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CYTOKINE PROFILES AND THEIR RELEVANCE TO

HUMAN TRANSPLANTATION

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Dedication

I would like to dedicate this thesis to Stella Birch to thank her for all of the support she has given me over the last three years. I would also like to make this dedication to my cat, Sox.

Abstract

NICOLA HELEN CARTWRIGHT
CYTOKINE PROFILES AND THEIR RELEVANCE TO HUMAN TRANSPLANTATION

The aim of this project was to develop an *in vitro* functional assay for the prediction of allograft rejection following renal transplantation. This assay was also used to study acute GVHD following identical sibling bone marrow transplantation. Lymphocyte cytokine profiles were measured by ELISA (protein secretion) and flow cytometry (cytokine expression) following mitogen stimulation and MLR. In normal individuals, considerable inter-individual variations were found in both protein secretion (IL-2, IL-4, IL-10 and IFN-γ) and cytokine expression (IL-2 and IFN-γ). Strong relationships were found between IL-2 protein and expression, IL-2 and IL-10 protein, and IL-10 and IFN-γ protein secretion. Analysis of cytokine gene polymorphisms showed no correlation between IFN-γ protein secretion, frequency or gene polymorphism.

Pre-transplant MLRs were set up between renal transplant patient/donors pairs and cytokine protein secretion (IL-2, IL-4, IL-6, IL-10 and IFN-γ) measured by ELISA. Analysis was performed to ascertain predictive factors of allograft rejection. Inter-individual variations were found for all cytokine profiles. Significant correlations were found between individual cytokine protein profiles including IL-10 and IFN-γ. In addition, a correlation was found between HLA-DR mismatching and both IL-10 and IFN-γ protein secreted in the MLR.Primary univariate analysis revealed that HLA and HLA-DR mismatching, female donor sex, MLR-stimulated IL-10, MLR-stimulated IFN-γ and spontaneous IL-4 secretion were associated with an increased risk of rejection. Multivariate analysis showed the strongest correlations for predicting risk of rejection to be female donor sex, HLA mismatching and MLR-stimulated IL-10 secretion. A combination of high HLA mismatching and high IL-10 secretion in MLR gave the highest risk of rejection (RR=25.5). Finally, cytokine secretion decreased when measured post-transplant. Prediction of graft survival could not be analysed due to the low number (n=6) of patients that suffered graft failure in the group.

Cytokine protein secretion (IL-2, IL-4, IL-10 and IFN-γ) in MLR was also studied for prediction of GVHD after bone marrow transplantation. There was a very low MLR response by all BMT pairs, therefore analysis could not be performed.

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Abbreviations

α	alpha
A°	angstrom
aa	amino acids
ACE	angiotensin converting enzyme
AD	anno domini
ADA	adenosine deaminase
ALG	antilymphocyte globulin
ALL	acute lymphoblastic leukaemia
AML	acute myeloblastic leukaemia
APC	antigen presenting cell
ATG	antitthymocyte globulin
β	beta
β2-m	beta 2-microglobulin
BMT	bone marrow transplantation
BSA	bovine serum albumin
BSHI	British Society of Histocompatibility and Immunogenetics
CD	cluster of differentiation
CGL	chronic granulocytic leukaemia
CMV	cytomegalovirus
CO$_2$	carbon dioxide
Cr	chromium
CsA	cyclosporin A

CT	cadaveric transplantation
CTL	cytotoxic T lymphocyte
CTL-p	cytotoxic T lymphocyte precursor
CV	coefficient of variation
°C	degrees celsius
dATP	di adenosine triphosphate
DB	dilution buffer
DMSO	dimethylsulphoxide
DNA	deoxyribosenucleic acid
dNTP	di nucleotide triphosphate
DTH	delayed type hypersensitivity
ECG	electrocardiogram
EDTA	ethyldiamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ESRF	end stage renal failure
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
g/l	grams per litre
GSR	graft survival rate
GVHD	graft versus host disease
H_2O_2	hydrogen peroxide
HA	histocompatibility antigen
HCl	hydrochloric acid
HLA	human leucocyte antigen

HPV	human papillomavirus
HSP-70	heat shock protein-70
HTL-p	helper T lymphocyte precursor
HVG	host versus graft
ICAM-1	intracellular adhesion molecule-1
Ig	immunoglobulin
IFN-γ	interferon gamma
IvIg	intravenous immunoglobulin
IL-	interleukin
IR	immune response
IU/ml	international units per millitre
IV	intravenous
KDa	kilodalton
kg	kilogram
LDA	limiting dilution analysis
LFA-1	leucocyte functional antigen
LMP	large multifunctional protein
LRT	live related transplantation
M	moles
MBq	mega bequerels
$MgCl_2$	magnesium chloride
MHC	major histocompatability complex
ug	microgram
ul	microlitre
um	micrometre
uM	micromole

mg	milligram
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mls	millilitres
mM	millimole
mRNA	messenger ribonucleic acid
M. vaccae	*Mycobacterium vaccae*
NaOH	sodium hydroxide
ng	nanogram
nm	nanometres
nmol/l	nanomoles per litre
NK	natural killer
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
%	percentage
PE	phycoerythrin
pg/ml	picograms per millilitre
PHA	phytohaemaglutinnin
PMA	phorbol myristate acetate
+	positive
PLT	primed lymphocyte testing
PNP	purine nucleoside phosphorylase
PTLD	post-transplant lymphoproliferative disease
R	responder

RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
S	stimulator
SDS	sodium dodecyl sulphate
Std Dev	standard deviation
TAP	transporter associated with antigen processing
TBE	tris borate electrophoresis
Tc	T cytotoxic
TCR	T cell receptor
Th	T helper
TMB	tetramethyl benzidine
TNF-α	tumour necrosis factor alpha

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Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. History of transplantation

1.1.1. Early experiments

Transplantation may be described as "the removal or partial detachment of a part of the body and its implantation to the body of the same or different individual". Accounts of transplantation date back as far as 250AD with the famous legend of Cosmas and Damian. This legend describes transplantation as one of the miraculous feats of these two medical martyrs, where they performed numerous healings until their deaths in 287AD.

Later, in 348AD, the miracle of the black leg occurred, whereby an elderly parishioner lay sleeping with a gangerous leg. It is believed that saints came and removed his leg with a saw, and replaced the destroyed tissue with a fresh leg obtained from a Moor buried that day. The man awoke to find himself free of pain and able to walk with his new healthy black leg (Kahan, 1983).

A new Western tradition of transplantation surgery arose during the Renaissance in Bologna, where the 16th century anatomist and surgeon Gasparo Tagliacozzi developed his technique for reconstruction using a flap of skin from the inner side of the upper arm. He carved the flap of skin into the shape of the patients nose and stitched it to the forehead and inner surface of the cheek, leaving a slender attachment to the arm to maintain the blood supply. After three weeks of healing, the attachment was severed. The technique is currently still in use, and is known as the Tagliacotian flap or *Italian method* (Converse and Casson, 1968).

The Scottish surgeon John Hunter (1728-1793) is also famous for his experimental procedures in transplantation. One of his grafts includes the transplant of a cocks claw to its comb. Not only did the claw survive, but actually grew towards the beak of the animal. John Hunter managed to revive the practice of transplanting teeth, which had been utilized in ancient Egypt, Greece, Rome, Arabia and pre-Columbian

America, as well as by Ambrose Pare of Paris in the 16th century (Converse and Casson, 1968).

1.1.2. Organ transplantation in the twentieth century

During the early years of this century, it had been shown that autotransplantation (where donor and recipient are the same individual) was virtually always successful and homo- (donor and recipient of the same species) and heterotransplantation (donor and recipient are of different species) were always unsuccessful. Organ transplantation has developed during the 20th century, with transplantation of organs including the kidney, liver, heart, lungs and intestine being performed.

One of the first autotransplants was performed in 1902 by Emmerich Ullmann where he removed both the kidneys of a dog, and replanted one of them. The interruption of the blood circulation did not interfere with the function of the kidney, and from this experiment, it was viewed that grafting of an organ was a possibility (Carrel, 1908). Homotransplantation using the same technique was not as successful. The recognition that blood vessel anastomosis was required in order to move major vascularised organs such as the kidney from one animal to another was made by Alex Carrel in 1902. His method has been used since that time, with few modifications (Carrel, 1908). Later, in 1936, a Ukranian surgeon, Yu Yu Voronay treated six patients with renal failure by the transplantation of a human kidney. However, these transplants were unsuccessful (reviewed by Brent and Sells, 1989). The first successful human renal transplant in modern times was performed by Hufnagel and colleagues, who, in 1946, transplanted a cadaveric kidney to the arm of a woman suffering from septicaemia. The kidney was rejected after three days (Hufnagel, 1947).

Transplantation of long-term surviving kidneys was finally put into practice in 1954 by Murray and Colleagues in Boston, Massachusetts. A kidney was transplanted between identical twin brothers, and immediate function was seen. The organ continued to function for a further eight years (Merrill, 1956). Following their success, the group had many cases of renal failure where non-identical relatives offered to donate kidneys.

1.1.3. The second set response

Although there was some success in transplantation, it was soon realised that the loss of the grafted organ was due to something quite distinct from processes such as infection or inflammation. The term "rejection" indicated a process by which the new host was refusing to grant the "right of abode" to the transplanted organ. Following these observations, during World War II, the British Medical Council focused on the problems of skin grafting. Dr Peter Medawar, a British Lebanese, was asked to work with a plastic surgeon, Dr Thomas Gibson in order to perfect skin grafting. It was noticed that if a skin graft was placed from one animal to another, it survived for about one week. Then, if a second set of skin was applied in exactly the same fashion, the second set of skin rejected in about half that period of time. This was termed the "second set response", which now has major historical importance in transplantation science (Converse and Casson, 1968).

1.1.4. Clinical immunosuppression

Since 1962, all tissue transplantations between unrelated individuals have been carried out with the patient under the influence of a chemical reagent to suppress the immune system of the recipient to the graft. Many investigators were studying whole body or whole graft irradiation as a means of immunosuppression. The breakthrough

came when Schwartz and Dameshek made clinical observations on the effect of a compound called 6-mercaptopurine on protein. They discovered that by radioactively labelling albumin given to a laboratory animal, it was possible to study its disappearance curve. It was rapidly removed from the blood system by circulating antibodies, but when the animals were given 6-mercaptopurine, the foreign protein had a normal half-life in the body fluids of the recipient (Schwartz and Dameshek, 1959).

A young surgeon, Mr Roy Calne, followed these findings by applying 6-mercaptopurine to the kidney graft model. Following the success of his experiments, a derivative of this drug, now known as azathioprine, was developed. Improved graft survival in kidney transplants was demonstrated (Calne *et al*, 1978), and organ transplantation was considered to be the treatment of choice for a wide variety of end stage diseases.

1902 First successful experimental (canine autograft) transplantation

1912 Alexis Carrel awarded Nobel Prize for describing techniques on organ grafting

1933 First human allograft kidney transplant performed by Yu Yu Voronay, Russia

1943 Rejection observed by Medawar and Gibson and recognition of "second set response"

1946 First functioning human allograft performed in Boston, USA
to 1953 First long term surviving human renal isograft performed in Paris, France

1960 Observation of effectiveness of 6-mercaptopurine in canine renal tranplantation

1962 Widespread use of azathioprine and steroids in renal transplantation

1967 Machine hypothermic perfusion of kidneys providing seventy two hours of storage

1967 Clinical introduction of anti-lymphocyte globulin for prophylaxis against rejection in Denver, USA

1968 Brain death criteria determined by committee of Harvard Medical School

1968 First prospective histocompatibility locus antigen matching of cadaver kidney recipients in Melbourne, Australia

1969 Cold storage of kidneys for twenty four hours

1978 First clinical use of cyclosporin in Cambridge, England

1981 First clinical use of mouse monoclonal anti-CD3 antibody (OKT3)

1990 First clinical use of FK506 in Pittsburgh, USA

Table 1.1. Experimental chronology of transplantation in the twentieth century (adapted from Allen and Chapman "A Manual of Renal Transplantation"; 1993).

1.2. Allogeneic renal transplantation in man

1.2.1. Introduction

Kidney transplantation is now considered the treatment of choice for a variety of end-stage renal diseases. There are several steps which have to be taken before the transplant can take place. Not all patients receiving dialysis are suitable for transplantation. Careful assessment of potential renal transplant recipients both by those who are responsible for their long term care and by the team responsible for transplantation is essential for all renal units. The patient needs to consider both the options available and to meet those involved. The process of assessment is critical to a successful outcome of the transplant.

1.2.2. Procedure

Explicit clinical criteria are applied when determining a patients' suitability for transplantation. A full pre-operative assessment is made of the potential renal transplant recipient, including a notification of full medical history eg; history of renal disease, family history, allergies etc. A physical examination is also carried out together with routine laboratory and clinical investigations, including full blood counts, cross-matching, chest X-ray and electrocardiogram (ECG). When the patient goes into theatre, he/she is anaesthetised. The donor kidney is prepared and transplanted in a heterotopic position in one or other of the iliac fossa, with vascular anastomoses to the recipient iliac vessels, and ureter anastomosis to the urinary bladder (Belzer, 1991, Hayes, 1993). Once transplantation has been performed, if the patient is in a stable respiratory and cardiovascular state, they may be transferred directly to the transplant ward where they will be monitored for cardiopulmonary and transplant function, and started on immunosuppressive therapy as soon as possible.

1.2.3. Indications for renal transplantation

The causes of end stage renal failure may include glomerulonephritis (15.7% of total) (Heaf et al, 1999, Couser, 1999), diabetes mellitus (9.5%) (Klein et al, 1999), reflux nephropathy (15.5%) (Vallee et al, 1999), polycystic disease (9.3%) (Kohno and Yunoki, 1999), hypertension (5.3%) (Dasgupta et al, 1999) and renal vascular disease (2.6%) (Christensson, 1999). According to the UK renal registry report, other causes, either uncertain (34%) or causes with no information (24%) may be associated (Ansell and Feest, 1999). Viral infections may be associated, for example, Hepatitis C has been shown to be associated with glomerulonephritis (Okada et al, 1996). Transplantation is contraindicated in the presence of malignancy or active infection. There are few situations where transplantation is not possible, and in some there are difficulties. Oxalosis represents a relative contra-indication, although this condition may be considered to be rare (Farreli et al, 1997, Gluck et al, 1998). However, it has been shown that patients with this condition may be transplanted successfully if perioperative management is applied (Allen et al, 1996). Conditions such as Fabry's Disease (Sirvent et al, 1997, Wuthrich et al, 1998) and amyloidosis (Druecke et al, 1995) may also be considered contraindications for transplantation. Although transplantation is not strictly a contra-indication in patients with renal amyloidosis, it carries high post-operative risks such as cardiac complications (Turkmen et al, 1998, Kaaroud et al, 1999). Age may also be classed as a relative contra-indication. Patients up to the age of seventy are now being regularly transplanted, although mortality in the years after transplantation is much greater, primarily due to cardiovascular disease. However, more recently, it is thought that chronological age is not a major factor in determining the short term (<five years) survival of grafts because an increase in deaths with a functioning graft is counterbalanced by a lower rejection rate in the elderly (Cameron et al, 1994, Cantarovich et al, 1994, Tesi et al, 1994, Sanchez-

Fructuoso *et al*, 1998). A worse graft survival, however, has been shown in older patients when receiving a kidney from an older donor, possibly due to a series of changes characterised by glomerular, vascular and tubular senescence, which may be aggravated by artherosclerosis, hypertension, or diabetes, which are highly prevalent in older individuals (Basar *et al*, 1999).

1.2.4. Preservation

Effective preservation is essential in all kidney transplant programmes. In the course of renal transplantation, the kidney, once removed from the donor, starts to cool towards room temperature. Damage may be caused to the organ, termed ischaemia. It is the length of ischaemia time that is said to determine the long term behaviour of the organ (Calne, 1965). This can be reduced either by continuous perfusion on a machine or by flushing with one of several appropriate solutions and then storage in ice, which will allow preservation for at least twenty four hours (Matsuno *et al*, 1996, Kozaki *et al*, 1997). Solutions used are usually citrate-based, which contain high concentrations of potassium, phosphate and mannitol. This process may cause injury to the organ, and has been reviewed that this may even be associated with decreased graft survival and/or rejection (Szabo and Heeman, 1998, Chandraker, 1999). However, despite methods employed to minimize damage to the kidney (Brasile *et al*, 1997), it remains essential that the renal transplantation is performed as quickly as possible.

1.2.5. Live versus cadaveric transplantation

The use of organs donated from live related (and unrelated) donors has been suggested as a solution to the shortage of cadaveric organ donation (Thiel, 1998). The limitation of cadaveric donation is due to the availability of cadaveric donors, which is falling due to improvements in road safety and changes in medical practice, with the

added complication of the continued refusal for organ donation (between 20 and 30%). Therefore the need continues for donation by living donors (Terasaki *et al*, 1997, Foss *et al*, 1998, Haberal *et al*, 1998, Cecka, 1999). There has been a great improvement in short-term survival in organ allografts. Recent transplant registry data shows that five year graft survival for recipients of first cadaveric kidneys has increased to over 60% (Cecka, 1998). These advances resulted from a combination of factors including more powerful and less toxic immunosuppressive drugs, better tissue matching and improved surgical techniques (Gorski, 1996). In previous years, the expected half life of a cadaveric allograft that remained functional for twelve months was an additional eight and half years, a duration that has not changed appreciably since (Turka, 1998). Donation from live related or unrelated donors can be considered an effective alternative to cadaveric donation (Alfani *et al*, 1996, 1998, Peters *et al*, 1999). Registry data also shows that graft survival at five years is higher in recipients receiving grafts from living donors compared to cadaveric donors (Cecka, 1998). Long term observations reveal that survival and function of kidneys transplanted from living-related donors are better than cadaveric kidneys (Stryjecka-Rowinska and Wataszewski, 1996, Fangmann *et al*, 1999). This may be due to several factors:

a) Better immunological compatibility between donor and recipient

b) Better pre-operative preparation of donor and recipient

c) Application of less aggressive immunosuppressive protocols

d) The possibility of immunizing the future recipient with the donors' blood

Despite a substantial increase in the number of living donor transplants performed in recent years, graft survival rates are still superior to all but HLA-identical cadaver transplants (Cecka, 1997). Allograft survival at ten years have been recently shown to be over 70% for HLA-identical sibling transplants, and 55% for other living donor transplants (eg; HLA-non-identical) and cadaveric transplants

(Cecka, 1997). Slightly earlier data also showed an improved graft survival rate at both five and ten years in live-related transplants (5=87%; 10=85%) compared to cadaveric transplants (5=75%; 10=57%; Mouquet *et al*, 1996). (Figure 1.1). Both patient and graft survival has also been shown to be up to 10% higher in live donor transplants compared to cadaveric (Recio *et al*, 1996, Cecka, 1998).

As well as the long term survival in live-related transplants being better than cadaveric, first acute rejection episodes have been shown to be lower in live related transplants compared to cadaveric (Cecka and Terasaki, 1993, Cecka, 1998, Offner, 1994). This may not primarily be due to a better HLA compatibility in live related transplants, but the higher extent of graft tissue damage in cadaveric grafts might contribute to the susceptibility to alloimmune reactions leading to more rejection episodes. A higher rate of chronic rejection has also been shown in cadaveric grafts compared to live related (Fangmann *et al*, 1999). Although the number of rejection episodes are lower in live-related transplantation, immunosuppressive therapy remains necessary for the prevention of allograft rejection.

1.2.6. Complications of renal transplantation

1.2.6.1. Technical

The majority of the serious technical complications are either vascular or urological. Renal artery or vein thrombosis occurs within the first week after transplantation, and generally within the first forty eight hours (Ghekiere *et al*, 1998). It may be suggested that this complication may not arise very often, a 5% incidence has been reported and that these are occurring due to the increased use of cyclosporin therapy (Beyga and Kahan, 1998). Presentation of thrombosis involves painful enlargement of the renal transplant, haematuria followed by graft rupture and hypotension. The transplant itself presents as being enlarged, oedematous and is
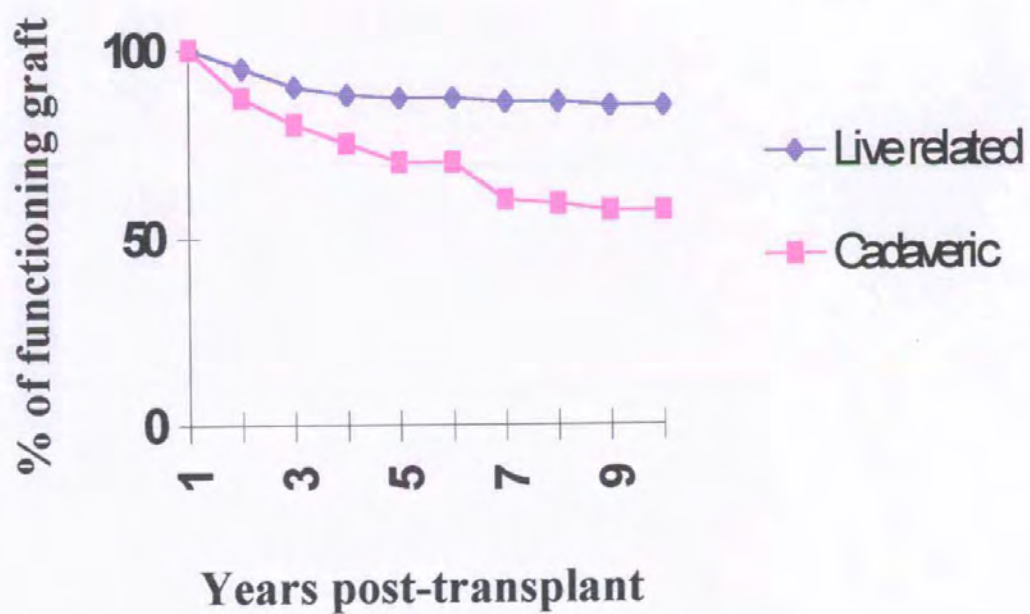
Figure 1.1. Graft survival in live related and cadaveric transplantation (data from Mouquet *et al*, 1996).

surrounded by fresh blood clots (Hussein *et al*, 1999). However, early diagnosis of vascular thrombosis may enable graft salvage by surgical or thrombolytic treatment (Ismail *et al*, 1997).

Renal artery stenosis has been reported to develop in quite a low percentage of patients of patients (Merkus *et al*, 1993, Lopes *et al*, 1998). This complication can occur anytime between three months and years after transplantation. The occurrence of this complication is technical in origin, although it may reflect initial damage during the nephrectomy or perfusion (Rigg, 1995, Preston and Epstein, 1997). In general, the patient will present with hypertension and deteriorating renal function. Surgery is usually the approach to correct this complication (Henry *et al*, 1999, Sekkarie, 1999, Dowling *et al*, 1999).

Urinary tract complications comprise of either urinary leaks or obstructions. Urine leaks (or ureteric stenoses) generally occur from the lower end of the ureter which may have necrosed during ischaemia. This ischaemia is almost inevitably related to damage to the blood supply to the ureter during removal of the kidney. The incidence of urological complications following renal transplantation is 2-10%, and most of these complications occur within the first year, leading to a deterioration of renal function (Maier *et al*, 1997). Surgery may be performed to reconstruct the ureter, as with renal artery stenosis (Insall *et al*, 1995, Peregrin and Lacha, 1998).

Obstruction of the ureter in the early period after transplantation is uncommon, but can be associated with kinking when a redundant length of ureter has been used. It may also be caused from external compression by a transplant lymphocele, which may be treated with omentorplasty/omentopexy (Bry *et al*, 1990, Ovnat *et al*, 1995). Urinary tract haemorrhages are common in the first week post-transplant, but are an uncommon cause of impaired renal function. A bleeding vessel may be found at the end of the ureter, or in the bladder wall (Jaskowski *et al*, 1987, Shoskes *et al*, 1995).

1.2.6.2. Infections

Infection of any form is common in immunosuppressed transplant recipients and has non-specific effects on transplant function. During the first month post-transplant infections are mostly conventional bacterial including wound infections, pneumonia and urinary tract infection (Schmaldienst and Horl, 1998), with the only viral infection occurring during this time being herpes simplex virus (Schlupen *et al*, 1995, Gomez *et al*, 1999).

Cytomegalovirus (CMV) is now considered to be one of the most important infectious complication after organ transplantation (Daniel, 1999). A variety of clinical manifestations such as fever, leucopenia, hepatitis, ureteritis and glomerulopathy have been described (Rao *et al*, 1994, Zhang and Chan, 1994, Moudgil *et al*, 1997, Yeung *et al*, 1998). The infection is usually treated with the anti-viral agent ganciclovir, either orally (Ahsan *et al*, 1997) or intravenously (Sancho *et al*, 1999). An association between either acute or chronic rejection and CMV infection has been reported in renal (Kashyap *et al*, 1999, Yeung *et al*, 1998) and liver transplantation (Lautenschlager *et al*, 1997, Rosen *et al*, 1998). However, there is evidence to suggest that enhancement, but not induction of rejection is associated with CMV in heart transplantation (Boriskin *et al*, 1996, Decoene *et al*, 1996).

Interestingly, some reports suggest that increased HLA class II antigen expression is associated with increased CMV infection, in particular HLA-DR7 (Blancho *et al*, 1992, Kraat *et al*, 1993, 1994), thus triggering the anti-allograft response. However, other work has suggested this not to be the case (Gomez *et al*, 1993). Several *in vitro* studies have supported the latter (Sedmak *et al*, 1994, Ng-Bautista and Sedmak, 1995, Knight *et al*, 1997, Scholz *et al*, 1997). It has also been reported that glycoproteins encoded by the CMV genome exhibit homology with both HLA class I (Bankier *et al*, 1991) and class II antigens (Fujinami *et al*, 1988, Plachter

et al, 1993). Rejection is known to precede clinical evidence of CMV, and this may be explained by the long incubation time of several weeks, with the virus being of donor origin and received within the graft at the moment of transplantation (Grundy *et al*, 1988, Lauzurica *et al*, 1992, Costa *et al*, 1994). It has also been shown in murine models that latent CMV may be reactivated by allogeneic stimulation and immunosuppression (Bruning *et al*, 1989, Bruggeman, 1993, Yagku *et al*, 1994). In contrast, there is now evidence to suggest that factors like immunosuppression may not be associated with CMV reactivation, but instead cytokines such as tumour necrosis factor (TNF) may be responsible (Koffron *et al*, 1999). It therefore appears that association with CMV infection and rejection is involved with HLA antigens and the immune response against those antigens.

1.2.6.3. Long term

Hypertension is common after transplantation and the prevalence of hypertension has increased with the use of immunosuppression (Gerhardt *et al*, 1999, Taler *et al*, 1999). Immunosuppressants like tacrolimus have been shown to induce less hypertension than drugs like cyclosporin (Canzanello *et al*, 1998, Taylor *et al*, 1999). It has been suggested that cardiovascular complications are the major cause of morbidity following renal transplantation (Kasiske *et al*, 1996, Aker *et al*, 1998, Aakhus *et al*, 1999). Drugs such as angiotensin converting enzyme (ACE) inhibitors and beta blockers are highly effective in reducing blood pressure and may help to preserve graft function in the long term (Grekas *et al*, 1996, Hausberg *et al*, 1999).

The prevalence of all types of cancer is increased in patients on immunosuppressive therapy after transplantation. The most common cancers include squamous cell carcinomas (Shafqat *et al*, 1997, Hiesse *et al*, 1997), lymphomas (Johnson and Herzig, 1999) and even cervical cancer (ter Haar-van Eck *et al*, 1995).

Human papilloma virus infections are associated with both skin and cervical cancers (Mansat-Krzyanowska *et al*, 1997, Ozsaran *et al*, 1999), whilst Epstein Barr virus infections are mainly affiliated with skin lymphomas (Chai *et al*, 1999). In the early stages of the lymphoma (termed post-transplant lymphoproliferative disease or PTLD), the B cell proliferation is polyclonal and diffuse (Tessler *et al*, 1998). This disorder is potentially reversible on reduction of immunosuppression (Balfour *et al*, 1999, Rees *et al*, 1998).

1.3. Immunosuppressive therapy

1.3.1. Introduction

In an ideal clinical situation, all organ donor-recipient pairs would be HLA-identical, and therefore the incidence of rejection would be small (de Mattos *et al*, 1999). However, this is rarely the case due to the shortage of donors combined with the difficulty of HLA matching. Therefore, donor-recipient pairs usually exhibit a degree of HLA incompatibility, and therefore acute rejection may occur (Reisaeter *et al*, 1998). Immunosuppressive therapy is used to try and prevent or reverse acute rejection by selectively abrogating the patients' reactivity to the allograft (Keown, 1998). Immunotherapy should, preferably, be donor specific and targeted to minimise the risks of non-specific immunosuppression. An ideal immunosuppressant would specifically block the activities of only the small population of antigen-specific cells actually engaged in the events of rejection, for instance, Basiliximab (Onrust and Wiseman, 1999, discussed later). There are perhaps four key needs which, if not met, could marginalise transplantation as a form of therapy: i) Achieving logical immunosuppression (striking a balance between maximizing therapeutic effectiveness and minimizing side effects) ii) overcoming chronic rejection iii) new therapeutic

targets and iv) increasing the number of organ donations. However, although the ideal immunosuppressant has not been found as of yet, great progress has been made.

1.3.2. Cyclosporin/Sandimmune

1.3.2.1. Mode of action

This drug was originally isolated from two strains of fungi imperfecti by the Department of Microbiology at Sandoz in Basle, Switzerland as an antifungal agent of limited activity. The compound increased in interest when Borel and colleagues discovered that it exerted pronounced immunosuppressive activity *in vivo* without causing myelotoxicity or other severe side effects (Borel *et al*, 1976). Following Borels' initial description of the immunosuppressive properties of this drug, it was then described to suppress rejection of vascularised organ allografts in a variety of species (Morris, 1981). Clinical trials of the drug in renal transplantation began in Cambridge in 1978 (Calne *et al*, 1979) and when cyclosporin became generally available in the early 1980's, it quickly became the major immunosuppressive drug used in organ transplantation.

Cyclosporin is a fat soluble cyclic peptide antibiotic derived from the fungus *Tolypodadium Inflatum*. Of the eleven amino acids, seven are non-methylated and one is a unique derivative of threonine (Verheyden *et al*, 1994). Due to its lipophilic properties, cyclosporin readily diffuses across cell membranes and binds to two intracellular proteins, calmodulin and cyclophilin. Calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase has been shown to be inhibited by the complex of cyclosporin and its receptor, cyclophilin (Li and Handschumacher, 1993, Ivery, 1999). Cyclosporin inhibits early stages of lymphocyte proliferation in response to a variety of activation signals. It specifically blocks the activation of not only IL-2 production (Matsuda *et al*, 1998), but also IL-2 receptor at the mRNA level (Li *et al*,

1992), thus inhibiting IL-2 gene transcription and subsequent protein secretion. The decrease in IL-2 production has been shown to cause a shift towards tolerance inducing cytokines, thus attempting to dampen down the allogeneic rejection response (Van den Berg, 1998). The drug appears to affect CD4+ T cells in particular (Fischer *et al*, 1991), as CD4 plays a major role in IL-2 induction (Oyaizu *et al*, 1992) although some effects on CTL activity, B cell differentiation and HLA antigen expression has been shown (Roelen *et al*, 1993, Vaessen *et al*, 1994)

New formulations of cyclosporin have been made, attempting to improve adsorption and decrease side effects. One such formulation is known as Neoral and has been subjected to many random double blind studies to establish its safety and tolerability (Barone *et al*, 1996, Feutren *et al*, 1996). Neoral is a novel microemulsion formulation of cyclosporin, and all studies performed have shown there to be an improved outcome with this new formulation, not only with tolerability, but also with decreased incidence of acute rejection (Keown, 1996, Warrens *et al*, 1996, Cossu *et al*, 1998).

1.3.2.2. Side effects

The major side effect of cyclosporin is nephrotoxicity, which is ironic in the sense that the clinical features distinguishing cyclosporin kidney toxicity from other causes of graft dysfunction are not clearly established (Barone *et al*, 1996). Although pharmacokinetic considerations have been taken into account in tailoring dosage of cyclosporine (Kahan, 1996), inter-individual differences in drug sensitivity can still lead to chronic nephropathy (Povlsen *et al*, 1991). This side effect has been defined as a clinicopathologic entity produced by exposure of a patient to cyclosporin (Bennett *et al*, 1996). Cyclosporin vasoconstricts the vessels of the kidney, and as these are much more sensitive to the constrictive effects of cyclosporine compared to non-renal blood

vessels, this leads to renovascular injury (Curtis, 1996). The potent vasoconstriction seen after administration is mediated by reduction in vasodilatory prostaglandins and nitric oxide, leading to subsequent systemic hypertension (Bobadilla *et al*, 1998).

Surprisingly, although this drug is used as part of an anti-rejection regimen, extensive use of cyclosporin may be one of the leading causes of long-term renal allograft failure due to its nephrotoxicity (Vanrenterghem and Peeters, 1996). It has even been shown that in some transplants, there has been an increased frequency in kidney allograft rejection due to this risk factor (Bowes *et al*, 1990), with cyclosporin monotherapy leading to increased risk of rejection compared to combined cyclosporine-steroid immunosuppression (Oppenheimer *et al*, 1994).

1.3.3. Corticosteroids

1.3.3.1. Mode of action

Corticosteroids remain one of the main classes of drugs used for induction, maintenance and rescue therapy. Steroids have been shown to lead to a better histological and functional outcome in transplant recipients (Rush *et al*, 1998). Synthetic compounds (prednisone, prednisolone or methylprednisolone) are the most widely prescribed agents, and are usually given in combination with other immunosuppressive agents (Opelz, 1995, Lafferty *et al*, 1997). Corticosteroids bind to intracellular, cytoplasmic high affinity receptors with which they form corticosteroid-receptor complexes that interact with DNA and modulate gene expression. mRNA concentrations are modified and protein synthesis is consequently initiated or blocked (Bach and Viard, 1993). Corticosteroids provide the proximal block in the T cell activation cascade. They are able to do this by interfering with T cell proliferation through blocking the activation of the IL-1 and IL-6 genes (Vella and Sayegh, 1997). The fever associated with allograft rejection is probably caused by the secretion of IL-

1, and therefore the ability of steroids to prevent fever in septic states almost certainly derives from the blocking of IL-1 release. As IL-2 release is dependent upon IL-1 stimulated IL-6 release, corticosteroids also indirectly block IL-2 production (Crabtree, 1989, Brattsand and Linden, 1996).

The anti-inflammatory effects of corticoids are probably mediated by proteins of the lipocortin family, which are synthesised by macrophages in response to natural and synthetic corticosteroids (Almawi et al, 1996). Lipocortin is an inhibitor of phospholipase A_2, an enzyme involved in transmembrane signal transduction and in the synthesis of inflammatory mediators such as prostaglandins and platelet activating factor (Flower, 1990). Corticosteroids preferentially affect CD4+ T cells, inhibiting cytokine synthesis. B cells appear to be more resistant.

Glucocorticoids have broad non-specific immunosuppressive and anti-inflammatory effects (Cohn, 1997). Glucocorticoids have been suggested to inhibit the production of Th1 type cytokines, and in turn, enhance the production of Th2 type cytokines (Almawi et al, 1998, 1999, Ramirez, 1998). Besides their effects on cytokines they reduce the migration of monocytes to sites of inflammation . A major drawback to these drugs is that they inhibit the entire immune system, which can lead to some undesirable side effects.

1.3.3.2. Side effects

Due to the initial high dose protocols, inflammatory and phagocytic capacities are decreased, resulting in increased susceptibility to infection, impaired wound healing, hyperglycaemia, increased capillary fragility, osteoporosis in adults and growth suppression in children.

1.3.4. Azathioprine (Imuran)

1.3.4.1. Mode of action

Azathioprine has been shown to be successful in reducing acute rejection episodes after transplantation (Bergan *et al*, 1998), with azathioprine monotherapy being safe and effective (Bartucci *et al*, 1999). Azathioprine is an anti-metabolite and is a derivative of 6-mercaptopurine. It has non-specific effects on the immune system, but is known to have an anti-proliferative effect on lymphocytes and exerts a toxic effect on endothelial cells after metabolism by vascular endothelium (Weigel *et al*, 1999). The main active metabolite of azathioprine, thionosinic acid, inhibits purine synthesis via a complex mechanisms involving numerous enzymes. However, T cells appear to be the major target of this drug, although, unlike 6-mercaptopurine, azathioprine inhibits proliferation by a mechanism independent of purine ribonucleotide depletion (Dayton *et al*, 1992). The imidazole ring of azathioprine is probably involved in its mode of action, perhaps through fixation of the drug to certain amino acid residues of membrane proteins. High concentrations of azathioprine tends to decrease the number of migratory cells such as neutrophils by inhibition of IL-8-activated chemotaxis (Elferink *et al*, 1997). Early reports suggest that lymphocyte blast transformation and natural killer cell activity is inhibited (Ramsey *et al*, 1984, Musiani *et al*, 1985). As a result, the number of circulating monocytes capable of differentiating into macrophages is decreased.

1.3.4.2. Side effects

The major side effect of azathioprine is myelotoxicity, leading to severe leucopenia and depression of the bone marrow. Among the other possible deleterious effects of azathioprine administration are thrombocytopenia, gastrointestinal disturbances, fever, hepatotoxicity and an increased risk of neoplasia (Heaf, 1993).

1.3.5. Tacrolimus/FK506/ProGraf

1.3.5.1. Mode of action

FK506 is a macrolide antibiotic of fungal origin which possesses similar immunosuppressive properties to cyclosporin, which although used in lower doses, may lead to an increased risk of infection (Cao *et al*, 1999). FK506 has been used since 1989 as a primary immunosuppressive agent in both adult and pediatric renal transplantation (Shapiro *et al*, 1996). This drug has been suggested to have the additional advantage over cyclosporin of permitting the tapering and, in some cases, the cessation of steroid therapy allowing for FK506 monotherapy in up to 40% of patients (Jordan *et al*, 1996). The mechanism of action is similar to that of cyclosporin, with the blockade of calcineurin-mediated T cell receptor signal transduction (Salowe and Hermes, 1998). It also has the advantage of being more water soluble than cyclosporin, and therefore is less dependent on bile salts for adsorption.

In vivo, like cyclosporin, FK506 inhibits allograft rejection, delayed type hypersensitivity reactions, and antibody production against thymus-dependent antigens. FK506 has been reported to affect the production of cytokines, including IL-4 and IL-10 (Wang, 1992), by inhibiting cytokine gene transcription. The number of IL-2 producing cells are lower with FK506 compared to cyclosporin, which may explain the higher efficiency *in vivo* (Rostaing *et al*, 1999a).

1.3.5.2. Side effects

FK506 can cause hypertension and late renal insufficiency. FK506 can also cause nephrotoxicity, similar to that of cyclosporin (Finn, 1999). One potential benefit of FK506, however, is as rescue therapy (Jordan *et al*, 1996). This study reported how FK506 was able to be used as rescue therapy of rejecting allografts under primary cyclosporin immunosuppression.

1.3.6. Mycophenolate mofetil

1.3.6.1. Mode of action

Mycophenolate is a morpholinoethyl ester of mycophenolic acid. Mycophenolate has the ability to reduce the incidence of acute rejection, in particular if used in conjunction with other therapies (Kim *et al*, 1999, Puig *et al*, 1999). The drug has a selective effect on lymphocyte activation. Mycophenolate mofetil causes the inhibition of purine synthesis, followed by the selective inhibition of the proliferation of T and B cells, which subsequently inhibits antibody formation and the generation of CTLs (Sollinger and Rayhill, 1997). Multicentre studies have shown the effect of mycophenolate on graft and patient survival, renal function etc to be similar to drugs such as azathioprine (US Renal Transplant Mycophenolate Mofetil Study Group, 1999). Studies have also demonstrated mycophenolate as rescue therapy for biopsy-proven acute rejection in patients (Sollinger *et al*, 1992, Birkeland, 1996).

1.3.6.2. Side effects

It is not classed as a nephro- or hepatotoxic drug, although it can cause some bone marrow toxicity. In studies of liver transplant patients, major side effects of mycophenolate observed included gastrointestinal problems, which usually manifest as gastritis and diarrhoea (McDiarmid, 1996, Hebert *et al*, 1999).

1.3.7. Monoclonal antibodies

1.3.7.1. Mode of action (OKT3)

These drugs offer a more sophisticated approach to immunosuppressive protocols. The constant domains of the T cell receptor (TCR) complex is directly targeted for therapy by the use of monoclonal antibodies. OKT3 is a monoclonal antibody which is directed at the CD3 antigen on the surface of the T cell. This acts as

a transducer of signal from the T cell receptor, in conjunction with co-stimulatory molecules, to intracellular activation events (Alegre *et al*, 1995, Kawaguchi and Eckels, 1995). When the agent binds to the TCR, each of the latter's six proteins is stripped from the cell surface, either by shedding or internalization. OKT3-treated T cells therefore become literally blinded to the antigens of the allograft, and the rejection process comes to a halt. Studies have undertaken experiments and have suggested that the mechanism of inter-T cell bridging occurs, and therefore leads to cell mediated cytolysis (Wong *et al*, 1990, Lwin *et al*, 1995) The CD3+ T cells rapidly disappear from the circulation (Chatenoud *et al*, 1990, Reinke *et al*, 1997). T cells bearing the usual array of cell surface molecules ie; CD2, CD4, CD8 reappear but these cells are devoid of the CD3 molecule, therefore become immunoincompetent (Callait-Zucman *et al*, 1990, Norman, 1995). Anti-CD3 monoclonal antibodies, together with anti-CD2 antibody, have been shown to induce alloantigen specific tolerance (Punch *et al*, 1998). OKT3 is a powerful immunosuppressant and if used for the treatment of steroid-resistant allograft rejection, the drug has been shown to reverse a high percentage of acute rejection episodes (Jogose *et al*, 1997), although this is not the case in every study (Rowe *et al*, 1994). Much interest has been shown in targeting particular antigens or receptors as selective immunosuppressive therapy. One recent approach has been to target the IL-2 receptor, which is upregulated during a rejection response.

1.3.7.2. Mode of action (anti-IL-2 receptor)

Humanized monoclonal antibodies that recognize the alpha (α) chain of the IL-2 receptor have been used to prevent allograft rejection, since the chain is expressed by T cells participating in allograft rejection but not by resting T cells. The high affinity IL-2 receptor is composed of three distinct membrane components, including the 55

KDa IL-2 receptor-α chain, CD25 (Taniguchi, 1992, Minami et al, 1993). Antibodies to the IL-2 receptor inhibit proliferation of antigen-activated T cells and prevent the generation of cytotoxic T cells (Yasuda et al, 1998). Examples of these types of drugs include Daclizumab (Waldmann and O'Shea, 1998, Nashan et al, 1999) and Basiliximab (Nashan et al, 1997, Onrust and Wiseman, 1999). The approval of the new antibody was based on evidence that its use could reduce the rate of rejection episodes to about 20% (Auchincloss and Wood, 1998).

Although anti-IL-2 receptor directed therapy has been used with considerable success, there are limitations. Antibodies to the IL-2 receptor alpha subunit do not inhibit IL-2 mediated activation of natural killer (NK) cells. In their resting state, NK cells do not express IL-2 receptor alpha but do express the other subunits, IL-2 receptor-β/IL-15 receptor-β and γ (Waldmann and Tagaya, 1999). Therefore, these antibodies do not inhibit the action of IL-15. Anti-IL-2 receptor α antibodies alone do not provide the complete immunosuppression of T cell function that could be achieved if the actions of all of the cytokines that stimulate T cells were inhibited. A recent trial however, has attempted to administer an anti-IL-2 receptor drug which is specific for all three subunits, and was shown to be capable of suppressing the induction of CTLs and NK cells, resulting in prolongation of graft survival (Yasuda et al, 1998).

1.3.7.3. Side effects

In particular for OKT3, within a few hours of the first dose, fever, chills, and general malaise can be seen. This response is thought to be due to the release of TNF from lysed T cells, with toxicity increasing with drug dosage (Parlevliet et al, 1995, Richards et al, 1999). This is usually treated with intravenous anti-histamines. The major side effect is an influenza-like syndrome (Embrey and Geist, 1995). Nausea may also be suffered.

OKT3 has been used as an induction regime as a cyclosporin sparing drug, although this has been proved to have no long term benefit. This approach provides a window of immunosuppression until graft function is established, followed by the introduction into the therapeutic protocol (Vanderwerf, 1996).

1.3.8. Polyclonal antibodies

1.3.8.1. Mode of action

Due to the frequency and severity of adverse events as well as high costs of these drugs, they are only usually applied after treatment with methylprednisolone (Hilbrands *et al*, 1996). Polyclonal antilymphocyte globulin (ALG) or antithymocyte globulin (ATG) preparations available for use in organ transplantation since the 1970's, have proved effective for reversing renal allograft rejection (Burdick, 1986, Thibaudin *et al*, 1998). ALG therapy has even been shown to be more effective that OKT3 when used concomitantly with cyclosporin (Grino *et al*, 1992). Such polyclonal immune globulins are obtained by injecting animals (eg; horses or rabbits) with human lymphoid cells and then separating the resulting immune sera to obtain purified gamma globulin fractions. Polyclonal immune globulins represent a heterogeneous group of antibodies, only a minority of which are specific to T cells.

There are several possible mechanisms by which globulins may exert an immunosuppressive effect. These include classic complement mediated lysis of lymphocytes, resulting in the decrease in T cell numbers (Strom, 1994, Lange *et al*, 1999). The specific antibodies bind to lymphocytes, and this usually results in a prompt and profound lymphopenia. In short, the resolution of cell-mediated graft rejection results from the elimination of circulating T cells (main cellular target), and the subsequent inhibition of proliferative responses maintains the immunosuppressive effect (Burdick, 1986).

1.3.8.2. Side effects

As mentioned, because the specific antibodies bind to lymphocytes, lymphopenia may occur. Fever, chills, rash and increased susceptibility to infection may also occur (Min and Monaco, 1991). Anaphylactic shock may occur in a few cases due to the formation of xenogeneic serum protein antibodies, but this is extermely rare. Minimising the toxicity of these antibodies is being discussed (Pascual *et al*, 1996).

1.3.9. Multiple drug protocols

A regimen of azathioprine and prednisone in combination was the standard treatment for recipients with organ allografts prior to the introduction of cyclosporin. Almost all protocols have included adjunctive steroid treatment. Triple therapy is now used, consisting of cyclosporin, prednisone and azathioprine and has been shown to be more beneficial to the patient (Pfaff *et al*, 1996). Quadruple therapy has also shown to be beneficial after renal transplantation (Hiesse *et al*, 1999). Results have shown quadruple therapy to be more effective at reducing the incidence of allograft rejection compared to triple therapy (Cantarovich *et al*, 1998). One practical advantage of multiple drug therapy is that it permits more flexible immunosuppression. The dosage of the individual components can be altered to minimize complications or adverse effects while maintaining adequate overall immunosuppression.

The use of intravenous immunoglobulins (IvIg) have recently come into use in patients receiving the basic cyclosporin regimen either for prevention of infectious complications or as an additional prophylaxis of GVHD. The addition of IvIg has been shown to cause a cyclosporin sparing effect, therefore leading to a possible combined immunosuppressive protocol (Schanz *et al*, 1996). In another study, IvIg was used as rescue therapy of renal allograft recipients on the standard cyclosporin protocol

(Casadei *et al*, 1997). It revealed that 84% of the patient sample biopsies obtained after intravenous Ig treatment showed no evidence of rejection. However, IvIg may induce adverse effects such as hyperviscosity and renal insufficiency (Nydegger and Sturzenegger, 1999). The exact mechanism of IvIg action is still unknown, but it may involve anti-idiotypic antibodies that are present. These anti-idiotypic antibodies block anti-HLA antibodies, which may explain the ability of IvIg to reverse a positive cross-match.

Other combinations of drugs have been investigated to attempt to improve the clinical outcome, including a combination of anti-CD4 and anti-IL-2 receptor therapy in addition to the usual regimen (Mousson *et al*, 1995, Racadot *et al*, 1995). Even adding additional drugs to further block the calcium channel along with cyclosporin have been attempted, with no significant improvement (Citterio *et al*, 1996). In conclusion, immunosuppression significantly improves both patient and graft survival, although a drug which is able to completely inhibit the rejection response has not yet been found.

1.4. Graft rejection

1.4.1. Introduction

The immune response to an allograft (a graft between animals of the same species, but of different genotype) will almost invariably result in rejection if not prevented by the appropriate treatment (Morris, 1989). Not only are grafts lost in the early weeks or months after transplantation, but in surviving grafts the lesions frequently caused by early rejection reactions may lead to permanent damage and to a gradual deterioration of function, months to years later (Morris, 1989).

Rejection remains the major cause of transplant failure regardless of the tissue or organ grafted. Numerous host, donor and clinical factors can affect the level and

type of reactions to allografts (Sanfillipo *et al*, 1984). In organ transplants such as kidney transplantation, functional decline is usually the first sign of a rejection reaction. It is important to establish a prompt diagnosis either of rejection or of any unrelated complication that might be the cause of deteriorating function, so that prompt and appropriate treatment can be commenced to reverse the process. The principles of diagnosis are essentially a deterioration of organ function accompanied by appropriate histological changes of rejection.

Several types of rejection have been recognised for many years, namely hyperacute, acute and chronic. These terms are essentially based on the timing of the rejection and the immunological mechanisms causing the rejection.

1.4.2. Hyperacute rejection

Hyperacute rejection may be seen within minutes to hours after surgery. The kidney may become swollen and cyanotic, with renal circulation therefore being compromised. Within an hour of revascularization, the kidney becomes blue and flabby, and if a biopsy was taken, there would be evidence of widespread polymorphonuclear leucocyte infiltration (Wada *et al*, 1998). Subsequent glomerular and arterial thromboses would also be evident (Bakir *et al*, 1996). Histologically, severe vascular damage is typical and includes thrombosis (as mentioned), congestion and necrosis.

1.4.3. Acute rejection

This is the most frequent type of rejection seen after kidney transplantation. Acute rejection may develop between one week and three/six months post-transplant. Since the era of cyclosporin A, reactions may now be seen within two to four weeks of transplantation and manifest by a moderate rise in serum creatinine (Castro *et al*,

1998) and often, but not always, a moderate fever (Toogood *et al*, 1994), usually with some swelling and tenderness of the kidney (Chung *et al*, 1991). What now complicates the diagnosis of rejection is the acute cyclosporin nephrotoxicity that can become evident at the same time. As can be seen in Table 1.2, cyclosporin nephrotoxicity may also be associated with a rise in serum creatinine. Cellular infiltration of the graft is the most characteristic histopathological feature of the acute process (Vanrenterghem, 1995). As infiltration can also occur in well functioning grafts, it has been determined that it is the *degree* of cellular infiltration that provides a diagnostic indication of rejection (McWhinnie *et al*, 1985). This has been used to distinguish between clinical and subclinical rejection (Grimm *et al*, 1999). Cellular infiltration is discussed later. When inflammation is widespread and prolonged, blood flow is compromised and ischaemic tissue damage results, therefore acute graft rejection causes many of the pathological signs that is characteristic of tissue ischaemia (Orosz and VanBuskirk, 1998). Numbers of acute rejection episodes have been shown to among the best predictors of allograft survival (Tesi *et al*, 1993). Unusually, in this study, traditional measures of immunological risk eg; HLA mismatches and panel reactive antibodies did not correlate with long term graft survival. HLA mismatching has been widely implicated as being a significant contributing factor towards increased acute rejection (discussed later). A more recent study has shown that in adult renal transplant recipients, the co-variate that best predicts the impact of acute rejection on graft survival is not the number of acute rejection episodes, but instead the impact of acute rejection on graft function (Cosio *et al*, 1997). Recent work has also shown there to be a significant association between delayed graft function and acute rejection (Troppmann *et al*, 1995), and that acute rejection remains a risk factor not only associated with the allograft outcome (Peeters

	Acute Rejection	CsA Toxicity
Serum creatinine	Elevated	Elevated
Urine output	Falling	Unchanged
Temperature	Elevated	Normal
Graft	Swollen and tender	Unchanged
Biopsy	Cellular infiltrate	Normal or minimal cellular infiltrate
	Oedema	
	Vascular changes: fibrinoid necrosis interstitial haem thrombosis	Vacuolization of tubules
		Giant cell mitochondria
		Arteriolar thrombosis
Immunohistology	HLA class II induction	Normal class II expression
Cyclosporin trough levels	Low	High

Table 1.2. Distinguishing features of acute rejection and cyclosporin nephrotoxicity (information adapted from Morris, 1989).

et al, 1995) but also for incidence of chronic rejection (Matas, 1998, Matas *et al*, 1999).

1.4.4. Chronic rejection

Chronic rejection is the designated term for the slow, progressive and uncontrollable loss of renal transplant function. This stage presents as insidious deterioration in renal function at any time from six months to many years post-transplant, and there is little or no response to additional treatment with high dose steroids. The histological picture shows interstitial fibrosis, marked intimal fibrosis of arteries and arterioles and minimal to moderate cellular infiltration (Hayry, 1996). Studies in rat models have shown cellular infiltration to include macrophages by preventing changes in the pathophysiology of chronic rejection by using specific macrophage inhibitors (Leendert *et al*, 1992, Heemann *et al*, 1993, Azuma *et al*, 1995, Ishibashi *et al*, 1995). Macroscopically, the surface of the kidney is pale, and with increasing time after transplantation, the kidney becomes smaller. Risk factors for the incidence of chronic rejection have been discussed (Azuma and Tilney, 1994), including both immunologic and non-immunologic factors (Mourad *et al*, 1996, Kasiske, 1997), including ischamia time and re-perfusion. Previous studies have implied, however, that the main cause of chronic rejection is the extensive damage caused by earlier episodes of acute rejection (Almond 1993a, 1993b, Koskinen *et al*, 1996, Matas *et al*, 1999).

Cyclosporin toxicity has also been linked to chronic rejection (Bennett *et al*, 1996, Pascual *et al*, 1998). The histopathological lesions of chronic CsA toxicity share similarities with that of chronic rejection, although chronic CsA toxicity predominantly affects small vessels, whereas rejection affects larger arteries (Curtis, 1996). General criteria used to classify graft rejection may be found in Table 1.3.

