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MD Thesis

January 2006

**Immune reconstitution of B cell and T cell compartments
following reduced intensity allogeneic stem cell transplantation
for myeloma**

Dr Shirley Patricia D'Sa



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Abstract

Immune reconstitution following conventional allogeneic transplantation is a major determinant of survival. A detailed investigation of T and B cell immune reconstitution and clinical outcome in 19 patients with myeloma undergoing reduced intensity stem cell transplantation using in vivo T cell-depletion with alemtuzumab was undertaken. The rate of recovery of lymphocyte numbers and function following transplant was studied using immunophenotyping with 3-colour flow cytometry and intracellular cytokine staining. In addition, T and B cell spectratyping were used to study the repertoire of immune recovery. The patients in this study experienced delayed T cell recovery and T cell receptor spectratype analysis showed a reduced repertoire diversity, which improved rapidly following the administration of DLI and subsequent conversion to full donor T cell chimerism. Post transplant recovery of B cells was also significantly delayed. Spectratype analysis of IgH CDR3 repertoire revealed a gradual normalisation in spectratype complexity by 6-12 months post transplant. There was a high incidence of viral infection, particularly CMV reactivation but the regimen related mortality was low, perhaps due to the very low incidence of severe acute graft-versus-host disease (GVHD). A total of 10 patients experienced GVHD. Of these patients, 8 eventually demonstrated a disease response alongside clinical evidence of GVHD, demonstrating that the graft-versus-myeloma effect is frequently obtained at the expense of GVHD. Over 80% of all patients have relapsed at a median of 9 months following transplant, suggesting that the initially low rate of GVHD has been achieved at the expense of the desired graft-versus-myeloma effect.

Acknowledgements

Dr Kwee Yong, in gratitude for the unstinting personal support, practical help and understanding that I have received over the past few years that has gone well beyond project supervision.

Dr Kirit Ardeshta for being a great source of advice, support and encouragement.

My husband, Ken for his encouragement of my professional and personal development without whose support I would not be where I am now.

My children, Lisa, James & Joseph for sharing me with the NHS.

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Chapter 1. Introduction

1.1. Introduction

Myeloma results from clonal proliferation of idiotypic B lymphocytes, leading to accumulation of plasma cells in the bone marrow and accompanied by bone destruction and concomitant suppression of normal haematopoiesis. The malignant plasma cells may also infiltrate non-haematopoietic tissues, causing extramedullary disease. The hallmark of myeloma is the production of monoclonal immunoglobulins by the malignant plasma cells, although rarely, neither a paraprotein nor Bence-Jones protein (BJP) is produced (non-secretory myeloma). This is typically accompanied by a reduction of normal immunoglobulin production (immunoparesis). The diagnosis is confirmed by examination of the bone marrow, imaging of the skeleton by a skeletal survey or magnetic resonance imaging to identify lytic lesions, and identification of biochemical disturbances such as hypercalcaemia or critical organ involvement such as renal impairment. Myeloma comprises about 1% of all malignant disease and 10% of haematological malignancies (Kyle 1990). It has an incidence of 40 per million (about 2500 new cases) per annum in the United Kingdom. It is characteristically a disease of middle and later life with a median age of 65 years at diagnosis. Myeloma is relatively infrequent in younger age groups, with an incidence of 0.3% in those under 30 years and 2.2% in those under 40 years. There is a slight male predominance and a greater incidence in both Black males and females compared with the white population (Blade, *et al* 1992).

2.1.1 Normal immunoglobulins

An important component of the humoral arm of adaptive immunity is the production of antibodies (immunoglobulins) by terminally differentiated B cells (plasma cells) in response to antigenic stimulation. These immunoglobulins consist of 2 heavy chains (μ , δ , γ , α or ϵ) and 2 light chains (κ or λ). Only one class of heavy chain and one type of light chain are present in any given immunoglobulin molecule, and the name is taken from the combination of the heavy and light chain designations, for example IgG κ or IgA λ . Immunoglobulins are detected by electrophoresis of serum, where they migrate in the γ globulin component and consist of a heterogeneous group of molecules whose

structural features enable them to bind firmly to a spectrum of foreign antigens. This property is due to the presence of approximately 110 residues at the amino terminal ends of each light and heavy chain known as the variable region. Not every variable residue is equally involved in the process of antigen binding and three regions stand out as being 'hypervariable' (the so-called complementarity-determining regions). The structure and function of this region will be discussed in detail in Chapter 6. The remainder of the immunoglobulin molecule, which is not involved in antigen recognition is termed the constant region and is identical to other immunoglobulin molecules of the same class, subclass and allotype. The properties of the major immunoglobulin molecules are shown in Table 1.1.

Properties	IgG	IgA	IgM	IgD	IgE
Molecular weight	150 000	170 000	900 000	180 000	196 000
Subclass	IgG 1-4	IgA 1-2	None	None	None
Light chain isotype	κ & λ	κ & λ	κ & λ	κ & λ	κ & λ
Half-life	21 days	5.8 days	5.1 days	2.8 days	2.3 days
Daily synthetic rate (mg/kg)	33	24	6.7	0.4	0.02
Complement fixation	IgG1, IgG3	No	Yes	Yes	Yes
Normal adult levels (g/l)	6.5-15.0	0.6-4.0	0.5-3.2		

Table 1.1. Properties of the five major immunoglobulin molecules.

The production of immunoglobulin classes does not reach adult levels until after the first decade. Because maternal IgG crosses the placenta, neonatal levels of IgG are within the normal adult range but fall rapidly to reach their nadir at about 4-6 months according to the half-life of IgG, and the onset of endogenous synthesis. Light chains have a molecular weight of 22 000 daltons and contain

210-220 amino acids. The variable region is responsible for antigen binding, as in the heavy chain, and also the unique thermal solubility of the light chain molecule. Two-thirds of serum light chains are κ and one-third λ . Light chains are synthesised *de novo* in slight excess by plasma cells. They are catabolised by the kidney, and when produced monoclonally in great excess may leak out in the urine as so-called Bence Jones protein. This protein is best detected by electrophoresis of a concentrated specimen of urine, in which the monoclonal molecules form a band.

1.1.2 Monoclonal immunoglobulins

The finding of a monoclonal serum (paraprotein) or urine protein (BJP) is not diagnostic of myeloma, because such proteins may be found in a variety of conditions, including connective tissue diseases, autoimmune diseases, malignant diseases such as lymphomas, amyloidosis, cutaneous disorders such as pyoderma gangrenosum and psoriasis, infectious diseases such as infective endocarditis, HIV infection, tuberculosis, and as a consequence of treatment such as stem cell transplantation and chemotherapy. In the absence of abnormal findings to identify a specific cause, the presence of a paraprotein in the serum or urine is termed a monoclonal gammopathy of unknown significance (MGUS).

Once a serum or urine paraprotein is detected by cellulose acetate or agarose electrophoresis, it is further characterised by immunofixation, using specific antibodies to the Fc portions of the IgG, IgA, IgM, IgD and IgE immunoglobulin molecule or to κ or λ light chains to identify the isotype of the paraprotein. Thereafter, the monoclonal proteins are quantified by a technique such as densitometry, in which the turbidity produced by the antibody-antigen interaction in liquid media produces a reduction in light transmission that is proportional to the original concentration of antigen (which in this case is the amount of immunoglobulin present). Once a serum or urine paraprotein has been identified and quantified, the same method is used for sequential quantification of the protein to determine response to therapy.

1.1.3 The bone marrow in myeloma

Bone marrow infiltration by malignant plasma cells is confirmed by taking an aspirate from the bone marrow, as well as a trephine biopsy. Typically, the posterior iliac crest is sampled and the diagnosis made based on cytological and histological features, including the percentage of plasma cells present. Normal plasma cells constitute about 1% of nucleated cells in adult marrow aspirates. The cells are typically evenly distributed throughout the red marrow with no significant differences between various skeletal sites. The standard criterion for diagnosis of myeloma is a plasma cell infiltrate of greater than 10% of nucleated cells. Myeloma does not have pathognomonic cytological or histological features, but some characteristics may be suggestive of a malignant process. These include variation in plasma cell size, nuclear size and shape, multinuclearity, the presence of Dutcher or Russell bodies, Mott cells and flaming cells. The histotopography of plasma cells is also useful in distinguishing between reactive and neoplastic plasma cells (Bartl *et al* 1988). In the reactive setting, many typical plasma cells are located around small blood vessels, whereas in a malignant plasma cell infiltrate, although there is initial random interstitial infiltration among fat and haematopoietic cells, subsequently denser aggregates of myeloma cells accumulate along endosteal surfaces and around ectatic sinusoids and arteries, eventually forming nodules or sheets which replace the haematopoietic and fat tissues.

In addition, immunological characterisation of the plasma cells can demonstrate monoclonality by means of antibodies to heavy or light chains. Myeloma cells also have a characteristic pattern of antigen expression: CD38+, CD138+, CD56+ and cytoplasmic Ig+. They have variable expression of CD40, and lack of CD19, CD20, CD45 and membrane Ig (Leo, *et al* 1992). No significant correlation has been found between the pattern of surface markers, the M-type and clinical stage in myeloma or MGUS.

1.2 Myeloma- the disease

1.2.1 Clinical presentation of myeloma

The establishment of the diagnosis of myeloma may rarely follow an incidental finding of a paraprotein or raised erythrocyte sedimentation rate (ESR) during the investigation of another disorder. It more commonly presents with a constellation of symptoms or signs. One of the commonest presenting features is bone pain (Kyle, *et al* 1975), which is usually of insidious onset, although it may be more abrupt following a strenuous activity such as lifting which results in a fracture of vertebral body or bodies, or a fall, causing the fracture of a long bone. Fractures usually occur at sites of lytic bone lesions that are frequent in myeloma patients due to the unequal balance between osteoclast and osteoblast activity, resulting in a relative increase in bone resorption. This process also results in diffuse osteopenia in a fifth of patients, including around 5% of patients who do not have lytic bone disease. Weakness and fatigue are often prominent symptoms and may result from the frequent presence of anaemia, which is typically normochromic and normocytic (Kyle 1975). Bone marrow infiltration may result in reduced levels of other lineages, with concomitant clinical features such as bruising and bleeding due to thrombocytopenia. Leucopenia may contribute to a greater incidence of infections such as recurrent pneumonia (Barasch, *et al* 1986), recurrent maxillary sinusitis or urinary tract infections. The immunosuppressive features of myeloma are discussed in a later section. Less common presenting features include neurological symptoms such as loss of sensation or paraesthesiae (peripheral neuropathy) or more dramatically the effects of spinal cord compression due to a fractured vertebra or plasmacytoma extending into the spinal canal (Spiess, *et al* 1988). Renal dysfunction is common in myeloma (about a third of patients have an elevated serum urea or creatinine), but presenting symptoms such as thirst, polyuria and oedema are relatively uncommon. Hypercalcaemia may be a presenting feature of myeloma in 20-30% of cases (Kyle 1975, Riccardi, *et al* 1991). The clinical manifestations of hypercalcaemia are extremely variable, ranging from no symptoms to a pre-coma state accompanied by dehydration that may result from inadequate fluid intake or increased fluid excretion. The hyperviscosity syndrome is a group of symptoms and signs related to increased blood viscosity due to monoclonal

immunoglobulins, particularly IgM, but also polymers of IgG and IgA. It may rarely be a presenting feature of myeloma, with symptoms such as headache, blurred vision, drowsiness, vertigo, tinnitus and ataxia. Its presence is confirmed by the finding of a raised plasma viscosity, and if severe, may warrant specific management in the form of plasma exchange.

The diagnostic criteria for of myeloma are shown in Table 1.2.

Major Criteria
<ul style="list-style-type: none"> • Plasmacytosis in tissue • Bone marrow plasmacytosis >30% • Monoclonal IgG >35 g/l or IgA > 20 g/l • Bence Jones protein > 1 g/24h
Minor Criteria
<ul style="list-style-type: none"> • Bone marrow plasmacytosis 10-30% • Monoclonal IgG >35 g/l or IgA > 20 g/l • Osteolytic lesions • Suppression of normal immunoglobulins
<p>Diagnosis of myeloma can be made if one major and one minor criterion are present. Essential minor criteria are either bone marrow plasmacytosis or an 'M' component in the serum.</p>

Table 1.2. Diagnostic criteria for myeloma according to Durie (1986)

2.2.2 Durie-Salmon staging and clinical course of myeloma

The clinical features of myeloma usually reflect the tumour burden of the disease. The Durie-Salmon staging system is widely used to stage the disease at presentation. Three stages are described that correspond to the tumour mass (Durie 1986). The details of the staging criteria are shown in Table 3. Stage I is thought to represent a low tumour mass ($<0.6 \times 10^{12} / \text{m}^2$) and Stage III a high tumour mass ($>1.2 \times 10^{12} / \text{m}^2$), with Stage II reflecting an intermediate tumour mass ($0.6 - 1.2 \times 10^{12} / \text{m}^2$). In addition, the criteria for an asymptomatic stage termed smouldering myeloma are shown in Table 1.3.

<p><u>Smouldering Myeloma</u></p> <p><i>All the following:</i></p> <ul style="list-style-type: none"> • Normal FBC • Serum calcium normal • Paraprotein: IgG <35g/l IgA<20g/l • BJP <1g/day • BM < 20% plasma cells • No evidence of bony or renal disease
<p><u>Stage I</u></p> <p><i>All the following:</i></p> <ul style="list-style-type: none"> • Hb >10g/dl • Serum calcium normal • Paraprotein: IgG<50g/l IgA<30g/l • BJP<4g/day • X-ray: Normal or solitary lytic lesion only
<p><u>Stage II</u></p> <ul style="list-style-type: none"> • Fitting neither Stage I or III
<p><u>Stage III</u></p> <p><i>One or more of the following:</i></p> <ul style="list-style-type: none"> • Hb <8.5g/dl • Serum calcium >3mmol/l • X-ray: Advanced lytic lesions • Paraprotein: IgG>70g/l IgA>50g/l • BJP>12g/day
<p>Subclassification: A = creatinine <180mmol/l; B = creatinine>180mmol/l</p>

Table 1.3. Durie-Salmon Staging System

Typically, the course of the disease is one of relentless progression in the absence of treatment. Prior to the introduction of chemotherapy, the median survival was 6-9 months (Osgood 1960). The use of chemotherapy has improved prognosis such that alkylating agents with or without steroids produce a response in half the patients treated and improves the survival in an unselected series to 26 months in Stage III patients (Durie and Salmon 1975). A

number of markers of poor prognosis have been identified and are in regular clinical use. These include a raised β_2 -microglobulin, which is associated with a shorter survival, but does not predict response to chemotherapy (Bataille, *et al* 1983), a raised C-reactive protein, which is a surrogate marker for IL-6, an important growth factor for myeloma cells is also predictive of shorter survival (Bataille and Klein 1992). Both markers correlate with tumour burden. More recently, cytogenetic analyses have led to the discovery that complete or partial deletion of chromosome 13 may be an important adverse prognostic indicator, whilst the significance of this finding is less certain when laboratory techniques such as interphase cytogenetics using fluorescence *in situ* hybridisation (FISH) are employed (Desikan, *et al* 2000).

Gene expression profiling (GEP) is increasingly being used to identify genes that might play a role in the initiation and progression of myeloma (Zhan, *et al* 2003b). This methodology is still in a state of evolution, but preliminary results suggest that newly diagnosed myeloma might fall into 4 subgroups. Two of these resemble MGUS, whilst the other 2, which also tended to have poor-risk features at presentation such as raised β_2 -microglobulin resemble myeloma cell lines. Analysis of GEP signatures have also demonstrated that whilst it is possible to differentiate MGUS and myeloma from normal plasma cells, MGUS is not distinguishable from myeloma by virtue of the GEP signature (Zhan, *et al* 2003a).

2.2.3 Infections in myeloma

Infection is a major cause of morbidity and mortality in myeloma patients. Patients with myeloma have 15 times more infections per year than the normal population (Twomey 1973). The rate of infection and risk factors for infection vary with the stage of the disease as well as other factors such as hospitalisation, chemotherapy and neutropenia (Table 1.4).

At diagnosis	Chemotherapy-related period	Off-treatment observation phase	Phase of disease progression
Humoral deficiency	Neutropenia, humoral deficiency, transient cell-mediated deficiency	Humoral deficiency	Humoral and cell-mediated complex immunodeficiencies
Encapsulated bacteria <ul style="list-style-type: none"> • <i>Streptococcus pneumoniae</i> • <i>Haemophilus influenzae</i> • <i>Neisseria meningitidis</i> 	Bacterial infections GRAM POSITIVE: <ul style="list-style-type: none"> • <i>Staphylococcus aureus</i> • Coagulase-negative <i>Staphylococcus</i> • <i>Streptococcus viridans</i> GRAM NEGATIVE: <ul style="list-style-type: none"> • Enteric gram negatives • <i>Pseudomonas aeruginosa</i> Viral infections: <ul style="list-style-type: none"> • <i>Herpes simplex</i> • <i>Herpes zoster</i> Fungal infections: <ul style="list-style-type: none"> • Superficial infections with <i>Candida spp.</i> • Systemic candidiasis • Disseminated aspergillosis with prolonged neutropenia 	Encapsulated bacteria	Encapsulated bacteria Gram negative bacteria <i>Staphylococcus aureus</i> Viral infections Fungal infections

Table 1.4. Infections in different stages of the disease

The serious infection rate is much higher at disease onset, during periods of active disease and in the terminal stages (Goranov 1994, Savage, *et al* 1982, Twomey 1973). The major underlying immune defect inherent to myeloma patients is a polyclonal hypogammaglobulinaemia (Jacobson and Zolla-Pazner 1986). This immunoglobulin deficiency is present in more than 80% of patients at the onset of the disease and is often unaltered by therapy (Oken 1984). Patients with myeloma also show a decreased antibody response to bacterial and viral antigens, suggesting primary humoral immunosuppression (Stoll, *et al*

1985). They are particularly susceptible to bacterial infection with encapsulated organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Kyle 1975).

Non-bacterial causes of infection in myeloma patients are not uncommon (Kyle 1975). Invasive fungal infections are rare outside the setting of HDT, since patients with myeloma rarely experience prolonged periods of severe neutropenia. *Pneumocystis carinii* pneumonia has been reported in myeloma patients without other predisposing causes (MacKenzie, *et al* 1991) and cutaneous *Herpes zoster*, which occurs in 4.5-11 cases per 1000 of the normal elderly population, has been reported to occur in 2% of myeloma patients (Kost and Straus 1996).

1.3. Treatment of myeloma

1.3.1 Conventional dose chemotherapy

The median survival of a newly diagnosed myeloma patient is about 30 to 36 months from the start of treatment with standard conventional dose chemotherapy, but the course of the disease can be extremely variable. Some patients have rapidly progressive, resistant disease to which they may succumb within a few months despite treatment with a variety of induction chemotherapy regimens. Others present with asymptomatic disease that remains stable for several years. The choice of initial cytoreductive therapy depends in part on whether the patient is considered suitable for high dose therapy and stem cell transplantation (see Section 1.3.2).

The current strategy is to treat secondary organ damage and complications such as renal failure and severe infections, in parallel with cytoreductive therapy using chemotherapy and/or radiotherapy. The most effective agents in the treatment of myeloma are alkylating agents, corticosteroids, radiotherapy, and with more recent experience, thalidomide and other biological agents such as proteasome inhibitors. For many years, the standard treatment for myeloma has been melphalan at a dose of 5-7 mg/m²/day with prednisolone (40 mg/day) for 5 days each month (MP). This strategy produces an overall response rate of 50%, most of which are partial responses; in fact the attainment of complete remission (CR), namely the disappearance of the monoclonal protein from urine

or serum by immunofixation, and normalisation of bone marrow appearances is rare with this treatment regimen. The response duration is approximately 18 to 24 months, before further treatment is needed. Another agent, cyclophosphamide given weekly at a dose of 400 mg/ m² produces similar response rate and response duration. Attempts have been made at improving the response rate or duration by combining these agents with other chemotherapy drugs, such as VMCP/VBAP (vincristine, melphalan, cyclophosphamide, prednisolone/ vincristine, BCNU, Adriamycin, prednisolone). A meta-analysis of the results of 18 trials involving 3814 myeloma patients comparing MP with combination chemotherapy showed no significant difference between the two forms of treatment (Gregory, *et al* 1992). The VAD (vincristine, Adriamycin and dexamethasone) regimen was developed as a salvage treatment for relapsing or refractory myeloma patients (Barlogie, *et al* 1984). It differs from other treatments in that the vincristine and adriamycin are infused continuously via a central venous catheter over 4 days rather than given as bolus injections. The rationale for infusion in this way was to kill a greater proportion of the more slowly dividing myeloma cells by giving the drugs over a longer period. The VAD regimen was found to be more effective than any previous regimen in relapsed patients, with a response rate of 40% and a survival of over 1 year. Subsequent studies of VAD as first-line therapy showed that over 80% respond, with 10-20% achieving a CR. Unfortunately, the duration of these responses is not long, lasting 18 months on average, even in those achieving CR (Samson, *et al* 1989). The advantages of the VAD regimen include its utility in patients with renal failure, (since none of the component drugs are renally excreted), lack of myelosuppression and lack of stem cell toxicity, thus permitting subsequent autologous stem cell transplantation. There are a number of variations of VAD (termed VAD-type chemotherapy), including VAMP (methyl prednisolone instead of dexamethasone), with or without the addition of cyclophosphamide, and Z-Dex (the anthracycline idarubicin instead of Adriamycin). These regimens are broadly considered to be equivalent in terms of efficacy.

1.3.2 High dose chemotherapy and autologous stem cell transplantation

A report that high-dose melphalan (HDM) could induce a high response rate even in patients refractory to conventional doses of melphalan (McElwain and Powles 1983) led to the current standard of consolidating the response achieved by conventional dose chemotherapy with a high dose melphalan-conditioned autologous stem cell transplant. The global standard for this approach is melphalan 200 mg/m² followed by infusion 2 days later of a minimum of 2 x 10⁶ CD34+ cells/kg (in the case of a peripheral blood stem cell harvest) or 2 x 10⁸ mononuclear cells/kg (in the case of a bone marrow harvest). HDM/ autologous stem cell transplant produces a 24-75% CR rate and a 5 year event free survival of 28%, and overall survival of 3 years (Attal, *et al* 1996, Palumbo, *et al* 1999). Transplant-related mortality (TRM) is around 3-4%, mainly due to severe infection with gram-negative bacteria resulting in multi-organ failure. This procedure requires a 3 to 4 week admission to hospital, during which patients require barrier nursing and active treatment of infection with broad-spectrum antibiotics as well as blood product support. Early haematopoietic regeneration of white blood count, a sustained haematocrit and platelet count without transfusion support takes 2-3 weeks.

New treatments are changing the rate and spectrum of infections encountered in myeloma patients, including autologous and allogeneic peripheral blood and marrow stem cell transplantation following high dose chemo/radiotherapy (Barlogie, *et al* 1995). Neutropenia and/or mucositis associated with high dose therapy (HDT) predispose to serious Gram-positive and Gram-negative bacterial infections. The infection rate is highest during the transplantation stage of HDT (1.3 infections per month), but within 1 month of transplant, the incidence of infection returns to levels experienced by non-transplant treated groups (1 infection per 9 months) (Donnelly 1995). Urinary tract infections caused by Gram-negative bacteria have become more frequent than pneumococcal pneumonia in the last 30 years (Rayner, *et al* 1991), perhaps reflecting the greater use of more intensive chemotherapeutic regimens in recent years (Alexanian, *et al* 1994). Reactivation of varicella zoster virus is more frequent after autologous transplantation (Schuchter, *et al* 1989) compared to the incidence in the normal population, but reactivation of other

infections (e.g. tuberculosis, Epstein-Barr virus and herpes simplex virus) do not seem to be more frequent.

1.4 Allogeneic stem cell transplantation

The rationale for using allogeneic transplantation is 3-fold. Firstly, the myeloma cell is highly sensitive to irradiation and many cytotoxic drugs, which form the basis for the conditioning therapy for a transplant procedure. Secondly, high dose therapy followed by stem cell rescue has been shown to have curative potential in other haematological malignancies such as acute leukaemia and chronic myeloid leukaemia. Thirdly, there is increasing evidence for a graft-versus-tumour effect in a number of haematological malignancies including myeloma (Tricot, *et al* 1996b, Verdonck, *et al* 1996). Importantly, as mentioned previously, conventional dose chemotherapy and even high dose therapy followed by autologous stem cell transplantation is not curative in the treatment of myeloma, so other strategies must be sought to improve outcome for young patients with this diagnosis.

Until 1998, when the efficacy of reduced intensity (non-myeloablative) conditioning regimens were first reported (Slavin, *et al* 1998), the conditioning regimens for allogeneic transplantation were designed to be myeloablative. The aim was to utilise the cytotoxicity of the conditioning regimen to kill the myeloma cells, and then rescue the patient from prolonged induced bone marrow failure by infusing HLA-matched stem cells (a tumour-free stem cell source) derived from a donor. This conventional allogeneic transplantation approach can induce a CR in 60% of patients and in the one-third that maintain evidence of molecular CR, the risk of relapse appears to be very low (Corradini, *et al* 2003). The 5-year survival is about 30% and important prognostic factors for survival are female rather than male recipient, early stage of disease at diagnosis irrespective of the time of transplantation, to have received only one line of treatment before transplantation and to have responsive disease. However, TRM is between 40-50%, and therefore survival is poorer than with autologous transplantation.

1.4.1 The major histocompatibility complex and HLA typing

In order for a patient to accept stem cells from a donor, there must be tissue compatibility (histocompatibility) between donor and recipient (Dickinson and Middleton 2005). The Human Leucocyte Antigen (HLA) system is the major histocompatibility complex (MHC) of humans. It consists of a cluster of genes located on the short arm of chromosome 6 that encode the cell surface production of proteins. These antigens are not specific to leucocytes, but are present on many tissues play an important role in immune regulation and of course tissue and organ transplantation. The HLA system consists of 3 classes of closely linked genes.

The class I genes encode the heavy (α) chains of the 6 class isoforms, HLA-A, -B, -C, -E, -F and -G molecules. The HLA-A, -B and -C heavy chain genes are highly polymorphic, hence their importance as transplantation antigens. There is limited polymorphism exhibited by the other 3 genes. Class I antigens are expressed on all nucleated cells.

There are 5 isotypes of class II proteins, namely HLA-DM, -DO, -DP, -DQ and -DR. Each class II antigen consists of 2 membrane-inserted glycosylated polypeptides designated α (34 kd) and β (28 kd) and bound together non-covalently; the polymorphism of the class II molecules can derive from both α and β chains. HLA-DRB1 is the most polymorphic region and is an important transplantation antigen. There is a high degree of polymorphism at both α and β chains of HLA-DP and DQ, but little polymorphism at HLA-DM or HLA-DO. Class II antigens are more restricted than class I in their tissue distribution, being found primarily on B lymphocytes, macrophages, monocytes, endothelial cells, activated T lymphocytes and Langerhans cells.

In order to determine histocompatibility between donor and recipient, HLA typing is performed. In view of the large degree of polymorphism exhibited at the HLA loci, it is important to perform DNA typing to uncover all allelic differences. Previously, low-resolution serological methods were used to identify tissue types. This meant that many mismatches remained 'hidden', and led to greater degrees of graft-versus-host disease (GVHD) than expected. The

inadequacy of serological methods can be demonstrated by the fact that although there are 225 described alleles at HLA-A, this is represented by only 24 serological specificities.

In the patient group within this study, molecular typing for class I and class II was used (Shaw, *et al* 2001). Class I typing was performed by a method known as sequence-specific oligonucleotide probes (SSOP) In this method, DNA is amplified and then incubated with an oligonucleotide probe (Hao and Xiao 2002). The probe is designed to detect a particular polymorphic motif. Only if the amplified DNA contains the sequence complementary to that of the oligonucleotide will it bind the probe. Class II typing was performed using a combination of SSOP and sequence-specific primers (SSP). The latter method uses sequence-specific primers for the PCR. To distinguish between a set of alleles requires a different set of PCRs, the number being at least half of that of the alleles to be typed. The HLA type is then inferred from the presence or absence of specific bands when the products are run on an agarose gel. If there are ambiguous results from SSOP or SSP in class II typing, then a higher resolution method, sequence-based typing (SBT) is used. This method employs PCR to amplify all the alleles of a locus. The alleles are then sequenced as a mixture and analysed using a computer programme that identifies the positions of heterozygosity. From the comparisons of the patterns obtained with those expected for all combinations of alleles, the programme determines the possible tissue types.

The presence of a mismatch at both class I and II is associated with increased complications and decreased overall survival following transplantation (Petersdorf, *et al* 1998a). The relative importance of mismatches at individual loci remains controversial. For example, matching at HLA-DRB1 and –DQB1 reduces the risk of acute GVHD and improves survival following unrelated SCT (Petersdorf, *et al* 1998b).

1.4.2 Conditioning therapy

Conditioning regimens have 2 purposes: eradication of disease and eradication of the patient's immune system to permit engraftment. In general, the degree of immune suppression is proportional to the intensity of the regimen and

subsequently to the toxicity of the procedure, so it is important for the intensity of the conditioning therapy to be appropriate for the clinical setting in terms of the disease being treated and the degree of mismatch between donor and recipient. With regard to tumour eradication, the purpose of transplantation is to permit intensification of therapy and subsequent curative potential. There are a wide variety of conditioning regimens, each with its own set of advantages and disadvantages. No single approach has been found to produce a superior survival. The use of more intensive regimens may reduce the relapse rate, but those benefits may be offset by increasing the rate of lethal complications caused by the intensified treatment. Regimens that have been used to condition patients with myeloma include cyclophosphamide and total body irradiation (TBI), melphalan and TBI, and less commonly busulphan and cyclophosphamide and melphalan alone. GVHD prophylaxis measures have also varied, and include ciclosporin and / or methotrexate, and T cell depletion of the graft with or without other measures (Gahrton, *et al* 2001). In a recently published evidence-based review, no recommendations can currently be made about the use of the preferred myeloablative conditioning regimen for allogeneic transplantation in myeloma (Hahn, *et al* 2003).

1.4.3 Sources of stem cells

Stem cells may be procured from a patient or donor in one of 2 ways. Bone marrow (BM) itself can be directly harvested by puncture and aspiration of the posterior iliac crests under general anaesthesia. Alternatively, cytokines can be used to mobilise stem cells in to the peripheral circulation, which can then be obtained by leucapheresis. The lymphocyte and monocytes composition and function of cytokine-mobilised peripheral blood stem cell harvests (PBSC) vary from that of directly aspirated BM. An 11-fold increase in CD19+ B cells, a 19.4-fold increase in CD56+ NK cells, a 16-fold increase in CD3+ cells, a 13-fold increase in CD4+ cells and 27.4-fold increase in CD8+ cells have been found on cytokine-mobilised PBSC compared to BM harvests (Korbling, *et al* 1995). These differences in cell content could conceivably impact on immune reconstitution as well as the severity of GVHD and even probability of disease relapse. In the patient group reported here, BM-derived stem cells were obtained from unrelated donors and PBSC were harvested from sibling donors following a series of growth factor injections to the donor. The basis for this

difference is based on the acceptability of administering growth factor injections to normal donors. Unit policy was to not administer growth factor injections to unrelated donors, whereas sibling donors were consented to receive growth factor injections. Whilst PBSC products may be cryopreserved in DMSO and infused at a later date, BM-derived products are freshly infused. A minimum of 2×10^8 mononuclear cells/kg are necessary to ensure engraftment of BM-derived stem cells, whereas a PBSC containing at least 2×10^6 CD34+ cells/kg are required.

1.4.4 Toxicity of allogeneic stem cell transplantation

As mentioned previously, the morbidity of allogeneic stem cell transplantation increases as the histocompatibility differences between donor and patient increases. This morbidity is largely due to alloreactivity, the recognition of self/non-self by the donor and patient immune systems, which are co-mingled during transplantation. Alloreactivity increases in proportion to differences in histocompatibility and causes graft-versus-host disease (GVHD), in which donor alloreactivity dominates or graft rejection if the balance is reversed. However, the total absence of alloreactivity (as in syngeneic allo-transplantation) is a disadvantage, with a higher rate of relapse due to a lesser graft-versus-tumour effect. Thus the success of allogeneic transplantation depends partly on effective modulation of alloreactivity. The manifestations and treatment of GVHD, and its influence on immune reconstitution are addressed in detail in Chapter 7.

The toxicity of allogeneic transplantation also relates to damage to tissues and organ damage by the conditioning therapy. The complications that may occur within the first 100 days of myeloablative allogeneic stem cell transplantation are shown in Table 1.5.

Complication	Frequency of occurrence	Clinical manifestations	Treatment/ Outcome
Mucositis	Universal	Painful oral mucosal with ulceration Dysphagia Nausea & vomiting Diarrhoea Abdominal distension	Antiseptic mouth care Analgesia Parenteral nutrition and fluid replacement Treatment of super-infection
Pneumonitis and carditis	2% May be related to conditioning regimen	Dyspnoea Cough Hypoxia	Diuretics for fluid overload Oxygen Ventilation
Veno-occlusive disease of the liver	5-50% of patients depending on conditioning	Poorly understood phenomenon Weight gain/ fluid retention Hepatomegaly Hepatic pain Hyperbilirubinaemia	Careful maintenance of fluid and electrolyte balance A variety of empirical treatment including anticoagulants
Haemorrhagic cystitis	5-50% following cyclophosphamide-containing conditioning	Haematuria of varying degree Bladder spasms Urinary obstruction	Symptomatic treatment Hydration Bladder installation of aluminium hydroxide, silver nitrate or formalin Stents Treatment of viral super-infection (BK, JC and adenovirus)
Infections			
• Bacterial	Universal	Neutropenic fever Septicaemia	Broad spectrum antibiotics Supportive care for haemodynamic instability
• Viral	Variable	Dependent on viral aetiology. EBV associated lymphoproliferative disorder HSV/ VZV dermatological infection or viraemia	Rituximab, EBV-specific cytotoxic lymphocytes (CTLs) Aciclovir in high dose
	CMV reactivation occurs in 50% of cases of recipient and/ or donor seropositivity	CMV reactivation may result in persistent fever, pneumonitis, gut infection, hepatitis, ocular infection	Ganciclovir or foscarnet CMV-specific CTLs
• Fungal	Variable, commonest organisms are <i>Aspergillus spp.</i> , and <i>Candida spp.</i>	Persistent fever Pneumonitis Hepatitis	Antifungal therapy: fluconazole, itraconazole, amphotericin
GVHD	See Chapter 7		

Table 1.5. Early complications (within 100 days) of allogeneic stem cell transplantation

It is the combination of these morbidities that result in the considerable TRM following allogeneic stem cell transplantation. In addition, patients have frequently received several lines of prior therapy, which leads to pre-transplant organ damage including cardiac and renal function, making them more susceptible to the rigors of the post-transplant period. For that reason, other strategies have been pursued in order to take advantage of the benefits of myeloablative allogeneic transplantation whilst reducing the toxicities. They include the use of non-myeloablative conditioning therapy, which causes less direct critical organ damage and a more gradual transition to donor haematopoietic chimerism.

1.4.5 Reduced intensity conditioned allogeneic stem cell transplantation

Several studies have been performed to assess the efficacy of reduced intensity conditioned allogeneic stem cell transplantation (RIT) in myeloma. These studies have included patients with refractory disease (Giralt, *et al* 2002), heavily pre-treated patients who received RIT as salvage therapy (Crawley, *et al* 2005), patients with progressive disease who had received prior therapy including an autograft (Ando, *et al* 2005, Badros, *et al* 2002) and patients who have received RIT following an autograft (Kroger, *et al* 2002). The clinical heterogeneity of the patient groups, as well as variations in conditioning therapy, including the use of fludarabine, melphalan and ATG in one (Ando, *et al* 2005, Kroger, *et al* 2002) and melphalan and low dose TBI in another (Badros, *et al* 2002) make the relative efficacy of these various studies difficult to compare. Overall, RIT is feasible in myeloma patients, with successful donor engraftment and acceptable toxicity. However the effect on long-term disease control is still being evaluated.

GVHD and disease recurrence particularly in-patients with refractory or relapsed disease remain significant obstacles that need to be overcome. Kroger *et al* found that day 100 TRM was 11%, and at a median follow-up post allografting of 13 months, the estimated 24 month OS was 74% and disease-free survival 56%. In the study by Badros *et al*, 58% developed acute GVHD and 61% a CR or near CR. Median OS was 15 months and better in patients who received the allograft as planned consolidation of a single autograft. The findings of Giralt *et al* were a non-relapse mortality of 19% at 100 days and 40%

at 1 year. Survival at 2 years was estimated to be 30% with progression-free survival of 19%.

We have recently reported on the use of RIT, with fludarabine, melphalan and CAMPATH-1H (alemtuzumab) as front line therapy for myeloma patients with chemosensitive disease (Peggs, *et al* 2003a). Alemtuzumab is the humanised form of the CAMPATH-1 monoclonal antibody (Riechmann, *et al* 1988), directed against the CD52 antigen, which is expressed on more than 95% of lymphocytes (T cells more than B cells), monocytes and macrophages, but not granulocytes, platelets or erythrocytes (Ginaldi, *et al* 1998). CAMPATH-1 is thought to induce cell death via complement-mediated lysis and antibody-dependent cellular cytotoxicity (ADCC) (Xia, *et al* 1993) and has been used with useful therapeutic effect in autoimmune disorders (Isaacs, *et al* 1992), bone marrow transplantation (Hale and Waldmann 1994) and lymphoproliferative disorders (Bowen, *et al* 1997, Hale, *et al* 1988). Alemtuzumab at a dose of 60 mg over 10 days is known to produce prolonged (> 18 months) depletion of CD4+ cells, and a lesser degree of depletion of CD8+ cells, but quicker recovery of NK cells and B cells (within 2 to 3 months) following its use in patients with rheumatoid arthritis (Isaacs, *et al* 1992). Another study using alemtuzumab intravenously, also in patients with rheumatoid arthritis showed similar results, with dramatic effects seen particularly on CD4+ cells, which remained depleted (<20% pre-treatment levels) for more than 500 days of follow up (Brett, *et al* 1996). A further study of patients with low grade lymphoma (Tang, *et al* 1996) was closed prematurely because of marked lymphopenia and an unacceptably high frequency of serious viral infections when treated intravenously with 25 mg alemtuzumab three times a week.

