexed and centrifuged at 12,000 rpm for 10 minutes at 4 ° C. The aqueous phase was carefully transferred to a fresh tube and then two volumes of 100% ethanol was added, mixed and precipitated at -20 ° C at least 2 hours or preferably freezing at -20 ° C by overnight. The RNA pellet was recovered by centrifugation at 12,000 rpm for 10 minutes at 4 ° C and the supernatant was discarded. The RNA pellet was allowed to air dry before dissolving in 10µl to 20µl of RNase free water by vortexing for approximately 5 minutes.

The concentration of RNA was measured using GeneQuant DNA/RNA calculater. The samples were diluted 1:250 by adding 2µl of dissolved RNA to 498µl of RNase free water in a clean eppendorf. A reference of RNase free water was used to zero the spectrophotometer. A GeneQuant cell was filled with

0.5ml of diluted RNA and the absorbance at 260nm measured. The concentration of RNA was calculated using the formula:

Concentration (μg/ml) = A_{260} x 250 x 40 μg/ml (A_{260} of 1 = 40 μg/ml)

2.12.2. Reagents used for RT

All the reagents were obtained from Perkin-Elmer (PE Applied Biosystems Ltd), Warrington, Cheshire, UK.

10x PCR buffer II:

500mmol/l KCl

100 mmol/l Tris/HCl

25 mmol/l $MgCl_2$ solution

dNTPs: 10mmol/l deoxyribonucleoside triphosphates

Random Hexamers (50μmol/l)

RNase inhibitor (20 units/μl)

M-MLV reverse transcriptase (50 units/μl)

Reverse Transcription (RT)-PCR

The thermostable polymerases used in the basic PCR process require a DNA template. In order to apply PCR methodology to the study of RNA sample must first be transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called reverse transcription (RT), hence the name RT-PCR (Becker-Andre and Hahlbrock,1989).

2.12.3. Method

Total RNA 1μg was used as a template for RT-PCR. The RT reaction step was set up in a 20μl mixture containing 50 mmol/l KCl, 10mmol/l Tris/HCl, 5 mmol/l $MgCl_2$, 1 mmol/l of each of dNTPs, 2.5 μmol/l random hexamers, 20 units RNase inhibitor and 50 units of M-MLV reverse transcriptase. Incubations

were performed in a DNA thermal cycler (Perkin-Elmer 9600) for 10 minutes at room temperature, followed by 30 minutes at 42°C and 5 minutes at 99 ° C. After cDNA synthesis by RT, the incubation mixture was split in to two 10µl aliquots for separate amplification of cDNA using specific primers in the LightCycler™ instrument (Roche Molecular Ltd.).

2.13. REAL TIME RT-PCR

2.13.1. Reagents

Light Cycler™ DNA master SybrGreen 1

Human beta-Actin primer set for LightCycler™

Human beta-Actin primer mix: Ready to use with Light Cycler™ DNA master SybrGreen 1. It contains optimal $MgCl_2$ concentration.

Standard β-actin cDNA: 346,000 copies /µl

Human IL-10 primer set for LightCycler™

Human IL-10 primer mix: Ready to use with Light Cycler™ DNA master SybrGreen 1. It contains optimal $MgCl_2$ concentration.

Standard IL-10 cDNA: 16,000 copies /µl

2.13.2. Procedure

To quantify the number of cDNA copies for specific cytokines present in each cDNA sample, a LightCycler™ instrument (Roche Molecular Ltd.) was used for PCR amplification and detection. cDNA was amplified in the presence of cytokine specific primers and a SYBR green 1 dye. This dye binds to the minor groove of double stranded DNA. The fluorescence of the dye is enhanced by its binding to DNA and can be detected by the instrument after exciting the dye with an LED. During the PCR reaction, SYBR green binds to the DNA products as they are synthesised and thus the increase in SYBR green

fluorescence, when measured at the end of each elongation cycle, indicates the amount of PCR product formed during that cycle. By comparing the fluorescence of a PCR product of unknown concentration with fluorescence of several serial dilutions of an external standard, the LightCycler™ was able to perform a quantification analysis.

To quantify the copies of cytokine cDNA, amplification kit for the cytokines and the beta-actin house keeping gene (Search-LC GmbH) were used. A dilution series of (1:10, 1:100, 1:1000) of the standard contained within the kit was prepared using standard stabilizer solution. These were prepared by sequentially taking 2μl of standard or the previous dilution and combining it with 18μl of stabilizer. For IL-10 this produced standards containing 16, 160, and 1600 cDNA copies and for beta-actin this produced standard containing 346, 3460, and 34600 cDNA copies.

The PCR reaction mixture for each cDNA sample contained 6μl of water, 2μl of primer set (Search-LC GmbH) and 2μl of LightCycler™ DNA master SYBR green 1 (Roche Molecular Ltd.). The reaction mix was aliquoted into LightCycler™ capillaries and 10μl of cDNA template was added. 10μl of PCR mix was also aliquoted into 4 capillaries and 10μl of each standard was added into the first three capillaries. 10μl of standard stabilizer solution was added to the fourth capillary as a negative control. Each capillary was sealed with a stopper and centrifuged at 2000rpm for 30 seconds. The capillaries were then placed into the rotor of the LightCycler™ instrument. The samples were cycled with parameters shown in table 2.9.

Table.2.9. Thermocycling parameters for PCR on the LightCycler™

Parameter	Value		
Cycles	35		
Temp.Targets	segment 1	segment 2	segment 3
Target temperature (^{0}C)	95	68	72
Incubation time (seconds)	10	10	16
Temp. Transition rate (^{0}C/sec)	20	20	20
Secondary target temperature	0	58	0
Step size	0	0.5	0
Step delay	0	1	0
Acquisition mode	none	none	single

To quantitate PCR product the LightCycler™ software compares the fluorescence of a PCR product of unkown concentration with the fluorescence of several serial dilutions of an external standard. The software uses the fluorescence that is measured during the log-linear phase of amplification (figure 2.6).

The software plots individual graphs for each sample of the increase in fluorescence with PCR cycle number (figure 2.5). At the end of the PCR reaction, the software produces a line of best fit- a line traverses through all of the log-linear regions of the curves. The point at which the line of best fit

intercepts each curve may be used to calculate the concentration of the sample that generated the curve.

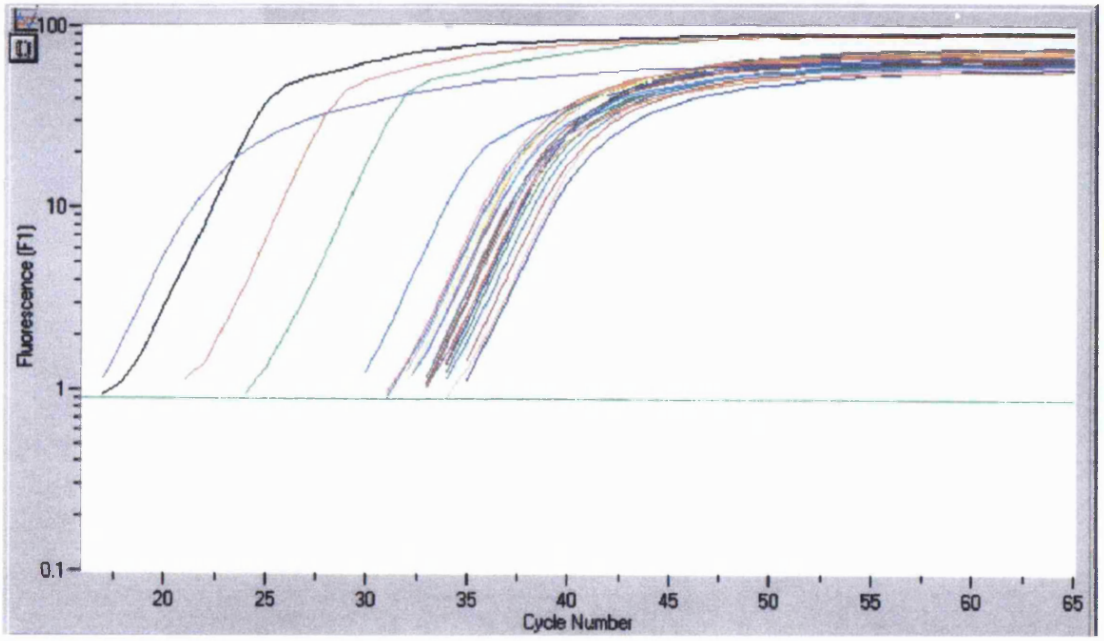


Figure: 2.5. Readout from the Roche LightCycler™ for quantification of IL-10 mRNA levels in LPS stimulated PBMCs.. The graph shows increase in fluorescence with increasing number of cycles. Each sample is represented by different colour on the graph.

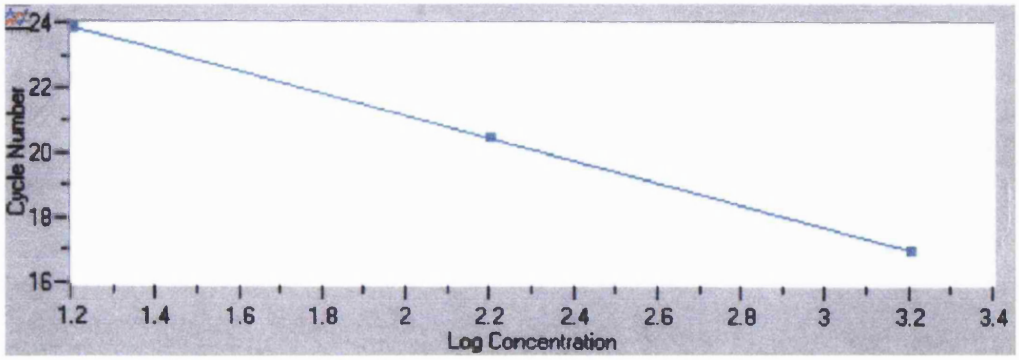


Figure: 2.6. The three points are serially diluted external standards of IL-10 cDNA, which are used to construct a calibration curve in order to quantify IL-10 mRNA of unknown samples.

2.14. MEASUREMENT OF ACTIVATED NUCLEAR FACTOR -KB

PBMCs were isolated from genotyped normal healthy individuals. From each individual, $1x10^6$ cells/ml of cell culture medium containing 10% heat inactivated human AB serum in RPMI 1640 supplemented with L-glutamine (2mM), 100 units/ml penicillin and 100μg/ml streptamycin were cultured at 37°C in 5% CO_2 for 24 hours with 100ng/ml LPS. The controls were treated in the same way without any addition of stimuli. Cells were collected after 24 hours and nuclear extracts were prepared from the cell pellets for the measurement of activated nuclear factor -κB.

2.14.1. Preparation of cellular nuclear extracts:

Materials

Buffer A

10 mM HEPES pH 7.9

10 mM KCl

1.5 mM $MgCl_2$

0.5 mM DTT

0.4 % NONIDET-P40

1 µg/ml Protease inhibitors: Antipain, leupeptin, Bestatin, Chymotrypsin (PI) or

1:100 (v/v) Sigma Protease inhibitor cocktail (Sigma product No. P8340)

0.5 µM PMSF

Buffer B

20 mM HEPES pH 7.9

420 M NaCl

1.5 mM $MgCl_2$

25 % Glycerol

0.2 mM EDTA

0.5 mM DTT

1 µg/ml PI

0.5 mM PMSF

2 mM Benzamidine

Method

Trypsinise the suspended mononuclear cells were pelleted by centrifugation and supernatant was discarded.1.5 ml ice cold buffer A was added into the pellet and disrupted by cells were passing repeatedly through a 21 G

needle. Disrupted cells were placed on ice for 10 mins to ensure complete cell lysis, and lysates were transferred into 1.5 ml microcentrifuge tubes and centrifuged at 6000 rpm at 4 ^{0}C for 10 mins. The supernatant was discarded; 150 µl of buffer B was added to the nuclear pellet and extraction of nuclear proteins was enhanced by vortexing and passing repeatedly through a pipette tip. Then it was placed on ice for 15 mins and centrifuged at 13000 rpm for 10 mins at 4 ^{0}C. Supernatants containing nuclear proteins were aliquoted into fresh tubes and stored at –70 ^{0}C

2.14.2. Electrophoretic mobility shift assay

The gel shift or electrophoretic shift assay provides a simple and rapid method of detecting DNA-binding proteins. This method has been widely used in the study of sequence-specific DNA-binding proteins, such as transcription factors (Briggs et al. 1986; Lee et al. 1987). The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein or a complex mixture of proteins (such as cell or nuclear extracts) with ^{32}P end-labelled DNA fragment containing the putative protein-binding site. The reaction products are then analysed on a nondenaturing polyacrylamide gel and exposed to X-ray film. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using non-radioactive DNA fragments or oligonucleotides containing a binding site for the protein of interest, or other unrelated DNA sequences.

Reagents:

Binding Buffer:

10 mM HEPES pH 7.9

50 mM KCl

0.2 mM EDTA

2.5 mM DTT

10 % Glycerol

0.05% NP-40

Protease inhibitor cocktail

For 100 ml

0.238 g HEPES

0.373 g KCl

0.1 ml 200 mM EDTA stock solution

10 ml Glycerol

50 µl NP-40

Make up to 100 ml autoclave and store at 4 ^{0}C

Add DTT and protease inhibitors immediately prior to use.1:100 (250 mM stock

DTT).

0.386 g DTT per 10 ml H_2O, freeze aliquots at –40 ^{0}C

Gel loading buffer (10 X):

250 mM Tris-HCl pH 7.5

0.2 % Bromophenol blue

40 % Glycerol

For 100 ml :

3.94 g Tris

0.2 g Bromophenol blue

40 ml Glycerol

Running Buffer (5 X) pH 8.3

25 mM Tris Base

190 mM Glycine

For 2000 ml :

3.028 g TRIS

28.527 g glycine

Dilute 1:5 for working buffer: 400 ml 5 × buffer in 1600 ml H_2O.

Method:

DNA binding reactions and electrophoretic mobility shift assay (EMSA)

Protein concentrations in nuclear extracts were measured using the method of Bradford (Bradford 1976) and 5 μg of extract used in binding reactions. A double stranded NF-κB binding consensus oligonucleotide, 5'-*AGT TGA GGG GAC TTT CCC AGG C*-3' (Promega, Southampton, UK) was end labelled with ^{32}P using Ready-To-Go ™ T4 polynucleotide kinase (Amersham Biosciences, Bucks, UK). Unincorporated nucleotides were removed using sephadex G-25 microspin columns (Amersham Biosciences).