1.5. Graft versus host disease

1.5.1. Introduction

Graft versus host disease (GVHD) is the clinical manifestation of the graft versus host reaction. Human GVHD is composed of two distinct clinical entities, acute and chronic GVHD, with both of these conditions having a different pathogenesis (Parkman, 1998). There are three requirements for the development of GVHD:

1. The graft must contain immunocompetent cells capable of recognising and reacting against host antigens

2. The host must be immunocompetent and incapable of rejecting the donor graft

3. Alloantigenic differences must exist between donor and recipient, and these must be capable of inducing an allogeneic response.

These criteria were first described more than thirty years ago in lethally irradiated mice given homologous (allogeneic) spleen cells (Billingham, 1966). Human GVHD was first observed in patients with primary immunodeficiences following whole blood or maternal-fetal transfusions which led to the patients' death (Walknowska et al, 1969).

Factors involved in GVHD have been investigated both in animal (Shenoy et al, 1998, Drobyski et al, 1999) and human studies (Przepiorka et al, 1999, Quaranta et al, 1999, Liem et al, 1999). Extensive fibrotic changes in the skin is characteristic for GVHD, and is known to be continuing major complication in transplantation, especially with the increasing use of related and mismatched donors (Vogelsang and Hess, 1994).

It is now felt that GVHD is more complex than simple alloreactivity and is now suggested that even cytokines may play a role in mediating many of the clinical and experimental manifestations of GVHD (Jadus and Wepsic, 1992, Ferrara et al, 1993, Krenger et al, 1997, Ferrara and Krenger, 1998).

1.5.2. Acute GVHD

Acute GVHD can manifest within the first one hundred days post-transplant. Acute GVHD is produced by the attack of donor immunocompetent T or null cells against recipient histocompatibility antigens. It is characterised clinically by dysfunction of the skin, liver and gastrointestinal tract (Ghosh *et al*, 1998). The most frequent clinical manifestation is an erythrematous skin rash (Moreb *et al*, 1997). In more severe cases, generalised erythroderma can occur (Symington and Storb, 1989, Suzuki *et al*, 1993). Liver involvement is manifested by hepatocellular enzymes and an increase in alkaline phosphatase. GVHD of the gastrointestinal tract presents as diarrhea. Cells, presumably of donor origin are found in the epidermis, with epithelial cells displaying signs of cellular damage including basal cell vacuolization and dyskeratosis, thus supporting the hypothesis that acute GVHD is a lymphocyte mediated disorder (Kohler *et al*, 1997).

Acute GVHD is usually graded (grades 0-IV) by the pattern of organ involvement using the classic Glucksberg-Seattle criteria (Glucksberg *et al*, 1974). A new severity index has now has been developed by re-grouping the patterns of organ involvement into five indeces, O, A, B, C and D (Martino *et al*, 1999). These can be seen in Table 1.4. O represents no acute GVHD, grades A and B represent the early stages of GVHD (equivalent to the former grades I-II). Grade A is usually mild, with grade B being moderatley severe. Grades C and D represent the advanced stages, equivalent to former grades III and IV, with C representing severe multi-organ GVHD, and D being life-threatening.

Classification	Kinetics	Target Structures	Immune Mediators	Inflammatory Pattern
1. Hyperacute	Minutes/hours post-transplant	Vascular (large and small)	Humoral	Fibrinoid necrosis granulocytic infiltration
2. Acute:	Rapid onset	Vessels (large and small)	Humoral, cellular	Monocyte, Granulocytic, Mononuclear cell infiltrate
3. Chronic:	Late post-transplant	Vessels (large and small)	Cellular, humoral	Ischemic damage

Table 1.3. Classification of rejection.

1.5.3. Chronic GVHD

Chronic GVHD is caused by immunocompetent cells that differentiate in the recipient (Parkman, 1998). Chronic GVHD is a multi-organ, autoimmune-like disorder with clinical manifestations including malar erythema, photosensitivity, oral ulcers, polyserositis and sicca (Sjorgen's) syndrome (Sullivan et al, 1981, Sherer and Shoenfeld, 1998). Chronic GVHD can be classified as limited or extensive. Limited chronic GVHD involves localised skin involvement or hepatic dysfunction due to chronic GVHD. Extensive chronic GVHD involves either i) Generalized skin involvement ii) Localized skin involvement and/or hepatic dysfunction due to chronic GVHD, plus positive liver histology, eye biopsy, positive salivary gland or oral mucosa biopsy or involvement of any other target organ (Shulman et al, 1980, Woo et al, 1997). If untreated, after six to eighteen months, dermal thickening, scleroderma and joint contractures will develop (Chosidow et al, 1992). The absence of clinical and pathological renal disease resembling lupus or scleroderma kidney is another significant difference. Although chronic GVHD features common to naturally occurring autoimmune disorders, several differences can be clearly defined.

1.6. Overview of the immune system

1.6.1. Introduction

Transplant rejection occurs due the recognition of foreign molecules and activation of various components of the immune system. The immune system in mammals has evolved to protect the host from pathogens and is dependent on the ability to recognise foreign molecules, especially proteins. The immune system has two functional divisions that are involved in a normal reaction to foreign antigens, non-specific (innate) immunity and specific (acquired) immunity. The two

Grade	Skin	Liver	Gut	Functional impairment
0 (none)	0	0	0	0
A (mild)	+ to ++	0	0	0
B (moderate)	+ to +++	+	+	+
C (severe)	++ to +++	++ to +++	++ to +++	++
D (life - threatening)	++ to ++++	++ to ++++	++ to ++++	+++

Table 1.4. Severity index developed by the IBMTR (Martino *et al*, 1999)

systems work in unison, and it is known that many cellular and soluble components function in both systems (Carroll and Prodeus, 1998). Innate immunity is the initial non-specific response to tissue injury produced by the body in response to stimuli. Acquired immunity is a mechanism provided for the body to selectively detect and eliminate foreign antigens. It could be said that activation of innate immunity is a prerequisite for an adaptive immune response (Parish and O'Neill, 1997). Antigens may either be exogenous eg; pathogens or endogenous eg; damaged cells. A response is provoked when the body detects components recognised as "non-self". Acquired immunity consists of a humoral component (antibodies) and a cellular component (lymphocytes).

1.6.2. Cellular effectors of the immune system

The cells involved in the immune response are derived from the bone marrow, probably from a common precursor. These include the granulocytes ie; neutrophils, eosinophils and basophils, the mononuclear cells ie; monocytes/macrophages and the lymphocytes ie; B cells, T cells and NK cells. The different cell types can be determined by cell surface markers used to divide the cells into different phenotypes. These cell surface markers have been conventionally defined by CD (cluster of differentiation) numbers as agreed by 6th International Worshop (Sixth International Workshop and Conference on Human Leucocyte Differentiation Antigens, 1996).

1.6.3. Granulocytes

Polymorphonuclear granulocytes mainly consist of neutrophils (95%) which are released from the bone marrow at a rate of approximately seven million per ml of blood. Granulocytes make up about 70% of the total blood leucocytes, and like

monocytes (discussed later), can adhere to endothelial cells lining the blood vessels and extravasate by squeezing between the endothelial cells to leave the circulation (Jagels *et al*, 1999). This process is known as diapedesis.

1.6.3.1 Neutrophils

These cells are involved in acute inflammation. They possess phagocytotoic activity and are granular. These granules contain substances such as lysozyme, antibiotic proteins, and myeloperoxidase (Tapper, 1996). They exhibit chemotactic activity and express high levels of receptors for a family of chemoattractants known as chemokines eg; IL-8 (Siddiqui *et al*, 1999). Products from the complement system, products of bacteria and factors from fibrinolytic systems may act as chemotactic agents. Chemotactic stimuli results in neutrophil margination (adhesion to endothelial cells) and diapedesis.

1.6.3.2. Eosinophils

These cells comprise approximately 2-5% of blood leucocytes. They also exhibit phagocytotic activity. Their mode of activity may be by release of their granular content into the surrounding area or by production of toxic reactive oxygen intermediates (Fabian *et al*, 1992). Eosiniophils are attracted by chemotactic factors such as ECF-A (eosinophil chemotactic factor of anaphylaxis), which is released from T cells, mast cells and basophils. Eosinophils also release histaminase, which inactivates the mast cell products histamine and the slow reactive substance of anaphylaxis (SRS-A) (Abelson et al, 1995).

1.6.3.3.Basophils and mast cells

Basophils are found in very small numbers in the circulation, approximately making up 0.2% of the leucocyte population. This cell type is indistuinguishable from the mast cell in a number of properties.

Mast cells may be found near blood vessels in all connective tissues, and contain randomly distributed granules which contain SRS-A, histamine and ECF-A. The stimulus for basophil or mast cell degranulation is IgE-mediated and is enhanced by cytokines such as IL-4 (Bischoff *et al*, 1999). Degranulation can only be effective if allergen (an antigen causing an allergic reaction) cross-links IgE molecules bound to the surface of the cell via its high affinity Fc receptors for IgE (FcεRI). Mediators of allergy such as histamine are released by degranulation.

1.6.4. Monocytes/Macrophages

Macrophages mature from circulating monocytes. Macrophages may be found in the tissue or as migratory cells. They secrete inflammatory intermediates including nitric oxide. Macrophages secrete a variety of pro-inflammatory cytokines, including tumour necrosis factor alpha (TNF-α, IL-1 and IL-6), with these cells being involved in the early stages of inflammation known as the acute phase response (Koj, 1996).

As with dendritic cells, macrophages are able to express HLA class I and class II molecules (Laupeze *et al*, 1999). This only occurs during an inflammatory reaction, so that autoimmunity does not develop.

1.6.5   Endothelial cells and adhesion molecules

The main immunological role of the endothelium is to aid the migration of cells and proteins to the sites of inflammation. The movement of leucocytes from the

blood circulation into sites of inflammation requires co-operative interaction between signalling and adhesion molecules (McEver, 1992). The selectin family (L-, P- and E-selectin) mediate the initial rolling contacts of leucocytes with the endothelium. Following activation, integrins (CD11, CD18) strengthen adhesion by binding to members of the immunoglobulin super gene family-intercellular adhesion molecule (ICAM)-1 and ICAM-2 (Malik, 1993).

The other important adhesive interaction between leucocytes and the endothelium involves binding of selectins to their endothelium carbohydrate counter-receptors on leucocytes. The latter molecules also facilitate transendothelial migration, diapedesis and migration along connective tissue (Penberthy et al, 1997). As well as adhesion molecules, endothelial cells also express cytokines including IL-1, IL-6 and IL-8 (Krishnaswamy et al, 1999). Adhesion molecule expression may be induced by cytokines such as IL-1 and TNF-α (Krishnaswamy et al, 1999), transcription factors like NF-kappa-B (NF-κB) and AP-1 (Ledebur and Parks, 1995) and lipopolysaccharides (Fries et al, 1993).

1.6.6. Lymphocytes

The lymphocyte population consists of NK cells and null cells, with the two main kinds of lymphocytes being T and B cells. T and B cells express antigen receptors and other surface molecules or markers important for their different functions.

1.6.6.1. T cells and the TCR

The T cell population is primarily derived from the bone marrow and matures in the thymus, which has recently been shown in adults (Poulin et al, 1999). Discrimination from self and non-self antigens and HLA molecules is achieved by

negative and positive selection (von Boehmer and Kisielow, 1990). T cells may be divided into two subsets, CD4 and CD8. Cells expressing CD4 cell surface marker are known to recognise peptide associated with HLA class II molecules. CD8+ T cells are known to recognise peptide associated with HLA class I molecules (Krensky, 1997). This difference in expression allows the differentiation of T cells into their two major subsets. CD4 cells function as helper cells while CD8 cells function as cytotoxic cells. The main pan T cell marker is, however, CD3.

T helper cells (CD4+), after recognition of a HLA class II associated peptide, undergo clonal proliferation. These clones activate B cells, cytotoxic T cells (CD8+) and other effector cells by the secretion of soluble mediators known as cytokines. T helper cells may be divided into two subpopulations, T helper (Th) 1 and Th2, which are determined by the secretion of certain cytokines (Mossman and Coffman, 1989) (discussed in detail later). Th1 responses are associated with the activation of a cell mediated immune response. This, in turn, activates cells such as macrophages and cytotoxic T cells. Th2 responses activate a B cell, and an anti-inflammatory response. Cytotoxic (CD8+) T cells, on association with HLA class I, produce IL-2 and stimulate the T cell surface molecule, CD28 (Hornell *et al*, 1999). The CTLs initiate apoptosis and lysis of altered cells by the activation of soluble effector mediators.

T cells recognise antigen by their heterodimeric T cell receptor (TCR). This receptor always occurs in close association with the CD3 complex of molecules which are important in intracellular signalling. The TCR recognises short peptides in association with HLA molecules, and due to significant structural differences in the TCR between different T cell clones, it has a wide range of specificity for different peptides (Litman *et al*, 1999). Like immunoglobulin (section 1.6.6.2), the TCR consists of α and β chains (or γ and δ chains), which are linked by disulphide bonds, which has been analysed by X-ray crystallography (Fields and Mariuzza, 1996,

Garcia *et al*, 1996, Garboczi *et al*, 1996). The genes encoding these chains undergo similar rearrangement as immunoglobulin during T cell maturation which leads to a diverse family of TCRs (Shortman and Wu, 1996).

1.6.6.2. B cells and immunoglobulin

B cells mature in the bone marrow and are defined by the presence of immunoglobulin on their surface. B cells also express HLA class II on their surface, which is important for interactions with T cells, and therefore may act as antigen presenting cells (APC). Immunoglobulin is constitutively produced and are inserted into the cell surface membrane, where they act as specific antigen receptors. The majority of human B cells in peripheral blood express two isotypes, IgM and IgD, and when mature, have been shown to migrate back to the bone marrow (Paramithiotis and Cooper, 1997). Interaction between antigen and receptor induces proliferation of both plasma and memory B cells, and the activation of kinases and the phosphorylation of proteins. It is the plasma cell population that can produce the soluble form of immunoglobulin (antibody). CD19, CD20 and CD22 are the main markers currently used to identify human B cells (Paloczi *et al*, 1998).

Diversity and heterogeneity are the characteristic features of immunoglobulin molecules. Like the TCR, there is extensive gene rearrangements which generate immunoglobulins capable of recognising many different antigens (Rao *et al*, 1999). The basic structure of all immunoglobulin molecules is a unit consisting of two identical light polypeptide chains and two heavy polypeptide chains containing variable and constant regions. The chains are linked by disulphide bonds. The class and subclass of immunoglobulins are determined by its heavy chain.

Immunoglobulins are a complex multigene family of proteins specified by genes encoding variable (V), diversity (D), joining (J) and constant (C) domains

(Marchalonis *et al*, 1993). Diversity in immunoglobulin antigen receptors is generated in part by V(D)J recombination (Sollbach and Wu, 1995). Recombination activation genes (RAG)-1 and RAG-2 gene products are known to be responsible for the activation of V(D)J recombination (Silver *et al*, 1993). Immunoglobulins are also known to have the ability to switch classes or isotypes in the presence of external factors eg; in the presence of IL-4, IgM may class switch to IgE (Hikida *et al*, 1999).

1.6.6.3. Natural killer (NK) cells

NK cells belong to a group known as null cells whereby neither T nor B cell markers are expressed. NK cells may become activated during an immune response via the secretion of cytokines including IL-2 and IL-12. These cytokines are thought to activate NK activity by enhancing the expression of lymphocyte-specific chemokines called single cysteine motif (SCM)-1α and SCM-1β (Hennemann *et al*, 1999). Human NK cells express receptors for HLA class I encoded by the killer cell inhibitory receptor (KIR) gene family present on chromosome 19q13.4 (Lanier, 1998). If the KIR receptor recognises HLA class I molecule, this then suppresses NK activity, hence preventing autoimmunity. If HLA class I expression is lost, then cytotoxicity may occur (Lopez-Botet *et al*, 1995). Functional studies of NK cells have revealed that in addition to KIR, another type of receptor is responsible for recognition of HLA class I (Houchins *et al*, 1997). This receptor, designated CD94/NKG2, is a heterodimer composed of a CD94 glycoprotein that is disulphide bonded to an NKG2 subunit. NK cells also express a number of activating receptors, which are regulated by the inhibitory receptors. These include p46, which is known to cooperate with other triggering receptors to induce NK activity (Sivori *et al*, 1997).

1.7. The major histocompatibility complex

1.7.1. Introduction

The major histocompatibility complex (MHC) is a set of genes found in all mammalian species. It encodes for the expression of a group of cell surface molecules, termed the human leucocyte antigen (HLA) system in humans. This system plays an important role in the initiation of the immune response and also in rejection responses in allograft transplantation.

1.7.2. History

The definition of the MHC locus of man, HLA, is intertwined with the evolution of typing and crossmatching for human blood donor selecton. The work of Landsteiner during the first four decades of this century with erythrocyte ABO and rhesus antigens was a necessary basis for blood banking and blood transfusion which came into extensive use during and after World War II (Landsteiner, 1901). The development of blood transfusion contributed to progress with the problem of graft rejection in three ways. Firstly, the A and B erythrocyte antigens are widely distributed in tissues and are transplantation antigens that must be considered in the selection of tissue and donor organs. Second, by analogy with typing and cross-matching for blood donor selection, one of the major approaches to the problem of graft rejection has been tissue compatibility testing. Thirdly, the serum of patients who have received multiple blood transfusions frequently contains antibodies to human leucocytes, now known as HLA antibodies.

Some of the first experiments to describe transplantation antigens were performed by Gorer and colleagues (1937), in which he illustrated the genetic basis of tumour rejection in mice, with the identification of anti-sera specific for MHC antigens. He discovered an erythrocyte antigen present on both normal and tumour

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cells, which was associated with tumour rejection. This was named "antigen II".
Later, in 1944, Medawar and colleagues illustrated the classical study in which it was
shown that the intradermal injection of buffy coat evoked homograft sensitivity
(Medawar, 1944). Seventeen years later, Rapaport and colleagues confirmed these
findings in man (Rapaport *et al*, 1961). In 1948, Snell and colleagues described the
histocompatibility system in mice. In this work, he showed that tumours will grow
progressively in mice carrying certain dominant genes present in the stock of origin
(Snell, 1948). In these experiments, he discovered a locus which determined
allogeneic tumour graft rejection. He termed this the H-locus. This locus was then
found to be responsible for the coding of antigen II previously discovered (Gorer *et
al*, 1948). Medawar and colleagues had previously described that allografts were
rejected as a result of an immune event (Medawar and Gibson, 1943). The suggestion
that graft rejection was due to cell-mediated immunity was strengthened by the
subsequent work of Mitchinson. Using a system now known as 'adoptive transfer',
he transferred lymphocytes from previously sensitized recipients into non-immunized
animals. The results of these experiments showed that specific immunity could be
transferred with cells from lymph nodes draining from the grafted tissue (Mitchinson,
1954).

The serological identification of transplantation antigens began in 1952 when
Dausset discovered a leucocyte antigen responsible for transfusion reaction (Dausset,
1954). These findings prompted a more extensive study, where sera of great
specificity were prepared by serial transfusions into normal male recipients, using
graded doses of blood from the same donor. The recipients produced high titres of
leucocyte antibodies which agglutinated leucocytes from the blood donor as well as
cells obtained from randomly selected individuals. This pioneering work of leucocyte
grouping was confirmed and extended by many others, notably by Payne (1957) and

van Loghem *et al* (1958). The hypothesis of the "second set response" was put forward (section 1.1.3) by Gorer and colleagues, whereby the presence of circulating antibodies against the H_2 antigens were present after graft rejection (Gorer and Gorman, 1956), with earlier findings by Amos and colleagues (1953) demonstrating the presence of leucocyte agglutinins after skin grafts in mice, thus proving that skin and leucocytes share antigens. On the basis of his own work in 1954, Dausset was able, by immunizing a number of recipients with leucocytes from single donors, to produce agglutinins which, even though they were not pure, seemed to recognise the same antigen. This antigen, termed Mac (now known as HLA-A2) occurred in about 60% of the French population (Dausset, 1958). In 1962, a study of renal homotransplant recipients was performed, demonstrating that "a close genetic relationship increased the probability of graft tolerance, presumably as a result of an increase in the number of identical transplantation antigens in the recipient and donor" (Hamburger *et al*, 1962).

In 1964, the first International Workshop on Histocompatibility was organized by D Bernard Amos in Durham, North Carolina. In the same year, Payne reported the first clear evidence that these leucocyte antigens segregated in families as a genetic system. Whereas the original serological identification of transplantation antigens was done by leucoagglutination, Terasaki introduced a much more sensitive and specific microlymphocytotoxicity test (Terasaki and McClelland, 1964). Also in the same year, work by Colambani *et al* (1964) and Walford *et al* (1964) demonstrated similar findings to that of earlier work by Amos (1953) and Gorer (1956) in man. Indirect evidence that leucocyte antigens and transplantation antigens are related was also provided by the work by Van Roods group (Rapaport *et al*, 1964) whereby microsomal fractions containing the transplantation antigens also contained leucocyte antigens. The following year, studies illustrated that injection of

white cell extracts sensitised an individual against grafts of foreign skin (Rappaport *et al*, 1965). In the same year, Dausset and colleagues showed that by skin grafting of six randomly chosen volunteers that at least five identifiable leucocyte antigens or antigen complexes affect the survival of skin grafts (Dausset *et al*, 1965). Attention therefore focused on analysing the iso-antigen systems of human white cells.

The history of the discovery of leucocyte antigens has been reviewed by Van Rood and colleagues (1966). In the same year, sera was prepared from forty five multiparous women, two polytransfused patients and three post-graft volunteers in order to further investigate the complex system they had now termed Hu-1 (Dausset *et al*, 1966). In this study, they managed to define ten leucocyte antigen using adsorption studies and complex statistical analyses. In 1967, isoantisera was also prepared by immunizing voluteers with a series of small skin grafts, with adsorptions of isoantisera carried out as previously described (Batchelor and Chapman, 1966). From the seventy four individuals screened, twenty six antigens were able to be recognised (Batchelor and Chapman, 1967). Studies on the inheritance of these antigens in eighteen families have shown that a large proportion are inherited *en bloc* in a manner similar to that of the H-2 antigens of the mouse. It was concluded from this work that most of the antigens detected belong to a single pseudo-allelic system.

In 1968, Morris and colleagues used the method of leucocyte typing in cadaver renal transplantation (Morris *et al*, 1968). Leucocytes were typed from donor and recipient in twenty seven renal allotransplants. They reported that twenty two of the mismatched recipients underwent moderate to severe rejection episodes, and found that the remaining five patients who were matched for eight of the presently defined antigens fared better. Influence of HLA incompatibility in renal transplantation was also examined by Batchelor and Joysey (1969). Fifty two cases were studied, and it was shown that the number of graft incompatibilities

significantly correlated with the subsequent clinical status of the patient. In the same year, skin grafts experiments were performed (Ceppelini *et al*, 1969). This illustrated that the graft survival time decreased with increasing mismatches (identical siblings>haploidentical>mismatched).

Later, in 1972, McDevitt and colleagues identified the gene(s) in mice that are responsible for genetically determined variation in immune reactivity to the MHC region (McDevitt *et al*, 1972). These were the immune response (IR) genes and were localized in the H-2 system. T cells are known to only recognise antigen by the interaction of the TCR with processed antigen presented by HLA molecules. This is known as HLA/MHC restriction. This was first demonstrated by Zinkernagel and Doherty (1974) where in order to trigger an immune response, T responder cells must bear the same HLA molecule as the antigen presenting cell. This was demonstrated by showing that T cells recognising lymphocytic choriomeningitis virus antigen in association with $H-2K^k$ could not recognise the same antigen associated with $H-2K^d$ or $H-2D^b$ alleles. In 1976, Terhorst and colleagues (1976) managed to determine the partial protein sequence of HLA-A2. cDNA cloning and sequencing of class I genes were performed later in 1980 (Ploegh *et al*, 1980) whereby a clone (pHLA-1) containing HLA-specific cDNA was constructed by reverse transcription of partially purified HLA mRNA. The pHLA-1 cDNA insert was found to correspond to the COOH-terminal forty six amino acids of an HLA-A, -B or –C antigen. Two years later, cDNA cloning was also performed, but this time, for the HLA-DRα chain (Lee *et al*, 1982). In the late 1980's, X-ray crystallograpy was being used to determine structures of HLA molecules, which allowed a more detailed study of the presentation of antigens by HLA molecules eg; HLA-A2 (Bjorkman *et al*, 1987). Due to these findings, it is now known that the primary biological role of HLA molecules is the regulation of immune responses to foreign antigens, with the

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discrimination of self from non-self. These major findings up until this decade have allowed for a better understanding of the organisation and involvement of the HLA system in clinical disease, and classification of the HLA system has recently been described (Proceedings of the 12th International Histocompatibility Workshop, France, 1996).

1.7.3. Genetic organization and polymorphism

The HLA region in man is encoded on the short arm of chromosome 6, and spans four million base pairs of DNA. The orientation of the three major gene clusters and their relationship with one another was first clarified by genomic cloning and subsequently by pulse-field gene electrophoresis, a technique which allows separation of large DNA fragments for construction of restriction maps (Hardy *et al*, 1986, Lawrance *et al*, 1987, Dunham *et al*, 1987, 1989, 1990, Spies *et al*, 1989, Sargent *et al*, 1989). More than two hundred genes have been located in the human HLA, many of which have immunological functions in the antigen processing and presentation (Figure 1.2). The HLA is divided into class I, class II and class III regions (Trowsdale and Campbell, 1997). The class I region (distal) contains genes encoding the classic transplantation antigens, HLA-A, -B and –C. The non-classical genes HLA-E, -F and –G genes are also expressed in this domain (discussed later). The class II region is at the opposite (centromeric) end and contains genes such as HLA-DP, -DQ and –DR. The class III region, lying between the other two clusters, is packed with genes encoding a variety of functions, including complement eg; C2, C4A, C4B, heat shock (HSP-70) and cytokine genes (TNF). The class I antigens, HLA-A, -B and –C play a cental role in T cell recognition by binding peptides for recognition by CD8+ T cells (discussed later). HLA class I molecules are expressed

on virtually all nucleated cells, with their level of expression being subject to regulation (Geraghty, 1993, Le Bouteiller, 1994).

The human HLA class I region spans at least 2000 kilobases (kb) of DNA, and is larger than both the class II and class III regions. The nomenclature of HLA genes is being updated all of the time, with over two hundred and twenty five alleles being named since 1996 (Bodmer *et al*, 1998). As with class II and III, class I includes expressed genes, many of which have immunological functions in antigen processing and presentation (Campbell and Trowsdale, 1993). Other genes, known as pseudogenes, have various deleterious mutations in the DNA sequence which prevent transcription, or are only fragments of an entire gene.

HLA class I genes share a very similar organization. As previously mentioned, HLA class I region consist of the classical transplantation antigens (class 1a) -A, -B and –C (Trowsdale and Campbell, 1997). HLA-A, -B and –C have been studied in detail in a heterozygous individual from which the B lymphoblastoid cell line (LCL) 721 is derived (Geraghty *et al*, 1992a, 1992b). At least twelve pseudogenes and gene fragments constitute the bulk of class I-related sequences, and three functional class I genes, HLA-E, -F and –G, commonly referred to as non-classical or class iB genes, complete the histocompatibility complex gene family (Geraghty *et al*, 1992a). At the nomenclature meeting following the 12th International Workshop, one hundred and twenty four HLA-A, two hundred and fifty eight HLA-B and seventy four HLA-C alleles were recognised (Bodmer *et al*, 1998). The extreme polymorphism of class I antigens is reflected in the existence of multiple loci, the large number of alleles at each locus, many of which are present at significant frequencies in the population, and the multiple structural differences among alleles.
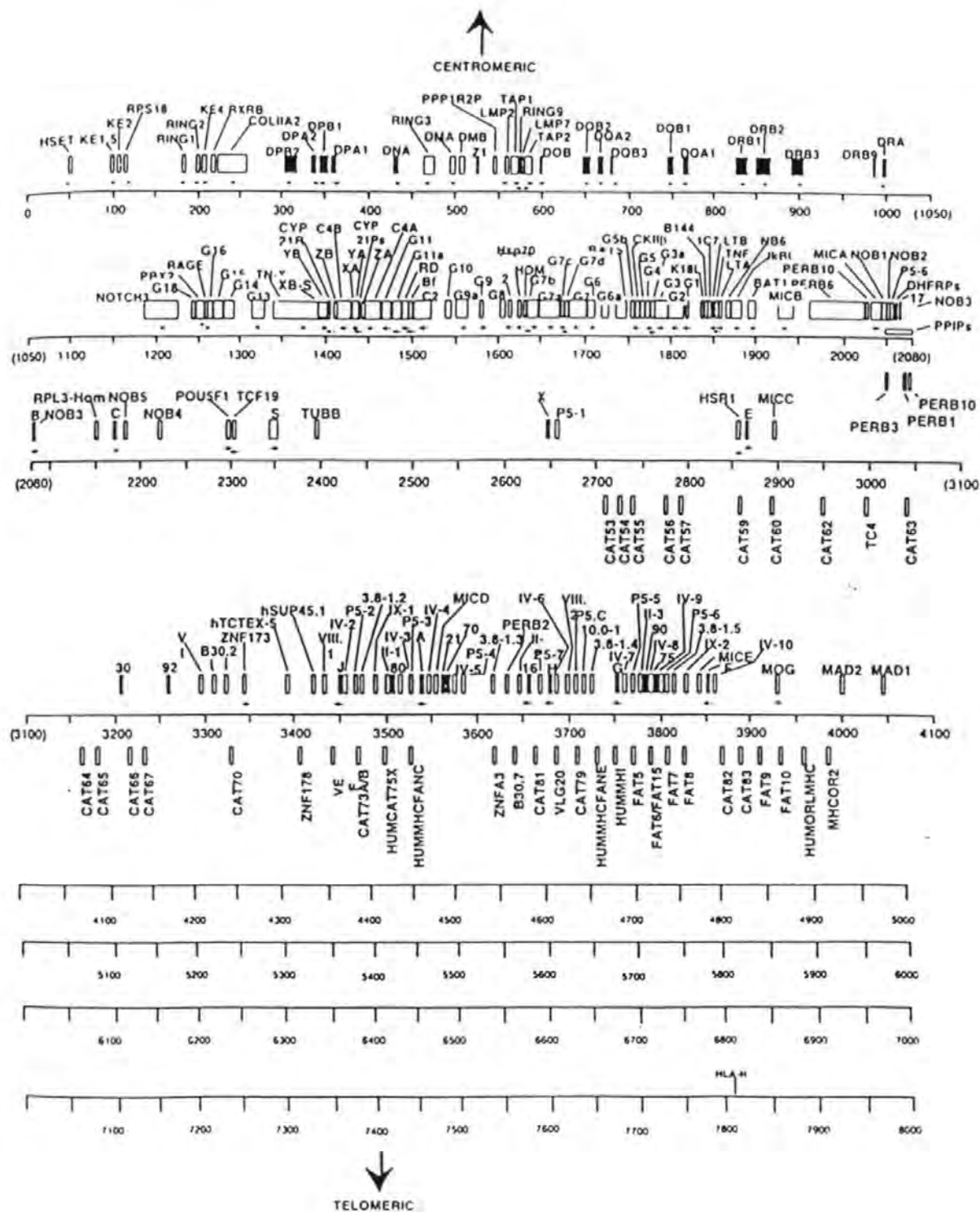
Figure 1.2. Map of the human HLA (as illustrated by Trowsdale and Campbell, 1997).

HLA-DR, DQ and DP are referred to as class II. The class II genes, as mentioned, are located at the centromeric end of the MHC, and span about one megabase (Stephens *et al*, 1999). Over thirty genes have been mapped in the class II region of the MHC (Trowsdale and Campbell, 1997). The class II region may be divided into genes that code for α and β chains. Additional class II genes, including DNA and DOB have also been previously identified (Kuhner and Peterson, 1992). Class II genes come in three different forms, functional genes, pseudogenes and genes of unknown status. Compared to class I genes, class II genes show very little polymorphism, with the exception of DRB gene (Blasczyk *et al*, 1998). The polymorphism of the DRB gene has mainly focused on the second exon, but in this study, the intron sequences were highly polymorphic. In contrast, HLA-DQ and -DP class II subregions are conserved (Andersson, 1998). Polymorphisms in the DQ subregion (DQA and DQB) have also now been identified (Kwok *et al*, 1995, Hashemi-Tavoularis *et al*, 1998). Polymorphism allows a high degree of variation of antigenic recognition at the population level. However, this causes problems for matching patients and donors for transplantation.

In addition, genes including TAP (transporter associated with antigen processing) and LMP (large multifunctional protein) are located in the class II region. The TAP1 gene is located between the DNA and DOB genes, while TAP2 is located between TAP1 and DOB genes. The protein products of both of these genes are involved in the functioning of HLA class I antigen processing (Pamer and Cresswell, 1998). TAP functions as the protein associated with the translocation of peptides, ions and small molecules from the cytoplasm into the endoplasmic reticulum (Momburg and Hammerling, 1998). Prior to loading antigenic peptides, assembled HLA class I molecules associate with TAP in a complex which also includes the endoplasmic reticulum chaperone, calreticulin, and a recently described

component, tapasin (Ortmann et al, 1997, Powis, 1997). Tapasin is a member of the immunoglobulin superfamily, which mediates the binding of HLA class I molecules to TAP, and stabilizes the HLA class I loading complex (Bangia et al, 1999). The Tapasin gene has been shown to be located within 500Kb of the TAP1 and TAP2 genes (Herberg et al, 1998).

There are two LMP genes that are located in the class II region, LMP2, which is immediately centromeric to TAP1, and LMP7, which is located between TAP1 and TAP2 (Beck and Trowsdale, 1999). Both LMP2 and LMP7 genes share homology with proteosome, which is a proteinase complex found in the cytosol. In addition to functioning in the complete degradation of cell proteins, proteosomes are the source of most antigenic peptides present in the immune system on HLA class I molecules (Gaczynska et al, 1993, Goldberg et al, 1995). IFN-γ has been suggested to play a role in the induction of both TAP and LMP gene expression (Seliger et al, 1997).

The class III region of the HLA contains genes encoding the complement proteins C2, C4A, C4B and Bf as well as the gene for the cytochrome P-450 enzyme 21-hydroxylase. C2 and Bf genes are less than 2000bp apart and are situated approximately 30Kb telomeric of the C4A and C4B genes (Trowsdale and Campbell, 1992). There is extensive protein polymorphism for C4A and C4B, with Bf, C2 and 21-hydroxylase exhibiting a degree of polymorphism (Brai et al, 1994). There are no genes which encode for histocompatibility products in class III region. Genes encoding for TNF-α and β and heat shock protein-70 (HSP-70) are also encoded for in the class III region. The TNF gene locus is located 250Kb centromeric to HLA-B and 350Kb telomeric to the C2/Bf gene complex. TNF is a pro-inflammatory cytokine and polymorphisms in this gene has been shown to associated with transplant rejection (Turner et al, 1997). HSP-70 is a gene that encodes for a protein that is involved in signalling the immune system of the presence of immunologically

dangerous situations against which an immune reaction shound be raised. It has been shown to be induced during tumour cell killing and the induction of Th1 type cytokines (Todryk et al, 1999).

HLA genes are inherited as co-dominant alleles. Recombination between the loci does occur and the frequency is about 1% between A and B, 0.8% between B and DR and about 10% between DQ and DP (Carrington, 1999). The abnormally high frequency of DQ-DP recombinants is thought to be due to a recombination "hot spot" for the loci are known to be in close proximity in this area (Begovich et al, 1992). Because the six loci are relatively closely linked they are usually inherited en bloc by the offspring from each parent. The HLA encoded by the genes of one chromosome are termed an HLA haplotype and each individual therefore has two haplotypes. Therefore, there are four haplotypes per family. Four different combinations can therefore be found eg; if the paternal haplotype is GH and the maternal haplotype designated as IJ, then the possible combinations are GI, GJ, HI and HJ.

1.7.4. Structure of HLA molecules

Both HLA class I and II are members of the immunoglobulin superfamily of molecules, which are transmembrane glycoproteins arranged as a series of domains at the exterior surface of the cell. HLA class I molecules may be found on virtually all somatic cells, with a precise meiotic map having been determined (Bouissou et al, 1995). Class I molecules are heterodimeric membrane-bound glycoproteins made up of a α heavy chain of 45 KDaltons (KDa), which is non-covalently associated with beta-2-microglobulin (β2m) (Figure 1.3) (Shields et al, 1999). The α chain is a glycoprotein consisting of a polypeptide and short carbohydrate chains. This chain is divided into three domains, extracellular (280 amino acids), hydrophobic

transmembrane (23 amino acids) and hydrophilic cytoplasmic region (25-32 amino acids). The extracellular domain has three domains, $\alpha1$, $\alpha2$ and $\alpha3$, each of which consist of approximately 90 amino acids, and are encoded on exons 2, 3 and 4 respectively (Stern and Wiley, 1994, Madden, 1995). The $\alpha2$ and $\alpha3$ domains have intra-chain disulphide bonds. $\beta2m$ is a 12KDa serum protein which is encoded by a gene on chromosome 15. The structure is stabilised by non-covalent associations between the α and β chains forming $\alpha\beta$ dimers (Madden, 1995). As previously mentioned, there is extensive polymorphism in the class I genes, mainly found in the $\alpha1$ and $\alpha2$ domains.

All of the components of the tri-molecular complex that is at the heart of T cell recognition had been defined in terms of primary sequence by 1985. This evidence was elicited by work that demonstrated peptide binding to purified HLA molecules by equilibrium dialysis (Babbit *et al*, 1985). The HLA molecule was then discovered as a molecule acting as a restriction element for the peptide in question (Buus *et al*, 1987). These results established that the ligand specifically recognised by T cells is a binary complex of HLA molecule and peptide. Following these experiments, the next major landmark was the crystallographic analysis of the structure of the human class I molecule, HLA-A2 (Bjorkman *et al*, 1987a, 1987b). This work then led onto the proposal of a hypothetical model for class II structure. (Figure 1.4). Other crystalline structures of class I molecules have also been previously determined (Krishna *et al*, 1992, Madden *et al*, 1993). More recently, HLA- B27, HLA-E, H-2κb and H-2Dd have been described (Rognan *et al*, 1997, O'Callaghan *et al*, 1998, Celia *et al*, 1999, Achour *et al*, 1999).
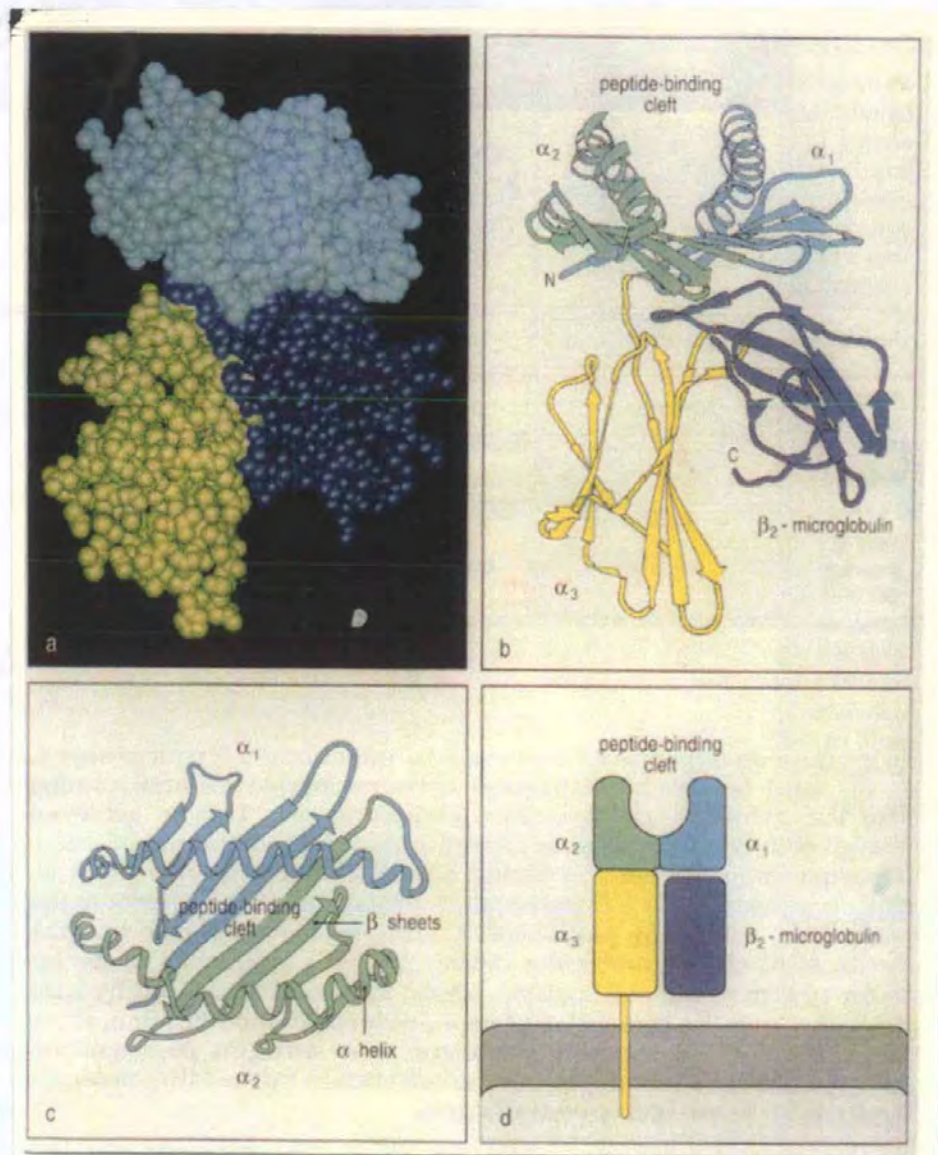
Figure 1.3. Structure of a HLA class I molecule. Panel a represents a computer graphic representation of human HLA-A2, which has been cleaved from the cell surface by the enzyme papain. Panel b shows the ribbon diagram of the structure. The α3 domain and β-2 microglobulin show similarities in amino acid sequence to Ig constant domains and have a similar folded structure (b and d) (adapted from Janeway and Travers, 1996).

The class I molecule has an antigen binding cleft which is approximately 25 angstroms (A°) by 10A°. It is formed by a floor of eight parallel β pleated sheets flanked by side walls of α helices. Both α1 and α2 domains contribute to the formation of the antigen binding site, and are supported by the α3 domain and β2m (Young et al, 1995). It is known that HLA class I molecules bind smaller peptides than class II, with the majority of HLA class I peptides being between 8-10 amino acids long (Blanden, 1995, Appella et al, 1995). As previously mentioned, both HLA class I and class II antigens bind peptides of diverse sequences with high affinity, in order to generate maximal immunological protection (Batalia and Collins, 1997).

The class II antigens comprise two polypeptide chains which associate themselves in the endoplasmic reticulum and are then transported to the cell surface (Solheim et al, 1995). HLA class II glycoproteins are also heterodimeric and consist of a heavy chain (α) of 35KDa and a light chain (β) of 28KDa (Figure 1.4). Each glycoprotein chain contains two domains, α1 and α2 or β1 and β2. The two chains are non-covalently associated (Jones, 1997). It is the α1 and β1 chains that fold to form the peptide binding cleft (Stern and Wiley, 1994). The amino acid sequence of the two chains indicate that they each have two membrane-external domains of approximately 90 aa, and a transmembrane region and cytoplasmic domain of approximately 40 aa. The β chain contains two intrachain disulphide bonds, and the α chain a single intrachain disulphide bond.

Crystallographic studies of HLA-DR1 showed that the structure of class II molecules is generally similar to that of class I. Studies included the analysis in complex with peptide (Jardetzky et al, 1996, Murthy and Stern, 1997) and without (Brown et al, 1993). These analyses suggested that the antigen binding site of class II molecules has a more open conformation than class I. The antigen binding groove in
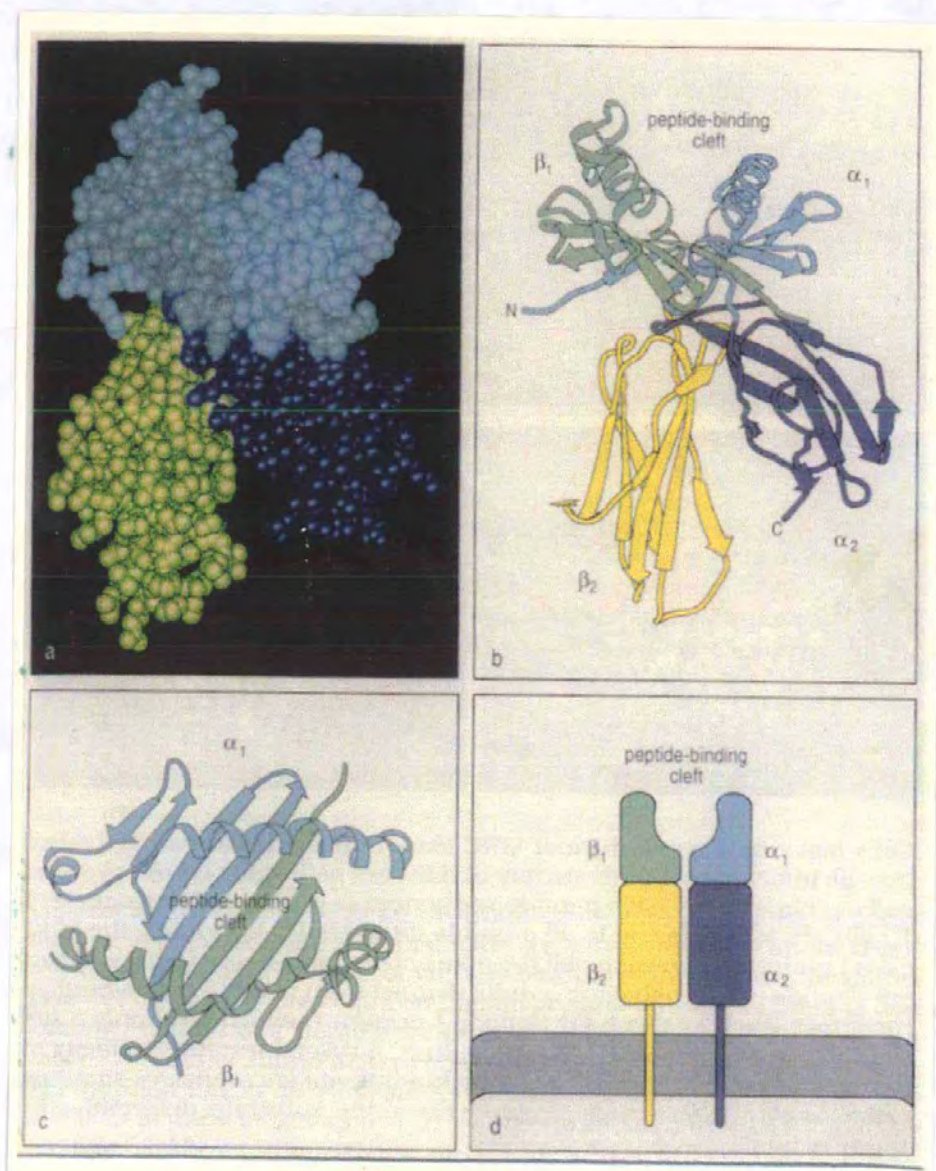
Figure 1.4. Structure of a HLA class II molecule. Panel a shows the computer graphic representation of the HLA class II molecule (HLA-DR1), and panel b shows the equivalent ribbon diagram. The α1 and β1 domains have amino acid sequence and structural similarites to the Ig constant domains, with the two domains forming the peptide binding cleft (c and d) (adapted from Janeway and Travers, 1996).

class I molecules is able to present antigens between 10-34 residues in length (Appella *et al*, 1995). Class II molecules present constitutively on the cell surfaces of B lymphocytes, monocytes, macrophages and dendritic cells, which all have common property of being antigen-presenting cells for T lymphocytes. In addition, class II molecules are also found on vascular endothelium, various ductal epithelia and kidney glomeruli (Daar *et al*, 1984).

1.7.5. Loading and transport of HLA molecules

1.7.5.1. The class I pathway

Cytoplasmic proteins are taken up by proteosomes. These organelles consist of seven α and seven β subunits. The genes for two of these subunits, LMP-2 and LMP-7, have been implicated in the early steps of this process (Belich and Trowsdale, 1995, Pamer and Cresswell, 1998). When induced by IFN-γ, these low molecular mass polypeptides replace two constitutive subunits of the 20S proteasome, presumably preparing the proteasome for antigen processing (Groettrup *et al*, 1996, Griffin *et al*, 1998). The IFN-γ-inducible 11S regulator (PA28) also plays a role in the peptide production (Groettrup *et al*, 1995, Kuehn and Dahlmann, 1997). Similarly, a third subunit, MECL-1, is also inducible (Nandi *et al*, 1996, Groettrup *et al*, 1997, Griffin *et al*, 1998). Generally, protein degradation within the cell is accomplished by proteosomes using the ubiquitin to target proteins towards the proteasome (Hochstrasser, 1995, 1996, D'Andrea and Pellman, 1998). However, it is unclear whether ubiquitin is required for the production of peptides in the class I loading pathway. Although some evidence for non proteasomal protein degradation exists, it has been shown by proteasomal inhibitors that inactivation attenuates the supply of peptides for class I molecules (Dick *et al*, 1996, Craiu *et al*, 1997).

In order for these peptide fragments to reach the cell surface, they must first move to the endoplasmic reticulum (ER), and then cross the membrane, consisting of a lipid bilayer, that is confined to the ER (Figure 1.5). The transport across the cytosol may involve shuttling of heat shock proteins but the actual mechanism is unclear. To traverse the ER membrane the peptides must associate with the TAP proteins (Koopman *et al*, 1997, Gabathuler *et al*, 1998, Uebel and Tampe, 1999). TAP, in common with other membrane transporters, is an ATP binding cassette (ABC) protein. The process by which TAP transports is ATP dependent. TAP not only functions as a transporter for these peptides, but it also seems that it is essential for the co-localisation of nascent HLA class I molecules with peptide to form the peptide:HLA complex. HLA molecules require peptide binding to stabilise the structure. Therefore, after synthesis of the class I α chain, it rapidly binds to a membrane bound protein, calnexin, which functions as a chaperone (Ho *et al*, 1999). Calreticulin, BiP and tapasin, are also involved in this process (Harris *et al*, 1998, Plemper *et al*, 1997, Grandea *et al*, 1997). The α chain then dissociates from calnexin when it binds to β-2 microglobulin (Sugita and Brenner, 1994). As previously mentioned, this complex binds to TAP-1 subunit until it is stabilised by the binding of the peptide. Once the peptide is bound, the HLA:peptide complex dissociates from TAP and it is shuttled to the cell surface via the Golgi apparatus.

1.7.5.2. The class II pathway

Although some microorganisms replicate in the cytosol, others are predominantly extracellular or reside in intracellular vesicles. These pathogens are seperated from the cytosol by membrane and so are not accessible to the proteasome-TAP pathway. An alternative antigen processing pathway has therefore developed

which involves the breakdown of proteins within these vesicles and delivery to the cell surface in association with class II molecules (Figure 1.6).

Internalised protein antigens are enclosed within acidic vesicles called endosomes (Pond and Watts, 1997, Brachet *et al*, 1999). The low pH is essential to activate proteases such as the cathespins. Like HLA class I, class II molecules are also synthesised in the ER and yet they do not bind peptide at this site. This is achieved by the binding of a monomorphic protein called invariant chain. This protein forms a homonymous trimer which associates with nascent class II molecules (Cresswell, 1994). The interaction and trimer formation occurs at the site of the invariant chain transmembrane domain (Ashman and Miller, 1999). Calnexin is also involved as a chaperone at this stage (Arunachalam and Cresswell, 1995). Each subunit of the trimer associates with as $\alpha\beta$ class II heterodimer. Cells that lack invariant chain retain many class II molecules in the ER as misfolded proteins. The invariant chain trimer (Ii) also functions to target the delivery of the complex to an appropriate low pH endosomal compartment. Leucine based signals in the cytoplasmic tail of invariant chain control targeting of newly synthesised HLA class II molecules to the endocytic pathway for acquisition of antigenic peptides (Zhong *et al*, 1997). The complex makes its way through early endosomal compartments on to a later, more acidic lysozome-like compartment. It is in this compartment, called MIIC (MHC class II compartment) that the invariant chain is cleaved so that the class II heterodimer now contains only CLIP (class II-associated invariant chain peptide) in its peptide binding groove, with particular methionine residues in Ii (Gautam *et al*, 1997). CLIP can bind all the different alleles and isotypes of class II (Gautam *et al*, 1997). The class II molecule now has to unload the CLIP fragment and load a peptide from the array that is present in the compartment. This is
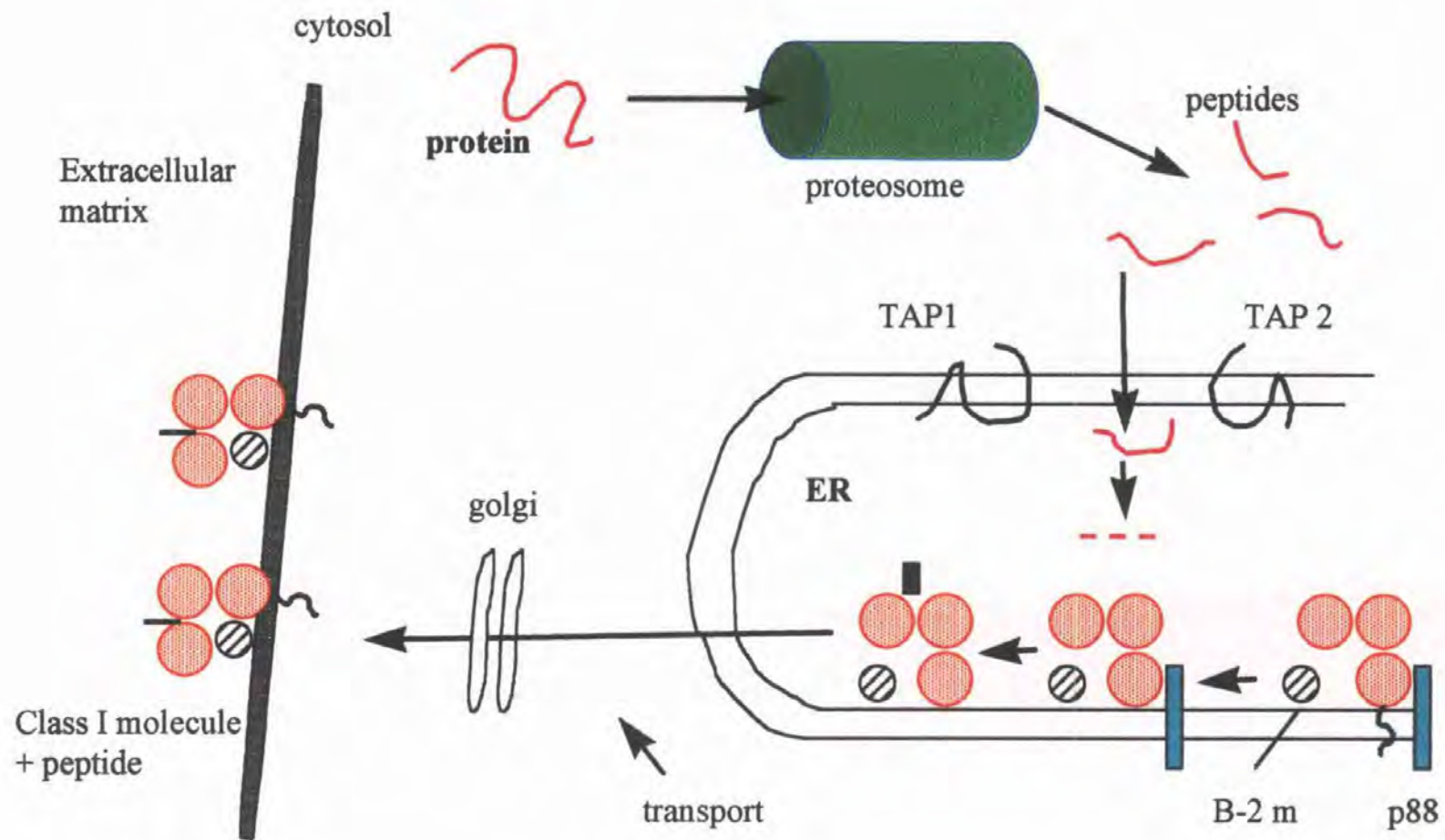
Figure 1.5. Loading and intracellular transport of HLA class I molecules.

performed by DM (Pierre *et al*, 1996). DM is an atypical class II molecule formed as a heterodimer from the products of the non-polymorphic DMA and DMB loci within the MHC class II region. DM does not require peptide to stabilise its structure and it is not expressed on the cell surface, but operates predominantly in MIIC. DM catalyses the removal of any peptide from class II in proportion to its intrinsic rate of dissociation (Denzin *et al*, 1996, Stebbins *et al*, 1996). This is accomplished by stabilising a transition rate that leads to full dissociation. Thus DM ensures that only stably-associated peptides remain bound to class II. Once the peptide is bound, the class II heterodimer passes on to the cell surface.