In our study, although the non-relapse mortality (15%) and acute GVHD rate were relatively low compared with conventional myeloablative allogeneic transplantation series, disease responses at 6 months post-transplantation were modest. Fourteen patients received escalating-dose DLI for residual/progressive disease. Three developed acute GVHD and 2 developed limited chronic GVHD. Seven demonstrated further disease responses, which appeared to be more common in those developing GVHD. Response durations

were disappointing (5 <12 months) and progression often occurred despite persisting full donor chimerism. Two-year estimated overall survival and current progression-free survival rates (intention to treat with DLI from 6 months) were 71% and 30%, respectively. Viral infections were common in this patient group, including 10 cases of CMV reactivation, 8 of RSV and 2 systemic adenovirus infections. Two others developed shingles shortly after stopping aciclovir prophylaxis.

More recently, investigators reported on autologous HCT combined with subsequent non-myeloablative allogeneic HCT to maintain the benefits of both approaches with acceptable toxicity (Maloney, *et al* 2003). Fifty-four patients with previously treated myeloma, of which half had refractory or relapsed disease received melphalan 200 mg/m² and autologous SCT. Subsequently, 52 patients received a single fraction dose of 2 Gy TBI and SCT from HLA-identical siblings and post-transplant immunosuppression with mycophenolate mofetil (MMF) and ciclosporin. Patients experienced medians of 0 days of hospitalization, neutropenia, and thrombocytopenia. Sustained engraftment was uniform. With a median follow-up of 552 days after allografting, overall survival is 78%. Thirty-eight percent of patients developed acute GVHD (grade II in all but 4 cases) and 46% chronic GVHD requiring therapy. Tumor responses occurred slowly. Thus far, 57% of patients have achieved complete remissions and 26% partial remissions for an overall response of 83%. The impact of this approach on long term outcome is awaited.

1.5 Assessing disease response to therapy

There are published criteria for assessing response to therapy in myeloma patients (Blade, *et al* 1998). These criteria are defined in Appendix 1, and were used throughout this study.

Chapter 2: Patient characteristics, treatment protocols and clinical outcome

2.1 Patient characteristics and chemotherapy treatment

Nineteen patients with myeloma were enrolled in this prospective study of immune reconstitution following RIT including 5 women and 14 men. This group of patients, with a median age of 47 years (range 34-59 years) is younger than the majority of patients with myeloma who have an age of 65 years at diagnosis. This reflects the specialist nature of the Centre to which they have been referred, namely a tertiary haematology unit which specialises in stem cell transplantation. Patient and donor details are summarised in Table 2.1. The disease isotypes were as follows: 10 had IgG κ , 1 had IgG λ , 4 had IgA κ , 2 had κ light chain myeloma, 1 had λ light chain myeloma and 1 had non-secretory disease. All patients received VAD-type therapy for initial cytoreduction. The number of cycles of chemotherapy ranged from 4 to 7, with a median number of 5 cycles. In 8 patients, the RIT procedure was upfront, following on from the initial VAD-type therapy. The other 11 patients received further lines of therapy prior to their allogeneic transplants, consisting of a non-cross reactive regimen, etoposide, methylprednisolone, high dose cytarabine and cisplatin (ESHAP) in 5, dexamethasone, cisplatin and etoposide (DCE) in 1, and higher dose of cyclophosphamide in 3 patients (4g/m² in 2 patients and 7 g/m² in 1 patient). Five patients had undergone an autograft prior to the allograft. In 2 cases, the conditioning therapy was melphalan 200 mg/m², in 1 case it was melphalan 220 mg/m², and in the other 2 it was melphalan 140 mg/m² and TBI. The most heavily pre-treated patient (Patient 16) required a further course of ESHAP after his autograft in order to prepare him for allograft. His disease was primary refractory. In summary, 42% of this patient group had a single line of prior therapy, 26% had 2 prior lines of therapy, 16% had 3 prior lines and 16% had 3 or more lines of therapy. At the time of allogeneic transplantation, 1 patient was in a complete remission, 15 patients had a partial response, 2 had a minor response and 1 had progressive disease.

Patient	Age/Sex	Isotype	Lines of prior therapy (including autograft)	Disease status at Tx	Donor	Stem cell source	Stem cell dose (x10 ⁵ CD34+ cells/kg)	CMV status R/D	No. of DLI (day given)
1	43/F	BJPk	1	PR	SIB	PB	21.1	Neg/Neg	3 (341, 446, 496)
2	46/F	NS	1	PR	SIB	PB	6.8	Neg/Neg	2 (197, 289)
3	47/M	IgGk	2	PR	MUD	BM	2.3	Neg/Pos	4 (211, 448, 552, 708)
4	40/F	IgGk	1	PR	MUD	BM	na	Pos/Pos	0
5	34/M	IgGk	2	MR	MUD	BM	na	Pos/Neg	5 (192, 290, 392, 489, 581)
6	44/M	IgGk	2	PR	SIB	PB	4.9	Pos/Pos	5 (183, 315, 434, 530, 623)
7	55/M	IgGk	2	PR	SIB	PB	3.8	Pos/Neg	5 (185, 283, 364, 458, 555)
8	38/F	IgGk	4 (Auto)	MR	MUD	BM	na	Neg/Pos	1 (140)
9	47/M	IgAk	1	PR	MUD	BM	na	Neg/Neg	1 (448)
10	58/F	IgGI	1	PR	SIB	PB	5.5	Pos/Pos	3 (264, 385, 468)
11	50/M	IgAk	2	PR	SIB	PB	3.6	Pos/Pos	3 (217, 301, 392)
12	53/M	IgGk	2	PR	MUD	BM	na	Neg/Neg	3 (211, 469, 806)
13	54/M	IgAk	3 (Auto)	PR	SIB	PB	1.9	Neg/Neg	2 (196, 285)
14	52/M	IgGk	1	PR	SIB	PB	6.9	Pos/Pos	3 (182, 277, 369)
15	35/M	IgAk	3 (Auto)	PR	SIB	PB	na	Pos/Neg	3 (238, 318, 405)
16	43/M	BJP	5 (Auto)	PD	MUD	BM	na	Pos/Pos	1 (155)
17	47/M	IgGk	1	PR	MUD	BM	na	Neg/Neg	1 (173)
18	47/M	BJPI	1	CR	SIB	PB	3.6	Neg/Pos	1 (343)
19	59/M	IgGk	3 (Auto)	PR	SIB	PB	2.7	Neg/Neg	1 (293)

Abbreviations:

PR, partial response; MR, minor response; PD, progressive disease; CR, complete response; NR, No response; BJP, Bence Jones Protein; NS, non-secretory; k, kappa light chain; I, lambda light chain; SIB, sibling; MUD matched unrelated donor; PB, peripheral blood; BM, bone marrow; CMV, cytomegalovirus; R, recipient; D, Donor; Neg, CMV seronegative; Pos, CMV seropositive; DLI, donor leucocyte infusion/s; na, not available

Table 2.1. Patient and donor details

2.2 Conditioning regimen, GVHD prophylaxis and anti-infective prophylaxis

The conditioning regimen used in this study was designed to be sufficiently immunosuppressive to ensure engraftment and at the same time to be non-myeloablative, hence reducing the toxicity of the preparative regimen. Suppression of the recipient's immune system was achieved by the use of fludarabine, a purine analogue that causes profound and prolonged lymphopenia, as well as alemtuzumab (section 1.3.8). The inclusion of alemtuzumab was also designed to produce *in vivo* depletion of the donor T cells within the infused stem cell graft with the intention of minimising GVHD (section 7.3.1).

The conditioning regimen in this study utilised a total alemtuzumab dose of 100mg, administered in a schedule of 20mg/day on days -8 to -4 and fludarabine 30mg/m²/day on days -7 to -3 as well as melphalan 140mg/m² on day -2. The effective *in vivo* T-cell depletion achieved by this dose of alemtuzumab, has been confirmed by the demonstration of persisting lympholytic concentrations of alemtuzumab for approximately 56 days post transplant when administered as part of this conditioning regimen in 10 patients with haematological malignancies (Morris, *et al* 2003).

G-CSF- mobilized peripheral blood stem cells were procured from HLA-identical sibling donors in 11 cases, while HLA matched unrelated donors underwent bone marrow harvesting under general anaesthetic in 8 cases. On day 0, patients received G-CSF-mobilised unmanipulated PBSC grafts from their HLA-matched siblings or unmanipulated bone marrow grafts from their unrelated donors. GVHD prophylaxis consisted of intravenous ciclosporin A, 3mg/kg starting on day -1, with a target level of 300 ng/ml. Intravenous Ciclosporin was converted to an oral dose when appropriate, and weaned by 3 months post-transplant in the absence of GVHD. Antiviral prophylaxis consisted of intravenous aciclovir (250 mg/m²/day) until engraftment, converting to oral aciclovir (200 mg twice daily) prior to discharge. Oral co-trimoxazole (960 mg twice daily) was administered

daily from day -8 to day -1 as prophylaxis against *Pneumocystis carinii* infection. Nebulised pentamidine was given prior to discharge and then monthly until cytopenias no longer precluded the use of prophylactic co-trimoxazole (960 mg twice daily on three days per week). Patients at high risk of fungal infection received intravenous itraconazole until discharge. Weekly surveillance for cytomegalovirus (CMV) was performed by PCR for CMV DNA on whole blood or plasma and those testing positive on 2 consecutive weeks were treated with intravenous ganciclovir or foscarnet according to institutional protocol.

2.3 Donor leucocyte infusions

In the absence of active GVHD, donor leucocyte infusions (DLI) were commenced at 6 months post transplant in patients with residual disease or mixed chimerism. CD3+ T cells were administered at a starting dose of 1×10^6 CD3+ cells/kg at 6 months. Escalating doses were administered at 3 monthly intervals (3×10^6 , 1×10^7 , 3×10^7 , and 1×10^8 CD3+ cells/kg) in the continued absence of GVHD. No patient received glucocorticoids prior to DLI administration. A total of 18 patients have received DLI. Sixteen patients received a median of 2 DLI (range 1-5) according to protocol. One patient received a higher dose (1×10^7 CD3+ cells/kg) at 4.5 months because of disease progression, and a second patient received a higher starting dose (3×10^6 CD3+ cells/kg) at 6 months for progressive disease at this point. One patient did not receive DLI due to donor withdrawal.

2.4 Clinical outcome

The patients in this study were followed up for a median of 469 days (range 278 to 741 days). Details of clinical outcome, including time to neutrophil engraftment, infections including viral reactivation, GVHD, disease status and mortality are shown in Table 2.2 and clinical grading systems for acute and chronic GVHD in Tables 2.3 & 2.4.

Table 2.2. Clinical Outcome

(shown overleaf)

Patient	ANC>0.5 (days)	CMV reactivation	Other infections	Acute GVHD (Site) / (Treatment)	GVHD post DLI (Site) / (Treatment)	Status pre-DLI/ Best response after DLI	No of days to disease progression	Outcome, Survival in days, (Current disease status or Cause of death)
1	12	N		N	N	PD/ NC	278	Dead, 522 (PD)
2	23	N	PFIII	N	N	PD/ NC	162	Dead, 558 (PD)
3	12	N	HHV7 myelitis	N	Grade II (s, g), LC(o,s) (Top steroids)	MR/ PR	-	Alive, 708 (PR)
4	10	Y	PFIII	Grade II (s) (Top steroids)	N	PR/ na	895	Alive, 941 (PD)
5	10	Y		N	N	MR/ PR	538	Dead, 937 (PD)
6	16	Y		N	N	PD/ PR	167	Alive, 1146 (PD)
7	14	Y	VZV	N	N	PD/ NC	182	Alive, 1142 (PD)
8	11	N	PFIII, HSV	N	Grade III (s, g, l) (Top steroids & thalidomide)	PD/ NC	126	Dead, 427 (PD)
9	14	N	Influenza A, VZV	Grade II (s) (Top steroids)	N	PR/ NC	448	Alive, 1097 (PD)
10	11	Y	PFIII, HSV, TB	N	N	NC/ NC	-	Alive, 1055 (SD)
11	13	Y	HSV	N	LC (o, s) (Top steroids)	PR/ CR	637	Alive, 1055 (PD)
12	12	N		N	LC (o, s) (Top steroids) Grade IV(s, l) (dacluzimab, infliximab)	NC/ PR	-	Alive, 978 (PR)
13	11	N		N	N	PR/PD	702	Dead, 791 (PD)
14	11	Y		N	N	MR/ MR	420	Dead, 855 (PD)
15	12	N		N	N	PR/NC	745	Alive, 963 (PD)
16	16	Y	RSV	N	Grade IV (s, g, l) (iv steroids & ATG)	PD/ NC	153	Dead, 327 (GVHD)
17	13	N	Systemic adenovirus	Grade I (s) (Top steroids)	Grade IV (s, l) (iv steroids & ATG)	PD/ PR	174	Dead, 303 (GVHD)
18	14	Y	RSV	Grade I (s) (Top steroids)	Grade III (l) (iv steroids)	PD/ PR	259	Dead, 475 (PD)
19	18	N		Grade II (s) (Top steroids)	N	PR/PR	287	Alive, 887 (PD)

Abbreviations:

ANC>0.5, absolute neutrophil count >0.5 x 10⁹/L; PFIII, parainfluenza III virus; HHV7, human herpes virus 7; VZV, varicella zoster virus; HSV, herpes simplex virus; TB, tuberculosis; GVHD, graft-versus-host disease; s, skin; g, gut; l, liver; o, oral; LC, limited chronic; po, per oral; iv, intravenous; ATG, anti-thymocyte globulin; PD, progressive disease; CR, complete remission; PR, partial remission; MR, minimal response; NC, no change; SD, stable disease; na, not applicable

Table 2.2 Clinical Outcome

Individual organ system grading:

Skin	Gastro-Intestinal Tract	Liver	Grade
Rash	Diarrhoea	Bilirubin ($\mu\text{mol/l}$)	
<25%	0.5l	12-20	1
25-50%	1.0l	29-50	2
>50%	1.5l	>50	3
Desquamation	Pain/Ileus	Raised AST/ALT	4

Overall grading:

Skin	Gastro-Intestinal Tract	Liver	Grade
1-2	-	-	I
1-3	1	1	II
2-3	2-3	2-3	III
2-4	2-4	2-4	IV

Table 2.3. Grading of acute graft versus host disease (based on Glucksberg criteria, 1974).

Subclinical GVHD
Histologically positive, but no clinical symptoms
Limited chronic GVHD
<i>Either or both:</i> Localised skin involvement Hepatic dysfunction (due to chronic GVHD)
Extensive chronic GVHD
<i>Either:</i> Generalised skin involvement <i>Or:</i> Localised skin involvement or hepatic dysfunction due to chronic GVHD or both <i>Plus:</i> Liver histology showing chronic aggressive hepatitis, bridging necrosis or cirrhosis <i>Or:</i> Involvement of the eyes (Schirmer's test <5mm wetting) <i>Or:</i> Involvement of minor salivary glands or oral mucosa demonstrated on labial biopsy <i>Or:</i> Involvement of other target organ (lung or kidney)

Table 2.4. Classification of chronic GVHD (according to Shulman et al, 1980)

All patients experienced neutropenic fevers and received broad spectrum intravenous antibiotics as per unit protocol. Clinical course throughout the neutropenic period was unremarkable in this patient group, and no different in character from the unit's clinical experience with autologous stem cell transplantation. All patients engrafted successfully, with a median time to neutrophil count $> 0.5 \times 10^9/l$ of 12 days (range 10-23 days). Median hospital stay was 23 days (range 17-55 days).

2.4.1 Viral Infections

Infection due to cytomegalovirus (CMV) remains a significant cause of morbidity and mortality after allogeneic SCT. After myeloablative allogeneic transplantation, 60-70% of patients who are CMV seropositive will experience reactivation and without ganciclovir prophylaxis or pre-emptive therapy, 20-30% of these will develop end-organ disease (Hakki, *et al* 2003). In the study reported here, CMV reactivation occurred in 9 out of 12 patients (75%), in whom either recipient or donor was seropositive prior to transplant, which is comparable to the unit's experience with myeloablative allogeneic transplantation. In all cases, reactivation occurred before 100 days post transplant. Detection of CMV reactivation was based on weekly molecular surveillance of CMV nucleic acid in patient whole blood samples. Patients with CMV reactivation (2 consecutive qualitative PCR assays being positive or CMV genome copies exceeding 500/ml) received pre-emptive outpatient treatment with intravenous ganciclovir 5mg/kg, which was continued until the genome copy number by PCR was undetectable. Patients who did not tolerate ganciclovir (due to neutropenia or renal impairment) or who failed to respond to it received intravenous foscarnet (180 mg/day) instead, until detectable virus was eradicated.

Factors that are thought to influence the recovery of CMV-specific CD4+ and CD8+ function following transplant include the source of stem cells (BM vs. PBSC), age, GVHD, steroid use, conditioning regimens, ganciclovir use, HLA matching, circulating CMV antigenaemia, absolute CD4+ and CD8+ cell counts and donor CMV serology. In the group of patients reported here, 8 out of the 9 patients who reactivated CMV were seropositive, whereas only 1 was seronegative, but had a seropositive donor (Table 2.5).

Patient	Recipient CMV status	Donor CMV status	Stem cell source	CD8+ count at 3 months	CD4+ count at 3 months	Acute GVHD
4	+	+	BM	Low	Low	Grade II
5	+	-	BM	Low	Low	No
6	+	+	PB	Normal	Low	No
7	+	-	PB	Low	Low	No
10	+	+	PB	Low	Low	No
11	+	+	PB	Normal	Low	No
14	+	+	PB	Low	Low	No
16	+	+	BM	Low	Low	No
18	-	+	PB	Low	Low	Grade II

Low CD8+ or CD4+ count refers to a value below the normal range.
Abbreviations:
CMV: cytomegalovirus; BM: bone marrow; PB: Peripheral Blood

Table 2.5. CMV status, stem cell source, CD4+ and CD8+ cell counts and incidence of GVHD in patients who reactivated CMV within the first 100 days post transplant.

In this study, the source of stem cells did not show a significant association with incidence of CMV reactivation. Other studies have shown improved qualitative and quantitative T cell restoration after PBSCT compared to BMT, due to the higher lymphocyte content of PBSC (Ottinger, *et al* 1996), although this finding was not demonstrated to influence the risk of infectious complications. In the present study (refer to section 4.3.2 for full discussion), every patient demonstrated persistently low absolute numbers of CD4+ cells (including those who did not reactivate CMV), and all but 2 patients who reactivated CMV had low absolute numbers of CD8+ cells for up to a year after transplant. This prolonged lymphopenia is likely to be due to the alemtuzumab used in the conditioning therapy. With regard to acute GVHD, only 2 out of the patients who reactivated CMV experienced GVHD (grade II), both of whom responded to topical steroids. No patient received systemic steroid therapy within the first 100 days of transplantation. There was no incidence of CMV end-organ disease in this study, and no deaths due to CMV.

Other documented viral infections included parainfluenza III (PFIII) virus infection in 4 patients, herpes simplex virus (HSV) infection in 3, varicella zoster virus (VZV) infection in the form of shingles in 2, respiratory syncytial virus (RSV) infection in 2 patients and adenovirus, influenza A and human herpes virus 7 (HHV7) infection in one patient each. These viral infections occurred in 5 of the 9 patients who reactivated CMV, and 5 patients who did not. All episodes of infection occurred within the first 100 days of transplant. None of these infections resulted in significant morbidity, and there were no deaths as a result.

2.4.2 Other infections

One patient (Patient 10) developed tuberculosis (TB) affecting a lymph gland at 10 months post transplant; she received and has responded to standard anti-TB therapy.

2.4.3 Chimerism status and GVHD

Trilineage full donor chimerism was achieved in 15 patients after a median of 9 months post transplant (range 3-18 months). Of these, 13 patients converted after a median of 1 DLI (range 1-4) and 2 converted before receiving DLI. Four patients (3 post DLI, 1 pre-DLI) remain mixed chimeras at a median of 12 months (range 8-18 months) post transplant. The patient who did not receive DLI showed trilineage chimerism at 3, 6 and 9 months post transplant. At 12, 16 and 19 months, she had mixed chimerism in the T lineage, before spontaneously reverting back to full donor chimerism at 22 months post transplant. Throughout this period, she had stable disease, but then showed disease progression at 29 months post transplant.

Five patients developed grade I-II acute skin GVHD post transplant, all of whom responded to topical steroid therapy. No patient developed grade III/IV GVHD post transplant. Five out of 18 patients who received DLI developed grade II-IV acute GVHD following DLI, including 2 who had experienced grade I acute GVHD post transplant. With regard to stem cell source, 5 out of 8 unrelated donor/ bone marrow stem cell recipients experienced some form of GVHD following transplant or DLI compared to 2 out of 11 sibling donor/ peripheral blood stem cell recipients. Those who experienced up to grade II GVHD of the skin following transplant or DLI responded to topical steroid application. Two

patients with grade III GVHD of the gut and liver following DLI responded to thalidomide and intravenous steroids respectively, whilst 2 patients who developed grade IV GVHD of the liver following DLI died of their GVHD despite treatment with high dose intravenous steroids and ATG. The third patient who developed grade IV GVHD of the skin and liver responded to a combination of high dose intravenous steroids, infliximab, dacluzimab and reinstatement of ciclosporin therapy, and remains in a PR.

The incidence and treatment of GVHD following transplant and DLI in this patient group, and its influence on immune reconstitution are addressed in detail in Chapter 7.

2.4.4 Disease response

Six patients achieved a PR following transplant. Five of these received DLI, resulting in a CR in one case, a further PR in another, no change in 2 patients and disease progression despite DLI in 1 patient. One patient did not receive DLI due to donor withdrawal, but achieved a PR following transplant and maintained this level of response without further intervention until day 895, when she showed evidence of disease progression.

Following transplant, 2 patients showed no change (NC) in their disease status, one of whom subsequently achieved a PR following DLI administration. Three patients had a MR to the transplant, of which 2 achieved a PR and 1 showed NC after DLI. Of the 8 patients who showed evidence of PD following the transplant, but before DLI, 3 showed NC in disease status after DLI and 2 progressed further in spite of DLI, whereas 3 patients achieved a PR following DLI administration.

Ten patients experienced GVHD in this study. Out of these 10 patients, 8 eventually demonstrated a disease response alongside clinical evidence of GVHD. This included those with GVHD following transplant (n=5): 3 (patients 4, 9, 19) had a PR following transplant, whereas 2 (patients 17, 18) initially had PD. These 2 patients with PD subsequently went on to show a response to DLI (being converted to a PR), accompanied by worsening of their GVHD

(developing grade III & IV GVHD following DLI). Patient 17 subsequently died of GVHD, whereas patient 18 subsequently died from PD. Out of the other 3 patients, 1 showed a further PR following DLI, another showed no change in disease status and the third did not receive DLI due to donor withdrawal. Three responding patients developed GVHD for the first time following DLI (patients 3, 11, 12). In all 3 cases, the disease response was improved by DLI as well as being associated with GVHD. These findings demonstrate that the GVM effect is frequently obtained at the expense of GVHD, which can be dangerous and life-threatening. The latter may also occur without evidence of the former such as the 2 patients (patients 8 & 16) who showed progressive disease following transplant in the absence of any GVHD, but went on to develop grade III or IV GVHD following DLI, without a demonstrable disease response. One subsequently died of GVHD and the other of progressive disease.

On the other hand, 2 patients in this study showed evidence of the GVM effect in the absence of clinical demonstrable GVHD (patients 5 & 6). These 2 patients showed little or no clinical response to the conditioning procedure/ transplant, but went on to demonstrate a PR following DLI in the absence of GVHD. In 3 other patients (patients 13, 14 & 15), there was evidence of a disease response to transplantation in the absence of GVHD, but this response may in part be due to the cytoreductive effects of the conditioning therapy rather than a GVM effect post transplant.

Overall, 16 patients (84%) have shown evidence of disease progression following transplant and/ or DLI after a median of 283 (range 153-895) days. Of these, 7 have died from their progressive disease and 7 remain alive on thalidomide-containing salvage therapy. Two patients have achieved a PR, and 1 has stable disease following the transplant/ DLI protocol and continue to show freedom from progression at 708, 978 and 1097 days respectively.

2.5 Discussion

The rationale behind the use of reduced intensity conditioning regimens for allogeneic transplantation in myeloma is to combine reduction of toxicity with the exploitation of an allogeneic graft-versus-myeloma response. In order to

determine if such strategies have a place in improving the clinical outcome of myeloma, a clear understanding of the kinetics and quality of immune recovery following such procedures is needed. This novel treatment modality was explored in this group of patients because of their relatively young age and because many of them had required more than 2 lines of prior therapy to induce a degree of remission or had relapsed following previous autograft. The non-relapse mortality of this regimen was low, confirming its potential applicability in patients up to the age of 59 years, an age which is well beyond the limit of myeloablative stem cell transplantation.

In keeping with the delayed CD4+ and CD8+ T cell recovery, this patient group experienced a high incidence of viral infections, especially CMV reactivation, which occurred in 75% of seropositive patients and this was a main source of morbidity. However, there was no incidence of CMV end-organ disease or CMV-related deaths. In the context of non-myeloablative conditioning, the degree of immune impairment and the number of infectious complications post transplant is likely to reflect the immunosuppression used, and the incidence and severity of GVHD. Thus, while studies on a fludarabine-based low intensity regimen reported lower infection-related mortality and CMV disease compared with myeloablative conditioning (Giralt, *et al* 2002) others have found no difference between non-myeloablative and myeloablative protocols in the incidence of CMV antigenemia requiring treatment or invasive fungal infections (Oh, *et al* 2004). The low infection-related morbidity and mortality in our series probably reflects the low incidence of post transplant GVHD in this group, as well as the reduced toxicity of the conditioning regimen.

Although this report describes clinical and immunological outcome in a patient group with a single disease entity, there are heterogeneities within the patient group that deserve mention. Eight patients received bone marrow-derived stem cells from an unrelated donor (of whom 5 experienced some form of GVHD), whereas 11 patients received peripheral blood stem cells from HLA-identical siblings (of whom 2 experienced GVHD). The higher incidence of GVHD in unrelated donor transplants compared to sibling donor transplants probably reflects the greater degree of HLA disparity rather than the stem cell source.

Although T cell subset recovery was delayed in patients who experienced GVHD compared to those that did not, there was no significant difference in clinical outcome in terms of infection rate, disease response or survival after transplant attributable to stem cell source.

Reduced T cell function post transplant however has critical implications for the generation of an effective GVM effect, and may compromise disease control. Conversely, less immunosuppressive regimens may achieve more disease responses, but at the expense of increased rates of GVHD. The effective in vivo T-cell depletion achieved by this alemtuzumab-containing conditioning regimen, confirmed by the demonstration of persisting lympholytic concentrations of alemtuzumab (Morris, *et al* 2003) resulted in a low incidence of GVHD, but a high rate of disease progression.

In conclusion, this study indicates that this alemtuzumab-containing nonmyeloablative preparative regimen results in a low incidence of GVHD, a high incidence of viral infections and poor disease control. However, the infection-related mortality is low, perhaps due to the reduced toxicity of the conditioning regimen, and the low incidence of GVHD. If RIT is to have a place in the overall management of patients with MM, conditioning regimens need to be developed, that are less immunosuppressive thus allowing the earlier recovery of T cell immunity. A detailed analysis of immune reconstitution (discussed in Chapters 4, 5 and 6) has been undertaken in this study with the intention of providing information about the kinetics of immune recovery and its influence on the clinical outcome, including disease response, the incidence of infective complications and GVHD.

The influence of immune reconstitution on the generation of a graft-versus-myeloma effect will be discussed in more detail in Chapter 7.

Chapter 3. General Methods and Materials

In this chapter, the general methods and materials employed in the study are described. More specialised techniques are described in detail in the chapter pertaining to the subject under discussion.

3.1 Isolation of peripheral blood mononuclear cells

Blood samples were obtained from patients prior to transplant, and at 3-month intervals thereafter and anticoagulated with ethylenediaminetetraacetic acid (EDTA). All the laboratory methods outlined below were carried out using peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation through Ficoll-Paque (Pharmacia, St Albans, UK).

3.2 B, T and NK cell quantification by flow cytometry

PBMC were isolated by density centrifugation and washed and resuspended in Hanks/ 2% fetal calf serum (FCS) at a cellular concentration of 5×10^6 /ml. Monoclonal antibodies (mAbs) specific for CD19, CD3 and CD56 antigens conjugated to fluorescein isothiocyanate (FITC) and phycoerythrin (PE) or peridinin chlorophyll protein (PerCP) were used to identify B cells, T cells and NK cells respectively. T cell subsets were further characterised using three-colour flow cytometry to evaluate the expression of CD4 and CD8, and CD45RA (naïve cells) and CD45RO (memory cells). 50 μ l aliquots of PBMC suspension were incubated with 5 μ l of each antibody for 20 minutes at room temperature (or on ice if the incubation time was longer). In the case of identifying CD45RA+ and CD45RO+ cells, the volume of PBMC and antibody aliquots were doubled to take account of the anticipated lower numbers of these subsets. Following incubation with the mAbs, 2 mls of **cold** phosphate buffered saline (PBS) /0.5% bovine serum albumin (BSA)/ 0.02% azide were added; the tubes were then spun at 1500 rpms for 5 mins, decanted, blotted and resuspended. Fixation was achieved by the addition of 250-300 μ l 2% buffered paraformaldehyde (PF) (PBS/2%PF).

All mAbs were obtained from Becton Dickinson (Oxfordshire, UK). The stained and fixed samples were analysed on a Coulter EPICS Elite Flow Cytometer (Beckman Coulter, Buckinghamshire, UK) following acquisition of 50000 events per sample. The absolute numbers of each phenotypically defined cell type per

microlitre was determined by multiplying the numbers of lymphocytes per microlitre by the percentage of nucleated cells that fell within the appropriate gate.

For each sample, the tubes were set as shown in Table 3.1:

	*PMT2	*PMT3	*PMT4
	FITC	PE	PERCP
TUBE 1	CD3	G1	G1
TUBE 2	G1	CD4	G1
TUBE 3	CD3	CD4	CD8
TUBE 4	CD3	HLADR	CD4
TUBE 5	CD19	CD4	CD8
TUBE 6	CD3	CD56	CD8
TUBE 7	CD3	CD14	CD4
TUBE 8	G1	G1	CD4
TUBE 9	G1	G1	CD8
TUBE 10	CD45RA	CD45RO	CD4
TUBE 11	CD45RA	CD45RO	CD8

Table 3.1 Lymphocyte subsets phenotype panel
*PMT= Photo-multiplier tube

3.3 Chimerism analysis

This was undertaken pre-transplant, then at 3-monthly intervals following transplant, to determine the pattern of recipient, mixed and donor chimerism following transplantation.

3.3.1 Proteinase K digestion: isolation of genomic DNA

Prior to transplant, donor and pre-transplant recipient PBMC were isolated for the analysis. Following transplant, recipient PBMC and buffy coat were used. T cell, B cell fractions were isolated from PBMC by incubation on ice with anti-CD3 and anti-CD-19 conjugated beads (Dyna) for 30 minutes at a bead-to-cell ratio of 4:1, followed by positive selection using a magnet. The myeloid fraction was obtained from buffy coat. Genomic DNA was isolated from the various cell fractions using Proteinase K digestion:

The lysis buffer (total volume 1ml) consisted of:

20 mM DTT (400 µl)

1.7 μ M SDS (200 μ l)
50 μ g/ml Proteinase K (200 μ l)
VNTR buffer (200 μ l).

The VNTR buffer was composed of:

45mM Tris HCl (450 μ l)
11mM NH_4SO_4 (110 μ l)
6.7 mM 2-mercaptoethanol (134 μ l)
4.5 μ M EDTA (45 μ l)
110 μ g/ml BSA (110 μ l)
 H_2O to make volume up to 1ml.

10 μ l of PBMC or the isolated cell fractions were added directly to the PCR tubes (inclusive of Dyna Beads) at a concentration of 10^5 cells/tube, together with 10 μ l of lysis buffer. This mixture was covered with 50 μ l of mineral oil to prevent drying and incubated at 55°C for 1 hour followed by 5 minutes at 95°C to inactivate the proteinase K enzyme.

3.3.2 Microsatellite PCR method

A PCR reaction was then used to amplify microsatellite regions of genomic DNA using the following primer sets that flank highly polymorphic short tandem repeats on different chromosomes: VWA31 (Perkin Elmer), THO1 (Perkin Elmer), F13A1 (Perkin Elmer) and ACPP (forward: ACTGTGCCTAGCCTATACTT; backward: AGTGAGCCAAGAGTGC ACTA), HUMSTRX1 (forward: CTCCTTGTGGCCTTCCAAATGG; backward: CTTCTCCAGCCCCAAGGAAGTCA) as previously described (Mackinnon, *et al* 1992). Primers that gave rise to recipient/ donor-specific peaks were identified and used for post-transplantation determination of chimeric status in the different cell populations.

Two PCR master mixes were used depending on the primer sets.

The PCR master mix for primers VWA31, THO1, and F13A1 comprised the following reagents:

5 μ l Geneamp PCR II Buffer (Perkin Elmer)

3 μl MgCl_2 , 4 μl , dNTP
1 μl of each primer
0.2 μl Amplitaq DNA polymerase (Perkin Elmer)
25.8 μl H_2O
10 μl DNA.

Cycling conditions were as follows:

Stage 1: 94°C for 3 minutes 1 cycle

Stage 2: 94°C for 45 seconds
 54°C for 45 seconds 30 cycles
 72°C for 1 minute

Stage 3: 72°C for 1 minute 1 cycle

For the ACPP and HUMSTRX1 primers the reagents used were as follows:

4 μl VNTR Buffer
10 μl MgCl_2
8 μl dNTP
1 μl of each primer
0.2 μl Amplitaq DNA polymerase (Perkin Elmer)
15.8 μl H_2O
10 μl DNA.

Cycling conditions were as follows:

Stage 1: 95°C for 5 minutes 1 cycle

Stage 2: 95°C for 30 seconds
 58°C for 30 seconds 30 cycles
 72°C for 45 seconds

Stage 3: 72°C for 10 min 1 cycle

The forward primer of each pair were labelled with either JOE or FAM fluorescent dyes. Once the primers and Taq polymerase were thawed they were kept at 4°C. The PCR reaction volume was 50 µl. To each microfuge tube, 40 µl of master mix were added, followed by 10 µl of each DNA sample. Two drops of mineral oil were added and the tubes were centrifuged for 15 seconds at 13000 rpm. 1µl of PCR product was denatured in 12 µl of formamide and electrophoresed through Performance Optimised Polymer 4 (Perkin Elmer) on an ABI 110 automated sequencer (Perkin Elmer) in the presence of Rox 500 size standard (Perkin Elmer). Genescan software 2.1 (Perkin Elmer) was used to analyse the data.

Extensive measures were taken to minimize contamination. All samples were handled with disposable gloves, which were changed after any spillage and at frequent intervals. Preparation of blood samples, DNA extraction, and PCR preparation were performed in a laminar flow hood. All reagents and PCR reactions were prepared using pipette tips incorporating filters to prevent aerosol contamination. The thermal cycler and reaction tubes containing PCR product were kept in a separate room from where samples were handled, and separate pipettes and racks were used in the PCR-designated room. A negative control with no DNA was run with every assay.

Chapter 4: Recovery of NK cell numbers and T cell subsets and function

4.1 Introduction

Reconstitution of NK and T cell functions is essential to the success of stem cell transplantation. Natural killer (NK) cells and T cells play an important role in normal innate and adaptive immunity. NK cells are large granular lymphocytes that kill tumour cells and virus-infected cells in a non-specific manner; that is, they do not require previous sensitisation or the presence of antibody to be cytotoxic. Their granules contain pore-forming proteins that can mediate cell lysis. NK cells express a number of surface markers, including CD56 and CD16. The CD56 antigen is present on approximately 10-25% of peripheral blood lymphocytes. It is present on all resting and activated NK cells and approximately 5% of CD3+ peripheral blood lymphocytes (Lanier, *et al* 1986). CD3+CD56+ T lymphocytes comprise a unique subset of cytotoxic T lymphocytes that mediates non-MHC-restricted cytotoxicity (Lanier, *et al* 1986). So-called natural killer T cells (NKT cells) have been identified in both murine and human tissues and may have an important role in immune surveillance and regulation (Mendiratta, *et al* 1997, Yoshimoto, *et al* 1995). Two subsets have been identified to date: one subset expresses CD4 or is CD4 and CD8 double-negative and also co-expresses the NK marker NK1.1. This population is usually found in the liver and thymus and produces large amounts of IL-4 when stimulated (Baker, *et al* 2001). The other subset co-expresses CD8 and NK1.1 and has a more diverse and variable TCR repertoire (Baker, *et al* 2001). Little is known about the CD8+ population, but the CD4+/ CD4-CD8- population has been found to suppress GVHD and is therefore of potential interest in allogeneic transplantation (Zeng, *et al* 1999). More recently, the detailed characterisation of NK cell receptors (killer cell immunoglobulin-like receptor (KIR), natural cytotoxicity receptors (NCR) and C-type lectins) has contributed to the understanding of the graft-versus-disease effect, such that it may become possible to manipulate receptor/ligand interactions to prevent disease relapse post transplant (Farag, *et al* 2002).