Binding reactions were performed with 127 fmol of ^{32}P labelled oligonucleotide for 30 min at room temperature in 10 mM HEPES (pH 7.9): 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT and 10 % glycerol and 1 μg of poly (dI-dC). Protein-DNA complexes were resolved on 7 % native polyacrylamide gels using a TRIS-glycine running buffer and exposed to X-ray film. An IL-1β

stimulated HeLa cell nuclear extract (Promega) was used as a positive control and band specificity was confirmed by the addition of 30-fold excess of cold specific oligonucleotide.

End-labelling of DNA fragments for use in band-shift assays.

T4 Polynucleotide Kinase (PNK) is a tetrameric protein composed of four identical subunits. It is widely used in molecular biology to radiolabel nucleic acids and to add a phosphate to the 5'-end of dephosphorylated nucleic acids to facilitate ligations.

Ready-To-Go T4 Polynucleotide Kinase is a single-dose, ambient-temperature stable format of the enzyme. 25 µl of water was added to the tube containing the Ready-To-Go T4 PNK. The tube of reconstituted Ready-To-Go T4 PNK was incubated at room temperature for 2 to 5 minutes, then the contents of the tube were mixed by gentle pipetting up and down. 5-10 pmol of 5'-ends of oligonucleotide and sufficient water was added to bring the reaction volume to 49 µl.1 µl of (gamma-32P) ATP (3000 Ci/mmol, 10 µCi/µl) was added and mixed gently, then centrifuged briefly to collect the contents at the bottom of the tube. The tube was incubated 30 minutes at 37°C. The reaction was stopped by adding 5 µl of 250 mM EDTA.

Note: Using more or less than 50 µl to reconstitute the reaction mix will result in less than optimal buffer concentrations.

Free ^{32}P was removed by passage through a Sephadex G-25 spin column.

Removal of unincorporated ^{32}P using Sephadex microspin G-25 spin columns

(Apbiotech product no: 27-5325-01)

To remove the free or unbound ^{32}P from the labelled reaction mixture, the solution was allowed to pass through a column containing resin. The resin was resuspended in the column by vortexing. The top cap was removed and snapped off the bottom closure and both were discarded. The column was placed in a 1.5 ml screw-cap microcentrifuge tube for support. Alternatively, the cap was cut from a flip-top tube and this tube was used as a support. The column was placed in a variable speed microcentrifuge. A digital timer and the microcentrifuge were started simultaneously.

"Pulse spin" should not be used as this will override the variable speed setting.

The column was spun for 1 minute at 735 x g.

The column was transferred to a new 1.5 ml tube and the sample was slowly applied to the centre of the angled surface of the compacted resin bed without disturbing the resin. Any flowing samples around the sides of the bed should be avoided.

The column was spun for 2 minutes at 735 x g. The purified sample was collected in the bottom of the support tube. The column was discarded into a radioactive waste store.

2.14.3. Measurement of active NF-κB using TransAM kit:

This method combines a fast and user friendly ELISA format with a sensitive and specific assay for NF-κB. Trans AM NF-κB kit contains a 96 well plate on which has immobilized oligonucleotide containing the NF-kB consensus site (5'-GGGACTTTCC-3'). The active form of NF-kB contained in

cell extract specifically binds to this oligonucleotide. The primary antibodies used to detect NF-kB recognise an epitope on p65 or p50 that is accessible only when NF-kB is activated and bound to its target DNA. An HRP conjugated secondary antibody provides a sensitive colorimetric readout that is easily quantified by spectrophotometry.

Reagents:

All the reagents used for the assay were supplied with the kit.

Working complete lysis buffer:

1M DTT :5 µl

Protease inhibitor cocktail :10 µl

Lysis buffer :1ml

Mixed and used on the same day.

Working complete binding buffer:

1M DTT : 2µl

Herring sperm DNA (1mg/ml) : 10µl

Binding buffer : 1ml

1X washing buffer (100ml):

10X washing buffer : 10 ml

Distilled water : 90 ml

Mixed gently to avoid foaming.

1X antibody binding buffer (10ml):

10X antibody binding buffer : 1ml

Distilled water : 9ml

Mixed gently.

Developing solution

Stop solution

HeLa whole cell extract:

This was used as a positive control for the assay. 2.5 μg/well was used.

NF-κB wild type and mutated consensus oligonucleotides:

The wild type consensus oligonucleotide was provided as a competitor for NF-κB binding in order to monitor the specificity of the assay. The mutated consensus oligonucleotide should have no effect on NF-κB binding.

Procedure:

Binding of NF-κB to its consensus sequences:

30μl of complete binding buffer was added to each well except two sets of wells. 30μl of complete binding buffer which containing 20 pmol (2μl) mutant and wild type oligonucleotide are added to the appropriate wells. 20μl of sample which containing 5 μg of cell extract in complete lysis buffer was added to the well. 1μl of HeLa whole cell extract in 19 μl of complete lysis buffer was added into positive control wells. 20μl of complete lysis buffer alone was added into the blank wells. The plate was sealed and incubated at room temperature for 1 hour with mild agitation. Then each well was washed three times with 200μl of 1X washing buffer and dried by tapping on absorbent paper towels.

Binding of primary antibody:

100μl diluted NF-κB antibody (1:1000 dilution in 1X antibody binding buffer) was added to all wells. The plate was covered and incubated for 1 hour at room temperature without agitation. Then the plate was washed 3 times with 200μl 1X washing buffer.

Binding of secondary antibody:

100µl of diluted HRP-conjugated antibody (1:1000dilution in 1X antibody binding buffer) was added to all wells. The plate was covered and incubated for 1 hour at room temperature without agitation. After that the wells were washed 4 times with 200µl 1X washing buffer. Then 100µl of developing solution was added to all wells and incubated for 5 to 10 minutes at room temperature. Then 100µl of stop solution was added to each well to stop the reaction further. The absorbance was measured within 5 minutes at 450nm with a reference wavelengh of 655 nm.

2.14.4. Bio-Rad Protein Assay:

The Bio-Rad protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Bradford (Bradford 1976) first demonstrated the usefulness of this principle in protein assays. Spector (Spector 1978) found that the extinction coefficient of a dye-albumin complex was constant over a 10-fold concentration range. Thus Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration. Over a broader range of protein concentrations, the dye-binding method gives an accurate, but not entirely linear response.

Reagents:

Bio-Rad protein assay dye concentrate Cat No: 500-0006.

25 µg/ml bovine serum albumin (BSA) protein standard.

Method

The dilutions of BSA (as shown in table 2.10) in duplicate were prepared and placed in a 96 well ELISA plate. Samples were diluted in duplicate with distilled H_2O as required and 50 µl of dye concentrate was added to standards and samples, then mixed thoroughly using a plate mixer and incubated at room temperature for 5 minutes to one hour. Then OD was measured at 595 versus reagent blank using an ELISA plate reader. OD_{595} versus concentration of standards were plotted and unknowns were read from the standard curve.

Table.2.10. Concentrations of BSA standards used in the assay.

BSA µg/ml	Volume of BSA (µl)	Volume of H_2O (µl)
0.0	0	200
2.5	20	180
5.0	40	160
7.5	60	140
10.0	80	120
15.0	120	80
20.0	160	40
25.0	200	0

COSHH: Dye reagent contains phosphoric acid (corrosive) and methanol (toxic). Appropriate hand and eye protection was used.

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2.15. STATISTICAL ANALYSIS

The genotype and allele frequencies of the cytokine gene polymorphisms were calculated by direct counting and then dividing by the number of individuals to produce genotype frequency or by the number of chromosomes to produce allele frequency. The data were tested to fit Hardy-Weinberg equilibrium by calculating the expected frequencies of each genotype and comparing it to the observed frequencies. A chi-square test (SPSS™) was used in 2x2 contingency table to test the goodness of fit between the observed and expected values. Fisher's exact test (SPSS™) was also used if any of the observations were noted to be less than five. The p values were corrected for the number of comparisons made (Bonferonni correction)

The Kruskal-Wallis test (SPSS™) was used if the data were not normally distributed and if more than two groups were being analysed such as comparing cytokine production in different microsatellite alleles. The non-parametric Mann-Whitney U test (SPSS™) was used to compare cytokine production in single nucleotide polymorphism in the promoter region such as TNF-α production in TNFα -308 (A) positive and negative groups, IL-10 production in IL-10 -1082 (A) positive and negative groups, IL-2 production in IL-2 –330 (G) positive and negative groups, and IL-6 production in IL-6 –174 (G) positive and negative groups. When testing for differences in RNA or protein levels at different time points a Student's t-test (SPSS™) was used.

CHAPTER-3

CYTOKINE POLYMORPHISM AND ACUTE REJECTION IN RENAL TRANSPLANTATION

3.1 INTRODUCTION

Pro-inflammatory and anti-inflammatory cytokines are believed to influence the outcome of organ transplantation. Promoter region polymorphisms may affect the binding of transcription factors and consequently increase or decrease the production of mRNA and thus regulate cytokine production. Animal transplant models have suggested a role for interleukin –10 in promoting graft survival (Turner et al. 1997b) although it has been implicated in graft rejection (Wilson et al. 1997) as well. Microsatellite polymorphism may also influence protein production, possibly through a close linkage with a functional mutation (Pociot et al. 1993). The variation in TNF-alpha production has shown to be linked to TNF-microsatellite polymorphism (Pociot et al. 1993; Turner et al. 1995) in the TNF locus, which is located within the MHC region on chromosome 6. A genetic predisposition to high or low levels of specific cytokine may bias the patient to develop the incidence of acute rejection after transplantation. Alternatively, the mechanism by which cytokine polymorphism could be associated to different transplant outcomes may be unrelated to differential cytokine production but could simply be explained by their linkage disequilibrium to other, as yet, unknown loci.

Renal transplantation is the most effective mode of renal replacement therapy for patients with end stage renal disease. Despite the increasing success of renal transplantation over the past fifteen years, acute rejection and chronic rejection remain a problem. Presently there is no established method of tailoring immunosuppressive drugs for individual transplant recipients. It may be that for some patients too much immunosuppression is given exposing the patient to increased risk of side effects. Genotyping patients for markers such as cytokine

polymorphism may allow the identification of particular patients who are at high risk of acute rejection after renal transplantation may provide a tool to the clinician to regulate the immunosuppressive regime.

3.2 METHOD

In this part of the study, a number of renal transplant patients and healthy blood donors for a range of cytokine polymorphism were genotyped and examined for any association between these polymorphism and incidence of acute rejection that were observed in some patients following renal transplantation.

3.3 STUDY GROUP

3.3.1 Patients:

One hundred and thirteen renal transplant patients, from August 1985 to May 1999 being followed up at the Royal Free Hospital, London were genotyped for IL-10, TNF-α, IL-2, IL-6, TGF-β, and IFN-γ polymorphism. All the patients in this study received triple immunosuppression therapy with cyclosporin-A or tacrolimus, azathioprine and corticosteroids. The immunosuppressive therapy was modified later in some patients following the occurrence of acute rejection and adverse effects from drugs.

3.3.2 Diagnosis of acute rejection:

Acute rejection episodes were diagnosed on the basis of histopathological evidence and significant elevation in creatinine levels. Patients who had more than one episode of rejection in the first year after transplantation were included in the rejection group and the rest of the patients were included in the rejection free group.

Table 3.1.Comparison of two patient groups with regard to recipient demographic profile, graft's cold ischaemia time, DR matching, Panel reactive antibody. There was no significant difference between the group having more than two episodes of acute rejection (acute rejection group) and rejection free group with regard to the above parameters.

Recipient, graft details	Acute rejection group (n=35)	Rejection free group (n=78)	*P* value
Recipient mean age (range)	41yrs(25-65)	44yrs(19-68)	Not significant
Recipient sex Male Female	 20 15	 41 35	 Not significant
Cold ischaemia time ≤ 24 hours > 24 hours	 29 6	 63 15	 Not significant
HLA mismatches 0 DR mismatch 1 DR mismatch 2 DR mismatch	 16 18 1	 33 43 2	 Not significant
Panel reactive antibody ≤ 80% > 80%	 0 35	 2 76	 Not significant

3.3.3 Controls:

Control samples were obtained from healthy blood donors at National Blood Service, Colindale. Controls were genotyped to analyse the percentage frequency of each cytokine polymorphism and they were compared with the percentage frequencies of cytokine polymorphism in transplant patients.

3.4 RESULTS

3.4.1 Frequency of polymorphisms in control and patient groups:

3.4.1.1 TNF-a microsatellite

One hundred and thirteen renal transplant recipients and one hundred controls were typed for the TNF-a microsatellite (section 2.6). Alleles were numbered relative to cell line DNAs of known TNF-a type (Udalova et al. 1993).

All thirteen previously described alleles were observed. The frequencies of the thirteen alleles in the control and patient groups are shown in Table 3.2

Table 3.2 Frequencies of TNF-a microsatellite alleles in patients and control. No statistically significant differences were observed in the frequencies of the TNF-a microsatellite alleles in the control and patient groups. These allele frequencies were comparable with previously reported frequencies (Gallagher et al. 1997).

TNF-a allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
a1	1.5	1
a2	25.5	24
a3	2	2
a4	7	6
a5	5.5	8.5
a6	12	13
a7	7	9
a8	0.5	0.5
a9	2.5	3
a10	10.5	12
a11	20.5	13
a12	3	2
a13	2.5	6

3.4.1.2 TNF-d microsatellite:

One hundred and thirteen renal transplant recipients and one hundred controls were typed for TNF-d microsatellite (section2.6). Alleles were numbered relative to cell line DNAs of known TNF-d type (Udalova et al. 1993). All six previously described alleles were observed. The frequencies of the six alleles in the control and patient groups are shown in table 3.3.

Table 3.3. Frequencies of TNF-d microsatellite alleles in patients and control. No statistically significant differences were observed in the frequencies of the TNF-d microsatellite alleles in the control and patient groups.

TNF-d allele	Allele frequency (%) in Control (n=200)	Allele frequency (%) in Patients (n=226)
D1	5	4
D2	7.5	5
D3	50	53
D4	23.5	26
D5	12	11.5
D6	2	0.5

3.4.1.3 TNF-308 polymorphism

One hundred and thirteen renal transplant recipients and one hundred controls were typed for a single nucleotide polymorphism at position –308 in the TNF-α promoter. TNF1 and TNF2 alleles were identified following Nco1 digestion of PCR product and electrophoresis on an acrylamide gel, as previously described (section 2.3) (Wilson et al. 1992). Figure 2.1 shows an

example of a TNF-α(-308) acrylamide gel. The frequencies of the TNF1 and TNF2 alleles in the control and patient groups are shown in table 3.4

Table 3.4 Frequencies of TNFα-308 alleles in patients and controls. No statistically significant differences were observed in the frequencies of the TNFα-308 alleles in the control and patient groups.