1.7.6. HLA typing and matching

HLA antigens are conventionally typed by serological techniques using lymphocytes as the target cells. Peripheral blood lymphocytes or separated T cells are used for class I typing and B lymphocytes for class II typing, as DR and DQ antigens are found on B cells but not on resting T cells. The antibodies to these antigens may be found in the sera of repeatedly transfused patients and in multiparous women. It was in transfused patients that Jean Dausset was able to obtain antibodies for the detection of the first HLA antigen (Dausset, 1958). Nowadays, some laboratories still use the microdroplet lymphocyte cytotoxicity technique developed by Terasaki and McClelland (1964). HLA DP products were first detected by primed lymphocyte testing (PLT) (Shaw *et al*, 1980). The mixed lymphocyte reaction (MLC) is also of importance in HLA matching (discussed later). Identical HLA matching is regarded as a necessity in bone marrow transplantation and a bonus in organ transplantation, although, it has suggested that it may be acceptable that compatibiliity at the HLA-D locus only may be tolerated (Dupont *et*
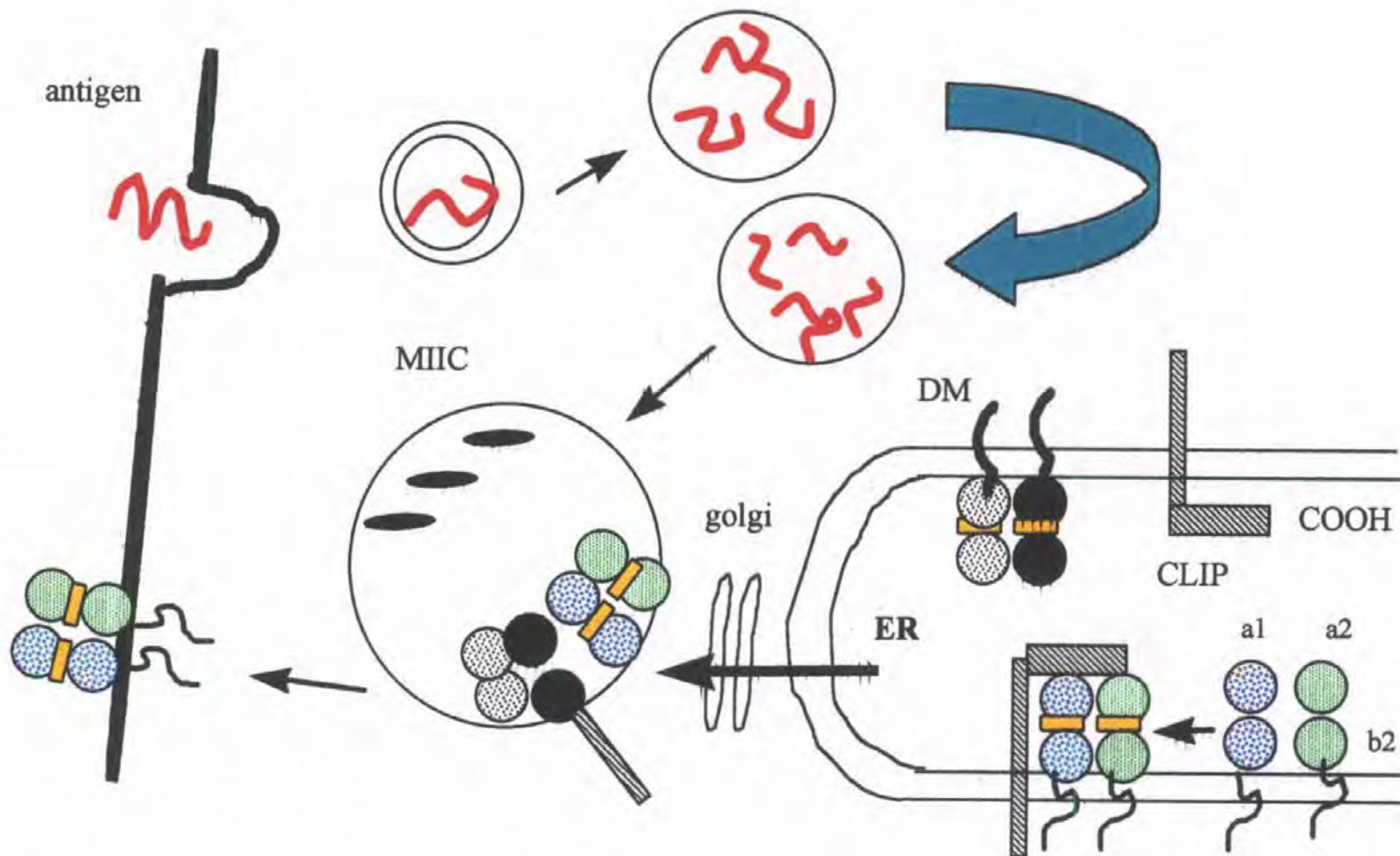
Figure 1.6. Peptide generation, loading and intracellular transport of HLA class II molecules.

al, 1980). As previous studies have shown, differences in HLA between individuals do elicit an immune response (Araten *et al*, 1993, Gorski, 1996), therefore it is important to ensure as close a match as possible in order to reduce the risk of allograft rejection. Histocompatibility antigens are those cellular determinants specific for each individual of a species that are responsible for immune rejection when attempts are made to transfer or transplant cellular material from one individual of the same species to another (Dyer and Class, 1997). It has been shown in both early and more recent studies that better matching does lead to a longer survival time for organ allografts (Morris *et al*, 1968, Batchelor and Joysey, 1969, Terasaki *et al*, 1989, Tesi *et al*, 1993, De-Lin *et al*, 1998, Ketheesan *et al*, 1999, Muro *et al*, 1999).

A high success rate in transplantation, however, has been seen even with partial matching, and this has been suggested to be due to a gradation of strength of the antigens encoded by the different loci, with the DR antigens being the strongest transplantation antigens. The differences in HLA antigen alloreactivity has been previously described, for instance, matching for HLA-B has been shown to be more important for allograft survival than matching for HLA-A (Zhang *et al*, 1990). There is little data to illustrate that either DQ or DP matching is crucial for transplantation. In one study however, DP matching, although had no deleterious influence on first cadaver transplants, did illustrate a statistically significant effect on re-transplants (Mytilineos *et al*, 1997) A study of HLA-DQ matching in cadaveric renal transplantation has also been performed, with the finding that HLA-DQ matching does not significantly affect renal allograft survival (Freedman *et al*, 1997). The impact of HLA mismatches on the survival of cadaveric kidney transplants has been shown (Held *et al*, 1994). In this work, over thirty thousand cadaveric transplants were studied over a seven year period, and it was shown that HLA matching has a statistically significant and clinically important impact on short and long term graft

survival. In another study, one hundred and eighty one patients receiving cadaveric renal transplants were monitored and it was shown that allografts with better HLA-DR and HLA-B matching showed significant lower rejection rates than those less well matched (Beckingham *et al*, 1994). It has even been shown that poor HLA-DR matching is associated with increased early rejection episodes, whereas HLA-B matching was associated with a reduced frequency of late rejection episodes with improved graft function at two years (McKenna *et al*, 1998). HLA-DR matching has been shown to beneficial not only in kidney transplantation (Opelz *et al*, 1998, De-Lin *et al*, 1998, Reisaeter *et al*, 1998), but also in heart transplantation (Sucia-Foca *et al*, 1998, Sheldon *et al*, 1999). In one study, it was even suggested that HLA-DR incompatibility could predict heart transplant rejection (Costanzo-Nordin *et al*, 1998). In the past decade or so, there has been developments of molecular methodology. Polymorphism can be detected at the DNA level and occurs primarily in the variable domains (α chain in class I; β chain in class II). The conventional way to determine HLA specificities is still based on immunological methods.

Recently, the polymerase chain reaction (PCR) has been used to analyse and characterise HLA class subtypes. Sequencing of these DNA variants allows the synthesis of primers complementary to the polymorphic HLA genes (Wade, 1996). It now appears that this method is used in tissue typing laboratories in conjunction with the established methods to increase the precision of recipient or live donor selection for transplantation. DNA analysis has been shown to be more precise in defining HLA DR alleles than serological typing (Olerup and Zetterquist, 1992), and has also been shown to illustrate a significant reduction in acute rejection episodes compared to the broad classification yielded by serology (Reviron *et al*, 1996). PCR may be used in conjunction with other methods such as restiction fragment length polymorphism (RFLP) analysis using sequence specific probes (SSP) (Sengar *et al*,

1995, Moribe *et al*, 1997, Nieto *et al*, 1997, Woszczek *et al*, 1997, Mitsunaga *et al*, 1998, Palou *et al*, 1999). Molecular PCR typing allows for precise antigen determination.

It is now generally accepted that a very important part of typing potential donors and recipients is the cross-match between recipient sera and donor lymphocytes (Ting, 1989). Since hyperacute rejection of an allograft in the presence of pre-existing donor specific cytotoxic antibodies (Terasaki and McClelland, 1964), a positive cross-match has been a contraindication in transplantation. However, there are exceptions to this hypothesis. For instance, the antibody causing a positive crossmatch may be an autoantibody which can be directed against either the donor B cells or donor B and T cells, which may be from some remote time in the past while a current serum is negative (historically positive, currently negative). The immunoglobulin (Ig) class of the antibody in the peak reactive serum may be an important predictor of graft outcome. IgG antibodies have been shown to associated with graft failure whereas success has been achieved in the presence of IgM antibodies (Chapman *et al*, 1986, Leavey *et al*, 1997, McCalmon *et al*, 1997). Antibodies are nearly always IgM whereas HLA antibodies are usually IgG, although IgM HLA antibodies may occur in patients on immunosuppression. Therefore, it may be concluded that transplantation against some positive crossmatches can be successfully performed, but the conditions under which the chances of success are optimal have still to be worked out fully.

Flow cytometry crossmatching before transplantation has been suggested to be more advantageous than serological crossmatching as it has been shown that flow cytometry is more sensitive in the detection of pre-formed antibodies (Sutton *et al*, 1995, Kimball *et al*, 1998, Przbylowski *et al*, 1999), and allows the simultaneous detection of complement-activating and nonactivating alloantibodies, the class of

donor specific antibodies (IgG and/or IgM) and the donor target cells (T and/or B lymphocyte). One study indicated that flow cytometry crossmatching combines sensitivity with specificity in evaluating the patients response to the graft, with the suggestion that this method may even be used as a prediction marker for rejection (Piazza *et al*, 1998).

1.8. The minor histocompatibility complex

1.8.1. Introduction

In human transplantation, donors and recipients are routinely screened for identification of the major histocompatibility genes, and although they may be fully matched at this genetic level (eg; identical siblings), rejection and GVHD may still occur. This is now thought to be due to other histocompatiblity antigens other than those coded for by the MHC (HLA). These products have been termed minor histocompatibility antigens (Goulmy, 1988). These antigens can be peptides derived from cellular proteins that are presented on the cell surface by HLA class I molecules, which are similar to viral antigens. These histocompatiblity antigens tend to elicit slower graft rejection responses compared to major antigens.

1.8.2. History

Minor antigens were described along with major antigens in 1940 where Snell defined thirteen genetic loci that were responsible for tumour graft rejection in inbred congenital mice (Snell, 1948). The H-2 locus was later named the major histocompatibility complex and was responsible for the most vigorous graft rejection. The other loci, termed minor, also mediated tumour graft rejection but to a lesser extent. Later, one minor H antigen identified independently of the use of congenic mouse strains was the male-specific antigen H-Y. This was discovered in

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experiments in which Eichwald and Silmser (1955) exchanged skin grafts between male and female mice of various inbred mouse strains. They found that in some (but not all) strains, females would reject syngeneic male grafts. Following this work, Snell (1956) interpreted these findings as evidence for a minor H antigen, encoded by a gene on the Y chromosome, hence the name H-Y. The ability of females of some but not all mouse strains to reject syngeneic male skin was subsequently found to be due not to allelism or failure to express H-Y, but to polymorphism of immune response (IR) genes (Bailey, 1971, Gasser and Silvers, 1972, Simpson, 1982) including both HLA and non-HLA IR genes (Simpson, 1984).

In vitro cell mediated cytotoxic responses to the male specific antigen H-Y and were found to be H-Y restricted (Gordon *et al*, 1975). This meant that cytotoxic T lymphocytes (CTLs) recognise foreign antigens, such as H-Y only when they are presented to the CTLs on cells which share the same homology of the H-2 region as expressed on the effector CTLs. Similarly, the recognition of other minor antigens by CTLs is also major HLA-restricted (Bevan, 1975). In 1979, CTLs of host origin were also shown to recognise minor histocompatibility alloantigens on tolerated H-2 allogeneic cells in H-2 tolerant mice (Forman and Streilein, 1979). Further murine studies also illustrated the development of GVHD in mice due to minor histocompatibility antigens (Korngold and Sprent, 1983, Hamilton and Parkman, 1983, Holda *et al*, 1985). In these studies, H-2 compatible strains of mice were used. Lethally irradiated recipients were transplanted with donor bone marrow cells. All experiments showed that induction of both acute and chronic GVHD was possible due to minor HA disparities.

The evidence that humans, like mice, express minor H antigens comes from the clinical studies involving transplantation of various organs and tissues. Ceppelini (1969) illustrated that even in HLA-identical siblings, grafts were rejected, although

at a slower pace than when HLA mismatches were present. Cytotoxicity in human transplantation was illustrated by Goulmy *et al* (1983), where severe GVHD occurred in a male patient suffering from acute myeloid leukamia who had received HLA-matched bone marrow from his sister. Strong cytotoxicity of the post-transplant donor lymphocytes against the patients pre-transplant lymphocytes were found, demonstrating that there was a possible role of minor HLA-mismatching, and that minor antigens were detected by HLA-restricted CTLs during GVHD. The same group produced more data further illustrating the impact of mismatching for minor HLA in bone marrow transplantation, correlating it with incidence of GVHD (Goulmy *et al*, 1985). However, conflicting evidence suggested that not all minor alloantigens were responsible for the elicitation of GVHD (Elkins *et al*, 1987). In 1988, Marrack and Kappler found evidence in mice that there is preferential usage of T cell $\alpha\beta$ receptor variable gene families by T cells reacting with minor antigens (Marrack and Kappler, 1988). The year before, an *in vitro* cellular cytotoxicity assay using haemopoietic progenitor cells as targets was developed by Voogt *et al* (1987). Using this assay, it was demonstrated that there was differential expression of the minor antigens on human progenitor cells (Voogt *et al*, 1988a, 1988b). It was found that the male-specific antigen H-Y and HA-1, HA-2, HA-4 and HA-5 were expressed on progenitor cells, while HA-3 is not. Studies following this revealed that H-Y and HA-3 are detectable on keratinocytes, while HA-1, HA-2, HA-4 and HA-5 are not (van Els *et al*, 1989). The findings from these early studies have led to a better understanding of the immunological mechanisms involving minor antigens in processes such as GVHD (Simpson *et al*, 1999).

1.8.3. T cell responses to minor antigens: MHC restriction

Graft rejection is mediated principally by T cells and not by antibodies, as was originally shown in cell transfer experiments by Mitchinson (1954) and by Billingham *et al* (1954). Analysis of class I and class II molecules has been possible because antibodies are made against them. Antibodies are not made to minor antigens, yet *in vivo* T cell responses (graft rejection) to individual minor H antigens has allowed the chromosomal mapping of the genes encoding them in mice, in which breeding experiments establish mouse strains differing from the parental strain at single minor H loci (Bailey, 1975). These loci are scattered throughout the genome, and a minimum of forty have been identified. The same is likely to be true for humans, but because it is not possible to study individual minor H responses *in vivo*, *in vitro* T cell responses are the principal means for study. T cells are HLA restricted ie; they recognise the minor H antigen in the context of self HLA molecules, and are either class I (HLA-A, B or C in humans, H-2K, D or L in mouse) or class II (HLA-D in humans, H-2IA or IE in mouse). The majority of T-cell defined minor antigens are determined by autosomal genes inherited in a Mendelian fashion and that they show either high or low phenotype frequencies (Goulmy *et al*, 1996). From genetic analysis of HLA-A2 restricted minor antigens, HA-1, HA-2, HA-4 and HA-5, it was shown that these four antigens can each be considered the product of a gene with one allele expressing the detected specificity, illustrating that the loci are independent of each other and independent of HLA (Schreuder *et al*, 1993). Several studies have attempted to establish the role of T cell responses to minor antigens (Tsoi *et al*, 1980, Van Els *et al* 1990a, 1990b, Nevala and Wettstein, 1996, Warren *et al*, 1998). It was established in all of the studies that T cells play a predominant role in the recognition of minor antigens, although other risk factors may be involved.

HLA restricted recognition of minor antigens strongly suggests that there is a physical association between major HLA and minor antigens. It is now postulated that these antigens are peptide fragments of processed cellular molecules, which have become lodged in the antigen binding groove of the restricting HLA molecule. The various polymorphic amino acid substitutions seen in the different alleles of class I molecules lie within the proposed antigen-binding groove (Bjorkman et al, 1987). HLA class II molecules may also be modelled on that of class I (Brown et al, 1993), and therefore present antigen fragments in a similar fashion. This would explain how both class I and class II restricted T cells can recognise minor antigens (Roopenian et al, 1993). It would also explain minor antigens are recognised by T cells and not by antibodies (Simpson and Roopenian, 1997).

1.8.4. Minor histocompatibility antigen responses in transplantation

As previously suggested, minor H antigens may be responsible for the continual elicitation of GVHD in HLA-matched recipient-donor pairs, and may even be considered to be a major risk factor for GVHD (Den Haan et al, 1995, Goulmy et al, 1996, Martin et al, 1998). It has been suggested that some minor antigens are expressed on the kidney and that they can trigger graft rejection (Perreault et al, 1990, Beck et al, 1993). Allograft infiltrating CTLs have been recognised to be present, and these are suggested to recognise minor antigens (Deckers et al, 1997, Poindexter et al, 1997). In bone marrow transplantation, an earlier study was able to detect HLA restricted anti-minor antigens from skin lesions from HLA-identical siblings (Reinsmoen et al, 1984). More recent studies have illustrated that minor antigen mismatches have contributed to the development of GVHD in HLA-identical pairs after bone marrow transplantation (Marijt et al, 1993, Goulmy et al, 1996, Martin et al, 1998). The role of minor antigens in rejection and GVHD appears to be

an important one, and further research may finally obtain a fuller picture as to their complete role in transplantation.

1.9. The immune system in rejection and GVHD

1.9.1. Introduction

Without special treatment to suppress its activity (immunosuppression), the immune system responds aggressively towards a transplant, as it does towards infections. This response, which is known as rejection, will destroy the transplant. Rejection occurs because inherited genes make each one of us an individual, and also makes the cells within the body different. The immune system perceives these different cells in a transplant as foreign, and therefore mounts an attack upon them. The immunological aspects of each stage of rejection will be discussed.

In vitro and *in vivo* studies have suggested lymphocytes to be an integral part of transplantation immunity (Wilson and Billingham, 1968). The response of T cells to antigen involves the participation of a number of distinct receptor-ligand engagements. Antigen presentation and recognition plays a major role in many diseases and conditions, including allograft rejection both in renal (Krensky *et al*, 1990) and bone marrow transplantation (Witherspoon and Storb, 1993) and other disease states such as atopy (Kapsenberg *et al*, 1998). Antigen receptors on lymphocytes play a central role in immune regulation by transmitting signals that positively or negatively regulate lymphocyte survival, migration, growth and differentiation (Healy and Goodnow, 1998). Work is constantly being undertaken to attempt to reach a better understanding of the cellular events that mediate incidences such as allograft rejection.

1.9.2. Overview of alloreactivity

Responses between genetically dissimilar humans, termed alloresponsiveness are directed against a cluster of antigens known as the Human Leucocyte Antigens or HLA (Amos and Sanfillipo, 1984). The HLA genes are differentially expressed on most mammalian tissues, and so these are important targets of immunoreactivity. This was first described by Snell and colleagues in mice (Snell, 1948) who went on to determine that histocompatibility genes are the genes which determine susceptibility and resistance to tissue transplants (Snell, 1964). The HLA molecules are the molecules involved in the presentation of antigens to T cells which mediate the immune responses of rejection. The critical role of T cells in allograft rejection was first established by the failure of athymic nude rodents to effect rejection (Corley and Kindred, 1977), and to react to foreign antigens (Hendry, 1979). Other early experiments also showed that a transplant from an allogeneic source could be transferred to nude mice, and the transplant tolerated (Rygaard, 1974). Experiments like these demonstrated that T cells were indeed involved in the rejection process.

In the rejection response, T cells are activated by HLA molecules presenting foreign antigens on the grafted organ (Figure 1.7). This activation leads to a cascade of immunological mechanisms. In short, helper/inducer T cells recognise foreign HLA class II antigens via the TCR and are activated to proliferate, differentiate, and secrete a panel of growth factors. These induce further expression of HLA class II, stimulate B cells to produce antibody against determinants on the transplant and help T cells develop effector functions such as cytotoxicity. Cytotoxic T cells, specific for foreign HLA class I antigens, damage the graft. CD4 and CD8 act as accessory molecules to increase the stability of the interaction (Kelso et al, 1991), and act as modulators of TCR recognition of antigen (Zamoyska, 1998). The CD4 molecule is a
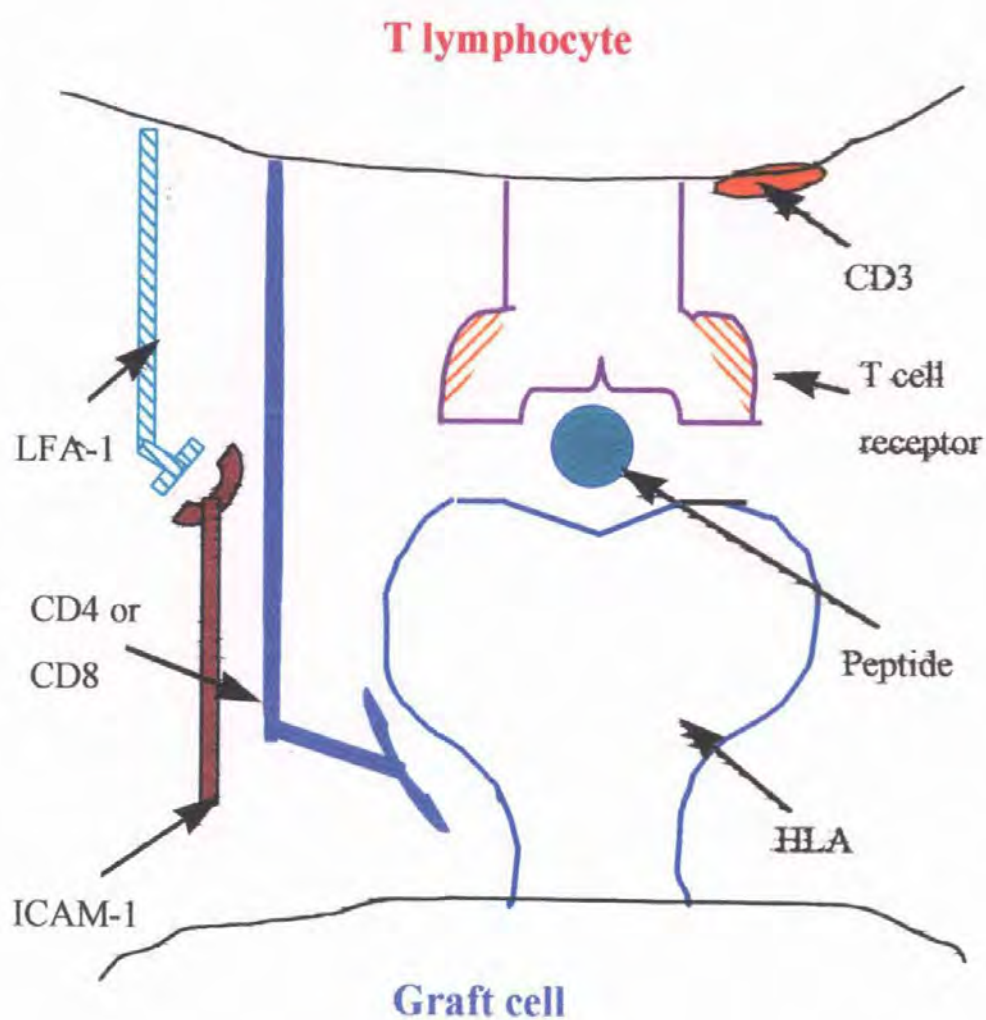
Figure 1.7. Interaction between host T cell and graft cell. The graft cell contains the HLA molecule presenting the foreign antigen. The TCR recognises the antigen, which in turn, activates the T cell.

ligand for the HLA class II molecules, which are distributed on lymphoid tissues (Hauss *et al*, 1995). Expression of class II is generally limited to "professional" antigen presenting cells such as as dendritic cells, circulating B lymphocytes, monocytes and vascular endothelial cells (Thomas and Lipsky, 1994, Meyers, 1995) The CD8 molecule is a ligand for HLA class I, which is expressed on most cell surfaces. The HLA antigens in the allograft activate the T lymphocytes to proliferate, differentiate and secrete a variety of cell mediators, known as cytokines (see cytokine section). Both the HLA molecule and an allogeneic (foreign) peptide are required for T cells to recognise graft "foreigness" (Hall, 1991). Accessory molecules are required for the activation event to occur (Figure 1.8). Other molecules such as ICAM-1 are also present. Without the involvement of these accessory molecules, unresponsiveness or "anergy" may occur (Lombardi *et al*, 1994). This interaction involves the approximation of the antigenic receptor of specific T cells to an antigenic peptide bound in the groove of the HLA molecule. The induction of expression of surface receptors for IL-2 on most cells, including activated CD4+ and CD8+ T cells, B cells and macrophages is of particular importance in the rejection process (Hutchinson, 1996).

The rejection process may be divided into two stages (Lechler and Wood, 1993, Krensky and Clayberger, 1994). Firstly, the allogeneic cell (APC) activates the recipients immune system, through the activation of CD4+ and CD8+ T cells through the HLA complex (Figure 1.8). This is termed the "afferent stage". In turn, other cells are then activated, inducing proliferation and subsequent cytokine release ("efferent stage"). This induces cell migration into the graft. Other cells including B cells are also activated, thereby inducing antibody synthesis. Antigen-induced interactions between macrophages, CD4+ and CD8+ lymphocytes and their humoral products are termed as critical for the completion of the allograft response
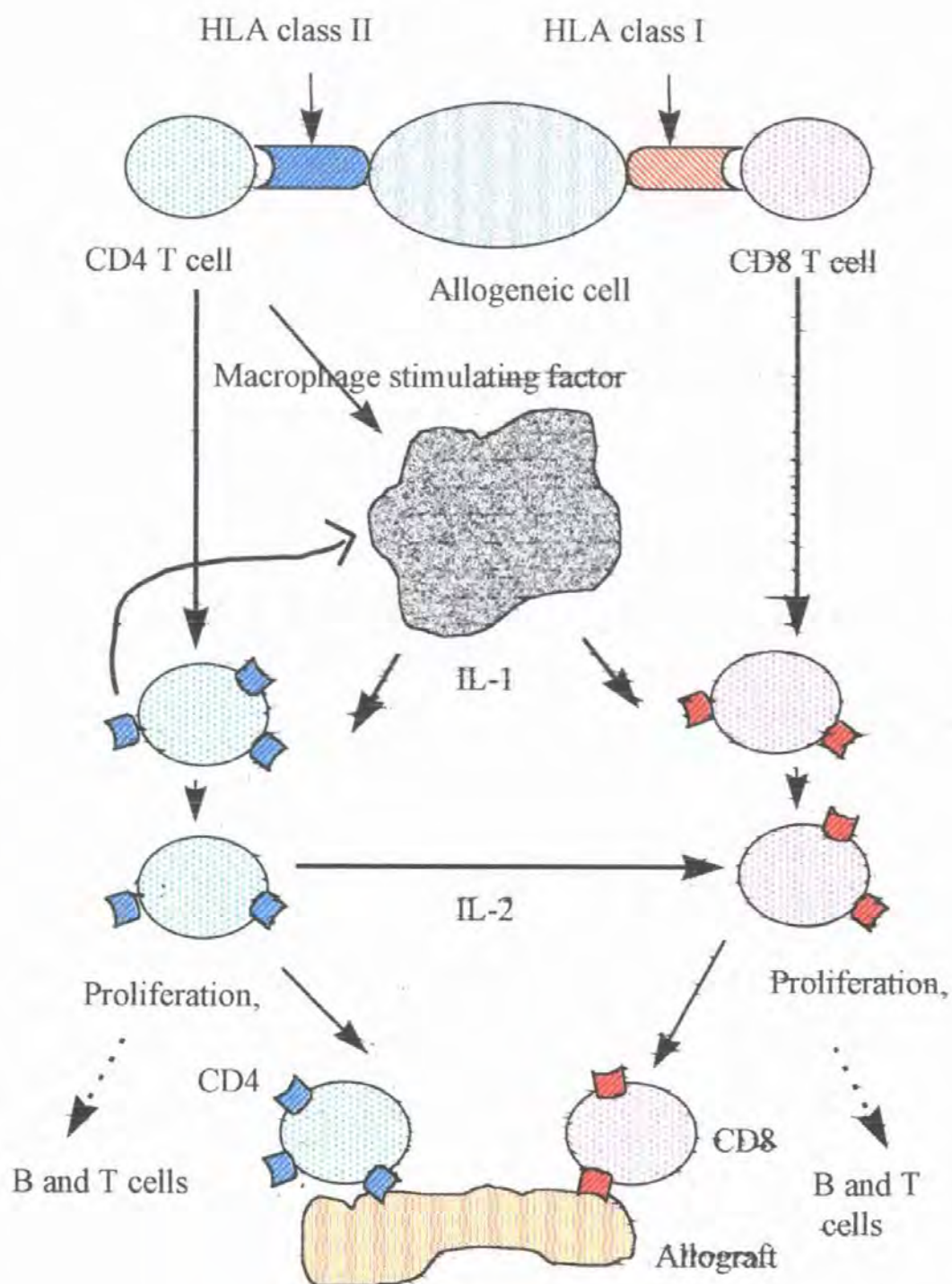
Figure 1.8. Overview of the allogeneic immune response. Activation of CD4+ and CD8+ T cells leads to activation of cells including macrophages, which in turn, leads to activation and proliferation of T and B cells, with upregulation of cytokine production.

(Hutchinson, 1996). This whole cascade of activation, proliferation and synthesis of mediators such as antibodies and cytokines is responsible for the rejection and inevitable destruction of the graft.

1.9.3. Indirect and direct allorecognition

Lechler and Batchelor (1982) performed experiments in which long surviving HLA mismatched renal allografts from rats were re-transplanted into recipients syngeneic with the first recipients. There was a dramatic reduction in the immunogenicity of the grafts because of the loss of passenger dendritic cells derived from the original donor. However, some of the kidneys were still rejected if immunosuppression was not given. Based on these experiments, the group proposed that there were two routes of allorecognition, the "indirect" and "direct" pathway (Figure 1.9).

In the direct pathway, T cells recognise intact allo-HLA on the surface of the donor cells The antigenic peptides presented are derived from endogenous proteins, which bind into the groove of the donor HLA (antigen presenting cell) and are then recognised by recipient T cells (Benichou, 1999). The T cell response that results in early, acute cellular rejection is caused mainly by direct allorecognition (Hornick and Lechler, 1997). Direct pathway T cells may make up as many as 5-10% of the total pool of peripheral T cells. The strength of alloreactivity is directly related to the number of activated allogeneic cells. This binary complex hypothesis was first described by Matzinger and Bevan (1977). If the ligand for alloreactive T cells is allo-HLA complexed with a specific peptide, this could account for the observed high frequency of alloreactive cells. HLA molecules on the foreign cells surface are occupied by a wide variety of peptides, thus leading to a wide range of allo-HLA-peptide complexes, and therefore stimulating a large number of T cells (Pattison and

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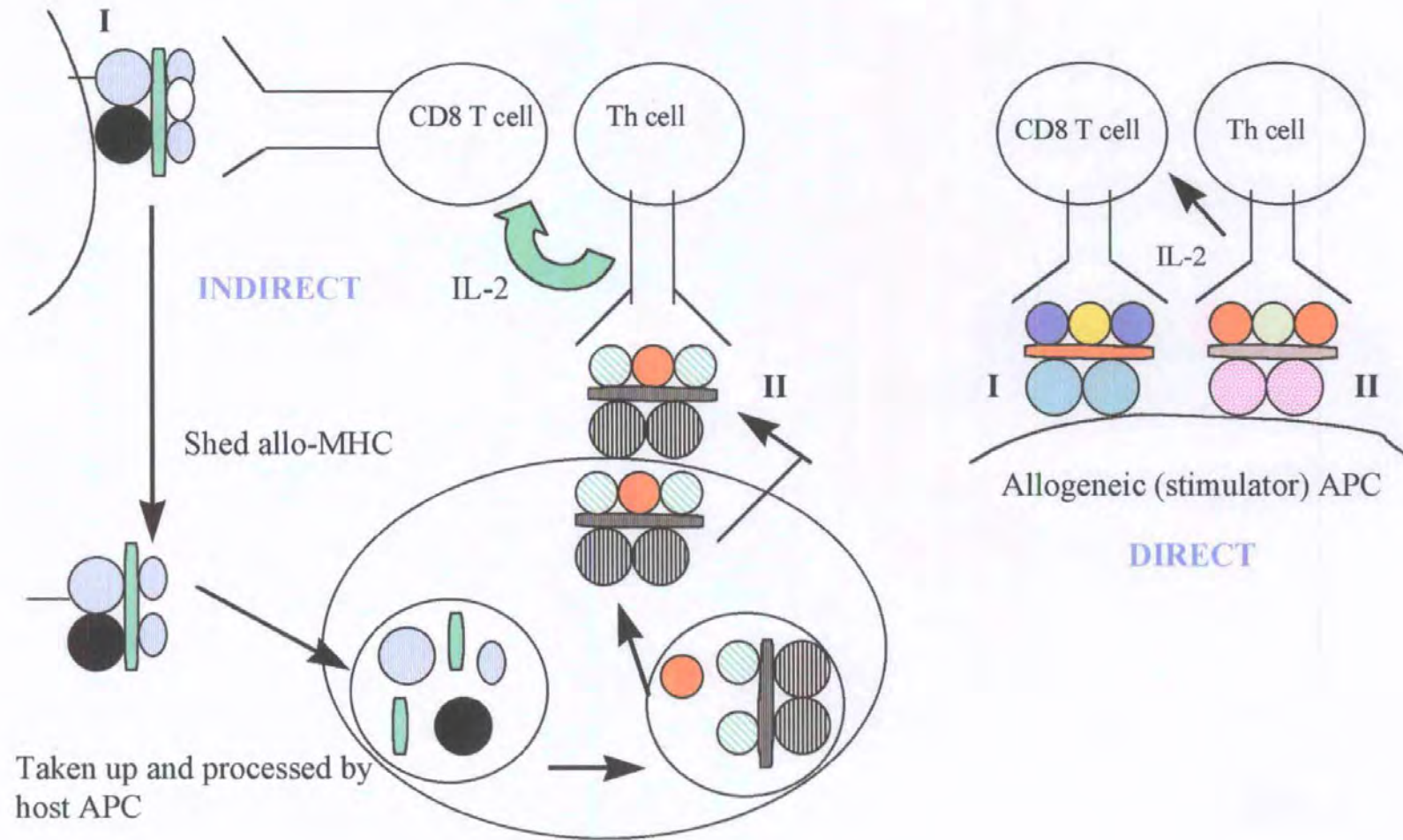
Figure 1.9. Indirect and direct allorecognition in T cell activation.

Krensky, 1997). The other hypothesis, known as the high ligand density hypothesis provides an alternative explanation for the high precursor frequency of alloreactive T cells. In this instance, the TCR ligand, which is the allo-HLA molecule itself, is present at a far higher density than would normally be achieved. Therefore, T cells with a lower specific affinity for the ligand than would be required for an antigen-specific response can be recruited for an alloresponse. This proposal may explain the gradation of strength of the HLA antigens (ie: HLA-DR, -A, and –B) as each of these antigens differ in the number of alloreactive cells that they can stimulate.

In the "indirect" pathway, T cells recognize processed alloantigen in the context of self-antigen presenting cells (Bradley, 1996). Donor alloantigens (either allo-major HLA, minor histocompatibility antigens, or tissue-specific antigens) are shed from the graft, taken up by recipient APCs and presented to CD4+ T cells (Suciu-Focia et al, 1996). These activated T helper cells secrete cytokines and provide the necessary signals for the growth and maturation of effector cytotoxic T lymphocytes (CTLs) and B cells (Vella et al, 1999). Indirect presentation may be important in amplifying and maintaining the rejection response. The consequences of T cell activation by these two routes are different (Hornick and Lechler, 1997).

Rejection may be divided into three stages, hyperacute, acute and chronic. The immunology of each stage will be discussed below.

1.9.4. Hyperacute rejection

This is the initial stage of allograft rejection. Hyperacute rejection may be seen within minutes to hours after surgery. The presumptive mechanism in hyperacute rejection is humoral pre-sensitisation, since there is a high correlation between the presence of pre-formed cytotoxic antibodies with the blood group or HLA antigens of the donor (Scornik et al, 1992, Toma, 1994, Ghasemian et al,

1998). Pre-formed antibodies bind to antigens on vascular endothelium, which in turn, activates complement, thrombosis and activates neutrophil activity. Although with the recognition that positive cross-matches for IgG antibodies and not IgM antibodies are a contraindication to transplantation, this classical type of rejection is now uncommon (Leavey *et al*, 1997, McCalmon *et al*, 1997). HLA class I antibodies are also known to be contraindicative for hyperacute rejection, but not HLA class II. In addition, blood group ABO incompatibility is an absolute contraindication to organ transplantation as this increases the incidence of hyperacute rejection (Chui *et al*, 1997). However, experiments performed using A2 kidneys have been shown to succesfully cross the ABO barrier, with the implementation of plasmapheresis to remove anti-AB antibodies pre-transplant, reducing the risk of hyperacute rejection (Nelson *et al*, 1998, Tanabe *et al*, 1998, Alkhunaizi *et al*, 1999).

1.9.5. Acute rejection

Acute rejection may develop between one week and three/six months post-transplant. The afferent limb of the immune response involves the release of antigenic material into the draining lymphatics, where it activates an immune response in the draining lymph nodes (central stage). Secondly, cellular and humoral effector mechanisms are generated which leave the lymph node, enter the blood, and are delivered to the graft, causing its destruction (efferent stage). Within a few hours of engraftment, neutrophils may enter the interstitium after interactions with selectins upregulated on vessel walls (Azuma *et al*, 1996). Macrophage infiltration has been shown to be present in renal allograft biopsies (Svalander *et al*, 1993). There is also evidence to show that cells such as monocytes infiltrate the allograft as early as forty eight hours after transplantation (Hughes *et al*, 1994).

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Acute rejection is primarily a T cell mediated host event, with both CD4+ and CD8+ T cells present (Dallman, 1997). Direct allorecognition is mainly associated with acute rejection (Shoskes and Wood, 1994, Hutchinson, 1996). From this stage, CD4+ T cells are activated by recognition of HLA class II antigens, which leads to the activation of NK cells, B cells and CD8+ cytotoxic T cells. Numbers of acute rejection episodes have been shown to be among the best predictors of allograft survival (Tesi *et al*, 1993), and it is well known now that HLA mismatching between individuals elicits immune responses (Gorski, 1996), therefore, as previously mentioned, it is important to ensure as close a match as possible in order to reduce the risk of acute allograft rejection.

1.9.6. Chronic rejection

This stage presents as insidious deterioration in renal function at any time from six months to many years post-transplant. The pathogenesis of chronic rejection is a complex network of immunological, metabolic and haemodynamic events leading to a cascade of cellular and molecular events (Koskinen *et al*, 1996). The immune and non-immune inflammation induces persistent low grade damage to the vascular endothelium, which in turn, leads to the induction of inflammatory mediators, such as IL-1, IL-6 and TNF-α (macrophages), IgM and IgG production (B cells) and TGF-β (T cells) (Hayry *et al*, 1993). This results in smooth muscle cell replication in the vascular wall, the migration of smooth muscle cells from the media into the intima, and generation of arteriosclerotic lesions throughout the entire length of the vessel wall (Lemstrom *et al*, 1995). The changes seen in chronic rejection may be immune-mediated, or may be the result of previous damage sustained during acute rejection.

1.9.7. GVHD

This includes acute GVHD, which occurs within the first one hundred days post-transplant, and chronic GVHD, which occurs following an acute episode or *de novo*. The mechanism of GVHD, in particular acute, begins with the pre-conditioning regimen (radiation, chemotherapy), which leads to injury of host tissues (Hill *et al*, 1997). This gives rise to the induction of cytokines such as TNF-α and IL-1 (Krenger *et al*, 1997, Hill *et al*, 1999), together with an increase in major and minor histocompatibility expression. The donor bone marrow is then infused, and this leads to the activation of Th1 cells (induction of IL-2 and IFN-γ) (Ochs *et al*, 1996). CTL and NK cells become activated, together with the subsequent release of nitric oxide (NO) and TNF-α and IL-1 (Vora *et al*, 1997). Lipopolysaccharide (LPS) leaks through the mucosa, damaged in the beginning, and stimulates further cytokine secretion, thus leading to target organ damage (Cooke *et al*, 1998). This cascade of events may be prevented if donor T cells are activated after they produce a Th2 type cytokine profile after transplantation.

1.9.8. Immunological tolerance

Graft specific immunosuppression has obvious prophylactic and therapeutic advantages over non-specific immunosuppression (see immunosuppressive therapy section). Experimental animal models have previously demonstrated that specific immunological tolerance of organ allografts may be achieved, and the hope is that similar states can be induced for clinical purposes (Calne *et al*, 1994, Dong *et al*, 1999, Noris *et al*, 1999). The methods for inducing tolerance is short term, but the tolerance achieved is long term. The term "T cell tolerance" was initially used to define antigen-directed unresponsiveness states resulting from deletion, anergy, ignomace or specific suppression (Lechler and Bluestone, 1997, Weigle and

Romball, 1997). It is immunologically specific, and can be transferred by T cells from tolerant donors to syngeneic naïve animals. Tolerance may be explained by the proposal that the tolerance inducing protocols generate a predominantly Th2 type of response, and that the suppressive T cells are specific for alloantigen, but secrete IL-4, IL-10 etc (Plain et al, 1999). This immune deviation towards a Th2 type response may lead to the induction of "infectious tolerance" (Cobbold and Waldmann, 1998, Reemtsen et al, 1999) and may be described as a form of immunoregulation, in the periphery, and is dependent on CD4+ T cells that suppress primary and secondary immune responses, such that these also become both tolerant and infectious (Cobbold and Waldmann, 1998). Another concept is that alloreactive T cells can be triggered by antigen either into an activated state where they can mediate allograft rejection, or into an anergic state. In this latter state, they may compete with, and inhibit the alloantigen activated T cells (Gillanders et al, 1997, Chen et al, 1998).

There is also an observed reduction in immunogenecity of HLA incompatible allografts with the passage of time after transplantation. This may be explained by the hypothesis that the original (allogeneic) population of passenger leucocytes present in the graft is replaced by leucocytes of the recipient (Perico and Remuzzi, 1997). Dendritic cells are widely accepted as the most potent APCs and have been suggested to play a role in tolerance induction (Steptoe and Thomson, 1996, Thomson and Lu, 1999). With dendritic cells being within the passenger leucocyte population, and being highly immunogenic, when they are replaced by recipient-genotype dendritic cells, the allograft becomes much less immunogenic.

1.9.9. CD4 and CD8 T cells

The relative roles that CD4+ and CD8+ T cells have in the mediation of allograft rejection is determined in part by the dependence of CD8+ cells on CD4+

cells (Schulick *et al*, 1993). In humans, the ability of CD8+ cells to respond to alloantigen without CD4+ help is very limited (Bachetoni *et al*, 1993). If this is the case, then the rejection response is totally dependent upon class II MHC in the graft-activating host CD4+ cells, suggesting that the CD4+ T cell dependent pathway may be dominant (Hall, 1991). It also appears that CD4+ T cells provide the majority of help not only to CD8+ cells, but also to B cells for alloantibody production. Experiments have been performed in humans indicating that T cell subsets may even be helpful as a marker of rejection (Olausson *et al*, 1992) and even for predicting long term loss of allografts (Schulick *et al*, 1993). Experiments to investigate T cell subsets in acute renal allograft rejection also suggested that there is a shift from naïve to memory T cell phenotype in both CD4+ and CD8+ subsets in patients with confirmed acute graft rejection, and that the study of T lymphocyte subsets may be useful in the monitoring of recipients with acute decline in renal function (Beik *et al*, 1998). The hypothesis that CD4/HLA class II complex is involved in the rejection process has been shown in rodents (Ito *et al*, 1997). This study illustrated that a bi-directional blockade of CD4/HLA class II complex using monoclonal antibodies in heart transplant recipients prolonged graft survival compared to untreated animals. The antibody combination was also proved to be more effective than using anti-CD4 or anti-HLA antibodies alone, illustrating that the complex is fundamental in rejection.

CD4+ T helper cells may be divided into two subpopulations, Th1 and Th2. Th1 and Th2 cells are known to secrete different panels of soluble mediators or cytokines (Street and Mossman, 1991). Th1 cells produce pro-inflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-2 and tumour necrosis factor (TNF)- α. There has been suggestive evidence to indicate that there is a role for pro-inflammatory cytokines in the activation of antigen-specific CD4+ T cells (Pape and

Jenkins, 1998). Th2 cells, on the other hand, produce anti-inflammatory cytokines including IL-4, IL-5 and IL-10 (Nickerson *et al*, 1994). CD8+ T cells may also be divided into two subsets determined by their cytokine secretion (Mossman *et al*, 1997, Van Emmerik, 1997). Type 1 cytotoxic cells are associated with the production of IFN-γ. Type 2 cells may suppress the killing of infected cells, and produce IL-4, IL-5 and IL-10 (Salgame *et al*, 1991).

1.10. Cytokines

1.10.1. Introduction

T cells are important immunoregulatory cells involved in the activation of B cells, macrophages and other T cells, of which many of the functions are mediated by soluble mediators or cytokines (Cohen *et al*, 1994). Cytokines are soluble proteins or glycoproteins produced by lymphocytes, and in many cases other cell types, which act as chemical communicators between cells, but generally not as effector molecules in their own right (Corrigan, 1994). Most are secreted, but some can be expressed on the cell membrane, and others held in reservoirs in the extracellular matrix. Cytokines bind to specific receptors on the surface of target cells, which are coupled to intracellular signal transduction and second messenger pathways.

Over the last three decades, cytokines have been given various names. "Lymphokines" were originally defined as cell free soluble factors generated by sensitised T cells in response to specific antigen (Dumonde *et al*, 1969). The terms "cytokine" and "interleukin" served to broaden this definition to include factors originating from many different cell types (Aarden *et al*, 1979). Cytokines were defined initially on the basis of their activities, but the cloning of the genes for these products has greatly facilitated their classification, and cytokine expression both *in vitro* and *in vivo* may now be studied at the gene, mRNA or protein level.

Cytokines interact in a self regulating network and any disruption of this network may have dramatic effects on the outcome of the immune response (Dallman, 1993). Cytokines are major components of both cellular and humoral immune responses and of their effector mechanisms. As previously described, cytokines may be used as target sources of immunosuppressive therapy and play a central role in recipient defence against infectious episodes and tumours (Soulillou, 1993, Leimenstoll *et al*, 1993).

The physiological role of cytokines is governed by redundance (ie; several factors control cells in any one lineage) and pleiotropic effects (ie; most factors can influence more than one cell lineage) (Digiovine and Duff, 1990). Cytokines may act in a synergistic fashion eg; IL-4 and IL-5 for immunoglobulin switching or as antagonists eg; IL-4 and IFN-γ on Igγ2a production. Cytokines are now known to be involved in the majority of immune responses, being implicated in both acute (Schmouder and Kunkel, 1995) and chronic rejection (Stein-Oakley *et al*, 1995).

1.10.2. Cytokine production and regulation

A widely accepted working model for T cell regulation of the immune response has developed from the interpretation of many experimental and clinical observations in transplantation, infection, autoimmunity and other immunological conditions. The model suggests that signals received from the antigen presenting cell and bystander cells during T cell activation direct the development of the T cell cytokine response towards the production of either type 1 or type 2 cytokines (discussed later). The longer the immunogen persists, the more polarized the T cell cytokine response becomes. The preferential production of type 1 or type 2 cytokines in turn regulates the development and activation of effector cells of the inflammatory and immune systems, and therefore determines the outcome of the response

(Fitzpatrick and Kelso, 1998). This model is supported at the T cell population level by the demonstration that cytokine profiles are polarized in immune responses to certain infectious agents, allergens and other persistent immunogens in both mouse and man, and that these polarized profiles play a key role in antigen clearance and pathogenesis (Mossman and Sad, 1996).

Most cytokines are not constitutively expressed but are rapidly synthesised in response to stimulation. The extent of stimulation may elicit qualitatively different patterns of cytokine gene expression (Imada *et al*, 1995). For most cytokines, the nature and extent of the transcription factors which regulate expression of their genes are not yet fully characterised. The specific consensus nucleotide recognition sequences for known transcription factors can be identified in genomic DNA. The precise biochemistry of the regulatory processes imposed even by the known transcription factors, is extremely complex. For example, the induction of IL-2 gene transcription requires both constitutive (NF-AT1 and NFIL-2a) (Banerji *et al*, 1991, Chow *et al*, 1999) and inducible factors (AP-1 and NFκB) (Mortellaro *et al*, 1999), although other work has shown apoptosis-inhibiting factors such as AP-1 and NFκB not to be required for IL-2-mediated cell cycle progression (Iacobelli *et al*, 1999). Several mediators also act to limit cytokine gene expression. The classical inhibitors are the glucocorticoids, known to be used as anti-inflammatory drugs. The glucocorticoid receptor complex binds to elements in the promoter regions of the cytokine genes, therefore modulating cytokine gene transcription.

Control of cytokine function may also be achieved by regulating the processing of precursors. Many cytokines are produced as integral membrane proteins which require processing enzymes for proteolytic cleavage for the release of the active cytokine molecule. Experiments are being done to identify these enzymes (Thornberry *et al*, 1992, Thornberry and Molineaux, 1995). Finally, modulating the

numbers of cytokine receptors on the cell surface may also act as a form of regulation. The modulation may be performed by internalization or shedding of the receptors. Modulation of the affinity of the receptor or its function may also be brought about by receptor phosphorylation, or through competition for shared receptor chains or signal transduction molecules (Ullrich and Schlessinger, 1990, Glenney, 1992).

1.10.3. The Th1/Th2 paradigm

CD4+ T helper cells may be divided into two subpopulations, T helper 1 (Th1) and T helper 2 (Th2) cells. This was first proposed by Mossman and co-workers (1989), where murine CD4+ helper T cell clones were divided based on differences in their pattern of cytokine production. This work gave rise to the "Th1/Th2 paradigm". These two polarized forms of the specific cellular immune responses provide a useful model for explaining not only the different types of protection, but also the pathogenic mechanisms of several immunopathological disorders. Th1 dominated responses are potentially effective in eradicating infectious agents. In contrast, Th2 responses are apparently insufficient to protect against the majority of infectious agents, but can provide some protection against parasites (Del Prete, 1998).