4.1.1 T cell development

T cell development in the thymus begins with the lodgement of circulating bone marrow-derived stem cells in cortex of the thymus. From the cortex the developing thymocytes migrate to the medulla, and finally arrive in the peripheral lymphoid system via the lymphatics or veins (Figure 4.1).



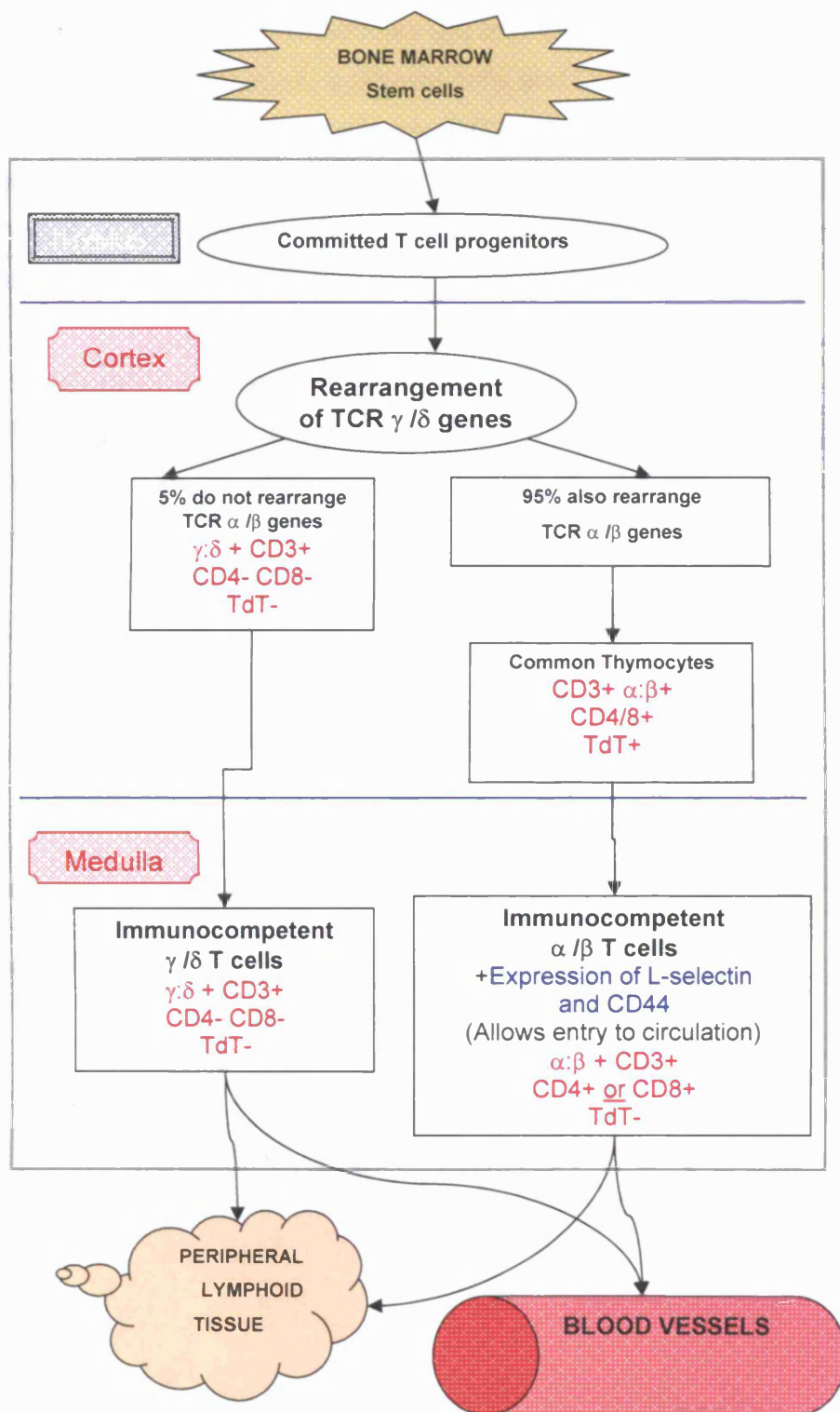


Figure 4.1. The role of the thymus in T cell development

The thymic environment provides stimuli that are required for the proliferation and development of thymocytes. Many of these stimuli come from non-lymphoid cells also found in the thymus, including thymic epithelial cells, bone marrow-derived macrophages and dendritic cells (DC's). Major histocompatibility complex (MHC) molecules and cytokines are important for T cell maturation.

Different classes of MHC molecules are expressed by non-lymphoid thymic cells: cortical macrophages, epithelial cells and DC's express high levels of class II; medullary epithelial cells and DC's express both class I and II; and medullary macrophages express high levels of class I molecules. Cytokines, such as IL-4, IL-6 are produced by thymic stromal cells and act to stimulate the proliferation of immature T cells.

T cell maturation proceeds through sequential stages that mirror T cell receptor (TCR) gene rearrangement (Figure 4.2):

Figure 4.2. The stages of gene rearrangement in α : β T cells. (overleaf)

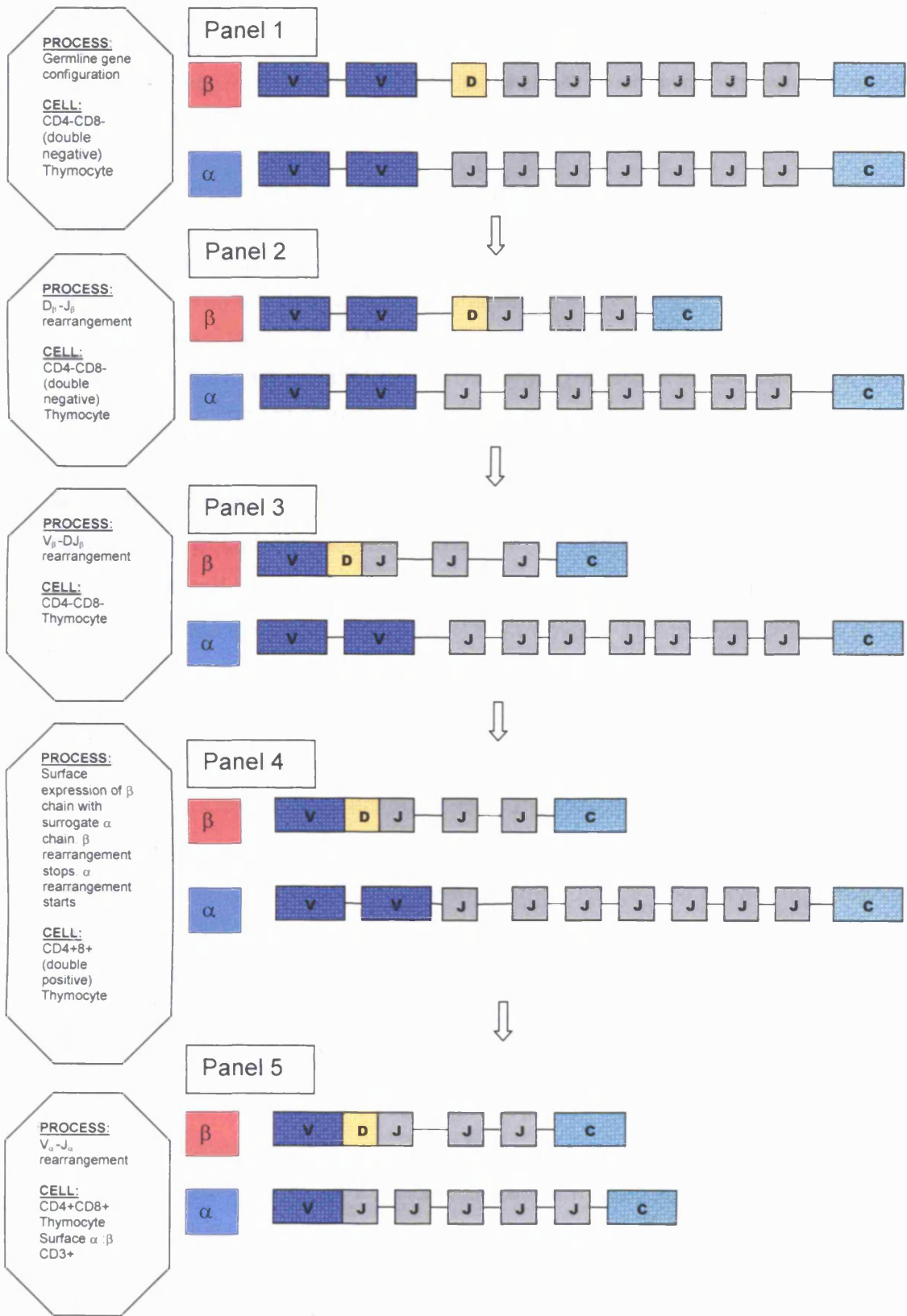


Figure 4.2. The stages of gene rearrangement in α : β T cells. (Figure legend overleaf)

Figure 4.2 Figure legend. The stages of gene rearrangement in α : β T cells. The sequence of gene rearrangement is shown, together with an indication of the stage at which events take place and the nature of the cell surface receptor molecules expressed at each stage:

The T cell receptor (TCR) β - chain genes rearrange first in CD4-8- double-negative thymocytes. Diversity (D) to joining (J) rearrangements precede variable (V) to DJ rearrangements. The productively rearranged gene is expressed initially within the cell and then at low levels on the cell surface in a complex with CD3 chains (Panels 2 and 3).

The expression of the TCR β : CD3 complex signals via the tyrosine kinase Lck to the developing thymocyte to express CD4 and CD8 and to rearrange the α chain genes as well as to halt β chain rearrangement (Panel 4 and 5).

which may be defined by the expression of the TCR and CD4 and CD8 co-receptors or accessory molecules (Blue, *et al* 1988). Once in the thymus, bone marrow-derived committed progenitors do not express TCR, CD3, CD4 or CD8, and are termed double-negative (DN) thymocytes. The majority (>90%) of DN thymocytes will give rise to $\alpha\beta$ TCR- rather than $\gamma\delta$ TCR -expressing, MHC-restricted CD4⁺ and CD8⁺ cells. At the next stage of maturation, thymocytes express both CD4 and CD8 (double-positive, DP, thymocytes). The expression of CD4 and CD8 is essential for subsequent selection events and is regulated by TCR binding to antigen. The selection of developing T cells is stimulated by recognition of antigen-MHC complexes in the thymus and serves to preserve cells that recognise foreign peptides presented by self-MHC and to eliminate potentially harmful cells (those that recognise ubiquitous self-antigens with high avidity and the potential for autoimmunity). Positive selection is the process whereby low avidity binding of TCR to self-peptide-MHC complexes stimulates thymocyte survival, while lack of binding by TCR leads to cell death. This ensures that mature T cells are self-MHC restricted. Positive selection also determines class I or class II MHC restriction of T cell subsets, such that CD8⁺ cells are specific for class I and CD4⁺ cells for class II-associated peptides (Cosgrove, *et al* 1992). Negative selection is the process by which thymocytes whose TCR's bind with high avidity to self-MHC molecules are killed. The end result of these selection processes is that the repertoire of mature cells is self-MHC restricted and tolerant to many self-antigens. Cells that successfully undergo these selection processes go on to mature into CD4⁺ or CD8⁺ (single-positive) T cells. CD4⁺ cells acquire the ability to produce cytokines in response

to subsequent antigen stimulation and to express effector molecules such as CD40 ligand that 'help' B cells to produce antibody. CD8⁺ cells on the other hand become capable of producing perforins and granzymes that lyse other cells.

The rate of T cell development in the thymus is greatest before puberty, after which the thymus begins to shrink and the production of new T cells in adults is lower. The role of the thymus in T cell reconstitution in adults after stem cell transplantation is controversial (Mackall, *et al* 1995), but Douek *et al* have recently provided evidence for a more substantial contribution to T cell immune reconstitution in this setting than was previously thought, using an assay which allows accurate measurement of thymic output using TCR excision circles (Douek, *et al* 2000).

4.1.2 Circulating CD3⁺, CD4⁺ and CD8⁺ T cells

The CD3 antigen is present on 61-85% of normal peripheral blood lymphocytes and 60-85% of normal thymocytes (Reichert, *et al* 1991). It consists of a complex of proteins, which is stably associated with the TCR on the surface of T cells. Ligation of the TCR/CD3 complex by antigen leads to signalling via the cytoplasmic tail of CD3 that results in the activation of tyrosine phosphorylation-dependent signalling pathways. Support for the role of the CD3 complex in signal transduction is strengthened by the observation that mAb to CD3 can activate T cells in the absence of antigen-MHC recognition by the TCR (Kaneoka, *et al* 1983). The CD3 complex is also required for the cell surface expression of the TCR. CD4 and CD8 molecules also play an essential role in T cell activation (see section 4.1.3).

The CD4 antigen is normally found on the surface of 28-58% of peripheral blood lymphocytes (Reichert, *et al* 1991) and 80-95% of normal thymocytes and is also present in low density on the cell surface of monocytes and macrophages (CD3-CD4⁺) (Evans, *et al* 1981). CD4 T cells recognise antigens presented by MHC class II molecules. CD4 T cells are mainly cytokine-secreting helper cells, and can be divided in to two major types, based on patterns of cytokine secretion (see section 4.1.5).

CD8 is normally expressed on 19-48% of peripheral blood lymphocytes (Reichert, *et al* 1991) and 60-85% of normal thymocytes (Evans, *et al* 1981). The CD8 antigen is also expressed on a subset of natural killer (NK) cells (Lanier, *et al* 1986). CD8 cells recognise antigen presented by MHC class I molecules, which are expressed on all nucleated cells. Cytotoxic cells bind to the antigen-MHC complex and then kill the infected cell by inserting perforins into the cell membrane and injecting granzymes or by binding to the Fas molecules to induce apoptosis. In addition to killing infected cells directly, CD8 cells also produce a number of cytokines, including tumour necrosis factor (TNF)- α and INF, and appear to have distinct subsets akin to Th1 and Th2 subtype of CD4 cells, termed Tc1 and Tc2 cells (Maggi, *et al* 1994, Salgame, *et al* 1991) but their respective roles remain unclear.

4.1.3 Role of accessory molecules in T cell activation

T cells express several integral membrane proteins other than members of the TCR complex, which play a crucial role in antigen recognition and T cell activation. These molecules are called accessory molecules and were first characterised by the use of mAbs raised against T cells. Unlike the TCR, each accessory molecule is identical on all T cells in all individuals and so have no capacity to specifically recognise antigens. The majority of these molecules are members of the immunoglobulin, integrin and selectin families of proteins. Ligand binding to these accessory molecules results in signal transduction that is thought to act in concert with signals generated by the TCR-CD3 complex due to antigen binding. Another important property of accessory molecules is their binding to endothelial cells (EC) and extracellular matrix (ECM) proteins and consequent homing of T cells to tissues and the retention of T cells in tissues. Table 4.1 shows the properties of the principal accessory molecules of T cells.

Name of Molecule	Biochemical Features	Cellular Expression	Ligand	T cell Signalling
CD4	55-kD monomer	Class II-restricted T cells	Class II MHC	+
CD8	78-kD alpha chain 32-kD beta chain	Class I-restricted T cells	Class I MHC	+
CD28	Homodimer of 44-kD chains	>90% CD4+ T cells 50% CD8+ T cells	B7-1 (CD80) and B7-2 (CD86)	+
CTLA-4 (CD152)	Homodimer of 33 kD chains May be monomer >90% is intracellular	Activated T cells	B7-1 (CD80) and B7-2 (CD86)	+
CD2	50 kD monomer	>90% T cells NK cells	Leucocyte function-associated antigen (LFA)-3 (CD58)	+
CD44	80-200 kD monomer	Lymphocytes Granulocytes	Matrix proteins	+
L-selectin (CD62L)	150-kD monomer	Leucocytes	Carbohydrate ligands on endothelial venules	-
CD40	Homodimer of 44-kD chains	B cells Macrophages Endothelial cells Dendritic cells	CD40 ligand (CD154)	-

Table 4.1. Principal accessory molecules of T cells.

- CD4 and CD8.** Apart from their important role in selectively binding class II or class I MHC molecules, CD4 and CD8 participate in the early signalling events that occur in T cell recognition of peptide-MHC complexes on antigen presenting cells (APC's) (Gallagher, *et al* 1989). These signal-transducing functions are mediated by a T cell-specific Src family tyrosine kinase called Lck that is closely associated with the cytoplasmic tails of both CD4 and CD8 (Kiefer, *et al* 2002). When a T cell recognises peptide-MHC complexes by its antigen receptor, simultaneous interaction of CD4 or CD8 with the MHC molecule brings the co-receptor and its associated Lck close to the

TCR complex. Lck then phosphorylates tyrosine residues in an important conserved sequence motif of the CD3 cytoplasmic domain known as the immunoreceptor tyrosine-based activation motif (ITAM), thus initiating the T cell activation cascade.

2. **CD28 and CTLA-4.** CD28 is a membrane protein that transduces signals that function together with signals delivered by the TCR complex to activate naïve T cells. Naïve T cells need 2 distinct extracellular signals in order to initiate their proliferation and differentiation into effector cells, (Janeway and Bottomly 1994) the first of which is provided by binding peptide-MHC to the TCR. The second signal for T cell activation is provided by so called costimulatory molecules, the best defined being B7-1 (CD80) and B7-2 (CD86) for T cells (June, *et al* 1994). These B7 costimulators are expressed on APC's, and are specifically recognised by receptors on T cells, CD28 and CTLA-4 (CD152). CD28 is constitutively expressed on >90% of CD4+ T cells and 50% of CD8+ T cells. Binding of B7 molecules to CD28 delivers signals to the T cells that induce the expression of anti-apoptotic proteins, stimulate production of growth factors and other cytokines and promote T cell proliferation and differentiation (Linsley and Ledbetter 1993). The second receptor, CTLA-4 is structurally homologous to CD28, but expressed on recently activated CD4+ and CD8+ cells. Its function is to inhibit T cell activation by counteracting signals delivered by CD28, and terminating T cell responses (Walunas, *et al* 1996).
3. **CD2.** This glycoprotein is present on >90% of mature T cells, 50-70% of thymocytes and on NK cells. The principle ligand for CD2 is a molecule called leucocyte function-associated antigen-3 (LFA-3), which is expressed on a wide variety of haematopoietic and non-haematopoietic cells. CD2 functions as an intercellular adhesion molecule and a signal transducer.
4. **CD40 and CD40 ligand.** Initially recognised for its role in B cell activation, the CD40: CD40 ligand system has been acknowledged as a key pathway for T cell activation as well (Durie, *et al* 1994) CD40 is expressed on B cells, macrophages and DC's as well as other cell types such as EC's (Reul, *et al* 1997). The ligand for CD40 (CD40 ligand or CD154) is expressed on activated CD4+ cells. Stimulation of CD40 provides important signals for antibody production and isotype switching by B cells and induces B7 expression on APC's. Thus the CD40: CD40 ligand system may have an

important role in T cell co-stimulation. Activation of APC's through CD40 also induces the expression of adhesion molecules and inflammatory cytokines that participate in T cell activation (Guo, *et al* 1996).

4.1.4 T cell activation

T cell activation refers to the immune activation of mature T cells in peripheral blood, lymphatic system or tissues. During this process, which is initiated by MHC-restricted binding of foreign antigen, T cells undergo a sequence of genetic and phenotypic changes, which results ultimately in clonal expansion and the induction of effector functions (Collins 2000). The antigen specificity of the response is dictated by the TCR, and accessory molecules play a crucial role in amplifying signals that contribute to T cell activation as stated above. Activated T cells then differentiate into effector cells, including the Th1 and Th2 subsets of CD4+ cells and CD8+ cytotoxic lymphocytes (CTL's). The differentiation of T cells is associated with transcriptional activation of genes encoding (1) effector molecules, such as cytokines and CTL granule proteins, which are released from the effector cells, and (2) surface molecules such as CD40 ligand and Fas ligand that have their own effector functions.

4.1.5 Th1 and Th2 classification of immune responses

The identification of 2 polarised T cell subsets, which produce certain combinations of cytokines (Mosmann and Sad 1996) has provided a useful framework for understanding immunological processes such as GVHD. This polarisation was first described in mouse helper cells, but has since been verified in humans and other species.

Type 1 helper (Th1) cells secrete interferon- γ (IFN- γ). Type 2 helper (Th2) cells secrete IL-4, 5, 6 and 10. IL-2 is ubiquitously produced by both Th1 and Th2 cells at some time during their development, and is not considered an exclusively Th1 cytokine. Rather, IFN- γ is regarded as the quintessential type 1 cytokine. Another essential type 1 cytokine is IL-12, which is produced by antigen presenting cells (APC's) such as monocytes and dendritic cells, and is a powerful inducer of IFN- γ production by T cells (Trinchieri 1998). IFN- γ also promotes the production of IL-12, whilst suppressing IL-4 production, so down regulating type 2 cytokines in favour of type 1. IL-10 is the most important Th2

cytokine. It is produced by T cells and monocytes and is strongly anti-inflammatory with potent anti-IFN- γ and IL-12 effects (Hsieh, *et al* 1992).

It is now clear that individual T cells may express various combinations of cytokines and that the Th1/Th2 paradigm may be a simplification of the reality. To date no other phenotypic markers provide definitive classification. Th1 and Th2 subsets do show differences in expression of various cytokine receptors, but such differences may reflect the activation status of the cells and may not be stable. Th1 and Th2 subsets develop from the same precursor, namely naïve CD4⁺ T cells (termed Thp) (Rocken, *et al* 1992). As the maturing T cell develops, the genes for several effector cytokines such as IL-4, IFN- γ and IL-10 start to be expressed (Abehsira-Amar, *et al* 1992). The final response depends on external factors including cytokines, chemokines and adhesion molecules from other T cells and APC's. In most cases these stimuli lead to a skewing of the response in a Th1 or Th2 direction. This is not always the case; the balance of stimuli may result in a clonal T cell population producing both type 1 and type 2 cytokines (the Th0 stage) (Firestein, *et al* 1989). The Th0 stage was initially thought to be a transitory stage between Th1 and Th2, but it is now recognised that mature antigen-specific differentiated type 0 cells do exist, e.g. cells that simultaneously produce IL-10 and IFN- γ (Gerosa, *et al* 1996).

The principal function of Th1 cells is to stimulate phagocyte-mediated defence against intracellular microbes. IFN- γ produced by Th1 cells enhances the microbicidal activities of phagocytes, and the production of opsonising and complement-fixing IgG antibodies, which promote phagocytosis of microbes. IL-2 secreted by Th1 cells acts as their autocrine growth factor and together with IFN- γ , stimulates the proliferation and differentiation of CD8⁺ CTL's, which kill virus and bacteria-infected cells. The principal effector function of Th2 cells is IgE and mast cell-mediated immune reactions. These reactions are induced by IL-4, IL-5 and IL-13. Th2 cells are responsible for defence against helminthic and arthropod infections and for allergic reactions. The antibodies stimulated by Th2 cells do not promote phagocytosis or activate complement efficiently. In addition, several of the cytokines produced by Th2 cells antagonise the actions of IFN- γ and inhibit macrophage activation. Th2 cells may therefore function as

suppressor cells to control immune responses, particularly inflammatory Th1 responses.

Of particular relevance to the immunology of allogeneic transplantation is the role of the Th1/Th2 paradigm in GVHD (Krenger and Ferrara 1996). There is evidence to suggest that acute GVHD (acute GVHD) is the result of Th1 polarisation, with a critical role for IL-2, whereas chronic GVHD (chronic GVHD) may be due to Th2 polarised cells. There are also some studies that suggest Th2 cells can inhibit acute GVHD, but evidence for this is conflicting. This subject will be discussed in further detail in Chapter 7.

Also of importance in the context of this study is the nature of the immune response that prevails in the presence of the myeloma disease state. The reasons for the failure of the immune system to eradicate myeloma cells, especially in the setting of minimal residual disease remains unclear. Myeloma cells may suppress immune responses via mediators such as Fas ligand, sMUC-1 and TGF β (Cook and Campbell 1999). Myeloma cells can stop the initiation of the IL-2 autocrine growth pathway; mitogen or alloantigen induced proliferation, and down regulate activated T cell responses to IL-2. Many of these responses are regulated by TGF β . It is possible that myeloma cells turn off immune responses that could otherwise eradicate the tumour (Cook, *et al* 1999).

4.1.6 Cytokine production by T cells

Fine control of immune reactions by T cells is achieved by the delivery of cytokines in appropriate quantities and combinations to the correct target cells. T cells are the principal producers of most cytokines as well as the autocrine, paracrine or endocrine cellular targets of their actions.

Interleukin-12

Interleukin-12 (IL-12) secreted by activated mononuclear phagocytes and DC's is a pleiotropic cytokine that plays a pivotal role in the induction of a Th1 response, and hence cell mediated immunity (Paul and Seder 1994). Its other immunoregulatory functions include a synergistic action with interleukin-2 (IL-2) in augmenting allogeneic CTL responses, LAK activity and interferon- γ (IFN- γ)

production by peripheral blood lymphocytes. It may also stimulate IFN- γ production from NK and T cells directly, increase the lytic activity of NK cells and expand activated NK and T cells (Tahara and Lotze 1995).

Tumour necrosis factor- α

Tumour necrosis factor (TNF- α) is the principle mediator of the acute inflammatory response to gram negative bacteria and other infectious agents. It is responsible for many of the systemic complications of severe infections and is produced by macrophages/ monocytes, activated T cells and NK cells. The most potent stimulus for TNF- α production by macrophages is bacterial LPS or endotoxin augmented by IFN - γ secreted by T cells and NK cells in response to the same stimulus.

TNF- α acts on vascular EC and leucocytes to stimulate the recruitment of neutrophils and monocytes to sites of infection, and activates these cells to eradicate microbes. It achieves this by inducing vascular endothelial cells to express adhesion molecules, particularly ligands for leucocyte integrins and endothelial selectins. This makes the endothelial cell (EC) surface adhesive to neutrophils, monocytes and lymphocytes. In addition, TNF- α stimulates EC and monocytes to secrete chemotactic cytokines (chemokines), which direct leucocyte migration (Murdoch and Finn 2000).

TNF- α has systemic effects when produced in high enough quantities to enter the blood stream. It induces fever by increasing prostaglandin synthesis by the hypothalamus; it increases synthesis of acute phase proteins by the liver; very large concentrations of TNF- α inhibit myocardial and smooth muscle tone, causing the clinical syndrome of shock. These systemic effects are frequently seen in the post-transplant and other clinical settings related to infection with or without an intact immune system.

Interleukin-2

Interleukin-2 (IL-2) is a growth and differentiation factor for antigen-stimulated T cells and is responsible for T cell clonal expansion after antigen recognition. As detailed above, T cell activation results in the modulation of the surface

phenotype and the regulation of the secretion of critical cytokines (Lustgarten, *et al* 1991). IL-2 is the principal cytokine produced by naïve CD4⁺ (Th1) T cells. It is also produced to a lesser extent by CD8⁺ cells together with costimulators, which promote transcription of the IL-2 gene. T cell growth and clonal expansion is a tightly regulated process and the extent of the proliferative response is determined by the concentration of IL-2 and IL-2 receptor (IL-2R) expression on the cell surface (Taniguchi and Minami 1993). Production is transient (peaking at 8-12 hours after activation) and it acts mainly in an autocrine manner.

IL-2 causes proliferation of antigen-specific cells by the induction of cyclin D and cyclin E, proteins that associate with and activate cyclin-dependent kinases. The kinases phosphorylate and activate a variety of cellular proteins that stimulate transition from G₁ to the S phase of the cell cycle, thus promoting cell cycle progression. IL-2 also promotes cell survival by inducing the anti-apoptotic protein Bcl-2. IL-2 also promotes the proliferation and differentiation of other immune cells; it stimulates the proliferation of NK cells and enhances their cytolytic function producing lymphokine activated killer (LAK) cells. IL-2 is a growth factor for B cells and stimulates antibody synthesis. It also contributes to the termination of the immune response by making activated T cells sensitive to apoptosis by the Fas pathway.

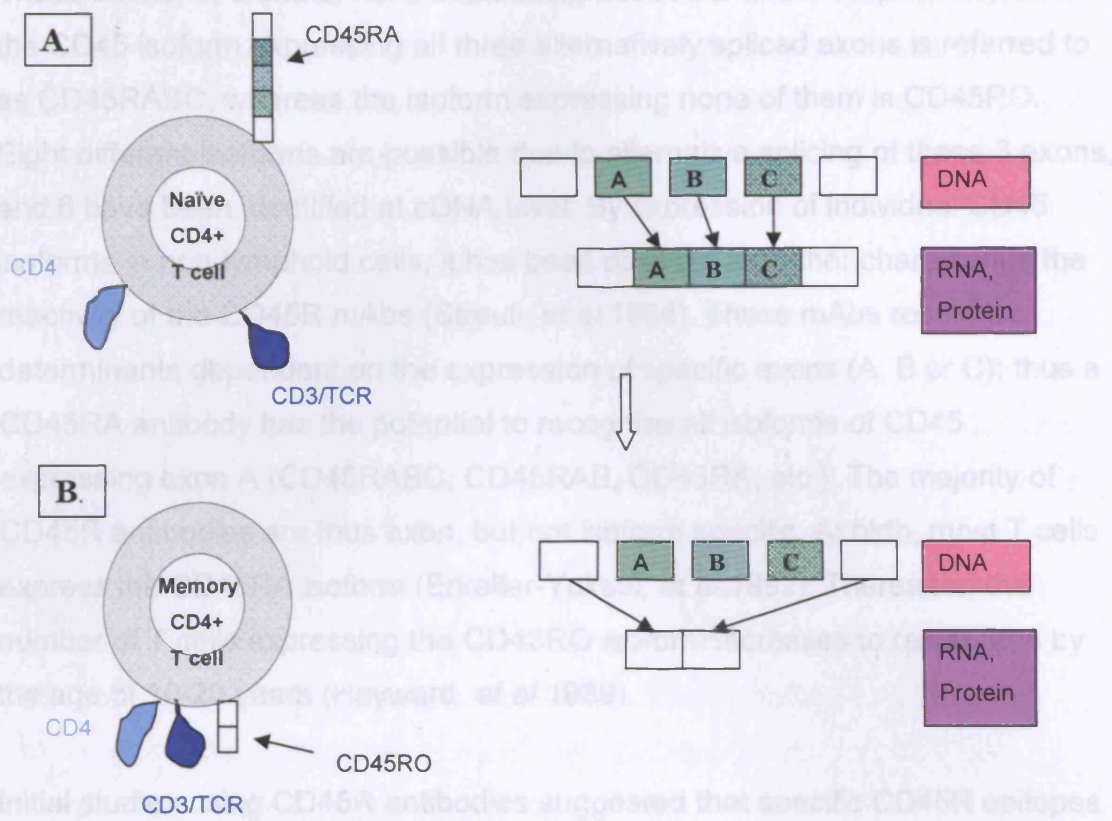
Interleukin-4

Interleukin-4 (IL-4) has important immunoregulatory functions and is the signature cytokine of the Th2 subset (Li-Weber and Krammer 2003). In addition to Th2 cells, activated mast cells and basophils also produce IL-4. IL-4 acts on monocytes and macrophages to change morphology, regulate surface antigen expression and to inhibit antibody-dependent cellular cytotoxicity (ADCC). IL-4 downregulates IL-12 and interferon- γ (IFN- γ) production, thus favouring the development of a Th2 response. IL-4 functions as an autocrine growth factor for differentiated Th2 cells. IL-4 also stimulates B cell immunoglobulin heavy chain class switching to the IgE isotype, which provides defence against helminthic infections, and is the principle mediator of allergic reactions. Evidence that IL-4 does not play a role in lymphopoiesis come from experiments showing that IL-4-deficient mice show normal lymphocyte development and function, but Th2 responses are blocked (Kopf, *et al* 1993).

4.1.7 CD45 isoforms: memory and naïve T cells

To provide protection against new infectious agents as well as recurrence of infections, the immune system maintains separate populations of naïve and memory cells. In addition, it needs to ensure an appropriate balance of CD4 and CD8 cells in both naïve and memory pools. Hence analysis of these separate populations of T cells is critical to the understanding of immune recovery after allogeneic stem cell transplantation.

CD45 (formerly known as the leucocyte common antigen) is expressed on all leucocytes and is a protein tyrosine phosphatase (Tonks, *et al* 1988). Its cytoplasmic tyrosine phosphatase domain is thought to play a role in T cell activation (Ledbetter, *et al* 1988). Characterisation of this major lymphocyte antigen has shown that the CD45 antigens are a family of molecules that show heterogeneity in molecular weight (MW), antigenicity and carbohydrate structures (Andersson, *et al* 1980). Monoclonal antibodies to CD45 can be divided into 2 classes: those that recognise common CD45 determinants on all isoforms of CD45, and those that recognise a restricted determinant present on only a subpopulation of CD45 molecules (CD45R antibodies). When the genomic sequence of CD45 was determined (Hall, *et al* 1988), it was apparent that the differences in MW were due to the differential expression of additional protein sequences close to the amino terminus of the molecule. Addition of this extra protein sequence also resulted in the addition of extra carbohydrate structures (Jackson and Barclay 1989) and created new antigenic determinants. The protein heterogeneity occurred as a result of alternative splicing of 3 exons close to the 5' end of the gene (Figure 4.3).



CD45 is a transmembrane tyrosine phosphatase with 3 exons (A, B and C) that encode part of its external domain. In naïve T cells, high molecular weight isoforms (CD45RA) are found that do not associate with either the T cell receptor (TCR) or its co-receptors, CD4 and CD3, as shown in Figure A. In memory T cells, the variable exons are removed by alternative splicing of CD45 RNA, and this isoform (CD45RO) associates with both the TCR and CD4 and CD3 as shown in Figure B. This assembled receptor appears to transduce signals more effectively than the receptor on naïve cells.

Figure 4.3. Expression of CD45R isoforms on naïve and memory CD4⁺ T cells.

These exons, 4, 5 and 6, have been designated A, B and C respectively, and the CD45 isoform expressing all three alternatively spliced exons is referred to as CD45RABC, whereas the isoform expressing none of them is CD45RO. Eight different isoforms are possible due to alternative splicing of these 3 exons, and 6 have been identified at cDNA level. By expression of individual CD45 isoforms in non-lymphoid cells, it has been possible to further characterise the reactivity of the CD45R mAbs (Streuli, *et al* 1988). These mAbs recognise determinants dependent on the expression of specific exons (A, B or C); thus a CD45RA antibody has the potential to recognise all isoforms of CD45 expressing exon A (CD45RABC, CD45RAB, CD45RA, etc.). The majority of CD45R antibodies are thus exon, but not isoform specific. At birth, most T cells express the CD45RA isoform (Erkeller-Yuksel, *et al* 1992). Thereafter, the number of T cells expressing the CD45RO isoform increases to reach 50% by the age of 10-20 years (Hayward, *et al* 1989).

Initial studies using CD45R antibodies suggested that specific CD45R epitopes might be present on a particular functional T cell population (Bottomly, *et al* 1989, Rudd, *et al* 1987). However further work suggested that isoform expression might correlate better with the activation state of the cell (Bell and Sparshott 1990). For example, loss of CD45RA and gain of CD45RO was shown to occur upon T cell activation, implying that CD45RA reactive isoforms were present on naïve T cells and that CD45RO was a marker for primed or memory T cells (Akbar, *et al* 1988). The different CD45 isoforms on naïve and memory T cells may serve to facilitate the interaction of the T cells with APC's, and their activation by antigen. Recent evidence suggests that the CD45RO, but not the CD45RABC isoforms form heterodimers with CD4 and CD8 and augment TCR signalling phosphorylation events (Dornan, *et al* 2002). In addition, CD45RO preferentially associates with the TCR and enhances T cell activation (Leitenberg, *et al* 1999). One complicating factor is that the loss of CD45RA and gain of CD45RO, which occurs on T cell activation, can be reversible, particularly in the CD8 subset. Continued monitoring of activated cells shows that they can re-express CD45R determinants when these cells enter a resting stage and that these determinants can be lost again upon reactivation (Faint, *et al* 2001, Rothstein, *et al* 1991, Warren and Skipsey 1991)

4.1.8 In vitro assays of T cell function

There are a several techniques for assessing T cell function and activation in vitro. T cells can be stimulated to proliferate in response to polyclonal mitogens such as phytohaemagglutinin (PHA), concavalin A (con A) and pokeweed mitogen (PWM), or when activated by polyclonal stimuli such as anti-CD3 antibodies or phorbol 12-myristate 13-acetate (PMA) with calcium ionomycin. Alternatively, the activation response to a specific antigen, such as CMV may be measured. The readout assays for cell proliferation or cytokine production utilise one of several techniques, such as limiting dilution analysis (LDA), which measures the frequency of lymphocytes for a specific antigen, an enzyme-linked immunoassay (ELISA)-based assay for detecting cytokine-producing cells (ELISPOT), reverse-transcription (RT)-PCR, in situ hybridisation (ISH), and intracellular cytokine staining (ICCS). LDA and ELISPOT assays are laborious procedures that require high levels of technical expertise but give limited information about the nature of activated cells and their abilities to simultaneously secrete multiple cytokines.

ICCS combines immunofluorescent staining with multiparameter flow cytometry and has a number of advantages over the other techniques. By this method, cytokine expression is characterised at the level of the single cell, and the resulting analysis provides information on both the frequency of cytokine-producing cells as well as the cytokine production by individual cells.