TNFα-308 allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
TNFα allele 1- G	82.5	87
TNFα allele 2- A	17.5	13

3.4.1.4 IL-6 promoter (-174) polymorphism

One hundred and thirteen renal transplant recipients and one hundred controls were typed for the single nucleotide polymorphism in the 5' flanking region of IL-6 gene at position –174 from the transcription start site. The frequencies of IL-6 (-174) alleles in the control and patient groups are shown in table 3.5.

Table 3.5 Frequencies of IL-6-174 alleles in patients, and controls. There was no statistically significant difference in the frequencies of the IL-6 -174 alleles between the control and patient groups.

IL-6 –174 allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
IL-6 –174 allele G	60	65
IL-6 –174 allele C	40	35

3.4.1.5 IL-2 promoter (-330) polymorphism

One hundred and thirteen renal transplant recipients and one hundred controls were typed for the single nucleotide polymorphism in the 5' flanking region of IL-2 gene at position –330 from the transcription start site. The frequencies of IL-2 (-330) alleles in the control and patient groups are shown in table 3.6.

Table 3.6 Frequencies of IL-2-330 alleles in patients and controls. No statistically significant differences were observed in the frequencies of the IL-2 -330 alleles in the control and patient groups.

IL-2 –330 allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
IL-2 –330 allele T	73	64
IL-2 –330 allele G	27	36

3.4.1.6 IL-10 promoter gene polymorphisms

One hundred and thirteen renal transplant patients and one hundred controls were typed for the three single nucleotide polymorphisms in the IL-10 promoter at positions -1082, -819 and –592 using an SSOP method (section 2.4). Figure 2.2 shows an example of an IL-10 SSOP photographic film.

For the single base substitution at position –1082, alleles were designated IL-10 1*G and IL-10 1*A for the G and A variants respectively; for the C/T polymorphism at –819, alleles were designated IL-10 2*C and IL-10 2*T; and for the C/A polymorphism at –592, alleles were designated IL-10 3*C and IL-10

3*A. The frequencies of the alleles of the three polymorphisms in the control and patient group were analysed (table 3.7).

Table 3.7 Frequencies of IL-10 (-1082, -819, -592) alleles in patients and controls. No statistically significant differences were observed in the frequencies of the three single nucleotide variants at position –1082, -819 and –592 between the control and patient groups.

IL-10 allele Position relative to cDNA start	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
IL-10 1*G (-1082)	59	49
IL-10 1*A (-1082)	41	51
IL-10 2*C (-819)	82	74
IL-10 2*T (-819)	18	26
IL-10 3*C (-592)	82	74
IL-10 3*A (-592)	18	26

Three putative haplotypes were identified for alleles of three polymorphisms; IL-10-1082 G, IL-10-819 C, IL-10-592 C (GCC), IL-10-1082 A, IL-10-819 C, IL-10-592 C (ACC) and IL-10-1082 A, IL-10-819 T, IL-10-592 A (ATA). The frequencies of these haplotypes were compared between the control and patient groups (table 3.8).

Table 3.8 Frequencies of IL-10 (-1082, -819, -592) haplotypes in patients and controls. No statistically significant difference was found in the frequencies of haplotypes between controls and patients.

Haplotype	Control frequency(%)(n=100)	Patient frequency(%)(n=113)
GCC	59	49
ACC	23	25
ATA	18	26

3.4.1.7 IL-10-G microsatellite polymorphism

IL-10 G, CA-repeat microsatellite located close to the IL-10 gene and were genotyped by capillary electrophoresis of PCR product on an ABI™ 310 analyser (section 2.7). The frequencies of IL-10 G alleles in the control and patient groups are shown in table 3.9.

Table 3.9 Frequencies of IL-10 G microsatellite alleles in patients and controls. No statistically significant differences were found in the frequencies of IL-10 G alleles between controls and patients.

IL-10G allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
G1	0	0
G2	0	0
G3	0	0
G4	0	0
G5	0	0
G6	1.5	0
G7	4.5	6
G8	3.5	2
G9	43	48
G10	9	8
G11	8.5	8
G12	5	5
G13	17	19
G14	7	4
G15	1	0

3.4.1.8 IL-10 R microsatellite polymorphism

IL-10 R, CA-repeat microsatellite located close to the IL-10 gene and were genotyped by capillary electrophoresis of PCR product on an ABI™ 310 analyser (section 2.7). The frequencies of IL-10 R alleles in the control and patient groups are shown in table 3.10.

Table 3.10 Frequencies of IL-10 R microsatellite alleles in patients and controls. No statistically significant difference was found in the frequencies of IL-10 R alleles between controls and patients.

IL-10R allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
R 1	1	2
R 2	69	63
R 3	29	32
R 4	1	3

3.4.1.9 IFN-gamma polymorphism

IFN-γ, CA-repeat microsatellite located in the first intron were genotyped by capillary electrophoresis of PCR product on an ABI™ 310 analyser (section 2.5). The frequencies of IFN-γ alleles in the control and patient groups are shown in table 3.11.

Table 3.11 Frequencies of IFN-γ microsatellite alleles in patients and controls. No statistically significant differences were observed in the frequencies of IFN-γ microsatellite alleles in the control and patient groups

IFN-γ allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
Allele 1	0	4
Allele 2	43.5	39
Allele 3	45.5	46
Allele 4	6	6
Allele 5	3.5	4
Allele 6	1.5	1

3.4.1.10 TGF-β1 polymorphism

The TGF-β polymorphisms in the codon 10 and codon 25 were genotyped by PCR-SSP (section 2.8). The frequencies of TGF-β alleles in the control and patient groups are shown in table 3.12.

Table 3.12 Frequencies of TGF-β alleles in patients and controls. No statistically significant differences were observed in the frequencies of TGF-β1 alleles in the control and patient groups

TGF-β1 allele	Allele frequency (%) in control (n=200)	Allele frequency (%) in patients (n=226)
Codon 10 allele T	57	54
Codon 10 allele C	43	46
Codon 25 allele G	88.5	91
Codon 25 allele C	11.5	9

3.4.2 IL-10 (-1082) polymorphism and acute rejection

IL-10 (-1082 A) in the promoter has been associated with decreased IL-10 production *in vitro* (Turner et al. 1997b). They have showed the IL-10 (-1082 A/A) homozygous individuals, IL-10 (-1082 G/A) heterozygous individuals, IL-10 (-1082 G/G) homozygous individuals may be predicted to be low, moderate and high IL-10 producers respectively. Therfore, the influence of IL-10 (-1082) polymorphism on acute rejection was analysed in the first year post renal transplant (table 3.13).

Table 3.13. IL-10 (-1082) genotypes in renal transplant recipients; The occurrence of IL-10 (-1082 GG) genotype was significantly higher in the rejection free group than in the acute rejection group.

Cytokine genotype	% Frequency in patients	Acute rejection (n=35)	Acute rejection free (n=78)	χ^2	*P*value
IL-10 (-1082GG)	28	5	27	4.9	<0.03
IL-10 (-1082GA)	42	13	34		
IL-10 (-1082AA)	29	17	17		

3.4.3 TNF-a and TNF-d microsatellite polymorphism and acute rejection

The variation in TNF-α production has been linked to TNF microsatellite polymorphism (Pociot et al. 1993; Turner et al. 1995) in the TNF locus. Therefore, we have analysed the influence of these microsatellite polymorphisms on acute rejection in the first year post renal transplant recipients to test whether any of these alleles act as a marker of increased chance of acute rejection. We have noted a significant association between TNF a9 allele and acute rejection episodes (table 3.14). There was no significant difference between the group having more than two episodes of acute rejection (acute rejection group) and rejection free group with regard to the degree of HLA matching, percentage of panel reactive antibodies or the graft cold ischaemia time (table 3.1). There was no significant association found between any of TNF-d microsatellite alleles and acute rejection (table 3.15).

Table 3.14 Correlation of TNF-a microsatellite alleles with acute rejection. The occurrence of TNF-a9 allele in the recipients was significantly higher in the group with more than one episode of acute rejection (acute rejection group) than in the rejection free group.

TNF allele	% Frequency in patients	Acute rejection group (n=35)	rejection free group (n=78)	X^2	*P*value
a1	1	0	2		
a2	24	12	42		
a3	2	0	4		
a4	6	3	11		
a5	8.5	5	14		
a6	13	9	22		
a7	9	10	10		
a8	0.5	0	1		
a9	3	7	1	12.86	*0.003
a10	12	12	18		
a11	13	8	21		
a12	2	0	3		
a13	6	4	7		

Corrected P value *P_c < 0.04, when Fishers's exact test was used with correction for number of comparisons made.

Table 3.15. Correlation of TNF-d microsatellite alleles with acute rejection. There was no significant association between any of the TNF-d microsatellite alleles and acute rejection

TNF allele	% Frequency in patients	Acute rejection group (n=35)	Rejection free group (n=78)	*P* value
D1	4	3	6	
D2	5	4	7	
D3	53	36	84	
D4	26	18	41	
D5	11.5	8	18	
D6	0.5	1	0	Not significant

3.4.4 Other cytokine gene polymorphisms and acute rejection

Polymorphisms in recipient TNF-∝ (-308), IL-2 (-330), IL-6 (-174), TGF-β1 codon 10 and codon 25, IL-10G and R microsatellites and IFN-γ microsatellites were analysed in relation to acute rejection episodes in the first year after renal transplantation. None of these above mentioned polymorphisms, analysed individually, correlated with acute rejection (tables 3.16, 3.17, 3.18, 3.19, 3.20, 3.21, 3.22).

Table 3.16. Correlation of TNF-308 genotypes with acute rejection in transplant recipients. No significant association was found between any of TNF-308 genotypes and patients having more than one episodes of acute rejection (acute rejection group) compared to rejection free group.

Cytokine genotype	% Frequency in patients	Acute rejection group (n=35)	Rejection free group (n=78)	*P* value
TNF-A(-308GG)	75	25	60	
TNF-A(-308GA)	23	9	17	
TNF-A(-308AA)	2	1	1	Not significant

Table 3.17. Correlation of IL-2-330 genotypes with acute rejection. No significant association was found between the IL-2-330 genotypes and patients having more than one episode of acute rejection (acute rejection group) compared to rejection free group.

Cytokine genotype	% Frequency in patients	Acute rejection group (n=35)	Rejection free group (n=78)	*P* value
IL-2(-330 TT)	41	16	30	
IL-2(-330 TG)	46	15	37	
IL-2(-330 GG)	13	4	11	Not significant

Table 3.18. Correlation of IL-6-174 genotypes with acute rejection in transplant recipients. There was no significant association found between any of IL-6-174 genotypes and patients having more than one episodes of acute rejection (acute rejection group) compared to rejection free group.

Cytokine genotype	% Frequency in patients	Acute rejection group (n=35)	Rejection free group(n=78)	*P* value
IL-6(-174GC)	52	18	41	
IL-6(-174GG)	39	15	29	
IL-6(-174CC)	9	2	8	Not significant

Table 3.19. Correlation of TGF-β codon 10 and codon 25 genotypes with acute rejection in transplant recipients. No significant association was found between any of TGF-β codon 10 or codon 25 genotypes and patients having more than one episodes of acute rejection (acute rejection group) compared to rejection free group.

Cytokine genotype	% Frequency in patients	Acute rejection group (n=35)	Rejection free group(n=78)	*P* value
TGF-beta Codon10 CC	13	4	11	
TGF-beta Codon10 CT	66	25	49	
TGF-beta Codon10 TT	21	6	18	Not significant
TGF-beta Codon 25 GG	82	32	61	
TGF-beta Codon25 GC	17	3	16	
TGF-beta Codon 25 CC	1	0	1	Not significant

Table 3.20. Correlation of IFN-γ microsatellite alleles with acute rejection in renal transplant recipients. No significant association was found between any of IFN-γ microsatellite alleles and patients having more than one episodes of acute rejection (acute rejection group) compared to rejection free group.

IFN-γ allele	% Frequency in patients	Acute rejection group(n=35)	Rejection free group(n=78)	*P* value
1	4	2	6	
2	39	27	61	
3	46	33	71	
4	6	4	9	
5	4	2	8	
6	1	2	1	Not significant

Table 3.21. Correlation of IL-10 G microsatellite alleles with acute rejection. No significant association was found between any of IL-10G microsatellite alleles and patients having more than one episodes of acute rejection (acute rejection group) compared to rejection free group.

IL-10G allele	% Frequency in patients	Acute rejection group (n=35)	Rejection free group (n=78)	*P* value
G1	0	0	0	
G2	0	0	0	
G3	0	0	0	
G4	0	0	0	
G5	0	0	0	
G6	0	0	0	
G7	6	5	9	
G8	2	2	2	
G9	48	32	77	
G10	8	5	12	
G11	8	4	13	
G12	5	3	8	
G13	19	15	28	
G14	4	4	7	
G15	0	0	0	Not significant

Table 3.22. Correlation of IL-10R microsatellite alleles with acute rejection in renal transplant recipients. No significant association was found between any of IL-10R microsatellite alleles and patients having more than one episodes of acute rejection (acute rejection group) compared to rejection free group.

IL-10R allele	% Frequency in patients	Acute rejection group(n=35)	Rejection free group (n=78)	*P*value
R1	2	0	5	
R2	63	46	97	
R3	32	23	48	
R4	3	1	6	Not significant

3.5 DISCUSSION

Influence of cytokine gene polymorphisms in relation on acute rejection by analysing IL-10, TNF-α, IL-2, IL-6 promoter regions, TGF-β (codon 10 and codon 25) polymorphisms and IFN-γ, TNF-a, TNF-d, IL-10G, and IL-10R microsatellite polymorphisms in 113 renal transplant patients was studied. Percentage frequencies of each allele for each cytokine polymorphism were analysed. The frequency of the IL-10 (-1082) GG genotype was high in the rejection free group when compared to rejection group ($p \leq 0.03$). The frequencyof TNF–α, IL-6and IL-2 polymorphism did not show any significant difference between the two groups. Among the low IL-10 producer genotype, 44% patients had zero DR mismatches and less than 24 hours of cold ischaemia.

31% of these patients were live related donor (LRD) transplants with haplotype matches, while 13% a further had 1A, 1B, 1DR mismatches.

The frequency of TNF-a9 allele with fourteen adenine, cytosine (AC) dinucleotide repeat microsatellite was higher in the rejection group, when compared to the rejection free group ($p< 0.04$). 87% of patients with the TNF-a9 allele had multiple rejections in the first year after transplantation. 33% of these transplants were zero DR mismatches and the other 67% were LRD transplants. The frequency of CA repeat microsatellite polymorphism in the first intron of IFN-γ. IL-10 (G or R) microsatellite and TNF-d microsatellite polymorphisms did not show any significant difference between the two groups.