Central to this paradigm is the role of certain cytokines which can act as cross-regulators of Th1/Th2 function. Th1 cells are associated with the secretion of cytokines including IFN-γ, IL-2 and TNF-α, while Th2 cells are associated with cytokines such as IL-4 and IL-10 (Romagnani, 1991, Del Prete *et al,*1994, Rincon and Flavell, 1997). Initial stimulation of IL-2 evokes IL-2, IL-4 and IFN-γ production from non-Th1/Th2 cells (ie; Th0). If the primary stimulation is accompanied by exogenous IL-4, CD4+ T cells commit to a Th2 type profile (Abehsira-Amar *et al,*

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1992). If levels of Th1 type cytokine predominates ie; IL-12, then the CD4+ T cell commits to Th1 lineage (Palm *et al*, 1996). IFN-γ has also been suggested to play an important role in generation and commitment of Th1 type cells (Miner and Croft, 1998). This can be seen in Figure 1.10. As previously mentioned, there are several factors which have been proposed to push a T cell response predominantly towards Th1 and Th2, including dose of antigen, site of exposure and ongoing immune reponses in the host (Openshaw *et al*, 1995). CD8+ cytotoxic T cells may also be divided into two subpopulations Tc1 and Tc2, and secrete the same patterns of cytokines as CD4+ T helper cells do (Cerwenka *et al*, 1998), with the same cytokines helping to commit to either a Tc1 or Tc2 lineage (Gish *et al*, 1995).

IFN-γ, a Th1 product, can inhibit the expression of the Th2 program, while a cytokine such as IL-4, namely a Th2 product can act to block the Th1 program (Parronchi *et al*, 1992). The Th2-derived cytokine, IL-10, has also been implicated in the crossregulation and inhibition of Th1 cytokine production (Fiorentino *et al*, 1991, Mossman and Moore, 1991). This has been illustrated in murine studies, but evidence now suggests that IL-10 is a Th1 and Th2 derived cytokine, and in humans, this cytokine can down-regulate the function of both subpopulations (Del Prete *et al*, 1993) (Figure 1.11). Th1 and Th2 cytokines also have different functional properties. Th1 type cytokines are proposed to be pro-inflammatory, and therefore are involved in cell mediated inflammatory responses. Th2 cytokines, however, are anti-inflammatory, and therefore involved in humoral immunity and may encourage antibody production (Mossman and Coffman, 1989). It has been shown that Th1 clones induce delayed type hypersensitivity (DTH) reactions, with IFN-γ commonly expressed at sites of DTH reactions (Ode-Hakim *et al*, 1991). Th2 cytokines can also enhance eosinophil proliferation and function, and are mainly found in association with strong antibody and allergic responses (Mossman and Sad, 1996).
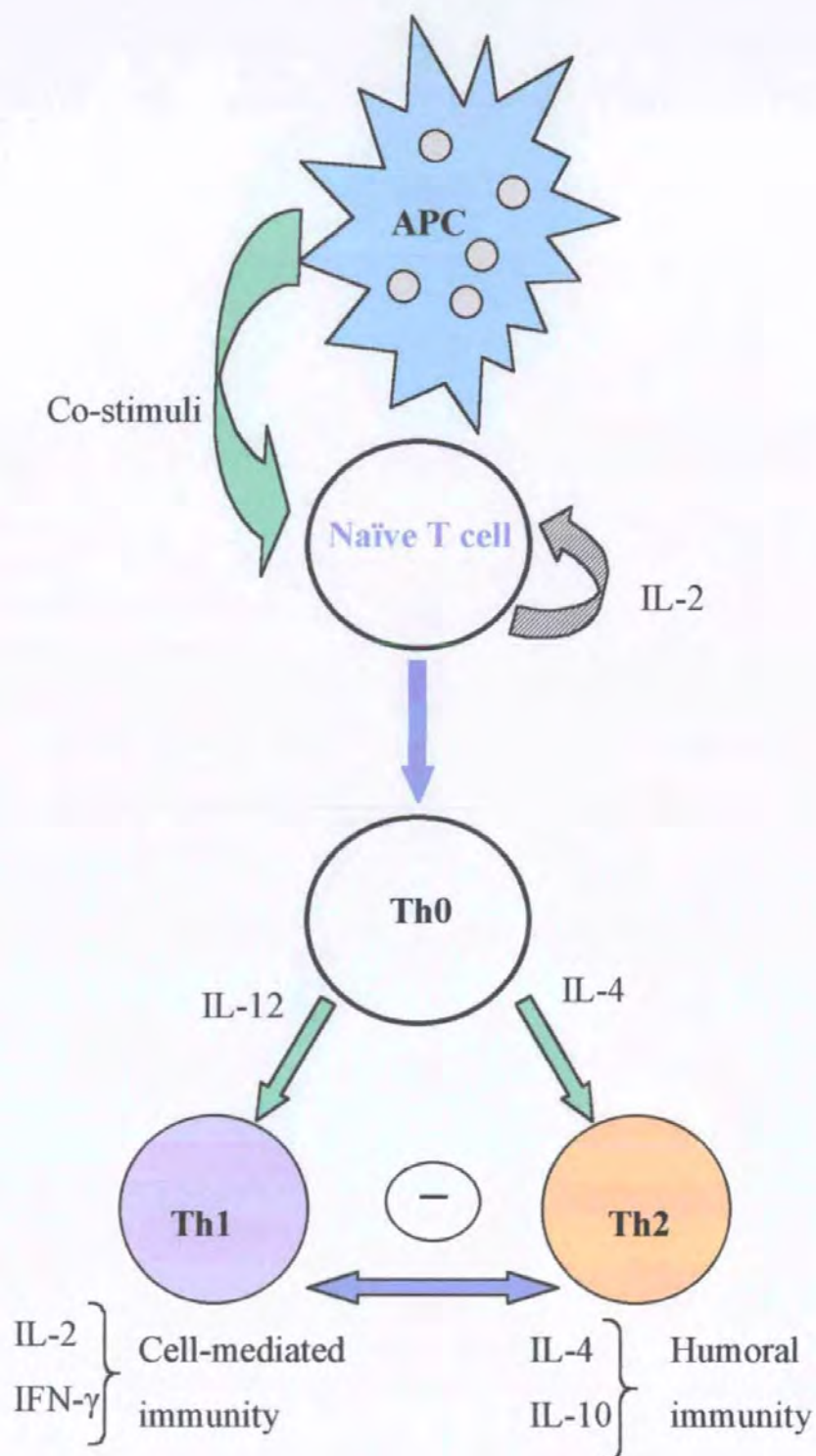
Figure 1.10. The Th1/Th2 paradigm. Initial stimulation of T cells evokes IL-2, IL-4 and IFN-γ production. If the primary stimulation is accompanied by IL-12, CD4+ T cells commit to a Th1 type lineage. However, if accompanied by IL-4, CD4+ T cells commit to a Th2 type lineage

It has therefore become clear that early events in an immune response stimulate the production of cytokines that direct the subsequent development of T helper subsets producing discrete patterns of cytokines. These findings are currently under extensive investigation in the context of targeting these immune events for therapeutic intervention, with the hope of clinically managing the immune response in various diseases (O'Garra and Murphy, 1994).

1.10.4. Th1, Th2 cytokines and disease

1.10.4.1. Transplantation rejection: pro-Th1

The Th1/Th2 paradigm has been extensively associated with the rejection process in transplantation and GVHD (Smith *et al*, 1991, Jadus and Wepsic, 1992, Nickerson *et al*, 1994, 1997, Dallman, 1995, Moller, 1995, Krenger *et al*, 1997, Ferrara and Krenger, 1998). The introduction of allogeneic tissue stimulates a wide range of immunological responses in the recipient. In solid organ transplantation, the phenomenon is primarily cell-mediated, and cytokines have been suggested to play an integral role in the regulation and co-ordination of acute allograft rejection (Gaston, 1994). As T cells are activated, there is enhanced production of IL-2, formation of high affinity receptors, and ultimately proliferation of an antigen specific T cell response.

During rejection, cytokines can facilitate tissue damage by immunological mechanisms, with the ultimate result of the response being rapid allograft destruction. The rejection response leads to an array of inflammatory responses. The rejection response may even be described as a "cytokine storm" where a whole array of cytokines are produced (Ferrara *et al*, 1993, Merville *et al*, 1993a, 1993b), with the activation of specific and non-specific effector mechanisms being dependent on the production of these cytokines (Bachetoni *et al*, 1993). Th1 cytokines, as
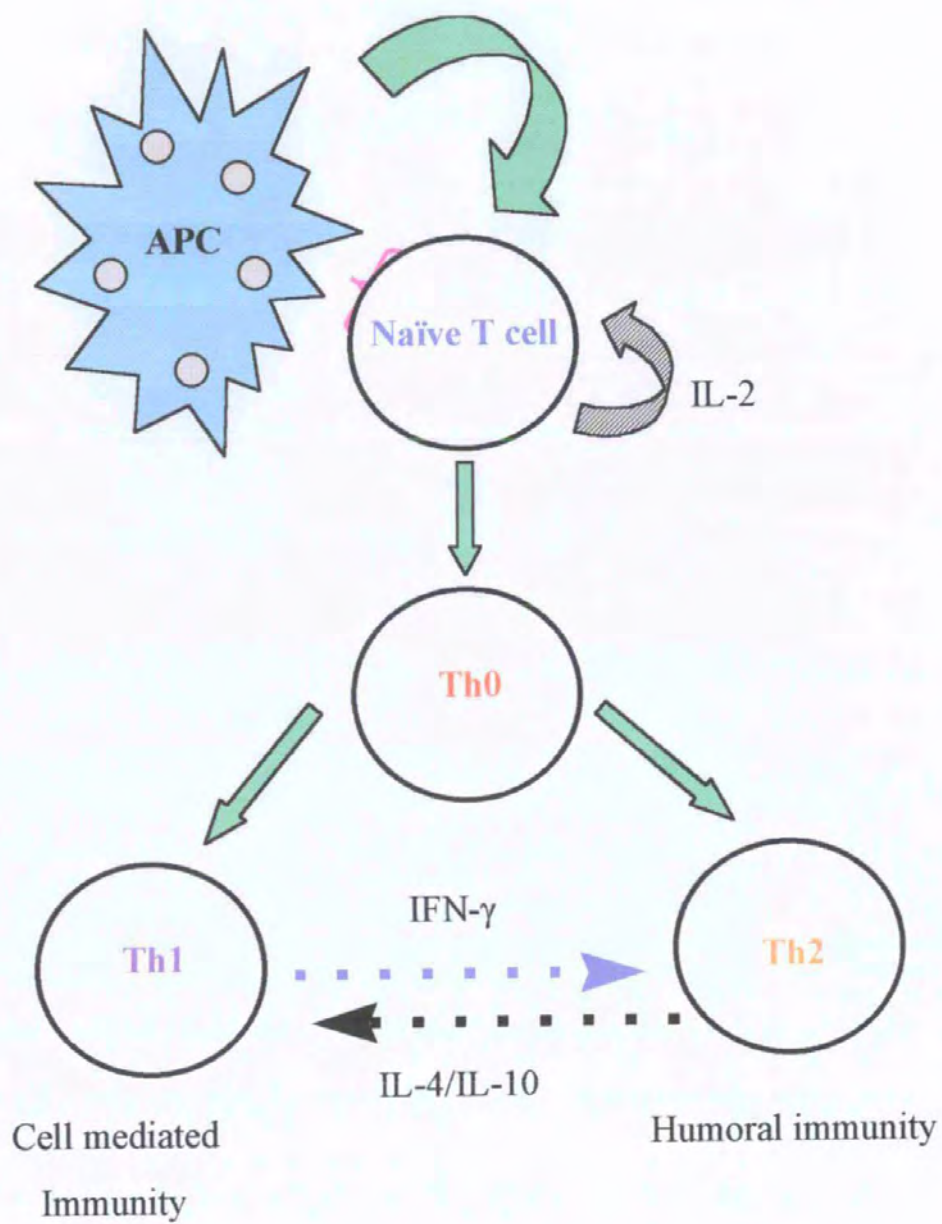
Figure 1.11. Regulation of Th1 and Th2 responses.

mentioned previously are pro-inflammatory and have been widely associated with the association of rejection episodes in transplantation eg; kidney (D'Elios *et al*, 1997), small bowel (Galvao *et al*, 1996), liver (Gaweco *et al*, 1995) and heart (Van Besouw *et al*, 1997). Studies using the famous skin explant model for predicting GVHD and investigating the aetiology of the disease have demonstrated the role of Th1 cytokines as important mediators of cellular damage in the GVH reaction (Dickinson *et al*, 1991, 1994a, 1994b). Th1 cytokines such as IL-6 and TNF-α have been shown to be significantly elevated in acute rejection biopsies (Cho *et al*, 1998). Another study also illustrated the elevated expression of Th1 cytokines, IL-6 and TNF-α in biopsies taken from patients undergoing acute renal allograft rejection, identifying that these cytokines do play a role in rejection (Vandenbroecke *et al*, 1991). IL-6 has also been associated with chronic rejection in kidney allograft recipients (Kaminska *et al*, 1996). However, there have been conflicting results, suggesting that the Th1 cytokine proteins are present but are not significantly elevated in both heart (Azzawi *et al*, 1996, Grant *et al*, 1996). and kidney transplants (Callait-Zucman *et al*, 1991), suggesting that Th1 cytokines do not act alone in the process of rejection.

Cytokines such as IL-2 and IFN-γ have also been suggested to be associated with the development of cellular rejection and that these inflammatory cytokine networks are active throughout the development of rejection (Stein-Oakley *et al*, 1995). Enhanced production of IFN-γ has been found in T cell clones from rejected kidney grafts (Benvenuto *et al*, 1991). In this study, CD4+ and CD8+ T cell clones were generated. It was found that IFN-γ was up-regulated in the clones made from rejected allografts compared to clones synthesized from normal peripheral blood. IL-2 and IFN-γ levels have also been shown to correlate with grades of cellular

rejection (Norohna *et al*, 1993). Manipulation of Th1 cytokine production such as IFN-γ have also been shown to suppress allograft reactivity, thus inducing tolerance (Landolfo *et al*, 1985, Dallman *et al*, 1991). However, as before, it has been suggested that cytokines such as IL-2 and IFN-γ may be necessary, but not sufficient for the development of cellular rejection (McClean *et al*, 1997).

1.10.4.2. Transplantation tolerance: pro-Th2

Th2 cells have been suggested to produce cytokines that may be associated with tolerance (Lowry *et al*, 1993). The induction of tolerance to alloantigen is accompanied in many cases by a decrease in the production of cytokines such as IL-2 and IFN-γ, with a sustained increase in Th2 cytokines IL-4 and IL-10 (Dallman *et al*, 1993). Patterns of Th1 and Th2 cytokines have been studied (Lambert *et al*, 1995; Joseph *et al*, 1995). Lambert *et al* (1995) illustrated that differential Th1/Th2 production corresponds with different outcomes of grafts eg; IL-10 and IFN-γ, while Joseph and colleagues illustrated that elevated Th2 cytokine expression was not found in rejecting allografts, thus confirming that Th1 and Th2 cytokines have opposite roles in transplantation rejection.

IL-10 and IL-4 have both been shown to have a protective role in the immune system (Holan *et al*, 1994, Bromberg, 1995, Kusaka *et al*, 1995), and are shown to be associated with tolerance in transplantation, for instance of the kidney (Burke *et al*, 1996, Weimer *et al*, 1996) and the heart (Bushell *et al*, 1994, Grant *et al*, 1996, He *et al*, 1998). Removal of Th2 cytokines, such as IL-4 have been suggested to even prevent the induction of transplantation tolerance (Bushell *et al*, 1994). On the other hand, blocking of Th1 cytokines and expansion of Th2 immune responses by possible immunomodulation could possibly encourage the tolerance response (Chen *et al*, 1996). Preliminary data already shows that the administration of recombinant

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Th2 cytokine (in particular IL-10) has a significant effect on the reduction of pro-inflammatory mediators in both normal volunteers (Fuchs *et al*, 1996) and transplant patients (Wissing *et al*, 1997). However, some conflicting evidence has been published stating that Th2 cytokines may be both beneficial, deleterious (Picotti *et al*, 1997) and even irrelevant in transplantation (Carayol *et al*, 1997, Piccotti *et al*, 1997).

In addition Th2 cytokines such as IL-10 have also been found to be deleterious as they have been detected in rejecting allografts of the liver (Lang *et al*, 1995, 1996), heart (Cunningham *et al*, 1994, Krieger *et al*, 1996, El Gamel *et al*, 1998), and kidney (Merville *et al*, 1995). However, the presence of Th2 cytokines such as IL-10 may not necessarily mean that they are associated with transplantation rejection. As rejection is an inflammatory response, it is possible that anti-inflammatory mediators will be present in the graft in order to either try to suppress the allogeneic response or even cross-regulate the effector functions of the Th1 cytokines (Tadmori *et al*, 1994, Alfrey *et al*, 1995). The presence of anti-inflammatory mediators may therefore lead to a mechanism of tolerance. The likes of IL-10 may even serve a function of immune regulation at the site of inflammation, rather than in the immune suppression of the rejection response (Azzawi *et al*, 1995). With all of this conflicting evidence, the Th1/Th2 paradigm may not be as clearly divided into pro- and anti-rejection cytokines. Th1 and Th2 cytokines may both be present in the rejecting allograft, but it may be the ratio of Th1:Th2 cytokines that may determine a pro- or anti-rejection response (Katsikis *et al*, 1994).

1.10.5. Th1 and Th2 cytokines in other clinical manifestations

The Th1/Th2 paradigm may be linked to many clinical situations. Pregnancy, for instance has been shown to be associated with a skewing towards a Th2 type

response (Bjorksten, 1999). A shift towards the Th1 type response has been associated with unsuccessful preganacy and recurrent abortions (Raghupathy, 1997, Piccini *et al*, 1998). Other conditions such as Bechets disease (Raziuddin *et al*, 1998) and in particular, allergy (Kapsenberg *et al*, 1998) have also been associated with divergent cytokine profiles.

There is increasing evidence that T cell reactivity to the environment in the development of allergy may occur during early childhood. Initial T cell priming may even occur in utero (Holt *et al*, 1997, Prescott *et al*, 1997). Clinical manifestations such as allergy and atopy have been associated with a pro-Th2 response (Nakazawa *et al*, 1997, Bjorksten, 1999) with evidence suggesting that Th2 polarization may occur during chilhood (Piccini *et al*, 1996, Yabuhara *et al*, 1997). An increase in Th2 cytokines such as IL-4, IL-6 and IL-13 have been shown, with a dominant increase in IL-10 (Esnault *et al*, 1996, Prescott *et al*, 1998). There has also been evidence that an increase in IL-4 may be accompanied with a decrease in cytokines like IFN-γ (Warner *et al*, 1993, Prescott *et al*, 1999). The upregulation of cytokines like IL-4 in atopy is due to the fact that IL-4 acts as an isotype switch cytokine which activates the IgE pathway in B cells (crucial for mast cell activation and histamine release), while IFN-γ inhibits this transformation (Jujo *et al*, 1992).

The increase in allergies has been almost blamed on the lack of dirt and disease since the elevated use of anti-bacterial products and vaccination in childhood. The first suggestions of this hypothesis was in 1989, where David Strachan, an epidemiologist in London, noticed that children from extensive families were less likely to develop eczema, asthma or hay fever. It was suggested that older siblings bring their younger family members into contact with more infections than in smaller families, thereby exposing their immune systems to antigens (reviewed by Hamilton, 1998). Immunologically, this has been explained by the Th1/Th2 responses. Th1

cells are predominantly activated by Th0 cells being exposed to external antigens such as bacteria and viruses. If exposure to antigens is minimal, Th2 cells predominate. Therefore, individuals exposed to fewer antigens are more likely to have a Th2 dominated immune response (Rook and Stanford, 1998).

At present, there is much work being carried out in order to find ways to compensate for the lack of Th1 immunity. Vaccination has been suggested, with the intent to replace infections which stimulate the Th1 response in order to re-address the cytokine balance. Previous work by Erb *et al* (1998) reported that inhaling mycobacteria can stave off allergy attacks in mice. Allergic mice were injected with egg white which was intended to boost their Th2 responses. When the vaccine was injected before the second dose of egg white, there was no allergic response. In a second study, Rook and Stanford (1998) showed the same effect when dead *M. vaccae* was injected into mice. Trials of this vaccine are currently underway on allergic patients. Extensive research is constantly being carried out in order to find ways to modify the immune response in conditions such as allergy (Allen and Maizels, 1997).

1.11. Functional tests

1.11.1. Mixed lymphocyte culture (MLC)

It has been known that while normal individuals do not react against their own tissue, a vigorous response can be mounted against foreign tissue leading to graft rejection or GVHD. It is now known that the cellular basis for alloreactivity is the recognition by alloreactive lymphocytes of alloantigens encoded for by the HLA and when allogeneic lymphocytes are cultured together, proliferation occurs (Dozmorov *et al*, 1995).

When a mixture of two populations of mononuclear cells are cultured together, this is termed a mixed lymphocyte culture (MLC) or mixed lymphocyte reaction (MLR) (Bain *et al*, 1964, Bach and Hirschhorn, 1964). If the two populations of cells arise from HLA-identical individuals or single individual, the cells will eventually die. However, if the individuals are mismatched, the cells will undergo DNA synthesis, blast transformation and proliferation. This would be termed a two way MLC, but the standard method to use would be a one way MLC, where one population (stimulator) is treated either by irradiation or mitomycin C in order to render them unresponsive (see materials and methods). In such a culture, the one way MLR may be set up in either the host-versus-graft (HVG) or graft-versus-host (GVH) direction. In this assay, the cells are cultured together and then tritiated (^{3}H) thymidine is added to the cultures (thymidine incorporates into the DNA of dividing cells only, and therefore will radiolabel the responding cell population). The cells are the harvested and the amount of thymidine uptake is measured by a scintillation counter. The response is compared to both a positive (response to HLA-mismatched cells) and a negative control (response to autologous cells). If there is a significant increase above the negative control, then the reaction is considered to be positive.

During the MLR, two distinct lymphocyte populations are stimulated, proliferating and cytotoxic populations, both of which are stimulated by different HLA differences (Bach *et al*, 1973). In an MLR, the responding cells are mainly alloreactive CD4+ T helper cells responding to HLA class II differences on foreign cells. There is also a weak response by CD8+ T cells against HLA class I differences. The stimulatory cells are antigen presenting monocytes or B cells which have HLA class II antigens on their surface. HLA-DR mismatches predominate in the MLC (Al-Daccak *et al*, 1990), with HLA-DR and DQ mismatches defined by (RFLP) analysis

correlating with a positive MLC reaction (Clay *et al*, 1989). It has also been shown in macaques, who share close DNA homologies with humans, that multiple DR mismatches show a trend towards a higher MLC proliferative response (Lobashevsky *et al*, 1998). However, positive reactions can occur in HLA-DR and DQ matched pairs suggesting that other antigens may be involved, such as HLA-A and B (Termijtelen and Van Rood, 1981a) and/or HLA-DP (Clay *et al*, 1989). The role of HLA-DP remains controversial in that studies have demonstrated an influence (Termijtelen and Van Rood, 1981b) and no influence on the MLR (Pawelec *et al*, 1982). Other studies have also shown that certain DP mismatches may induce a response in the MLC, although the response is not as strong as HLA-DQ or DR mismatches (Cesbron *et al*, 1990, Olerup *et al*, 1990, Moreau *et al*, 1993).

The MLC is a semiquantitative HLA matching test which confirms HLA class II identity between donor and recipient. Although it has played a role in selection of unrelated donors for bone marrow transplantation (BMT), the assay is now thought of as being relatively imprecise and has been shown not to predict GVHD and/or clinical outcome after BMT in identical sibling (Lim *et al*, 1988, DeGast *et al*, 1992), related haploidentical (Mickelson *et al*, 1993a) and unrelated donors (Hows *et al*, 1986, Mickelson *et al*, 1993b, 1996). In the case of renal transplantation, there has been evidence for (Harmon *et al*, 1982, Langhoff *et al*, 1985, Ghobrial *et al*, 1994, Agrawal *et al*, 1998, Mizutani *et al*, 1998, Salomao *et al*, 1998) and against (Cullen *et al*, 1977, Burke *et al*, 1993, Steinmann *et al*, 1994) the MLC predicting rejection and/or clinical outcome. The reasons for this assay possibly not predicting clinical outcome may be that it predominantly detects CD4+ HLA class II and not CD8+ class I reactive lymphocytes, which are an important effector cell population, and that the assay is only semiquantitative. The MLC therefore, remains an uncertain method for the prediction of transplant outcome.

1.11.2. Cytotoxic T lymphocyte precursor (CTL-p) analysis

A cytotoxic T lymphocyte precursor (CTL-p) is a naïve cell, that has the potential to develop and differentiate into a mature CTL. This occurs after encountering a specific foreign antigen. The CTL-p assay is used in order to determine the frequency of alloreactive CTL-p's in a population of mononuclear cells by the method of limiting dilution. Limiting dilution analysis (LDA) for CTL-p frequency has been previously described (Lefkovits and Waldmann, 1984a, 1984b, Sharrock et al, 1990, Kaminski et al, 1991). Limiting numbers of responder cells (usually 5 x 10^4 - 0.125 x 10^4) are cultured with a constant number of irradiated stimulator cells (5 x 10^4). Recombinant IL-2 is added to the culture and on the day of harvest, ^{51}chromium (^{51}Cr) labelled stimulator target cells (PHA blasts) are added to the cultures. The supernatants are then harvested and ^{51}Cr release is measured. Positive wells are scored if the ^{51}Cr release is greater than the mean plus three standard deviations of the control (irradiated stimulator cells only). It is estimated that at high concentrations, there will be more CTL-p's (>1/well) than at low concentrations (<1/well). Using the Poisson distribution model, it is possible to estimate the frequency of alloreactive CTL-p in peripheral blood.

The CTL-p response is attributed to predominantly CD8+ CTLs recognising HLA class I differences of foreign cells. As in the MLC, CTL-p frequencies have also been shown to correlate with the degree of HLA matching (Kaminski et al, 1988) and severity of acute GVHD in unrelated bone marrow transplantation (Kaminski et al, 1989). In the case of organ transplantation, no correlation has been between CTL-p frequencies and rejection after renal (Steinmann et al, 1994, Bouma et al, 1995, Mestre et al, 1996), heart (Reader et al, 1990) or liver transplantation (Eberspacher et al, 1994). There is conflicting evidence, however, which suggests

that CTL-p frequencies do correlate with graft outcome in heart transplantation (Hu *et al*, 1994).

It therefore appears that the CTL-p assay is of use for measuring GVHD in unrelated bone marrow transplantation. This does not appear to be the case for organ transplantation, and it seems that it has proved difficult to correlate pre-transplant CTL-p reactivity with organ graft rejection. However, it has been demonstrated using the CTL-p assay that a selective reduction of donor-specific CTL-p's were found in patients with a well functioning renal allograft (Herzog *et al*, 1987), therefore this assay may be of value in post-transplant monitoring.

1.11.3. Helper T lymphocyte precursor (HTL-p) analysis

HTL-p analysis is based on the measurement of IL-2 secretion by helper T lymphocytes. HTL-p frequencies, like CTL-p, are quantitated by limiting dilution analysis. The assay is set up in a similar way to that of the CTL-p assay. Graded numbers of untreated responder cells are added to microtitre plates. A constant number of treated stimulator cells are then added to the responder cells and cultured as an MLC. Following incubation, a fixed concentration of an IL-2-dependent cell line eg; CTLL-2 is added to each well. After further incubation, tritiated thymidine (^{3}H) is added to the culture and incubated. The cells are then harvested, and ^{3}H incorporation by the IL-2-dependent cell line is measured. HTL-p frequencies are calculated from the proportion of wells which are negative for IL-2 production at each responder cell dilution.

Studies of HTL-p analysis have been done in order to study human peripheral blood mononuclear cells for alloresponsiveness (Theobald *et al*, 1989, 1990, Reisaeter *et al*, 1996). Because anti-recipient HTL's have been implicated in the control and amplification of alloreactive immune responses that initiate GVHD,

HTL-p analysis has been shown to be useful for the selection of HLA-identical donors in bone marrow transplantation (Schwarer *et al*, 1993). Combined CTL-p and HTL-p limiting dilution assays have also been shown to be useful in predicting GVHD in unrelated donor bone marrow transplantation (Wang *et al*, 1996), but not in HLA-identical sibling bone marrow transplantation (Dickinson *et al*, 1998). The HTL-p assay has also been shown to be of use in determining frequencies of IL-4 secreting HTL-p's (Imami *et al*, 1997), thus illustrating that IL-4 producing HTL-p frequencies are associated with reduced risk of GVHD in bone marrow transplantation.

However, the HTL-p assay has not been of much use, in organ transplantation compared to bone marrow transplantation. No correlations have been found between HTL-p frequencies and rejection after renal transplantation (Bouma *et al*, 1995, Beik *et al*, 1997), although a positive correlation has been found between T cell sensitisation and HTL-p frequencies in chronic renal failure patients (Deacock and Lechler, 1992). This assay, like CTL-p has a use for prediction of GVHD in bone marrow transplantation, but its' use for prediction in organ transplantation is still unclear.

1.11.4. Cytokines as functional tests

Cytokine profiling may be performed *in vitro* in several ways:

a) Precursor frequencies of cytokine-secreting helper T cells using HTL-p analysis (see HTL-p section)

b) Quantification of numbers of cells expressing intracytoplasmic cytokines by flow cytometry

c) Cytokine mRNA detection by PCR or *in situ* hybridisation

d) Cytokine protein secretion by ELISA

Limiting dilution analysis of cytokine secreting lymphocytes has demonstrated large inter-individual variations in the frequencies of both alloreactive IL-2 and IL-4 secreting HTL-p's (see HTL-p section). Large inter-individual variations in both the amount of cytokine secretion by ELISA (IL-2, IL-4 and IFN-γ) and the numbers of cytokine secreting lymphocytes by flow cytometry (IL-2 and IFN-γ) have been demonstrated in mitogen stimulated cultures (Kaminski *et al*, 1995, Ferry *et al*, 1997). It should be mentioned that different stimuli may have different effects on the cytokine profile (Imada *et al*, 1995).

The MLC has been widely used for the measurement of cytokines including IFN-γ (Manger *et al*, 1981), IL-2 (Ilonen and Kartunen, 1984), IL-2 and IL-3 (Bishara *et al*, 1991, 1993), TNF-α and IL-6 (Toungouz *et al*, 1993) and IL-10 (Toungouz *et al*, 1996). There is also now evidence to suggest that some cytokine gene polymorphisms may determine the amount of protein secreted (Daser *et al*, 1996, Turner *et al*, 1997). There has been extensive work studying the clinical relevance of cytokine secretion in the MLC. Measurement of cytokine secretion in the MLC has been shown to detect HLA-DRB1 and DQB1 mismatches (Danzer *et al*, 1994a) and variations in HLA-DR4 polymorphisms (Toungouz *et al*, 1994). Quantitation of IL-2 and IFN-γ protein secretion and/or detection of IL-2, IL-5 and IFN-γ gene expression in the MLC has been shown to predict acute GVHD after identical sibling or mismatched BMT (Tanaka *et al*, 1994, 1995a, 1995b). *In vitro* skin explant studies have demonstrated the role of IFN-γ and TNF-α as important mediators of the cellular damage observed in the skin (Dickinson *et al*, 1991, 1994a, 1994b). High levels of both of these cytokines in MLC supernatants have also been shown to correlate with the severity of acute GVHD after HLA-identical sibling

BMT (Dickinson *et al*, 1994b), with this data suggesting the recognition of minor histocompatibility antigens.

In renal transplantation, cytokines have also been examined. Flow cytometric analysis has shown a positive correlation with increased IL-2 receptor expression and the onset of renal allograft rejection and rejection episodes (Reed *et al*, 1989, Langley *et al*, 1993). Cytokine secretion studies have shown IFN-γ and IL-6 as useful markers in the prediction of allograft rejection, while another study demonstrated a correlation between high levels of IFN-γ protein and a high risk of acute rejection (Kaminski *et al*, 1995). Low levels of IL-10 has been found to correlate with low risk of rejection (Weimer *et al*, 1996), however, in contrast, high IL-10 (and TNF-α) cytokine genotypes have been associated with renal allograft rejection (Sankaran *et al*, 1999). In addition, high levels of IL-10 mRNA transcripts have been detected in rejecting renal allografts (Strehlau *et al*, 1997). DNA studies have demonstrated that polymorphisms of particular cytokine genes (TNF-α and IL-10) are associated with high/low secretor status as illustrated in both heart (Turner *et al*, 1995, 1997), and bone marrow transplantation (Middleton *et al*, 1998), and even in conditions such as meningococcal disease (Westendorp *et al*, 1997). The influence of cytokine genotypes on allograft rejection is currently being extensively studied (Hutchinson *et al*, 1998a, 1998b).

1.12. Aims and objectives

In view of all of these findings, the general aim of this study was to develop a simple and reliable *in vitro* functional assay for the prediction of both acute and chronic rejection and thus improve the clinical outcome of allogeneic renal transplantation. The major aims and objectives include:

i) To develop *in vitro* assays for quantifying cytokine protein secretion and numbers of cytokine secreting lymphocytes (IL-2, IL-4, IL-6, IL-10 and IFN-γ).

ii) To compare the quantity of protein secretion with numbers of cytokine secreting cells in normal individuals.

iii) To compare IFN-γ protein secretion with numbers of IFN-γ secreting cells and IFN-G polymorphisms in normal individuals.

iv) To compare cytokine protein secretion and numbers of cytokine secreting cells in the MLC with acute rejection after allogeneic renal transplantation.

v) To compare cytokine protein secretion in the MLC with acute GVHD after HLA-identical sibling BMT.

CHAPTER 2

MATERIALS AND METHODS

2.1. Study population

2.1.1. Renal transplant recipients

One hundred patients with end stage renal failure were included in the study. Fifty seven of the patients attended clinics at Derriford Hospital in Plymouth, with forty three of the patients attending clinics at Aberdeen Royal Infirmary, Scotland. All of the patients selected received an allogeneic renal transplant. Due to the inconclusive results gained from the Aberdeen patients, clinical details were only gathered for the fifty seven Plymouth transplant patients (appendix 2). Of these patients, thirty two were male (age range: 24-76) and twenty five were female (age range: 29-71). All renal transplant recipients were HLA typed by serology (appendix 3).

2.1.2. Bone marrow transplant recipients

Eight patients undergoing bone marrow transplantation for chronic granulocytic leukaemia (CGL) (n=4), acute lymphoblastic leukaemia (ALL) (n=2) and acute myeloblastic leukaemia (AML) (n=2) were also studied. Bone marrow transplant recipients were HLA typed by serology (appendix 4).

2.1.3. Transplant donors

The donors chosen for renal transplantation were both unrelated cadaverics (n=56) and live-related (n=1). The bone marrow transplant donors were HLA-identical siblings. Renal transplant and bone marrow transplant donor HLA typing are shown in appendices 3 and 4. Renal transplant donors were selected on the basis of beneficial matching where possible and a negative serum cross-match.

2.1.4. Normal volunteers

This group consisted of normal healthy volunteers randomly chosen from Derriford Hospital and from around the Plymouth area.

2.2. Donor-recipient matching

2.2.1. HLA serotyping

The determination of an individuals' HLA type was determined by the standard micro-lymphocytotoxicity test, based upon the original method by Terasaki and McClelland (1964). Donor lymphocytes were isolated from peripheral blood for class I (HLA-A/B) and class II (HLA-DR) matching. The lymphocytes were incubated with HLA typing sera in Terasaki microtitre plates for thirty minutes at room temperature. Rabbit complement was then added to each well and incubated for sixty minutes at room temperature. Quench working solution (acridine orange/ethidium bromide) is then added to each well and incubated for a further fifteen minutes **in the dark** at room temperature. This dye is added in order to assess the degree of cell lysis. The wells are then read using a fluorescent microscope and the results are recorded as a percentage of dead (red fluorescence; positive reaction) to live cells (green fluorescence; negative reaction). From these results, the HLA type of the cells may be determined.

2.2.2 Cross-matching (Micro-lymphocytotoxicity)

This method was employed to establish if there were any pre-existing antibodies in the patient with specificity for the donor HLA antigens. Donor lymphocytes were isolated from the lymph nodes (for class I) and spleen (class II) matching. The lymphocytes from the lymph node (mainly T cells) and spleen (mainly B cells) were seperately incubated with the most reactive serum from the patient in

Terasaki microtitre plates for sixty minutes at room temperature. Rabbit complement was added as above and incubated for ninty minutes at room temperature. Quench working solution was added and incubated as in section 2.2.1. Red fluorescence indicates a positive cross-match, while green fluorescence indicates a negative cross-match.

2.3. Immunosuppressive therapy

2.3.1. Renal transplant recipients

No immunosuppressive therapy was administered to any of the patients pre-operatively. 500mg methylprednisolone was given during the transplant operation. Postoperatively, 10mg/kg cyclosporin (as Neoral) was orally given in divided doses (ie: 5mg/kg twice daily). In addition, 1mg/kg azathioprine was administered together with 10mg prednisolone orally daily. Subsequent follow-up studies were undertaken. Cyclosporin doses were adjusted according to the blood trough level to achieve a range between 100-150ug/l after one year (Table 2.1). Prednisolone was given at 10mg daily for first three months, then reduced by 2.5mg every following three months. Steroids were withdrawn completely at one year in most patients.

Acute rejection episodes were determined by commonly accepted clinical and laboratory criteria, and confirmed by needle biopsy. For suspected acute rejection, an initial course of 500mg methylprednisolone was administered on three consecutive days. Second rejection episodes were treated with a further course of methylprednisolone and conversion of cyclosporin to tacrolimus, whilst third episodes were treated with OKT3 antibodies.

Time Interval	Concentration (nmol/l) at 12 hours
0-14 days	300
2-6 weeks	250
7-12 weeks	200
3-6 months	175
6-12 months	150
after 12 months	125

Table 2.1. Target cyclosporin blood concentrations from 0-1 year.

2.3.2. Pre-conditioning regimen for stem cell and bone marrow patients

Conditioning therapy consisted of 1200cGy total body irradiation, plus 60mg of cyclophosphamide per kg of body weight per day for two days (60mg/kg/day etoposide for ALL patients). This was followed by 5mg/kg cyclosporin A for one day. Additional GVHD prophylaxis consisted of 5mg/kg cyclosporin (days -1 to +4) and 8mg/m^2 methotrexate (days +1, +3, +6 and +11). Infection prophylaxis included ciprofloxacin (500mg), itraconazole (400mg) and nebulised pentamidine (300mg) from day +28 monthly.

2.4. Materials

2.4.1. Reagents

Acetic acid and sulphuric acid were purchased from Analar Chemicals. Disodium ethylene diamine tetra-acetic acid, formamide, hydrochloric acid, magnesium chloride, sodium chloride, sodium dodecyl sulphate (SDS), sucrose, tris-hydroxy-methyl-methylamine and urea were purchased from BDH Laboratory Supplies-Merck Limited UK. Sodium hydroxide and nucleon silica were purchased from Scotlab UK. Chloroform and ethanol was purchased from Rathburns Limited UK. Dimethyl sulphoxide (DMSO), hydrogen peroxide, paraformaldehyde, phosphate buffered saline tablets, saponin, sodium acetate sodium bicarbonate, sodium carbonate, sodium perchlorate, 3,5,3',5'tetramethyl benzidine, triton X-100 and tween 20 (poly-oxethylenesorbitanmonolaurate) were all purchased from Sigma Chemicals UK.

2.4.2. Culture reagents and specialised materials

The following reagents were used for cell culture: Fetal calf serum (FCS), ionomycin, L-glutamine, mitomycin C, monensin, phorbol myristate acetate (PMA)

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and phytohaemaglutinnin (PHA) were purchased from Sigma Chemicals, UK, recombinant human IgG (Lorne Laboratories, UK), OKT3 (anti-CD3) Novartis), bovine serum albumin (BSA) (Lorne Laboratories UK), ^{32}P dATP (Amersham Life Sciences, UK), ficoll-hypaque ("Lymphoprep") (Robbins Scientific), human AB serum (Blood Transfusion Service, Bristol, UK), penicillin/streptomycin (ICN Pharmaceuticals Ltd), RPMI 1640 medium (Gibco Ltd, Scotland), dNTP's and T4 polynucleotide kinase (Pharmacia, UK) and thermostable DNA polymerase (HT Life Technology, UK). Nucleon DNA extraction kit was purchased from Scotlab UK. IFN-γ primers were purchased from MWG-Biotech Ltd.

Cryovials and sodium heparin vacutainers were purchased from Greiner Labortechnik. Eppendorf tubes were purchased from Elkay Products. Twenty four well plates (CoStar UK Ltd) were used for the incubation of cell cultures. Conjugated monoclonal antibodies used for flow cytometry were purchased from Pharmingen/Becton Dickinson except for rat anti-human IL-10 and isotype control (Serotec) (Table 2.2). ELISA kits for IL-4, IL-6, IL-10 and IFN-γ (Eurogenetics Ltd) and IL-2 (R and D Systems Europe Ltd). CD4 and CD8 pre-coated magnetic beads (Dynal UK Ltd).

2.5. Stock solutions

2.5.1. Flow cytometry

i)   **PBS**

Dissolve one tablet in 200mls distilled water.

ii)   **Staining Buffer**

PBS (pH 7.4) + 1% heat inactivated (56°C, 30 minutes) FCS.

Antibody	Concentration of Antibody
CD45 FITC/CD14 PE "Leucogate"	$20ul/10^6$ cells
Mouse anti-human CD3 (IgG1; FITC)	$10ul/10^6$ cells
Mouse anti-human CD16 FITC/CD56PE (NK cell marker; IgG1)	$20ul/10^6$ cells
Mouse IgG1 FITC isotype control immunoglobulin	$20ul/10^6$ cells
Mouse anti-human CD3 (IgG1, PECy5)	$5ul/10^6$ cells
Mouse anti human CD4 (IgG1, PE-Cy5)	$5ul/10^6$ cells
Mouse anti-human CD8 (IgG1 PECy5)	$5ul/10^6$ cells
Mouse anti-human CD3FITC/CD25PE dual stain	$20ul/10^6$ cells
Mouse IgG1 PECy5 isotype control immunoglobulin	$5ul/10^6$ cells
Mouse anti human IFN-γ (IgG1; PE)	$20ul/10^6$ cells
Mouse anti-human IL-2 (IgG1; PE)	$20ul/10^6$ cells
Mouse anti-human IL-4 (IgG1, P	$20ul/10^6$ cells
Mouse IgG1 PE isotype control immunoglobulin	$20ul/10^6$ cells
Rat anti-human IL-10 (IgG2a; PE)	$10ul/10^6$ cells
Rat IgG2a PE isotype control immunoglobulin	$10ul/10^6$ cells

Table 2.2. Flow cytometry antibodies and concentrations.

iii)   *0.5%/4% Fixation Buffer*

PBS + 0.5%/4% paraformaldehyde. Warm in 50°C waterbath until dissolved.

iv)   *Permeabilisation Buffer*

PBS + 10% heat inactivated human AB serum + 0.1% saponin.

2.5.2. ELISA (for IL-4, IL-6, IL-10 and IFN-γ)

i)   *Coating Buffer (0.1M carbonate/bicarbonate buffer)*

Solution A: 1.24g $Na_2CO_3.H_2O$ in 100mls distilled water (Baxter UK).

Solution B 1.68g $NaHCO_3$ in 200 mls distilled water.

Take 70mls of solution A and add solution B until pH is 9.6.

ii)   *PBS Wash Buffer*

PBS (pH 7.4) + 0.005% TWEEN 20).

iii)   *Substrate Buffer*

0.11M acetate buffer: 15g sodium acetate/800mls water, pH 5.5 with glacial acetic acid.

iv)   *3,5,3',5' Tetramethyl benzidine*

6mg/ml TMB in DMSO.

v)   *Hydrogen Peroxide Solution*

3% H_2O_2 in distilled water.

vi)   *Substrate Solution*

Per ELISA plate: 12mls substrate buffer; 200ul TMB solution; 12ul H_2O_2 solution.

vii)   *Stop Solution*

1.8M sulphuric acid in distilled water.

Component	IL-2	IL-4	IL-6	IL-10	IFN-γ
Coating antibody	Pre-coated	anti-IL-4, 1/100	anti-IL-6 1/100	anti IL-10 1/100	anti-IFN-γ 1/100
Sample/standard diluent	Dilution Buffer (DB) provided	DB	DB	DB	DB
Initial standard dilution	1:2	1:50	1:9	1:16	1:25
Initial standard concentration	2000pg/ml	100pg/ml	450pg/ml	300pg/ml	500pg/ml
Sample dilution	Neat	Neat	1:20	1:2	1:5 and 1:10
Biotin antibody dilution	Pre-prepared	1:100	1:100	1:100	1:100
Detection limit	7pg/ml	0.4pg/ml	2pg/ml	3pg/ml	2pg/ml

Table 2.3. ELISA components and concentrations.

2.5.3. Dynabead wash buffer

PBS (pH 7.4) + 2% heat inactivated FCS.

2.6. Preparation of medium

2.6.1. Wash medium

Wash medium was prepared using RPMI 1640 supplemented with 100IU/ml penicillin, 100ug/ml streptomycin, 2mM glutamine and 0.2% sodium bicarbonate.

2.6.2. Culture medium

Culture medium was prepared using RPMI 1640 supplemented with 100IU/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine, 0.2% sodium bicarbonate and 10% pooled heat inactivated human AB serum.

2.6.3. Cryopreservation medium

Freezing medium used for the cryopreservation of isolated mononuclear cells was prepared using 80% FCS and 20% DMSO.

2.7. Preparation and storage of peripheral blood mononuclear cells (PBMCs)

2.7.1. Whole blood

20-40mls of peripheral blood was collected into sodium heparin vacutainers from normal volunteers and renal transplant recipients (immediately pre-transplant, three months and twelve months post-transplant). Blood samples were also taken from bone marrow donor and recipients. The blood was diluted 1:1 in RPMI 1640 cell culture wash medium and mixed. 10mls of the diluted blood was carefully layered onto an equal volume of lymphoprep and centrifuged (Heraeus Labofuge 400; 2000rpm, twenty minutes at room temperature). This method is known as

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density gradient centrifugation. The mononuclear cell interface was then transferred into a sterile collection tube and washed once in RPMI 1640 wash medium to successfully pellet the cells (1500-1800 rpm, ten minutes). The supernatant was discarded, and the cells were washed a further two times in order to remove any remaining Ficoll/Hypaque (1200rpm, ten minutes). After washing, the cells were resuspended in 1ml of cell culture medium and counted using a haemocytometer. The cell density was adjusted to 1×10^6 cells/ml for culturing or 5×10^6 cells/ml for cryopreservation. These cells were used as the responder cell population in the MLC.

2.7.2. Donor spleen

Spleen from the cadaveric donor was collected from the operating theatre and immediately refrigerated on arrival. The spleen was then transferred to a sterile laminar for mononuclear cell isolation. The spleen was processed by gently teasing the tissue apart and resuspending the liquified tissue in cell culture wash medium. The suspension was then filtered through a sterile 0.22um filter (Sartorius Minisart®) to remove any debris. The filtered suspension was then layered onto an equal volume of Ficoll-Hypaque and centrifuged as above. The cells were then prepared using the whole blood method and used as stimulator cells in the MLC.

2.7.3. Cryopreservation of samples

1ml of isolated cells were pipetted into 2ml cryovials at a final concentration of at least 5×10^6 cells/ml/vial and kept on ice for fifteen minutes. Cells were then diluted 1:1 with freezing medium (final concentration: 50% cell suspension, 40% FCS, 10% DMSO) and stored at -70°C overnight. The cells were transferred to liquid nitrogen within twenty four hours.

2.8. Depletion of CD4+ and CD8+ T cells

2.8.1. Washing of dynabeads

The dynabeads in the vial were resuspended and the required volume transferred to a test tube. The tube was placed on the magnet for one minute and the fluid pipetted off. The vial was removed from the magnet and 1-2mls of PBS/FCS wash buffer was added. The protocol was repeated and the dynabeads were resuspended in a volume of PBS/FCS wash buffer equal to that originally in the vial.

2.8.2. Depletion

PBMCs were isolated as described in Section 2.7.1. The cells were then centrifuged and resuspended in PBS/FCS wash buffer at a concentration of 10-20 x 10^6 cells/ml. Dynabeads (72ul of CD4; 141ul CD8 per 1 x 10^7 PBMCs) were added, the suspension was mixed thouroghly and incubated for thirty to forty five minutes on rotator at 2-8°C. 5mls of PBS/FCS wash buffer was added and the tube was placed in the magnet for three minutes to collect the rosetted cells and the unbound dynabeads. The depleted supernatant was transferred to a second tube and the protocol was repeated for a complete depletion. The purity of the T cell subset populations were analysed by flow cytometry and was >98% pure (<2% debris, $^+/_-$ 0.5).

2.9. Cell culture

2.9.1. *In vitro* stimulation of PBMCs using phorbol myristate acetate (PMA)

PBMCs were pipetted into a twenty four well plate at a final concentration of 1 x 10^6 cells/ml/well. PMA was added to corresponding wells at a final concentration of 20ng/ml, together with 3ug/ml monensin and 1uM/ml ionomycin as described by

Jung *et al* (1993). Negative cell culture wells contained 3ug/ml monensin alone. The cultures were then incubated for six hours at 37°C in 5% CO_2 (Prussin and Metcalfe, 1995). Following incubation, the cells were harvested and stained for flow cytometric analysis.

2.9.2. *In vitro* stimulation of PBMCs using phytohaemagglutinnin (PHA)

PBMCs were plated at a concentration of 1 x 10^6 cells/ml/well into a plastic twenty four well plate. PHA was added to the respective wells at a final concentration of 1ug/ml. Negative wells contained mononuclear cells alone. The cells were then incubated for forty eight hours at 37°C in 5% CO_2 (previously optimised). The cultures were then harvested into sterile collection tubes and centrifuged at 1200rpm for ten minutes. The supernatants were dispensed into sterile cryovials and stored at -20°C for ELISA analysis.

2.9.3. *In vitro* stimulation using OKT3 (anti-CD3 monoclonal antibody)

Culture plate wells were coated with OKT3 (final concentration: 5ug/well) and incubated for two hours at room temperature. The wells were then washed three times with PBS and air dried. PBMCs were then added to the respective wells and incubated for forty eight hours at 37°C in 5% CO_2 (Kaminski *et al*, 1995). Negative wells contained mononuclear cells alone. The cultures were then harvested and centrifuged at 1200rpm for ten minutes. The cell free supernatants were pipetted into sterile cryovials and stored at -20°C for ELISA analysis.

2.9.4. Mixed lymphocyte culture

PBMC isolation was performed as previously described (Section 2.7.1). Cell concentrations were adjusted to 1×10^6 cells/ml. Donor stimulator cells were then irradiated at 30 Gy and plated out in twenty four well culture plates with the untreated responder lymphocytes. The plates were incubated for the calculated optimal time for each cytokine at 37°C in 5%CO_2. Following incubation, cell free supernatants were harvested and stored at -20°C until required for cytokine analysis.

2.10. Cellular analysis of samples

2.10.1. Quantification of CD3/CD25 (IL-2 receptor) expression by two colour flow cytometry

The MLC cultures were harvested and washed twice (1200rpm, ten minutes) in 1-2mls of PBS/0.2% BSA. 20ul of recombinant human IgG (final concentration: 5ug/10^6 cells) was added to each tube and incubated at room temperature for ten minutes. The cells were then washed in PBS/BSA (1200rpm, ten minutes). Conjugated monoclonal antibodies were then added to relevant tubes (Table 2.4) and incubated for fifteen minutes at room temperature in the dark. The cells were then washed twice in PBS/BSA and resuspended in 1ml PBS for analysis using Coulter Epics Elite flow cytometer.

NB: Cells may be resuspended in 0.5% fixation buffer and analysed within twenty four hours.

2.10.2. Quantification of intracytoplasmic cytokine expression by two/three colour flow cytometry

PMA stimulated (and unstimulated) cells were harvested and washed twice in staining buffer (1200rpm, ten minutes). The cells were then aspirated and

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resuspended in 100ul of 4% fixation buffer as described by Sander *et al* (1991), and incubated for fifteen minutes at room temperature. Cells were then stained with relevant cell surface antigen monoclonal antibodies (Table 2.4) and incubated for fifteen minutes at room temperature (in the dark). The stained lymphocytes were then washed twice in permeabilisation buffer. The cell pellet was resuspended in 100ul of permeabilisation buffer and incubated for one hour at room temperature (the saponin allows the cell membranes to become permeabilised while the AB serum acts as a blocking agent). Relevant conjugated anti-cytokine monoclonal antibodies (Table 2.4) were added and incubated for fifteen minutes at room temperature (in the dark). The samples were then washed twice in staining buffer and resuspended in 1ml PBS for flow cytometric analysis.

2.10.3. Quantification of cytokine protein by ELISA

2.10.3.1. Eurogenetics® method (IL-4,IL-6, IL-10 and IFN-γ)

Concentrations may be found in Table 2.3:

1. Coating antibody was diluted and 100ul added to each microtitre plate well. The plate was incubated overnight at room temperature.

2. The plate was washed five times in PBS alone.

3. Blocking buffer was diluted and 200ul added to each well. The plate was incubated for one hour at room temperature.

4. The plate was washed five times in PBS/TWEEN 20.

5. Leaving the substrate blank wells empty, 100ul of prepared standard and sample dilutions were added to the appropriate wells and incubated as in step 3.

6. Step 4 repeated.

Intracytoplasmic (IC) and MLC (Two Colour)	Responder Cells Alone (MLC)/Unstimulated (IC)	MLC Culture / PMA Stimulated (IC)
	Leucogate	Leucogate
	*FITC + PE antibody controls	*FITC/PE antibody control
	FITC + PE antibodies	**FITC antibody + PE control
		**PE antibody + FITC control
		FITC + PE antibodies
Three Colour	**Unstimulated**	**PMA Stimulated**
	Leucogate	Leucogate
	*FITC + PE + PECy5 controls	*FITC + PE + PECy5 controls
	FITC + PE + PECy5 antibodies	**FITC antibody + PE and PECy5 controls
		**PE antibody + FITC and PECy5 controls
		**PECy5 antibody + FITC and PE controls
		FITC + PE + PECy5 antibodies

Table 2.4. Staining protocols for two and three colour flow cytometry experiments (* negative control; ** compensation analysis).