Multicolour flow cytometric analysis enables the simultaneous detection of the light-scattering characteristics (forward- and side-scatter) of cells as well as their cytokine profiles and cell surface. In this way, the analysis enables the characterisation of individual cytokine-producing cells. These characteristics may define a cell's activation status, lineage or subset identity, or its capacity to bind other cells and tissues or home into sites of inflammation. Another important advantage of multicolour flow cytometry is that it permits the high-resolution analysis of particular cell types (as defined by the chosen parameters) within heterogeneous cell populations without the need for laborious cell-separation procedures. Since flow cytometry is a high throughput technique, large numbers of cells from different cell samples can be quickly analysed and compared resulting in statistically significant results. This was felt to be an important advantage in studying T cell function in stem cell transplant

recipients, who frequently have low T cell numbers. There are a number of important pitfalls, however. Permeabilisation frequently causes high autofluorescence and many antibodies that usually bind well to their target in other conditions do not work well when cells are permeabilised and/or fixed. Some cytokines seem to be expressed at low levels and appear as a continuous shoulder on a histogram of fluorescence, instead of being a well-separated bimodal distribution. Thus careful use of appropriate positive and negative controls is of paramount importance. Some of these factors are addressed in the later section on optimisation of the technique for this study.

4.2 SPECIAL METHODS

Isolation of peripheral blood mononuclear cells and quantification of CD3, CD4 & CD8 subsets, including naïve and memory subsets, and NK cell numbers by immunophenotypic analysis are described in Chapter 3, General Methods.

4.2.1 T cell function by intracellular cytokine staining and 3-colour flow cytometry

Interleukin (IL)-2, IL-4 and TNF- α production was assessed in the CD4 and CD8 subsets of 5 control subjects and 6 patients.

Since there is little or no spontaneous cytokine production in resting lymphocytes, PBMC were stimulated with PMA and calcium ionomycin to induce cytokine production. This was done in the presence of a protein secretion inhibitor to increase the amount of intracellular cytokine, which would otherwise be secreted out of the cell, beyond detection. Following incubation with surface mAbs, the cells are fixed and permeabilised and incubated with cytokine-specific mAb. Then 3-colour flow cytometry was used to determine the percentage of cytokine-secreting cells.

Optimisation of conditions for cell stimulation

A number of optimisation steps were required. They were adapted from technical reviews of this methodology (Collins 2000, Pala, *et al* 2000).

1. **Duration of incubation with protein transport inhibitor +/- PMA & ionomycin.** Cell stimulation in the presence of protein secretion inhibitors for longer than 24 hours can lead to cell death. Cell death has 2 major

consequences: DNA strands are released from dead cells, leading to clumping, and death of the most activated cells will affect the analysis. It is therefore important to optimise the incubation time with these reagents in order to capture maximal cytokine production together with minimal cell death. Initial studies utilising a propidium iodide (PI) and annexin-V staining assay to quantify the degree of cell death in the presence of the stimulants and the protein secretion inhibitor monensin were performed.

2. **The Annexin-V-Fluos assay:** Annexin-V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). It is suited to detecting apoptotic cells, since it acts as a sensitive probe for PS exposure in the outer leaflet of the cell membrane, which is exposed upon apoptosis of the cell (Vermes, *et al* 1995). Since PS is also exposed in necrotic cells, a means to distinguish between apoptotic and necrotic cells needs to be utilised. PI is a DNA stain; it binds to exposed DNA following cell necrosis. The test cell suspension of 1×10^6 /ml cells was washed in PBS. The cells were incubated with Annexin-V-Fluos (Annexin-V-Fluos, Roche) in a buffer containing PI, according to the manufacturer's instructions. This assay was performed on the test cells prior to incubation with PMA, ionomycin and monensin. Then the cell suspension was divided in 2, and one aliquot incubated with monensin alone, and the other with monensin plus PMA and ionomycin. The Annexin-V-Fluos assay was then performed after a 16-hour incubation. As seen in Figure 4.4a, prior to incubation with monensin and/or stimulants, 84.5% of cells are annexin-V and PI-negative, and hence viable, while 8.4% are annexin-V positive but PI-negative (apoptotic), 7.1% are PI-positive and/or annexin-V positive (necrotic).

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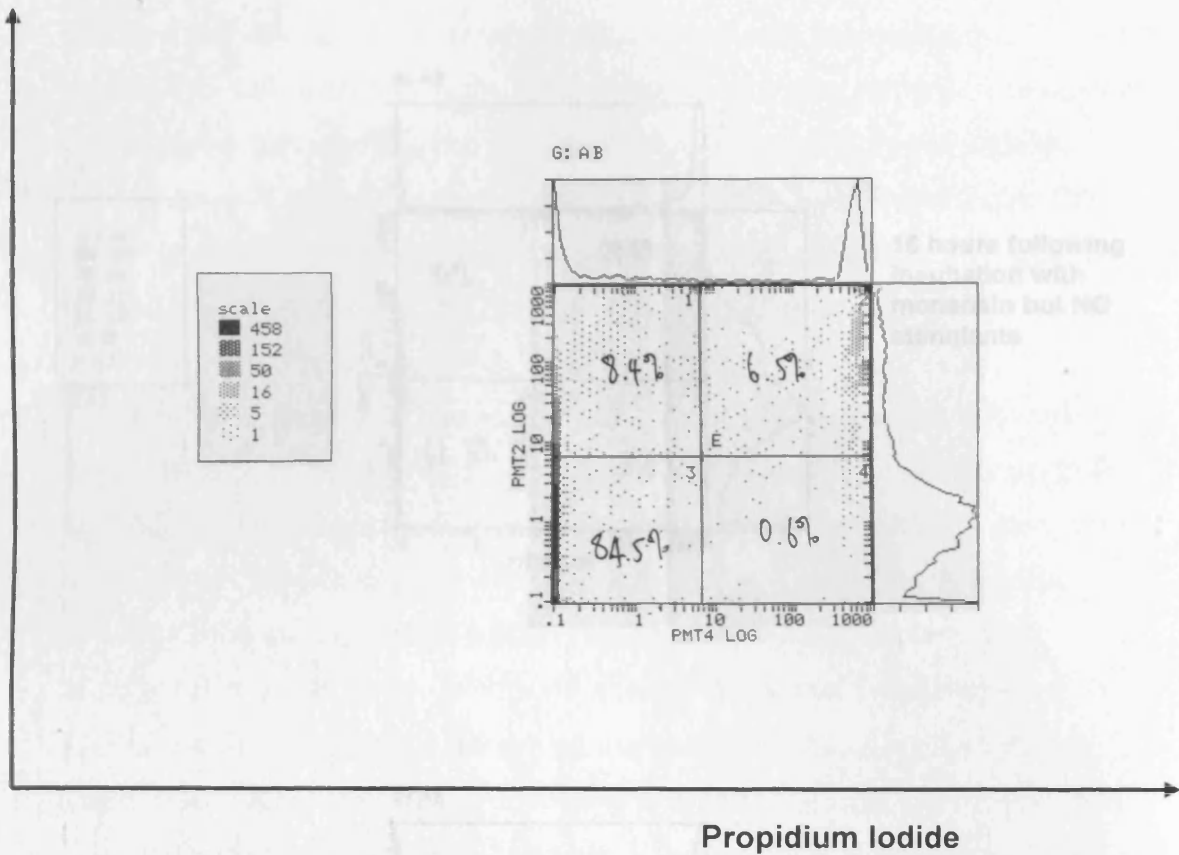


Figure 4.4a. The Annexin-V Fluorescence Assay prior to incubation with monensin and/or stimulants.

This demonstrates that 84.1% of cells are annexin-V and propidium iodide negative and hence viable.

The cells that were incubated with monensin, but not the stimulants showed 61.3% viability, and those incubated with both the stimulants and monensin showed 58.4% viability (Figure 4.4b on next page).

Propidium Iodide (PI)

Figure 4.4b. The Annexin-V Fluorescence Assay following incubation with monensin, with or without the stimulants, PMA and Ionomycin.

The 2 panels show similar cell viability in both situations, demonstrating that the inclusion of monensin does not adversely affect cell viability.

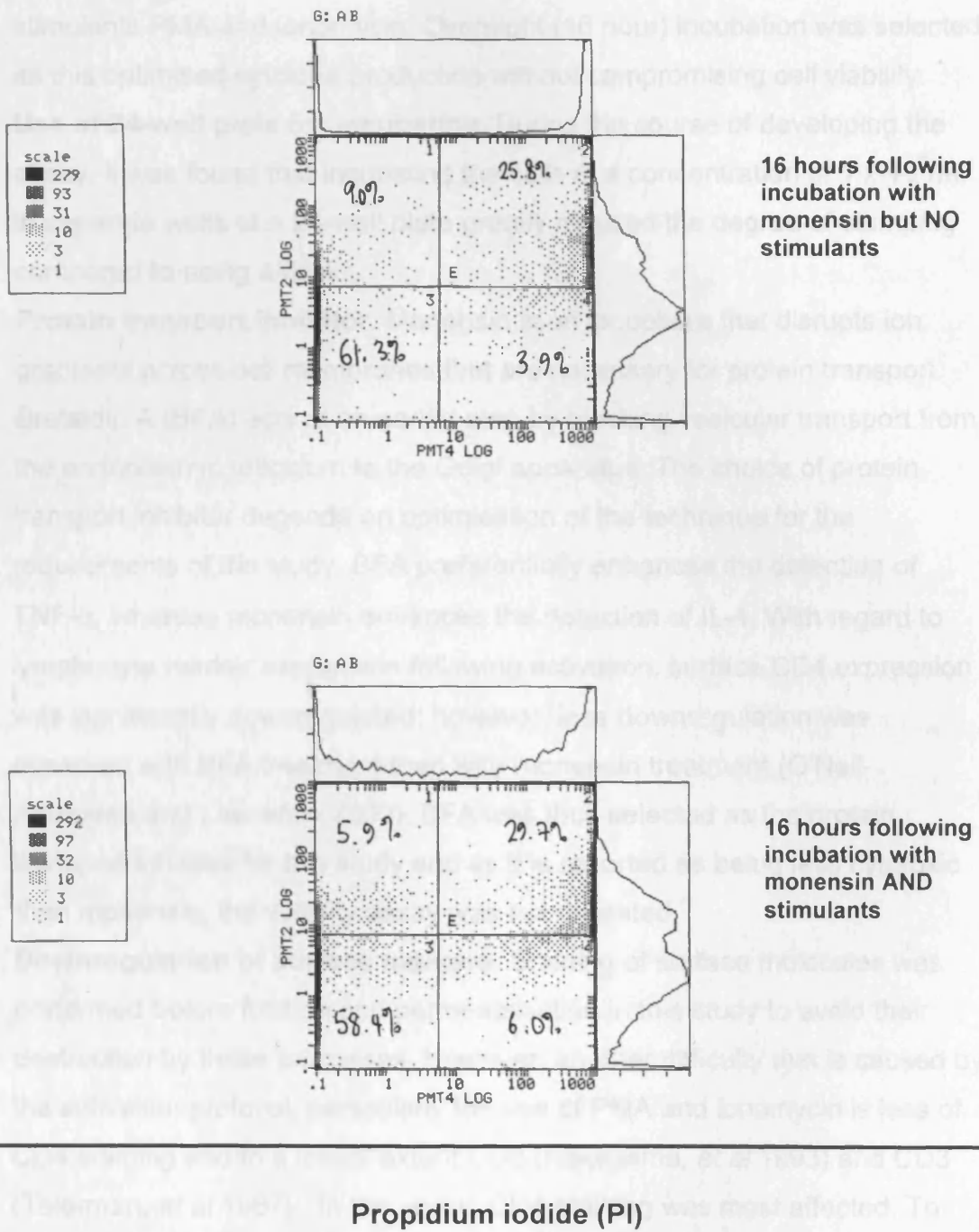


Figure 4.4b. The Annexin-V Fluos Assay following incubation with monensin, with or without the stimulants, PMA and ionomycin.

The 2 panels show similar cell viability in both situations, demonstrating that the inclusion of monensin does not adversely affect cell viability.

In conclusion, this viability assay showed that the monensin on its own reduced cell viability to the same extent as in combination with the stimulants PMA and ionomycin. Overnight (16 hour) incubation was selected as this optimised cytokine production without compromising cell viability.

3. **Use of 24-well plate for incubation.** During the course of developing the assay, it was found that incubating the cells at a concentration of 1×10^6 /ml in separate wells of a 24-well plate greatly reduced the degree of clumping compared to using a flask.
4. **Protein transport inhibitor.** Monensin is an ionophore that disrupts ion gradients across cell membranes that are necessary for protein transport. Brefeldin A (BFA) acts at an earlier step by blocking vesicular transport from the endoplasmic reticulum to the Golgi apparatus. The choice of protein transport inhibitor depends on optimisation of the technique for the requirements of the study. BFA preferentially enhances the detection of TNF- α , whereas monensin enhances the detection of IL-4. With regard to lymphocyte marker expression following activation, surface CD4 expression was significantly downregulated; however, less downregulation was observed with BFA treatment than with monensin treatment (O'Neil-Andersen and Lawrence 2002). BFA was thus selected as the protein transport inhibitor for this study and as it is reported as being less cytotoxic than monensin, the viability assay was not repeated.
5. **Downregulation of surface markers.** Staining of surface molecules was performed before fixation and permeabilisation in this study to avoid their destruction by these processes. However, another difficulty that is caused by the activation protocol, particularly the use of PMA and ionomycin is loss of CD4 staining and to a lesser extent CD8 (Nakayama, *et al* 1993) and CD3 (Telerman, *et al* 1987). In this study, CD4 staining was most affected. To overcome this, a FITC-conjugated mAb (Multi-CloneTM CD4, Becton Dickinson, Oxfordshire, UK) against 2 non-cross-blocking epitopes (Leu-3a and Leu-3b) on the CD4 molecule was used (Hennessy, *et al* 2001). This enabled successful co-assessment of CD4 expression and cytokine expression in PMA- and ionomycin stimulated cells.

Optimised method for ICCS

PBMC from patients and controls were washed twice in PBS with 0.1% bovine serum albumin (BSA) and resuspended at a concentration of 1×10^6 cells/ml in RPMI-1640 supplemented with 10% fetal calf serum (FCS), streptomycin and penicillin. After overnight stimulation in a 24-well plate with PMA, 5 ng/ml) and ionomycin (75 ng/ml) in the presence of brefeldin-A (10 μ g/ml, GolgiPlug™, BDPharMingen, Oxfordshire, UK), stimulated cells were washed twice in PBS with 0.1% BSA and incubated with mAbs against CD3, CD8 and Multi-Clone™ CD4 (Becton Dickinson, Oxfordshire, UK) at 4°C for 15 minutes. After washing in PBS with 0.1% BSA and 0.1% azide, the cells were fixed and permeabilised according to the manufacturer's protocol (Cytotfix/ Cytoperm™ Kit, BDPharMingen, Oxfordshire, UK) followed by incubation for 30 minutes in the dark at 4°C with antibodies specific for IL-2, IL-4 and TNF α . Species-specific isotype-matched antibodies (BDPharmingen, Oxfordshire, UK) were used as controls then resuspended in PBS with 0.1% BSA and 0.1% azide. The stained and fixed samples were analysed on a Coulter EPICS Elite Flow Cytometer (Beckman Coulter, Buckinghamshire, UK) following acquisition of 50 000 events per sample, and absolute numbers of cells calculated as described in general methods.

4.3 RESULTS

The reference ranges employed for CD3+, CD4+, CD8+ and CD56+ lymphocyte subsets were adopted from Reichert *et al*'s work on the distributions of lymphocyte populations (Reichert, *et al* 1991). Those for CD45RA and RO subsets were taken from Bisset *et al* (Bisset, *et al* 2004).

4.3.1 NK-cell reconstitution

NK cells (CD56+) were within normal limits, when first measured at 3 months post transplant (median 179 cells/ μ l, range 18-560 cells/ μ l), in the all but one patient, whose count was normal when measured at 6 months. Thereafter, NK cell levels remained normal or high throughout the period of follow up (median 539 cells/ μ l, range 373-994 cells/ μ l at 12 months) as shown in Figure 4.5.

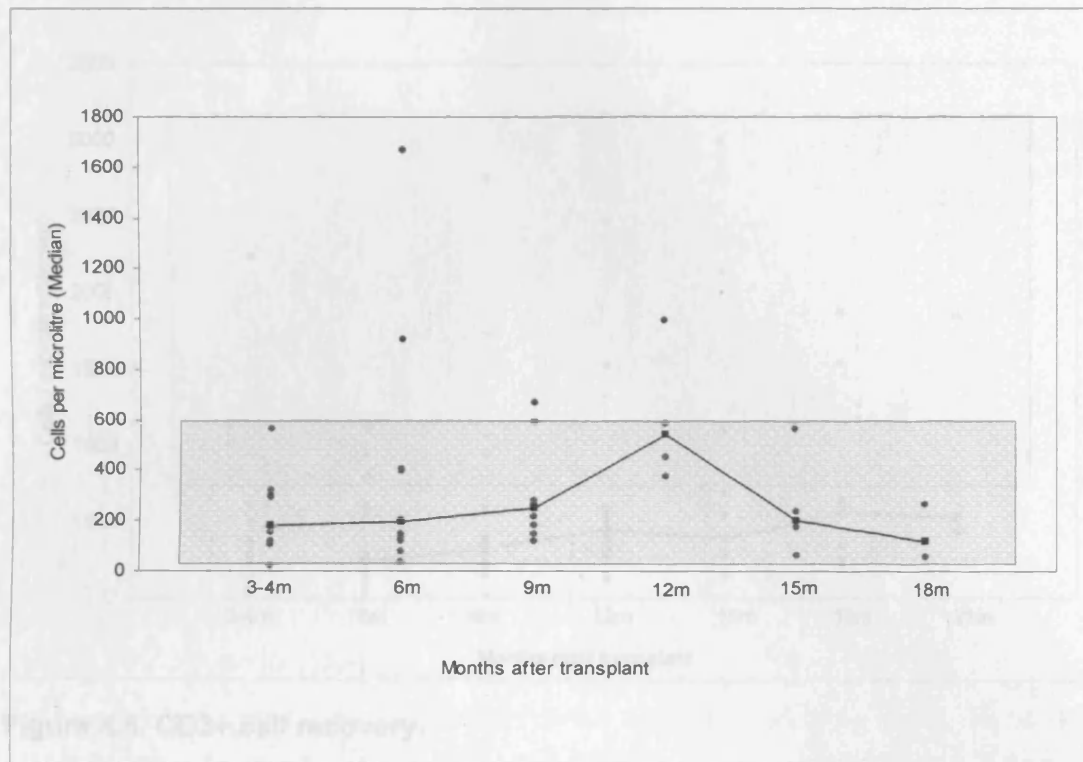


Figure 4.5. CD56+ cell recovery.

Cell numbers are given in absolute numbers per microlitre of blood. Individual as well as median values (given as line graph) are shown at 3 monthly intervals post-transplantation. The normal range is 50-600 cells per microlitre, indicated by the grey box.

4.3.2 Recovery of T cell subsets

Median CD3+ cells numbers gradually rose with time post-transplant, but remained below the normal range for up to 21 months, with just 17% of patients in the normal range at 6 months (n=18), 25% at 12 months (n=12), 36% at 15 months (n=11) and 43% at 18m (n=7). (Figure 4.6)

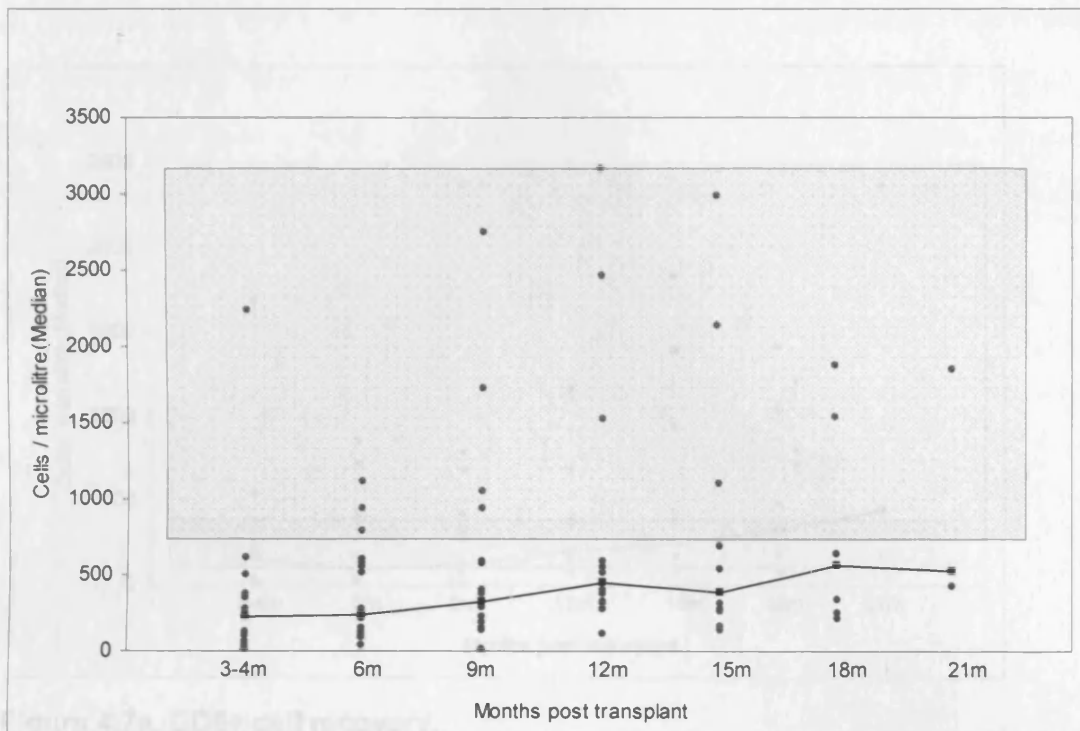


Figure 4.6. CD3+ cell recovery.

Cell numbers are given in absolute numbers per microlitre of blood. Individual as well as median values (given as line graph) are shown at 3 monthly intervals post-transplantation. The normal range is 600-3200 cells per microlitre, indicated by the grey box.

The majority of CD3+ cell recovery was accounted for by an increase in CD8+ cell numbers. By 6 months, 33% of patients (n=18) had normal CD8+ cell numbers, rising to 42% at 12 months (n=12) and 57% at 18 months (n=7) (Figure 4.7a).

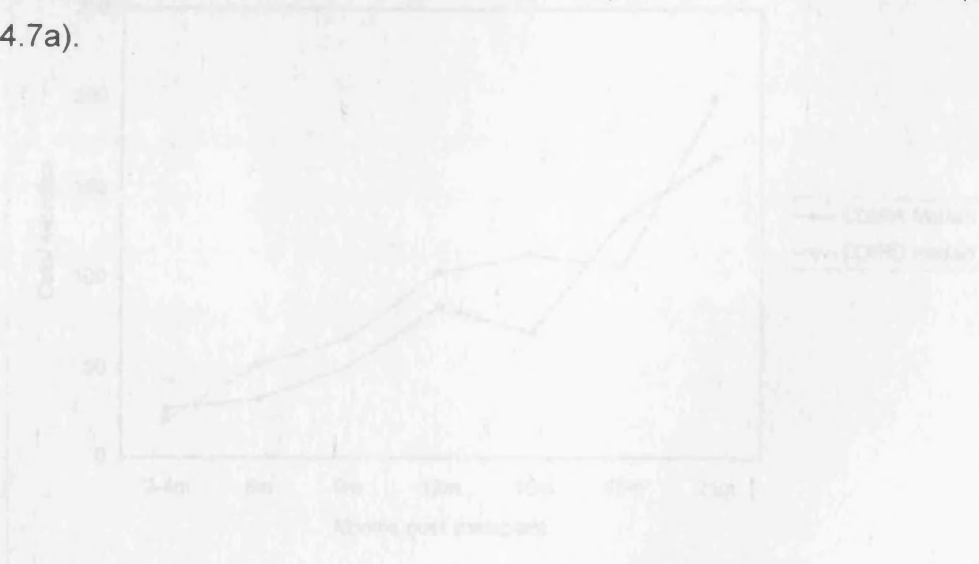


Figure 4.7a. CD8+CD45RA+ and CD8+CD45RO+ cell recovery.

Median values per microlitre of blood are shown for CD8+CD45RA+ cells (Normal range 42-100 cells per microlitre) and CD8+CD45RO+ cells (Normal range 72-377 cells per microlitre).

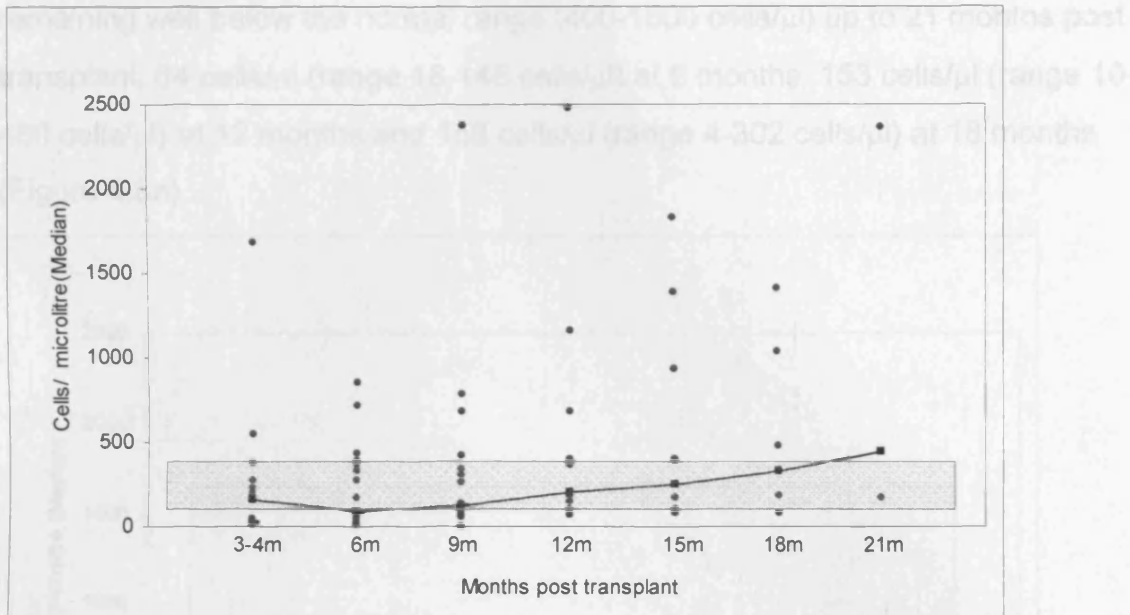


Figure 4.7a. CD8+ cell recovery.

Cell numbers are given in absolute numbers per microlitre of blood. Individual as well as median values (given as line graph) are shown at 3 monthly intervals post-transplantation. The normal range is 120-350 cells per microlitre, indicated by the grey box.

Both naïve (CD45RA) and memory (CD45RO) subsets of CD8+ cells showed similar recovery, in parallel with increasing overall CD8+ cell numbers. At 12 months, 42% of patients had normal CD8+ CD45RA+ numbers and 33% had normal CD8+ CD45RO+ numbers (Figure 4.7b).

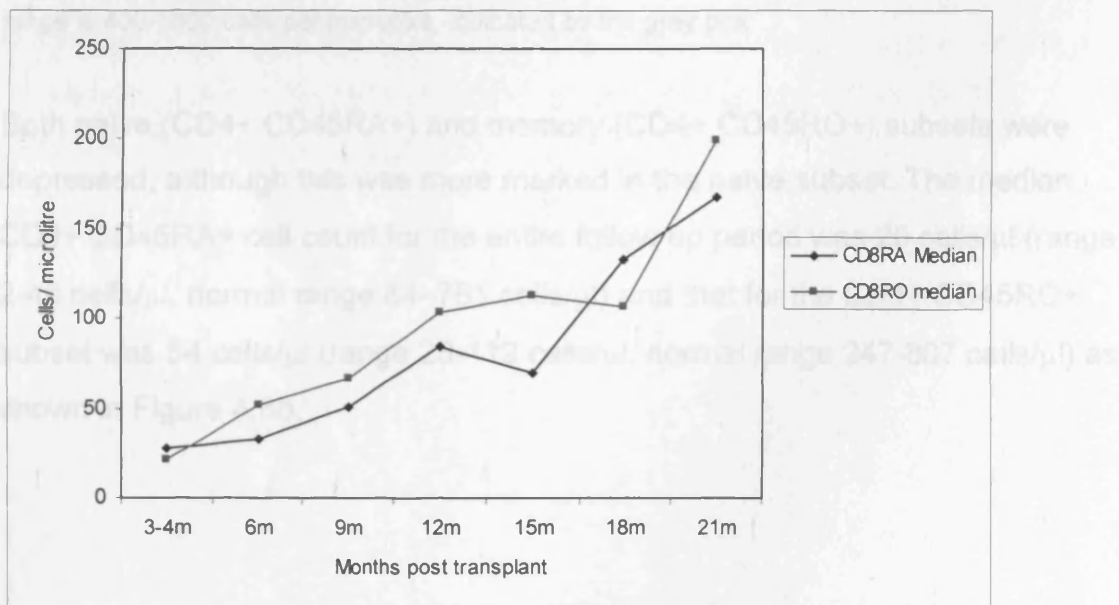


Figure 4.7b. CD8+CD45RA+ and CD8+ CD45RO+ cell recovery.

Median values per microlitre of blood are shown for CD8+CD45RA+ cells (Normal range 42-360 cells per microlitre) and CD8+CD45RO+ cells (Normal range 72-377 cells per microlitre).

In contrast, CD4+ cells showed much slower recovery, with median cell numbers remaining well below the normal range (400-1600 cells/ μ l) up to 21 months post transplant: 64 cells/ μ l (range 18-148 cells/ μ l) at 6 months, 153 cells/ μ l (range 10-480 cells/ μ l) at 12 months and 158 cells/ μ l (range 4-302 cells/ μ l) at 18 months (Figure 4.8a).

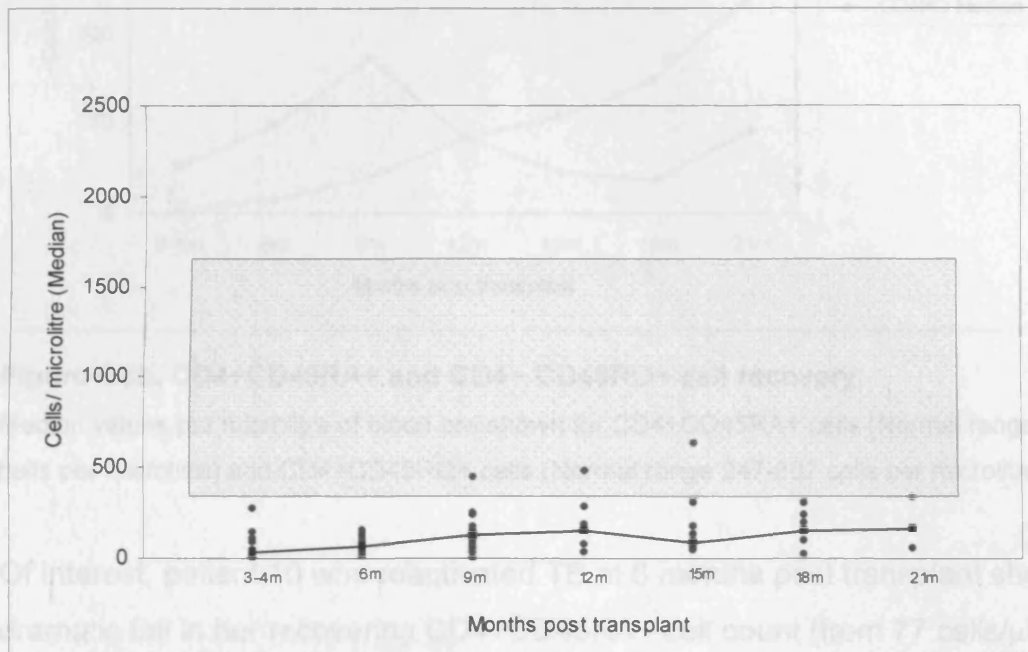


Figure 4.8a. CD4+ cell recovery.

Cell numbers are given in absolute numbers per microlitre of blood. Individual as well as median values (given as line graph) are shown at 3 monthly intervals post-transplantation. The normal range is 400-1600 cells per microlitre, indicated by the grey box.

Both naïve (CD4+ CD45RA+) and memory (CD4+ CD45RO+) subsets were depressed, although this was more marked in the naïve subset. The median CD4+ CD45RA+ cell count for the entire follow up period was 20 cells/ μ l (range 2-46 cells/ μ l, normal range 84-761 cells/ μ l) and that for the CD4+ CD45RO+ subset was 54 cells/ μ l (range 25-119 cells/ μ l, normal range 247-807 cells/ μ l) as shown in Figure 4.8b.

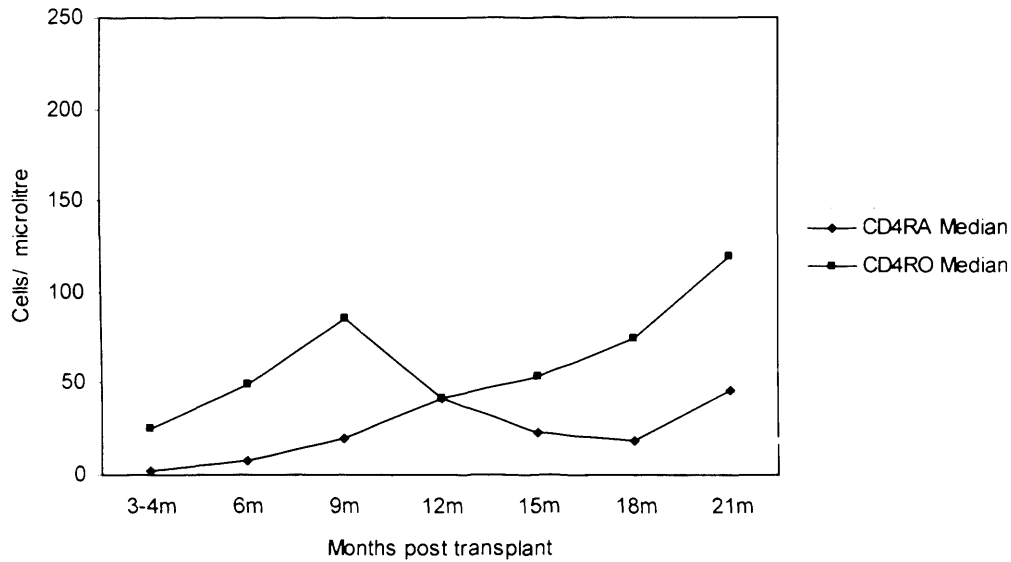


Figure 4.8b. CD4+CD45RA+ and CD4+ CD45RO+ cell recovery.

Median values per microlitre of blood are shown for CD4+CD45RA+ cells (Normal range 84-761 cells per microlitre) and CD4+CD45RO+ cells (Normal range 247-807 cells per microlitre).

Of interest, patient 10 who reactivated TB at 6 months post transplant showed a dramatic fall in her recovering CD4+CD45RA+ cell count (from 77 cells/ μ l at 6 months to 3 cells/ μ l at 9 months) with a concomitant rise in her CD4+CD45RO+ cells (from 1 cell/ μ l at 6 months to 235 cells/ μ l at 9 months). CD8+cells were 560 cells/ μ l for CD45RA+and 1523 cells/ μ l for CD45RO+ cells at 9 months post transplant. Thereafter, her CD4+CD45RA+ cells remained below 14 cells/ μ l up to 15 months post transplant, with continued elevation of the CD4+CD45RO+ subset and both CD8+ subsets (Figure 4.9).

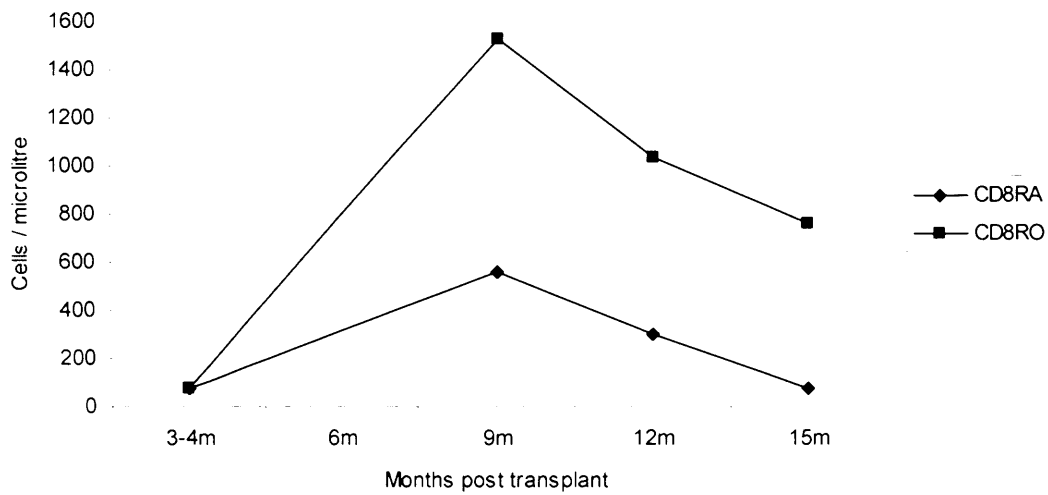
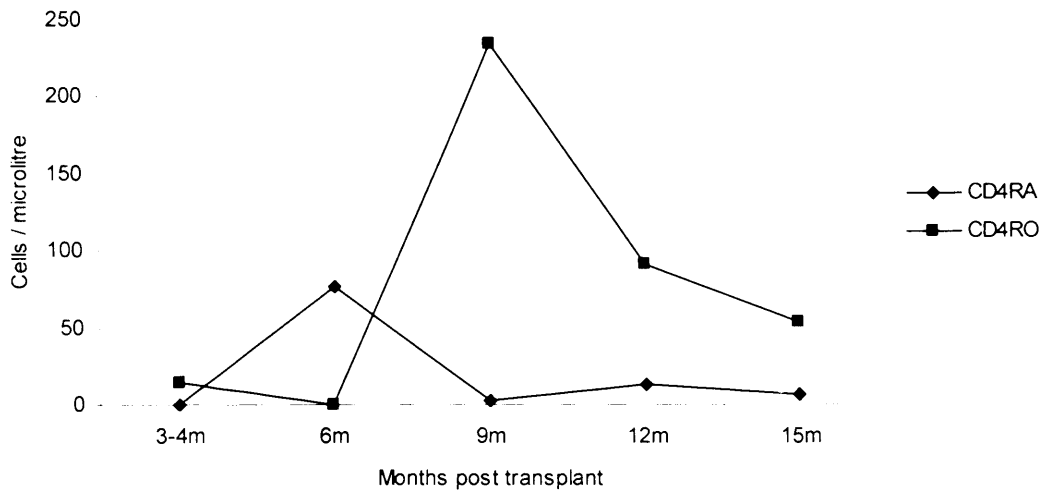


Figure 4.9. Recovery of naïve and memory subsets of CD4+ and CD8+ cells in patient 10.