This study showed an association between IL-10 genotype and acute rejection episodes. In this study, even in recipients with HLA-DR matched transplants, IL-10 (-1082 AA and GA), low producer genotypes were associated with acute rejection. The frequency of the IL-10 (-1082GG) high producer genotype was low in the group with multiple episodes of acute rejection. Our results are consistent with the observations of Poole *et al* (Poole et al. 2001) that the frequency of recipient IL-10 (-1082GG), high producer genotype in combination with low producer donor IL-10 (-1082 AA, GA) genotype was significantly reduced in renal transplant recipients experiencing mutiple acute allograft rejection episodes. Tan *et al*, who monitored peripheral T lymphocyte cytokine gene expression in the early post transplant period, noted a reduction in the expression of IL-10 during acute renal allograft rejection (Tan et al. 2001). These results are consistent with the experimental work done so far suggesting the dominant role of IL-10 as an anti-inflammatory cytokine (de Waal Malefyt et al. 1991b; Lowry et al. 1995). However, a previous study by Sankaran et.al

(Sankaran et al. 1999) showed IL-10 (-1082 GG) genotype was associated with increased incidence of rejection. A significant occurrence of rejection was observed in this study in the IL-10 (-1082 AA, homozygous and GA heterozygous) group when compared to the IL-10 (-1082 GG homozygous) group. Marshall *et al* failed to detect any association between this polymorphism and acute rejection, possibly because they limited their study period to rejection episodes within the first thirty days (Marshall et al. 2000).

TNF-α (308), IL-6, IL-2 (-330), and TGF-β gene polymorphisms were analysed as well, in all recipients individually but there was no relationship between the genotype and acute rejection episodes. IFN-γ microsatellite polymorphism (CA repeat in the first intron) did not show any association with acute rejection episodes in this analysis. Analysis of TNF-a microsatellite in the TNF locus showed that TNF-a9 allele was associated with acute rejection and this is consistent with the previous study in Japanese LRD transplant recipients (Asano et al. 1997). The frequency of this allele in our patient group (mixed population) was less when compared to Japanese population (3 % Vs 11 %). TNF-d microsatellites were also analysed, but there was no association with acute rejection. It will be necessary to correlate TNF microsatellite alleles and TNF-α production in this study population. Furthermore, it is also possible that the presence of this allele does not correlate with functional differences in TNF-α regulation and thus may instead be a marker for other closely linked genes in MHC region that are of primary importance.

The current analysis shows that the significance of these polymorphisms needs to be evaluated with a larger number of patients, if possible on a multicentre basis. The occurrence of multiple rejections in the LRD transplants

also suggests that this allele needs to be looked for and if present, either better matching or optimum immunosuppression needs to be given.

In summary, this study suggested that IL-10 promoter (-1082) and TNF-a microsatellite polymorphisms in the transplant recipient have predictive value for acute rejection in renal transplantation.

CHAPTER-4

CYTOKINE POLYMORPHISM AND CHRONIC ALLOGRAFT DYSFUNCTION IN RENAL TRANSPLANTATION

4.1. INTRODUCTION

During recent years, the understanding of the development of chronic allograft dysfunction has improved considerably. As a result, renal transplant half-lives, which was static at around 8 years until 1988, has started to increase to 11.6 years in1995.

Major advances in immunosuppression and a reduction in the rates of acute rejection have led to increasing graft and patient survival rates during the past two decades. Chronic dysfunction of the renal allograft, however remains a major clinical problem and probably represents the end result of the complex interplay between donor and recipient factors which are immunological or non immunological (allo-antigen independent) factors. Furthermore, evidence suggests that initiating factors which are mainly immunological are different from perpetuating factors which are mainly non-immunological. Immunological factors include HLA incompatibility, number and resolution of acute rejection episodes, sensitisation, CMV infection, and adequecy of immunosuppression. Non immunological factors include cold and warm ischemia time, reperfusion injury, donor age, kidney size, hyperlipidemia, hypertension, and diabetes. These risk factors act through cellular and molecular mediators that initiate and perpetuate chronic graft dysfunction. Alloantigen dependent mechanisms include both cell mediated and antibody mediated responses involving complement activation, cytokines, chemokines and growth factors. Cytokines are believed to play an important role in this phenomenon and cytokine polymorphisms may have the potential to influence these clinical outcomes.

4.2. METHODS:

In this part of the study, on the basis of long-term graft survival and histological evidence of chronic allograft nephropathy, eighty-eight renal transplant recipients were selected from the previous one hundred and thirteen renal transplant recipients. This group was assessed for any association between the cytokine gene polymorphisms and chronic allograft dysfunction or long term allograft survival. Renal transplant recipients who were included in this study were divided into two groups – patients who had biopsy proven chronic rejection and graft loss within five years (chronic rejection group) were compared with patients who had at least five or more than five year of graft survival after renal transplantation were included in this group (survival group). All the patients in this group, received cyclosporin-A or tacrolimus based therapy as described earlier (section 3.3.1).

Table 4.1.Comparison of two patient groups with regard to recipient demographic profile, graft's cold ischaemia time, DR matching, Panel reactive antibody. There was no significant difference between the group of patients having chronic rejection and the group having more five years of graft survival with regard to the above parameters.

Recipient, graft details	Chronic rejection (n=27)	Group with >5yrs graft survival (n=61)	*P* value
Recipient mean age (range)	42yrs(27-65)	46yrs(22-63)	Not significant
Recipient sex Male Female	 17 10	 36 25	 Not significant
Cold ischaemia time ≤ 24 hours > 24 hours	 23 4	 50 11	 Not significant
HLA mismatches 0 DR mismatch 1 DR mismatch 2 DR mismatch	 12 14 1	 29 30 2	 Not significant
Panel reactive antibody ≤ 80% > 80%	 0 27	 1 60	 Not significant

4.3. RESULTS

4.3.1. IL-10 (-1082) polymorphism and chronic rejection

The influence of the IL-10 (-1082) polymorphism on chronic rejection was analysed in renal transplant recipients. Patients were divided into two groups on the basis of biopsy proven chronic rejection (group 1) and recipients who had more than five years of graft survival (group 2). The frequency of the IL-10 (-1082GG) genotype was significantly higher in the recipient group who had more than five years of graft survival when compared to chronic rejection group (table 4.2).

Table.4.2 Correlation of IL-10 (-1082) genotypes with chronic allograft dysfunction in renal transplant recipients. The occurrence of IL-10 (-1082 GG) genotype was significantly higher in the long-term graft survival group than in the chronic rejection group.

Cytokine genotype	Percentage frequency in patients	Chronic rejection group(n=27)	Group with long-term graft survival (n=61)	χ^2	*P* value
IL-10 (-1082GG)	25	2	20	6.4	0.015
IL-10 (-1082GA)	48	14	28		
IL-10 (-1082AA)	27	11	13		

4.3.2 Other cytokine gene polymorphisms and chronic rejection

Polymorphisms in recipient TNF-α (-308), IL-2 (-330), IL-6 (-174), TGF-β1 codon 10 and codon 25, IL-10G and R microsatellites and IFN-γ microsatellites were analysed in relation to chronic rejection following renal transplantation. None of these polymorphisms, analysed individually, correlated

with chronic rejection, or long term survival of the graft (tables 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11).

Table 4.3 Correlation of TNF-a microsatellite alleles with chronic allograft dysfunction. No significant association was found between any of the TNF-a microsatellite alleles and occurrence of chronic rejection or long term survival of the graft.

TNF allele	% Frequency in patients	Chronic rejection group(n=27)	Group with long-term graft survival (n=61)	*P* value
A1	1	0	2	
A2	25	11	33	
A3	2	0	4	
A4	5	1	8	
A5	10	5	12	
A6	12	7	14	
A7	6	4	7	
A8	0.5	0	1	
A9	4.5	6	2	
a10	15	7	18	
a11	12.5	8	14	
a12	0.5	1	0	
a13	6	4	7	Not significant

Table 4.4 Correlation of TNF-d microsatellite alleles with chronic allograft dysfunction after renal transplantation. No significant association was found between any of the TNF-d microsatellite alleles and occurance of chronic rejection or long term survival of the graft.

TNF allele	% Frequency in patients	Chronic rejection group (n=27)	Group with long-term graft survival (n=61)	*P* value
d1	2	2	2	
d2	5	3	5	
d3	52	26	66	
d4	26	16	30	
d5	12	5	16	
d6	3	1	4	Not significant

Table 4.5 Correlation of TNFα-308 genotypes with chronic allograft dysfunction. No significant association was found between any of the TNFα-308 genotypes and occurance of chronic rejection or long term survival of the graft.

Cytokine genotype	% Frequency in patients	Chronic rejection group (n=27)	Group with long-term graft survival (n=61)	*P* value
TNFα (-308GG)	77	22	46	
TNFα (-308GA)	21	5	13	
TNFα (-308AA)	2	0	2	Not significant

Table 4.6 Correlation of IL-6 -174 genotypes with chronic allograft dysfunction in renal transplant recipients. No significant association was found between any of the IL-6-174 genotypes and occurance of chronic rejection or long term survival of the graft.

Cytokine genotype	% Frequency in patients	Chronic rejection group(n=27)	Group with long-term graft survival (n=61)	*P* value
IL-6(-174GG)	39	14	20	
IL-6(-174GA)	51	12	33	
IL-6(-174 AA)	10	1	8	Not significant

Table 4.7 Correlation of IL-2-330 genotypes with chronic allograft dysfunction in renal transplant recipients. No significant association was found between any of the IL-2-330 genotypes and occurance of chronic rejection or long term survival of the graft.

Cytokine genotype	% Frequency in patients	Chronic rejection group (n=27)	Group with long-term graft survival (n=61)	*P* value
IL-2 (-330TT)	40	9	26	
IL-2 (-330TG)	45	15	25	
IL-2 (-330GG)	15	3	10	Not significant

Table 4.8 Correlation of TGF-β codon 10 and codon 25 genotypes with chronic allograft dysfunction. No significant association was found between any of the TGF-β codon 10 or codon 25 genotypes and occurance of chronic rejection or long term survival of the graft.

Cytokine genotype	% Frequency in patients	Chronic rejection group (n=27)	Group with long-term graft survival (n=61)	*P* value
TGF-β(codon10 CC)	14	0	12	
TGF-β(codon10 CT)	65	18	39	
TGF-β(codon10 TT)	21	9	10	Not significant
TGF-β(codon25 GG)	83	25	48	
TGF-β(codon25 GC)	16	3	11	
TGF-β(codon25CC)	1	1	0	Not significant

Table 4.9 Correlation of IFN-γ microsatellite alleles with chronic allograft dysfunction. No significant association was found between any of the IFN-γ microsatellite alleles and occurrence of chronic rejection or long term survival of the graft.

IFN-γ allele	% Frequency in patients	Chronic rejection group (n=27)	Group with long-term graft survival (n=61)	*P* value
1	3	3	2	
2	42	22	52	
3	45	25	54	
4	5.5	2	8	
5	4.5	2	6	
6	0	0	0	Not significant

Table 4.10 Correlation of IL-10 G microsatellite alleles with chronic allograft dysfunction in renal transplant recipients. No significant association was found between any of the IL-10G microsatellite alleles and occurrence of chronic rejection or long term survival of the graft.

IL-10G allele	% Frequency in patients	Chronic rejection group(n=27)	Group with long-term graft survival (n=61)	*P* value
G1	0	0	0	
G2	0	0	0	
G3	0	0	0	
G4	0	0	0	
G5	0	0	0	
G6	0	0	0	
G7	6	6	5	
G8	1	0	2	
G9	50	25	63	
G10	7	6	6	
G11	8	5	9	
G12	3	1	5	
G13	19	8	24	
G14	6	3	7	
G15	0	0	0	Not significant

Table 4.11 Correlation of IL-10R microsatellite alleles with chronic allograft dysfunction in renal transplant recipients. No significant association was found between any of the IL-10R microsatellite alleles and occurrence of chronic rejection or long term survival of the graft.

IL-10R allele	% Frequency in patients	Chronic rejection group (n=27)	Group with long-term graft survival (n=61)	*P* value
R1	2	0	4	
R2	62	38	71	
R3	35	16	45	
R4	1	0	2	Not significant

4.4. DISCUSSION

Despite the use of immunosuppressive drugs after transplantation, which block the binding of important transcription factors to cytokine gene promoters, cytokines are still expressed by infiltrating immune cells during rejection. A complex network of cytokines influences the immune response against the graft. Among these interleukins, transforming growth factor-β, interferon-γ, and tumour necrosis factor-α play important roles.

The effect of polymorphism in the recipient TNF, IL-10, IL-6, IL-2, IFN-gamma and TGF-beta genes on chronic rejection in renal transplantation was investigated in this chapter. Individual analysis of TNF, IL-6, IL-2, IFN-gamma and TGF-beta failed to show any significant association with chronic allograft rejection. However in this study, the high producer IL-10 (-1082GG) genotype

had a protective effect for chronic renal allograft function, suggesting that the anti-inflammatory role of IL-10 is beneficial in the longer term. This observation confirms the finding of Asderakis *et al*, who showed an association between IL-10 (-1082GG) genotype and five years of graft survival after renal transplantation (Asderakis et al. 2001). IL-10 is a pleiotropic cytokine with a range of different functions. It is anti-inflammatory and suppresses the synthesis of many other cytokines including IFN-gamma (Opelz 1998).

Muller-Steinhardt *et al* reported a significant association of IL-6-174 polymorphism with long-term kidney allograft survival, which implied a protective effect of the –174G allele in the multifactorial pathogenesis of allograft rejection (Muller-Steinhardt et al. 2002). A previous investigation focused on patient cohorts treated predominantly with cyclosporin-A monotherapy failed to detect an influence of the IL-10 (-1082), TNF (-308), IFN-γ (CA repeat) polymorphisms on kidney allograft survival (Sankaran et al. 1999). Likewise, there was no significant association of the TGF-β1 genotype with long-term kidney graft survival (Asderakis et al. 2001), even though it appears to be a major factor in lung transplantation (El-Gamel et al. 1999) and has recently been proposed to be relevant in kidney transplantation (Hutchinson et al. 1998). These investigations revealed variable results that may be attributed to the different study designs including selection of patient groups, immunosuppressive protocol, definition of rejection and the period of observation. Thus, a comparison of the results from this study from different centres is difficult.

The study presented in this chapter suggests that genetic polymorphism in the IL-10 gene may influence the chronic clinical events following kidney

transplantation. This finding does not, however, indicate that identification of a cytokine genotype can replace the prognostic value of either matching or cold ischemic time. Genotyping transplant recipients for cytokine polymorphisms may allow the identification of particular patients who are at increased risk of chronic allograft dysfunction and this may be a tool for adjusting the immunosuppressive regime.

CHAPTER-5

THE EFFECT OF CYTOKINE GENE POLYMORPHISM ON CYTOKINE PRODUCTION

5.1 INTRODUCTION

Certain polymorphisms in the regulatory region of cytokine genes have been correlated with the *in vitro* production of cytokines by lipopolysaccharide (LPS) or mitogen stimulated peripheral blood mononuclear cells (PBMCs) from healthy individuals (Turner et al. 1997b).