7.   Leaving the substrate blank wells empty, 100ul of diluted biotinylated antibody was added to all wells and incubated as in step 3.

8.   Step 4 repeated.

9.   100ul of substrate solution was added to all wells and incubated in the dark for thirty minutes at room temperature.

10.   100ul of stop solution was added to all wells. The plate was read at 450nm using a Dias Microplate Reader.

2.10.3.2. R and D Systems® method (IL-2)

1.   100ul of assay diluent RD1A was added to each well.

2.   100ul of standard and sample was added to appropriate wells and incubated for two hours at room temperature.

3.   The plate was washed three times in prepared wash buffer.

4.   200ul of conjugate was added to each well and incubated for two hours at room temperature.

5.   Step 3 repeated.

6.   200ul of substrate solution was added to each well and incubated for twenty minutes at room temperature.

7.   50ul of stop solution was added to each well. The plate was read at 450nm (wavelength correction 570nm).

2.11. DNA analysis

These methods were performed by Mr Mohammed Jahromi for the IFN-γ gene polymorphism study:

2.11.1. Preparation of DNA

DNA was extracted using the Nucleon kit. 4mls of Nucleon A (10mM Tris-HCl, 320mM sucrose, 1% Triton X-100, 5mM $MgCl_2$, pH 8.0 using concentrated NaOH) was added to 1ml of blood (taken into 5% EDTA). The sample was shaken for four minutes at room temperature and then centrifuged (1300rpm, five minutes). The supernatant was discarded and 2mls of Nucleon B (400mM Tris-HCl, pH 8.0 using concentrated NaOH, 60mM EDTA, 150mM NaCl, 1% SDS) was added to the tube to disrupt nuclear membranes. The pellet was then resuspended and incubated at 37°C for ten to fifteen minutes. The suspension was then pipetted into a 15ml polypropylene tube, and 500ul of sodium perchlorate (5M) was added and the tube was inverted ten to fifteen times. 2ml of ice cold chloroform was then added and the tube again inverted ten to fifteen times. After centrifugation (1300rpm, three minutes), 200ul of Nucleon silica was added to the mixture and centrifuged at 1300rpm for three minutes at room temperature. The upper aqueous phase containing the DNA was then transferred to a clean plastic tube. The aqueous phase was again centrifuged to remove any remaining silica and the supernatant decanted into a fresh tube. Two volumes of ice cold 100% ethanol was then added in order to precipitate the DNA. The DNA was then extracted using a hooker pasteur pipette into an eppendorf tube containing 70% ethanol. The DNA was then air dried and finally resuspended into an eppendorf tube containing 500ul sterile water. Absorbency of the DNA was measured between 260nm and 280nm, using a Cecil 5500

Spectrophotometer (Cecil UK) and the concentration calculated (formula: ug/ul = OD_{260} x 50 x dilution factor/1000). DNA samples were then stored at -70°C.

2.11.2. Analysis of IFN-γ microsatellite polymorphism

2.11.2.1. Polymerase chain reaction (PCR)

A 100-300ng aliquot of extracted DNA was used in the amplification reaction. A pair of amplimers flanking a 123 base pair region of the IFN-γ gene containing a $(CA)_{12}$ dinucleotide repeat were used for the amplification of the microsatellite. The forward amplimer was labelled with $[^{32}P]$ dATP by T4 polynucleotide kinase. 2pmol of the unlabelled amplimer together with genomic DNA was mixed with 1pmol of labelled amplimer, 0.8nmol/l dNTPs, 2mmol/l Magnesium (Mg+) and 0.2 units of thermostable DNA polymerase in 1 x super buffer. The reaction was made up to a final volume of 20ul with double distilled water and PCR was performed using a Techne Thermal Cycler (Fischer, UK).

Forward

5'   **TCA CAA TTG ATT TTA TTC TTA C**   *3'*

Reverse

5'   **TGC CTT CCT GTA GGG TAT TAT T**   *3'*

The PCR reaction conditions were as follows:

95°C denaturation step (60 seconds), 48°C annealing step (30 seconds) and a 72°C elongation step (40 seconds), followed by a 72°C extension step (5 minutes).

2.11.2.2. Gel electrophoresis and autoradiography

An aliquot of the PCR products was electrophoresed through a 6% formamide/urea gel in 0.5% Tris/borate electrophoresis (TBE) buffer (10x solution: 0.89mM Tris base, 0.89M Boric acid, 2mM EDTA (pH8.0)) for three hours at 90 watts. The gel was then subsequently dried, and autoradiography was performed using Kodak X-Omat film with intensifying screens at -85°C for twenty four hours.

2.12. Analysis of data

Protein secretion and expression over time in normal volunteers was analysed using Analysis of variance. Univariate analysis of cytokine comparison data was performed using Pearsons correlation and Spearmans rank analysis (for non-normal data). IFN-G polymorphism was performed using Logistic regression analysis. For the analysis of the rejection data, cytokine levels were divided into groups using the median value as a cut-off. Patients were divided into two groups, those who did not undergo rejection, and those with at least one acute rejection episode. A preliminary analysis using Chi squared (χ^2) tests was undertaken to find associations between rejection and the following parameters: cytokine levels, patient and donor sex and age, HLA sensitisation, patient and donor CMV status, and HLA and HLA-DR mismatching. Variables significant at the $p=<0.2$ level were entered into "backward stepping" multivariate Logistic regression analysis. All quoted p values are two-sided. In addition, relationships between cytokine protein secretion and HLA/HLA-DR mismatching were analysed using the Kruskal-Wallis test. Significant differences in median cytokine protein secretion between rejectors and non-rejectors were also calculated using the Mann-Whitney U test.

CHAPTER 3

OPTIMISATION AND ANALYSIS OF CYTOKINE PROTEIN, FREQUENCY AND GENE POLYMORPHISMS IN NORMAL INDIVIDUALS

3.1. Optimisation of cultures

3.1.1. Introduction

It has been known for some time that the MLC can be used as a tool for studying cytokine production (Ilonen and Kartunen, 1984, Danzer *et al* 1994a, 1994b, Takahara *et al,* 1993). Such studies have demonstrated that several cytokines may be produced in an MLC response (Bishara *et al*, 1991, Toungouz *et al*, 1993, 1994, 1996). The standard procedure for an MLC is to set up a one way culture, where the donor cells are nulled by treatment and cultured together with untreated responder cells. Although previous studies have illustrated that cytokine secretion occurs within the MLC, it was considered important to optimise the reaction response in terms of incubation times and methods of inactivating the stimulator cells. This included examining peak levels of cytokine protein secretion, sensitivity of the MLC and optimal treatments for the production of a one way response. The project also included measurement of cytokines in PMA and PHA stimulated cultures. It was therefore also imperative that cytokine secretion profiles were optimised for mitogen stimulation.

3.1.2. Kinetics of cytokine protein secretion

The MLC cultures were set up using irradiated stimulator lymphocytes and untreated responder cells (Materials and Methods, Section 2.9.4), The cultures were incubated from day zero up to day eight. Cell free supernatants were harvested every twenty four hours and analysed for cytokine protein (IL-2, IL-4, IL-10 and IFN-γ) by sandwich ELISA. IL-6 was also optimised in the latter part of the project. It was found that there was a variation in optimal secretion times between the cytokines. Although protein levels varied between individuals on repeating the experiments (n=3), the optimal secretion times remained constant for each cytokine. Baseline

levels (ie: responder cells alone) remained below detectable limits. For all five cytokines, there appeared to be an increase in protein production up to day three. After day three, IL-2 (Figure 3.1a) and IL-10 (Figure 3.1b) protein levels decreased. However, for both IL-4 (Figure 3.2a) and IFN-γ (Figure 3.2b), peak secretion occurred on day four of the culture. IL-6 was found to be optimally secreted on day five of culture (Figure 3.3). For IL-2, IL-4, IL-10 and IFN-γ, the optimal cytokine times obtained from these experiments were then used in further studies. However, for IL-6, as the patient MLC cultures had already been harvested on days three and four for ELISA analysis, IL-6 levels had to be measured on day four.

3.1.3. Comparison of cell free and cell based ELISAs

These experiments were carried out in order to determine whether there were any significant differences between the measurement of cytokine secretion in culture supernatant by standard ELISA and direct cytokine secretion from cells by capture ELISA (capture ELISA involved the addition of MLC cells direct onto the ELISA plate). Taking the optimal cytokine secretion data into account, MLCs were only incubated for up to six days in these experiments. As before, these experiments were repeated for reproducibility (n=3). The cytokines appeared to peak at the same times as before, but it can be seen for IL-4, as an example (Figure 3.4), that there was no significant difference between the cell free and cell based method. This was also the case for IL-2, IL-10 and IFN-γ (data not shown). Future ELISA experiments were therefore carried out using cell free supernatants. As IL-6 was optimised at a later date, this experiment was not performed for this cytokine.

a)
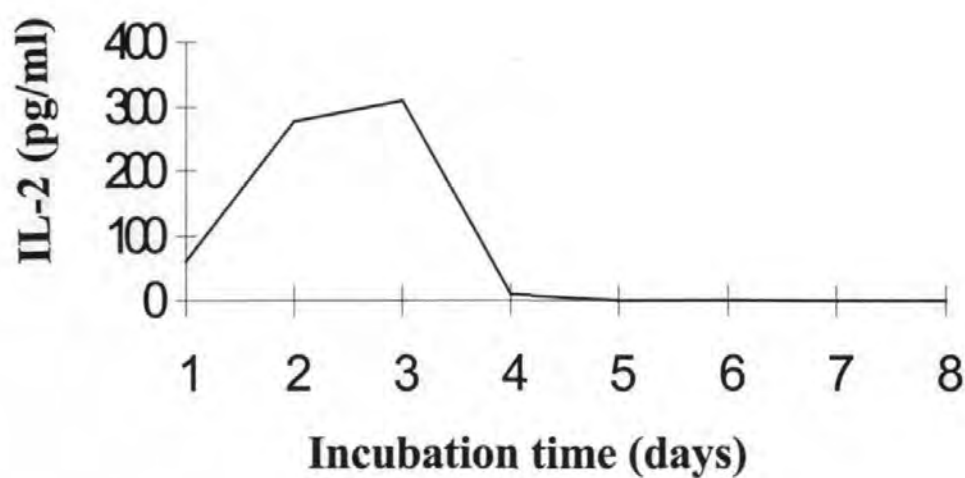
b)
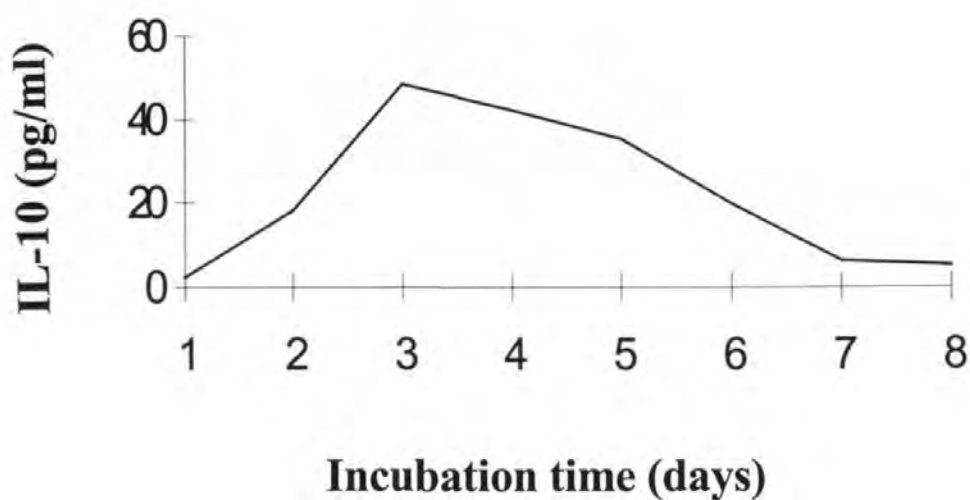
Figure 3.1. Cytokine secretion profile for IL-2 (a) and IL-10 (b) in one way MLC (data shown from single experiment).

a)

Incubation time (days)

b)

Incubation time (days)
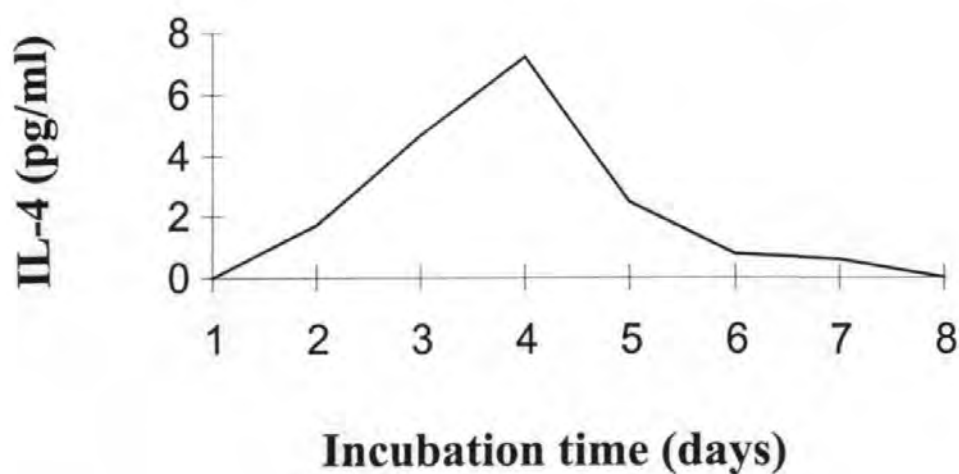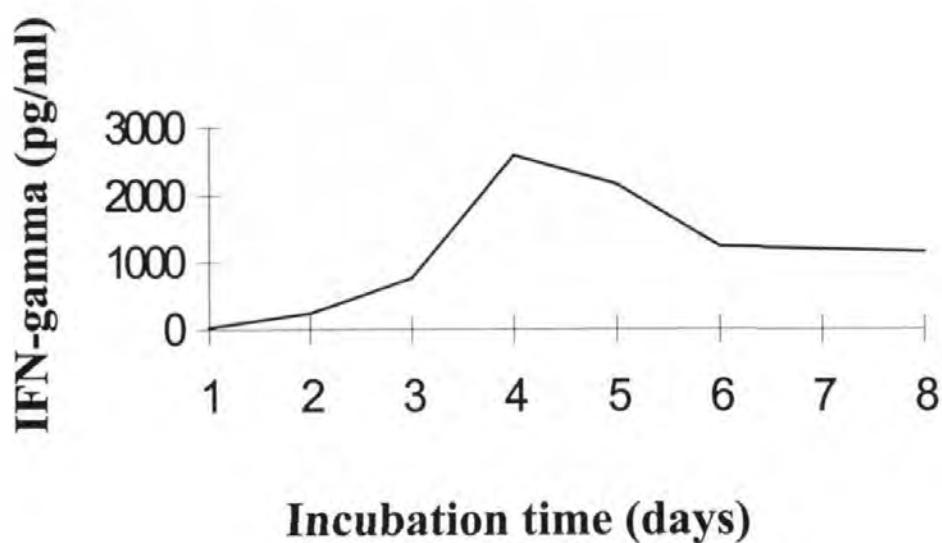
Figure 3.2. Cytokine secretion profile for IL-4 (a) and IFN-gamma (b) in one way MLC (data shown from single experiment).
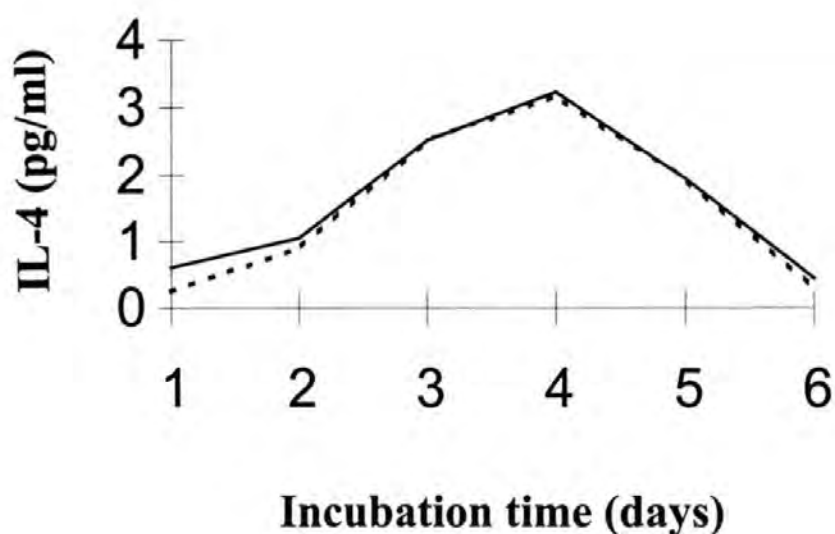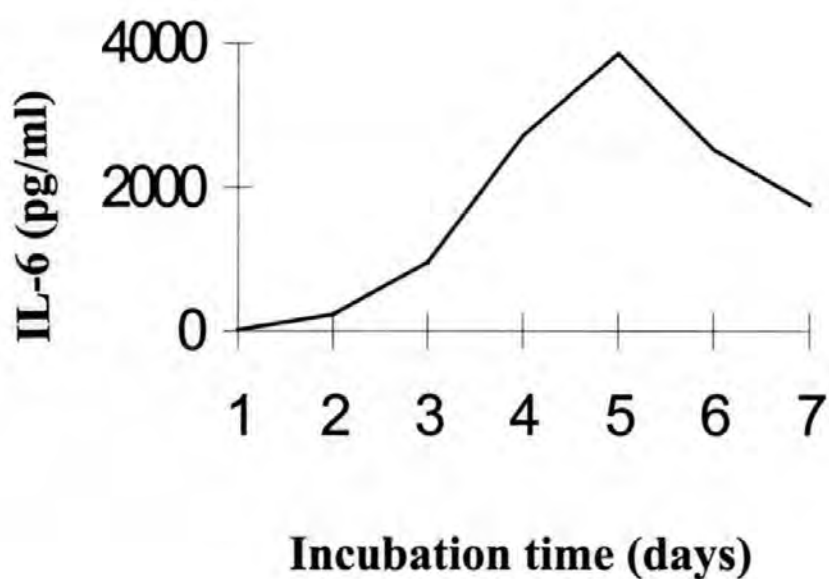
Figures 3.3 and 3.4. Cytokine secretion profile for IL-6 in one way MLC (upper figure) and IL-4 protein secretion in cell free and cell based ELISAs (broken line-cell free; solid line-cell based) (lower figure). Data shown from single experiment.

3.1.4. Treatment of stimulator cells

It was important to establish the most suitable method of treatment for the stimulator cells in the MLC. Two treatments were compared, gamma irradiation (caesium source) and chemical treatment by mitomycin C. Not only did the most suitable method have to chosen, but also the optimal dose or concentration in order to ensure that the stimulator cells were unresponsive.

Both populations of cells (stimulator (S) and responder (R) cells) were initially treated with various concentrations of mitomycin C (5-50ug/ml). The positive control included untreated cells (R+S). Cell free supernatants were harvested at optimal time points as previously established for each cytokine and analysed by ELISA. Again, these experiments were not performed for IL-6. With the other four cytokines, no pattern of decrease in cytokine protein response with increasing concentration was found (Figure 3.5 illustrates pattern for IL-10). Therefore, as a dose could not be determined by this method, irradiation treatment was considered.

Both populations of cells (ie: stimulator and responder cells) were treated with gamma irradiation at several doses (5-50 Gy). Controls again included untreated cells (R+S). Cell free supernatants were again harvested at optimal time points. It was established that between 20-30 Gy was the suitable dose range for all four cytokines (Figure 3.6 illustrates dose response for IFN-γ). Therefore, mitomycin C treatment was considered unreliable at any of the concentrations used, and that irradiation was the method of choice.
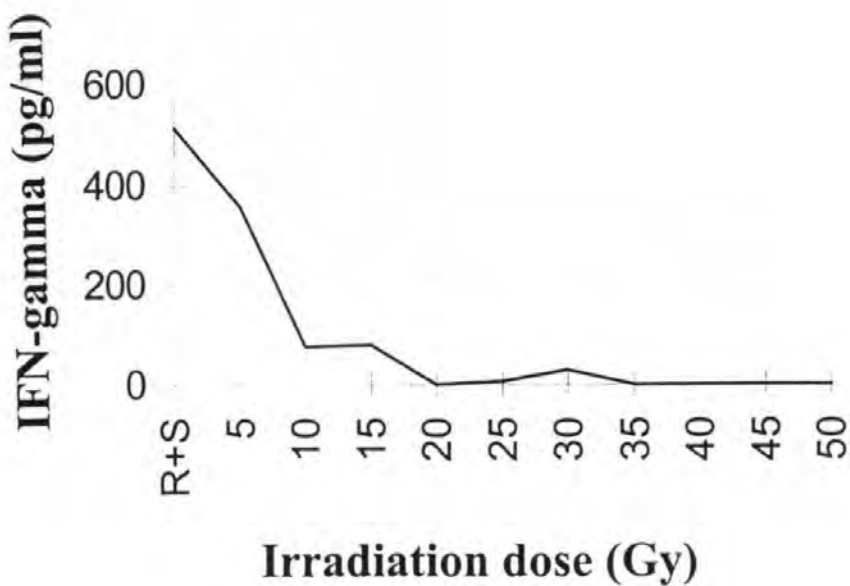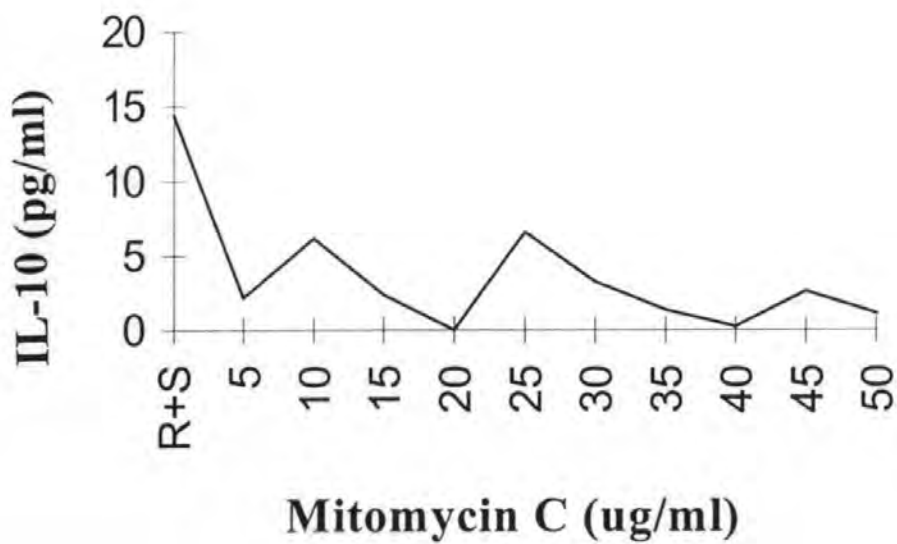
Figures 3.5 and 3.6. Effect of mitomycin C treatment on IL-10 secretion (upper figure) and effect of irradiation treatment on IFN-gamma secretion (lower figure). Data shown from single experiment.

3.1.5. Cytokine and CD3/CD25 (IL-2 receptor) expression in MLC

Once the conditions had been optimised for the MLC, the next step was to quantify peak cytokine (IL-2, IL-4, IL-10 and IFN-γ) expression in T cells (not performed for IL-6). The peak IL-2 receptor (CD25) expression was also measured in order to study levels of activation in T cells. Flow cytometric analysis was performed at two, four, six, ten and twenty four hours for the first day, and then every twenty four hours up to five days. At every time point, it was found that, due to the nature of the culture, it was impossible to gate a clear lymphocyte region, and therefore neither the cytokine or CD25 expression could be analysed (data not shown). It was therefore decided that only cytokine protein would be measured by ELISA.

3.1.6. PHA and OKT3 stimulated cultures

PHA and OKT3 cultures were included in the study of cytokine protein secretion by normal volunteers for ELISA analysis (all cytokine protein data may be found in appendix 1). Optimisation experiments concluded that peak secretion for all four cytokines (IL-2, IL-4, IL-10 and IFN-γ) was around forty eight hours. This time point was therefore used in all PHA and OKT3-stimulated cultures.

3.1.7. PMA stimulated cultures

PMA cultures were also included in the study of cytokine expression by normal volunteers for flow cytometric analysis (all cytokine expression data may be found in appendix 1). The optimal time point used in these experiments was six hours for IL-2 and IFN-γ, as previously described by Prussin and Metcalfe (1995). Experiments were also carried out to determine peak cytokine expression for IL-4 and IL-10. PMA stimulated cultures were set up and cells were harvested at various time points within a twenty four hour period (four, six, ten and twenty four hours).

Both cytokines were found to be below detectable limits at all four time points (data not shown). Therefore, only IL-2 and IFN-γ were investigated in further experiments.

3.2. Cytokine expression in normal volunteers

3.2.1. Introduction

It has been shown that many different cytokines may be secreted during an immune response. However, little is known about the variance of cytokine secretion and frequencies of cytokine expression between individuals and what factors may have an influence on these levels. As previously mentioned, inter-individual variations in both cytokine secretion and numbers of cytokine secreting cells have been demonstrated suggesting that there may be differences between random individuals and that there may be factors that affect these differences (Kaminski *et al*, 1995; Ferry *et al*, 1997).

In this part of the study, flow cytometry and ELISA were used as the methods of choice to establish the level of variation in both numbers of cytokine secreting cells and levels of secreted cytokine protein in mitogen stimulated cultures respectively in normal volunteers.

3.2.2. Cytokine expression in CD3+ T cells

The aim of this part of the study was to analyse the frequencies of cytokine expression using two colour flow cytometric analysis in normal volunteers. Lymphocytes were stimulated with PMA and ionomycin and incubated for six hours as previously optimised (Prussin and Metcalfe, 1995). Cultures were the harvested and stained for T cell expression of both IL-2 and IFN-γ. As previously mentioned, kinetic studies were performed for both IL-4 and IL-10, but expression appeared to be below detectable limits on the flow cytometer.

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A group of twenty normal healthy volunteers were randomly chosen. Figure 3.7 illustrates the frequency distribution of both IFN-γ and IL-2 in CD3+ T cells. It can be seen that there is a wide distribution of expression with both cytokines, illustrating a clear inter-individual variation between the individuals within the group (IFN-γ range: 0.8-48.7%; IL-2 range: 0.8-37.3%). Twelve (60%) of the individuals expressed less than 10% CD3/IFN-γ, forming a cluster of "low expressors". Only three individuals (15%) expressed less than 10% CD3/IL-2. Only the distribution pattern yielded by IL-2 conformed to a normal distribution.

As previously mentioned, there were clear high and low cytokine expressors within the group studied. Figures 3.8 and 3.9 illustrate flow cytometric data of both a low and high expressor for IL-2 and IFN-γ respectively. A distinct difference in cytokine expression may be observed between the individuals. An increase in both IL-2 and IFN-γ expression with PMA stimulation was clearly identified in each individual when compared to unstimulated cells.

3.2.3. Cytokine expression in T cell subsets and natural killer (NK) cells

Two colour flow cytometry was also used to measure IL-2 and IFN-γ expression in CD4+ and CD8+ T cell subsets. Four individuals were randomly chosen to determine the predominance of expression. PBMCs were stimulated with PMA and ionomycin as before (Section 2.9.1). Figures 3.10 and 3.11 illustrates the percentage of expression in one of the individuals for IL-2 and IFN-γ in CD4+ and CD8+ T cells respectively, analysed by flow cytometry. In this individual, it can be seen that IL-2 is predominantly expressed by CD4+ T cells, as was the case for the
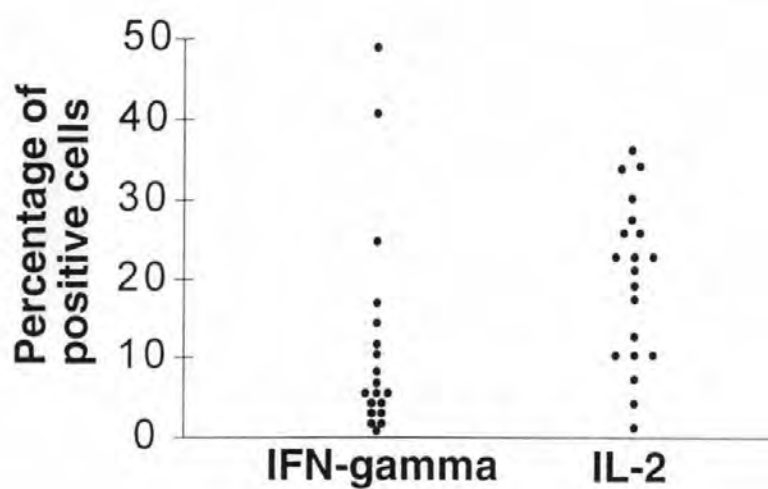
Figure 3.7. Frequency distribution of IFN-gamma and IL-2 secreting CD3+ T cells. Inter-individual variations were observed for both cytokines

Unstimulated　　　　　　　　　**Stimulated (+PMA)**

a)

b)

CD3 FITC
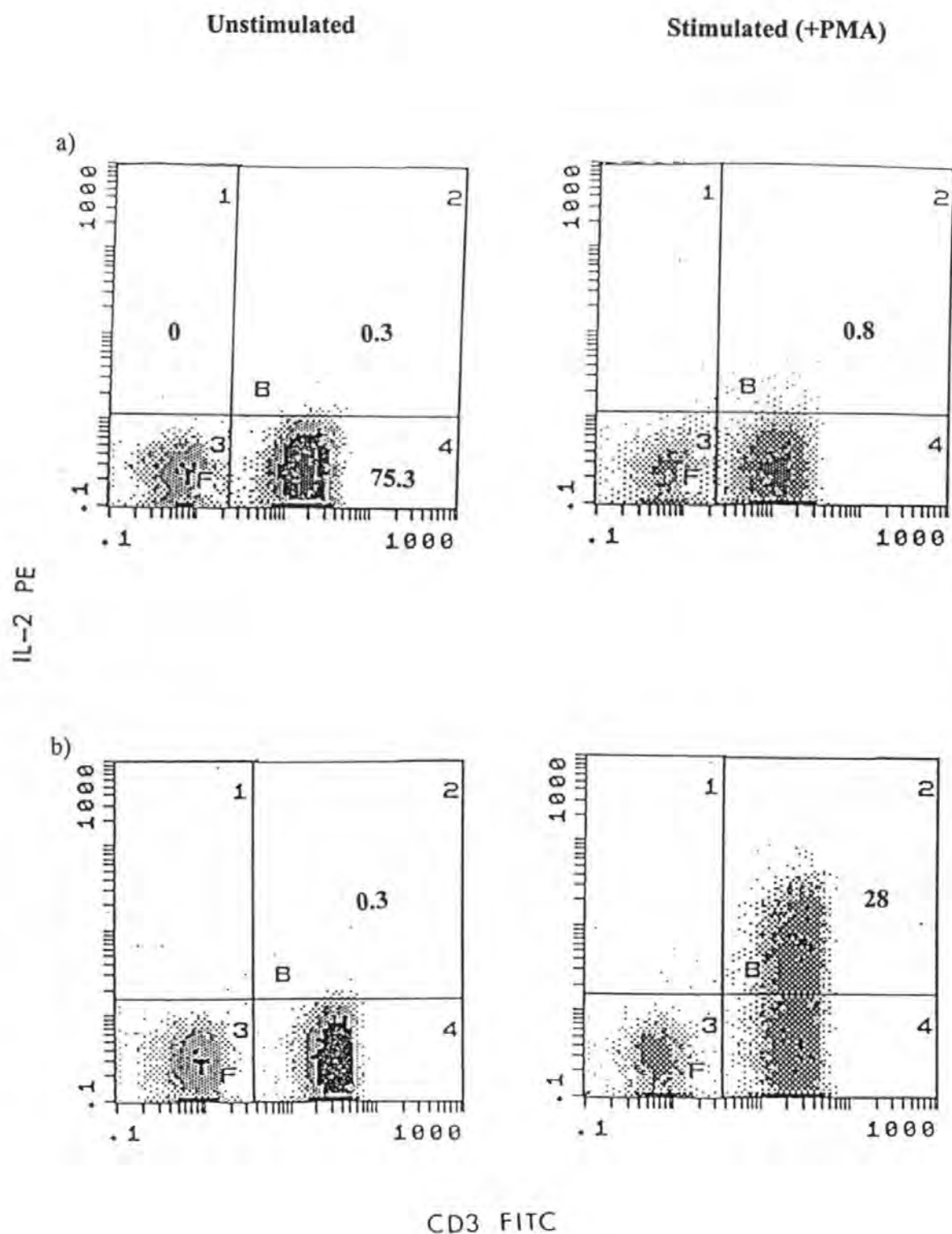
Figure 3.8. IL-2 expression in normal volunteers. Figure 3.8a represents low cytokine expression in unstimulated and stimulated cells. Figure 3.8b illustrates the relative high expression of IL-2 in unstimulated and stimulated cells. FITC= fluorescein isothiocyanate, PE=phycoerythrin. Quadrant 1=IL-2+; quadrant 2= double positive (CD3+/IL-2+); quadrant 3=negative (CD3-/IL-2-); quadrant 4=CD3+.
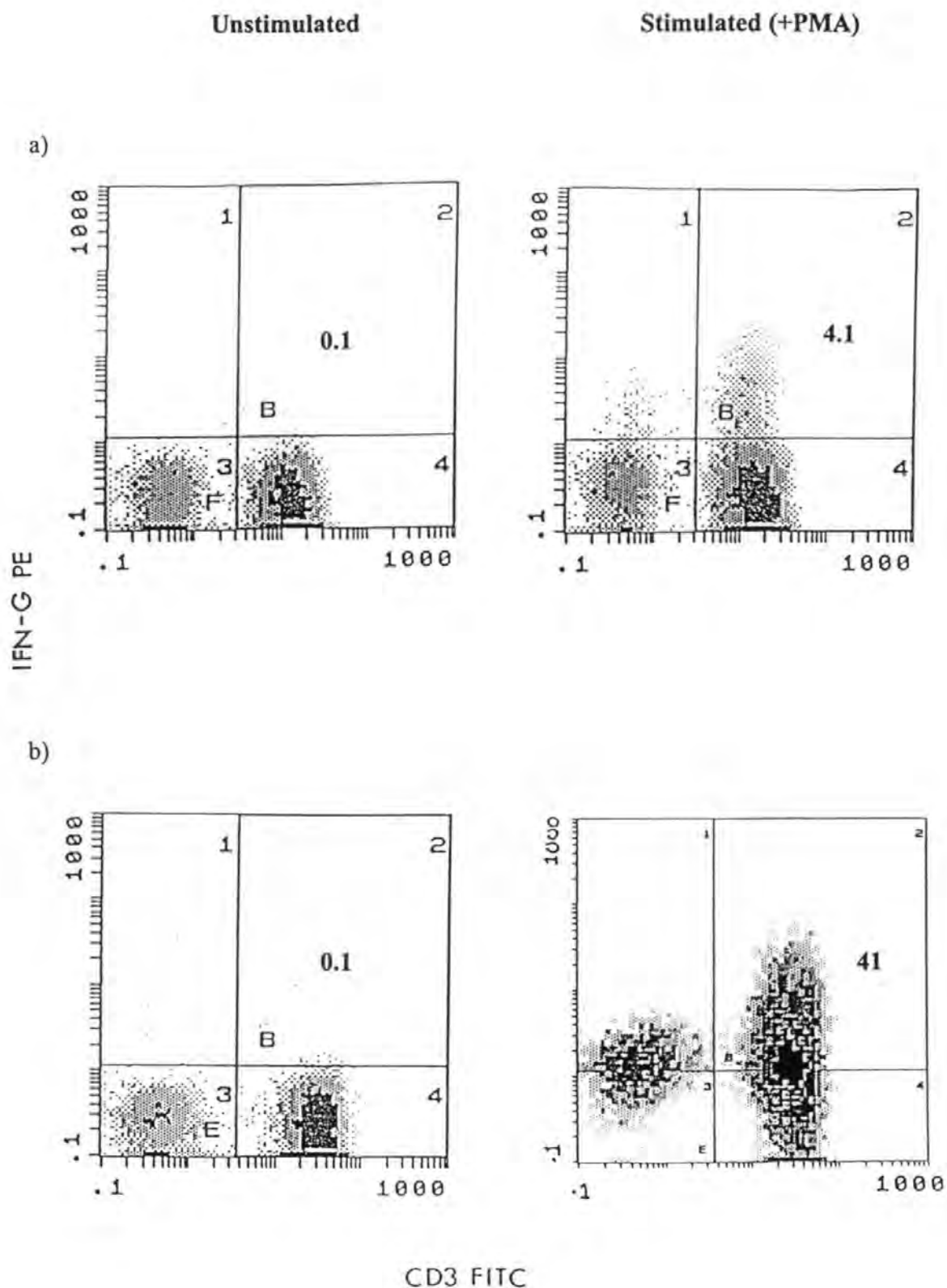
Figure 3.9. IFN-gamma expression in normal volunteers. Figure 3.9a illustrates low cytokine expression in unstimulated and stimulated cells. Figure 3.9b represents relative high expression of IFNγ in unstimulated and stimulated cells. Quadrant 1=IFN+; quandrant 2=CD3+/IFN+; quandrant 3=CD3-/IFN-; quandrant 4=CD3+.

other individuals analysed (Figure 3.10). IFN-γ, however appeared to yield a different profile, with this cytokine being predominantly expressed by CD8+ T cells (Figure 3.11).

It was also of interest to analyse whether natural killer (NK) cells contributed to cytokine expression in normal individuals. A negligible contribution was observed to both IL-2 and IFN-γ expression by NK cells (0.1-0.2%, data not shown).

3.2.4. Co-expression of cytokines in T cell subsets

Co-expression of IL-2 and IFN-γ in both CD4+ and CD8+ T cells in the same individuals was then studied. Attempts were originally made using three colour flow cytometry on PMA stimulated PBMCs. Results, however showed that CD4 expression was down-regulated by PMA and therefore another method had to be found in order to carry out these experiments (data not shown). It was therefore decided that both CD4+ and CD8+ T cell subsets would be isolated by precoated magnetic bead selection. Purity was determined by flow cytometry and each T cell subset then stimulated with PMA. Two colour flow cytometry was then used to determine IL-2/IFN-γ expression in each subset.

Figure 3.12 illustrates the expression of IL-2 and IFN-γ in (a) CD4+ and (b) CD8+ T cells. It can be seen that co-expression is relatively low (<2%) in each subset, compared to single cytokine expression (Figures 3.10 and 3.11). There was also no statistical significance between the level of co-expression in CD4+ and CD8+ T cells (p = 0 3).
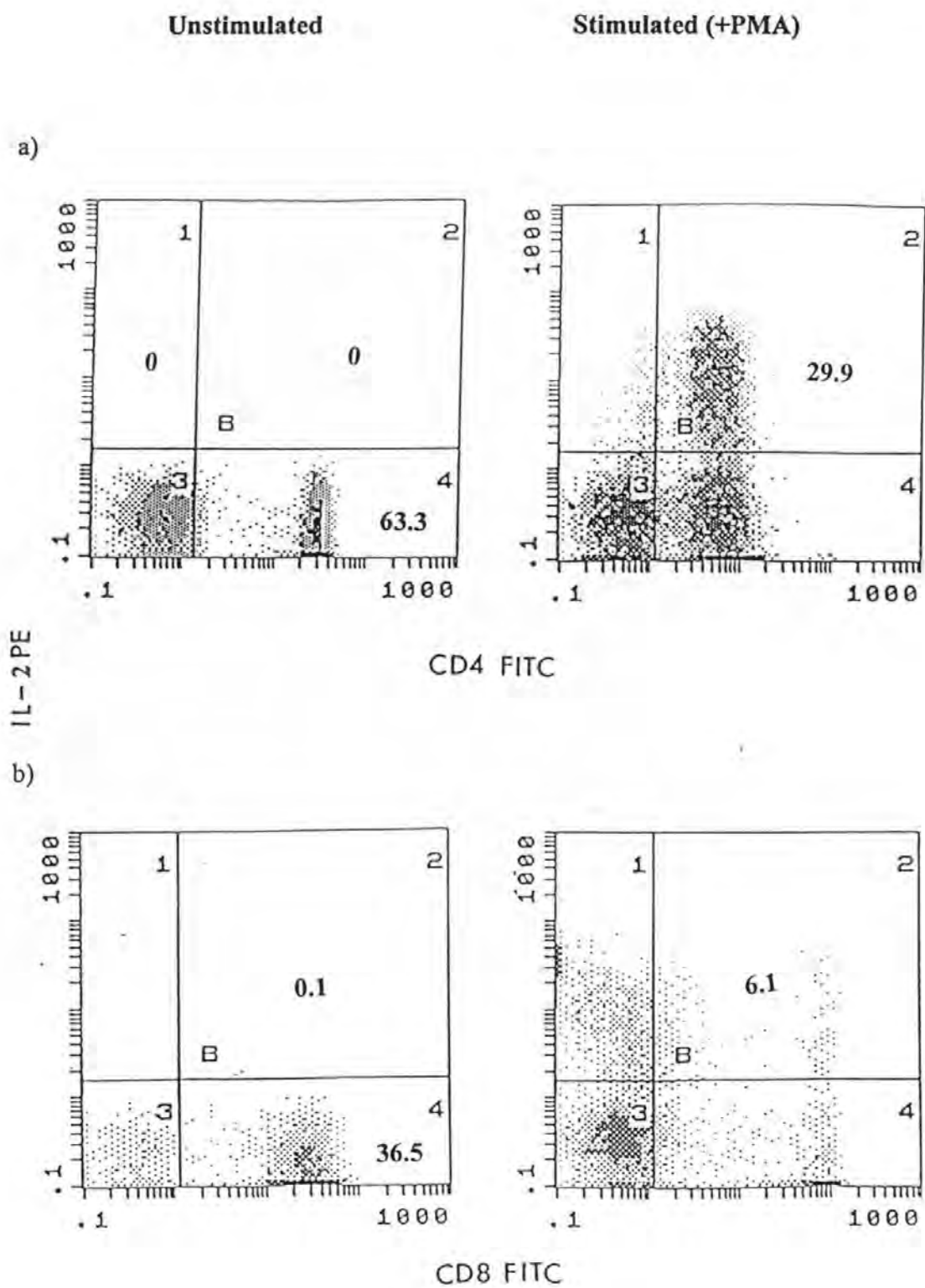
Figure 3.10. IL-2 expression in CD4+ (a) and CD8+ (b) T cells. IL-2 expression was shown to be predominantly expressed in CD4+ T cells.

Unstimulated **Stimulated (+PMA)**

a)

b)

CD4 FITC

CD8 FITC
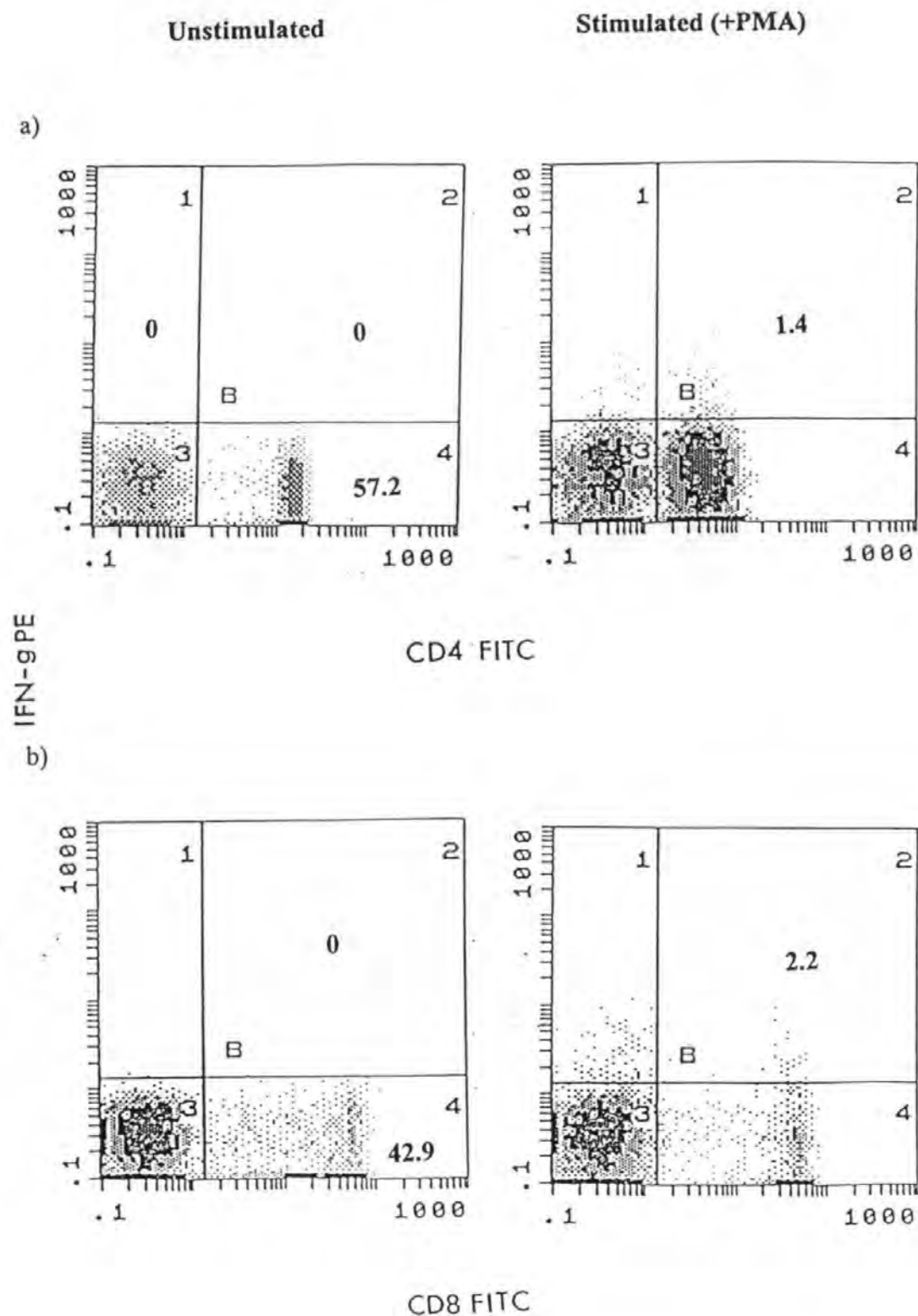
Figure 3.11. IFN-gamma expression in CD4+ (a) and CD8+ (b) T cells. Lower levels of expression were observed with IFN-γ compared with IL-2. Predominant expression was seen in CD8+ T cells.

a)

b)

IL-2 FITC
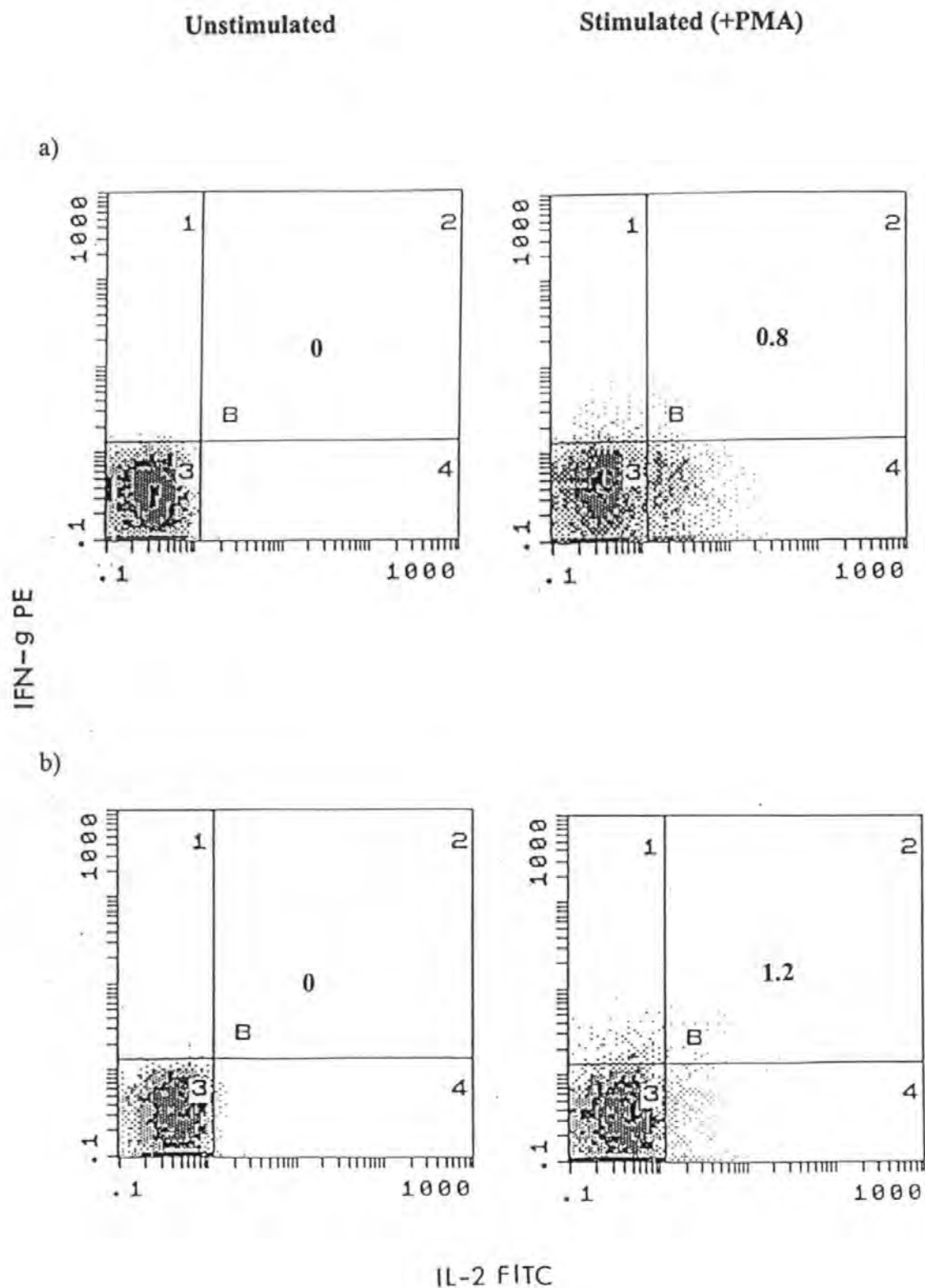
Figure 3.12. Co-expression of IL-2 and IFN-gamma in CD4+ (a) and CD8+ (b) isolated T cells. Low levels of co-expression in both CD4+ and CD8+ T cells were observed.

3.2.5. Variation of cytokine expression over time

In order to establish whether cytokine expression varies with time in the same individuals, five volunteers were assayed on six separate occasions over a twelve month period. This group included individuals with both high and low numbers of cytokine expressing cells.

Analysis of variance did not demonstrate a diurnal effect (p=0.893). Of the five individuals, two were found to be consistent over time for both IFN-γ (CV: 0.05 and 0.01) and IL-2 (CV: 0.15 and 0.25), whilst there was a modest degree of variation in the remaining three individuals for IFN-γ (CV: -1, -0.25, -0.5) and IL-2 (1.5, 2.0 and 4.1).

3.3. Cytokine secretion in normal volunteers

3.3.1. Cytokine secretion by CD3+ T cells using PHA stimulation

PHA stimulated cultures were set up using the same group of twenty normal healthy volunteers and supernatants harvested for analysis of cytokine (IL-2, IL-4, IL-10 and IFN-γ) protein by ELISA. Time points for peak cytokine secretion had been previously optimised. Low, medium and high secretion was based on calculated ranges for each individual cytokine.

Inter-individual variation of secretion was observed for IFN-γ (Std Dev: 3426.28). As seen in Figure 3.13a, a large cluster of individuals (n=12) were classed as "low secretors" (<2000pg/ml). Two individuals appeared to be "intermediate secretors" (2-8000pg/ml), while six remaining subjects were classed as "high secretors" (these individuals secreted at least 8000pg/ml, exact data not shown). In contrast, IL-4 yielded a different pattern (Std Dev: 16.017). The majority (n=17) of the individuals secreted low-medium amounts of IL-4 (<30pg/ml), again yielding a

large cluster at the lower end of the scale. Only three individuals secreted high levels of this cytokine (>30pg/ml; Figure 3.13b).

In the case of IL-2, (Std Dev: 430.691), a cluster of "low-medium secretors" was again evident (n=18; <1000pg/ml). Only two individuals within the group secreted high levels (>1000pg/ml; Figure 3.14a), and may be classed as outliers. Finally, for IL-10, there appeared to be a more widespread distribution of secretion (Std Dev: 205.37). Just under half (n=8) of the group secreted higher (>200pg/ml) amounts of IL-10 cytokine (Figure 3.14b).

3.3.2. Variation of cytokine secretion over time

In order to establish whether cytokine secretion varies with time in the same individual, four volunteers were assayed every two months over a twelve month period, as with cytokine expression. This experiment was only performed with PHA stimulated data as OKT3 experiments were performed at a much later stage of the project. These particular individuals were chosen as they represented a group of high and low secretors for IL-2, IL-4, IL-10 and IFN-γ. Analysis of variance was again performed.