Absolute values for naïve (CD45RA+) and memory (CD45RO+) subsets of CD4+ and CD8+ cells are shown where available for each time point.

This pattern of T cell recovery occurred in conjunction with DLI, and hence may reflect transfer of donor immunity, followed by peripheral expansion of TB-specific memory T cells.

4.3.3 Effects of T lineage chimeric status, GVHD and disease status on T cell subsets.

1. **T lineage chimeric status.** There was no correlation between CD3+, CD4+ or CD8+ cell numbers on T cell lineage chimeric status.

2. **GVHD**. T cell subset recovery was assessed in the 10 patients who experienced GVHD either after transplant and/ or after DLI and compared to those patients who did not have GVHD (n=9). Median CD3+, CD4+ and CD8+ cell numbers were lower in patients with GVHD than in those without (Figure 4.10). At the 12 month timepoint, using the Mann-Whitney test, this was not a significant finding ($p=0.21$, $p=0.47$ and $p=0.37$ respectively).

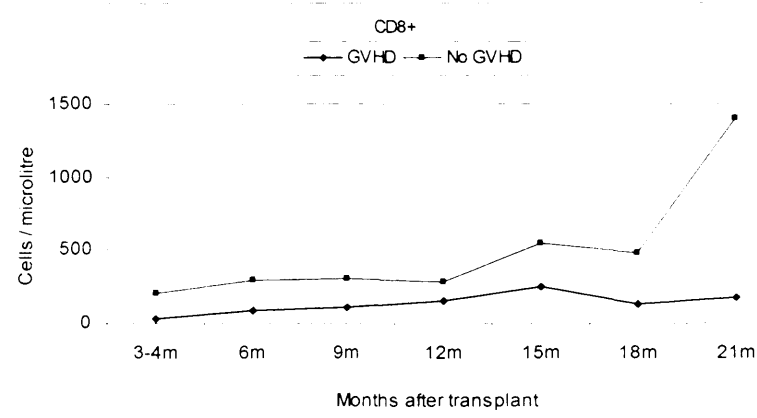
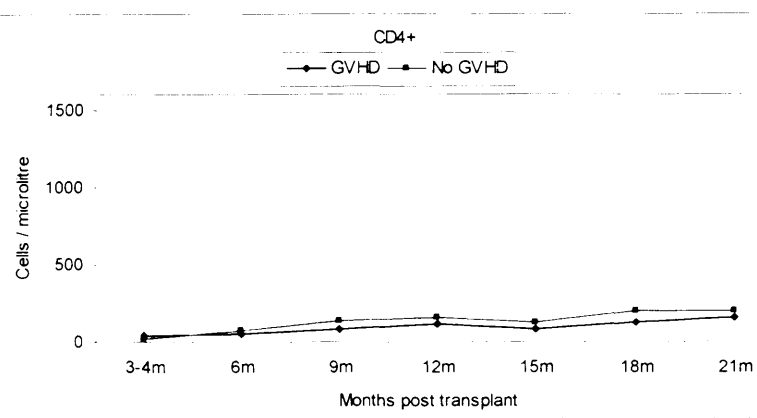
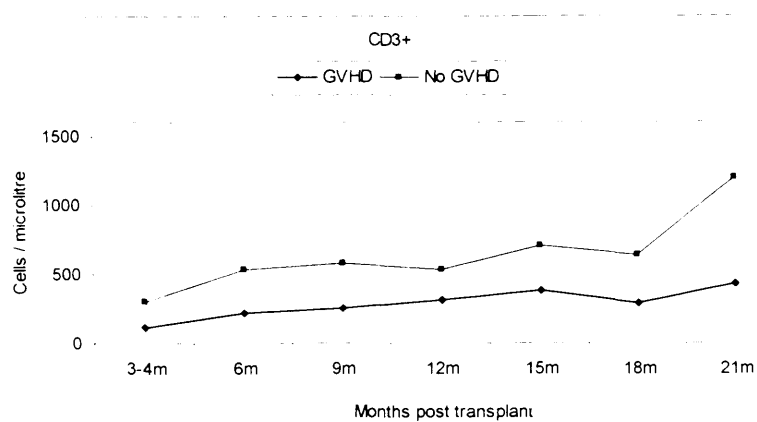


Figure 4.10. Effect of GVHD on CD3+, CD4+ and CD8+ cell recovery

Median values of CD3+, CD4+ and CD8+ cells are shown for 10 patients with GVHD and 9 patients without GVHD. At the 12 month timepoint, using the Mann-Whitney test, there was no significant effect of GVHD on CD3+, CD4+ and CD8+ cell recovery ($p=0.21$, $p=0.47$ and $p=0.37$ respectively)

Of the 9 patients who had GVHD, only 1 had CD3 numbers within the normal range, but 6 out of 10 patients without GVHD had normal CD3 numbers.

3. **Disease status post transplant.** Nine patients with progressive disease and 10 patients with responding/ stable disease were assessed at 12 months post transplant. Figure 4.11 shows that disease status had no influence on CD3+ or CD4+ cell recovery. Patient numbers were too small for statistical analysis.

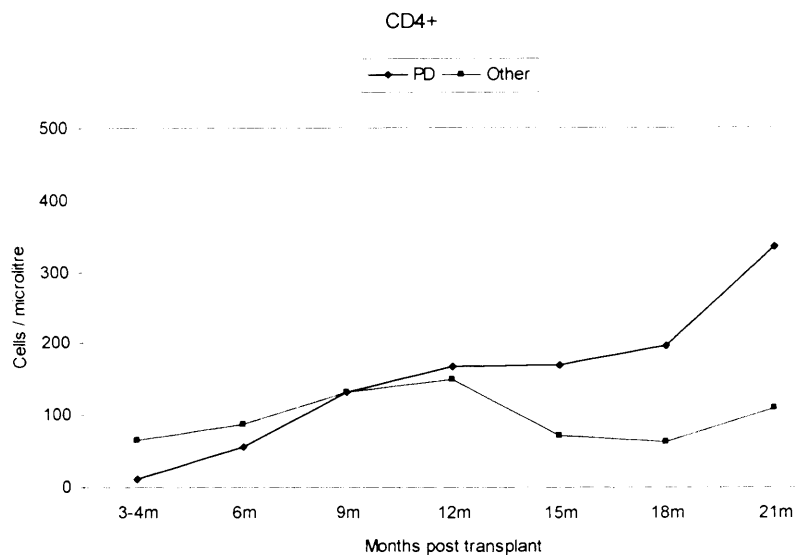
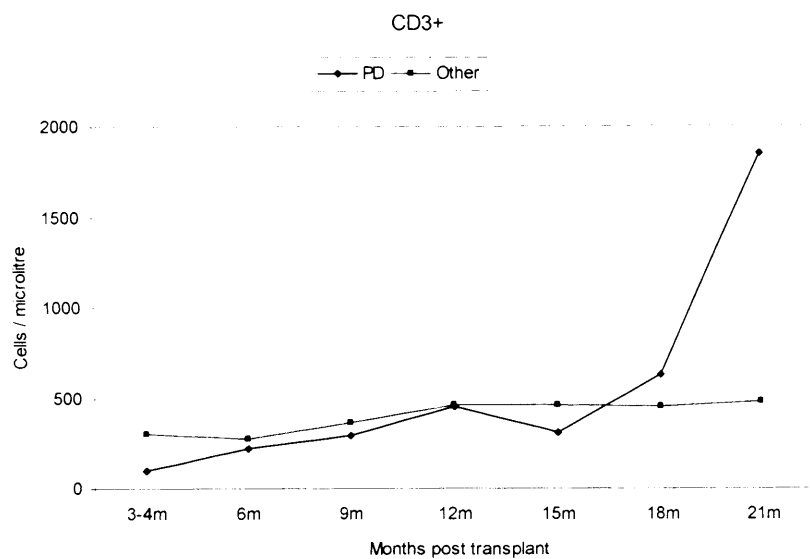


Figure 4.11. Effect of progressive disease on CD3+ and CD4+ cell recovery.

Median values are shown for CD3+ cells, and CD4+ cells in 9 patients with progressive disease (PD) and 10 patients with responding or stable disease (Other) post transplant. The numbers were too small for statistical analysis in this sample.

4.3.4 Cytokine production by T cells

This was analysed in 6 patients at 6 to 20 months post transplant and 5 age-matched controls using PBMC stimulated with PMA and calcium ionomycin. Results are shown in Figure 4.12.

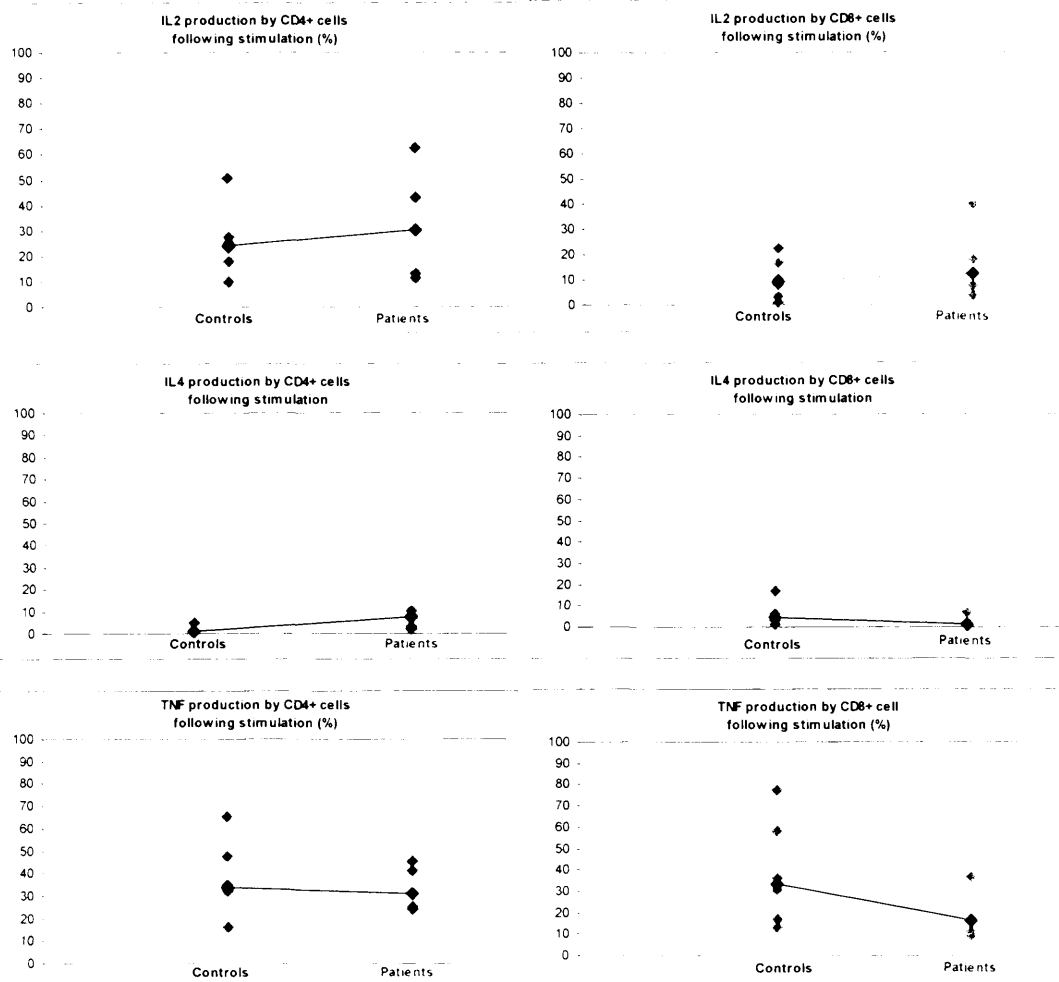


Figure 4.12. Intracellular cytokine production by CD4+ and CD8+ cells.

In each case, the y-axis represents the percentage of cells showing positivity for the cytokine of interest. Data regarding IL-2, IL-4 and TNF-alpha secretion for 5 normal controls is shown on the left column, and that for 6 patients is shown in the right column. The median values are shown by black dots, connected with a black line.

The proportion of CD4+ cells producing IL-2, TNF α and IL-4 in patient samples was comparable to that in controls. In the patient group, 24% (range 10.1-50.8%) of CD4+ cells produced IL-2, compared to 30.4% (range 11.5-62.7%) in the control group. There were similar findings for TNF α production by CD4+ cells: 34% (16.2-65.4%) in the patient group and 30.8% (24.1-45.6%) in the control group. IL-4 production by CD4+ cells was lower in the patients (8%, range 2.2-15.9%) and controls (1%, range 0.4-6.5%).

Similarly, the percentage of cytokine-producing CD8+ cells was also comparable between patients and controls. In the patient group, 9% (range 0.9-22.5%) of CD8+ cells produced IL-2, compared to 12.2% (range 3.9-40%) in the

control group. There were similar findings for TNF α production by CD8+ cells: 33% (13-77.6%) in the patient group and 16.5% (9.1-36.5%) in the control group. IL-4 production by CD8+ cells was also lower in the patients (4.5%, range 0.9-16.8%) and controls (0.9%, range 0.4-7%).

There was no difference in results of cytokine measurements done before versus those done after 12 months post-transplant. Overall, these results indicate that the recovering T cell population, though reduced in number, is functionally normal in terms of cytokine production. It is worth noting, however, that the actual number of cytokine-producing cells, particularly in the CD4+ subset will be lower than that of normal controls, because of markedly reduced cells numbers. In all cases of patients and control subjects, more CD4+ cells were positive for IL-2 rather than IL-4 production, possibly due to a greater abundance of Th1 than Th2 cells. Apart from this observation, it was not possible to draw any conclusions about the relative frequencies of Th1 & 2 or Tc1 & 2 subsets.

4.4 DISCUSSION

In summary, while NK cell numbers recovered rapidly following RIT, the recovery of T cell numbers especially the CD4+ subset and most prominently the naïve (CD4CD45RA+) compartment was markedly delayed. Recovery of CD8+ cell was also delayed, but to a lesser degree. The proportion of mitogen-stimulated cytokine-producing cells was comparable in patients and control subjects.

Reconstitution of the lymphoid system after myeloablative allogeneic stem cell transplantation (ASCT) has been extensively studied, and comprehensively reviewed by Storek and Witherspoon (Storek 2000). Since nonmyeloablative conditioning regimens are relatively novel, there are fewer reports of immune recovery in this setting. There are few data on T cell recovery and the evidence is conflicting, largely owing to the varied conditioning regimens used. One study of T cell subset recovery found that T cell recovery was rapid and comparable following RIT as well as T cell depleted myeloablative transplants, with marked skewing of T cell repertoire (as shown by TCR spectratyping- refer to Chapter 5 for results related to the present study) following TCD myeloablative transplant

but not after RIT (Bahceci, *et al* 2003). Another group found that CD4+CD45RA+ and CD4+CD45RO+ T cell recovery was significantly lower in the RIT group (conditioned with the purine analogue cladribine, busulphan & antithymocyte globulin) compared with myeloablative transplant group, but this did not translate into an increased incidence of infection (Saito, *et al* 2003). Significantly, neither of these groups employed alemtuzumab in their conditioning regimen.

The present study addresses NK and T cell recovery in patients with myeloma undergoing RIT using an alemtuzumab-containing regimen which importantly has acceptable toxicity. The rate and quality of T cell recovery is an important determinant of the toxicity of a conditioning regimen and the incidence of infective complications. Myeloma itself is associated with an intrinsic immune dysregulation, with evidence of phenotypic and functional changes in T cells, B cells, macrophages and NK/LAK cells (Lauria, *et al* 1984). Previous studies have shown that CD4 cell counts are low in myeloma patients, and that this is associated with advanced clinical stage and a shorter survival (San Miguel, *et al* 1992). It is possible therefore that these intrinsic immune defects would influence the reconstitution of the immune system following allogeneic transplantation.

NK (CD56+) cell recovery in this study is comparable to data from myeloablative immune reconstitution studies. Normal NK cell numbers are reported to occur within 1 month of transplant, regardless of donor type and patient age, (Small, *et al* 1999) or stem cell source, (Ottinger, *et al* 1996) although another study found delayed recovery in patients with grade II-IV acute GVHD (Fujimaki, *et al* 2001). In this study, NK cells were measured at 3 months post transplant, and showed that all but one patient had normal levels despite the incidence of acute GVHD in 5 of them.

The profound and prolonged CD4+ T cell lymphopenia seen in this study is comparable to that reported in recipients of myeloablative allogeneic peripheral blood stem transplants (Shenoy, *et al* 1999) and myeloablative allogeneic bone marrow transplants (Fujimaki, *et al* 2001). For example, in a study of patients undergoing T cell depleted myeloablative transplantation, CD3+CD4+ cell

recovery was delayed for up to and even beyond a year after transplant and was particularly marked for the CD4+CD45RA+ (naïve) subset (Small *et al* 1999). In fact Fujimaki *et al* found that CD3+CD4+ cells remained below the normal range up to 5 years after myeloablative ASCT, including the CD4+CD45RA+ subset, although the memory subset (defined by the CD4+CD29+ phenotype) recovered to normal levels within 2 years post transplant.

In contrast, CD3+CD8+ cell numbers are reported to return to normal levels from 3 to 6 months post transplant following myeloablative transplantation in most series (Fujimaki, *et al* 2001, Shenoy, *et al* 1999). The rise in memory CD3+CD8+ cells occurs faster than naïve CD3+CD8+ cells (Storek, *et al* 1995). In the present study, recovery of CD3+CD8+ cells took somewhat longer with nearly 60% of the patients had normal CD3+CD8+ cell numbers by 18 months. Recovery of CD8+CD45RA+ and CD45RO+ cells occurred in parallel. Reconstitution of the T cell compartment occurs via 2 pathways (Storek 2000). One pathway is thymus-dependent, in which naïve T cells that are the progeny of engrafted stem cells are produced in the recipient's thymus. These cells have a CD45RA phenotype and a diverse TCR repertoire (discussed in detail in Chapter 5) (Dumont-Girard, *et al* 1998). The earlier pathway of reconstitution, however, is the result of expansion of mature T cells that were co-transfused with the graft. These T cells express the CD45RO phenotype and have a limited TCR repertoire (Roux, *et al* 1996). The peripheral expansion of infused donor memory T cells probably accounts for the early recovery in CD8+ cells in this study. As mentioned previously, T cell activation leads to a switch from expression of the CD45RA to the CD45RO isoform. This pattern of expression following activation is maintained in the CD4+ subset but CD8+CD45RO+ cells can revert back to the CD45RA phenotype (Faint, *et al* 2001). It is likely therefore that the majority of the recovering CD8+ population post transplant comprises memory T cells, irrespective of their CD45R phenotype (Heitger, *et al* 1997). On the other hand, CD4+ cells, once activated, retain expression of the CD45RO phenotype. Hence CD4CD45RA can be reliably taken to represent naïve thymus-derived CD4+ cells. In the present study, such cells remained at very low levels for up to 21 months post transplantation, indicating that during

this time, regenerating T cells are derived mainly from the oligoclonal expansion of CD4CD45RO+ cells co-transfused with the graft.

The functional status of recovering T cells, assessed in this study by intracellular cytokine production in response to mitogenic stimulation showed good recovery despite low cell numbers. Cytokine production in response to mitogen stimulation was proportionally within normal limits for both CD4+ and CD8+ subsets in the patient group, although whether this predicts for normal responses to antigenic challenge in vivo remains unclear. In addition, the absolute number of cytokine-producing cells was much reduced, particularly in the CD4+ subset. Previous studies of lymphoproliferative responses to antigens and polyclonal stimuli have shown subnormal responses both early and late post-transplant, with inferior responses seen after bone marrow versus peripheral blood stem cell transplantation (Ottinger, *et al* 1996, Shenoy, *et al* 1999, Talmadge, *et al* 1996). Others have shown that T cell proliferative responses to mitogens were positive in almost all patients tested after ASCT, but responses to antigenic stimulation were reduced (Maury, *et al* 2001) and the proliferative response to PHA, and HSV and VZV was similar in BM and PB stem cell recipient groups (Storek, *et al* 2001). Cytokine production by cytokine mRNA expression and intracellular cytokine staining by flow cytometry has also been studied. Shenoy *et al* found that cytokines including IL-2, IL-4 and TNF- α were strongly upregulated and easily detected in all allogeneic stem cell transplant recipients in response to stimulation with PHA (Shenoy, *et al* 1999). Another study showed that T cell function in peripheral blood mononuclear cells is depressed despite high levels of cytokine mRNA expression, and suggested that high levels of type 2 cytokines (IL-4 and IL-10) may contribute to the immune dysfunction seen after high dose therapy and PBSCT (Singh, *et al* 2000). There are few reports on T cell function after nonmyeloablative conditioned ASCT.

The prolonged depression of T cell numbers, but relative preservation of T cell function seen in this study is may be due to the significant degree of T cell depletion caused by the humanised monoclonal antibody alemtuzumab, which would not otherwise affect T cell function. Alemtuzumab is directed against the CD52 antigen and is abundantly expressed on most peripheral blood

lymphocytes (Hale, *et al* 1990). The dose of alemtuzumab used in this conditioning regimen exceeds that used to treat a variety of disease states in which prolonged lymphopenia and a high incidence of infections has been reported (Isaacs, *et al* 1992, Tang, *et al* 1996). The present study demonstrates a relatively low incidence of GVHD, accompanied by a high incidence of viral infections. This is in keeping with the prolonged lymphopenia found in this study. Further studies are planned to de-escalate the dose of alemtuzumab used in this conditioning regimen, with a view to offsetting the delay in lymphocyte recovery however, whether this can be achieved without a concomitant increase in GVHD and/or graft rejection remains to be established.

Chapter 5: Evaluation of T cell VB repertoire post transplant by T cell receptor spectratyping

5.1 Introduction

The diversity of T cell antigen receptors in an individual determines both the response to antigenic challenges from the external environment and the response to self-antigens. It therefore plays a critical part in determining immune competence and may also be important in determining susceptibility to a range of diseases resulting from immune activation. Several methods are used to assess T cell repertoire, including monoclonal antibodies and flow cytometry, and molecular techniques. Together, these techniques have provided information on normal T cell repertoire patterns as well as alterations caused by common infections and antigenic challenge. Skewing of T cell repertoire may be associated with pathological states, such as infections, autoimmune diseases and stem cell transplantation.

To some extent, the T cell repertoire must reflect previous exposure to antigenic stimuli. Conserved responses to a range of viral pathogens such as influenza, EBV and CMV are likely to have substantial effects over time in moulding the T cell repertoire. In addition to such external antigenic stimuli, the selection of T cells during maturation and development in the thymus is also driven by antigenic selection. Thymic selection, both positive and negative, is driven by MHC-peptide combinations. High affinity binding of the T cell receptor (TCR) to self antigens bound to MHC molecules leads to negative selection in the thymus. Low affinity binding to self MHC imparts a survival signal and hence leads to positive selection.

5.1.1 The T Cell Receptor

The TCR is a transmembrane heterodimeric molecule, which recognises peptide presented by HLA class I or II molecules. Greater than 90% of T cells have receptors composed of an α and a β chain, with the remainder bearing a γ and a δ chain (Hall and Lanchbury 1995). Mature α and β chains result from the productive rearrangement of variable (V), diversity (D) (β chain only), joining (J) and constant (C) region segments, which are encoded as non-contiguous elements within the germline. Diversity of the TCR is generated by random

combination of V, (D), J and C region segments; nibbling of junctional V, (D) and J regions; and the addition of nontemplate nucleotides which give rise to N region diversity (Kimura, *et al* 1987). The V regions of the TCR α and TCR β chains contain short stretches of sequences where the variability between different TCRs is concentrated, and these form the hypervariable or complementarity-determining regions (CDRs), of which the third hypervariable region (which forms CDR3) contains the most sequence variability (Liu, *et al* 1995). The 3 CDRs in the α chain are juxtaposed to 3 similar regions in the β chain to form the part of the TCR that specifically recognises peptide-MHC complexes. In some TCRs, parts of all 3 CDR loops make contact with the MHC molecule, whereas in others recognition of the MHC molecule is mainly the function of CDR1 and CDR2, while CDR3 makes contact with the peptide. The β chain V domain contains a fourth hypervariable region, which does not appear to participate in antigen recognition but is the binding site for microbial products called superantigens. Ultimately the specificity of the T cell is determined by the pairing of permitted α and β chains while cell survival is dictated by thymic selection events.

The $\gamma\delta$ TCR is a second type of diverse CD3-associated heterodimer expressed on a small subset of $\alpha\beta$ -negative T cells (Yoshikai, *et al* 1987). T cells expressing the $\gamma\delta$ TCR are a lineage distinct from the more numerous $\alpha\beta$ -expressing, MHC-restricted T cells. The percentages of $\gamma\delta$ T cells vary widely in different tissues, but overall less than 5% of all T cells express this form of TCR. The functions of $\gamma\delta$ T cells and the question of what the $\gamma\delta$ TCR recognises remain largely unresolved. $\gamma\delta$ T cells do not recognise MHC-associated peptide antigens and are not MHC restricted. The majority of $\gamma\delta$ T cells do not express CD4 or CD8. The limited diversity of the $\gamma\delta$ T cells in many tissues suggests that the ligands for these receptors are invariant and conserved. It is possible that $\gamma\delta$ T cells recognise antigens that are frequently encountered at epithelial boundaries between the host and the external environment. Thus they may initiate immune responses to a small number of common microbes at these sites, before the recruitment of antigen-specific $\alpha\beta$ T cells. The TCR δ locus is embedded in the TCR α gene in between V_α and J_α . The joining of V_α to J_α completely deletes the TCR δ gene on that chromosome. There is evidence that

TCR α rearrangement occurs only if initial TCR δ rearrangement fails to produce a viable transcript (Livak, *et al* 1995). How the rearrangement of TCR δ influence TCR α expression is not clear. Mice lacking $\gamma\delta$ T cells have little or no immunodeficiency and only a modest increase in susceptibility to infections by intracellular bacteria.

5.1.2 TCR gene rearrangement in the course of T cell maturation

T cell maturation with regard to surface molecule expression was addressed in detail in Section 4.1.1. Here, the process of TCR gene rearrangement that occurs during T cell maturation will be discussed. The mechanisms by which TCR germline DNA is rearranged to form functional receptor genes appear to be similar to those used in immunoglobulin gene (IgH) rearrangement. Similar recognition signal sequences (RSS) have been found flanking each V, D and J segment in TCR and IgH germline DNA. All TCR gene rearrangements follow the 12/23 joining rule found in IgH rearrangement. Both pre-B and pre-T cells express recombination activating genes (RAG-1 and RAG-2). As with the IgH genes, rearrangement of TCR genes also exhibits allelic exclusion, such that the rearrangement of one allele is inhibited, once a productive rearrangement occurs for the other allele.

Although TCRs are encoded by far fewer V gene exons than immunoglobulin molecules, a number of features contribute to generating even greater diversity in the TCR (Davis and Bjorkman 1988). Although the number of V gene segments is just 75 for the α chain and 25 for the β chain (compared to 300 for the IgH chain), there are greater numbers of J gene segments (50 for TCR- α and 12 for TCR- β , compared with 6 for the IgH chain), making the possible combinations after combinatorial V-J or V-D-J joining in the region of 10^{15} for $\alpha\beta$ TCR, versus 10^{11} for IgH. Other mechanisms, such as N region diversification and junctional diversity are similar for both TCR and IgH gene rearrangement.

In developing cells destined to express $\alpha\beta$ TCRs, the β chain locus undergoes recombination before the α chain locus. D β -to-J β rearrangements are followed by V β -to-DJ β rearrangements. Up to the point of D β -to-J β rearrangement, the thymocyte is CD4-CD8- (double negative) and resides in the subcapsular zone

of the thymic cortex. Once V_{β} -to- DJ_{β} rearrangement occurs, the thymocyte remains CD4-CD8-, but now expresses the productively rearranged β chain gene initially in the cytoplasm and later on the surface of the cell as a heterodimer with pT α , where pT α is a surrogate α chain. The genomic sequences between the rearranged elements are deleted during the rearrangement process. Poly-A tails are added to consensus polyadenylation sites located at the 3' end of the C_{β} RNA and the sequences between the VDJ and C RNA are spliced out to form mature mRNA in which VDJ segments are juxtaposed to either of the two C genes ($C_{\beta 1}$ and $C_{\beta 2}$). The cell now becomes CD4+8+ (double positive) and moves towards the thymic medulla, but still remains in the cortex at this point. This is the site and timing of positive selection. Negative selection occurs next, as the cell approaches the corticomedullary junction of the thymus. Translation of the mRNA gives rise to the nascent polypeptide, which after processing and glycosylation becomes the functional TCR chain.

The steps in α chain gene rearrangement are similar to those in the β chain gene. Because there are no D segments in the α locus, rearrangement consists solely of the joining of V and J segments. Unlike in the β chain locus, where production of the protein suppresses further rearrangement, there is little or no allelic exclusion in the α chain locus. Therefore, productive TCR α rearrangement may occur on both chromosomes, and if this happens, the T cell will express two α chains. In fact, up to 30% of mature peripheral T cells do express two different TCRs, with different α chains but the same β chain. The functional consequence of this dual receptor expression is unknown. The process of β chain gene rearrangement is depicted alongside α chain gene rearrangement in Figure 5.1a and b respectively.

Figure 5.1a. TCR β chain gene recombination and expression
(page 95)

Figure 5.1b. TCR α chain gene recombination and expression
(page 96)

TCR β chain

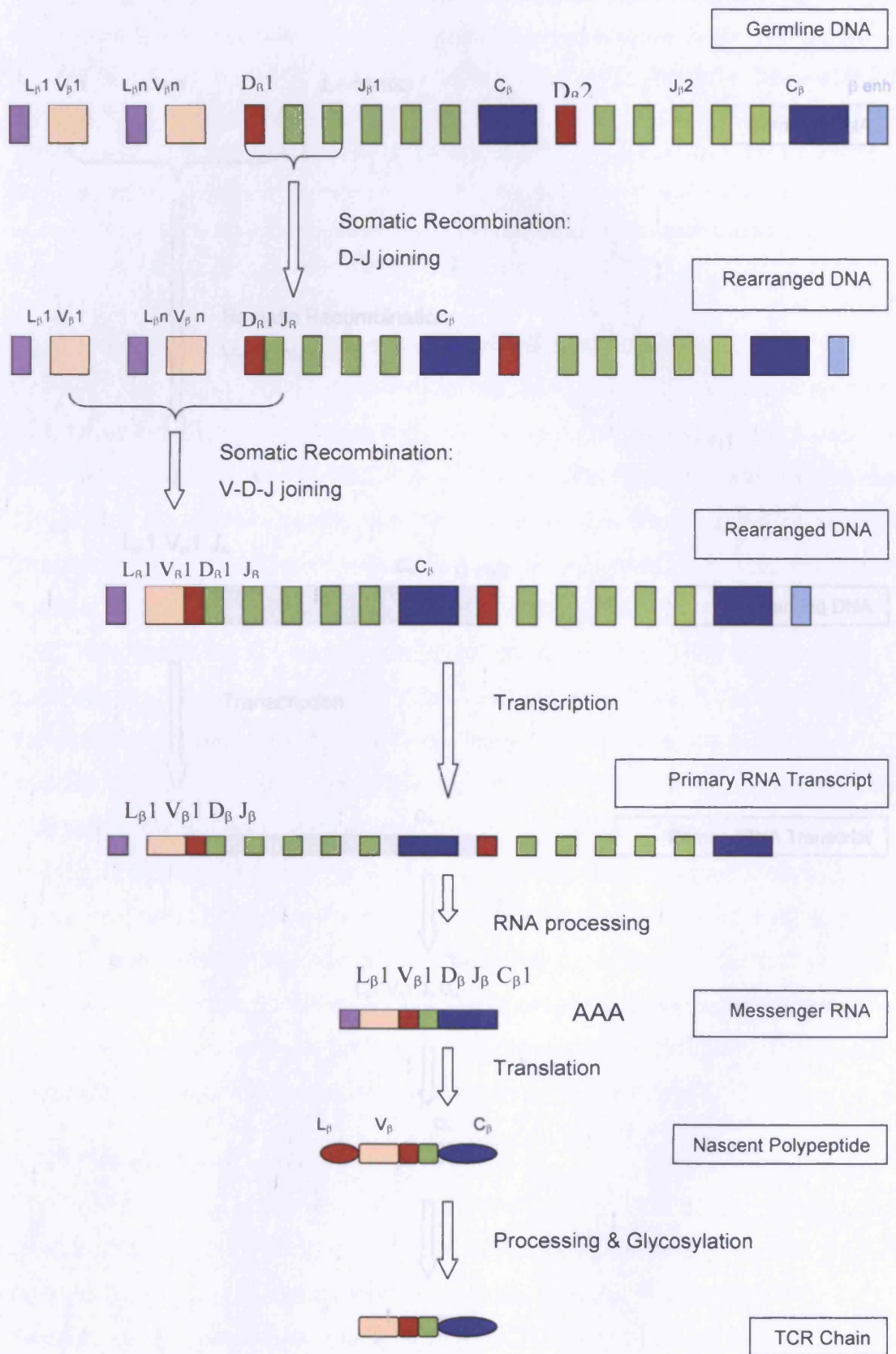


Figure 5.1a. TCR β chain gene recombination and expression (legend after Fig 1b)

TCR α chain

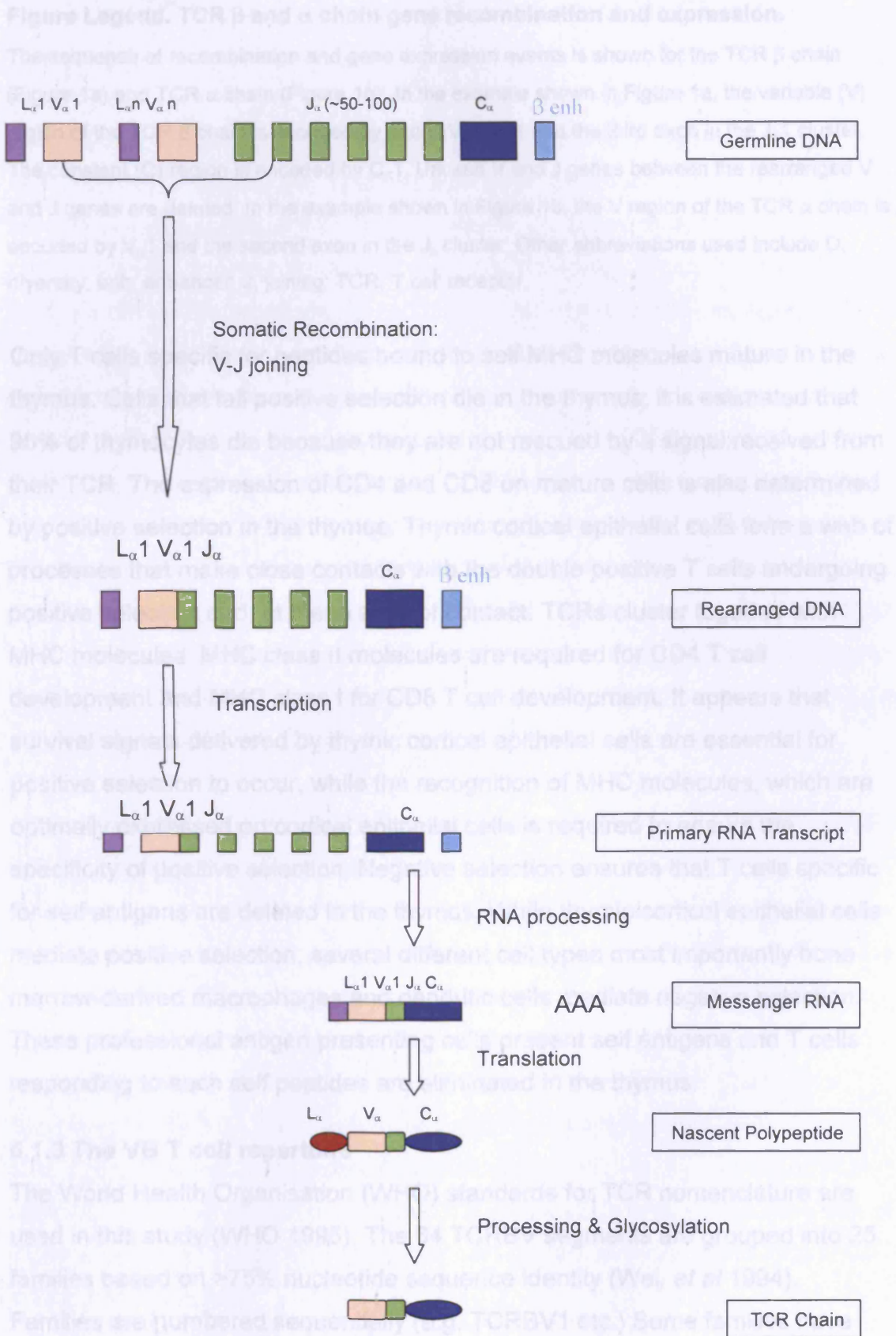


Figure 5.1b. TCR α chain gene recombination and expression (legend overleaf)

Figure 5.1a and b

Figure Legend. TCR β and α chain gene recombination and expression.