Cytokines play an important role in the post transplantation immune response. The levels of cytokine release and their interactions can also be studied using an *in vitro* allo-MLR model. In the human MLR, responder cells recognise alloantigens on the surface of stimulator cells, undergo proliferation and release various cytokines. The amount of cytokine released by each individual may vary depending on the genetic variation in the regulatory region of the cytokine gene. These polymorphisms can be found within the coding regions, introns, or promoter regions.

The aim of the work in this chapter was to establish whether cytokine gene polymorphisms result in differential cytokine production. Therefore, the secretory status of TNF-α, IL-10, IL-6, IL-2, TGF-β and IFN-γ were investigated in relation to their different genotypes.

5.2 Materials and Methods:

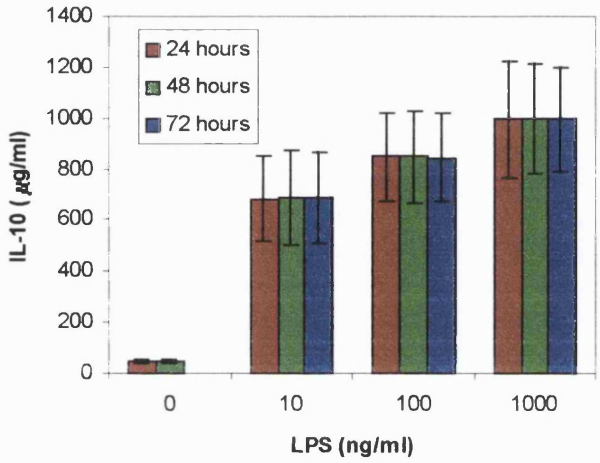
PBMCs from thirty-three healthy individuals were genotyped for the above mentioned cytokine polymorphisms. PBMCs from these individuals were then cultured with different stimuli such as lipopolysaccharide (LPS), phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA) and ionomycin or allogeneic PBMCs (one-way allo-MLR). The *in vitro* secretion of cytokines was then measured using ELISA.

5.3 Results:

5.3.1 Standardisation experiments

LPS was used to stimulate PBMCs to produce IL-10, IL-6, and TNF. LPS is a good stimulus for monocytes, from which these cytokines are produced. Usually doses of 10 to 1000ng/ml LPS are used when stimulating PBMCs. PBMCs were used in these experiments as they would include a combination of cell types that would closely match with the *in vivo* environment. Dose response and time course experiments were carried out in order to find the optimal dose and time point for collecting the supernatant for cytokine assay. The graphs in figure 5.1 show that PBMCs stimulated with a dose of 100ng/ml LPS for 24 hours give a good, measurable amount of each cytokine. Similarly, PBMCs from three healthy donors were used to find the optimal combination of doses of PMA and ionomycin and the optimal time point at which the IFN-gamma could be measured. PMA and Ionomycin together are good stimulators of T lymphocytes from which IFN-gamma, IL-2 and TGF-beta are produced.

a)



b)

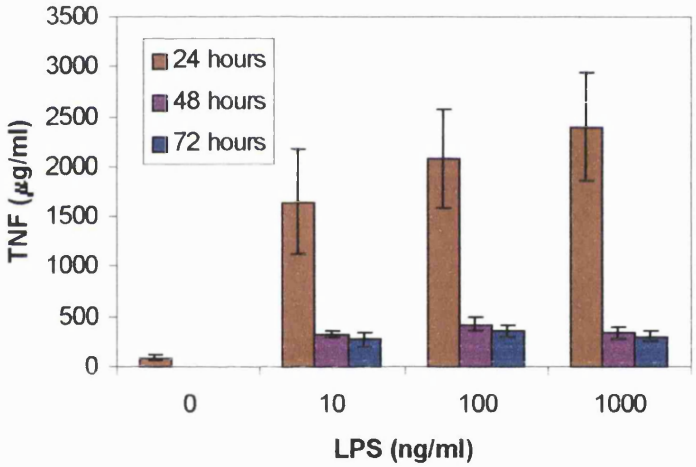


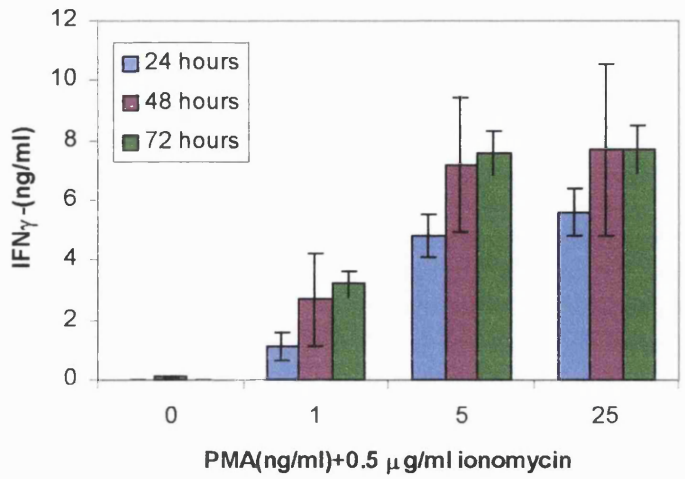
Figure.5.1 Dose response graphs for the production of cytokines TNF-α and IL-10 following LPS stimulation of PBMCs. The graph shows the levels of production at different time points for a) IL-10 and b) TNF-α (n=3, mean ± standard deviation).

PHA is also a good stimulator of lymphocytes therefore cytokine profiles in the PBMCs were also measured after stimulating with optimal dose of PHA at the optimal time point.

The graphs in figure 5.2 show that PBMCs stimulated with a combined dose of 5ng/ml of PMA and 1μg/ml of ionomycin for 48 hours gives a good measurable amount of IFN-γ. This production was not improved by an additional 24 hours of culture.

The figure 5.3 shows that PBMCs stimulated with a dose of 1μg/ml of PHA for 24 hours gave an optimal level of IL-2.

a)



b

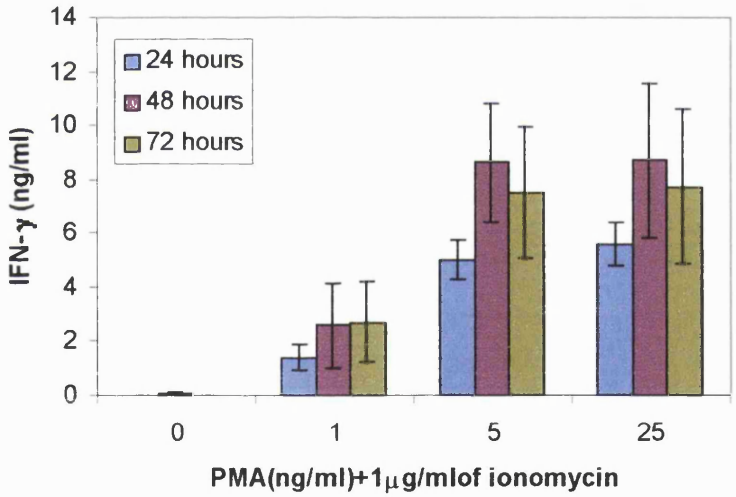


Figure.5.2 Time course and dose response graphs for the production of IFN-γ by a) 0 to 25ng/ml PMA and 0.5 μg/ml of ionomycin and b) 0 to 25 ng/ml PMA and 1μg/ml of ionomycin (n=3, mean ± standard deviation).

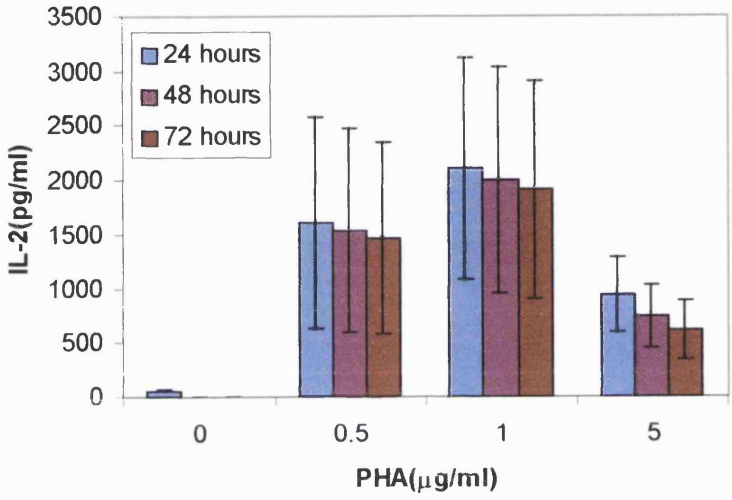


Figure.5.3 Dose response curves for the production of IL-2 by PHA at three different time points (n=3, mean ± standard deviation).

Secretion of different cytokine proteins in the supernatant of allo-MLR cultures was measured using ELISA.

For the reliability of MLC results, the critical steps were evaluated. We confirmed that the irradiated stimulator cells were not able to proliferate or respond to any mitogenic stimuli and then found the ratio of responder cells to stimulator cells, which gave the maximum response by measuring tritiated thymidine (^{3}H-TdR) uptake using a beta-counter. PBMCs stimulated with PHA (1μg/ml) were used as a positive control for the experiment. Experiments were carried out to find the best incubation period and appropriate cell density (2.5 X 10^6 cells) in order to give a good measurable amount of cytokines. Each experiment was repeated with five individuals.

The graph in figure 5.4 shows that cell density of each 2.5 X 10^6 cells of responders and 2.5 X 10^6 cells of stimulators resulted a good measurable response for the production of cytokine. The stimulators were pooled irradiated peripheral blood mononuclear cells from ten different individuals to ensure HLA disparity for the maximum alloresponse.

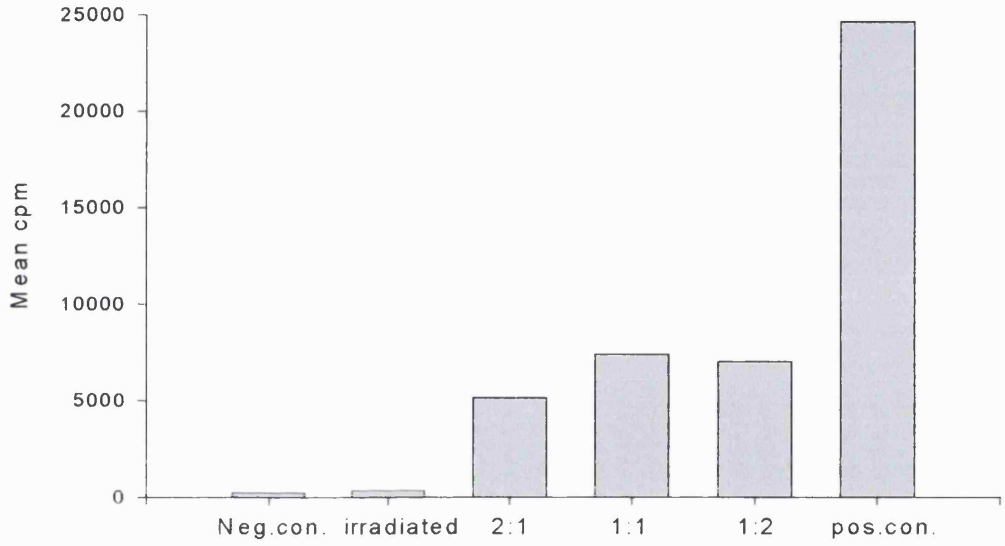


Figure 5.4 Response of allogeneic PBMCs to different ratios of gamma irradiated stimulator cells. Ratio 1 indicates 2.5 X 10^6 cells. The proliferative response of responders was measured in cpm. Mean cpm was calculated from five experiments.

5.3.2 Kinetics of production of various cytokines in culture supernatants of allo MLR

In one-way MLR, cytokine production was detected at 24 to 72 hours. Maximum secretion was obtained by 24 hours and there was no significant difference at 48 hours and 72 hours.

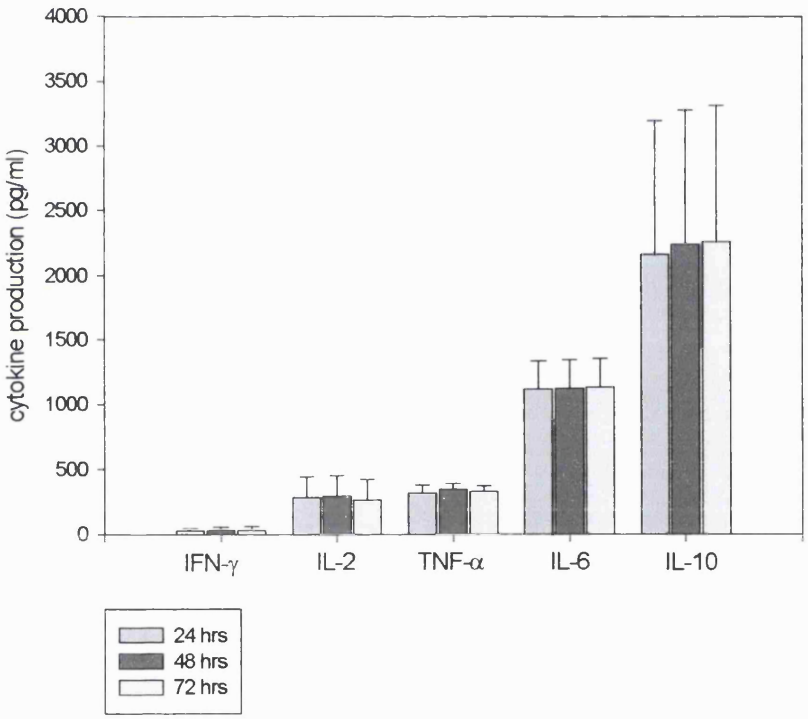


Figure 5.5 Cytokine production in allogeneic MLR. IFN-gamma, IL-2, TNF-alpha, IL-6, IL-10 production showed no significant increase after 24 hours, when further measurements were taken at 48 or 72 hours in one-way mixed lymphocyte culture.

5.3.3 *In vitro* production of TNF by PBMCs in relation to the -308 promoter polymorphism following stimulation by LPS, PHA or allo-MLR.

TNF production was assayed 24 hours after culturing the PBMCs with LPS or PHA. Twelve individuals with TNF-α (-308 A positive) polymorphism were shown to have increased TNF-α production in the supernatants of PBMCs stimulated with LPS compared to twenty two individuals with TNF-alpha (-308 A negative) polymorphism, although it failed to reach statistical significance (two tailed p value 0.066) (figure 5.6). However, there was no significant difference in production of TNF in PBMCs of different TNF-308 genotypes when stimulated with PHA or in allo-MLR (see table 5.1).