Analysis of cytokine secretion demonstrated no diurnal effect as with cytokine expression (p=0.545). For low secretion of the cytokines, three of the cytokines (IFN-γ, IL-4 and IL-10) were relatively consistent over time (CV: 0.2, 0.15 and 0.3 respectively). IL-2 appeared to exhibit a modest degree of variation (CV: 0.7). For high secretion, two of the cytokines were consistent over time (CV for IFN-γ: 0.25; IL-10: 0.1.6), with IL-2 and IL-4 varying slightly (CV: 0.62 and 0.55 respectively).

a)
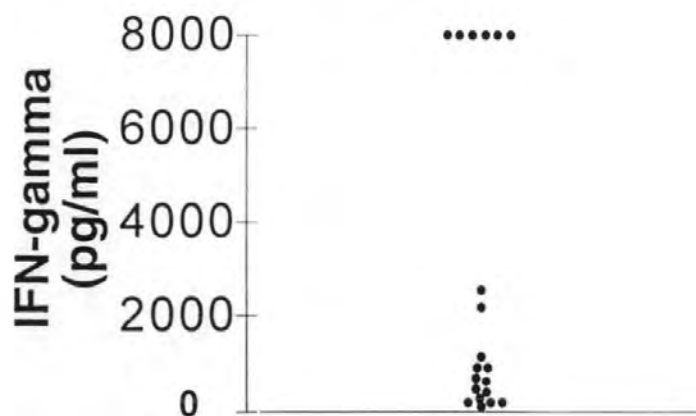
b)
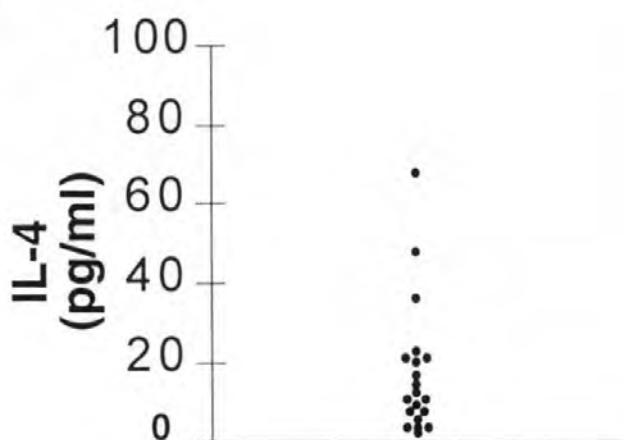
Figure 3.13. Variation in IFN-gamma (a) and IL-4 (b) secretion with PHA stimulation.

a)
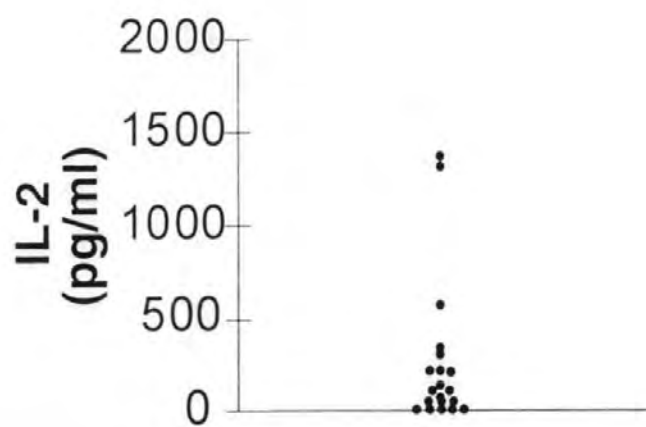
b)
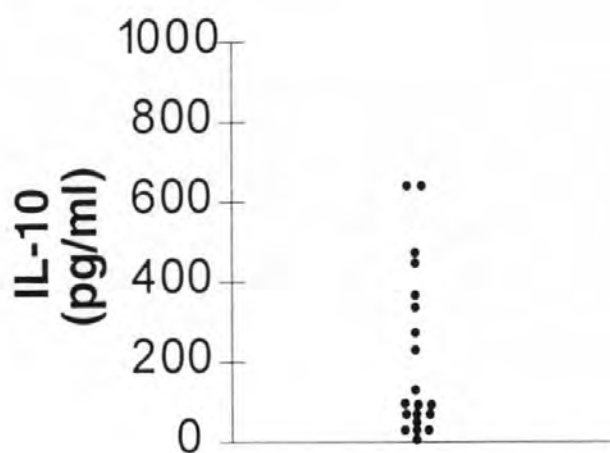
Figure 3.14. Variation in IL-2 (a) and IL-10 (b) secretion with PHA stimulation.

3.3.3 Cytokine secretion by CD3+ T cells using OKT3 stimulation

OKT3 was coated onto culture plates for two hours (room temperature) followed by incubation with PBMCs isolated from sixteen of the group subjects (four of the subjects were unavailable for analysis). Cell free supernatants were then harvested and assayed by ELISA. Optimal time points for each cytokine had been previously determined (Kaminski *et al*, 1995).

Inter-individual variations were also seen for IFN-γ with OKT3 stimulation (Figure 3.15a). Although, nearly all of the individuals could be classed as "low-medium secretors" (n=15), secretion levels for ten of those individuals secreted more than 2000pg/ml. Only one individual secreted a very high amount of IFN-γ (16330pg/ml), and may be classed as an outlier. OKT3 stimulation also yielded inter-individual variation for IL-4 secretion (Figure 3.15b). However, although there appeared to be a more widespread distribution with this mitogen, levels of cytokine secretion were generally lower than those reached with PHA stimulation. With OKT3, all (n=16) of the group yielded between 12pg/ml and 22pg/ml of IL-4. There were no clear outliers with this cytokine.

The pattern yielded by OKT3 again differed for IL-2 secretion (Figure 3.16a). Levels achieved were extremely low with this mitogen, with the highest amount only being 103pg/ml. The majority of the group (n=15) again formed a cluster of low-medium secretors (4-23pg/ml), with only the one individual secreting the highest amount (103pg/ml; outlier). However, a very large inter-individual variation was observed with IL-10 secretion (Figure 3.16b). As with PHA, a wide distribution could be seen, and therefore high or low secretion could not be determined. Only one individual secreted less than 200pg/ml, with ten individuals secreting between 2-800pg/ml. The remaining four subjects managed to secrete more than 800pg/ml.

a)
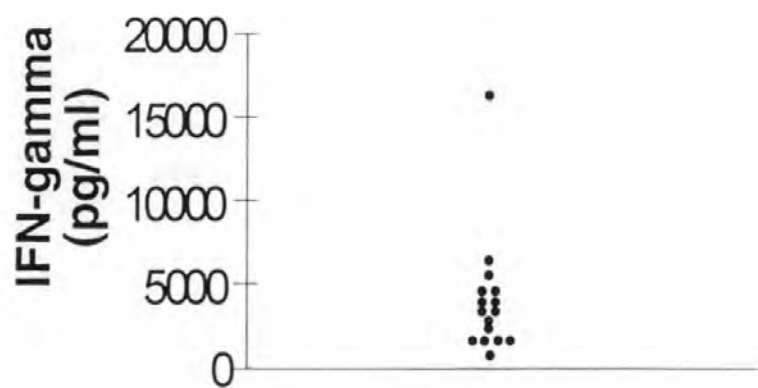
b)
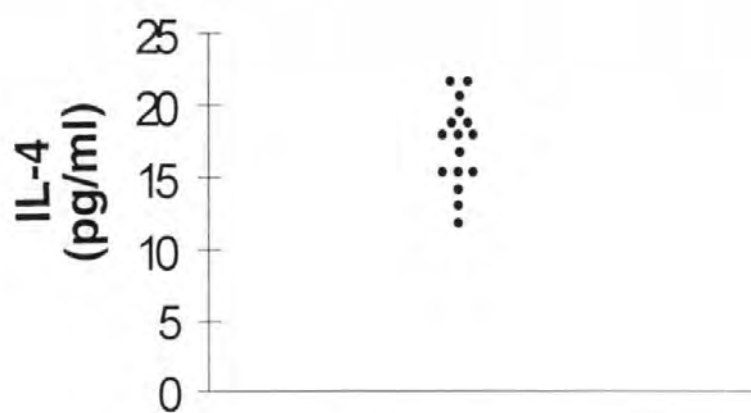
Figure 3.15. Variation in IFN-gamma (a) and IL-4 (b) secretion with OKT3 stimulation.

a)
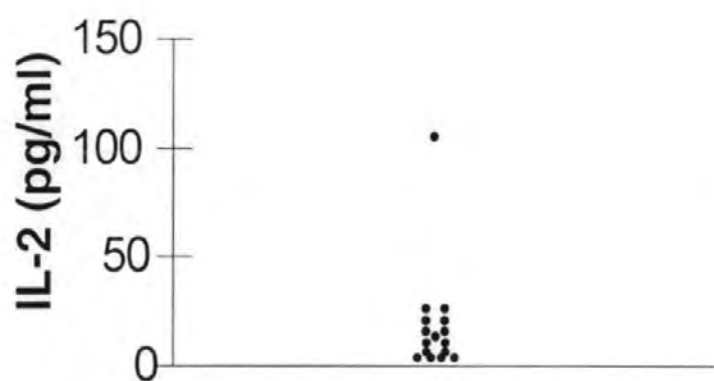
b)
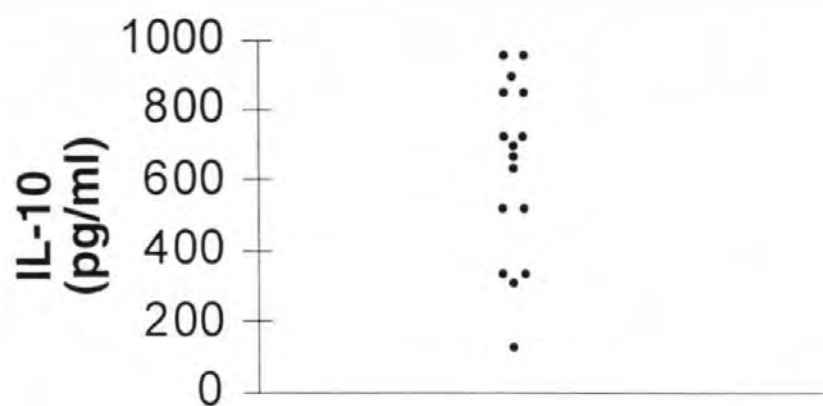
Figure 3.16. Variation in IL-2 (a) and IL-10 (b) secretion with OKT3 stimulation.

3.4. Relationship between Th1 and Th2 cytokines

3.4.1. Introduction

In view of the inter-individual variations which were found in both the numbers of cytokine secreting cells and protein secretion, it was important to determine whether there was a relationship between frequency and protein. It has also been suggested that within the Th1/Th2 paradigm, inter-regulation may occur, and therefore a possible inverse correlation could be expected. Therefore the relationship between the Th1 (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-10) protein levels was examined.

3.4.2. Cytokine expression and PHA-stimulated protein

In order to determine whether a correlation existed between IL-2 protein and frequency of IL-2 secreting cells, the data was analysed by Pearsons correlation analysis. A significant relationship was found between the amount of IL-2 protein secreted and numbers of IL-2 expressing cells (p=0.02; r=0.5). A sigmoidal relationship was also present between the two variables (figure not shown), log y transformation was performed (Figure 3.17). This generated a highly significant and linear relationship between the two parameters (p=0.0007; r=0.7). There also appeared to be two outliers (marked *), and therefore analysis was again performed without these subjects to see if they made a difference to the significance of the relationship. Analysis revealed there to be no strong influence by the two subjects, therefore these data points were not omitted (data not shown). Statistical analysis used for IL-2 could not be used for IFN-γ as the data did not conform to a normal distribution. The data for IFN-γ was therefore analysed using Spearmans rank analysis. The data was also plotted out of interest to see if there was any kind of relationship between IFN-γ protein and frequency. For any of the data analysed by

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Spearmans rank analysis, the plots were not representative of the statistical analysis as the data is ranked. Figure 3.18 illustrates this plot. Interestingly, although there appears to be no relationship between these two variables (p=0.762; r=0.07), it can be seen from the plot that there is a bi-modal distribution formed by this data. The majority of the individuals (n=12) were both low secretors and expressors, with the second group (n=6) secreting high levels of protein but expressing low numbers of cytokine-positive cells.

3.4.3. Cytokine expression and OKT3-stimulated protein

This data was also analysed by Spearmans rank analysis due a to a non-normal distribution. The data was again plotted. Figures 3.19 and 3.20 illustrate the relationships between protein and frequency for IL-2 and IFN-γ respectively. It can be seen in Figure 3.19 that the majority of the individuals are low secretors (hence the non-normal distribution). Analysis revealed there to be no relationship between IL-2 protein and frequency (p=0.29; r=-0.54). Only one individual secreted a higher amount of IL-2 (>100pg/ml). IFN-γ appeared to give a similar pattern, with thirteen of the individuals forming a cluster of both low secretors and expressors (Figure 3.20). The overall pattern was similar to that of the relationship between IFN-γ expression and PHA stimulated protein. there was also no relationship found between IFN-γ protein and frequency (p=0.7; r=0.1).
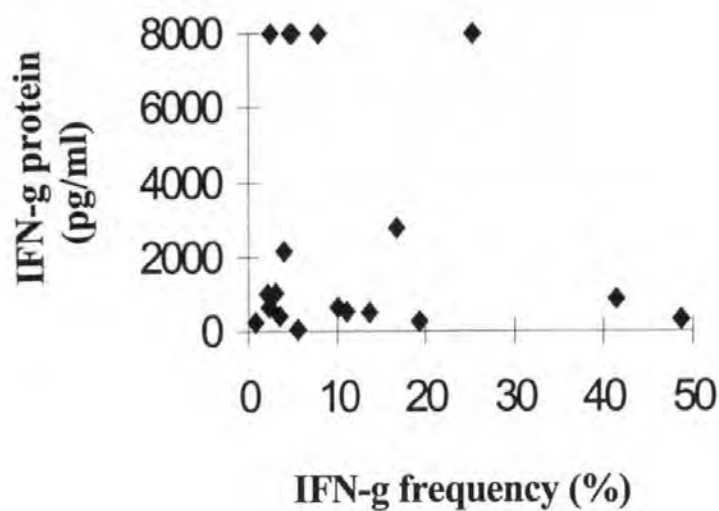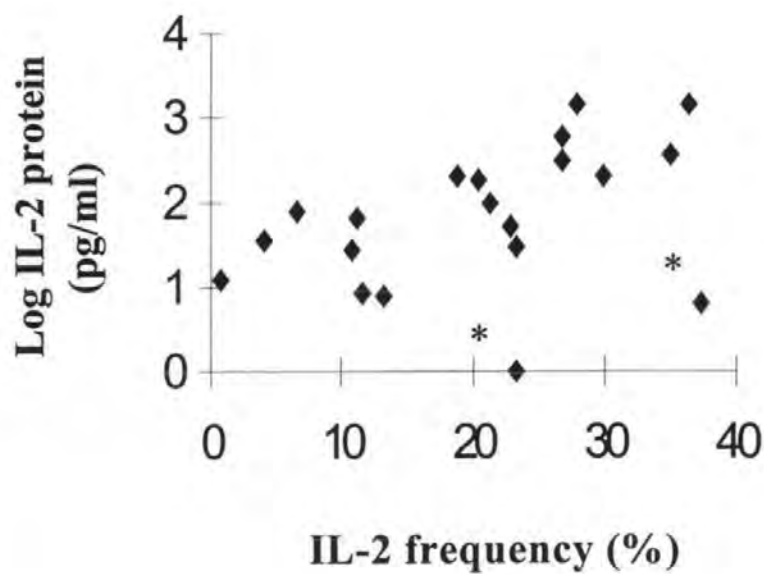
Figures 3.17 and 3.18. Correlation between IL-2 protein (PHA) and frequency (upper figure) and relationship between IFN-gamma (g) protein (PHA) and frequency (lower figure).
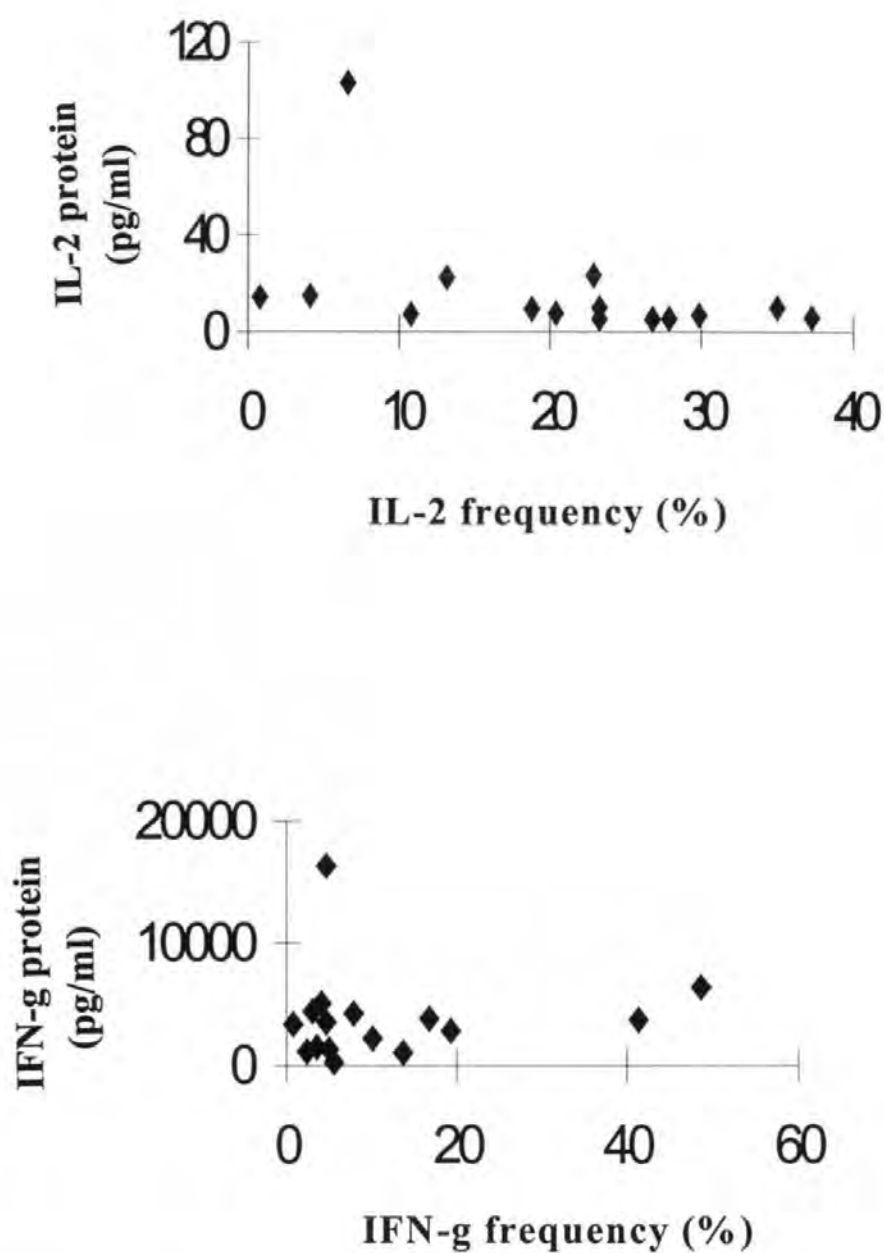
Figures 3.19 and 3.20. Relationship between IL-2 protein (OKT3) and frequency (upper figure) and relationship between IFN-gamma protein (OKT3) and frequency (lower figure).

3.4.4. Th1 versus Th2 protein (PHA)

3.4.4.1. IFN-γ versus IL-4

All analysis of this data was performed using Spearmans rank analysis. Figure 3.21 illustrates the observed relationship between IFN-γ and IL-4 protein. Analysis revealed there to be no relationship (p=0.563; r=0.13). From the plot, a large cluster of individuals (n=11) appeared to be low secretors for both cytokines. A second cluster is also evident, whereby a group of individuals secreted high levels of IFN-γ and low levels of IL-4 (n=6). The remaining three subjects secreted lower levels of IFN-γ with increasing levels of IL-4.

3.4.4.2. IL-2 versus IL-4

The second Th1 cytokine IL-2 was then analysed against IL-4 to see whether there was a relationship between these two cytokines. No relationship was found (p=0.275; r=0.25). Plotting the data revealed there to be a random relationship between these two proteins (Figure 3.22).

3.4.4.3. IFN-γ versus IL-10

The second Th2 cytokine, IL-10, was then plotted against IFN-γ. Analysis revealed there to be a strong relationship between the two cytokines (p=0.001; r=0.9155). This was reflected in the diagrammatic plot (Figure 3.23), which would suggest that there was a positive correlation between the two cytokines.
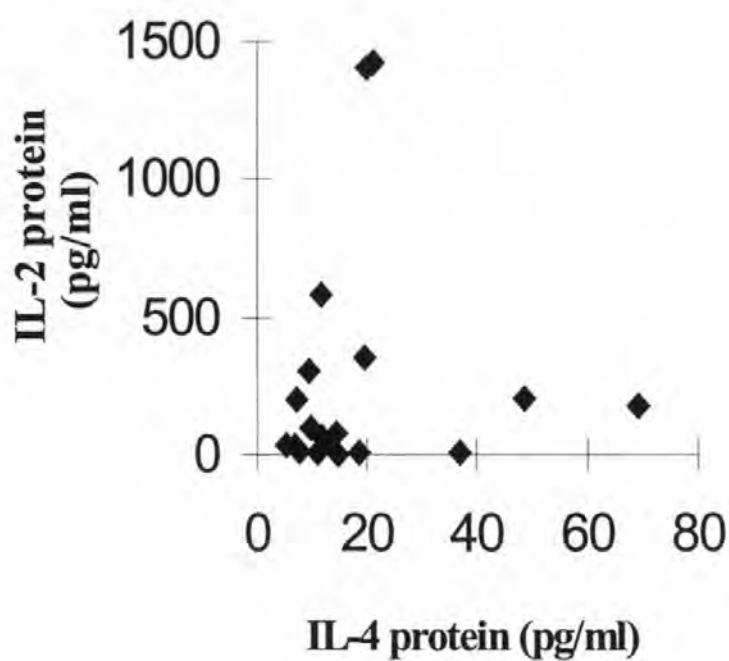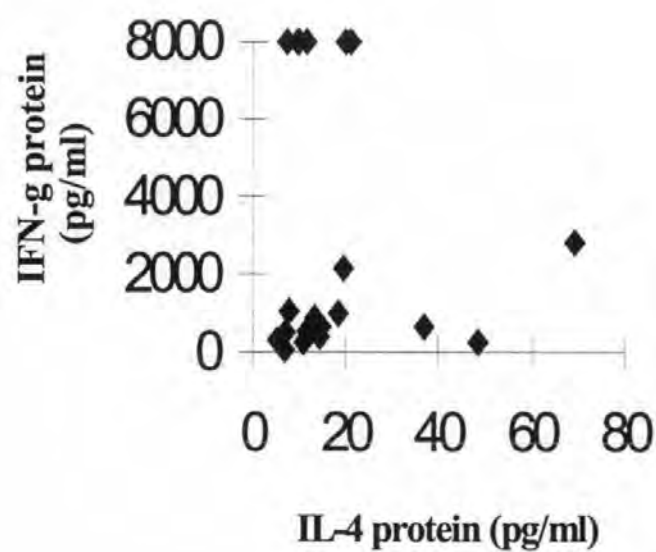
Figures 3.21 and 3.22. Relationship between IFN-gamma and IL-4 protein (PHA) (upper figure) and relationship between IL-2 and IL-4 protein (PHA) (lower figure).

3.4.4.4. IL-2 versus IL-10

Finally, IL-2 was plotted against IL-10, but this time, Pearsons correlation analysis could be performed as they both conformed to a normal distribution. Figure 3.24 illustrates the relationship between these two cytokines. A sigmoidal relationship was initially observed, therefore log y transformation was performed. This yielded a linear but non-significant relationship between this Th1 and Th2 protein (p=0.07; r=0.408).

3.4.5. Th1 versus Th2 protein (OKT3)

3.4.5.1. IFN-γ versus IL-4

The relationship between Th1 and Th2 proteins with OKT3 stimulation also required analysis. As only one set of data (IL-10) conformed to a normal distribution, Spearmans rank analysis was performed. However, it was also still of interest to observe the visual relationship between the proteins. The relationship between IFN-γ and IL-4 may be seen in Figure 3.25. It is obvious from this plot that there is no real relationship between these two cytokines, which was reflected by the analysis (p=0.34, r=0.12). Interestingly there appears to be a cluster of low-medium IL-4 secretors, which are also low secretors for IFN-γ (n=14).

3.4.5.2. IL-2 versus IL-4

The relationship between IL-2 and IL-4 was then plotted and analysed. This plot may be seen in Figure 3.26. Again, there appeared to be no obvious relationship between these two proteins. Analysis revealed there to be no relationship (p=0.096; r=0.42). However, the observed relationship did appear similar to that of IFN-γ and IL-4, with the majority of the individuals (n=14) forming a cluster in the lower half of the plot.
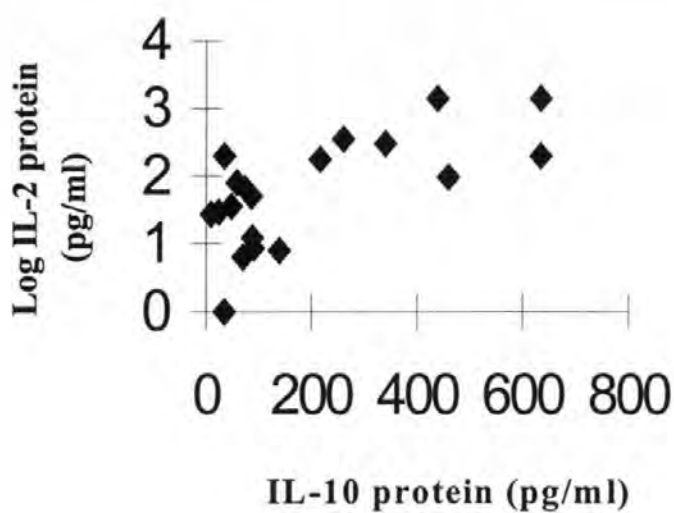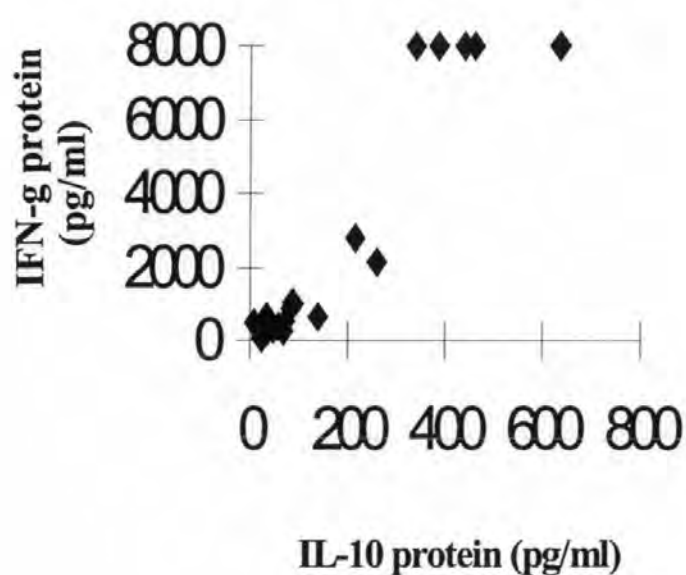
Figures 3.23 and 3.24. Relationship between IFN-gamma and IL-10 protein (PHA) (upper figure) and relationship between IL-2 and IL-10 protein (PHA) (lower figure).
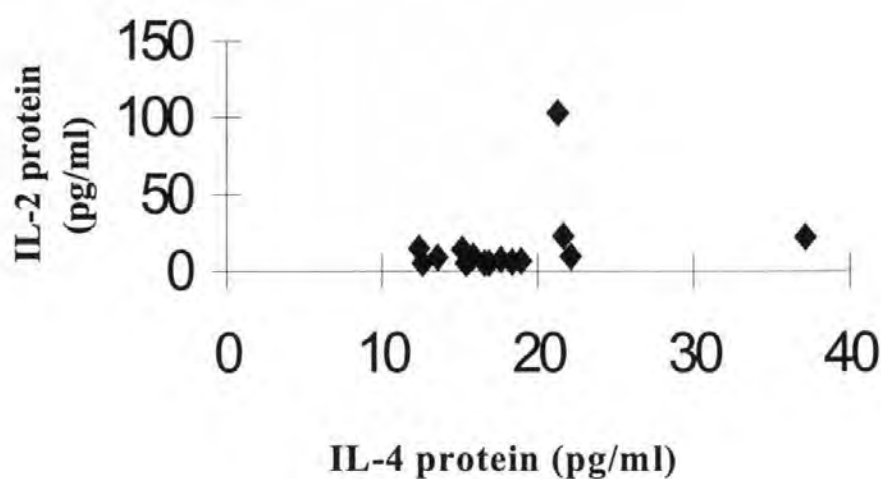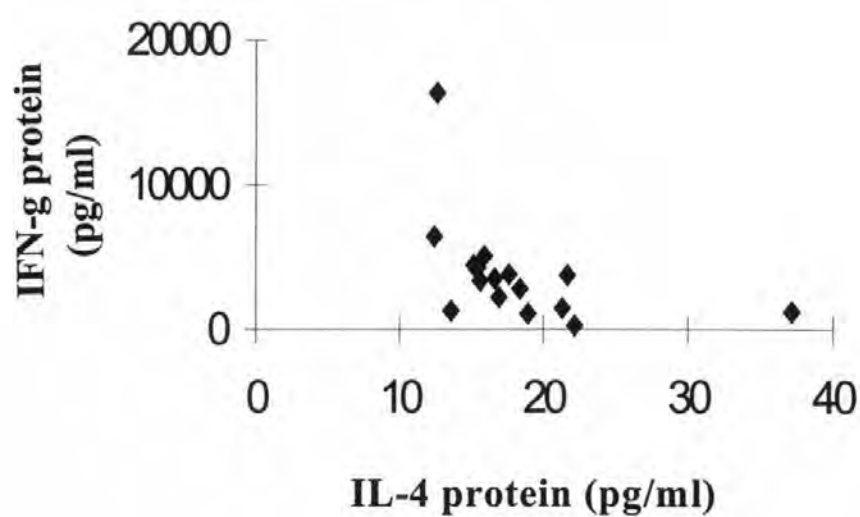
Figures 3.25 and 3.26. Relationship between IFN-gamma and IL-4 protein (OKT3) (upper figure) and relationship between IL-2 and IL-4 protein (OKT3).

3.4.5.3. IFN-γ versus IL-10

The second Th2 cytokine, IL-10, was then plotted against IFN-γ. This can be seen in Figure 3.27. The observed relationship between these cytokine proteins is again random, with the analysis showing no relationship at all (p=0.31; r=-0.27). For the majority of the individuals (n=15), there was a normal distribution of IL-10 secretion, with IFN-γ secretion levels remaining low-medium. Only one individual secreted high levels of IFN-γ protein.

3.4.5.4. IL-2 versus IL-10

Finally, IL-2 protein data was plotted against IL-10 (Figure 3.28). As the group consisted of mainly low IL-2 secretors (n=15), there was no obvious relationship between these two cytokines. Analysis also illustrated no relationship (p=0.57; r=-0.16). Only one individual was seen to secrete medium-high levels of IL-2 protein.
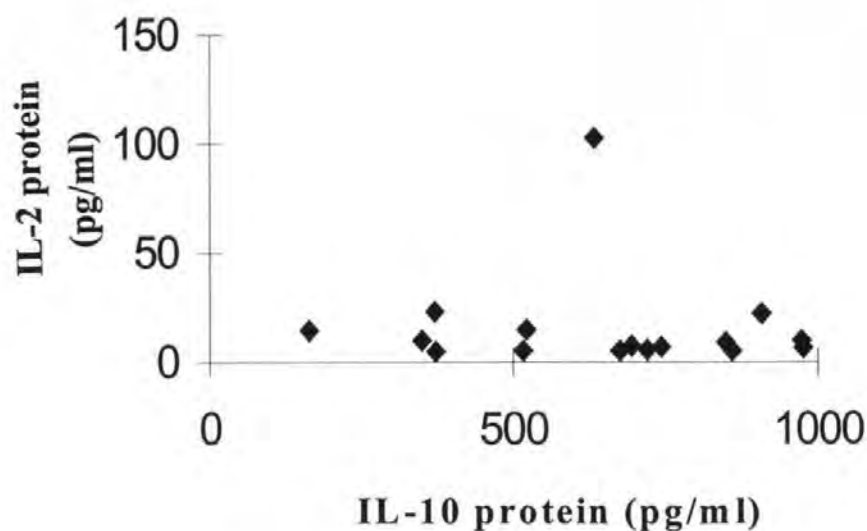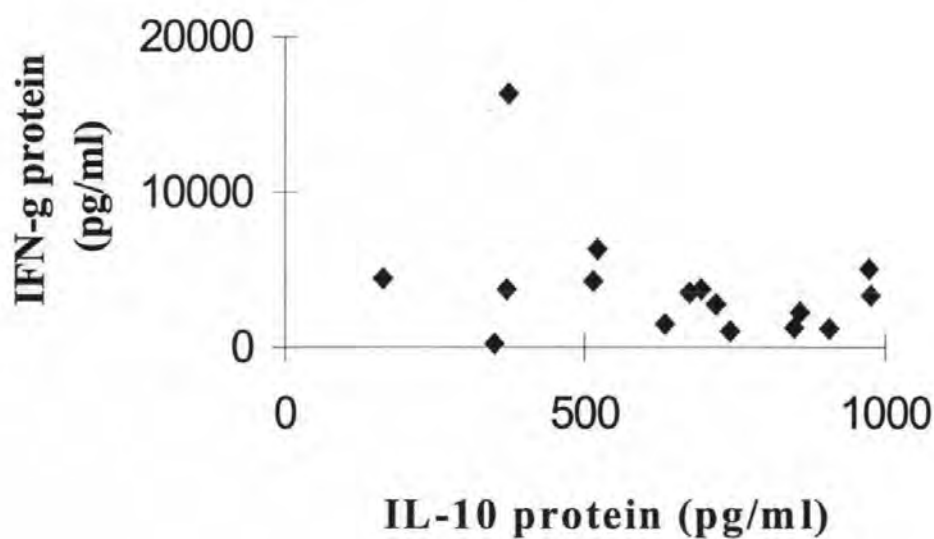
Figures 3.27 and 3.28. Relationship between IFN-gamma and IL-10 protein (OKT3) (upper figure) and relationship between IL-2 and IL-10 protein (OKT3) (lower figure).

3.5. Relationship between IFN-γ gene polymorphisms (IFN-G), IFN-γ protein secretion and expression

3.5.1. Introduction

DNA studies have demonstrated that polymorphisms of particular cytokine genes (TNF-α and IL-10) are associated with high/low secretor status (Turner *et al*, 1995, 1997). Due to the interesting bi-modal nature of the IFN-γ protein data, analysis was also performed to determine the role of IFN-G gene polymorphisms on IFN-γ expression and protein secretion.

3.5.2. IFN-G gene polymorphisms, protein secretion (PHA) and frequency

Analysis was performed using logistic regression analysis to determine the role of IFN-G gene polymorphisms on expression and secretion of this cytokine. Although relationships have been found for other cytokines, in this case, there was no statistically significant relationship between these three parameters (p value range: 0.252-0.588). A summary of the data is shown in Table 3.1.

3.5.3. IFN-G gene polymorphisms, protein secretion (OKT3) and frequency

Analysis was again performed using logistic regression analysis to determine whether stimulation with OKT3 made any difference to the relationship between IFN-γ protein, frequency and/or gene polymorphisms. The analysis was performed on sixteen individuals that were available. Again, there was no significant relationship between any of these parameters, as was seen with PHA-stimulated IFN-γ protein (p value range: 0.083-0.328). A summary of the data is also shown in Table 3.1.

Patient	Protein (PHA) pg/ml	Protein (OKT3) pg/ml	Expression (%)	Allele 1	Allele 2
1	267	2786	19	123	127
2	646	1046	14	123	119
3	501	2236	10	127	127
4	8000	4250	8	123	127
5	640	1188	2	129	133
6	534	ND	11	123	123
7	998	ND	2	133	127
8	400	1470	4	121	127
9	1040	4441	3	127	127
10	2798	3757	17	127	127
11	863	3718	41	123	123
12	308	6341	49	127	129
13	2159	5048	4	125	130
14	243	3355	1	123	129
15	53	226	6	123	133
16	8000	16330	5	123	133
17	8000	ND	25	127	127
18	8000	1290	5	123	127
19	8000	ND	3	123	123
20	8000	3491	5	123	123

Table 3.1. Summary of IFN-γ protein (OKT3 and PHA-stimulated), expression and IFN-G gene polymorphism data.

3.6. Summary

The experiments designed for the optimisation of the MLC were performed using blood from normal healthy volunteers. The initial experiments involved the incubation of "donor" and "recipient" lymphocytes together for up to eight days and the cytokine (IL-2, IL-4, IL-10, IFN-γ and IL-6) responses measured to establish peak cytokine secretion. The results showed there to be peak IL-10 and IL-2 protein secretion on day three, with peak IFN-γ and IL-4 secretion on day four. Peak secretion for IL-6 was found on day five, although for practical reasons, day four was used to collect supernatants. Further experiments showed there to be no significant difference in cytokine secretion between cell free and cell based culture samples. Titration experiments for the treatment of the stimulator cell population in the MLC found a 20-30 Gy dose to be sufficient to abrogate cytokine secretion and therefore have a real one way MLC. Neither cytokine or cell surface marker expression was able to be measured by flow cytometry, therefore only measurement of protein secretion was used.

In the case of PHA and OKT3 stimulated PBMCs from normal volunteers, inter-individual variations were found in both cytokine expression (IL-2 and IFN-γ) and protein secretion (IL-2, IL-4, IL-10 and IFN-γ). For PHA stimulation, a significant relationship was found between IL-2 protein secretion and frequency. A strong yet non-significant relationship was found between IL-2 and IL-10 (p=0.07), with a significant relationship being found between IL-10 and IFN-γ. No relationship was found between IFN-γ gene polymorphisms, protein secretion and/or frequency.

For OKT3 stimulated protein, there was no relationships between IL-2 protein and frequency or IFN-γ protein and frequency. As IL-10 protein was the only data to conform to a normal distribution, Spearmans rank analysis was performed.

No relationships were found between any of the cytokines. In addition, no relationship was found between IFN-γ gene polymorphisms, frequency and/or protein.

CHAPTER 4

PREDICTION OF RENAL ALLOGRAFT REJECTION

BY CYTOKINE SECRETION IN MIXED LYMPHOCYTE

REACTION

4.1. Introduction

The mixed lymphocyte reaction (MLR) has played a historical role in organ transplantation. In renal transplantation, there is evidence for (Harmon *et al*, 1982, Langhoff *et al*, 1985) and against (Cullen *et al*, 1977, Burke *et al*, 1993, Steinmann *et al*, 1994) the MLR predicting rejection and clinical outcome. However, the concensus is that it is not of clinical value. Kaminski *et al* (1995) demonstrated a correlation between high levels of IFN-γ in mitogen stimulated culture and rejection in renal transplantation. In view of these findings, cytokine protein secretion was measured in MLR to determine whether such an assay is predictive for acute rejection after allogeneic renal transplantation.

4.2. Study group

This consisted of fifty six consecutive patients who received a cadaveric renal transplant from an unrelated donor, and one patient receiving a renal transplant from a live related donor (see materials and methods chapter for detail).

4.3. HLA and HLA-DR matching

The group consisted of one "fully HLA matched" patient (000), three patients with one mismatch, eleven patients with two mismatches, twenty one patients with three mismatches, thirteen patients with four mismatches, seven patients with five mismatches, and one "fully mismatched" patient (appendix 2). In addition, within the study group, there were fifteen patients with zero antigen HLA-DR mismatch, thirty two with one antigen DR mismatches and ten with full HLA-DR mismatching (appendix 3).

4.4. Cytokine secretion in MLR and HLA mismatching

Univariate analysis was performed using the Kruskal-Wallis test. This analysis was chosen as the cytokine protein data was of a non-normal distribution. Original analysis was performed with cytokine protein and HLA mismatching (0-6), but when plotted there were single individuals who had either 0 or 1 HLA antigen mismatch. Analysis showed there to be no significant relationship between increase in HLA mismatching and levels of cytokine protein in the MLR (p values for IFN-γ=0.47; IL-10=0.544; IL-6=0.723; IL-4=0.24; IL2=0.4). In order to attempt to even the data, the patients were placed into two groups, 0-3 HLA mismatches (group 1) and 4-6 HLA mismatches (group 2). This gave rise to a 50:50 split. When analysis was performed on the grouped data, there still appeared to be no correlation between cytokine protein and HLA mismatching (p values for MLC: IFN-γ=0.224; IL-10 MLC=0.24; IL-6 MLC= 0.77; IL-4 MLC= 0.61; IL-2 MLC= 0.64; p values for spontaneous: IFN=0.05; IL-10=0.88; IL-6=0.655; IL-4=0.3; IL-2=0.54).

Analysis was then performed to observe if there was any relationship between HLA-DR mismatching and cytokine protein. As the data was evenly distributed when looking at 0, 1 and 2 HLA-DR mismatches, the data was not grouped as it was for full HLA mismatching analysis. Analysis revealed there to be a significant correlation between an increase in HLA-DR mismatches and both MLR IL-10 (p= 0.03) and MLR IFN-γ (p= 0.019). This data may be seen in Figures 4.1a and 4.1b respectively. There was no correlation found between increase in HLA-DR mismatching and MLR IL-2 (p value= 0.07), MLR IL-4 (p= 0.07) or MLR IL-6 (p=0.65). Analysis was performed with spontaneous protein secretion data, and no correlations were found with any of the cytokines and HLA-DR mismatching (p values for IFN-γ=0.4; IL-10=0.42; IL-6=0.65; IL-4=0.8; IL-2=0.12).

a)
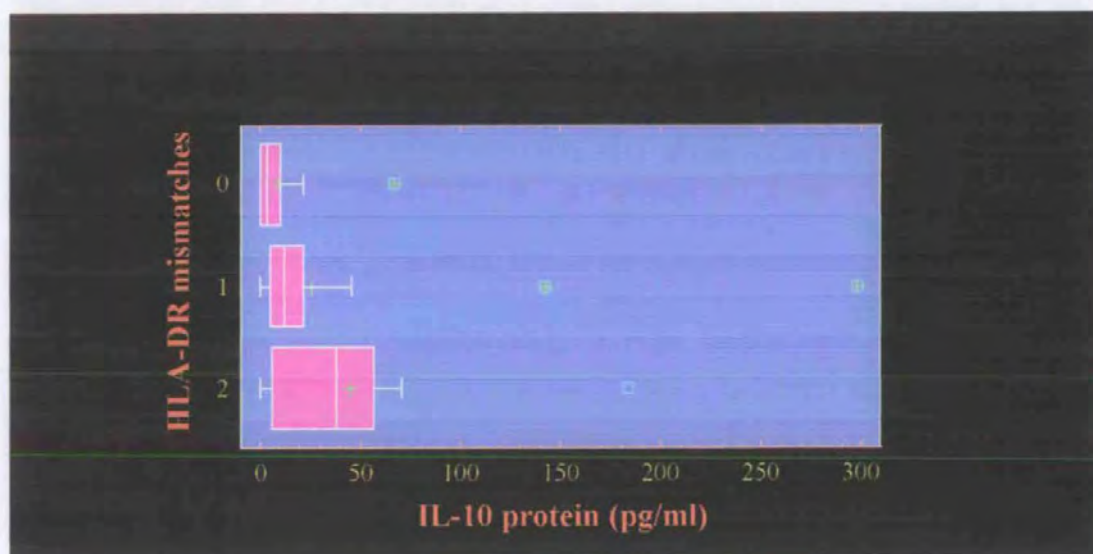
b)
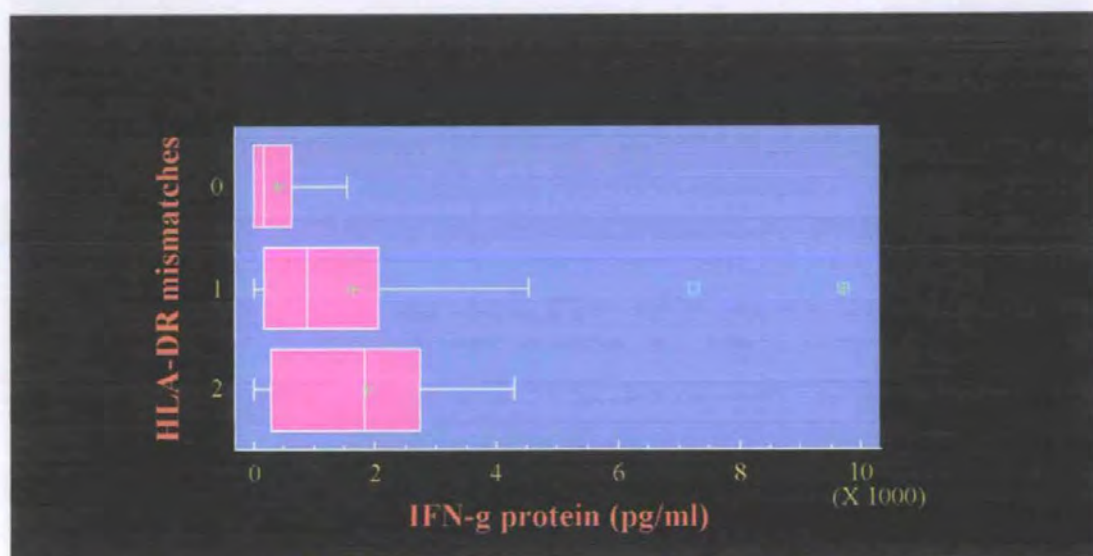
Figure 4.1. Relationship between HLA-DR mismatching and IL-10 (a) and IFN-gamma (b) protein secretion (Box and Whisker plot).

4.5. Inter-individual variations in cytokine protein in the MLR

Pre-transplant one way MLRs were set up between patient and donor and cytokine protein secretion (IL-2, IL-4, IL-6, IL-10 and IFN-γ) measured by ELISA. The data was analysed to determine whether there were differences in cytokine protein secretion between individuals and whether these differences predicted rejection. The clinical outcome was twenty seven rejectors and twenty six non-rejectors. Two patients were unevaluable for surgical reasons and two patients died. Significant differences were calculated using the Mann-Whitney U test. Figure 4.2 illustrates inter-individual variations in IL-2 (a) and IL-4 (b) protein secretion (protein secretion ranges: IL-2 – 0-1329pg/ml; IL-4 – 2-24pg/ml). It can be seen that there was no significant difference in median IL-2 levels between rejectors and non-rejectors (spontaneous p= 0.4, MLR p=0.09). There was a significant difference between rejectors and non-rejectors in median IL-4 secretion in spontaneous (p=0.03) and a strong, yet non-significant difference in MLR cultures (p=0.06).

Figure 4.3a shows the inter-individual variations in rejectors and non-rejectors for IL-10 protein secretion (range – 0-298pg/ml). Although there was no significant difference between spontaneous secretion in rejectors and non-rejectors (p=0.68), there was in the MLR cultures (p=0.018), thus illustrating that there is a association between increase in risk of rejection and increase in MLR-stimulated IL-10 secretion (discussed later). Figure 4.3b illustrates IFN-γ secretion (range – 0-9718pg/ml). As for all of the other cytokines, there are inter-individual variations in both patient groups, and there is also a significant increase in MLR-stimulated IFN-γ secretion in rejectors compared to non-rejectors (p=0.0126). There was no significant difference in spontaneous secretion (p=0.468). As for IL-10, this would suggest that there was also a correlation between increase in risk of rejection and MLR-stimulated IFN-γ

a)
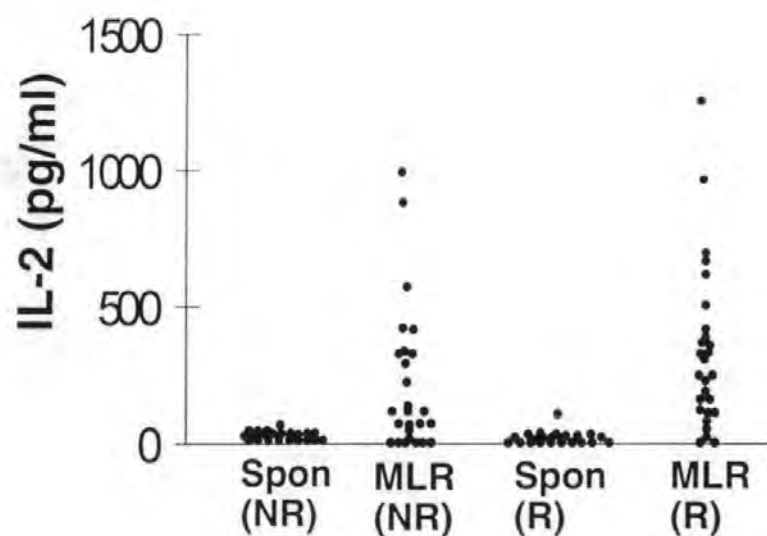
b)
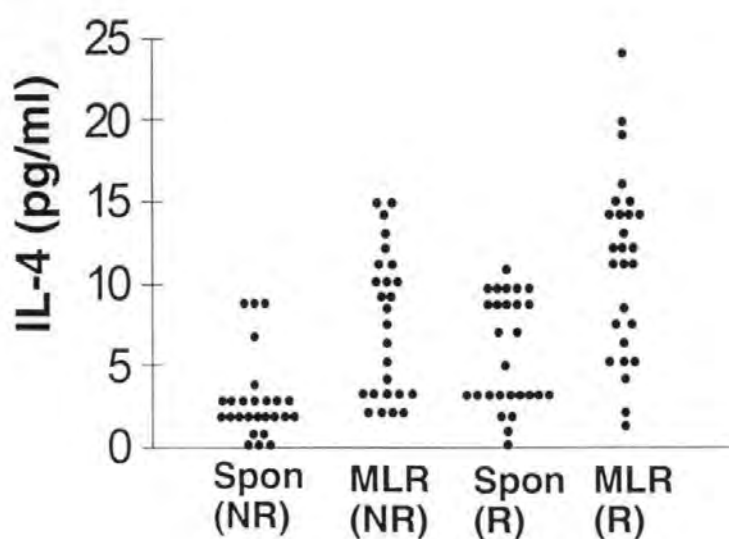
Figure 4.2. Inter-individual variations in IL-2 (a) and IL-4 (b) protein secretion in rejector (R) and non-rejector (NR) groups. Spon=spontaneous; MLR=mixed lymphocyte culture.

a)
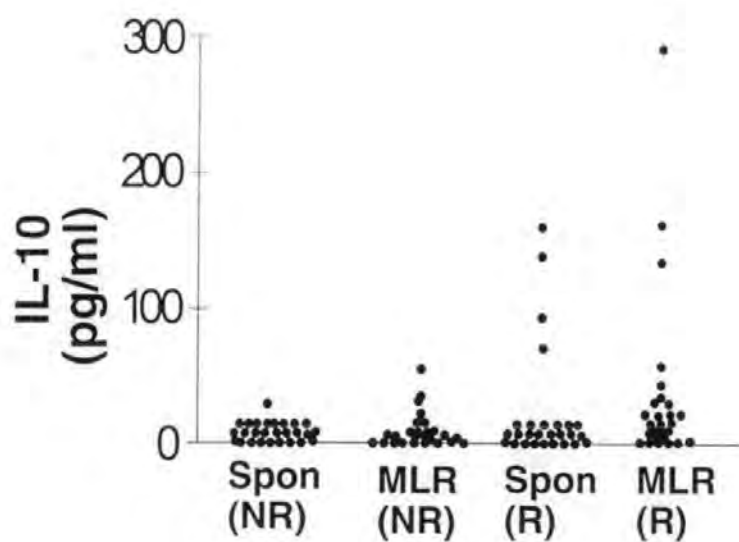
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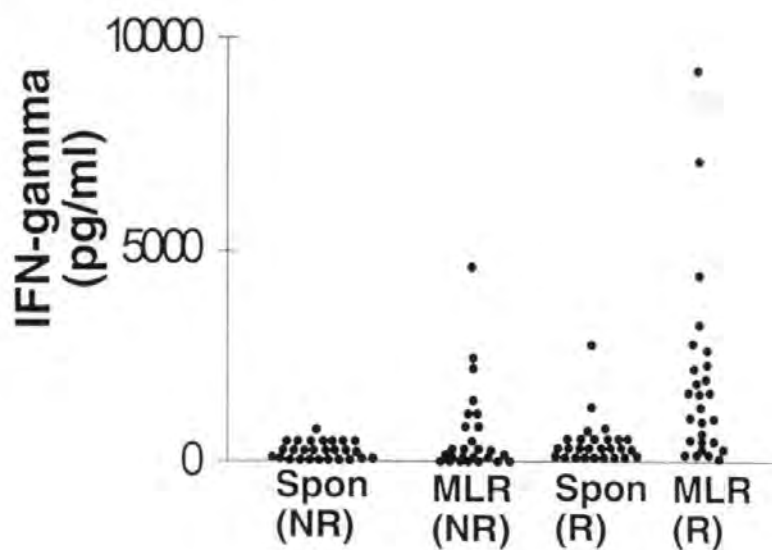
Figure 4.3. Inter-individual variations in IL-10 (a) and IFN-gamma (b) protein secretion in rejector (R) and non-rejector (NR) groups. Spon=spontaneous; MLR=mixed lymphocyte culture.

and increase in IFN-γ secretion (discussed later). Finally, IL-6 protein secretion was analysed (Figure 4.4; protein range – 7-13598pg/ml). From the analysis, there was no significant difference in either spontaneous or MLR-stimulated IL-6 protein secretion between non-rejectors and rejectors (spontaneous p=0.717, MLR p=0.235).

4.6. Th1 versus Th2 cytokines

As with the normal study group, analysis was performed to determine if there were any correlations between the cytokine proteins, both with spontaneous and MLR secretion. As all of the data did not conform to a normal distribution, standard regression analysis could not be performed. Therefore, the data was ranked and Spearmans rank correlation analysis was performed. Using this analysis, there were several correlations found. Observed data was not plotted for this part of the thesis as there were so many correlations found. Spontaneous IFN-γ secretion appeared to correlate with several other cytokine protein measurements including MLR-stimulated IFN-γ (correlation coefficient (CC)=0.272, p=0.01), spontaneous IL-10 (CC=0.45, p=0.001), MLR-stimulated IL-10 (CC=0.34, p=0.01) and spontaneous IL-2 (CC=0.47, p=0.001), although the correlation coefficients were not that strong. MLR-stimulated IFN-γ was also found to significantly correlate, although not that strongly with spontaneous IL-10 (CC=0.34, p=0.009). The strongest significant correlations were found between MLR-stimulated IFN-γ and MLR-stimulated IL-10 (CC=0.75, p=0.0001), and MLR-stimulated IFN-γ and MLR-stimulated IL-4 (CC=0.82, p=0.0001).