The sequence of recombination and gene expression events is shown for the TCR β chain (Figure 1a) and TCR α chain (Figure 1b). In the example shown in Figure 1a, the variable (V) region of the TCR β chain is encoded by exons $V_{\beta}1$, $D_{\beta}1$ and the third exon in the $J_{\beta}1$ cluster. The constant (C) region is encoded by $C_{\beta}1$. Unused V and J genes between the rearranged V and J genes are deleted. In the example shown in Figure 1b, the V region of the TCR α chain is encoded by $V_{\alpha}1$ and the second exon in the J_{α} cluster. Other abbreviations used include D, diversity; enh, enhancer; J, joining; TCR, T cell receptor.

Only T cells specific for peptides bound to self MHC molecules mature in the thymus. Cells that fail positive selection die in the thymus; it is estimated that 95% of thymocytes die because they are not rescued by a signal received from their TCR. The expression of CD4 and CD8 on mature cells is also determined by positive selection in the thymus. Thymic cortical epithelial cells form a web of processes that make close contacts with the double positive T cells undergoing positive selection and, at these sites of contact, TCRs cluster together with MHC molecules. MHC class II molecules are required for CD4 T cell development and MHC class I for CD8 T cell development. It appears that survival signals delivered by thymic cortical epithelial cells are essential for positive selection to occur, while the recognition of MHC molecules, which are optimally expressed on cortical epithelial cells is required to ensure the specificity of positive selection. Negative selection ensures that T cells specific for self antigens are deleted in the thymus. While thymic cortical epithelial cells mediate positive selection, several different cell types most importantly bone marrow-derived macrophages and dendritic cells mediate negative selection. These professional antigen presenting cells present self antigens and T cells responding to such self peptides are eliminated in the thymus.

5.1.3 The VB T cell repertoire

The World Health Organisation (WHO) standards for TCR nomenclature are used in this study (WHO 1995). The 64 TCRBV segments are grouped into 25 families based on >75% nucleotide sequence identity (Wei, *et al* 1994). Families are numbered sequentially (e.g. TCRBV1 etc.) Some families have more than one member and are designated BV6.1 and BV6.2, for example. Germline genes of the TCRBV chain are located on chromosome 7q, and a

cluster of six orphan genes is found on chromosome 9 (Robinson, *et al* 1993). The D, J and C segments are located at the 3' end of the TCRBV gene complex.

There are a number of molecular methodologies for the study of the human TCR repertoire, including V region PCR, 5' PCR, adapter PCR, RNase protection assays, anchor PCR and inverse PCR. These methods are cumbersome in the number of PCR reactions and gels needed for complete analysis of TCRBV repertoires. In addition, monoclonal antibodies against V regions can be usefully employed to monitor fluctuations in T cell numbers, and activation states. In the past, an incomplete panel of antibodies against all BV regions limited the use of this method in analysing the TCRBV repertoire, but more recently an increased number of TCRBV-specific mAb has become available, improving the potential for immunostaining-based TCR analysis (Muraro, *et al* 2000). However, this method does not distinguish between a monoclonal and polyclonal expansion of T cells, and fails to provide information on the composition of each BV family. This technique also requires a minimum number of T cells, whereas PCR can amplify TCR transcripts even from a single cell (Kurokawa, *et al* 1999).

Maslanka *et al* developed, and later refined a method for the molecular analysis of TCR BV region usage profiles, known as T cell spectratyping, which can delineate the composition of the T cell repertoire, indicate which BV families are involved in immune responses and provide individual profiles for each BV family (Maslanka, *et al* 1995). As a marker of sequence diversity, the TCR β chain also exhibits variability in length of its CDR3 region due to deletions and N nucleotide additions, which occur during genetic recombination (Davis and Bjorkman 1988). The T cell spectratyping method analyses this variability in CDR3 length, for each BV family and hence gives an overall measure of TCR diversity. This method has been employed in this study to study the evolution of TCR repertoire following RIT and the impact of clinical events and therapeutic interventions thereof.

5.1.4 Recovery of T cell repertoire following allogeneic stem cell transplantation

The outcome of ASCT is strongly influenced by the rate and quality of immune reconstitution. The alloresponses of donor lymphocytes create the balance between the desired graft-versus-disease effect versus GVHD, and can induce a significant morbidity and mortality through direct tissue damage. Post transplant the T cell compartment is reconstituted via 2 pathways (Dumont-Girard, *et al* 1998, Mackall, *et al* 1995, Mackall, *et al* 1997). Initially, there is antigen-driven peripheral expansion of a limited number of mature T cells that have been co-transfused with the graft, leading to a population with a TCR repertoire of limited diversity (Mackall, *et al* 1993, Roux, *et al* 1996). These mature T cells with a limited TCR diversity can be maintained in the periphery for up to 10 to 20 years. Subsequently, a thymus-dependent pathway produces T cells that have a very diverse TCR repertoire (Dumont-Girard, *et al* 1998). This mechanism involves selection of graft-derived precursor cells in the thymus (Collins, *et al* 1996). The contribution of peripheral expansion is particularly prominent when the function of the thymus is impaired. Because thymic function decreases with increasing age, thymic-dependent T cell reconstitution is thought to be most effective in paediatric ASCT recipients as reflected by delayed recovery of especially CD4+ T cells in adult ASCT recipients (Storek, *et al* 1995). The contribution of the thymus to T cell immune reconstitution in adults is not precisely known, but studies such as that by Dumont-Girard *et al* have suggested that thymic recovery post transplant does occur and contributes to the restoration of T cell recovery. More recently, studies using TCR excision circles (TRECs) to identify recent thymic emigrants post transplant have provided evidence for a significant role of the thymus in T cell reconstitution of in adults (Douek, *et al* 2000), particularly in the absence of chronic GVHD (Weinberg, *et al* 2001).

5.2 Special Methods

Blood samples were obtained from patients pre transplant, and then at 3 monthly intervals following transplant, including prior to and following DLI. The technique for isolation of peripheral blood mononuclear cells is described in Chapter 3, General Methods.

5.2.1 T cell receptor CDR3 spectratyping

RNA was extracted from PBMC using Ultraspec RNA (BiotecX Laboratories, Houston, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was generated from 1 μ g of RNA in a 20 μ l reaction using random hexanucleotide primers for reverse transcription with reverse transcriptase (Superscript, GibcoBRL, Paisley, UK). Each of 21 functionally rearranged TCRBV gene subfamilies was amplified across the CDR3-encoding regions using 23 BV subfamily-specific primers described previously by Maslanka *et al* (Maslanka, *et al* 1995) (omitting BV23 as this gave only weak product signals), and a fluorescent dye-conjugated (FAM, Perkin Elmer, Cambridge, UK) BC region-specific primer. BV primers were combined in duplex PCR reactions as follows: BV5.1 and 1; BV2 and 12; BV13 and 3; BV4 and 5.3; BV8 and 7; BV9 and 14; BV11 and 20; BV17 and 15; BV16 and 21; BV24 and 22. BV18, BV6.1 and BV6.2 were used unpaired. Hot start PCR amplifications were performed in a total volume of 20 μ l containing Genamp PCR buffer (Perkin Elmer), 2 mM MgCl₂, 0.2 mM each dNTP, 1 mM of each primer and 1 μ l cDNA. After a 5-minute denaturation step at 95°C, 0.5 U of Amplitaq DNA polymerase (Perkin Elmer) was added. Optimal cycling conditions were 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds, for 30 cycles, followed by a final extension at 72°C for 5 minutes. One microlitre of PCR product was denatured in 12 μ l formamide in the presence of 0.3 μ l Tamra 500 size standard (Perkin Elmer) and electrophoresed through Performance Optimized Polymer 4 (Perkin Elmer) on an ABI 310 automated sequencer (Perkin Elmer). Genescan software 2.1 (Perkin Elmer) was used to analyse the data. Data on peak sizes and areas were then exported directly to Excel spreadsheets for further analysis.

5.2.2 TCR repertoire diversity score

A number of systems have been devised to describe TCR diversity quantitatively and/ or qualitatively. There has been little consensus as to the most appropriate method. Some methods score the spectratype on the number of discrete peaks present per BV family ('complexity score') (Wu, *et al* 2000). Others have defined a normal spectratype as consisting of at least 6 peaks spaced 3 nucleotides apart without any gaps (Verfuerth, *et al* 2000). Orsini *et al* calculated 'Z scores' using means and standard deviations for the utilisation of BV gene segments in normal

subjects as a reference value against which patient data were compared (Orsini, *et al* 2000).

The present study involved the examination of large numbers of spectratypes from 19 patients obtained at multiple intervals post transplant. It was felt that a robust and reproducible system was needed to convert the visual data obtained in each spectratype (number of peaks, Gaussian distribution of peaks or the degree of skew or oligoclonality present) into statistical data that would accurately represent the evolution of the TCR repertoire. A novel scoring system was therefore devised to represent overall TCR repertoire diversity as derived from 23 BV spectratypes obtained for each timepoint per patient and termed the TCR diversity score (Peggs, *et al* 2003b). Spectratypes were produced for 12 normal adults. Numerical data from 4 spectratype parameters (peak number, peak area, kurtosis and skew) were recorded. Peak areas were converted to percentages of total peak area for each given BV subfamily to allow comparison between samples. For each BV family, the maximum possible score was four, making the maximum possible overall score 92 (23 x 4) for complete analysis. One point was deducted for each of the following: peak number < 6; 2 or more peaks showing a peak area > 3sd above the mean value; 1 or more peaks showing a peak area > 10% above the mean value; skew and kurtosis values both > 3sd outside the mean values. Using this diversity scoring system, normal adult samples scored a median of 80 (range 69 to 86). A similar analysis was carried out for patient spectratypes.

5.3 Results

Diversity scores were calculated for 16 patients pre-transplant, and at 3 monthly intervals post transplant. Results are shown in Figure 5.2.

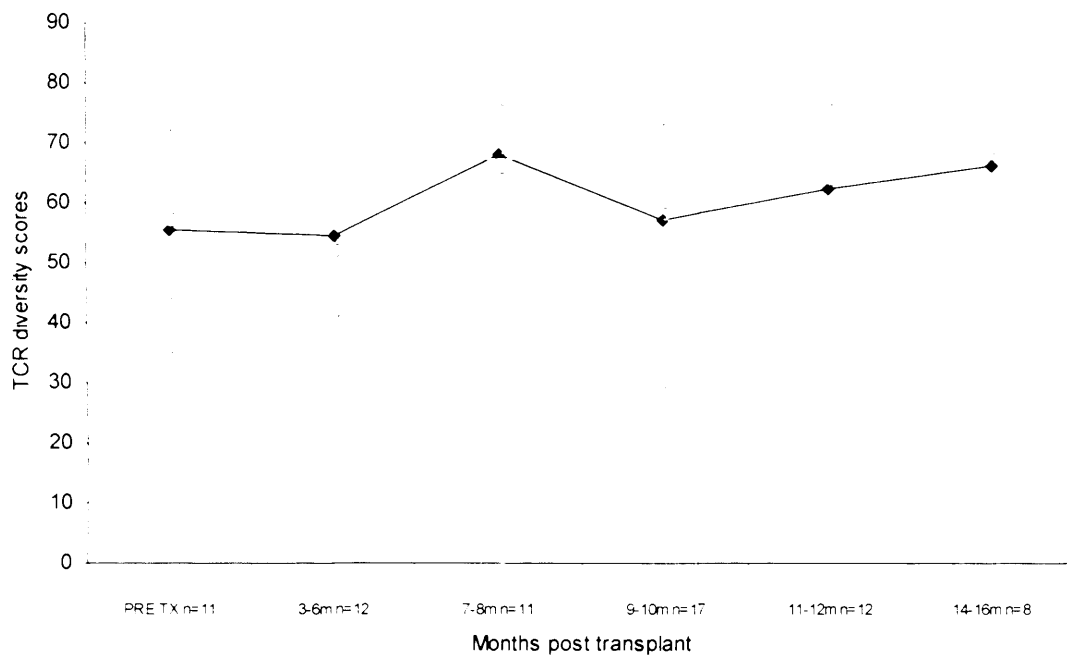


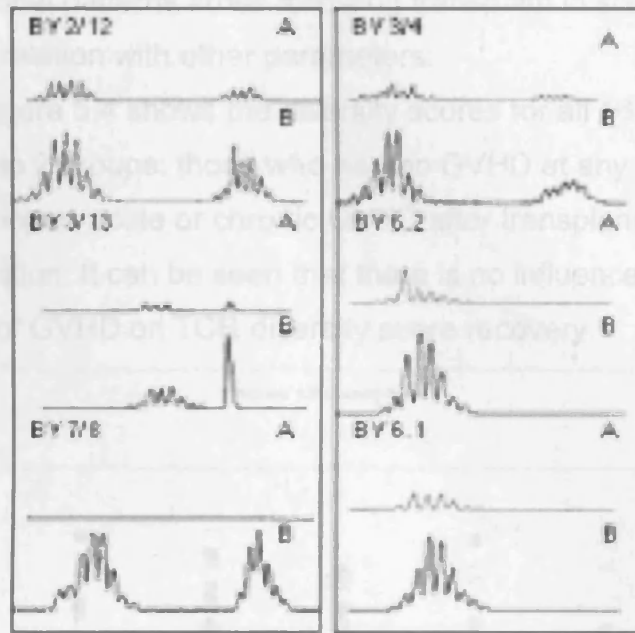
Figure 5.2. Median TCR diversity scores following transplant.

Median TCR diversity scores are shown in red prior to transplant, then at the timepoints indicated. Individual TCR diversity scores are shown as grey dashes. At each time point, the number of patients analysed is indicated. The normal range is indicated by a grey box.

Pre-transplant diversity scores were available in 11 patients and were below the normal adult range in 8 of them (median 56, range 26 to 78). Analysis of pre-transplant subfamily spectratype profiles and scores showed no relationship with myeloma isotype, prior therapy or previous autograft. In addition, there was no over- or under-representation of specific subfamilies. At 3-6 months following transplant, but before the administration of DLI, more than 90% of the patients' scores (median 55, range 38-77) remained below the normal adult range (69-82). Thereafter there was a trend to improvement in the median diversity score, and a greater overlap with the normal range. At 12 months post-transplant, approximately two thirds of patients remained below the normal range. Representative spectratypic profiles for patients 2 and 9 are shown in Figure 5.3 (next page). While many BV families show no or few representative members early after transplant, there is a tendency to a Gaussian pattern beyond the 9 month timepoint.

5.3.4 Effect of GVHD, stem cell source, donor type and disease status

Patient No.2



Patient No.9

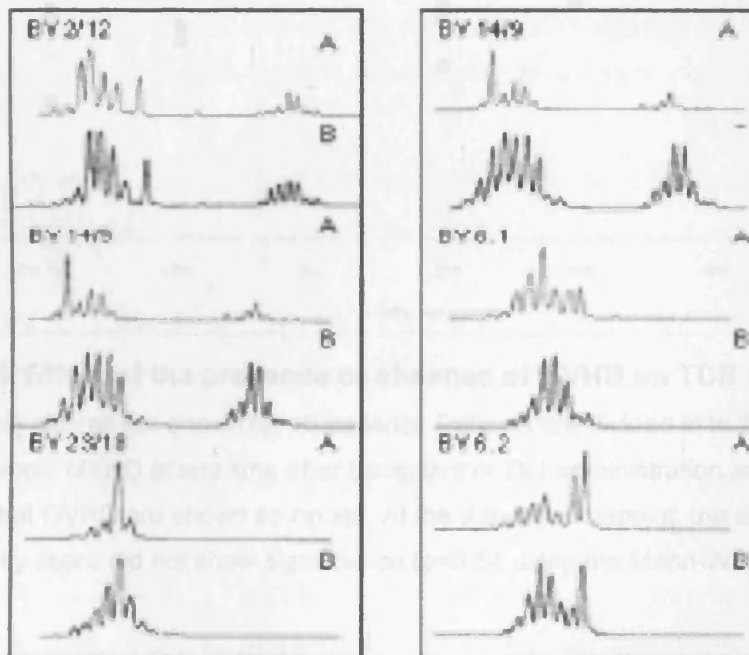


Figure 5.3. BV spectratype profiles in patients 2 and 9

The recovery of a Gaussian pattern in representative examples of BV family pairs is shown at 6 months (A) and 9 months (B) post transplant for patients 2 and 9.

5.3.1 Effect of GVHD, stem cell source, donor type and disease status

Although oligoclonal patterns arose following transplant in some patients, there was no clear correlation with other parameters:

1. **GVHD.** Figure 5.4 shows the diversity scores for all 16 patients analysed, divided into 2 groups: those who had no GVHD at any stage, and those who developed acute or chronic GVHD after transplantation or DLI administration. It can be seen that there is no influence of the presence or absence of GVHD on TCR diversity score recovery.

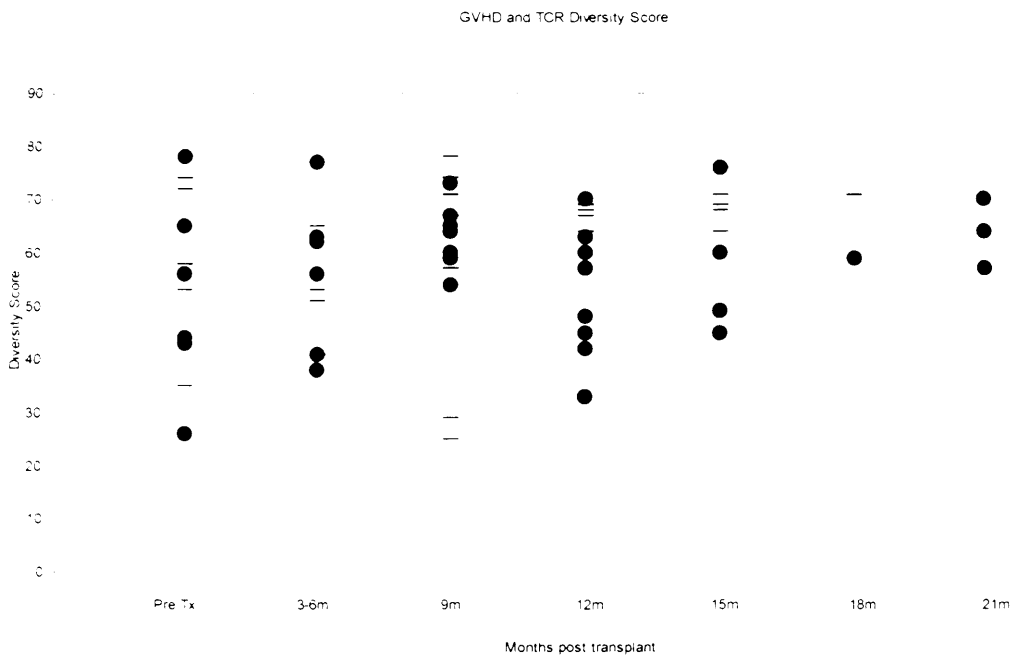


Figure 5.4. Effect of the presence or absence of GVHD on TCR diversity scores

TCR diversity scores are shown for 16 patients. Patients are divided into 2 groups: those with acute or chronic GVHD at any time after transplant or DLI administration are shown as dashes. Those without GVHD are shown as circles. At the 9 month timepoint, the effect of GVHD on TCR diversity score did not show significance ($p=0.54$ using the Mann-Whitney test).

2. **Stem cell source.** In this transplant protocol, the donor type determined the stem cell source. Sibling donors provided peripheral blood stem cells by apheresis whereas matched unrelated donors provided bone marrow stem cells. Figure 5.5 is a plot of diversity scores according to stem cell source, and shows no influence of this parameter on diversity score recovery.

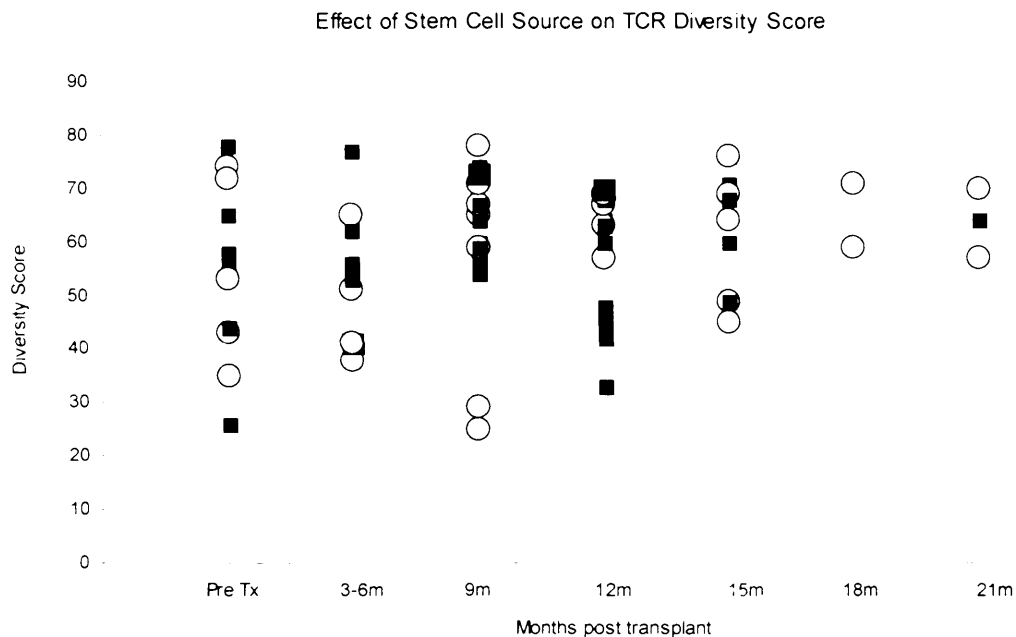


Figure 5.5. Effect of stem cell source on TCR diversity score

Data is shown for 16 patients at 3 monthly intervals. Patients who received peripheral blood stem cell transplants from sibling donors are shown as black squares, and those who received bone marrow transplants from matched unrelated donors are shown as open circles.

- Disease status.** Disease status post transplant did not alter the overall score in individuals.

5.3.2 Effect of T lineage chimeric status and donor leucocyte infusions

Table 5.1a shows the number of patients who received DLI, together with the proportion who were mixed or full donor chimeras in their T cell lineage at each time point, as well as the median diversity scores and ranges. Table 5.1b shows diversity score data for each patient, complete with an indication of when patients converted to full donor T lineage chimerism and whether or not they had received DLI at each time point. The majority (12 of 15 evaluable) of patients showed mixed chimerism in the T cell lineage at 6 months; at this time point, the median TCR diversity score was 55. By 15 months, by which time 14 out of 16 patients had received DLI, the median diversity score had risen to 63 (n=13), suggesting a possible effect of DLI on TCR repertoire. At this time, 6 patients had persistent mixed chimerism in at least one cell lineage and had lower diversity scores (median 60, range 49-71) compared with 4 patients who were full donor in all cell lineages (median 68, range 45-76, $p < 0.05$). This suggests that the administration

of DLI, and conversion to full donor T cell chimeric status may positively influence the restoration of TCR repertoire.

Timepoint	DLI in:	T cell chimeric status			TCR Diversity Score		
		Mixed	Full donor	Data available in:	Median	Range	Data available in:
Pre	0				56	26-78	11
3-6 m	0	12	3	15	55	38-77	12
9m	12	8	8	16	65	25-78	16
12m	14	5	9	14	63	33-70	13
15m	14	5	5	10	9	45-76	10
18m	15	2	2	4	65	59-71	3
21m	15	2	0	2	64	57-70	2

Table 5.1a. T lineage chimeric status post transplant and TCR diversity scores

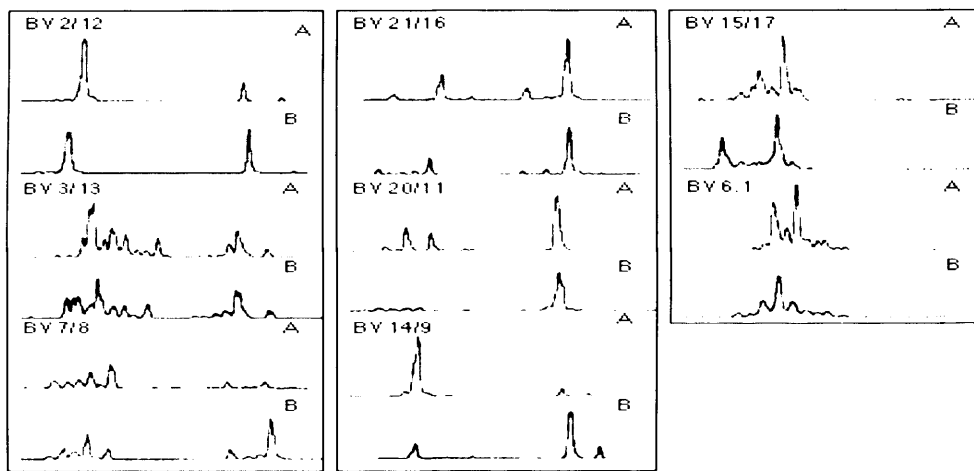
Data is shown for the specified timepoints. The proportion of patients who received DLI at each timepoint is indicated in the second column. The proportion of patients who were mixed chimeras or showed full donor chimerism is shown in columns three and four, and the median and range of TCR diversity scores in the fifth and sixth columns, together with an indication of the number of patients assessed.

Patient	pre Tx	3-6m	9m	12m	15m	18m	21m
1			60	42	76		
2			57	64	71		
3		51	29		64		
4			65	63	49		70
5	43	38	59	57	45	59	57
6	56		64	48			
7	65	63	59	60	49		64
8	74	41	78				
9	53	65	67	67	69	71	
10	44	58	67	45	60		
11	58	63	74	68	68		
12	72	65	71	69	76		
13	78	77	54	63			
14		41	73	70			
15	26	62	73	33			
16	35	41	25				
Median score	56	55	65	63	66	65	64

Table 5.1b. TCR diversity scores and T lineage chimeric status per patient, and the effect of DLI administration.

Data are shown for each of 16 patients, (who had a minimum of 3 analyses of their TCR repertoire by spectratyping) and the TCR diversity score at each timepoint. Chimeric status is also indicated: blue shading indicates mixed T cell chimerism and grey shading indicates full donor T cell chimerism. Red numbers indicate a score calculated prior to DLI administration, and black numbers indicate post DLI scores. Median TCR diversity scores are shown in the last row.

Patient 10, who reactivated TB 6 months post transplant, showed pronounced oligoclonality in individual spectratypes, at 6 and 8 months post transplant (Figure 5.6). Her diversity score at 6 months was 56. She received DLI soon after the 8-month timepoint. Thereafter, her diversity score fell to 45 at 3 months post DLI, with persistently oligoclonal spectratypes, and then rose to 60 at three months after her second DLI. However, oligoclonal spikes persisted in spite of receiving the second DLI.



The persistence of oligoclonal spikes in representative BV family pairs at 6 months (A) and 9 months (B) post transplant is shown for patient 10 who reactivated TB 6 months post transplant.

Figure 5.6. BV spectratype profiles in patient 10

5.3.3. TCR repertoire and T cell phenotype.

The size of the CD4+CD45RA+ cell compartment is a reliable indicator of the reconstitution of naïve thymus-derived CD4+ cells, as discussed in Section 4.4. The relationship between TCR repertoire (expressed as the diversity score) and CD4+CD45RA+ cell numbers was therefore assessed, both overall using median values (Figure 5.7a) and individually in 4 patients (patients 4, 5, 9 and

11, Figure 5.8), who had sufficient follow up data for correlation purposes. For comparison, median values of CD4+CD45RO+ cells were also plotted against TCR diversity score (Figure 5.7b).

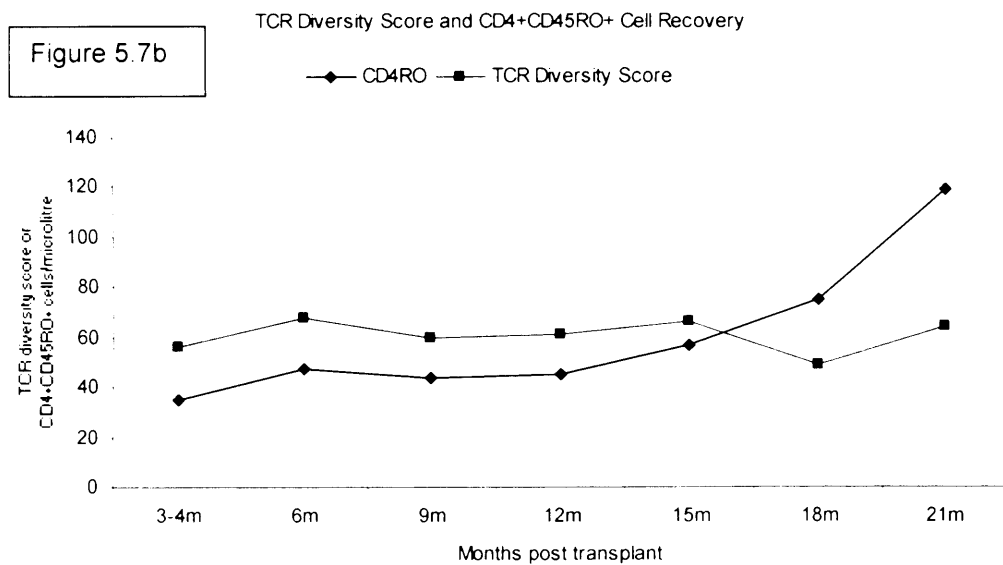
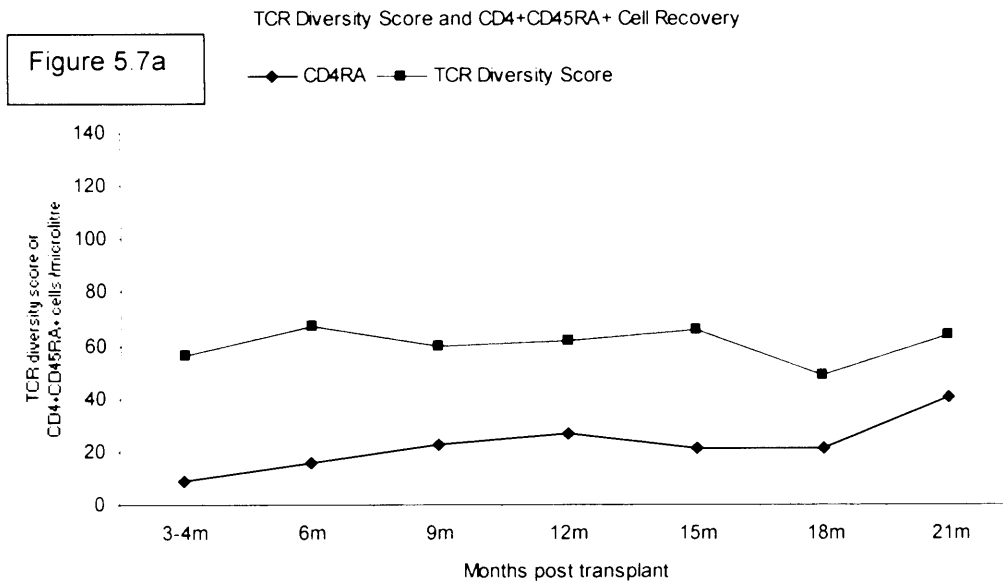


Figure 5.7. TCR diversity score and CD4+CD45R+ subset recovery

Median CD4+CD45RA+ cell numbers (Figure 5.7a) or CD4+CD45RO+ cell numbers (Figure 5.7b) are plotted with median TCR diversity scores to illustrate the relationship between CD4+CD45R+ subset recovery and T cell repertoire recovery.

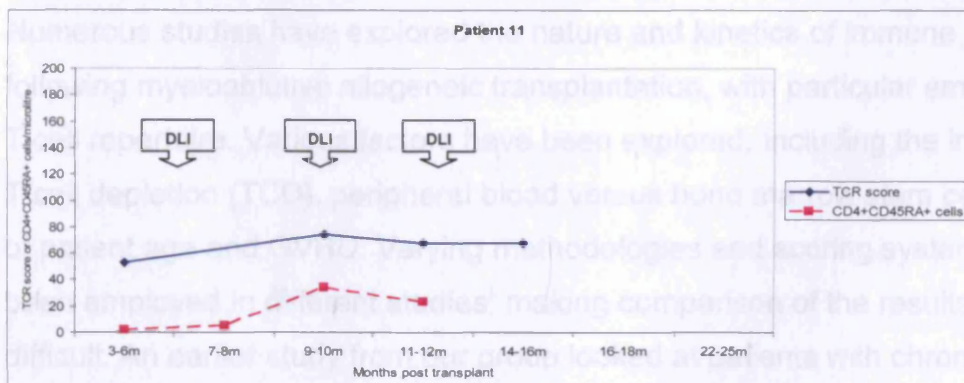
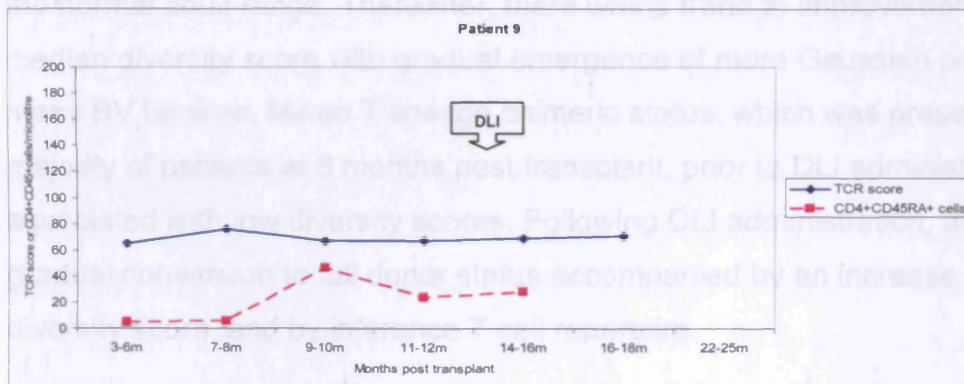
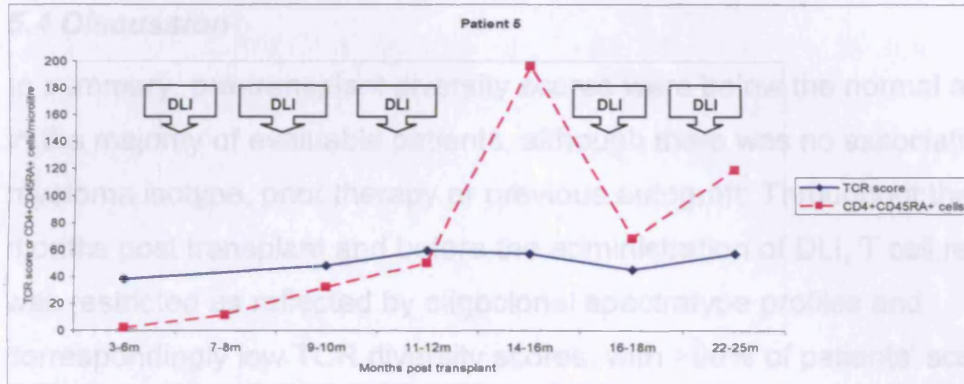
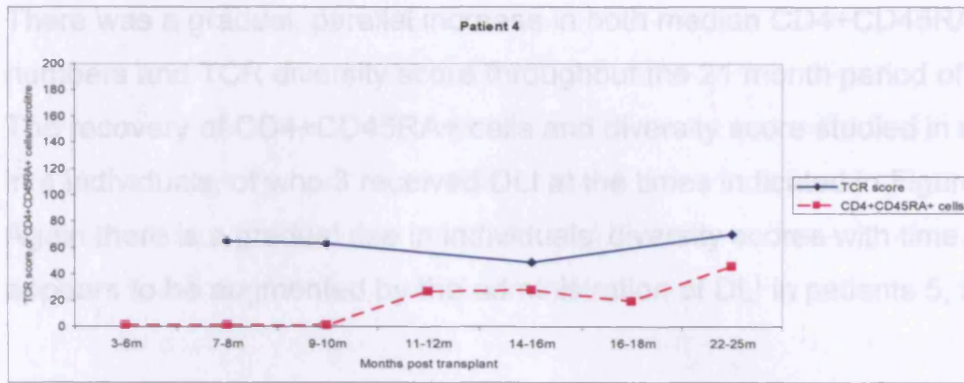


Figure 5.8. TCR diversity score and CD4+CD45RA+ cell recovery in individual patients.

TCR diversity scores and absolute CD4+CD45RA+ cell numbers are shown at the timepoints indicated for 4 patients following transplantation. Where administered, donor lymphocyte infusions are indicated by an arrow.

There was a gradual, parallel increase in both median CD4+CD45RA+ cell numbers and TCR diversity score throughout the 21 month period of follow up. The recovery of CD4+CD45RA+ cells and diversity score studied in more detail in 4 individuals, of who 3 received DLI at the times indicated in Figure 5.8. Again there is a gradual rise in individuals' diversity scores with time. This rise appears to be augmented by the administration of DLI in patients 5, 9 and 11.

5.4 Discussion

In summary, pre-transplant diversity scores were below the normal adult range in the majority of evaluable patients, although there was no association with myeloma isotype, prior therapy or previous autograft. Throughout the first 6 months post transplant and before the administration of DLI, T cell repertoire was restricted as reflected by oligoclonal spectratype profiles and correspondingly low TCR diversity scores, with >90% of patients' scores below the normal adult range. Thereafter, there was a trend to improvement in the median diversity score with gradual emergence of more Gaussian profiles in many BV families. Mixed T lineage chimeric status, which was present in the majority of patients at 6 months post transplant, prior to DLI administration, was associated with low diversity scores. Following DLI administration, there was a gradual conversion to full donor status accompanied by an increase in the diversity score, and by inference T cell repertoire.