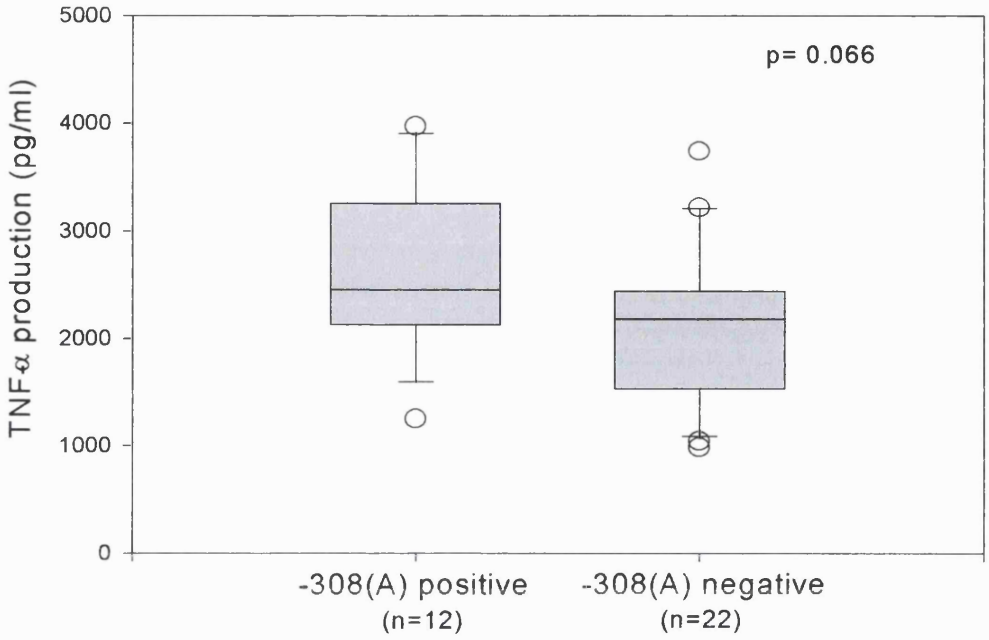


Figure 5.6 Cytokine production (TNF-alpha production) following LPS stimulation of human peripheral blood lymphocytes *in vitro*. Horizontal line in the box plot represents the median, the bottom and top bar represent 5th and 95 th percentiles of the data and circles represent the outliers. Twelve individuals with TNF-α (-308 A positive) polymorphism were shown to have TNF-α production ranging from 1250 pg/ml –39573 pg/ml with a median 2555.9 pg/ml compared to twenty two individuals with TNF-alpha (-308 A negative) polymorphism were shown to have TNF-α production ranging from 985.5 pg/ml – 3740 pg/ml with a median 2182.2 pg/ml .The Mann Whitney U test showed a two-tailed p value of 0.066 (see table 5.1) showing no significant differece between the two genotypes with regard to TNF-α production.

Table.5.1 Amount of TNF, IL-2, IL-6 and IL-10 produced by genotyped PBMCs when stimulated by allo-MLR, LPS and PHA. Significant reduction in IL-10 production was found with –1082A positive genotype in allogeneic MLR and using LPS stimulation. Other cytokine genotypes did not show any significant differences in cytokine production with the stimuli shown below.

Cytokine genotype of PBMC	**No:of subjects**	**Cytokine protein; (range) median pg/ml**	**p value**
MLR			
TNF-α(-308) A positive	11	(272-450) 349	
TNF-α(-308) A negative	22	(214-561) 353.5	NS
IL-2 (-330) G positive	16	(79-550) 313	
IL-2 (-330) G negative	17	(166-715) 431	NS
IL-6 (-174) C positive	28	(694-1546) 1324	
IL-6 (-174) C negative	5	(1102-1440) 1332	NS
IL-10 (-1082) A positive	20	(1080-3079) 1860	
IL-10 (-1082) A negative	13	(1444-4156) 2345	< 0.02
LPS stimulation			
TNF-α(-308) A positive	11	(1250-39753) 2555.9	
TNF-α(-308) A negative	22	(985.5-3740) 2182.2	NS
IL-2 (-330) G positive	16	(18.5-1735) 34.1	
IL-2 (-330) G negative	17	(17-1469) 45.9	NS
IL-6 (-174) C positive	28	(4493-26530) 18797	
IL-6 (-174) C negative	5	(13460-26530) 21060	NS
IL-10 (-1082) A positive	20	(121-3846) 421.8	
IL-10 (-1082) A negative	13	(186.8-2182) 593	< 0.02
PHA stimulation			
TNF-α(-308) A positive	11	(578-2525) 1870	
TNF-α(-308) A negative	22	(523-2567) 1333	NS
IL-2 (-330) G positive	16	(1.84-1201) 45.3	
IL-2 (-330) G negative	17	(1.5-660) 45.6	NS
IL-6 (-174) C positive	28	(4001-21054) 14426	
IL-6 (-174) C negative	5	(10500-21458) 16450	NS
IL-10 (-1082) A positive	20	(15-541) 142.8	
IL-10 (-1082) A negative	13	(63-431) 168	NS

5.3.4 *In vitro* production of IL-10 by PBMCs in relation to the -1082 promoter polymorphism following stimulation by LPS, PHA or allo-MLR.

IL-10 production was assayed 24 hours after culturing the PBMCs with LPS or PHA. Twenty individuals with IL-10 (-1082 A positive) polymorphism were shown to have significantly reduced levels of IL-10 production in the supernatants of PBMCs stimulated with LPS compared to thirteen individuals with IL-10 (-1082 A negative) polymorphism (two tailed p value = 0.015) (figure 5.7). Similarly in allo-MLR, levels of IL-10 in the supernatants of individuals with IL-10 (-1082 A positive) polymorphism was noted to be low when compared with IL-10 (-1082 A negative) polymorphism (two tailed p value = 0.0166) (figure 5.8). However, there was no significant difference in IL-10 production in PBMCs of different IL-10 (-1082) genotypes when stimulated with PHA (see table 5.1).

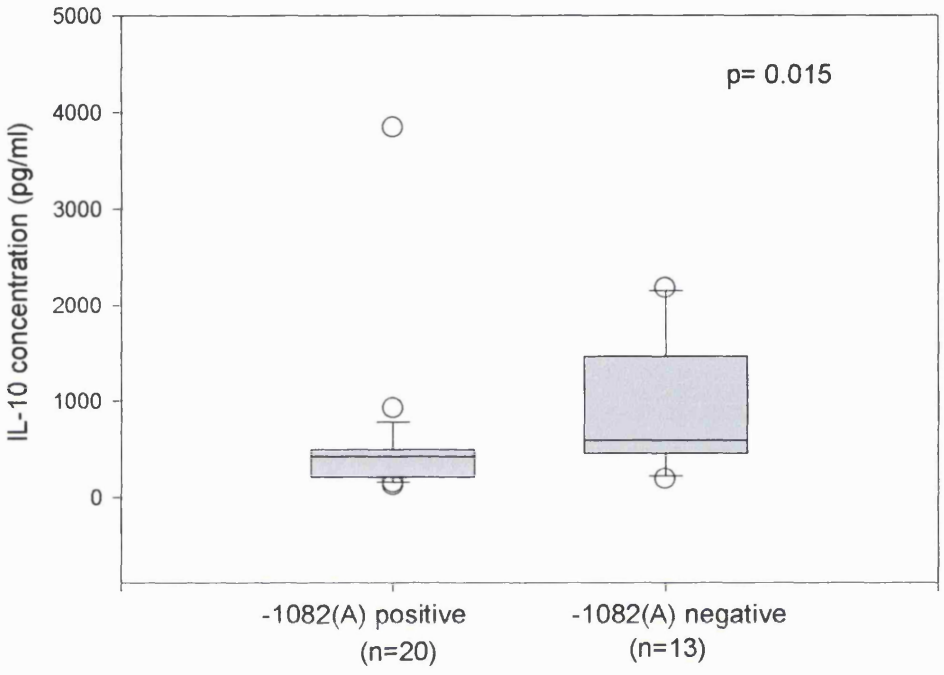


Figure 5.7. IL-10 production following LPS stimulation of human peripheral blood lymphocytes *in vitro*. Horizontal line in the box plot represents the median, the bottom and top bar represent 5th and 95 th percentiles of the data and circles represent the outliers. Significant difference in IL-10 production was found with –1082A positive genotype (ranging from 121 pg/ml - 3846 pg/ml with a median 421.8 pg/ml) compared to –1082A negative genotype (ranging from 186.8 pg/ml - 2182 pg/ml with a median 593 pg/ml). The Mann Whitney U test showed a two tailed p value of 0.015 (see table 5.1).

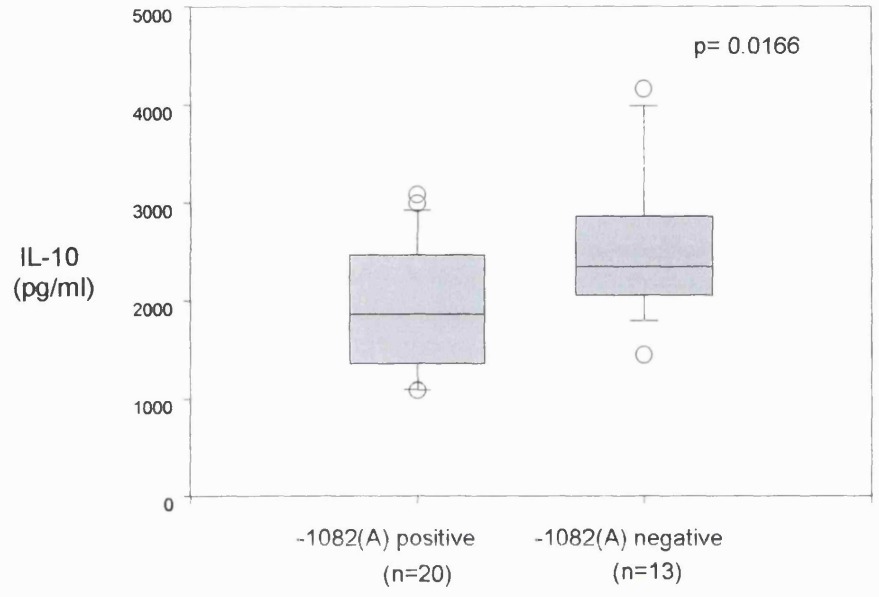


Figure 5.8. IL-10 production following one-way allogeneic MLR. Horizontal line in the box plot represents the median, the bottom and top bar represent 5th and 95th percentiles of the data and circles represent the outliers. Significant difference in IL-10 production was found with –1082A positive genotype (ranging from 1080 pg/ml - 3079 pg/ml with a median 1860 pg/ml) compared to –1082A negative genotype (ranging from 1444 pg/ml - 4156 pg/ml with a median 2345 pg/ml). The Mann Whitney U test showed a two-tailed p value of 0.0166 (see table 5.1).

5.3.5 *In vitro* production of IL-6 and IL-2 in relation to the gene polymorphisms when stimulated by LPS, PHA or allo-MLR.

IL-6 and IL-2 production by PBMCs was assayed after 24 hours culture with LPS or PHA. There was no significant difference in IL-6 production

between twenty nine individuals with IL-6 (-174 G positive) and four individuals with IL-6 (-174 G negative) polymorphism when PBMCs were stimulated with LPS, PHA or in allo-MLR (see table 5.1). Similarly, there was no significant difference in IL-2 production between sixteen individuals with IL-2 (-330 G positive) and seventeen individuals with IL-2 (-330 G negative) polymorphism when PBMCs were stimulated with LPS, PHA or in allo-MLR (see table 5.1).

5.3.6 Production of IFN-γ in relation to their microsatellite alleles when stimulated by PMA and Ionomycin.

IFN-γ was measured from each individual's PBMCs stimulated with PMA and ionomycin to assess whether there is any correlation between their microsatellite allele and protein production. Positivity for an individual allele was used for grouping the IFN-γ production. However, no significant association was found between any of the microsatellite alleles and IFN-γr production (data not shown).

5.3.7 TGF-β1 production

TGF-β1 was measured in the culture supernatants of PBMCs stimulated with PHA after 24 hours (table 5.2). Analysis of the production data was carried out by dividing them in to two groups according to the presence or absence of nucleotide variants, is cytosine in codon 10 (G to C) and in codon 25 (G to C). The Mann- Whitney tests showed that there were no significant differences in production in relation to their genotypes.

Table 5.2 TGF-β production by genotyped PBMCs when stimulated with PHA. No significant difference in TGF-β protein production was observed in TGF-β codon 10 or codon 25 allelic varients.

TGF-β genotype	No:of subjects	TGF-β protein; (range) median pg/ml	p value
TGF-β codon10-C positive	26	303-3978 (1057)	NS
TGF-β codon10-C negative	7	534-1774 (916)	
TGF-β codon25-C positive	11	303-3978 (1066)	NS
TGF-β codon25-C negative	22	309-2876 (927.5)	

5.3.8 Production of TNF in relation to microsatellite alleles when stimulated by LPS.

Production of TNF was analysed in TNF-a and TNF-d microsatellite alleles after stimulating the PBMCs of thirty three healthy individuals with LPS. Positivity for individual alleles of microsatellites was used for grouping the TNF production. However, using the Kruskal-Wallis test no significant association was noted between any of the microsatellite alleles and TNF production (data not shown).

5.3.9 Production of IL-10 in relation to microsatellite alleles when stimulated by LPS.

Production of IL-10 was tested in IL-10 G and IL-10 R microsatellite alleles after stimulating the PBMCs of thirty three healthy individuals with LPS. Positivity for each individual allele of microsatellites was used for grouping the IL-10 production. However, using the Kruskal-Wallis test, no significant

association was noted between the IL- 10 G or IL-10 R microsatellite alleles and IL-10 production (data not shown).

5.3.10 Production of IL-10 mRNA transcripts in relation to promoter (-1082) polymorphism

As the single nucleotide polymorphism in the IL-10 (-1082) promoter was found to be associated with the amount of IL-10 production, its effect on transcription was investigated. For this purpose, mRNA production was measured from genotyped individuals PBMCs that had been stimulated with 100ng/ml LPS *in vitro*. The appropriate time point for sampling was tested with three individuals.