Spontaneous IL-10, when analysed was found to weakly correlate with both MLR-stimulated IL-10 (CC=0.34, p=0.006) and MLR-stimulated IL-4 (CC=0.57, p=0.004). Finally, there appeared to stronger significant correlations between MLR-

stimulated IL-4 and spontaneous IL-4 (CC=0.64, p=0.002) and MLR-stimulated IL-2 (CC=0.7, p=0.001). As all of the correlation coefficients were positive, it could be concluded that there were no inverse correlations between any of the spontaneous or MLR-stimulated cytokines analysed.

4.7. Role of clinical factors and rejection

4.7.1. Total HLA and HLA-DR mismatching and rejection

Univariate analysis (χ^2 test) was performed to analyse whether there was relationship between both HLA or HLA-DR mismatching with incidence of acute rejection. HLA mismatching was represented as 0 (full house match) to 6 (full house mismatch). HLA-DR mismatching was represented as 0-2. As expected, when analysis was performed, both increases in HLA and HLA-DR mismatching correlated with increased incidence of acute rejection (p values-0.03 and 0.014 respectively).

4.7.2. Patient sex and age

It was also of interest to analyse the relationship between patient age and sex with incidence of acute rejection. Univariate analysis (χ^2 test) was again performed. Analysis revealed there to be no correlation between either patient sex or age and rejection (p= 0.87 and 0.97 respectively). The data therefore confirmed that patient age or sex as independent variables had no effect on risk of rejection.

4.7.3. Donor sex and age

The same analysis was performed to establish whether the age and/or sex of the donor had any bearing on the risk of rejection post-transplant. Results revealed there to be a non-significant relationship between rejection and donor age (p=0.36).

Figure 4.4. Inter-individual variations in IL-6 protein secretion in rejector (R) and non-rejector (NR) groups. Spon=spontaneous; MLR=mixed lymphocyte culture.
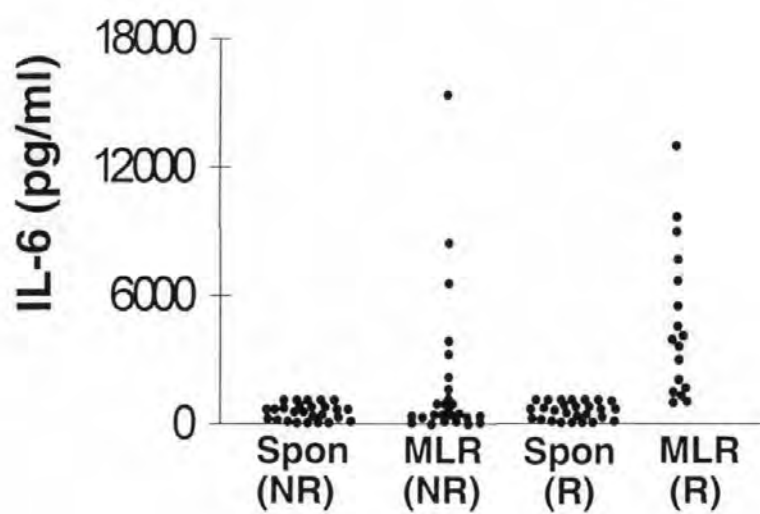
However, donor sex was found to be related to incidence of rejection, and therefore a factor associated with increased risk (p=0.009).

4.7.4. Cytomegalovirus (CMV) status

χ^2 analysis was performed to analyse whether patient or donor CMV status (ie: positive or negative) had any influence on the risk of rejection. Analysis revealed there to be no significant correlation between either patient (p=0.49) or donor (p=0.29) CMV status and incidence of rejection.

4.8. Effect of cytokine protein secretion on incidence of rejection

In order to determine whether cytokine protein secretion was independently related to incidence of acute rejection, univariate analysis was again performed using the χ^2 test, where rejection was compared with both baseline (or background) levels of cytokine and cytokine protein secreted in the MLR. Median cut-off points were calculated for each cytokine to determine high and low secretion groups. Interestingly, when the baseline data was compared to rejection, it was found that there were no significant correlations except for IL-4 background and rejection (p=0.005). The secreted levels for IL-4 background however, only ranged from 0-2 (low) and 3-7 (high).

When the MLC cytokine protein data was compared with incidence of rejection, more patterns were found. There appeared to be no relationship between rejection and IL-2 (p=0.21), IL-4 (p=0.22) or IL-6 (p=0.07). However, there was a different result for both IFN-γ and IL-10. With both of these cytokines, there was a strong correlation with rejection, suggesting that the higher the secretion of either IFN-γ (p=0.003) or IL-10 (p=0.009), the more likely the patient was to reject.

4.9. Multivariate analysis

Previous univariate analysis had shown that not only were increases in spontaneous IL-4 secretion, MLR-stimulated IL-10 and IFN-γ protein secretion, HLA and HLA-DR mismatching and donor sex associated with incidence of rejection, but also that increases in both MLR-stimulated IL-10 and IFN-γ protein secretion were also associated with an increase in HLA-DR mismatching. On the strength of these findings, multivariate Logistic analysis was performed. Multivariate analysis produced a model that included three variables, but analysis of interactions allowed a combination of HLA mismatching and IL-10 levels to produce a model with two variables acting as independent predictors of graft rejection (Table 4.1).

The relative risk of rejection was higher with female donors compared to male donors (p=0.006; relative risk(RR)=8.02). Compared with patients with mismatches at only 1-3 HLA antigens and an IL-10 level of >10pg/ml, patients with 4-6 mismatches and an IL-10 level of <10pg/ml or 1-3 mismatches and an IL-10 level of >10pg/ml had an increased risk of rejection (p=0.027, RR=6.04). Patients with 4-6 mismatches and an IL-10 level of >10pg/ml had a greatly increased risk of rejection (p=0.003; RR=25.5), indicating that the highest risk factors for rejection were high HLA mismatching and high IL-10 secretion.

4.10. Effect of clinical and immunological factors on graft survival

It was initially also of interest to analyse the effects of both clinical factors (ie: HLA mismatching, donor sex and age etc) and immunological factors (ie:cytokine profiles) on renal allograft survival. Unfortunately, this could not be performed due to the small number of patients who exhibited graft failure (n=6), therefore this has not been included in the study.

Variable	N	Relative Risk	p value
Donor sex	25	1.00	
Male			
Female	28	8.02	0.006
HLA mismatch and			
IL-10 level			
1-3 and low	19	1.00	
1-3 and low or			
1-3 and high	23	6.04	0.027
4-6 and high	11	25.5	0.003

Table 4.1. Model from a logistic analysis describing renal rejection following transplantation in 53 patients. 1-3 and 4-6 refer to the number of HLA mismatches, low and high refer to IL-10 secretion at the <10 and >10pg/ml levels in mixed lymphocyte reaction.

4.11. Comparison of pre- and post-transplant cytokine profiles

For some of the patients, follow-up clinical samples were able to be collected. These samples were taken at three and twelve months post-transplant. Forty four samples (twenty one rejectors, twenty three non-rejectors) were taken at three months post-transplant and seventeen samples (nine rejectors, eight non-rejectors) were taken at twelve months post-transplant. As with the fifty seven pre-transplant samples, one-way MLRs were set up between patient and donor and cytokine protein measured by ELISA (IL-2, IL-4, IL-10 and IFN-γ). Limited time did not permit the measurement of IL-6. Data was then plotted to analyse the pattern of cytokine secretion.

Figures 4.5 and 4.6 illustrate the patterns of cytokine secretion for IL-2 and IL-4 in both rejector (a) and non-rejector (b) groups respectively. Figures 4.7 and 4.8 illustrate the patterns of cytokine secretion for IL-10 and IFN-γ in rejector (a) and non-rejector (b) groups respectively. It can be seen for all cytokines and in both patient groups that there is an overall negative trend in cytokine secretion. Although some of the patients did show an increase in some cytokines but not others, there were two patients that increased in all four cytokines measured (pre-transplant and three month post-transplant measurements only). The first patient (patient 51, non rejector) significantly increased in IFN-γ (0-1001pg/ml), IL-10 (0-8pg.ml), IL-2 (33-257pg/ml) and IL-4 (3-20pg/ml). The second patient (patient 57, rejector) also significantly increased in IFN-γ (574-1600pg/ml), IL-10 (8-24pg/ml), IL-2 (357-988pg/ml) and IL-4 (11-24pg/ml). It was unclear as to why these two patients did not respond in the same way as most of the other patients.
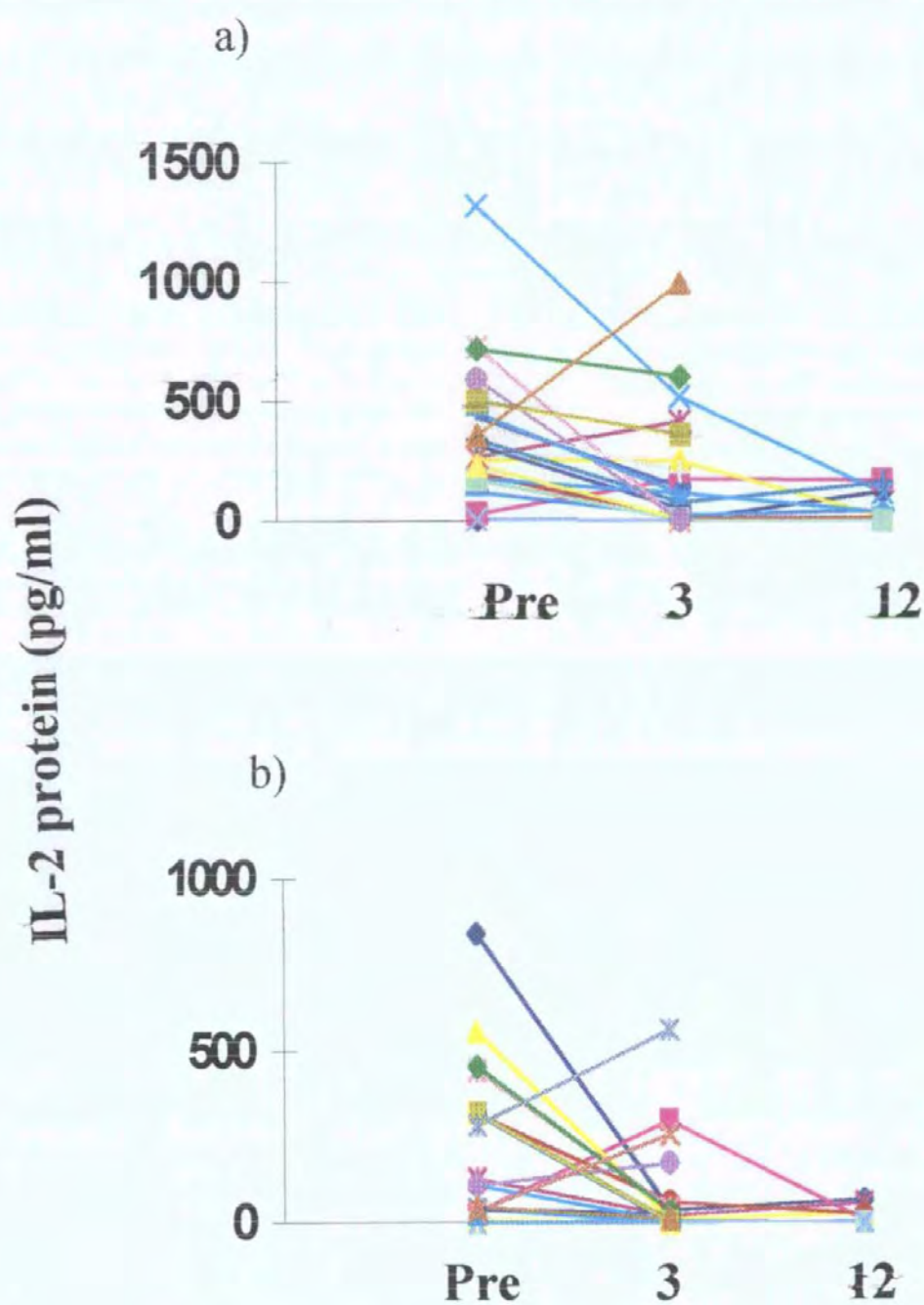
Figure 4.5. Pre- and post-transplant cytokine profiles (3 and 12 months) for IL-2 in rejector (a) and non-rejector groups (b).
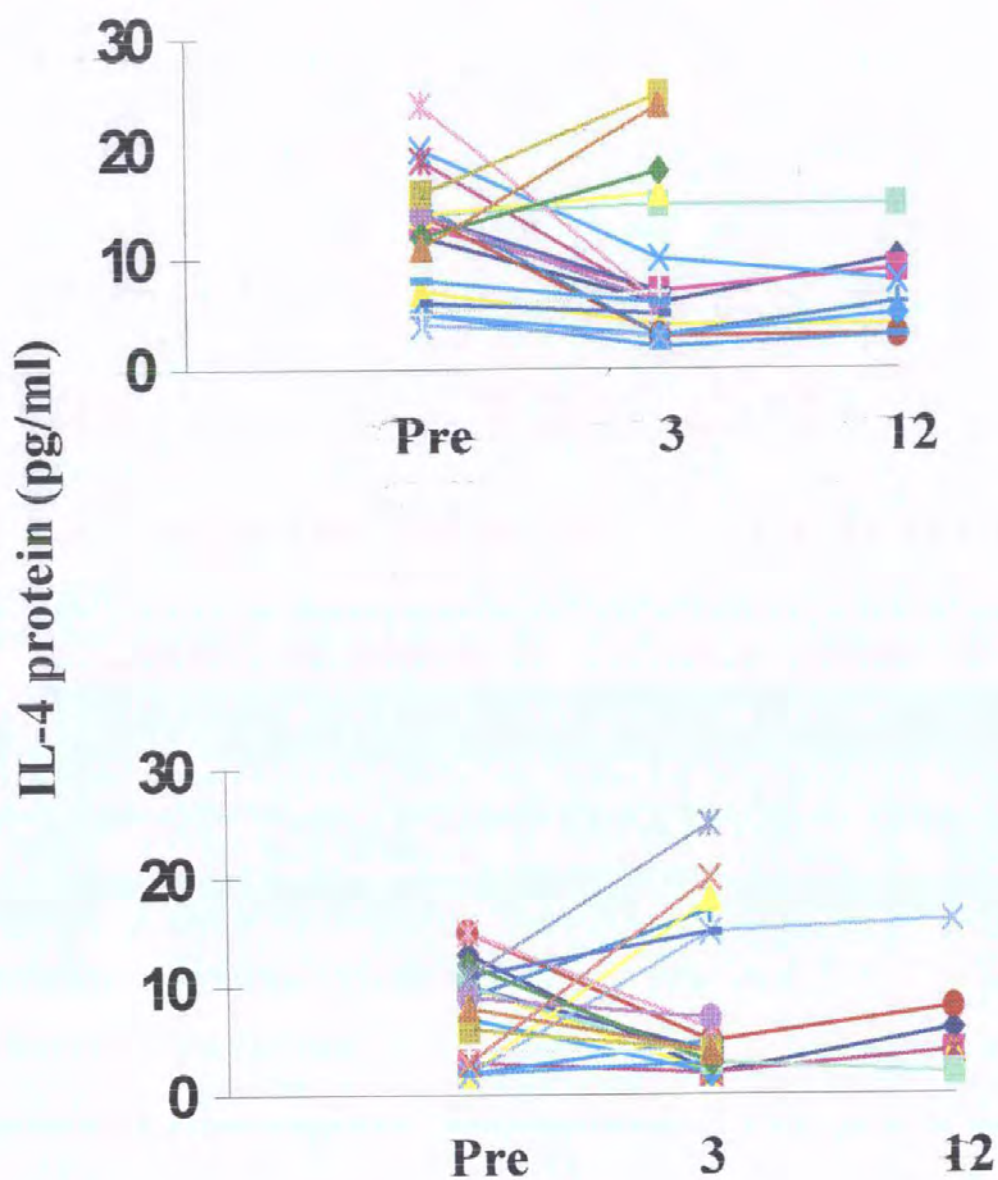
Figure 4.6. Pre- and post-transplant cytokine profiles (3 and 12 months) for IL-4 in rejector (a) and non-rejector groups (b).
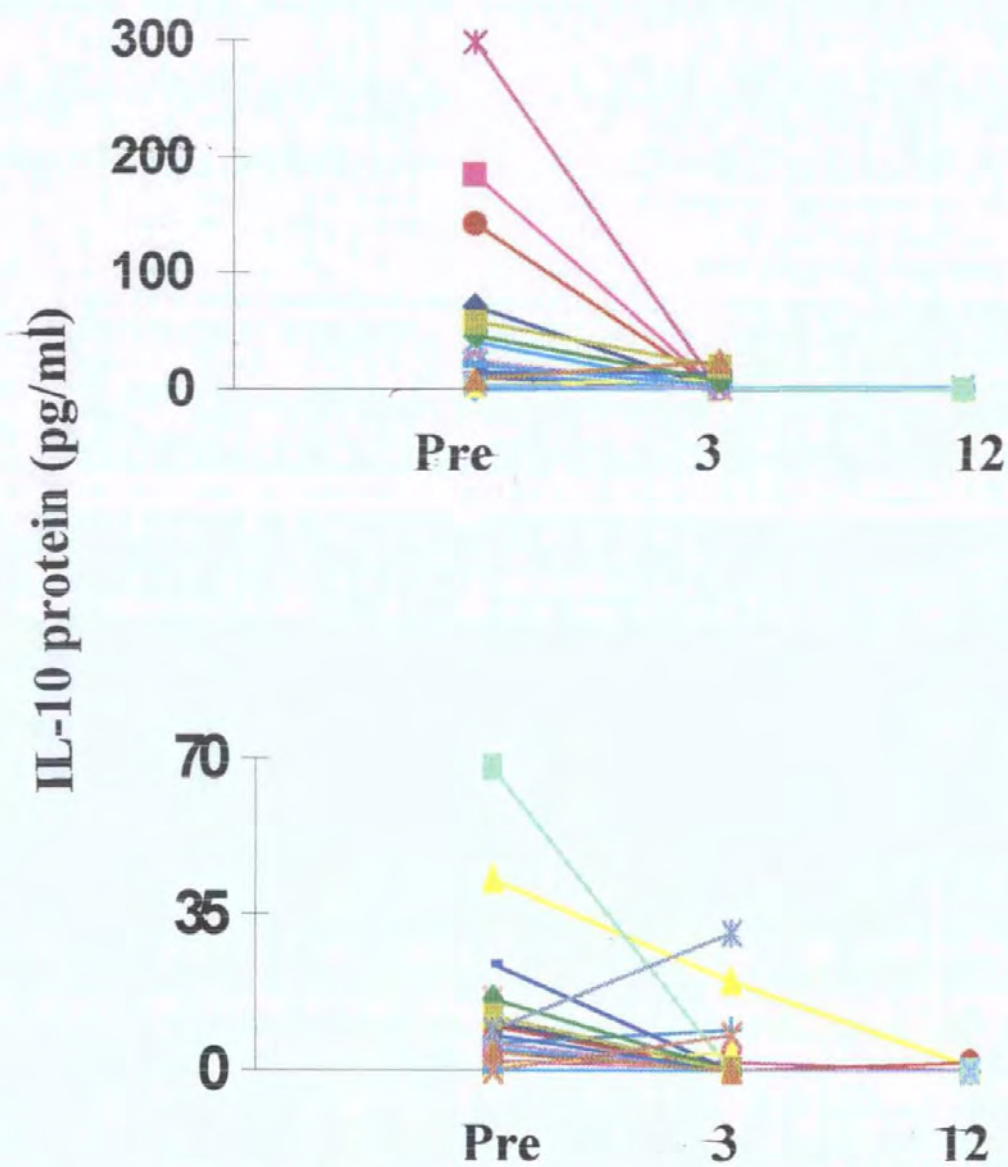
Figure 4.7. Pre- and post-transplant cytokine profiles (3 and 12 months) for IL-10 in rejector (a) and non-rejector groups (b).
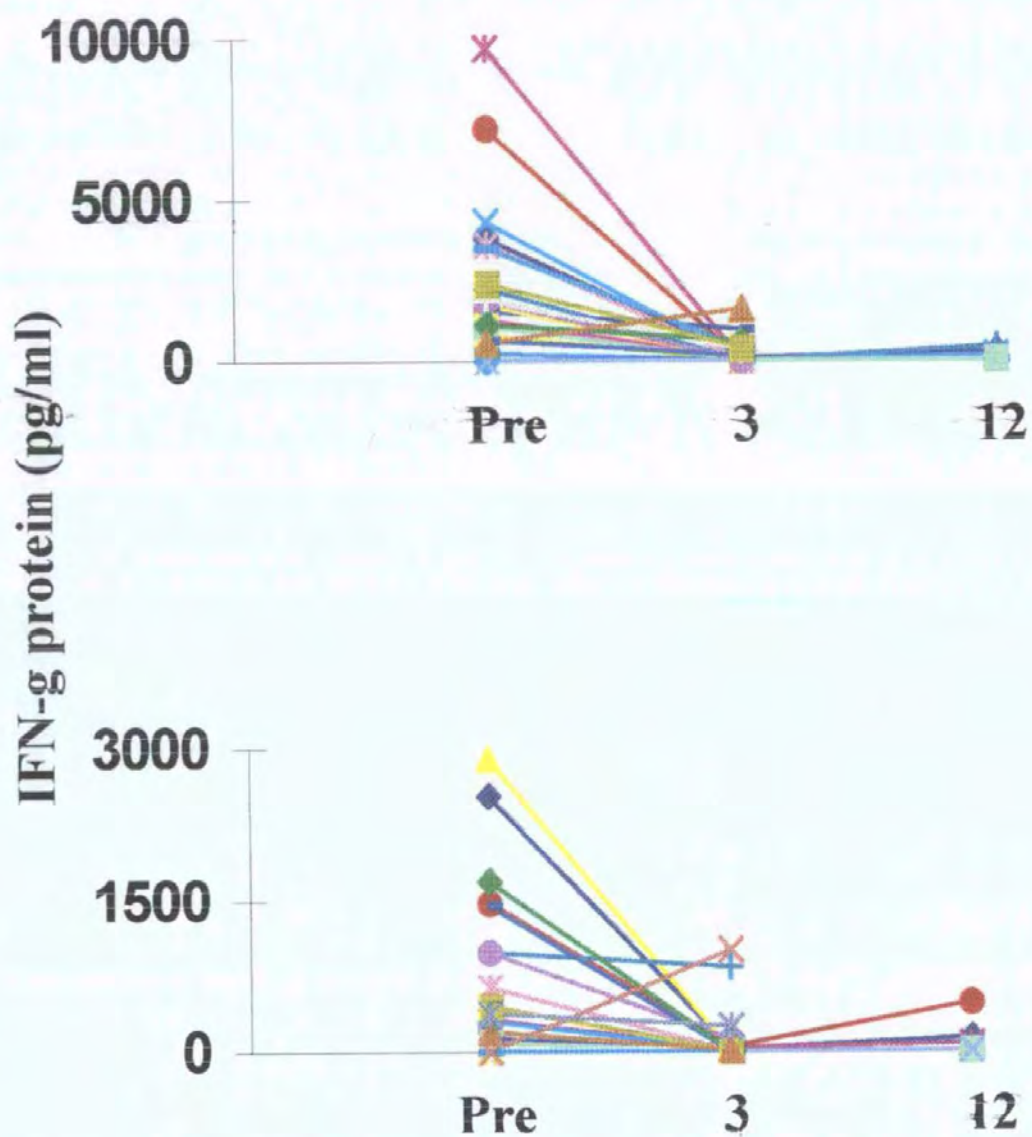
Figure 4.8. Pre- and post-transplant cytokine profiles (3 and 12 months) for IFN-gamma in rejector (a) and non-rejector groups (b).

4.12. Summary

MLRs were set up between fifty seven renal patient donor pairs and cytokine protein was measured by ELISA. Inter-individual variations were found in all cytokine profiles. Significant differences were found between rejectors and non-rejectors in spontaneous IL-4, MLR-stimulated IL-10 and MLR-stimulated IFN-γ protein secretion. Many significant correlations were found between the cytokines including MLR-stimulated IFN-γ and IL-10, MLR-stimulated IFN-γ and IL-4, and MLR-stimulated IL-4 and IL-2. A significant correlation was also found between HLA-DR mismatching and both MLR-stimulated IL-10 and IFN-γ protein secretion.

Univariate analysis was also performed to find which variables were related to incidence of rejection. Both HLA and HLA-DR mismatching were found to be associated. Female donor sex, MLR-stimulated IL-10, MLR-stimulated IFN-γ and spontaneous IL-4 protein secretion were also associated with risk of rejection. Multivariate analysis revealed that female donors, HLA mismatching and IL-10 protein secretion were the main factors associated with rejection. The highest risk of rejection occurred in patients who had both high HLA mismatching (4-6 antigen) and high IL-10 protein secretion (>10pg/ml; RR=25.5). In addition, it was generally found that cytokine protein secretion decreased following transplantation. This was found in some patients up to twelve months post-transplant.

CHAPTER 5

RELATIONSHIP OF CYTOKINE PROFILES TO CLINICAL

OUTCOME AFTER IDENTICAL SIBLING BMT

5.1. Introduction

Bone marrow transplantation (BMT) is preferentially carried out in cases where an HLA-identical sibling donor is available. GVHD may still occur in these transplants, and this is due to differences in minor histocompatibility antigens. The mixed lymphocyte reaction (MLR) has been used as a semiquantitative HLA matching test for confirming HLA class II identity between donor and recipient. Although this assay has played an important historical role in the selection of unrelated donors for BMT, it is not predictive of acute GVHD after HLA-identical sibling BMT (Lim *et al*, 1988, DeGast *et al*, 1992).

It is well known that cytokines are generated in the MLR, IL-2 and IFN-γ protein secretion and IL-2, IFN-γ and IL-5 gene expression have been detected in MLR between HLA-identical siblings (Tanaka *et al*, 1994, 1995a). However, these studies used a two-way MLR enhanced by Con A. Experiments using the skin explant assay have also demonstrated the role of the cytokines TNF-α and IFN-γ as important mediators of the *in vitro* cellular damage seen in the skin (Dickinson *et al*, 1991, 1994a, 1994b).

In view of these findings, MLRs were set up between HLA-identical sibling donor-recipient pairs and cytokine protein secretion was measured by ELISA. These pre-transplant cytokine profiles (IL-2, IL-4, IL-10 and IFN-γ) were then correlated with the severity of GVHD. IL-6 protein levels was not determined as there was not enough sample.

5.2. Patient group

Eight patients (four chronic granulocytic leukaemia (CGL), two acute lymphoblastic leukaemia (ALL), two acute myeloblastic leukaemia (AML)) who received an identical sibling transplant were used in this part of the study.

5.3. Cytokine profiles and their relevance to BMT clinical outcome

<u>5.3.1. IL-2</u>

Table 5.1 illustrates both the post-transplant clinical data and the cytokine profiles yielded. It can be seen that three of the patient-donor pairs secreted no IL-2 at all (pairs one to three). These pairs were found to have yielded a low grade (ie; 0-1) of GVHD. Four of the transplant pairs yielded between 2 and approximately 4pg/ml of IL-2 (pairs four, five, seven and eight). One of these pairs yielded the most severe grade of GVHD in the study group (grade III). Only one pair secreted around 16pg/ml of IL-2 (pair six). GVHD outcome was low risk (grade I) There appeared to be no correlation between levels of IL-2 and relative risk of GVHD. As there was an overall poor MLC response between the transplant pairs for IL-2 secretion it was decided that statistical analysis would not be performed.

<u>5.3.2. IFN-γ</u>

The MLC response for IFN-γ appeared to be even weaker than for IL-2. Only one BMT pair secreted any IFN-γ at all (pair three; 1.657pg/ml). This pair gave rise to grade 0 GVHD. With the results yielded for IFN-γ, it was again decided that analysis would not be performed.

<u>5.3.3. IL-4</u>

The results yielded for IL-4 were different to those found for both the Th1 cytokines. In all eight BMT pairs, both the controls (donor cells alone and irradiated recipient cells alone) and the MLC group gave rise to IL-4 levels exceeding 10pg/ml.

Patient-Donor Pair	GVHD Grade	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
1 Rec		0	0	13.362	0
Donor	I	0	0	14.361	0
MLC		**0**	**0**	**14.365**	**0**
2 Rec		0	0	12.488	0
Donor	0	0	0	13.496	0
MLC		**0**	**0**	**13.697**	**0**
3 Rec		0	1.011	11.335	0
Donor	0	0	0	13.026	0
MLC		**0**	**1.657**	**14.165**	**0**
4 Rec		0	0	12.892	0
Donor	0	2.660	0	14.231	0
MLC		**3.343**	**0**	**14.564**	**0**
5 Rec		0	0	12.082	0.470
Donor	III	0	0	12.161	1.124
MLC		**2.660**	**0**	**17.147**	**1.413**
6 Rec		0	0	15.650	0
Donor	I	0	0	16.467	0.324
MLC		**15.968**	**0**	**16.536**	**4.364**
7 Rec		0	0	15.172	0
Donor	II	0	0	16.604	1.557
MLC		**2.660**	**0**	**17.486**	**1.846**
8 Rec		0	0	14.143	0
Donor	0	0	0	15.582	0
MLC		**4.030**	**0**	**16.536**	**0.470**

Table 5.1. Clinical and laboratory data for HLA-identical sibling BMT pairs (Rec (Recipient) and donor data represent the background control samples).

However, in all pairs, there appeared to be little difference in IL-4 secretion between the control samples and the MLC responses and little inter-patient variation. The second highest overall response was yieded by pair five (17.147), whom gave rise to the highest grade of GVHD (III). The lowest IL-4 response gave rise to grade 0 GVHD (pair two; 13.697pg/ml). Again, due to the lack of significant response between donor and recipient, analysis was not performed.

5.3.4. IL-10

Finally, the culture supernatants were measured for IL-10 protein. Again, there was an overall poor response by the BMT pairs. Four of the eight donor pairs yielded no IL-10 cytokine protein at all (pairs one to four), all of which were 0 or low grade (I) GVHD . Three of the transplant pairs secreted between 0 and 2pg/ml (pairs five (grade III), seven (grade II) and eight (grade 0)). One pair (pair six) yielded in the excess of 4pg/ml of IL-10 protein (grade I). From the results shown, there appeared to be no advantage in analysing this data statistically.

5.4. Summary

The MLC was used to determine pre-transplant cytokine profiles (IL-2, IFN-γ, IL-4 and IL-10) in BMT HLA-identical sibling pairs. MLCs were set up and culture supernatants harvested at optimised time points for the cytokines in question. It was then hoped that statistical analysis could be performed to determine if the cytokine profiles correlated with acute GVHD.

With all four cytokines, there appeared to be a lack of response by the MLC, with the lowest response being seen for IFN-γ cytokine protein. Although there appeared to be a better response for IL-4 protein, there was little difference between the background control and MLC samples and inter-patient variation. It was decided

that analysis would not be performed to ascertain the relationship between the cytokine profiles and GVHD outcome as there was low responses for all four cytokines, and it was therefore felt that application of statistical analysis would not be informative.

CHAPTER 6

DISCUSSION

6.1. Optimisation and analysis of cytokine profiles in normal individuals

6.1.1. Introduction

Th1/Th2 polarisation is thought to be associated with allograft rejection and GVHD. In view of the fact that cytokines are secreted during mixed lymphocyte reaction (MLR) and mitogen stimulation, the aim of this project was to develop an *in vitro* functional assay for the prediction of acute (and chronic) rejection in renal transplantation. In the first instance, studies were performed to optimise and analyse cytokine profiles in normal individuals.

6.1.2. Optimisation of culture conditions

6.1.2.1. Mixed lymphocyte culture

Previous studies have shown several cytokines to be produced during an MLC response (Bishara et al, 1991, Toungouz et al, 1993, 1994, 1996). However, for the purposes of this study, it was considered important to optimise the conditions for the MLC in normal individuals. Firstly the kinetics of protein secretion was determined for cytokines IL-2, IL-4, IL-10 and IFN-γ (IL-6 at a later point) by ELISA. It was found that there was a variation in time for optimal secretion between each cytokine, which confirmed the importance of these experiments. These incubation times were then used in future experiments. Other experiments have also shown a difference in time points for cytokine secretion (Kohka *et al*, 1999) in which IFN-γ, IL-6 and Il-10 were included. It was observed that first detection of these cytokines was at twelve hours, with a marked increase thereafter. Secretion of IL-12 was not detected until forty eight hours. The levels of IFN-γ were downregulated with the addition of anti-IL-18 and anti-IL-12 antibodies, suggesting that the secretion of IFN-γ may be dependent on IL-18 in the early phase of the MLR, and dependent on both IL-18 and IL-12 in the late phase. This may well be the case for

the experiments performed in this project. Optimisation of cytokine protein and receptor expression in MLC was also performed in a similar manner by flow cytometry. This part of the project failed due to the incapability of gating a clear lymphocyte region within the PBMC population. As the stimulator cells were treated for this culture, there may have been an increased level of background debris, therefore making it more difficult to determine live from dead or dying lymphocytes. This did appear to become more apparent as the culture incubation time increased. In retrospect, a viability stain (eg; propidium iodide) may have been a way in which the dead or dying cells could have been designated from the live cells. If the cells are positive for propidium iodide, then the cells are dead. Knowing the approximate position of the lymphocytes on the screen, the live lymphocytes may then be gated (propidium iodide negative). However, the failure of detection of cytokine expression in the MLC was not a surprise as the number of alloreactive cells, and therefore cytokine expression were expected to be below the limit of detection of the flow cytometer (sensitivity=1%).

To determine the response of only the responder (or recipient) cells in this culture, the stimulator (or donor) cells had to be treated in some way in order to render them unresponsive. This culture, as previously mentioned, is termed a one way MLR or one way MLC. This may be performed by chemical treatment, using mitomycin C, or gamma irradiation. There is a standardised technique using the irradiation method (Dupont et al, 1980), although this is performed for proliferation measurement in the MLC. For the purposes of this study, I wanted to confirm the most suitable method of treatment for the measurement of cytokines in the MLR. Both irradiation and mitomycin C experiments were performed, using titrated doses in order to determine the cut-off points of cytokine secretion. In the irradiation experiments, there was a clear cut-off point at 20-30 Gy. However, for mitomycin C,

the cut-off point could not be determined as the cytokine secretion pattern was very sporadic. It is unclear as to the reason for this result, but from these experiments it was concluded that the irradiation technique was the most suitable method.

Both gamma irradiation and mitomycin C treatment of stimulator cells have been used in previous experiments in order to achieve unidirectionality of response in the MLR (Leenaerts *et al*, 1992, Malinowski *et al*, 1992). Both irradiation and mitomycin C treatment are known to block lymphocyte replication and downregulate HLA expression. As in the experiments performed in this project, it has been shown that gamma irradiation has a more profound effect on nulling stimulator cells in the MLR than mitomycin C (Malinowski *et al*, 1992).

6.1.2.2. Mitogen stimulated cultures

Experiments have been performed to attempt to enumerate cytokine profiles at both the protein (Hutchings et al, 1989, Mossman and Fong, 1989) and expression level (Sander et al, 1991, Jung et al, 1993). However, different mitogens may have different effects on cytokine profiles. Jason and Larned (1997) and Katial et al (1998) both demonstrated a broad spectrum of cytokine levels in normal individuals, varying with the mitogen used. PHA and OKT3 mitogens were used for the stimulation of protein secretion while PMA was used for protein expression studies. OKT3 had previously been optimised (Kaminski et al, 1995), therefore experiments were only perfomed for the other two mitogens. For the four cytokines of interest (IL-2, IL-4, IL-10 and IFN-γ), forty eight hours appeared to be the optimal incubation time for peak protein secretion. Optimisation of cytokine expression using PMA stimulation had previously been performed (Prussin and Metcalfe, 1995), and therefore these conditions were used. These experiments were repeated and confirmed. In addition, Mascher *et al* (1999) also studied the expression of these cytokines, together with

TNF-α. This study found that peak cytokine expression for IL-2 and IFN-γ was between six and eight hours. Although IFN-γ remained stable for twenty four hours, IL-2 declined markedly after eight hours. Interestingly, in optimisation experiments performed in this project, IL-4 and IL-10 were found to be below detectable limits. This may be due to both of these cytokines being expressed and secreted at much lower levels than IFN-γ and IL-2, or may be used up much more quickly in both an autocrine and paracrine fashion. Like all assays, flow cytometry has a baseline of positivity, and both IL-4 and IL-10 were found to be below this baseline (1%).

In an attempt to improve the detection of both of these cytokines, increased amounts of conjugated anti-cytokine antibodies were used. This only increased the background, and not the detection of intracellular cytokine. This also occurred when the length of permeabilisation incubation time was increased together with a decrease in blocking incubation time (to allow more anti-cytokine antibody to bind, and therefore increase the sensitivity). Therefore, amendments to the protocol did not seem to improve the detection of either IL-4 or IL-10 cytokine expression. In contrast, other studies have claimed that IL-4 (Andersson et al, 1990) and IL-10 together with IL-4 (Rostaing et al, 1999b) can be detected. However, as with this study, the Th2 cytokines (both IL-4 and IL-10) were found to be detected at lower levels than cytokines such as IL-2 and IFN-γ (Rostaing et al, 1999b).

6.1.3. Inter-individual variation in cytokine profiles in normal individuals

Frequencies of various cytokines have been suggested to vary (Merville et al, 1993a). Little is known about the variance of both cytokine secretion and frequencies of cytokine expression between individuals and what factors may have an influence on these levels. Mitogen stimulation of lymphocytes has demonstrated large inter-

individual variations in IL-2, IL-4 and IFN-γ cytokine protein secretion by ELISA (Kaminski et al, 1995) and numbers of IL-2 and IFN-γ cytokine expressing cells by flow cytometry (Ferry et al, 1997). In this project, a group of normal individuals were randomly chosen and PHA and OKT3-stimulated cytokine protein by ELISA (IL-2, IL-4, IL-10 and IFN-γ) and PMA-stimulated cytokine expression by flow cytometry (IL-2 and IFN-γ) measured. Large inter-individual variations were also found in both PHA and OKT3-stimulated protein secretion and PMA-stimulated expression, confirming these previous findings. Different levels of cytokine protein were secreted between OKT3 and PHA, confirming that different mitogens elicit different effects on cytokine production (Imada et al, 1994). Interestingly, there appeared to be a bi-modal distribution for PHA-stimulated IFN-γ protein secretion. It may be concluded from these results that a low, medium and high secretor status does exist in cytokine profiling. Although these experiments were performed using mitogen stimulation, this may also be seen in the MLR (Chapter 4), and therefore may play an important role in the determination of whether these inter-individual variabilities may determine clinical outcome. Use of other mitogens in mononuclear cell cultures, including Concanavilin A (ConA) and lipopolysaccharide (LPS) have also illustrated significant inter-individual variations in cytokine production, which is unaffected by the type of stimulus used or by time (Yaqoob *et al*, 1999).

6.1.4. Contribution to cytokine production by CD4+ and CD8+ T cells and NK cells

Additional experiments were also performed to determine the contribution of CD4+ and CD8+ T cells to cytokine expression. In conformation of previous findings (Prussin and Metcalfe, 1995, North et al, 1996), this study demonstrated predominant expression of IL-2 by CD4+ T cells illustrating that IL-2, on activation

of mainly T helper cells, is expressed (and secreted) as part of the cytokine cascade, and stimulates proliferation in an autocrine fashion, which leads to the paracrine activation of CD8+ T cells. IFN-γ.is also believed to activate CTLs, but is also suggested to be associated with the recruitment of macrophages to the site of immune response (Nickerson *et al*, 1994). IFN-γ expression, as with this study, has also been shown to be primarily expressed by CD8+ T cells (Sewell *et al*, 1997), although in this project, the increased expression was negligible. Experiments were also performed to study cytokine co-expression in T cell subsets. Results showed there to be low co-expression of both cytokines, sugesting that upon activation, CD4+ and CD8+ T cells produce a preponderance of either IL-2 or IFN-γ. Therefore, although there is a difference in the predominant cell subtype between these two cytokines, they are both known to play an equally important role in the cytokine cascade and the pro-inflammatory immune response. This is in accordance with previous data (Caruso *et al*, 1998), where a high percentage of cells expressing either IL-2 or IFN-γ was observed, but not with double expression. NK cells were found to express extremely low levels of IL-2 and IFN-γ. IFN-γ has been shown to be one of the cytokines produced by NK cells on activation (Perussia, 1996) and cross-linking of molecules such as CD45 (Shen *et al*, 1995) and NKR-P1 (Arase *et al*, 1996). The use of PMA stimulation was therefore not sufficient to observe cytokine expression, although this did illustrate that NK cells did not contribute to IL-2 and IFN-γ expression in these experiments.

6.1.5. Relationships between Th1 and Th2 cytokine profiles

Although it has been previously demonstrated that inter-individual differences do occur in both cytokine protein secretion and expression, little is

known about the factors responsible for the production of cytokine protein. Recently, there has been evidence to demonstrate that genetic polymorphisms may determine the amount of cytokine protein secreted (Daser *et al*, 1996) and that particular cytokine genes are associated with high/low secretor status (Turner *et al*, 1995, 1997). Analysis was therefore performed to determine the relationship between cytokine protein and secretion (for IL-2 and IFN-γ), between Th1 and Th2 cytokine protein secretion, and due to the bi-modal nature of the IFN-γ protein secretion profile, between IFN-γ protein, IFN-γ cytokine expression and IFN-γ gene polymorphisms (discussed later). The analysis illustrated a positive correlation between IL-2 protein secretion and frequency, suggesting that the number of IL-2 secreting cells determined the amount of IL-2 protein secreted. However, there did appear to be two individuals who could be classed as outliers. Although these individuals did not affect the analysis, it would have been of interest to observe if these points were included in a second population cluster. The study group was not extended due to limited time (see section 6.1.7).

Strong positive relationships were found between IFN-γ/IL-2 and IL-10 PHA-stimulated protein secretion. This result was surprising as an inverse correlation was expected between Th1 and Th2 cytokines. OKT3 stimulation demonstrated no relationships between any of the cytokine profiles. The use of mitogen stimulation may be regarded as rather synthetic compared to activation in culture conditions such as the MLC. The MLC may be regarded as a more true reflection of the *in vivo* immune response than mitogen stimulation. The fact that absent or unexpected relationships were observed between cytokine protein secretion patterns in particular may be due to this. Mitogen compounds tend to push the cell to its' maximum stimulatory capacity, and therefore, as previously stated, does not give a true reflection of the *in vivo* immune response, although does give an idea of what may

occur when lymphocytes are activated in different individuals. Inter-individual variation has been shown to exist in normal individuals, and this may have clinical relevance in cytokine profiling and clinical outcome in transplant patients.

6.1.6. Relationship between IFN-G gene polymorphisms, protein secretion and frequency

It has been shown that even a single base change in the DNA sequence can greatly increase or decrease the binding of a particular transcription factor (Wilson *et al*, 1992, Bailly *et al*, 1993). In this instance, the frequency of polymorphic dinucleotide repeats (CA) of the microsatellite region in the IFN-γ gene was studied. Unlike IL-2, no relationship was found between IFN-γ protein secretion and expression. This would therefore suggest that the number of IFN-γ expressing cells does not determine the amount of IFN-γ secreted. It is unsure as to why there is a relationship between expression and protein secretion with IL-2 and not IFN-γ. This may be due to that different IFN-γ expressing cells may vary in IFN-γ protein production, which is obviously less random than with IL-2. However, this finding does suggest that another factor may influence IFN-γ protein production. Therefore, IFN-γ gene polymorphisms were studied. No relationship was found between IFN-γ expression, protein production and gene polymorphisms. This finding was in contrast to Pravica *et al* (1999). In this study, the frequency of the IFN-γ CA repeat polymorphism was investigated in normal volunteers, together with the production of IFN-γ in the supernatants of PHA stimulated PBMCs isolated from the same individuals. They found that there was a correlation between IFN-γ genotype and *in vitro* IFN-γ production. It was unclear as to why no correlation between IFN-γ genotype and protein production was found in this study. One explanation could be

that either another polymorphism in the IFN-γ gene or even in another gene may be responsible for determining IFN-γ protein production. However, this would have to investigated.

6.1.7. Summary of statistical analysis

The aim of the analysis studying the relationships between protein secretion and expression was to observe whether the number of cytokine expressing cells determined the amount of protein secreted. The reason for analysing the relationships between Th1 and Th2 cytokine protein secretion profiles was to assess if there was an inverse correlation. This was expected due to the understanding that Th1 and Th2 cytokines regulate each other. It may be argued that if enough multiple comparisons are analysed, then a relationship will eventually be found. However, this not the case for this analysis, as when relationships were found eg; IL-2 protein and frequency, the level of significance was strong. Cytokine protein secretion data were catergorised as low-medium and high. For the majority of the cytokines, the group was split into three subgroups to obtain arbitary ranges for low, medium and high secretion. However, for cytokines such as PHA-stimulated IL-2 and OKT3 stimulated IFN-γ and IL-4, this was not so simple, as there were clear outliers within the data distribution. Therefore, as the outliers were included in the data analysis (see below), the majority of the individuals were classed as low-medium secretors.

Each of the sets of data were then tested for normality. Pearsons correlation analysis was performed on the data conforming to a normal distribution. Most of the data did not conform to a normal distribution, therefore Spearmans Rank analysis was used. When the spread of data was plotted, there were outliers (eg; PHA stimulated IL-2, OKT3 stimulated IFN-γ and IL-2). In normal circumstances, these outliers would be eliminated as they may skew the analysis. However, this was not

done with this study group as it was thought that they may have a biological significance in the variation of cytokine production. For all analysis, p values were corrected, but this did not affect the level (or lack) of significance. If time had permitted, the number of normal individuals would have been increased to ascertain whether a more normal overall distribution of protein secretion was present within the group. This would have been of particular interest with PHA-stimulated IFN-γ as a bi-modal distribution was observed.

6.2. Cytokine protein secretion in the MLR - predictive value for renal allograft rejection

6.2.1 Introduction

The MLR has been shown to be relatively imprecise in the prediction of GVHD in bone marrow transplantation (Lim et al, 1988, DeGast et al, 1992, Hows et al, 1986, Mickelson et al, 1994) or rejection in renal transplantation (Cullen et al, 1977, Burke et al, 1993, Steinmann et al, 1994). Although some studies have shown the MLR to predict rejection following renal transplantation (Harmon *et al*, 1982, Langhoff *et al*, 1985), it is now accepted that the standard MLR is of limited value. Based on this previous work, it was decided that that there was a need for a simple *in vitro* functional assay for determining clinical outcome in renal transplantation. I therefore set out to study the role of cytokine profiles and other clinical factors in the prediction of allograft rejection in renal transplantation.

6.2.2. HLA and HLA-DR mismatching and cytokine profiles

Cytokine protein data was compared with degree of HLA and HLA-DR mismatching using Kruskal-Wallis testing. Interestingly, there appeared to be no significant association between total HLA mismatching and any of the MLR

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cytokine profiles. There was a significant correlation however between HLA-DR mismatching and MLR-stimulated IL-10, and between HLA-DR mismatching and MLR-stimulated IFN-γ. No correlations were found between either total HLA or HLA-DR mismatching with any spontaneous cytokine secretion. This would suggest that alloactivation of lymphocytes in the MLR, when there is a higher degree of HLA-DR mismatching, induces higher secretion of both IL-10 and IFN-γ, and that these cytokines were associated as independent variables. Previous work has shown that measurement of cytokine secretion can detect both mismatches (Danzer *et al*, 1994) and polymorphisms in HLA genes (Toungouz *et al*, 1994). In the work by Danzer *et al* (1994), it was shown that cytokine determination in the MLR is suitable to detect identical alleles of both HLA-DRB1 and HLA-DQB1. It was shown that HLA-DP has no stimulatory effect on cytokine release and also suggested that HLA-DR is more effective in inducing IFN-γ secretion than HLA-DQ. This data would therefore explain as to why there is a positive correlation between HLA-DR mismatching and IFN-γ secretion. Toungouz *et al* (1994) also showed that HLA-DR mismatches may increase cytokine production in the MLR. However, in contrast, they found one of these cytokines to be IL-6. Later work by the same group also showed that an increase in HLA-DR mismatching (increase from one to two HLA-DR mismatches) led to an increase not only in IFN-γ, but also TNF-α production in the MLR (Toungouz *et al*, 1996). They concluded that a decrease in HLA-DR mismatching (ie: one mismatch) led to optimal IL-10 production, which in turn exerted efficient inhibition of the Th1 cytokines produced in the MLR.

IL-10 has been suggested to be associated with Th2 cells and therefore with tolerance in kidney transplantation (Romagnani, 1991). The finding that IL-10 is associated with HLA-DR mismatching, and that this mismatching is strongly

associated with rejection would suggest that IL-10 may be also associated with the rejection process. IL-10 has also been proposed to have the role of down-regulating both Th1 and Th2 subpopulations (Del Prete *et al*, 1993). Recent work has shown an association with HLA-DR mismatching and IL-10 secretion (Sankaran *et al*, 1999). This research also found an association with allograft rejection (discussed later). The relationship between IFN-γ, IL-10 and incidence of rejection will be discussed later.

6.2.3. Inter-individual variations in cytokine profiles

It was previously established (chapter 3) that inter-individual variations were present when normal individuals were stimulated with mitogen. However, it was also of interest to observe whether variations occurred in the MLR response. Cytokine protein data was gathered and the patient study group was divided into rejector (R) and non-rejector (NR) groups. As the cytokine protein data did not conform to a normal distribution, the medians of each group were compared instead of the means. The analysis was only performed for pre-transplant data. For all five cytokines (IL-2, IL-4, IL-6, IL-10 and IFN-γ), there did appear to be inter-individual variations in both rejectors and non-rejectors in the MLR response. Previous work also demonstrated similar variations, albeit in mitogen-stimulated cultures (Kaminski *et al*, 1995, Cartwright *et al*, 1999). However, the variations found in the MLR response were not as clear for spontaneous/background secretion. At present, there is no data to support variations in cytokine excretor levels *in vivo*. This would prove to be quite difficult to study as cytokines are suggested to have a relatively short half-life *in vivo*, and therefore would not accurately be able to be measured. Therefore, cytokines are only measured *in vitro*. Results also showed there to be no significant difference between rejectors and non-rejectors for IL-2, IL-4 or IL-6 secretion. However, a strong significant difference was seen for IL-10 and IFN-γ secretion in

the MLR response. This is in accordance with Kaminski *et al* (1995) where not only were inter-individual variations were seen in both rejectors and non-rejectors, but that the only significant difference that was found out of IL-2, IL-4 and IFN-γ secretion was IFN-γ. However, this study did not look at either IL-6 or IL-10 and the method of stimulation used was OKT3 mitogen. The important observation of inter-individual variability has also been shown by Turner *et al* (1997). It was suggested that there can be a 3-20 fold difference between the highest and the lowest cytokine production upon *in vitro* stimulation. No significant differences were found for any of the cytokines between the two groups in spontaneous cytokine release. The data found in this study indicates that not only are there are variations in cytokine protein secretion in response to alloactivation, but also that there are smaller yet present spontaneous inter-individual variations. These variations may be due to differing individual abilities to spontaneously secrete particular levels of cytokines, leading to the possibility of the involvement of cytokine gene polymorphisms.

6.2.4. Th1 versus Th2 cytokines

In the group of normal individuals (chapter 3), I wished to determine whether any relationships existed between Th1 and Th2 cytokines. As cytokine secretion was seen spontaneously as well as in the MLC response, analysis was also performed for this data. As previously mentioned, as the data did not conform to a normal distribution, the data had to be ranked and Spearmans rank correlation analysis performed. Using this analysis, there appeared to be quite a few correlations. Of major interest was the positive correlation between IFN-γ and IL-10. IFN-γ secreted in the MLR correlated with both spontaneously secreted and MLR-stimulated IL-10. This was of particular interest as a correlation was found between these two cytokines in mitogen stimulated cultures (see chapter 3). There are other publications

which have shown synergistic production of both IL-10 and IFN-γ in conditions other than renal allograft rejection, including heart allograft rejection (Van Hoffen *et al*, 1996) and even malaria (Wenisch *et al*, 1995), and therefore suggests that that stimulatory and inhibitory cytokines for macrophage activation and/or antibody production (ie; Th1 and Th2 immunoreaction respectively). There were also very strong significant correlations between MLR-stimulated IFN-γ and MLR-stimulated IL-4, and MLR-stimulated IL-2 and MLR-stimulated IL-4. There were no inverse relationships between any of the cytokines.

During an MLR, it is known that the cells undergo DNA synthesis, blast transformation and proliferation. During this reaction, as in allograft rejection, cytokines are also produced. The Th1/Th2 paradigm has been implicated in playing a role in transplantation (Soulillou, 1993) and that Th1 and Th2 derived cytokines may even regulate each other (Nickerson *et al* 1994, 1997). The positive correlations found between both Th1 and Th2 cytokines suggest that production of Th1 cytokines such as IFN-γ do not suppress Th2 cytokine production such as IL-4. As with mitogen stimulation (Katial *et al*, 1998), it appears that alloactivation in the MLR leads to a global up-regulation of cytokine synthesis, and that Th1/Th2 regulation may be a little more complex than originally thought.