Numerous studies have explored the nature and kinetics of immune recovery following myeloablative allogeneic transplantation, with particular emphasis on T cell repertoire. Various factors have been explored, including the influence of T cell depletion (TCD), peripheral blood versus bone marrow stem cells, effect of patient age and GVHD. Varying methodologies and scoring systems have been employed in different studies, making comparison of the results more difficult. An earlier study from our group looked at patients with chronic myeloid leukaemia who had received myeloablative conditioning and TCD grafts (Verfuerth, *et al* 2000). We showed using TCR spectratyping, that T cell repertoire is skewed in the early post-transplant phase, with oligoclonality in the first 3-6 months after transplant followed by a gradual trend towards more normal patterns by 12 months. In one-third of patients, the spectratype pattern

took 2 to 3 years to normalise, and in two-thirds some abnormality persisted even after several years. Half of the patients who received donor leukocyte infusions (DLI) showed no change in T cell repertoire after DLI, about a fifth showed an improvement, but a third showed a more restricted pattern after DLI (Verfuert, *et al* 2000). Another study using similar methodology looked at the effect of T cell depletion and found that the T cell repertoire was more restricted in recipients of TCD grafts than unmanipulated grafts (Roux, *et al* 1996). Similar results have been found using immunofluorescent methods (Gaschet, *et al* 1995, Villers, *et al* 1994). Wu *et al* used spectratyping to show that the reconstitution of a normal TCR repertoire following myeloablative TCD allogeneic BMT in adults is related to haematopoietic chimerism: complete donor chimerism was strongly correlated with the restoration of a diverse TCR repertoire, whereas persistent recipient haematopoiesis was associated with a restricted repertoire (Wu, *et al* 2000).

Nonmyeloablative regimens are designed to provide sufficient immunosuppression to achieve donor engraftment rather than eradicate the underlying malignancy by direct chemo/radiotherapy-induced cell-kill. There are few studies exploring the recovery of TCR repertoire following nonmyeloablative transplantation. Friedman *et al* have shown that recovery of a normal TCR repertoire was more rapid following nonmyeloablative than fully ablative SCT in the unrelated donor setting (Friedman, *et al* 2001). A study of adult recipients of nonmyeloablative umbilical cord blood stem cell transplantation showed that the TCR repertoire was markedly more diverse and robust compared with the repertoire in those receiving myeloablative regimens (Chao, *et al* 2002). The more rapid restoration of the T cell repertoire after nonmyeloablative transplantation reported in these 2 studies may relate to the reduced transplant-related acute toxicity. A number of mechanisms contribute to the reduced toxicity seen after nonmyeloablative procedures. Engraftment occurs more quickly after nonmyeloablative conditioning, with a median of 11-15 days to neutrophil recovery ($>0.5 \times 10^9/l$) in different studies (Giralt, *et al* 1997, Khouri, *et al* 1998, Slavin, *et al* 1998). This minimises the duration of the risk period for severe bacterial infections and their potential contribution to acute tissue damage in the immediate post transplant period. Mucositis is frequently absent, and veno-occlusive disease (VOD) is infrequent and exclusive to busulphan-

containing regimens (Barrett and Childs 2000). The reduction in the extent of tissue damage due to nonmyeloablative conditioning regimens abrogates the so-called 'cytokine storm' that is seen in myeloablative transplantation, in which inflammatory cytokines are released from damaged host tissues. These cytokines, including IL-1 and TNF- α upregulate the expression of adhesion molecules and host MHC antigens, and enhance recognition of the host tissue by mature donor T cells. On recognition of alloantigens, donor Th1 cells are activated and secrete IL-2 and IFN- γ , which recruit other T cells, cytotoxic T cells, NK cells, monocytes and macrophages (Via and Finkelman 1993). Subsequently, mononuclear cells primed by Th1 cells secrete more TNF- α and IL-1, which induce cellular damage or apoptosis, and restart the cycle of inflammation. Thus, in myeloablative transplantation, inflammatory and alloreactive immune processes serve to drive the oligoclonal expansion of memory T cells, leading to persistent skewing of the TCR repertoire.

The conditioning regimen in this study differs from the studies cited above in that it results in profound and prolonged TCD caused by alemtuzumab. As a result the recovery of CD4⁺CD45RA⁺ T cells was markedly delayed, with levels well below normal in the first year post transplant. Despite this, the spectratype profiles for most BV families, and the calculated diversity scores showed good recovery, the former with a greater tendency to the Gaussian by 9 months post transplant. The notable exception was patient 10, who had reactivation of TB infection at 6 months post transplant. In this patient, expansion of TB-reactive T cell clones, particularly following the administration of DLI, led to persistent skewing of many of her spectratype profiles, including BV 8, BV16 and BV17. It is possible that expanded TB-specific memory T cells occupied peripheral niches, thus preventing their repopulation by thymus-derived naïve CD45RA⁺ cells. Hence in this patient, the pattern of T cell recovery more closely resembles that seen in recipients of myeloablative regimens, where severe infections and GVHD lead to oligoclonal expansion of reactive T cell clones and hence a more prolonged skewing of the T cell repertoire.

In summary, despite the prolonged T cell depletion caused by this alemtuzumab-containing regimen, detailed analysis of T cell repertoire showed that although oligoclonal patterns predominated in the early post transplant

period, in the absence of the major toxicities of severe infections and severe acute GVHD seen in conventional allogeneic regimens, this group of patients demonstrated a fairly rapid restoration of normal TCR repertoire.

Chapter 6: B cell reconstitution: Circulating CD19 numbers, immunoglobulin levels and immunoglobulin heavy chain gene spectratype analysis

6.1 introduction

B cells play an important role in the defence against microorganisms. The main mechanism is via humoral immunity and the generation of antibodies against specific infectious antigens. Secreted antibodies are the effector molecules of humoral immunity, and the differentiation of B cells from antigen-recognising to effector cells involves a change in immunoglobulin expression from the membrane to the secreted form. Like B cell proliferation, antibody synthesis and secretion in response to protein antigens are stimulated by CD40-mediated signals and helper T cell-derived cytokines such as IL-2, IL-4 and IL-5, which activate transcription factors that enhance the transcription of immunoglobulin genes and therefore immunoglobulin synthesis. Interleukin-6, which is produced by macrophages, T cells, and many other cell types, is a growth factor for antibody-producing B cells that have already differentiated. Within lymphoid tissue, antibody-secreting cells are found mainly in extrafollicular sites, such as the red pulp of the spleen and the medulla of the lymph nodes. These cells also migrate to the bone marrow and at 2 to 3 weeks after stimulation by antigen, the bone marrow may be a major site of antibody production. Many of the antibody-secreting B cells differentiate into plasma cells that are morphologically distinct B cells committed to abundant antibody production. Secreted antibodies enter the circulation, but antibody-producing cells do not circulate actively. Antibodies in the blood and interstitial fluids bind antigens to initiate the effector phase of the humoral immune response. Memory B cells enhance the immune response to previously encountered antigens in the secondary immune response.

6.1.1 B Cell Development

In humans, the first cells to display cell surface antigens that mark commitment to the B lineage are detected in the fetal liver at approximately 8 weeks of gestation. B cell production ceases at this site in late pregnancy. Subsequently, B cells are also produced in the red marrow, and this production continues throughout adult life. Pro-B cells do not express H or L chains, since they have not yet begun the V(D)J gene rearrangement process that is the hallmark of B lymphocytes and is

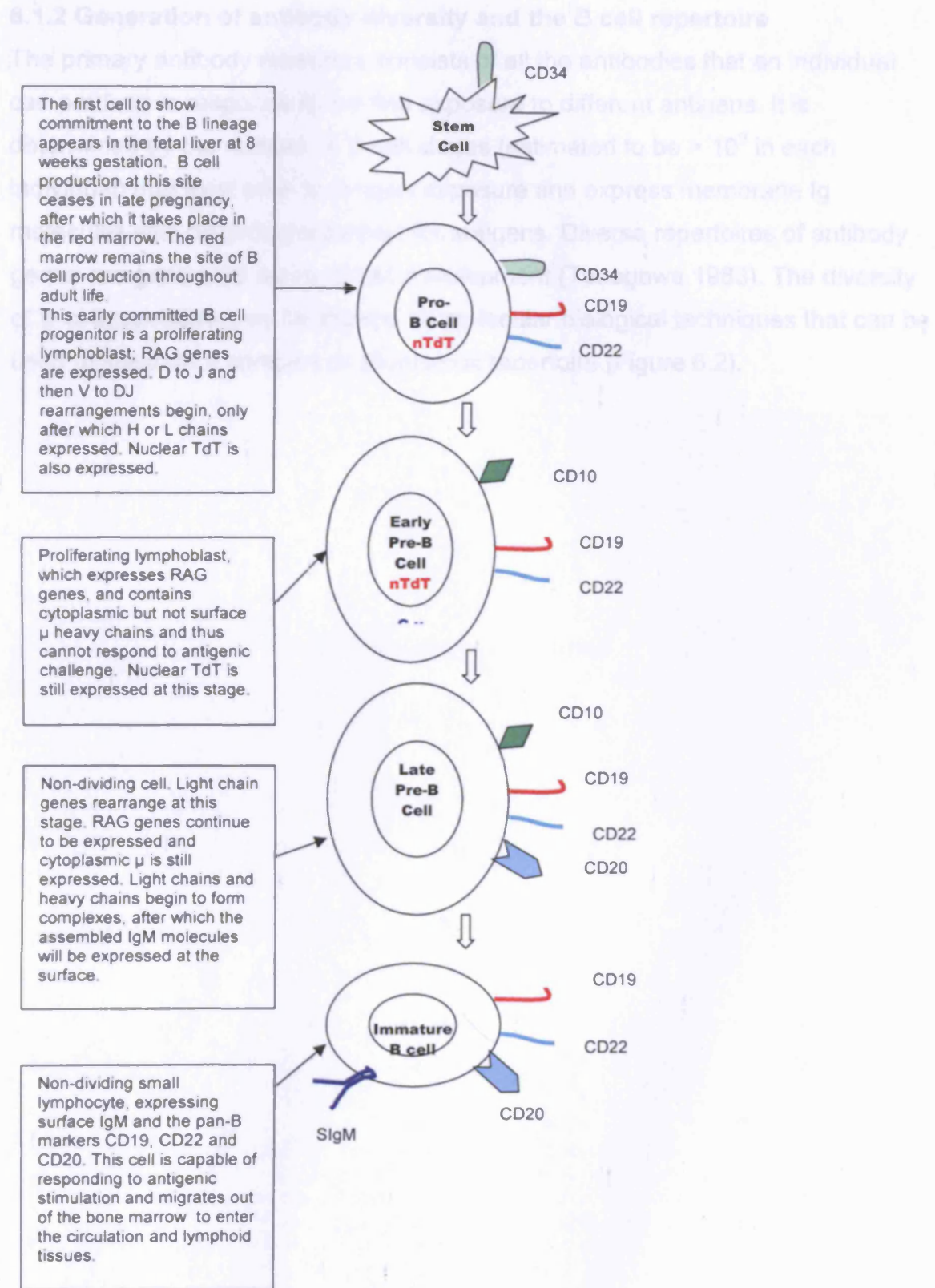
an obligate step in antigen receptor expression (Kuehl 1983). Although cellular differentiation along the B lineage pathway is most realistically viewed as a continuum, it can be divided into discrete developmental stages. The expression of surface Ig identifies the B cell stage. The earliest cell that synthesises a detectable IgG gene product (pre-B lymphocyte) contains cytoplasmic μ heavy chains composed of variable (V) and constant (C) regions and is found only in haematopoietic tissues such as the bone marrow and fetal liver. This cell does not express functional, fully assembled membrane IgM, since surface expression requires synthesis of both heavy and light chains. Thus pre-B cells cannot recognise or respond to antigen. At the next identifiable stage in B cell maturation, κ or λ light chains are also produced. These form a complex with μ heavy chains, and then the assembled IgM molecules are expressed on the cell surface, where they function as specific receptors for antigens. IgM-bearing B cells that are recently derived from marrow precursors are called immature B lymphocytes because they do not proliferate and differentiate in response to antigens. Once a B cell expresses a complete heavy or light chain, it cannot produce another heavy or light chain containing a different V region. Having acquired the ability to produce complete Ig molecules, and therefore specificity, B cells migrate out of the bone marrow and enter the circulation and lymphoid tissues.

The survival and function of newly formed B cells is initially dependent on the specificity of their immunoglobulin heavy chain gene. Autoreactive B cells can be eliminated or rendered tolerant ('anergised') at this stage of development. Mature B cells that survive negative selection co express μ and δ heavy chains in association with the original κ or λ light chain and therefore produce both membrane and IgM and IgD. Such cells are responsive to antigens and receive T cell help in their encounter with antigen. Mature B cells which encounter cognate antigen become activated B lymphocytes. Activated B cells proliferate and differentiate, producing an increasing proportion of their Ig in a secreted form and progressively less in a membrane-bound form, ultimately as plasma cells. Some of the progeny of activated B cells undergo heavy chain class (isotype) switching and begin to express Ig heavy chain classes other than μ and δ (e.g. γ , α and ϵ). Some of the antigen-activated B cells do not develop into antibody secretors, but

instead acquire the ability to survive for long periods of time as memory B cells that are available for anamnestic responses to recall antigens. Memory cells survive for weeks or months apparently without antigenic stimulation, and actively circulate between the blood, lymph and lymphoid organs. They are capable of mounting rapid responses to subsequent introduction of antigen.

The B cell developmental process can be usefully tracked using monoclonal antibodies against different cluster of differentiation (CD) molecules. A subpopulation of pre-B cells expresses CD34, also found on early haematopoietic cell precursors, including the haematopoietic stem cell. The earliest B cell progenitors express CD10, the common acute lymphoblastic leukaemia antigen. They also express CD19, which is found throughout the B lineage except in terminally differentiated plasma cells. The CD20 pan-B cell marker arises somewhat later as pre-B cells initiate Ig gene rearrangement. CD40 is expressed throughout B cell development except in terminally differentiated plasma cells. its ligand (CD40L) is expressed on activated helper T cells. The interaction of CD40 with CD40L is the conduit for T cell help, and the promotion of isotype switching. The sequential expression of cell surface molecules and intranuclear terminal deoxynucleotidyl transferase (TdT) expression during B lymphocyte maturation is schematically represented in Figure 6.1.

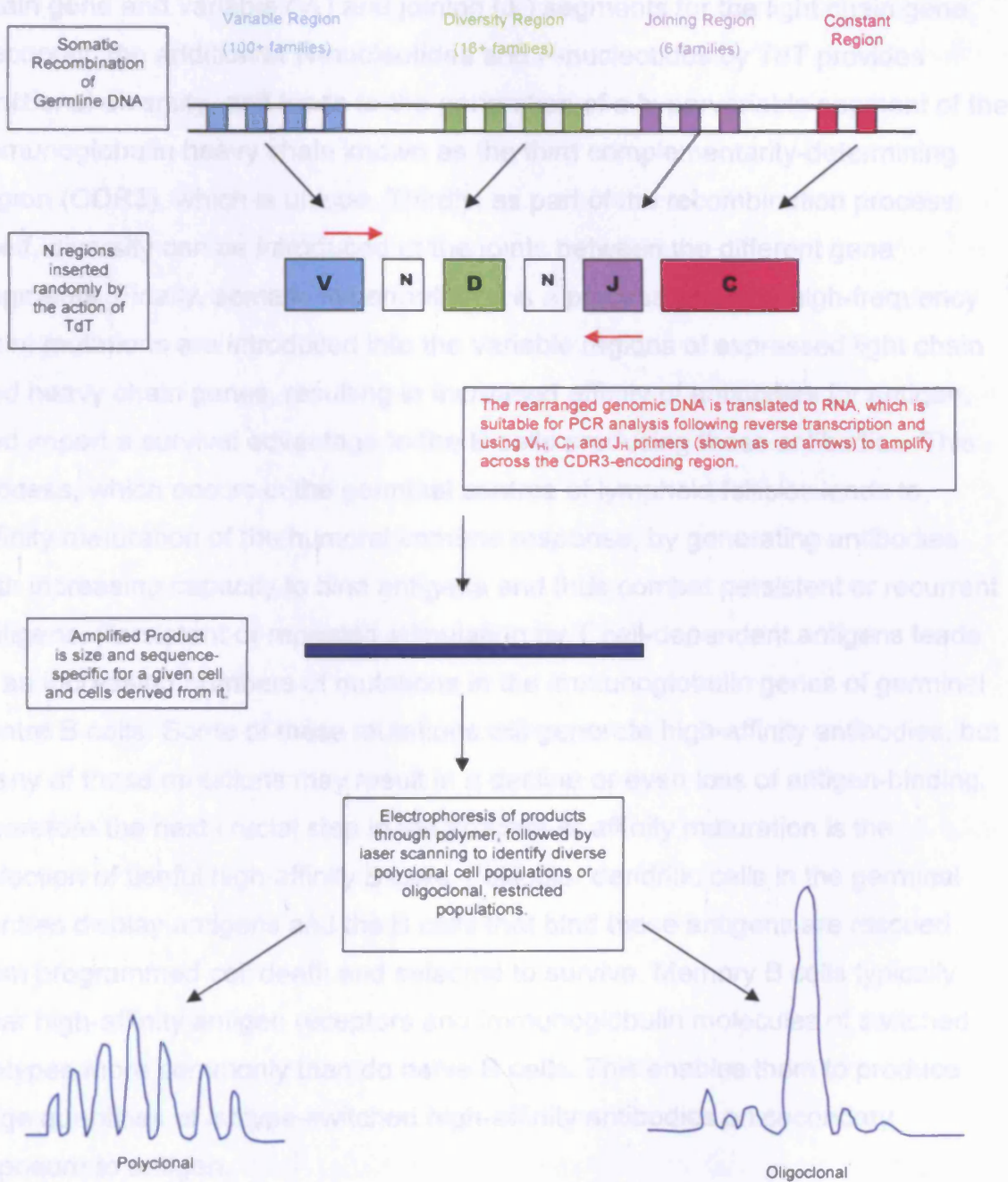
Figure 6.1. Expression of nuclear, cytoplasmic and surface markers during B cell development



6.1.2 Generation of antibody diversity and the B cell repertoire

The primary antibody repertoire consists of all the antibodies that an individual can produce in response to the first exposure to different antigens. It is determined by the number of B cell clones (estimated to be $> 10^9$ in each individual) that exist prior to antigen exposure and express membrane Ig molecules with distinct specificities for antigens. Diverse repertoires of antibody genes are generated during B cell development (Tonegawa 1983). The diversity of B cell repertoire may be studied by molecular biological techniques that can be used to illustrate a complex or oligoclonal repertoire (Figure 6.2).

Figure 6.2. Generation of B cell repertoire diversity and its study using PCR-based methodology



Combinatorial rearrangement of variable (V), diversity (D) and joining (J) segments of genomic DNA is one of the principle mechanisms of the generation of antibody diversity. Another crucial step, junctional diversity, by means of random N-nucleotide addition by TdT leads to the generation of the hypervariable segment of the immunoglobulin heavy chain gene (CDR3). The resulting variation in CDR3 size can be detected by a fluorescent PCR based method, with the resulting diverse or restricted 'spectratypes', as performed in this study.

This diversity is achieved by 4 main processes (Calame 1985). Firstly, combinatorial rearrangement of variable (V_H), diversity (D_H) and joining (J_H) segments (of which there are 100+, 15 and 6 genes respectively) for the heavy chain gene and variable (V_L) and joining (J_L) segments for the light chain gene. Secondly, the addition of N-nucleotides and P-nucleotides by TdT provides junctional diversity, and leads to the generation of a hypervariable segment of the immunoglobulin heavy chain known as the third complementarity-determining region (CDR3), which is unique. Thirdly, as part of the recombination process itself, diversity can be introduced at the joints between the different gene segments. Finally, somatic hypermutation is a process whereby high-frequency point mutations are introduced into the variable regions of expressed light chain and heavy chain genes, resulting in increased affinity of antibodies for antigen, and impart a survival advantage to the B cells producing those antibodies. This process, which occurs in the germinal centres of lymphoid follicles leads to affinity maturation of the humoral immune response, by generating antibodies with increasing capacity to bind antigens and thus combat persistent or recurrent antigens. Persistent or repeated stimulation by T cell-dependent antigens leads to an increasing numbers of mutations in the immunoglobulin genes of germinal centre B cells. Some of these mutations will generate high-affinity antibodies, but many of these mutations may result in a decline or even loss of antigen-binding. Therefore the next crucial step in the process of affinity maturation is the selection of useful high-affinity B cells. Follicular dendritic cells in the germinal centres display antigens and the B cells that bind these antigens are rescued from programmed cell death and selected to survive. Memory B cells typically bear high-affinity antigen receptors and immunoglobulin molecules of switched isotypes more commonly than do naïve B cells. This enables them to produce large quantities of isotype-switched high-affinity antibodies on secondary exposure to antigen.

Thus, within 4 to 7 days of antigen exposure, some of the activated B cells migrate deep into the lymphoid follicle and begin to proliferate rapidly, forming the germinal centre. The doubling time of these germinal centre B cells, also called centroblasts, is estimated to be 6 to 12 hours, so that within 5 days a single lymphocyte may give rise to almost 5000 progeny. Each fully formed germinal centre contains cells derived from only one or a few antigen specific B cell

clones. The progeny of the proliferating cells in the germinal centre are smaller cells, known as centrocytes, which undergo differentiation and selection processes outlined in Section 6.1.1. and above. The formation of germinal centres depends on the presence of helper T cells and the interaction between CD40 and CD40L and is therefore only observed in antibody responses to helper T cell-dependent protein antigens.

6.1.3 The immunoglobulin heavy chain variable region (V_H) gene families

Human V_H segments can be divided into 6 main families: V_H1 to V_H6 on the basis of nucleotide homology of 80% or above (Berman, *et al* 1991). V_H gene family usage in the normal adult B cell repertoire is proportional to the number of functional germline genes within each V_H family (Rettig, *et al* 1996). For example, the V_H3 family is the largest V_H family with approximately 22 functional members that account for 50 to 60% of rearrangements in the normal adult repertoire (Gokmen, *et al* 1998). The V_H2 family on the other hand has just 3 functional members, and contributes proportionately less (approximately 4-5% of rearrangements) to the normal adult repertoire (Brezinschek, *et al* 1995). A different situation occurs in the V_H repertoire of fetal and neonatal B cells as compared to adult B cells. B cell responsiveness to different antigens is programmed and appears at different points of ontogeny (Klinman and Linton 1988). Different repertoire restrictions also characterise subpopulations of adult B cells defined by their anatomical localisation and/or by their expression of various surface markers (Andrade, *et al* 1989, Freitas, *et al* 1990, Jeong and Teale 1989).

The pattern of reconstitution of cellular subsets and serum immunoglobulins post transplantation has raised the question of whether the immune deficiency state seen in this situation could be explained by restriction in utilisation of Ig genes. Several studies have therefore addressed the question of expression of V_H genes post transplantation. Fumoux *et al* found that V_H gene family usage is decreased twofold to threefold post transplant, compared to normal adults, and is compensated for by transient overexpression of V_H4 , V_H5 and V_H6 (Fumoux, *et al* 1993). They and others went on to conclude that V_H gene family usage recapitulated fetal B cell ontogeny (Storek, *et al* 1993) on the basis of relative overuse of V_H gene families such as V_H6 . Subsequently, Raaphorst found that V_H

repertoire analysis during reconstitution following transplantation could not clarify whether early post-transplant repertoire follows a fetal pattern, since V_H usage frequencies are poor markers of development owing to the overall similarity of fetal and adult V_H repertoires. In addition, he argued that V_H usage frequencies determined after transplant may not reflect actual recombination frequencies because (oligoclonal) expansions are frequent at this stage (Raaphorst 1999).

6.1.4 Consequences of B cell immunodeficiency

There are more than 70 recognised primary immunodeficiency states, which are the result of genetically mediated abnormalities in the development or function of the immune system. Five major categories of primary immune deficiency states are recognised by the World Health Organisation (WHO): deficient antibody production (eg Bruton's agammaglobulinaemia, hyper IgM syndrome, selective IgG deficiency), deficient antibody production combined with defective cellular immune responses (eg common variable immunodeficiency, severe combined immunodeficiency, adenosine deaminase deficiency), immunodeficiencies associated with other defects (eg Wiscott-Aldrich syndrome, Di George syndrome, ataxia telangiectasia), deficient complement production and defects of phagocyte function. Bruton's disease is the prototype for primary humoral immunodeficiencies. It is characterised by a virtual absence of serum immunoglobulins of all classes and clinically, and recurrent pyogenic infections dating from early childhood. Affected males are well during the first 6-12 months of life because of the protection afforded by the passive transfer of maternal antibodies. Thereafter, they experience recurrent pyogenic infections of the upper and lower respiratory tracts, sinuses, middle ears and skin, usually due to encapsulated bacteria.

Transplant recipients are susceptible to infection by pyogenic encapsulated bacteria due to absence of protective opsonising antibodies (Storek 2000). The post transplant state is also complicated by the impairment of multiple host protective mechanisms. For example T cell-dependent antibody responses to recall antigens (e.g. tetanus toxoid, diphtheria toxoid, polio vaccine, measles virus, hepatitis B surface antigen) are not detectable early after transplantation (Saxon, *et al* 1986, Wimperis, *et al* 1986), but can usually be elicited late (>1 year) (Storek and Saxon 1992). A recent study has elucidated some of the

factors influencing B cell lymphopoiesis following allogeneic transplantation (Storek, *et al* 2001). These authors found that the number of B cell precursors in the marrow on days 30 and 80 post transplant were at least 4-fold lower in patients with grade II-IV acute GVHD, compared to those with grade 0-I acute GVHD. In addition, the presence of extensive chronic GVHD reduced B cell precursor frequency by 18-fold. The number of B cell precursors was not affected by CD34 cell dose, source of stem cells (marrow or PB), donor age or patient age. An understanding of the kinetics of recovering humoral immunity following this alemtuzumab-containing conditioning regimen is important to the effective implementation of prophylaxis and management of infection in the recipients, including a programme of active and passive immunisations.

This study of B cell recovery focussed on:

1. Recovery of B cell numbers, using the CD19 surface marker.
2. Serum immunoglobulin levels.
3. B cell repertoire, using a RT-PCR-based method to assess usage of 2 selected immunoglobulin heavy chain gene variable region families (V_{H2} and V_{H3}).
4. An assessment of B cell chimeric status
5. The influence of persistent disease post transplant, GVHD and DLI on the above parameters.

6.2 Special Methods

6.2.1 Serum immunoglobulin levels

Serum immunoglobulins were measured by immunoturbidimetry on an Integra 700 analyser (Roche).

6.2.2 Immunoglobulin heavy chain gene (IgH) CDR3 spectratyping using fluorescent dye-labelled primers

Several methodological developments have contributed to the understanding of the V region repertoire. The application of PCR-based techniques (Feeney 1992, Gu, *et al* 1992) allowed the detailed analysis of individual gene sequences that has been of particular importance in the analysis of junctional diversity. This study utilises a modification of a RT-PCR-based method (White 1998) to determine V_H gene diversity after transplantation.

The method used agarose gel electrophoresis to identify first round and nested PCR products. Oligonucleotide primers with a fluorescent dye label were used in the nested step, and the products analysed by Genescan software 2.1 on an ABI 310 automated sequencer (Perkin Elmer). By this method, a typically diverse repertoire is characterised by 15-18 peaks separated from each other by 3 base pairs. Each peak corresponds to a specific CDR3 length. The peaks often show a Gaussian distribution in intensity (particularly in the IgM isotype), in which peak height correlates with the total amount of CDR3s of that length present (Figure 6.3). Following antigenic stimulation, this pattern becomes skewed by the presence of large peaks representing CDR3's of a particular size, and by inference, specificity.

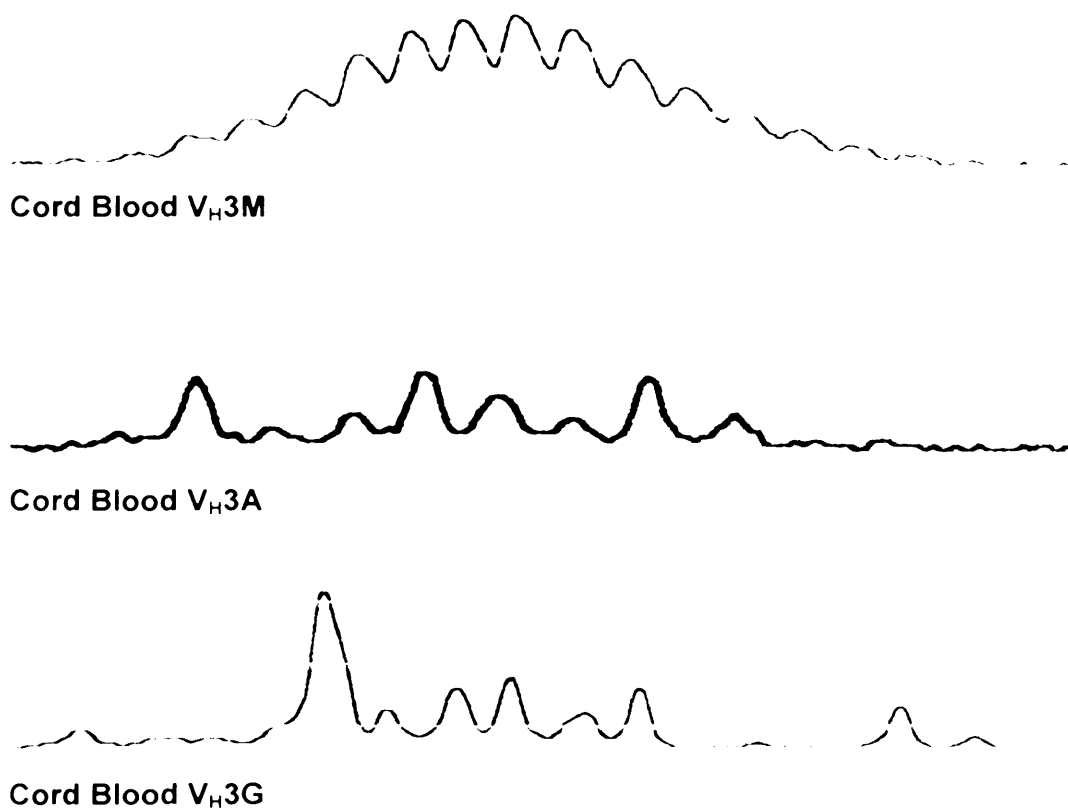


Figure 6.3. Example of cord blood V_H3 spectratypes for IgM, IgA and IgG isotypes.

The IgM isotype demonstrates the most Gaussian distribution, followed, in this case by the IgA and IgG isotypes.

The V_H2 and V_H3 families were selected in order to analyse patient usage of two differently represented V_H families following transplant compared to that of normal controls. The spectratyping method outlined below was used to study B

cell repertoire complexity in 8 patients at 6, 9, 12, 15 and 18 months post transplant and 8 age-matched normal controls.

6.2.3 RNA extraction and RT-PCR.

RNA was extracted from PBMC using Ultraspec RNA (BiotecX Laboratories, Houston, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was generated from 1 µg of RNA in a 20 µl reaction using random hexanucleotide primers for reverse transcription with reverse transcriptase (Superscript, GibcoBRL, Paisley, UK).

6.2.4 Spectratyping. Two V_H gene families (V_H2 and V_H3) were amplified across the CDR3-encoding regions in an isotype-specific manner using V_H/ constant region primer combinations (Oswel, Southampton, UK):

V_H2: CAGATCACCTTGAAGGAGTCTGGTCCT (forward) or

V_H3: GAGGTGCAGCTGGTGGAGTCTGGGGGAG (forward) and

IgM: TTTGTTGCCGTTGGGGTGTGCTGGAC (reverse),

IgA: CTGGGCAGGGCACAGTCACATCCT (reverse) and

IgG1: ACGGTGGGCATGTGTGAGTTTTGT (reverse)

in separate 50 µl PCR reactions containing Genamp PCR buffer II (Perkin Elmer), 1.5 mM/l MgCl₂, 0.2 mM/l each dNTP, 1 mM/ of each primer and 2 µl of cDNA. After a 3-minute denaturation step at 95°C, 0.5 U of Amplitaq DNA polymerase (Perkin Elmer) was added. Optimal cycling conditions were 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes, for 30 cycles, followed by a final extension at 72°C for 5 minutes. 2 µl of PCR product was subjected to a run-off reaction using a nested fluorescent FAM-conjugated J_H primer (0.1 mM/l) (TGAGGAGACGGTGACCAKGGTBCCHTGGCCCC, Oswel, Southampton, UK) in a 20 µl reaction containing Genamp PCR buffer II (Perkin Elmer), 1.5 mM/l MgCl₂, and 0.2 mM/l each dNTP. The hot start and cycling conditions were as described above, except that the number of cycles was 12 rather than 30.

1 µl of PCR product was denatured in 12 µl formamide in the presence of 0.4 µl Tamra 500 size standard (Perkin Elmer) and electrophoresed through Performance Optimized Polymer 6 (Perkin Elmer) on an ABI 310 automated

sequencer (Perkin Elmer). Genescan software 2.1 (Perkin Elmer) was used to analyse the data.

6.2.5 Optimisation of IgH spectratyping

A number of optimisation steps were undertaken to ensure reliability and reproducibility and to identify limitations of the assay. These experiments were carried out on a control subject's peripheral blood mononuclear cells:

1. **Sensitivity.** RNA extraction was performed from cell suspensions of varying concentrations, to ensure that the technique would be sufficiently sensitive to analyse samples from patients post transplant, who frequently had low lymphocyte counts. The PBMC concentrations used were $1 \times 10^6/\text{ml}$, $2 \times 10^6/\text{ml}$, $5 \times 10^6/\text{ml}$ and $10 \times 10^6/\text{ml}$. Actual CD19+ cell numbers were not taken into account, since they would be a fixed proportion of the PBMC cell aliquots. RNA extraction was carried out according to the manufacturer's protocol, followed by RT-PCR as described in Chapter 2. Spectratype analysis was then performed as outlined above for the IgA isotype of the V_H2 family and the IgM isotype of the V_H3 family. The result of this optimisation step for V_H2 A and V_H3 M are shown in Figure 6.4. At a cell concentration of $1 \times 10^6/\text{ml}$, the V_H2 IgA spectratype shows relative over-representation of some peaks and under-representation of others, due to the low cell concentration. This effect is less marked at a cell concentration of $2 \times 10^6/\text{ml}$, in which the spectratype shows better-defined peaks. The optimum cell concentration appeared to be $10 \times 10^6/\text{ml}$, with clear definition of peaks 3 base pairs apart. This optimisation step demonstrated that it is possible to obtain a spectratype profile from a cell concentration as low as $1 \times 10^6/\text{ml}$, but this is at the expense of optimal peak definition. Similar results were obtained for the V_H3 IgM isotype, although overall the spectratypes were less affected by the differences in cell concentrations due to the greater usage of this V_H family in the normal repertoire. Based on these preliminary experiments, 5-10 $\times 10^6/\text{ml}$ PBMC were used for IgH spectratype analysis whenever possible.

2. **Reproducibility.** The entire procedure, including isolation of mononuclear cells, RNA extraction, RT-PCR and first round and nested PCR reactions was carried out 2 separate times from the same subject to ensure the reproducibility of the results. It was confirmed that to be the case, with virtually identical spectra being obtained in the different experiments.

3. **Temporal stability of cDNA samples and nested PCR reactions** were carried out on the same samples at different time points to ensure degree of stability of the results throughout the assay. Subsequently, cDNA samples from the same subject could be analysed several weeks apart and yield identical results.

4. **Use of different cell concentrations for the first round product.** The first round PCR reaction was carried out using different cell concentrations, also from 5×10^5 cells, to verify whether better defined peaks could be obtained if the first round product was diluted.

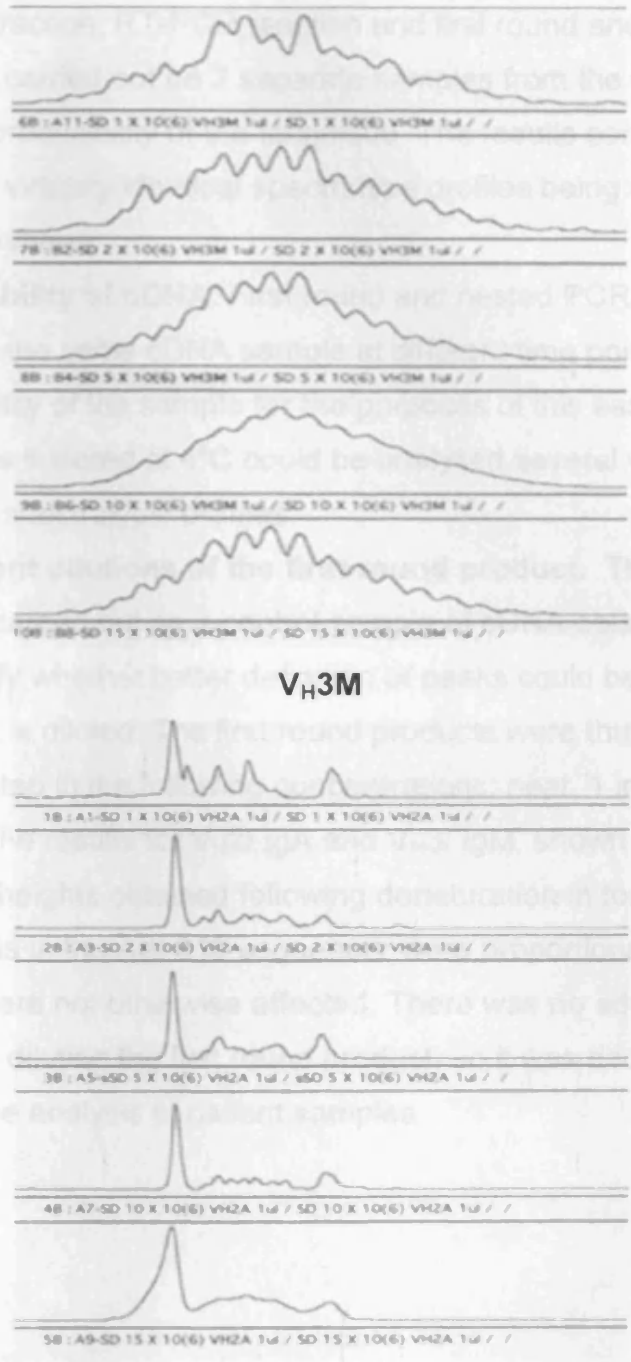
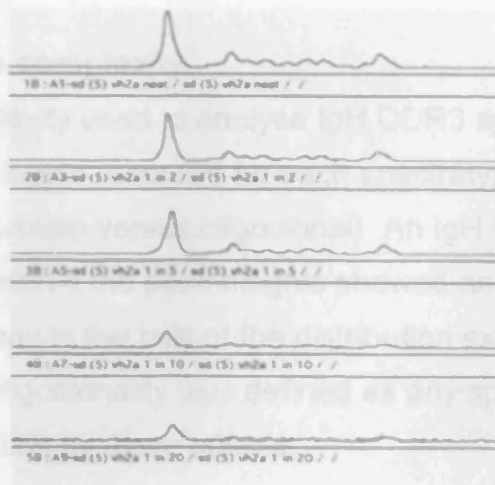


Figure 6.4. Optimisation of MNC concentration used for RNA extraction.
 A cell concentration of 10×10^6 /ml demonstrates optimal peak definition, more clearly seen in the V_{H2A} spectratype compared to the V_{H3M} spectratype, owing to the greater usage of the V_{H3M} family in the repertoire.