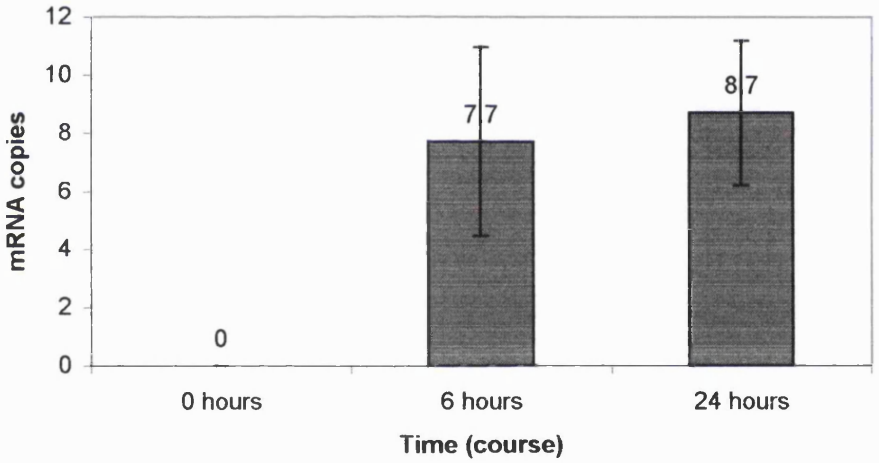


Figure 5.9. IL-10 mRNA copy numbers found in LPS stimulated PBMCs at different time points (n=3, mean ± standard deviation shown). No RNA copies were detected at time '0' hours. There was an increased production of IL-10 mRNA was observed at 6 hours and 24 hours after stimulating the PBMCs with LPS. Further experiments for mRNA quantification was carried out at 24 hours.

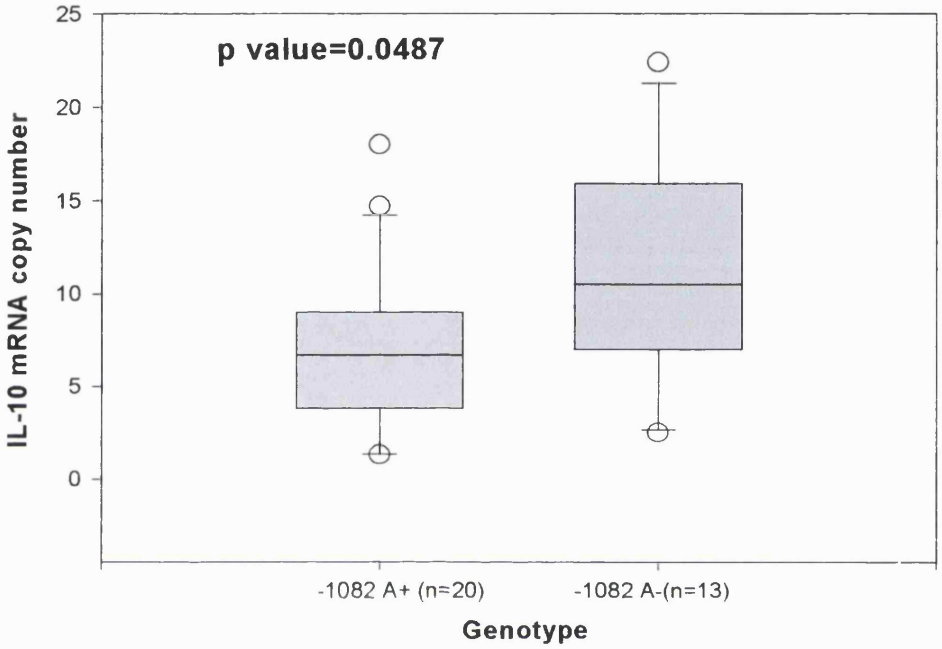


Figure 5.10 IL-10 mRNA copies following LPS stimulation of human peripheral blood lymphocytes *in vitro*. Horizontal line in the box plot represents the median, the bottom and top bar represent 5th and 95 th percentile of the data and circles represent the outliers. The Mann Whitney U test showed a two-tailed p value of 0.0487.

5.3.11 Cytokine promoter polymorphism and activation transcription factor NF-κB

As NF-κB is one of the major transcription factors involved in the transcriptional regulation of cytokines, it was useful to investigate whether cytokine gene polymorphism had any association with the activation or binding of NF-κB. Activated NF-κB binds to the promoter region of the cytokine gene, which in turn affects transcription and protein synthesis (Lenardo and Baltimore 1989).

The relationship between NF-κB binding activity and cytokine polymorphism was studied in twenty control individuals in an experimental system using LPS stimulated PBMCs.

Separate experiments were carried out using EMSA to find the appropriate time point for sampling. Using Trans ^{AM} NF-κB assay all the twenty individuals' samples were measured. There was no significant association between NF-κB activation and cytokine polymorphisms (data not shown).

5.4 DISCUSSION

The level of transcriptional activation of a gene depends on the binding of transcription factors to specific recognition sequences in the regulatory regions (Jain et al. 1995),(Kube et al. 1995). Polymorphisms in the cytokine gene promoter sequences may alter these transcription factor recognition sites and, therefore, influence transcriptional regulation and protein production. A correlation was observed between the presence of TNF (-308) single nucleotide guanine to adenine polymorphism in the promoter and was associated with increased TNF-alpha production (Wilson et al. 1992). These results were supported by transfection assays (Wilson et al. 1997).

In this study, the amount of IFN-γ, IL-2, TNF-α, IL-6 and IL-10 produced were measured after *in vitro* stimulation with LPS, PHA, PMA, or ionomycin and then compared with their genotypes. An allogeneic mixed lymphocyte reaction (MLR) was also used to demonstrate the differences in cytokine production.

These experiments showed a significant association between the IL-10 promoter (-1082 A positive) genotype and decreased IL-10 production in allogenic MLR as well as LPS stimulation. A significant association between the IL-10 promoter (-1082 A positive) genotype and decreased IL-10 mRNA transcripts were also noted following *in vitro* LPS stimulation of PBMCs from these individuals. This observation is consistent with a previous report by Turner et.al, where they showed a difference in IL-10 protein secretion in the presence or absence of 'A' nucleotide at position –1082 of human IL-10 promoter following concavalin A stimulation of PBMCs (Turner et al. 1997b).

A correlation was found between TNF (-308 A positive) genotype and increased TNF-α production following LPS stimulation when compared to TNF (-308 A negative) genotype, although it failed to reach statistical significance (p value 0.066). However there was no association between these genotypes and TNF-α production in allogeneic MLR or PHA stimulation. (Table 5.1).

This study failed to find any association between IFN-γ, TNF-a, TNF-d, IL-10G, IL-10R, IL-6 (-174), IL-2 (-330), TGF-β alleles and their protein production. Several of the microsatellite alleles were very rare in the general population and, therefore, it was difficult to obtain sufficient number of samples and to obtain definitive conclusions as to whether these alleles have any association with cytokine production. Failure to demonstrate any association

may also be possible due to the feedback mechanisms, which counterbalance any changes in cytokine levels. The influence of cytokine gene polymorphisms in addition needs to be investigated with *in vivo* models as it may have other effects in individuals where disease or immunosuppression alters normal physiology. The use of PBMCs in the MLR experiments for this thesis was based on the idea that it would reflect the mixture of interacting cells that would be found *in vivo*.

Some of the polymorphisms investigated in this study were examined previously for associations with differential cytokine production, and many of these studies reported conflicting results. The reason for these discrepancies may be due to the differences in the experimental design to investigate the effects of polymorphisms including the cell type and stimuli used in each study. Moreover, it is now known that the effect of a polymorphism in its ability to change the amount of cytokine production is dependent on the cell type and stimulus used (Kroeger et al. 2000).

This study also failed to show any association between activation of one of the transcription factors, NF-κB and IL-10 (-1082) gene polymorphism even though the polymorphism correlated with IL-10 mRNA and protein production. This suggests NF-κB activation may not be the major route via which the polymorphism affects differential production, and instead may be affinity of their binding to the polymorphic region or activation of other transcription factors such as c-jun/activating protein (AP-1), AP-2, or NF-AT.

In conclusion, the IL-10 (-1082) polymorphism was found to be associated with differential production of mRNA and IL-10 protein.

CHAPTER-6

EFFECT OF CYTOKINE GENE POLYMORPHISM ON ATHEROSCLEROTIC RENOVASCULAR DISEASE

6.1 INTRODUCTION

Atherosclerotic renovascular disease accounts for more than 90 percent of cases of renal artery stenosis, and is increasingly identified in patients with diabetes and hypertension (Safian and Textor 2001). In elderly patients it accounts for between 14 to 25% of end stage renal failure (Mailloux et al. 1988; Scoble et al. 1989). Current concepts of atherosclerosis are changing, and while it is still clear that abnormalities in lipid metabolism are of importance in pathogenesis, there is increasing evidence for the involvement of an inflammatory process. Atherosclerotic lesions are now believed to develop as a result of the interactions between circulating monocytes, T lymphocytes, and endothelium, ultimately resulting in the local migration and proliferation of vascular smooth muscle cells. Cytokines, through their pro- and anti-inflammatory effects, are thought to play a vital role in co-ordinating this process.

Genetic variation in cytokine production, which in part determines the balance between pro- and anti-inflammatory cytokines, may significantly influence the outcome of immune, inflammatory and proliferative responses. A large number of cytokine polymorphisms have been described (Bidwell et al. 1999; Pociot et al. 1993) and evidence is accumulating that some may be associated with functional differences in cytokine action or may influence cytokine production by modifying transcriptional activity (Fishman et al. 1998; Turner et al. 1997b; Wilson et al. 1997). Consequently, there has been considerable interest in studying these polymorphisms in a wide variety of inflammatory and immune mediated diseases. This study investigated the role

of polymorphisms in the cytokines TNF-alpha, IL-2, IL-6, and IL-10 in the development of atherosclerotic renal artery stenosis.

6.2. SUBJECTS AND METHODS

Sixty six patients with atherosclerotic renal artery stenosis attending the Royal Free Hospital, London were recruited for this study. In each patient, renovascular disease had been diagnosed by renal artery angiography. The average age of the patient group was 67yrs ± 9.5 (mean±SD). The plasma concentrations of creatinine and C-reactive protein were 175 μmol/l ±92 and 22 mg/l ± 39 (mean±SD), respectively. One hundred blood donors with normal plasma creatinine (<120μmol/l) from the National Blood Service, Colindale were used as controls. The age of control group was 46.5 yrs ± 11 (mean±SD).

PCR-SSP was used to analyse previously described single nucleotide polymorphism (SNPs) at nucleotide position -1082, -819 and -592 in the IL-10 promoter, at -308 in the TNF-α promoter, at -330 in the IL-2 promoter, and at -174 in the IL-6 promoter. Two previously described CA repeat microsatellites in the 5' flanking region of the IL-10 gene, IL-10 G and IL-10 R, and. at the TNF locus, TNF-a and TNF-d microsatellites, were analysed.

6.3 QUANTIFICATION OF CYTOKINE PRODUCTION

Peripheral blood mononuclear cells (PBMCs) were isolated from 22 patients using density gradient centrifugation (Lymphoprep, Nycomed Pharma As, Oslo, Norway). $1x10^6$ cells/ml from each individual were added to culture medium containing 10% heat inactivated human AB serum in RPMI 1640 supplemented with L-glutamine (2mM), 100 units/ml penicillin and 100μg/ml streptomycin, and plated in a 24 well tissue culture plate. Cells were incubated at 37°C in 5% CO_2 for 24 hours with 100ng/ml of LPS. Controls were treated in

the same way without addition of LPS to assess background cytokine production. To quantify mRNA production, total RNA was first isolated from cells using the acid guanidinium thiocyanate/phenol chloroform method. 1μg of total RNA was then used as a template for RT-PCR. To quantify cytokine protein production, cell supernatants were collected and analysed using cytokine specific enzyme linked immunosorbent assay (ELISA) (R&D systems, Abingdon, Oxon, UK).

6.4 RESULTS

Analysis of TNF-α, IL-6, and IL-2 gene polymorphisms revealed no difference in genotype frequencies between patients and controls (table 6.1). Similarly no difference was observed in allele frequencies of the TNF (table 6.2) or IL-10 microsatellite (table 6.3). However, significant differences were observed for IL-10 promoter SNPs. At nucleotide –1082, the GG genotype was reduced and GA and AA genotypes were increased in patients compared to controls ($p < 0.01$: table 6.1).

IL-10 –819 polymorphism is in linkage disequilibrium with –592 polymorphism. The AA-TT-AA homozygous genotype combination of the three SNPs at –1082, -819, and –592 in the IL-10 promoter was observed at a higher frequency in patients (15%) compared to controls ($p < 0.01$) (table 6.1). None of the other cytokine single nucleotide or microsatellite polymorphism, analysed individually or in combination with other cytokine polymorphisms, had any association with atherosclerosis. To correlate IL-10 genotypes with differences in IL-10 protein expression, *in vitro* mRNA and protein levels were analysed in LPS stimulated PBMCs from 22 patients with renal artery stenosis. Patients who were A positive at –1082 exhibited significantly lower protein production compared to

A-negative patients (figure 6.1). IL-10 mRNA copy numbers in A positive patients was also reduced but did not reach statistical significance (Figure 6.2).

Table 6.1. Comparison between percentage frequencies of cytokine promoter single nucleotide variant genotypes in controls and patients with renal artery stenosis. The frequency of IL-10 (-1082 GG), IL-10 (-819 TT), IL-10 (-592 TT) genotypes were significantly lower in the patient group who had renal artery stenosis compared to the control group.

Cytokine genotypes	% Frequency in control (n=100)	% Frequency in patients (n=66)	P value (χ^2)
TNF-alpha (-308) GG	69	70(46)	Not significant
GA	27	30(20)	
AA	4	0	
IL-10 (-1082) GG	37	12(8)	0.005 (16.9)
GA	44	40(61)	
AA	19	18(27)	
IL-10 (-819) CC	65	47 (31)	
CT	34	36(24)	
TT	1	15(10)	*0.008 (13.3)
IL-10(-592) CC	65	47 (31)	
CA	34	36(24)	
AA	1	15(10)	*0.008 (13.3)
IL-2 (-330) TT	54	43(28)	
TG	38	48(32)	
GG	8	9(6)	Not significant
IL-6 (-174) GG	34	36(24)	
GC	48	53(35)	
CC	18	11(6)	Not significant

Fisher's exact test was used since one value was less than 5 in the IL-10 (-819/-592) genotype in control group. *The corrected $p < 0.01$

Table 6.2. Comparison between percentage frequencies of TNF microsatellite alleles in controls and patients with renal artery stenosis. No significant difference was observed between the frequencies of TNF-a or TNF-d microsatellite allele in the patient group who had renal artery stenosis and the control group.