6.2.5. Effect of HLA and HLA-DR mismatching on incidence of rejection

The first part of the analysis to be performed was to establish which relevant factors play a role in clinical outcome involved analysing the relationship between HLA mismatching and risk of rejection. Univariate analysis was performed using the χ^2 test. This analysis showed there to be not only a relationship between increase in total HLA mismatching and risk of renal allograft rejection but also increase in HLA-DR mismatching and risk of rejection, both as independent variables. These findings

are in accordance with Beckingham *et al* (1994) who also found lower rejection rates with better HLA matching. Reviron *et al* (1996) also found that better HLA-DR matching reduced the risk of acute rejection after kidney transplantation, and even found that generic (ie; more specific) HLA-DR matching improved the risk of rejection even more than broad HLA-DR antigen matching. This is supported by an earlier study by Al-Daccak *et al* (1990) who found that a higher response was seen in the MLR with HLA-DR3 mismatches compared to some of the other HLA-DR haplotypes, suggesting that allorecognition does not necessarily have to be the recognition of the whole HLA molecule, but possibly a processed HLA molecule presented as a nominal antigen. More recent studies have also illustrated the benefits of HLA-DR matching (Reisaeter *et al*, 1998, McKenna *et al*, 1998). McKenna *et al* (1998) studied one hundred and five renal transplant patients, and it was found that poor HLA-DR matching was associated with an increase in both early (0-3 months post-transplant) and late (4-6 months post-transplant) rejection episodes. It was also found that poor HLA-A and B matching was only associated with late rejection episodes. However, in this study, we did not analyse HLA-A and -B mismatching. Reisaeter *et al* (1998) looked at a larger cohort of patients (655), and not only found an increase in rejection episodes with increased HLA-DR mismatches, but also worse allograft survival. In heart transplantation, it has been suggested that HLA-DR incompatibility may even predict rejection (Costanzo-Nordin *et al*, 1993). It has also been thought that HLA-DR matching is primarily more important than other HLA antigens. This is due to the hypothesis that HLA-DR may stimulate more alloreactive cells than mismatches at HLA-A or HLA-B, for instance, and therefore HLA-DR mismatches lead to higher risks of rejection. This would explain why this project and many other studies have found HLA-DR to be strongly associated with rejection. Therefore, it may be suggested that although overall HLA matching was found to

have a significant association with risk of rejection, it may have been influenced by the independently strong association between HLA-DR and rejection.

Much attention has been focused on the need for HLA-DR matching in transplantation, in particular HLA-DRB1 since the use of molecular HLA typing (Kobayashi *et al*, 1992, Nojima *et al*, 1996, Lardy *et al*, 1997). The method of PCR-RFLP analysis allows the analysis of the genotype for HLA-DRB1 alleles by digestion using restriction endonucleases in order to determine the "splits" at the DRB1 gene level. In each of these experiments, an increase in HLA-DRB1 mismatches were significantly associated with higher risk of graft rejection. These findings thus confirm the benefits of HLA genotyping for HLA-DRB1 in addition to serological typing for HLA-DR. It is thought that mismatches for HLA-DRB1 play a role in allograft rejection due to the mismatches localised in the beta-pleated sheet of the DRB1 molecule (Adorno *et al*, 1999a, 1999b).

6.2.6. Effect of sex and age on incidence of rejection

Univariate analysis was again performed to assess whether either patient or donor sex/age were related to incidence of rejection. Both donor and patient age were not associated with rejection. Patient sex was also not significantly associated. However, donor sex appeared to be independently strongly associated with the incidence of rejection (p value=0.009). It is well established that transplantation of male tissue into female recipients may lead to graft rejection in organ transplantation (and GVHD in bone marrow transplantation) due to the role of the male-specific minor histocompatibility antigen H-Y (Scott *et al*, 1997, Simpson *et al*, 1997). The recent identification of two peptides that in association with the mouse H-2K or human HLA-B7 MHC class I molecules, are recognised by H-Y specific T cells. The human peptide has been identified as a derivative from SMCY, an evolutionary

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conserved protein encoded on the Y chromosome (Wang *et al*, 1995). It was therefore a surprise to find that female donors led to a higher risk of rejection. This finding however is in accordance with Vereerstraeten *et al* (1999). This study was not only looking at incidence of rejection but also rate of graft failure due to rejection and technical failure. The poorest graft outcomes were kidneys from female donors to male recipients. In contrast to this finding was earlier data from Kawauchi *et al* (1993) where male donor into female recipients increased the risk of allograft rejection. However, this finding was not a direct comparison as this study included heart transplants in children. This particular finding was surprising, but it even may be due to the cohort of patients chosen, although this is unlikely. The nature of why female donors were a higher risk than male donors remains inconclusive.

6.2.7. CMV status and incidence of rejection

The study of transplant patients with CMV infection and its effect on incidence of graft rejection has led to a mixture of findings. Here, it was of interest to analyse whether either the donor or recipient CMV status had any bearing on the risk of rejection. Results showed that neither the patient CMV or donor CMV status was significantly correlated to rejection. An association between either acute or chronic rejection and CMV infection has been reported in renal transplantation (Yeung *et al*, 1998, Kashyap *et al*, 1999) but mostly in liver transplantation (Lautenschlager *et al*, 1997, Rosen *et al*, 1998). CMV has been shown to have been detected in renal allograft biopsies by PCR (Chen *et al*, 1992, Lee *et al*, 1992, Cunningham *et al*, 1995). Cytomegaloviral inclusions have also been detected by histological methods (Kashyap *et al*, 1999). The role of CMV infection in allograft rejection has been widely studied, and has been suggested to be involved in the induction of smooth muscle proliferation and intimal thickening (Borchers *et al*, 1999) and endothelial

cell damage (Toyoda *et al*, 1997). The finding that CMV status was not associated with risk of rejection in this study was surprising, although there has been previous suggestion that risk of rejection is associated with manifestation of CMV disease and not CMV infection alone (Bouedjoro *et al*, 1999).

6.2.8. Cytokine protein and rejection

Analysis was also performed to establish whether there were any significant associations between any of the cytokine proteins (both background and MLC) with risk of rejection. For the purpose of the analysis, median cut-off points had to be determined in order to produce a low and high secretor group (50:50). The results showed there to be a significant association between IL-4 background secretion and incidence of rejection (p value=0.005). This finding was surprising as Th2 cytokines such as IL-4 have been linked with allograft tolerance (Dallman *et al*, 1993, Nickerson *et al*, 1997). In addition, both MLR-IL-10 and IFN-γ were significantly correlated with rejection (p values=0.009 and 0.003 respectively).

There has been a lot of work that suggests that during transplant rejection, an array of soluble mediators or cytokines are secreted in response to the recognition of foreign antigens on the graft by activated T cells, and depending on which cytokines are produced, different clinical outcomes may occur. As previously described, the "Th1/Th2 paradigm" is said to play a role. T helper 1 (Th1) cells are said to secrete cytokines such as IFN-γ, IL-2 and IL-6, where these cytokines are suggested to be highly associated with transplant rejection. On the other hand, Th2 cells secrete cytokines including IL-4 and IL-10 and are proposed to be associated with tolerance (Mosmann and Coffman, 1989, Romagnani, 1991). These polarised Th1/Th2 cytokines are also said to regulate each other (Nickerson *et al*, 1994, Dallman, 1995), and that even the prevention of Th1 responses is critical for tolerance (Chen *et al*,

1996). However, more recently, it has become apparent that the Th1/Th2 model, while providing a valuable framework for models such as transplant rejection, has been oversimplified (Kelso, 1995, Mosmann and Sad, 1996, Zhai *et al*, 1999). As suggested by these three groups, this paradigm may not be sufficient to explain the recently demonstrated *in vivo* effects of cytokine manipulation on allograft rejection and survival.

Our results suggest that both IFN-γ and IL-10 are associated with incidence of rejection along with spontaneous IL-4 secretion. Th1 cytokines such as IFN-γ have been strongly associated with transplant rejection (Merville *et al*, 1993a, 1993b, 1995, D'Elios and Del Prete, 1998 (review), Oliveira *et al*, 1998, Heeger *et al*, 1999) and therefore this finding was expected. Merville *et al* (1993a) studied cytokine secretion (IFN-γ, IL-2, IL-4, IL-6 and IL-10) in rejecting human kidney allografts. The results showed spontaneous secretion of IFN-γ, IL-6 and IL-10 with induction of IFN-γ by IL-2 in rejecting allografts. IL-4 was found to be inhibitory. Although this study implicated both IFN-γ and IL-10 in allograft rejection, the study focused on spontaneous secretion, of which we found IL-4 to be of importance. As previously mentioned, the same group, in a later study (Merville *et al*, 1995) found high secretion of both IFN-γ and IL-10 in activated cells in rejected kidney allografts, supporting the results found in this project. Oliveira *et al* (1998) analysed fifty six adult cadaver renal transplants by fine needle aspiration biopsy and found a high proportion of acute rejection patients to have a high percentage of Th1 cytokine infiltration (IFN-γ). Heeger *et al* (1999) found that pre-transplant frequencies of donor-specific IFN-γ producing lymphocytes correlated with the risk of post-transplant rejection episodes. The fact that IFN-γ has been found to not only be associated with rejection in this study, but also in previous data, would allow the

suggestion that IFN-γ may well contribute, together with other factors, to the occurrence, and possibly even to the progression of allograft rejection.

Findings such as in the work of Joseph *et al* (1995) found using PCR that IL-10 was expressed in rejecting allografts while IFN-γ was absent. Xu *et al* (1995) demonstrated that IL-10 mRNA (together with IL-2 mRNA) significantly correlated with acute renal allograft rejection. These studies differ from the one here as they were looking at cytokine gene expression and not protein secretion. In addition, Lang *et al* (1996) measured levels of IL-2, IL-4, IL-10 and IFN-γ in post-transplant serum and bile samples in liver transplant recipients. Significant elevations of both IL-4 and IL-10 were found at the site of rejection, with increased circulatory levels of IL-2, IL-4 and IFN-γ. Weimer *et al* (1996) investigated the association of acute kidney graft rejection with factors including pre-transplant T helper/suppressor activity and *in vitro* cytokine profiles. The results showed that pre-transplant CD4 helper defects and low IL-10 responses predicted a low risk of graft rejection. Strehlau *et al* (1997) found that during acute renal allograft rejection, intragraft expression of cytokines, including IL-10, are significantly heightened. The finding that spontaneous IL-6 or MLR-stimulated IL-6 secretion did not appear to be associated with rejection was unusual. Kaminska *et al* (1996) studied baseline IL-6 serum and urine levels in rejected kidney allografts and found a significant positive association. Recent data presented by Marshall *et al* (British Transplantation Society Annual Congress, 1999) demonstrated a significant association between IL-6 low secretor polymorphism in the donor kidney and rejection. However, in the MLRs set up in this thesis project, IL-6 measured was that secreted by the recipient. Therefore the role of IL-6 in rejection is still not clearly understood, and further work needs to be carried out.

Cytokine secretion has also been studied in heart transplantation. Grant *et al* (1996) looked at serum cytokine secretion in twenty eight cardiac transplant

recipients. It was found that there was no systemic relationship between cytokine levels and histological rejection in cardiac transplant recipients. This study again slightly differed to this project as the MLR was not used as a stimulation model for cytokine secretion. The MLR itself is currently considered as an unreliable assay for the prediction of rejection as there is evidence for (Langhoff *et al*, 1985) and against (Cullen *et al*, 1977, Steinmann *et al*, 1994) the predictive nature of this assay. However, in BMT, a modification of the MLR (skin explant assay) has shown not only to demonstrate a direct involvement of cytokines (IFN-γ and TNF-α) in GVHD (Dickinson *et al*, 1991) but also as a predictive method for GVHD in identical sibling pair BMT (Dickinson *et al*, 1994b), thus suggesting that this modified cytokine-based MLR may play a predictive role in BMT. Dickinson *et al* (1998) have more recently demonstrated that the skin explant assay to be more predictive for acute GVHD in comparison to CTL-p and HTL-p frequency analysis. Although some of our findings (ie; association of rejection with spontaneous IL-4 and MLR-stimulated IL-10 secretion) do not confirm **original** hypotheses about the role of Th1/Th2 cytokines in rejection, it has still been illustrated that the pre-transplant cytokine-based MLR may possibly predict risk of rejection, using IL-10 and IFN-γ as predictive "markers". The association between spontaneous IL-4 secretion and rejection is also interesting as it may be possible that certain IL-4 gene polymorphisms may play a role in determining high/low IL-4 secretor status, and therefore risk of rejection.

6.2.9. Multivariate analysis

It has now been established that the cytokines IL-4 (spontaneous), IL-10 and IFN-γ (MLR) are associated with rejection together with increase in HLA and HLA-DR mismatching and donor sex as independent factors. In addition, it was shown that

both IL-10 and IFN-γ secretion were associated with HLA-DR mismatching, but not total HLA mismatching. Based on these findings, multivariate analysis was then performed to determine whether any of these variables were associated in any way. It was found that relative risk of rejection was higher with female donors (RR=8.02; discussed earlier). In addition, a high HLA mismatch together with high IL-10 secretion led to the highest risk of rejection (RR=25.46). As previously discussed, increase in HLA mismatching does correlate with increase risk of rejection. IL-10 has also been associated with increased rejection episodes (also previously discussed). However, this finding was of interest as risk of rejection appeared to increase even more when both factors were involved. It may be concluded therefore that measurement of IL-10 secretion in the MLR succesfully predicted the highest risk of rejection compared to other cytokines.

It has been shown that polymorphisms in the IL-10 gene promoter correlate with the amount of protein secreted (Turner *et al*, 1997). In this same study, it was shown that low IL-10 secretor genotypes were associated with rejection after heart transplantation. Although the group looked at heart and not kidney transplant patients, the findings by Turner *et al* (1997) were in contrast to this study, as high IL-10 secretion was found to be associated with increased risk of rejection. However, in agreement with the findings in this project is a recent publication by Sankaran *et al* (1999) where an association between high IL-10 (and TNF-α) producer genotype, HLA-DR mismatching and renal allograft rejection. In this study, rejection was also associated with high TNF-α genotype and HLA-DR mismatching. Genetic polymorphisms in the IL-10 promoter region is already being investigated with regards to renal transplantation (Kobayashi *et al*, 1999), and therefore immediate analysis should be carried out to correlate the IL-10 genotypes of the renal transplant patients in this study with IL-10 protein secretion and risk of rejection. From this

further work, it will hopefully be determined whether high IL-10 secretor genotypes are at more risk of rejection than low secretor genotypes.

As with previous studies and this project illustrating a correlation between IL-10 and rejection, this was a surprising result considering the anti-inflammatory functions of IL-10. As with Sankaran *et al* (1999), it was expected that high IL-10 production would act against the production of proinflammatory Th1 type cytokines, such as IFN-γ (in this project) and TNF-α (in Sankaran *et al*, 1999), and hence suppress acute graft rejection. However, recent reports have suggested that IL-10 may enhance antibody responses against the graft (Merville *et al*, 1995). Furthermore, IL-10 has been shown to be a potent stimulator, inducing differentiation and proliferation of B cells (Rousset *et al*, 1992), thus activating B cells to secrete immunoglobulins (Defrance *et al*, 1992, Banchereau *et al*, 1993), thus driving the immune response towards the humoral pathway, and therefore part of the acute rejection response may be antibody mediated.

6.2.10. Clinical and immunological factors on graft survival

Analysis could not be performed to analyse whether any of the factors discussed above had any effect on graft survival as there were only a small number of renal transplant patients that suffered graft failure (n=6). It has been shown that factors such as HLA mismatches play a role in graft survival (Farney *et al*, 1996, Freedman *et al*, 1997, Fuller *et al*, 1999, van der Meer *et al*, 1999), therefore it would have been of interest to analyse whether HLA-DR was relevant to graft survival in this study, along with the predominant cytokines, IL-10 and IFN-γ.

6.2.11. Pre- and post-transplant cytokine profiles

Finally, pre- and post-transplant cytokine profiles were compared in both rejectors and non-rejector groups. Unfortunately, not all of the patients had cytokine profiles measured at twelve months. In the four cytokines compared (IL-2, IL-4, IL-10 and IFN-γ), there appeared to be a general decrease in cytokine secretion after transplantation. There were no differences in cytokine profiles when comparing rejectors and non-rejectors. Only two patients (one rejector, one non-rejector) appeared to show an increase in all cytokine secretion. The fact that the majority of the patient group did show a decrease in cytokine production was almost certainly due to the effect of the immunosuppressive protocol. Immunosuppressive drugs such as cyclosporin A are known to suppress the activation of T cells and therefore cytokine secretion (Kahan, 1996, Bach and Viard, 1993, Rostaing *et al*, 1999a). It was unsure as to why there was a subsequent decrease in cytokine production after three months in some patients, when dosage on immunosuppression is progressively reduced before this time. This may indicate induced tolerance in these patients, and therefore their whole cytokine profile continues to decrease. The reduction in cytokine production in a few of the patients may also be due to an increased number of alloreactive cells compared to other patients, and therefore the cytokine levels in these particular patients took longer to decrease, irrespective of reduction in immunosuppressive drugs. It remains unclear as to why two patients showed an increase post-transplant in all of the cytokines secreted.

6.2.12 Summary of statistical analysis

As stated previously, univariate analysis performed included χ^2 test, Kruskal-Wallis test and Mann-Whitney U test. The χ^2 test was used as a preliminary analysis to find independent predictors of rejection eg; patient sex, HLA mismatching etc. To

establish whether cytokine protein levels were associated with rejection, low/high secretor status had to be determined. This was performed by dividing the groups so that a 50:50 split could be achieved. The data was handled in this way as it was easier for the analysis than to try and split the group into low, medium and high secretors as for the normal individuals (chapter 3). As before, there were outliers within the group, but these were again included in the analysis as they may be biologically significant. All of the data was again tested for normality, and it was found that each set of cytokine protein data did not conform to a normal distribution. Therefore, Kruskal-Wallis test was used when analysing the relationship between HLA and HLA-DR mismatching and protein secretion, and Mann-Whitney U test when analysing the significant difference in protein secretion between rejectors and non-rejectors as these analyses use the medians instead of the means (which would be used if the data was normally distributed).

In addition, Th1 and Th2 cytokine protein profiles were compared to analyse whether there were inverse relationships between the cytokines (as for the normal individuals) using Spearmans Rank analysis. Although some of the relationships appeared to be significant eg; spotaneous IFN-γ and MLR-stimulated IL-10 (CC=0.34, p=0.01), this was not really a strong correlation compared to the relationship between MLR-stimulated IFN-γ and MLR-stimulated IL-10 (CC=0.75, p=0.0001). Therefore, it may be suggested here that if enough multiple comparisons are performed, relationships may be found, even if they are weak. Finally, multivariate analysis was performed to establish the most significant factors associated with rejection. For this and the preliminary analysis, all p values were corrected, and again this did not affect the level (or lack of significance).

6.3. Th1 and Th2 cytokine profiles- relationship to clinical outcome after identical sibling BMT

6.3.1. Introduction

GVHD may still occur after HLA-identical sibling BMT, and this is thought to be due to differences in minor histocompatibility antigens. The mixed lymphocyte reaction (MLR) as measured by (^{3}H) thymidine incorporation has not been shown to be predictive for acute GVHD after identical sibling BMT. (Lim *et al*, 1988, DeGast *et al*, 1992). In this study, we wished to investigate whether the measurement of cytokines generated during the MLR were more predictive than (^{3}H) thymidine incorporation

6.3.2. Cytokine profiles and BMT clinical outcome

MLRs were set up between eight HLA-identical donor-recipient pairs in the donor-versus-patient direction and Th1 and Th2 cytokines (IL-2, IL-4, IL-10 and IFN-γ) measured by ELISA. As there is evidence that Th1 cytokines are thought to be implicated in acute GVHD (Imamura *et al*, 1994, Tanaka *et al*, 1995a, Krenger *et al*, 1997), we wished to correlate these cytokine profiles with acute GVHD. Very low levels of secretion of IL-2, IL-10 and IFN-γ were observed. In the case of IL-4, higher levels were detected but there was no inter-patient variation. No correlation was demonstrated between all four cytokines and acute GVHD. Donor-versus recipient cytokine production was essentially negative therefore, no real information could be evaluated from these results.

It could be suggested that this study has not demonstrated evidence of T cell responsiveness against minor histocompatibility antigens as measured by cytokine secretion in standard MLR, which is in accordance with previous studies using $^{(3)}$H thymidine incorporation (Lim *et al*, 1988, DeGast *et al*, 1992). It is well known that

cytokines are generated in the MLR. IL-2 and IFN-γ protein secretion and IL-2, IFN-γ and IL-5 gene expression have been detected in MLR between HLA-identical siblings (Tanaka *et al*, 1994, 1995a). However, these studies used a two-way MLR enhanced by Con A, and therefore these findings are not truly comparable to our results. In contrast to our findings, Dickinson *et al* (1994b) has demonstrated in the MLR section of the skin explant assay that high levels of Th1 cytokines (in this case, TNF-α and IFN-γ) correlate with the severity of acute GVHD after identical sibling BMT. This may be due to the difference in the way in which the MLR was set up in Dickinson *et al* (1994b) compared to this study. The MLR in Dickinson *et al* (1994b) was set up in 25ml culture flasks while the MLRs in this study were set up in 24 well culture plates. Although the culture plates in this study were tilted to enhance cell to cell contact, this may not have been as effective as if the cultures were set up in flasks for HLA-identical sibling pairs. Quantitative assays such as HTL-p frequency analysis (Schwarer *et al*, 1993), are currently the only predictive assays for identical sibling BMT available, although a more recent study by Dickinson *et al* (1998) has shown that this quantitative assay (also including HTL-p and CTL-p analysis) illustrates no predictive value compared to the much referenced skin explant assay. From the results in this project, it may therefore be concluded that cytokine secretion during standard MLR is not a predictor for acute GVHD after identical sibling BMT.

6.4. Future work

Future work that may arise from this project:

♦ Both IL-10 and IFN-γ were found to be strongly associated with risk of rejection. As cytokine secretor status has been linked to gene polymorphisms, it would be of interest to analyse IL-10 and IFN-γ gene polymorphisms in the renal patient group and correlate this with the protein secretion data obtained from this project and rejection status.

♦ As spontaneous IL-4 was also found to be associated with rejection, it would be of interest to look at IL-4 gene polymorphisms in this renal patient group. The role of IL-6 remains unclear in transplantation, and therefore IL-6 gene polymorphisms in this patient group may also be identified. All of this gene polymorphism data may then be correlated with protein secretion and rejection status.

6.5. Concluding points

The main conclusions from this project are as follows:

♦ Inter-individual variations in cytokine protein secretion and expression were found in mitogen stimulated and MLR cultures (protein secretion only).

♦ A positive relationship was found between mitogen stimulated IL-2 protein and expression.

♦ No relationship between IFN-γ protein secretion, expression and/or gene polymorphisms was observed.

♦ Univariate analysis showed an association between HLA-DR mismatching and both IL-10 and IFN-γ secretion in the MLR.

♦ Univariate analysis also illustrated an association between renal allograft rejection and the following factors: spontaneous IL-4 protein secretion, IL-10 and IFN-γ secretion in the MLR, donor sex, total HLA and HLA-DR mismatching.

♦ Multivariate analysis showed an association between increased risk of rejection and the following factors: female donor sex, and in particular high HLA mismatching and high IL-10 protein secretion in the MLR.

♦ A global decrease in cytokine protein secretion post-transplant was observed.

♦ The MLC was found to be of no predictive value for GVHD in identical sibling bone marrow transplantation.

APPENDICES

Appendix 1 Cytokine data from normal individuals

<u>**PHA stimulated cytokine protein (unstimulated in italics, stimulated in bold)**</u>

Volunteer	IL-2	IL-4	IL-10	IFN-γ
SB	*0*	*1*	*1*	*0*
	6	**11**	**70**	**267**
GH	*0*	*1*	*0*	*0*
	0	**15**	**35**	**645**
RS	*0*	*1*	*0*	*0*
	28	**7**	**9**	**501**
NC	*0*	*3*	*0*	*0*
	1424	**21**	**635**	**8000**
YW	*0*	*1*	*1*	*0*
	8	**37**	**139**	**640**
BY	*0*	*2*	*1*	*0*
	65	**12**	**73**	**534**
SL	*3*	*1*	*1*	*0*
	9	**19**	**90**	**998**
SB	*0*	*1*	*1*	*0*
	78	**14**	**58**	**400**
SP	*0*	*1*	*1*	*0*
	12	**8**	**87**	**1040**
PD	*9*	*1*	*1*	*0*
	178	**69**	**216**	**2798**
PC	*0*	*1*	*1*	*0*
	51	**13**	**85**	**863**
JC	*2*	*1*	*1*	*0*
	36	**5**	**48**	**308**
DR	*0*	*1*	*1*	*0*
	353	**20**	**260**	**2159**

Appendix 1 continued

Volunteer	IL-2	IL-4	IL-10	IFN-γ
AK	20	1	1	0
	202	49	34	243
NH	0	1	2	0
	30	7	24	53
SS	0	3	1	0
	301	10	340	8000
GJ	1	2	1	33
	97	10	460	8000
TH	39	2	1	40
	200	7	635	8000
CB	0	2	0	0
	1403	20	440	8000
VM	95	2	1	0
	581	12	386	8000

Appendix 1 continued

<u>OKT3 stimulated cytokine protein (unstimulated in italics, stimulated in bold)</u>

Volunteer	IL-2	IL-4	IL-10	IFN-γ
SB	*4*	*·14*	*0*	*0*
	6	**18**	**720**	**2786**
GH	*4*	*14*	*0*	*0*
	7	**19**	**742**	**1046**
RS	*3*	*15*	*0*	*3*
	5	**17**	**859**	**2236**
NC	*5*	*15*	*0*	*2*
	5	**15**	**516**	**4250**
YW	*5*	*13*	*0*	*6*
	22	**17**	**907**	**1188**
SB	*4*	*13*	*0*	*5*
	103	**21**	**634**	**1470**
SP	*4*	*13*	*0*	*6*
	14	**15**	**164**	**4441**
PD	*7*	*16*	*1*	*2*
	7	**18**	**694**	**3757**
PC	*5*	*15*	*0*	*5*
	23	**22**	**371**	**3718**
JC	*10*	*12*	*0*	*0*
	15	**- 12**	**523**	**6341**

Appendix 1 continued

Volunteer	IL-2	IL-4	IL-10	IFN-γ
DR	*5*	*14*	*0*	*4*
	10	16	973	5048
AK	*5*	*13*	*0*	*30*
	6	15	975	3355
NH	*4*	*16*	*0*	*5*
	10	22	351	226
SS	*5*	*12*	*0*	*20*
	5	13	374	16330
TH	*8*	*13*	*0*	*11*
	9	14	848	1290
VM	*4*	*15*	*0*	*7*
	5	17	675	3491

Cytokine expression using PMA stimulation

Volunteer	IL-2	IFN-γ
SB	37	19
GH	11	14
RS	23	10
NC	28	8
YW	13	2
BY	11	11
SL	12	2
SB	7	4
SP	1	3
PD	20	17
PC	23	41
JC	4	49
DR	35	4
AK	30	1
NH	23	6
SS	27	5
GJ	21	25
TH	19	5
CB	36	3
VM	27	5

Appendix 2 Clinical and laboratory details of renal transplant patients and donors

Patient	Donor	Patient age	Patient sex	Donor age	Donor sex
HC	AB	45	M	67	F
MP	AB	56	M	67	F
MK	GB	23	M	58	M
KR	AB	57	M	39	M
BC	AB	51	F	39	M
JM	VS	63	M	24	F
LF	MR	73	M	16	M
MH	AH	22	M	46	F
AD	LD	26	F	39	F
SJ	LF	34	F	26	M
GE	LF	49	M	26	M
SD	VH	48	F	46	F
SG	VH	49	F	46	F
JC	CT	64	M	37	F
RE	CT	59	M	37	F
PD	JJ	58	M	40	M
AB	TK	51	M	22	F
CH	JS	46	F	12	F
JB	JS	27	M	12	F
RJ	MB	62	M	57	F
JPW	SJ	44	F	26	M
MT	BG	54	F	50	M
NH	CB	29	F	39	F
BP	CB	60	F	39	F
MB	MP	38	M	47	F
RH	MP	47	M	47	F
ES	GR	34	F	41	M
BW	DS	33	F	56	M

Appendix 2 continued

Patient	Donor	Patient age	Patient sex	Donor age	Donor sex
EE	DM	69	F	38	F
EC	PC	69	M	45	M
GN	PC	58	M	45	M
KB	EW	20	F	23	F
TW	AG	33	F	55	M
AB	AG	30	M	55	M
JW	PL	54	F	69	F
AR	PL	61	M	69	F
LW	JW	42	F	34	M
PM	KS	54	F	23	M
NH	CL	39	M	40	F
RH	DM	42	M	51	F
JH	DM	32	F	51	F
JC	SP	28	M	38	M
JM	BW	55	F	50	M
AL	BW	29	M	50	M
SM	WH	29	F	58	M
CW	WH	43	M	58	M
DBR	JT	50	M	67	F
BS	BJ	63	M	59	F
JN	BJ	38	F	59	F
PG	AP	43	M	69	F
JT	NG	24	M	23	M
JL	MB	53	M	53	F
JA	MB	54	M	53	F
VE	AJ	35	F	66	M
CS	KB	34	F	60	F
NW	JN	33	M	32	M
WB	JN	57	M	32	M

Appendix 2 continued

Patient	Rejection	HLA anti-bodies (%)	Previous transplant	Delayed/Immediate function
HC	Y	0	0	Immediate (Imm)
MP	Y	0	0	Imm
MK	Y	0	0	Imm
KR	N	4	0	Imm
BC	Y	0	0	Imm
JM	N	0	0	Imm
LF	N	12	0	Imm
MH	N	0	0	Imm
AD	N	0	1	Imm
SJ	Y	0	0	Imm
GE	N	0	2	Imm
SD	Y	4	0	Delayed (Del)
SG	Y	0	0	Imm
JC	Y	0	0	Imm
RE	Y	0	0	Del
PD	N	0	0	Imm
AB	Y	0	0	Del
CH	N	0	0	Imm
JB	Y	40	0	Del
RJ	N	0	0	Del
JPW	Y	0	0	Imm
MT	N	0	0	Imm
NH	Y	8	0	Del
BP	Y	ND	0	Imm
MB	Y	0	0	Imm
RH	Y	0	0	Del
ES	N	0	0	Imm
BW	N	0	1	Imm

Appendix 2 continued

Patient	Rejection	HLA antibodies	Previous transplant	Delayed/Immediate function
EE	N	0	1	Imm
EC	N	0	0	Imm
GN	N	0	0	Imm
KB	N	0	0	Imm
TW	N	0	0	Imm
AB	N/A	0	2	Del
JW	Y	0	0	Del
AR	N	0	0	Imm
LW	N	0	1	Imm
PM	N	0	0	Del
NH	Y	8	0	Imm
RH	Y	0	0	Imm
JH	Y	0	0	Del
JC	N	0	0	Imm
JM	Y	0	0	Del
AL	Y	0	0	Del
SM	Y	0	0	Imm
CW	N	0	0	Del
DBR	N	0	0	Del
BS	N	0	0	Nephrectomy
JN	N/A	0	0	Imm
PG	Y	ND	0	Del
JT	N	0	0	Imm
JL	Y	0	0	Imm
JA	Y	0	0	Imm
VE	N	12	1	Del
CS	Y	0	0	Imm
NW	N	28	1	Del
WB	Y	0	0	Imm

Appendix 2 continued

Patient	Donor	Patient CMV	Donor CMV	HLA antigen mismatches	HLA-DR
HC	AB	Positive (P)	P	5	2
MP	AB	Negative (N)	P	4	2
MK	GB	N	N	5	1
KR	AB	P	N	5	2
BC	AB	P	N	4	2
JM	VS	P	N	1	0
LF	MR	N	N	4	1
MH	AH	N	N	1	0
AD	LD	N	P	2	0
SJ	LF	N	N	6	2
GE	LF	P	N	3	1
SD	VH	N	P	4	1
SG	VH	P	P	3	1
JC	CT	P	P	2	1
RE	CT	P	P	3	0
PD	JJ	P	P	4	1
AB	TK	N	P	4	1
CH	JS	N	P	3	1
JB	JS	N	P	3	1
RJ	MB	N	P	3	1
JPW	SJ	N	N	4	1
MT	BG	N	P	5	2
NH	CB	N	P	4	1
BP	CB	P	P	3	1
MB	MP	P	N	5	2
RH	MP	N	N	5	2
ES	GR	N	N	3	0
BW	DS	N	N	4	1

Appendix 2 continued

Patient	Donor	Patient CMV	Donor CMV	HLA antigen mismatches	HLA-DR
EE	DM	P	P	2	0
EC	PC	N	P	3	1
GN	PC	P	P	2	1
KB	EW	N	P	3	0
TW	AG	N	N	3	0
AB	AG	N	P	3	0
JW	PL	P	P	4	1
AR	PL	N	P	3	1
LW	JW	P	P	2	1
PM	KS	N	P	3	1
NH	CL	P	P	3	1
RH	DM	N	P	2	1
JH	DM	N	N	2	1
JC	SP	N	P	2	1
JM	BW	N	P	2	1
AL	BW	N	P	3	0
SM	WH	N	P	3	1
CW	WH	P	P	2	0
DBR	JT	N	P	3	1
BS	BJ	P	P	0	0
JN	BJ	N	P	2	0
PG	AP	N	P	3	1
JT	NG	N	N	4	0
JL	MB	N	N	5	2
JA	MB	N	N	4	2
VE	AJ	N	P	1	0
CS	KB	N	N	4	1
NW	JN	P	N	3	1
WB	JN	N	N	3	1

Appendix 2 continued

Patient	Creatinine at 12 months	Cold ischaemia time
HC	131	20hrs 45mins
MP	137	18hrs 45mins
MK	392	22hrs
KR	224	15hrs 45mins
BC	RIP	16hrs
JM	121	16hrs 10mins
LF	120	13hrs 50mins
MH	166	2hrs
AD	128	36hrs
SJ	87	16hrs 46mins
GE	138	21hrs 12mins
SD	RIP	17hrs 40mins
SG	265	20hrs 10mins
JC	168	18hrs 7 mins
RE	Return to haemodialysis	15hrs 22mins
PD	127	7hrs 40mins
AB	160	Unknown
CH	145	10hrs 10mins
JB	Unknown	17hrs 20mins
RJ	RIP	18hrs 35mins
JPW	163	14hrs 40mins
MT	114	43hrs 56mins
NH	Return to CAPD	23hrs 15mins
BP	181	18hrs
MB	219	16hrs 18mins
RH	Return to haemodialysis	Unknown
ES	150	19hrs 11mins
BW	117	15hrs 10mins

Appendix 2 continued

Patient	Creatinine at 12 months	Cold ischaemia time
EE	91	20hrs 40mins
EC	189	21hrs 53mins
GN	161	16hrs 5mins
KB	136	25hrs 40mins
TW	152	12hrs 27mins
AB	Return to haemodialysis	16hrs 58mins
JW	180	20hrs 18mins
AR	173	15hrs 39mins
LW	130	25hrs 20mins
PM	106	21hrs 4mins
NH	143	28hrs 39mins
RH	260	19hrs 12mins
JH	143	16hrs 57mins
JC	109	10hrs 9mins
JM	121	16hrs 40mins
AL	N/A	19hrs 35mins
SM	126	16hrs 2mins
CW	N/A	7hrs 2mins
DBR	RIP	21hrs 15mins
BS	Nephrectomy	22hrs 15mins
JN	Nephrectomy	18hrs 30mins
PG	Nephrectomy	17hrs 17mins
JT	N/A	11hrs 24mins
JL	N/A	19hrs 22mins
JA	N/A	21hrs 37mins
VE	N/A	43hrs 55mins
CS	N/A	25hrs 1min
NW	N/A	12hrs 35mins
WB	N/A	9hrs 52mins

Appendix 2 continued.

<u>Pre-transplant cytokine profiles (background in italics, MLC in bold)</u>

Pat/Don	IL-2	IL-4	IL-6	IL-10	IFN-γ
HC/AB	*0* pg/ml	*9*	*1060*	*65*	*2129*
	10	**12**	**5856**	**71**	**3804**
MP/AB	*0*	*10*	*355*	*149*	*649*
	31	**13**	**4165**	**184**	**1266**
MK/GB	*10*	*3*	*310*	*2*	*30*
	260	**7**	**545**	**19**	**799**
KR/AB	*4*	*2*	*1089*	*3*	*7*
	842	**13**	**1289**	**11**	**2531**
BC/AB	*6*	*3*	*304*	*8*	*7*
	917	**11**	**1244**	**36**	**2728**
JM/VS	*1*	*3*	*136*	*0*	*4*
	29	**3**	**364**	**2**	**319**
LF/MR	*4*	*3*	*1064*	*3*	*17*
	555	**10**	**8073**	**43**	**2896**
MH/AH	*14*	*2*	*1315*	*5*	*39*
	38	**3**	**1788**	**5**	**96**
AD/LD	*1*	*1*	*310*	*1*	*2*
	127	**3**	**951**	**2**	**313**
SJ/LF	*0*	*10*	*356*	*0*	*0*
	1319	**20**	**3986**	**40**	**4311**
GE/LF	*0*	*10*	*637*	*4*	*0*
	315	**15**	**3936**	**10**	**1476**
SD/VH	*4*	*10*	*1060*	*172*	*120*
	266	**19**	**13598**	**298**	**9718**
SG/VH	*0*	*1*	*1078*	*96*	*110*
	199	**14**	**9386**	**142**	**7221**
JC/CT	*0*	*9*	*1764*	*8*	*0*
	343	**15**	**7851**	**13**	**2246**

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-6	IL-10	IFN-γ
RE/CT	*0*	*10*	*1129*	*4*	*0*
	321	14	5022	10	1540
PD/JJ	*1*	*2*	*0*	*0*	*3*
	108	9	1127	6	985
AB/TK	*4*	*3*	*785*	*4*	*9*
	120	5	1608	14	220
CH/JS	*2*	*9*	*1341*	*11*	*0*
	37	10	6787	24	122
JB/JS	*3*	*10*	*1078*	*7*	*0*
	81	11	8749	13	377
RJ/MB	*0*	*1*	*0*	*0*	*0*
	1	2	16	0	0
JPW/SJ	*0*	*3*	*10*	*0*	*5*
	179	5	97	0	16
MT/BG	*0*	*4*	*184*	*0*	*0*
	110	7	485	6	291
NH/CB	*7*	*9*	*943*	*0*	*15*
	120	12	3562	9	2612
BP/CB	*0*	*9*	*562*	*1*	*0*
	174	14	1448	3	1085
MB/MP	*7*	*0*	*1*	*0*	*0*
	16	1	14	0	17
RH/MP	*0*	*0*	*0*	*0*	*3*
	0	2	11	0	0
ES/GR	*0*	*9*	*1396*	*26*	*1*
	1	10	16790	68	92
BW/DS	*0*	*2*	*900*	*9*	*0*
	12	2	950	1	11

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-6	IL-10	IFN-γ
EE/DM	0	0	0	0	0
	0	2	16	0	0
EC/PC	34	2	0	10	64
	442	15	993	16	642
GN/PC	10	0	265	5	10
	208	5	2086	10	429
KB/EW	1	2	502	0	0
	108	9	1127	6	985
TW/AG	5	3	340	0	0
	7	4	750	1	7
AB/AG	0	3	561	0	0
	1	4	30	0	0
JW/PL	0	9	61	1	4
	232	14	663	3	1725
AR/PL	1	3	1000	2	0
	316	11	1131	8	1447
LW/JW	0	2	893	0	0
	0	2	7	0	0
PM/KS	0	2	0	2	0
	455	12	3359	16	1690
NH/CL	3	3	446	1	0
	7	4	542	4	9
RH/DM	0	5	493	1	0
	728	24	3104	25	3606
JH/DM	7	2	178	0	0
	593	14	754	23	1866
JC/SP	13	0	249	0	5
	320	6	1473	12	446

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-6	IL-10	IFN-γ
JM/BW	*4*	*3*	*146*	*9*	*15*
	400	**5**	**6890**	**17**	**1850**
AL/BW	*0*	*2*	*1266*	*2*	*0*
	436	**6**	**2504**	**15**	**642**
SM/WH	1	3	77	0	20
	454	**8**	**1172**	**21**	**3504**
CW/WH	*0*	*3*	*612*	*0*	*0*
	44	**8**	**447**	**4**	**194**
DBR/JT	*3*	*3*	*52*	*4*	*4*
	1017	**14**	**966**	**46**	**4519**
BS/BJ	*5*	*4*	*455*	*5*	*17*
	159	**9**	**2472**	**8**	**505**
JN/BJ	*4*	*4*	*3896*	*5*	*2*
	572	**14**	**2692**	**22**	**1330**
PG/AP	*1*	*1*	*324*	*0*	*3*
	0	**2**	**75**	**0**	**0**
JT/NG	*0*	*4*	*34*	*0*	*1*
	33	**3**	**78**	**0**	**0**
JL/MB	*0*	*3*	*31*	*0*	*0*
	719	**12**	**1839**	**46**	**1107**
JA/MB	0	3	147	0	0
	503	**16**	**942**	**57**	**2386**
VE/AJ	*1*	*2*	*268*	*0*	*0*
	6	**3**	**137**	**0**	**0**
CS/KB	*111*	*7*	*91*	*6*	*26*
	130	**7**	**367**	**23**	**53**
NW/JN	*81*	*7*	*8*	*7*	*10*
	284	**11**	**116**	**9**	**382**
WB/JN	*0*	*7*	*64*	*0*	*0*
	357	**11**	**176**	**8**	**574**

Appendix 2 continued.

<u>3 month post-transplant cytokine profiles (background in italics, MLC in bold)</u>

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
HC/AB	*0*	*3*	*0*	*0*
	1	**6**	**0**	**76**
MP/AB	*0*	*3*	*0*	*0*
	179	**7**	**0**	**33**
MK/GB	*1*	*0*	*0*	*0*
	249	**4**	**0**	**1**
KR/AB	**0**	1	**0**	7
	34	**2**	**0**	**26**
BC/AB	*ND*	*ND*	*ND*	*ND*
	ND	**ND**	**ND**	**ND**
JM/VS	*6*	*1*	*0*	*0*
	298	**2**	**0**	**0**
LF/MR	*0*	*2*	*17*	*1*
	9	**2**	**20**	**0**
MH/AH	*0*	*1*	*0*	*0*
	1	**2**	**0**	**0**
AD/LD	*0*	*1*	*0*	*3*
	20	**2**	**2**	**35**
SJ/LF	*0*	*2*	*0*	*0*
	517	**10**	**1**	**2**
GE/LF	*0*	*3*	*0*	*0*
	60	**5**	**0**	**51**
SD/VH	*1*	*2*	*0*	*0*
	416	**6**	**0**	**9**
SG/VH	*3*	*2*	*0*	*0*
	7	**3**	**0**	**2**
JC/CT	*0*	*2*	*0*	*0*
	78	**3** -	**0**	**1**

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
RE/CT	0	3	0	0
	50	7	0	978
PD/JJ	0	15	2	22
	170	17	9	837
AB/TK	1	1	0	0
	20	2	0	0
CH/JS	0	2	0	0
	28	4	0	21
JB/JS	ND	ND	ND	ND
	ND	ND	ND	ND
RJ/MB	1	3	0	0
	1	5	0	1
JPW/SJ	4	2	0	0
	115	3	1	1
MT/BG	3	1	0	0
	3	2	0	12
NH/CB	ND	ND	ND	ND
	ND	ND	ND	ND
BP/CB	1	15	0	1
	3	15	1	4
MB/MP	ND	ND	ND	ND
	ND	ND	ND	ND
RH/MP	ND	ND	ND	ND
	ND	ND	ND	ND
ES/GR	0	2	0	18
	3	3	0	0
BW/DS	0	16	2	4
	0	18	4	5
EE/DM	2	14	0	2
	5	15	0	5

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
EC/PC	*3*	*3*	*0*	*0*
	41	6	0	0
GN/PC	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
KB/EW	*0*	*3*	*0*	*0*
	174	7	0	113
TW/AG	*0*	*3*	*0*	*2*
	0	4	0	8
AB/AG	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JW/PL	*2*	*15*	*0*	*2*
	10	16	8	2
AR/PL	*0*	*1*	*0*	*0*
	0	15	0	0
LW/JW	*0*	*2*	*0*	*0*
	1	3	0	1
PM/KS	*4*	*3*	*0*	*0*
	31	3	0	2
NH/CL	*1*	*2*	*0*	*0*
	2	3	0	0
RH/DM	*1*	*3*	*0*	*0*
	2	6	0	0
JH/DM	*1*	*3*	*0*	*0*
	4	6	0	0
JC/SP	*2*	*3*	*0*	*0*
	3	4	0	0
JM/BW	*2*	*2*	*0*	*0*
	204	7	3	337

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
AL/BW	*1*	*3*	*4*	*0*
	143	5	14	142
SM/WH	*2*	*2*	*2*	*0*
	118	6	3	389
CW/WH	*4*	*3*	*0*	*7*
	11	4	0	14
DBR/JT	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
BS/BJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JN/BJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
PG/AP	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JT/NG	*0*	*17*	*0*	*1*
	257	20	8	1002
JL/MB	*2*	*15*	*0*	*2*
	603	18	7	602
JA/MB	*0*	*16*	*1*	*0*
	359	25	19	359
VE/AJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
CS/KB	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
NW/JN	*234*	*24*	*29*	*193*
	561	25	31	249
WB/JN	*0*	*18*	*1*	*1*
	988	24	24	1600

Appendix 2 continued.

<u>12 months post-transplant cytokine profiles (background in italics, MLC in bold)</u>

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
HC/AB	*6*	*4*	*0*	*0*
	120	**10**	**0**	**274**
MP/AB	*4*	*4*	*0*	*0*
	169	**9**	**0**	**61**
MK/GB	*5*	*3*	*0*	*6*
	17	**4**	**0**	**6**
KR/AB	*1*	*3*	*0*	*0*
	65	**6**	**0**	**128**
BC/AB	*ND*	*ND*	*ND*	*ND*
	ND	**ND**	**ND**	**ND**
JM/VS	*2*	*3*	*0*	*0*
	6	**4**	**0**	**0**
LF/MR	*3*	*3*	*0*	*0*
	26	**4**	**1**	**5**
MH/AH	*1*	*3*	*0*	*0*
	3	**4**	**0**	**1**
AD/LD	*1*	*2*	*0*	*0*
	55	**4**	**0**	**71**
SJ/LF	*2*	*2*	*0*	*0*
	118	**8**	**2**	**149**
GE/LF	*3*	*2*	*0*	*14*
	24	**8**	**2**	**476**
SD/VH	*ND*	*ND*	*ND*	*ND*
	ND	**ND**	**ND**	**ND**
SG/VH	*1*	*3*	*0*	*0*
	12	**3**	**0**	**0**
JC/CT	*4*	*4*	*0*	*2*
	161	**6**	**0**	**408**

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
RE/CT	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
PD/JJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
AB/TK	*0*	*3*	*0*	*0*
	44	3	1	16
CH/JS	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JB/JS	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
RJ/MB	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JPW/SJ	*0*	*3*	*0*	*0*
	35	5	0	5
MT/BG	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
NH/CB	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
BP/CB	*0*	*16*	*0*	*2*
	0	15	0	14
MB/MP	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
RH/MP	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
ES/GR	*0*	*2*	*0*	*0*
	0	2	0	0
BW/DS	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
EE/DM	*0*	*15*	*0*	*0*
	1	16	0	0

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
EC/PC	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
GN/PC	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
KB/EW	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
TW/AG	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
AB/AG	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JW/PL	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
AR/PL	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
LW/JW	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
PM/KS	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
NH/CL	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
RH/DM	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JH/DM	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JC/SP	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JM/BW	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND

Appendix 2 continued.

Pat/Don	IL-2	IL-4	IL-10	IFN-γ
AL/BW	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
SM/WH	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
CW/WH	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
DBR/JT	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
BS/BJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JN/BJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
PG/AP	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JT/NG	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JL/MB	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
JA/MB	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
VE/AJ	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
CS/KB	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
NW/JN	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND
WB/JN	*ND*	*ND*	*ND*	*ND*
	ND	ND	ND	ND

Appendix 3 HLA typing data in HLA mismatched renal transplant pairs

HLA Mismatch	Patient/Donor	HLA Types (Patient/Donor)		
		A	B	DR
6 antigen	SJ/LF	2, 10	7, 14	1, 1
		1, 11	57, 27	4, 13
5 antigen	HC/AB	1, 24	8, 44	15, 4
		2, 3	7, 44	7, 16
	KR/AB	1, 28	8, 60	7, 17
		2, 26	7, 60	4, 12
	MT/BG	2, 28	27, 51	8, 13
		24, 25	18, 51	1, 103
	MB/MP	2, 3	7, 49	13, 15
		1, 2,	57, 50	7, 8
	RH/MP	2, 3,	44, 49	13, 13
		1 ,2	50, 57	7, 8
	BW/DS	1, 9	8, 27	1, 17
		1 2	35, 35	1, 4
4 antigen	JT/NG	3, 28	7, 27	4, 13
		2, 32	44, 61	4, 13
	JL/MB	2, 26	5, 62	4, 17
		1 ,2	8, 61	11, 11
	JA/MB	1, 1	61, 61	17, 17
		1, 2	8, 61	11, 11
	CS/KB	9, 29	13, 44	7, 11
		24, 29	14, 45	1, 11
	MP/AB	2, 2	44, 44	4, 4
		2, 3	7, 44	7, 16
	MK/GB	2, 2	35, 60	13, 15
		2, 28	39, 61	14, 15
	BC/AB	1, 2	7, 8	14, 17
		2, 26	7, 60	4, 12
	LF/MR	2, 11	35, 51	4, 15
		2, 23,	44, 62	4, 7
	SD/VH	2, 32	18, 35,	12, 17
		2, 24	8, 61	7, 17
	PD/JJ	2, 2	35, 44	1, 1
		1, 1	35, 38	1, 16
	AB/TK	1, 29	13, 35	4, 15
		1, 3	44, 44	4, 9
	JPW/SJ	3, 24	7, 62	4, 13
		23, 24	8, 50	4, 17
	NH/CB	24, 29	7, 44,	7, 13
		24, 26	38, 62	4, 13

Appendix 3 continued

HLA Mismatch	Patient/Donor	HLA Types		
		A	B	DR
4 antigen	JW/PL	1, 24	7, 8,	15, 17
		2, 29	7, 62	15, 14
3 antigen	AL/BW	2, 24	27, 57	4, 7
		2, 29	35, 44	4, 7
	SM/WH	3, 3	27, 44	1, 13
		3, 30	18, 27	1, 17
	DBR/JT	2, 28,	44, 37	11, 13
		1, 2	44, 57	1, 11
	PG/AP	2, 3	44, 60	7, 14
		3, 29	35, 44	7, 13
	NW/JN	1 ,2	8, 44	4, 4
		2, 26	44, 45	4, 7
	WB/JN	2, 2	44, 44	4, 4
		2, 26	44, 45	4, 7
	GE/LF	11, 28	27, 51	1, 4
		1, 11	27, 57	4, 13
	SG/VH	1, 24	8, 44	4, 17
		2, 24	8, 61	7, 17
	RE/CT	2, 11	49, 70	11, 13
		2 ,28	44, 62	11, 13
	CH/JS	A1, 2	8, 62	9, 17
		1, 11	8, 51	4, 17
	JB/JS	A1, 2	7, 8	15, 17
		1, 11	8, 51	4, 17
	RJ/MB	32, 32	5, 14	7, 7
		1, 32	8, 14	7, 17
	BP/CB	1, 24	8, 62	4, 17
		24, 26 ,	38, 62	4, 13
	ES/GR	3, 29	44 60	4, 15
		2, 2	44, 51	4, 15
	EC/PC	2, 11	35, 44	1, 12
		2, 3	7, 35	1, 15
	KB/EW	1, 30	35, 35	1, 13
		A1, 24	8, 24	1, 13
	TW/AG	3, 30	7, 13	11, 15
		1, 28	7, 44	15, 15
	AB/AG	2, 11	7, 13	15, 15
		1, 28	7, 44	15, 15
	AR/PL	3, 29	7, 44	7, 15
		2, 29	7, 62	14, 15

Appendix 3 continued

HLA Mismatch	Patient/Donor	HLA Types		
		A	B	DR
3 antigen	PM/KS	2, 3	14, 44	1, 1
		2, 26	27, 44	1, 16
	NH/CL	3, 3	15, 44	10, 13
		1, 3	37, 44	10, 12
2 antigen	JM/BW	2, 29	44, 44	7, 7
		2, 29	35,44,	4, 7
	CW/WH	30, 30	13, 18	1, 17
		3, 30	18, 27	1, 17
	JN/BJ	2, 2	8, 62	4, 17
		1, 2	8, 44	4, 17
	AD/LD	30, 32	13, 60	4, 7
		32, 32	14, 61	4, 7
	JC/CT	2, 28	44, 53	4, 13
		2, 28	44, 62	13, 11
	EE/DM	1, 2	8, 8	7, 15
		2, 11	7, 8	7, 15
	GN/PC	2, 3	35, 44	1, 4
		2, 3	7, 35	1, 15
	LW/JW	1, 24	7, 8	9, 17
		1, 2	7, 8	15, 17
	RH/DM	1, 2	8, 8	17, 17
		1, 1	8, 44	13, 17
	JH/DM	1, 1	8, 8	17, 17
		1 1	8, 44	13, 17
	JC/SP	1, 2	8, 8	17,52
		1, 2	8, 60	13, 17
1 antigen	VE/AJ	1, 25	8, 44	9, 17
		1, 3	8, 8	9, 17
	JM/VS	24, 24	7, 14	7, 15
		3, 24	7, 7	7, 15
	MH/AH	2, 2	7, 55	14, 15
		1, 2	7, 55	14, 14
0 antigen	BS/BJ	1, 2	8, 44	4, 17
		1, 2	8, 44	4, 17

Appendix 4 HLA typing data in identical sibling bone marrow transplant pairs

Donor/Patient Pair	HLA Types (Donor/Patient)			
	A	B	DR	DQ
KG/JH	2, 32	7, 15	4, 16	
	2, 32	7, 15	4, 16	
HJ/SJ	2, 3	7, 13	1, 7,	2, 5
	2, 3	7, 13	1, 7	2, 5
PW/GW	1, 30	55, 57	13, 15	
	1,30	55, 57	13, 15	
AW/LP	31, 32,	44, 60		
	31 32	44,60		
NB/AD	11, 32	27, 44	1, 1	5, 5
	11, 32	27, 44	1, 1	5, 5
MB/DB	3, 32	7, 14	4, 15	6, 8
	3, 32	7, 14	4, 15	6, 8
NC/AC	2, 25	18, 44	4, 15	
	2, 25	18, 44	4, 15	
IC/MC	2, 32	7, 61	4, 8	4, 8
	2, 32	7, 61	4, 8	4, 8

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