Cytokine genotypes	% Frequency in control (n=100)	% Frequency in patients (n=66)	P value (χ^2)
TNF-a Allele-1	1.5	3 (2)	
Allele-2	25.5	28 (21)	
Allele-3	2	0	
Allele-4	7	9(7)	
Allele-5	5.5	9 (7)	
Allele-6	12	19 (14)	
Allele-7	7	15 (11)	
Allele-8	0.5	1 (0.5)	
Allele-9	2.5	3 (2)	
Allele-10	10.5	16 (12)	
Allele-11	20.5	18 (14)	
Allele-12	3	2 (1.5)	
Allele-13	2.5	9 (7)	Not significant
TNF-d Allele-1	5	8 (6)	
Allele-2	7.5	14 (11)	
Allele-3	50	79 (60)	
Allele-4	23.5	23 (17)	
Allele-5	12	8 (6)	
Allele-6	2	0	Not significant

Table 6.3. Comparison between percentage frequencies of IL-10 microsatellite alleles in controls and patients with renal artery stenosis. No significant difference was observed between the frequencies of IL-10R or IL-10G microsatellite allele in the patient group who had renal artery stenosis and the control group.

Cytokine genotypes	% Frequency in control (n=100)	% Frequency in patients(n=66)	P value (χ^2)
IL-10 R Allele-1	1	0	
Allele-2	68.5	97 (74)	
Allele-3	30	32 (24)	
Allele-4	0.5	3 (2)	Not significant
IL-10 G Allele-1	0	0	
Allele-2	0	0	
Allele-3	0	0	
Allele-4	0	0	
Allele-5	0	0	
Allele-6	1.5	0	
Allele-7	5	5 (4)	
Allele-8	2.5	7 (5)	
Allele-9	41	61 (46)	
Allele-10	9	5 (4)	
Allele-11	8.5	11 (8.3)	
Allele-12	5	10 (8)	
Allele-13	17.5	23 (17)	
Allele-14	7	9 (7)	
Allele-15	2.5	1 (0.7)	
Allele-16	0.5	0	Not significant

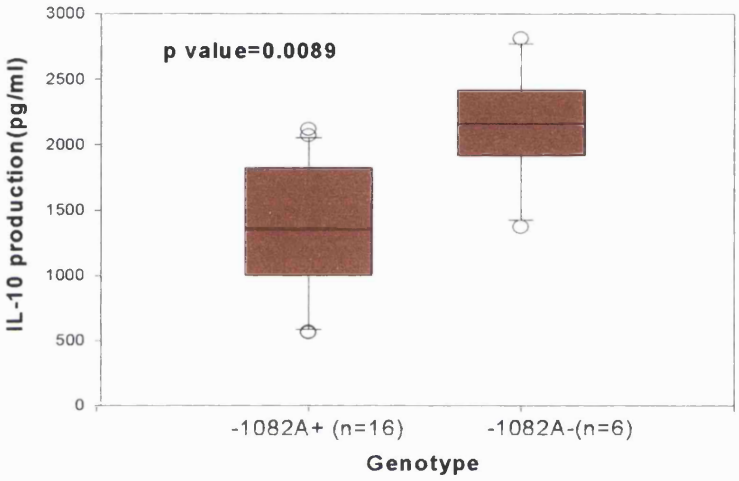


Figure 6.1. *In vitro* production of IL-10 protein in PBMCs from 22 patients after stimulating with LPS. The 16 patients with low producer genotype produced median 1357pg/ml (range 558-2118) and 6 patients with high producer genotype produced median 2159 pg/ml (1371-2813). The plot shows the median, 10^{th}, 25^{th}, 75^{th},and 90^{th} percentiles as vertical boxes with error bars and the circles represent outliers.

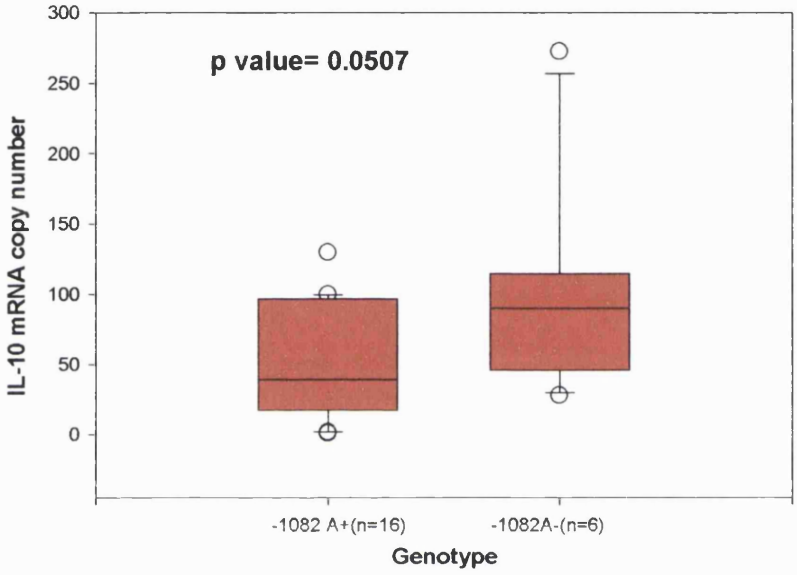


Figure 6.2. *In vitro* production of IL-10 mRNA in PBMCs from 22 patients and 33 controls after stimulating with LPS. The 16 patients with low producer genotype produced median 39.15 mRNA copy numbers (range1-129.9) and 6 patients with high producer genotype produced median 90.1 mRNA copy numbers (range 28-272.9). The 20 individuals with low producer genotype produced median 6.75 mRNA copy numbers (range 1.3-18) and 13 individuals with high producer genotype produced median 10.5 mRNA copy numbers (range 2.5-22.4).

6.5. DISCUSSION

In this study we observed that patients with renovascular disease had an increased frequency of GA and AA genotypes of the anti-inflammatory cytokine IL-10 promoter (-1082) polymorphism. In the IL-10 promoter three common haplotypes have previously been observed based on alleles at positions –1082, -819 and –592 respectively; GCC, ACC and ATA (Perrey et al. 1998; Turner et al. 1997b). The polymorphism at –819 is in linkage disequilibrium with –592 polymorphism. ATA-ATA (-1082AA/-819TT/-592AA) homozygous genotype frequency was also noted higher in patients compared to the controls ($p< 0.01$; Table 6.1). Previous studies have reported variability between individuals in IL-10 production after LPS stimulation of whole blood cultures, (Westendorp et al. 1997) suggesting that IL-10 production in humans is under genetic control and that high or low producer genotypes might be identified. Turner *et al* demonstrated a difference in IL-10 protein secretion in association with a presence or absence of 'A' nucleotide at position –1082 following concavalin A stimulation of PBMCs (Turner et al. 1997b). Therefore, the relationship of IL-10 (-1082) genotype to IL-10 protein and mRNA levels in the PBMCs of patients was examined. We found that in patients the absence of an A at position –1082 in the IL-10 promoter correlated with high levels of IL-10 protein and a high copy number of mRNA.

These observations are of interest because inflammation is thought to be an important mediator of atherosclerosis. A relative deficiency of an anti-inflammatory cytokine such as IL-10 may facilitate the development of atherosclerosis. IL-10 regulates Th1 cell activity, deactivates macrophages and modulates several factors that may be involved in the development, progression

and destabilisation of atherosclerotic plaques. These effects include activation of nuclear factor kB, increased tissue factor and cyclo-oxygenase-2 expression, enhanced metalloproteinase production and cell death. IL-10 is expressed in both early and advanced human atherosclerotic plaques and is thought to limit the local inflammatory response (Mallat et al. 1999; Uyemura et al. 1996). Evidence from animal studies has suggested that a deficiency of IL-10 could facilitate atherogenesis (Gupta et al. 1997; Mallat et al. 1999). IL-10 deficient mice exhibited a three fold increase in lipid accumulation indicating an anti-atherogenic role for this cytokine (Mallat et al. 1999). Consistent with this observation, transgenic overexpression of IL-10 in murine T cells using the human IL-2 promoter results in significant inhibition of lesion development in C57BL/6J mice fed an atherogenic diet (Pinderski Oslund et al. 1999). The potential for a genetically determined immunomodulatory role of IL-10 in humans is illustrated by the association between promoter polymorphism and immune function. For example, in haemodialysis patients receiving hepatitis B vaccination, the IL-10 (-1082) low producer genotype was found more frequently than in responders and was associated with impaired function of immune system as assessed by Girndt *et al* (Girndt et al. 2001).

In conclusion these results suggest that the IL-10 (-1082) promoter polymorphism may play an important role in facilitating the development of atherosclerosis. Primary and secondary preventive measures to minimise the complications of atherosclerosis may need to be more aggressive in individuals with a low IL-10 producer genotype.

CHAPTER-7

CONCLUSION

With the availability of sophisticated genotyping techniques, polymorphisms in the regulatory and coding regions of many cytokine genes are being identified along with their association with a range of diseases. In this thesis, cytokine gene polymorphisms in the TNF, IL-10, IL-6, IL-2, IFN-γ and TGF-β genes were examined for their influence on acute and chronic allograft rejection. The effect of these cytokine gene polymorphisms on their phenotypic expression, namely production was also examined *in vitro*. In addition, the influence of these genotypes on atherosclerotic renovascular disease was also investigated.

The effect of previously described cytokine gene polymorphisms in TNF-α, IL-10, IL-6, IL-2, IFN-γ, and TGF-β on acute and chronic rejection of renal allografts was examined. Two TNF gene region microsatellite markers TNF-a and TNF-d were also studied because of their position flanking the TNF genes and their high degree of polymorphism. Two previously described CA repeat microsatellites in the 5' flanking region of the IL-10 gene, IL-10 G and IL-10 R, were also analysed in these studies.

There was no statistical difference in the percentage frequencies of each cytokine polymorphism between the renal transplant recipients and control population. The low frequency of IL-10-1082 GG high producer genotype in the acute rejection group suggests that relatively low production of IL-10 could be a risk factor for acute rejection. The protective role of IL-10 in preventing rejection was further suggested by the reduced frequency of acute and chronic rejection in this high producer genotype. A positive association of TNF-a9 microsatellite allele and acute rejection was also noted in this study.

There was also a positive correlation between IL-10 (-1082) polymorphism and chronic allograft rejection after renal transplantation. The frequency of IL-10 (-1082GG) high producer genotype was significantly low in recipients who had chronic rejection with graft loss when compared to patients who had more than five years of graft survival.

Genotyping a group of patients with atherosclerotic renal artery stenosis showed that there was a difference in percentage frequency of IL-10 promoter polymorphism. The frequency of low producer IL-10 genotype was higher in patients with renal artery stenosis when compared to the control individuals. It must be pointed out that these individuals had evidence of atherosclerotic disease elsewhere in the vasculature as well. This result is of interest suggesting that an environment lacking endogenous anti-inflammatory factors such as IL-10 may facilitate the development of atherosclerosis. This finding gives additional support to the notion that atherosclerosis is an inflammatory disease and the graft vascular disease associated with chronic graft dysfunction may be attributable to inflammatory cytokines mediating renal injury (Ruan et al. 2003).

The results from these studies show an association between genotypic polymorphism and clinical outcomes with acute allograft rejection, chronic allograft dysfunction and atherosclerotic disease. As discussed before, promoter region polymorphism is located in the regions which are important in regulation of gene expression. A single nucleotide variant in the sequence may alter the affinity of binding of transcription factors and influence protein production by altering the rate of transcription. The other genotypic polymorphism, namely microsatellites, may be associated with differences in cytokine production by linkage disequilibrium to a locus that has a regulatory function in cytokine

production. Microsatellite alleles may have a direct effect on their gene expression. Different lengths of dinucleotide repeats in the microsatellites may also cause alteration in the conformation of DNA during the transcription and may alter the affinity of transcription factor binding sites.

In vitro experiments were performed using peripheral blood mononuclear cells from individuals with IL-10 (-1082) GG, GA and AA genotypes, stimulated using lipopolysaccharide and one way mixed lymphocyte culture. The results showed a significant increase in IL-10 protein production in IL-10 (-1082) GG genotype compared to GA and AA genotypes. A similar increase in IL-10 mRNA transcripts was also noted in these individuals confirming that this polymorphism has an influence on phenotypic expression. Investigating protein production with the TNF microsatellite a-9 allele was difficult due the low frequency of the allele in the population.

Three studies reported in this thesis suggests that IL-10 (-1082) polymorphism is associated with acute rejection, chronic rejection, and development of atherosclerosis. This polymorphism could either be in the susceptibility locus or in linkage disequilibrium with the susceptibility locus. There have been other reports confirming and contradicting the results in acute allograft rejection as discussed before (Poole et al. 2001; Sankaran et al. 1999). These differences are likely to be due to variable definitions for cases and controls in different studies, phenotypic heterogeneity of these phenomena, and differences in the genetic backgrounds of patients and controls.

It has to be appreciated that acute allograft rejection, chronic allograft dysfunction and atherosclerosis are complex diseases which evolve through multiple stages and there are interactions between several genetic and

environmental agents at each stage. It is possible that a biological link exists between environmental factors, gene polymorphism and the disease states.

Although a positive association was found between the IL-10 (-1082 GA and AA) low producer genotype and the TNF a9 microsatellite allele, we did not find an association between polymorphisms in other cytokine genes, IL-2, IL-6, TNF-α, IFN-γ and TGF-β. This does not mean, however, that there is no association between those gene products and development of disease. Only few alleles were examined and it is highly probable there are other alleles yet to be described which may be of importance.

This association needs to be evaluated by further population studies and further molecular genetic studies such as reporter gene assays to evaluate the promoter region, electrophoretic mobility shift assay to look at binding of transcription factors to the polymorphic sites and *in situ* hybridisation techniques on histological samples such as RT-PCR to evaluate expression of the cytokine at relevant time points. It is also important to explore the role of IL-10 *in vivo* using gene knockout and transgenic animal models with acute and chronic rejection and in the development of atherosclerosis although it has to be appreciated that there may be species differences in the inflammatory response patterns.

Despite the associations observed in this study between cytokine gene polymorphism and acute rejection in renal transplant recipients, the inclusion of other cytokine polymorphisms into this analysis and categorisation of patients into different rejection subgroups may give us additional insights. This would need a larger study population to withstand statistical scrutiny. Investigating each polymorphism on its own may not give any difference in production if

other functional polymorphisms that regulate their production co-exist. There have also been arguments for evaluating these gene polymorphisms as markers for pathological situations in a better characterised homogenous population matched for ethnicity, drug therapy etc. Further studies are also needed understand the functional effects of these polymorphisms.

In conclusion, the studies described in this thesis suggest an association between the presence of IL-10 (-1082 GA and AA) low producer genotype and the occurrence of acute and chronic allograft rejection as well as atherosclerotic renal artery disease. In addition, the TNF a9 microsatellite allele was found to be associated with acute renal allograft rejection. A greater understanding of these and other polymorphisms is important in understanding the variation in individual responses to pathological situations. This strategy may be of help in tailoring immunosuppression to individual transplant recipients as well as for identifying individuals at high risk for atherosclerotic diseases. Furthermore, in general, an understanding of the functional effects of these gene polymorphisms would help us, in a fundamental way, to understand the complex pathogenesis of acute and chronic allograft dysfunction.

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