2. **Reproducibility.** The entire procedure, including isolation of mononuclear cells, RNA extraction, RT-PCR reaction and first round and nested PCR reactions was carried out on 2 separate samples from the same subject to ensure the reproducibility of the technique. The results confirmed this to be the case, with virtually identical spectratype profiles being obtained in the different experiments.
3. **Temporal stability of cDNA.** First round and nested PCR reactions were carried out on the same cDNA sample at different time points to ensure temporal stability of the sample for the purposes of this assay. Subsequently, cDNA samples if stored at 4°C could be analysed several weeks apart and yield identical spectratypic profiles.
4. **Use of different dilutions of the first round product.** The first round PCR reaction was carried out on a control sample of cDNA obtained from 5×10^6 cells, to identify whether better definition of peaks could be obtained if the first round product is diluted. The first round products were thus subjected to the nested PCR step in the following concentrations: neat, 1 in 2, 1 in 5, 1 in 10 and 1 in 20. The results for V_H2/ IgA and V_H3/ IgM, shown in Figure 6.5 show that the peak heights obtained following denaturation in formamide and electrophoresis in the ABI 310 sequencer were proportional to the degree of dilution, but were not otherwise affected. There was no advantage to be obtained from diluting the first round product, so it was decided to use it undiluted in the analysis of patient samples.

3.2.6 IgH spectratype

The measure of complexity used in this IgH CDR3 spectratype was based on peak number (width) and appearance (new-Gaussian). An IgH CDR3 spectratype was defined as near-Gaussian if the distribution of peaks was unequivocally normal distribution, with no peak exceeding its more central neighbour in height. Otherwise, it was defined as any spectratype showing a non-Gaussian distribution.



3.2.7 Chimerism analysis by microsatellite PCR technique.

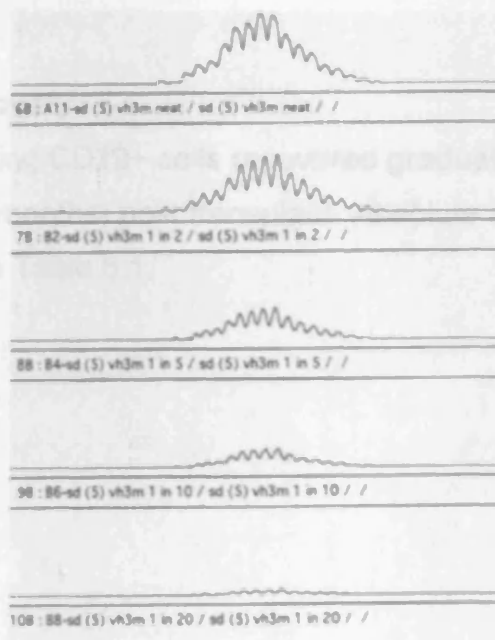
Refer to Chapter 2, General Method

V_H2A

3.3 Results

3.3.1 CD19+ cell count

In all patients, clonal CD19+ cells were present throughout the period of follow up (up to 21 months). The median CD19+ cell numbers are shown in



V_H3M

Figure 6.5. Use of different dilutions of first round product.

This example of the optimisation step of determining whether dilution of the first round product yielded better peak definition shows this not to be the case. The only change seen is that peak height varied proportionately to the degree of dilution of first round product.

6.2.6 IgH spectratype complexity.

The measure of complexity used to analyse IgH CDR3 spectratypes was based on peak number (which was recorded for each spectratype) and spectratype appearance (near-Gaussian versus oligoclonal). An IgH CDR3 spectratype was defined as near-Gaussian if the peak heights showed an unequivocally normal distribution, with no peak in the tails of the distribution exceeding its more central neighbour in height. Oligoclonality was defined as any spectratype showing a non-Gaussian distribution by these criteria.

6.2.7 Chimerism analysis by microsatellite PCR technique.

Refer to Chapter 2, General Methods.

6.3 Results

6.3.1 CD19+ cell recovery

In all patients, circulating CD19+ cells recovered gradually throughout the period of follow up (up to 21 months) post transplant. Absolute and median CD19+ cell numbers are shown in Table 6.1.

Patient	Time after transplant						
	3-4m	6m	9m	12m	15m	18m	21m
1				110	9	23	
2		214	6	14	16	5	
3	12	80	137		29		
4	0	3	4	32	39	130	87
5	3		49	202	36	180	
6	9	23	34	67		103	
7	2	12	11	32	38	48	23
8	34	87	105				
9	49	21	27	248	48	65	
10	1	134	391	97	102		
11	16	31		90	29		
12	128	38		53	26		
13	36	35	35	49			
14	156	165	380	44			
15		13	17				
16	3	8					
17	1	127	18				
18	69	212	141				
n=	15	18	16	10	8	4	3
Median	12	37	35	60	33	65	55
Min	0	3	4	14	9	5	23
Max	156	214	391	248	102	180	87

Table 6.1. CD19+ cell numbers

Absolute numbers (per microlitre of peripheral blood mononuclear cells) of CD19+ cells are shown for each patient at 3 monthly intervals post transplant. The normal range is 120-600 cells per microlitre. CD19+ counts that fall within the normal range are printed in red, and those below the normal range in black. Minimum, maximum and median values are also shown, together with the number of patients analysed at each time point.

Just 13% (n=15) of patients at 3 months had normal CD19+ cell numbers, rising to 31% of patients at 6 months and 29% at 9 months. The proportion of patients with normal CD19+ cell numbers at 12 months post transplant (n=12) fell to 17%, rising once more to 29% at 18 months (n=7). The levels of CD19+ cells fluctuated throughout the period of follow up, such that no single patient maintained normal

CD19+ cell numbers when followed up serially. Overall, median CD19+ cell numbers remained below normal for up to 21 months post transplant (Figure 6.6).

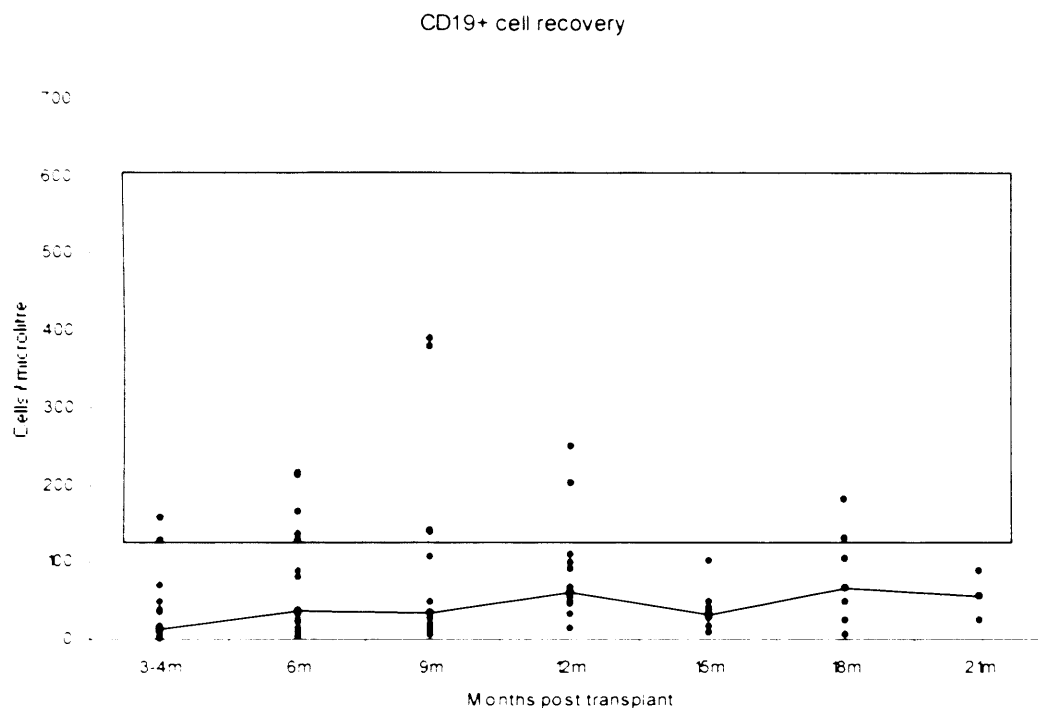


Figure 6.6. CD19+ cell recovery.

Cell numbers are given in absolute numbers per microlitre of blood. Individual as well as median values (given as line graph) are shown at 3 monthly intervals post-transplantation. The normal range is 120-600 cells per microlitre, indicated by the blue box.

CD19+ cell recovery was also assessed in the context of disease status, the presence of GVHD, B lineage chimeric status and DLI:

1. **Influence of disease status.** Eighteen patients were evaluated at 12 months post transplant for their disease status. Nine patients had progressive disease, and 9 had responding disease at this time point. There was no difference in CD19+ cell recovery between the 2 groups of patients, suggesting that the presence of progressive disease does not affect CD19+ cell recovery (Figure 6.7a).

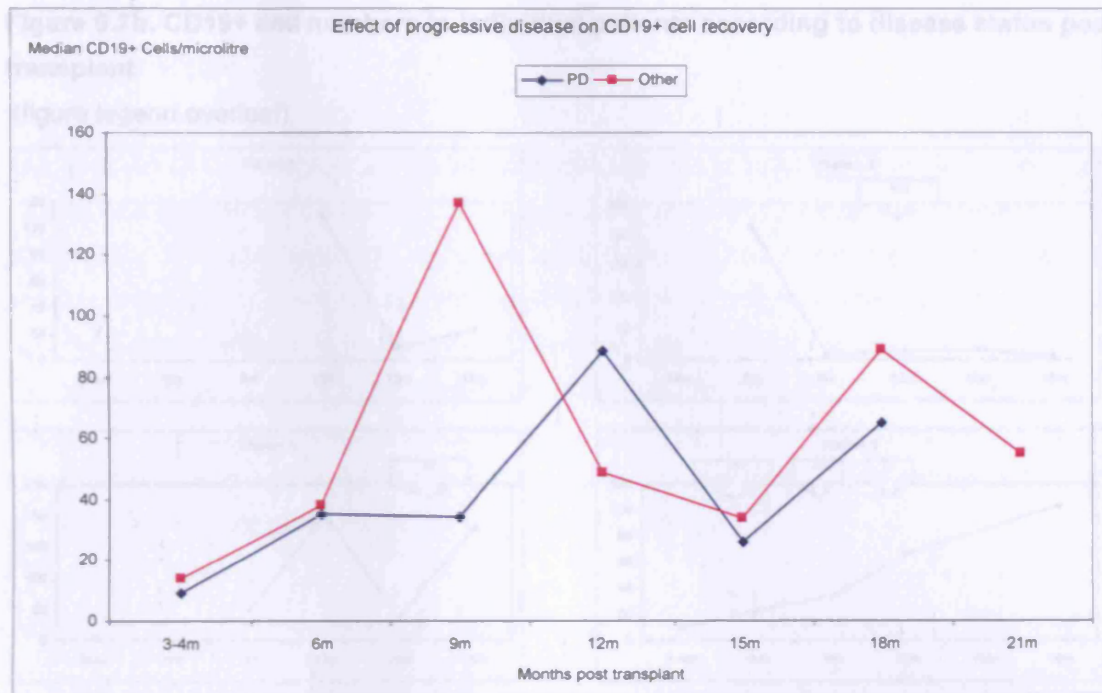


Figure 6.7a. CD19+ cell recovery and progressive disease

Median CD19+ cell numbers are shown for 9 patients with progressive disease (PD, blue line) and 9 patients with responding or stable disease (Other, pink line) at the timepoints indicated.

This was confirmed by analysis of individual patients' CD19+ cell recovery, as illustrated in Figure 6.7b.

Figure 6.7b. CD19+ cell numbers in individual patients according to disease status post transplant

(figure legend overleaf)

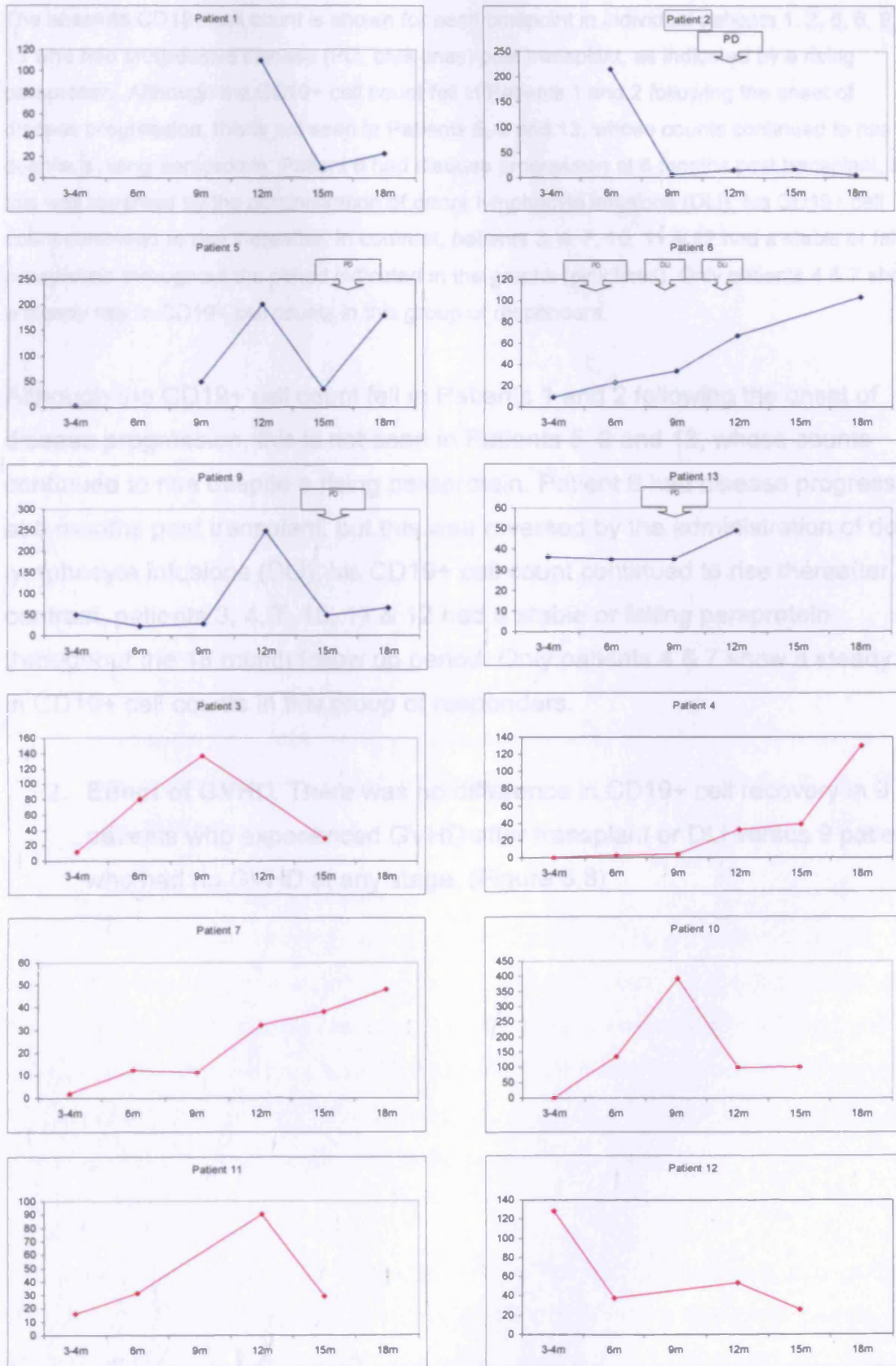


Figure 6.7b CD19+ cell numbers in individual patients according to disease status post transplant

Figure Legend:

The absolute CD19+ cell count is shown for each timepoint in individual patients 1, 2, 5, 6, 9 & 13 who had progressive disease (PD, blue lines) post transplant, as indicated by a rising paraprotein. Although the CD19+ cell count fell in Patients 1 and 2 following the onset of disease progression, this is not seen in Patients 5, 9 and 13, whose counts continued to rise despite a rising paraprotein. Patient 6 had disease progression at 6 months post transplant, but this was reversed by the administration of donor lymphocyte infusions (DLI); his CD19+ cell count continued to rise thereafter. In contrast, patients 3, 4, 7, 10, 11 & 12 had a stable or falling paraprotein throughout the period indicated in the graphs (pink lines). Only patients 4 & 7 show a steady rise in CD19+ cell counts in this group of responders.

Although the CD19+ cell count fell in Patients 1 and 2 following the onset of disease progression, this is not seen in Patients 5, 9 and 13, whose counts continued to rise despite a rising paraprotein. Patient 6 had disease progression at 6 months post transplant, but this was reversed by the administration of donor lymphocyte infusions (DLI); his CD19+ cell count continued to rise thereafter. In contrast, patients 3, 4, 7, 10, 11 & 12 had a stable or falling paraprotein throughout the 18 month follow up period. Only patients 4 & 7 show a steady rise in CD19+ cell counts in this group of responders.

2. **Effect of GVHD.** There was no difference in CD19+ cell recovery in 9 patients who experienced GVHD after transplant or DLI versus 9 patients who had no GVHD at any stage. (Figure 6.8)

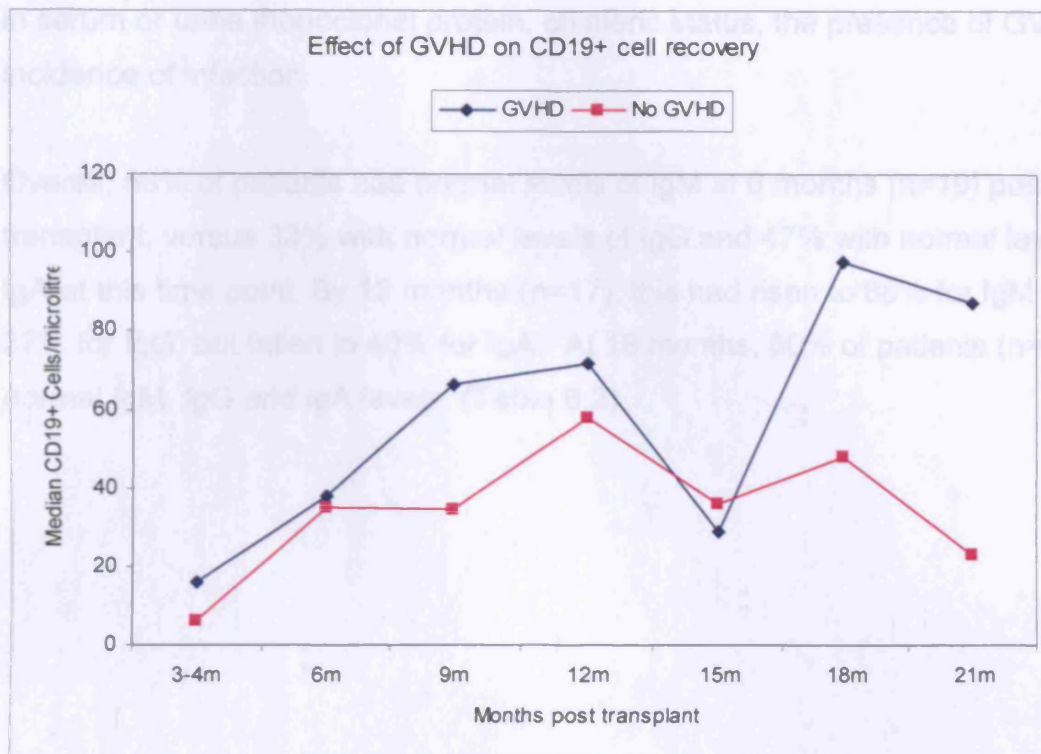


Figure 6.8. CD19+ cell recovery and GVHD

Median CD19+ cell numbers are shown for 9 patients who experienced GVHD following transplant or DLI administration (blue line) and 9 patients who did not experience GVHD at any stage following transplant (pink line).

- Influence of B lineage chimeric status and DLI.** Neither B lineage chimeric status nor the administration of DLI influenced the rate of CD19+ cell recovery.

6.3.2 B lineage chimeric status

When B lineage chimeric status was assessed, 16 of 19 patients converted to full donor B lineage chimerism within 6 months of transplant. Of the 3 patients who remained mixed B lineage chimeras at 6 months, 1 converted to full donor B lineage chimerism within a month of receiving DLI, another showed full donor B lineage chimerism at 8 months prior to receiving DLI, and the other remained a mixed chimera in the B lineage in spite of receiving 2 DLI's.

6.3.3 Immunoglobulin levels

Immunoglobulin levels were interpreted in the context of disease status since immune paresis is a well-known accompaniment of active myeloma. Levels were measured at 6, 9, 12 and 18 months post transplant and correlated with the trend

in serum or urine monoclonal protein, chimeric status, the presence of GVHD and incidence of infection.

Overall, 58% of patients had normal levels of IgM at 6 months (n=19) post transplant, versus 33% with normal levels of IgG and 47% with normal levels of IgA at this time point. By 12 months (n=17), this had risen to 88% for IgM and 37% for IgG, but fallen to 40% for IgA. At 18 months, 50% of patients (n=10) had normal IgM, IgG *and* IgA levels. (Table 6.2)

Patient	IgA				IgG				IgM				Viral Infections
	6m	9m	12m	18m	6m	9m	12m	18m	6m	9m	12m	18m	
1	N	N	N	low	N	N	low	low	N	N	N	low	
PP Trend	↓	↓	↔	↑	↓	↓	↔	↑	↓	↓	↔	↑	
2	N	N	N	low	low	low	low	low	N	N	N	low	
PP Trend	↓	↓	↑	CHEMO	↓	↓	↑	CHEMO	↓	↓	↑	CHEMO	PFIII
3	low	low	low	low	low	low	low	N	low	low	N	N	
PP Trend	↓	↓, GVHD	↓	↓	↓	↓, GVHD	↓	↓	↓	↓, GVHD	↓	↓	HHV7
4	N	low	low	low	PP overlaps beta				N	N	N	N	
PP Trend	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	PFIII
5	low	low	low	low	low	N	N	low	N	N	N	low	
PP Trend	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↑	
6	low	N	N	N	low	N	N	N	low	N	N	N	
PP Trend	↑	↓	↓	↓	↑	↓	↓	↓	↑	↓	↓	↓	
7	low	low	low	low	N	N	N	N	N	N	N	low	
PP Trend	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	VZV
8	low	low			low	low			N	N			
PP Trend	↓, GVHD	↓, GVHD			↓, GVHD	↓, GVHD			↓, GVHD	↓, GVHD			PFIII, HSV
9	Overlapping IgA paraprotein				N	N	N	low	N	N	N	low	
PP Trend	↔	↔	↔	↑	↔	↔	↔	↑	↔	↔	↔	↑	Inf A, VZV
10	N	N	N	N	N	N	N	N	low	N	N	N	
PP Trend	↓	↑	↔	↔	↓	↑	↔	↔	↓	↑	↔	↔	PFIII, HSV
11	N	N	N	N	low	low	low	N	N	N	N	N	
PP Trend	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	HSV
12	N	low	low		low	low	low		N	N	N		
PP Trend	↓	↑, GVHD	↓, GVHD		↓	↑, GVHD	↓, GVHD		↓	↑, GVHD	↓, GVHD		
13	Overlapping IgA paraprotein				low	low	low		low	low	N		
PP Trend	↔	↑	↓ CHEMO		↔	↑	↓ CHEMO		↔	↑	↓ CHEMO		
14	low	low	low		N	N	N		N	N	N		
PP Trend	↓, GVHD	↓	↓		↓, GVHD	↓	↓		↓, GVHD	↓	↓		
15	N	low	low		low	low	low		low	low	low		
PP Trend	↓	↔	↔		↓	↔	↔		↓	↔	↔		
16	low	low	low		low	low	low		low	low	N		
PP Trend	PD, GVHD	GVHD	GVHD		PD, GVHD	GVHD	GVHD		PD, GVHD	GVHD	GVHD		RSV
17	low	low	low		N	low	low		N	low	low		
PP Trend	↓	↑, CHEMO	↑		↓	↑, CHEMO	↑		↓	↑, CHEMO	↑		RSV
18	N	N	N		low	low	low		low	N	N		
PP Trend	↔, GVHD	↑	↑		↔, GVHD	↑	↑		↔, GVHD	↑	↑		
19	low	low			low	low			low	N			
PP Trend	↔	↓, GVHD			↔	↓, GVHD			↔	↓, GVHD			Adeno

n	17	17	15	9	18	18	16	9	19	19	17	10
%Normal Ig's	47	35	40	33	33	39	37	56	58	74	88	50

Table 6.2. Immunoglobulin levels

Normal (blue shading) or low (yellow shading) levels of IgA, IgG and IgM are shown at 6, 9, 12 and 18 months post transplant. A grey box indicates no measurement due to insufficient follow up time or death. In the second row for each patient, the trend of the paraprotein (PP), the presence of graft-versus-host disease (GVHD), & current use of chemotherapy (CHEMO) are indicated. The number of patients analysed at each timepoint and the percentage with normal immunoglobulins (Ig's) is shown in the smaller table. Proven (non-CMV) viral infections are indicated in the far-right column (HHV7, Human herpes 7; VZV, varicella zoster; PFIII, parainfluenza III; HSV, herpes simplex; Adeno, adenovirus; RSV, respiratory syncytial virus; Inf A, influenza A virus).

1. **Influence of disease status.** When individual patients' immunoglobulin levels were assessed in conjunction with disease status, it was found that there was no correlation between active disease, as indicated by a rising paraprotein (PP) and serum immunoglobulin level. 29% of patients with a falling PP had normal IgA levels, 43% had normal IgG levels and 100% had normal IgM levels. There were similar findings for those with a stable PP (33%, 50% and 83% respectively). Of the 3 patients with a rising PP, 2 each had normal IgA and IgM levels, but none had normal IgG levels (Table 6.3a), although it is difficult to draw firm conclusions as the number of patients is small.

PP trend	n=	Number of patients with normal Ig levels at 12 months (%)		
		IgA	IgG	IgM
Down (Responding disease)	7	2 (29%)	3 (43%)	7 (100%)
Stable (Plateau)	6	2 (33%)	3 (50%)	5 (83%)
Up (Progressive disease)	3	2 (66%)	0	2 (66%)

Table 6.3a. Immunoglobulin levels and disease status

Data is shown for patients at 12 months post transplant. The proportion of patients with normal Ig levels is shown, together with the percentage of the number of patients with a downward, stable or upward trend in the paraprotein (PP).

2. **Effect of GVHD.** All 7 patients with GVHD post DLI, when assessed at 9 months post transplant had persistently low levels of IgG and IgA but 43% of patients had normal levels of IgM. Those patients without GVHD (n=12), had significantly higher levels of IgA, IgG and IgM (33%, 50% and 83% respectively) (Table 6.3b).

GVHD	n=	Number of patients with normal Ig levels at 9 months (%)		
		IgA	IgG	IgM
Present	7	0	0	3 (43%)
Absent	12	4 (33%)	6 (50%)	10 (83%)

Table 6.3b. Immunoglobulin levels and GVHD

Data is shown for patients at 9 months post transplant. The proportion of patients with normal Ig levels is shown, for patients with and without GVHD.

3. **Influence of viral infections.** The correlation between IgG and IgA levels and the incidence of viral (non-CMV) infections was assessed. Out of 11 patients who experienced proven non-CMV viral infections post transplant, 64% had subnormal IgG and IgA levels versus 50% of 8 patients who did not have proven non-CMV viral infection. For IgM, the levels were normal in 73% of patients with non-CMV viral infections and in 75% of those without (Table 6.3c).

Non-CMV viral infection	n=	Number of patients with normal Ig levels at 9 months (%)		
		IgA	IgG	IgM
Present	11	4 (64%)	4 (64%)	8 (73%)
Absent	8	4 (50%)	4 (50%)	6 (75%)

Table 6.3c. Immunoglobulin levels and non-CMV viral infections

The proportion of normal immunoglobulin levels is shown for 11 patients who had proven non-CMV viral infection and the remaining 8 patients who did not, when assessed at 9 months post transplant.

4. **Influence of B cell chimeric status.** There was no correlation between immunoglobulin level recovery and B lineage chimeric status.

6.3.4 IgH CDR3 repertoire by spectratyping: complexity and spectratype appearance

Prior to transplantation, patient IgH CDR3 spectratype complexity was reduced in terms of peak number compared to that of normal controls in both V_{H2} and V_{H3} families. This may have been due to an effect of disease status or pre-transplant therapy, although there was no direct correlation between complexity and the actual number of lines of prior therapy. The reduction in spectratype complexity (their less-Gaussian nature) was more marked in the V_{H2} family, as would be expected in this less well-represented family. In addition, the IgA and IgG isotype spectratypes were frequently non-Gaussian, which is in keeping with the cellular composition that they represent namely heterogeneous populations of different clonal expansions at different stages of expansion or contraction.

Following transplant, there was a steady improvement in spectratype complexity, reflected by an increase in median peak number in both V_H families across the

isotype spectrum. Data (median peak number and range together with the proportion of subjects showing a Gaussian appearance) for the different V_H families and IgM, IgG and IgA isotypes in patients and controls are shown in Table 6.4.

Family/Isotype	Normal Controls	Patients					
		Pre Transplant	6 months	9 months	12 months	15 months	18 months
Number analysed	8	8	8	7	7	5	2
V_H2/ IgM							
Median	13	12	13	13	15	14	16
Range	8-16	7-14	10-16	10-16	11-16	10-16	16
%Gaussian	29%	43%	13%	29%	67%	60%	100%
V_H2/ IgA							
Median	9	7	10	11	12	12	15
Range	7-12	2-10	6-12	8-12	10-15	9-14	14-16
%Gaussian	0%	0%	0%	0%	0%	0%	50%
V_H2/ IgG							
Median	10	7	11	12	11	12	13
Range	7-14	2-14	6-14	9-14	10-14	9-14	11-14
%Gaussian	0%	0%	0%	0%	0%	0%	0%
V_H3/ IgM							
Median	16	12	13	14	14	14	14
Range	14-18	6-16	8-16	12-16	12-16	14-16	14
%Gaussian	75%	86%	88%	86%	86%	60%	50%
V_H3/ IgA							
Median	15	12	12	14	13	14	13
Range	13-16	2-14	4-14	10-16	10-14	10-14	12-14
%Gaussian	13%	0%	14%	14%	29%	0%	50%
V_H3/ IgG							
Median	15	11	13	12	12	12	13
Range	14-16	1-14	1-15	7-15	10-14	11-14	12-14
%Gaussian	0%	0%	38%	29%	0%	0%	0%

Table 6.4. IgH Spectratype complexity.

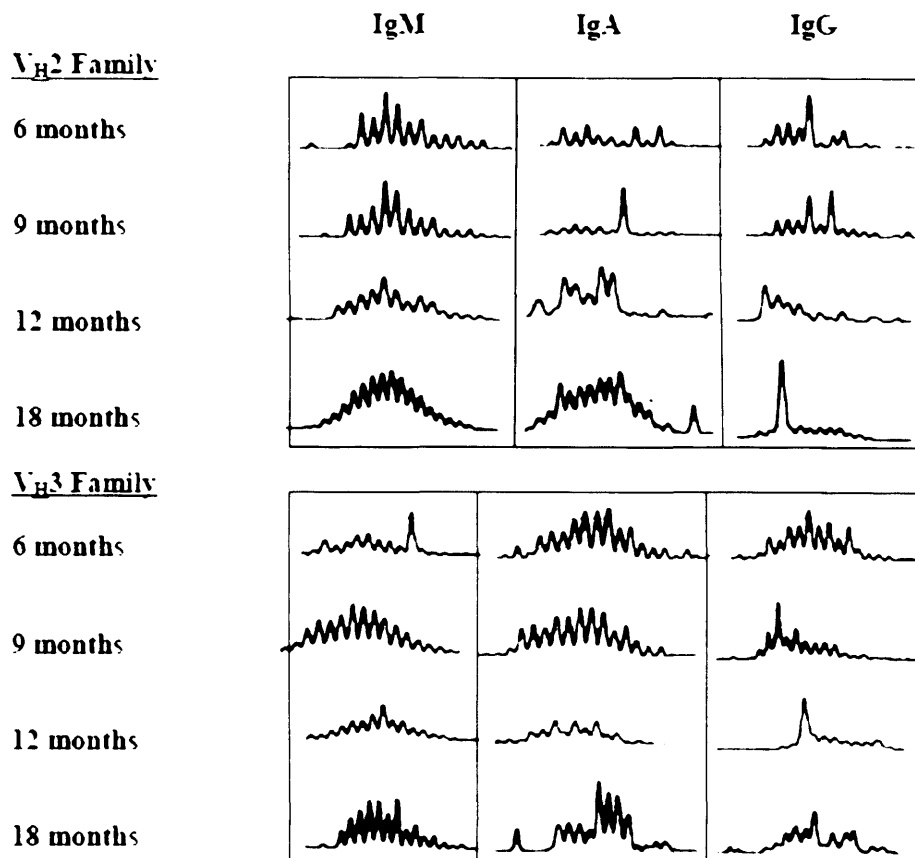
Median spectratype peak number and range and the proportion of subjects with a Gaussian spectratype appearance are shown for 8 patients and 8 control subjects for the different V_H families and IgM, IgA and IgG isotypes. The number of patients analysed at each timepoint is indicated in the 3rd row of the table.

Normal control subjects had fewer peaks per V_H2 family spectratype for all three isotypes (IgM > IgG > IgA) than that for the V_H3 family, as would be expected. In addition, the normal controls showed a low incidence of Gaussian appearance across both V_H families in all isotypes, except V_H3/ IgM, in which 75% of normal subjects showed a Gaussian distribution. By 6 months post transplant in the patient group, complexity of all isotypes of the V_H2 family had reached similar levels to normal controls, and remained at this level irrespective of the presence of GVHD (in 3 of 8 patients) and disease status (1 CR, 2 SD, 5 PD). The trend towards normality for the V_H3 family for all isotypes post transplant was slower, despite the larger contribution of this family to the overall repertoire. The median peak number in all isotypes increased with time, but remained below that of normal subjects throughout the period of follow up. There was no correlation

between IgH CDR3 spectratype complexity and stem cell source, donor type, GVHD or B lineage chimeric status.

For both V_H2 and V_H3 families, the IgM spectratypes, reflecting a larger B cell population in a more steady state showed a continuing trend towards a Gaussian distribution with time, reflecting normalisation of B cell repertoire post transplant. In contrast, the IgG & IgA spectratypes often remained non-Gaussian throughout the period of follow up, a finding that does not detract from a normalisation of B cell repertoire, given that they represent cellular populations of a heterogeneous nature. Representative examples of the change in IgH CDR3 spectratype complexity following transplantation are shown in Figure 6.9.

Figure 6.9. IgH spectratype profile complexity



A representative example of isotype-specific IgH CDR3 spectratype profiles is shown at the post-transplant time-points indicated. The IgM isotypes in both families show a progressively Gaussian distribution following transplant. Following antigenic stimulation, the patterns in the IgA and IgG isotypes demonstrate skewing due to the presence of large peaks representing CDR3's of a particular size.

6.4 Discussion

This study has examined the kinetics of CD19+ cell recovery and immunoglobulin production as well as the usage of 2 immunoglobulin heavy chain gene V_H families following transplant in patients who have received a significantly lymphocyte-depleting (alemtuzumab-containing) conditioning regimen prior to allogeneic stem cell reinfusion. The non-myeloablative nature of the conditioning therapy is also of particular relevance, with the reduced

incumbent tissue damage and hence lower acute toxicity and rate of acute GVHD, as well as the possible influence of mixed B cell chimeric status on the recipients' B cell immune recovery.

The kinetics and quality of B cell recovery following myeloablative transplantation are well documented: B cells are undetectable or low for the first 2 months after stem cell transplantation, and then rise to normal or supranormal levels by 1 to 2 years after transplant (Small, *et al* 1990, Storek, *et al* 1993). The rise in B cell numbers is faster in those without chronic GVHD compared to those with chronic GVHD. This may be due to the direct effect of GVHD and or its treatment on B cell development in the bone marrow (Storek, *et al* 1996). Early recovery is quicker following allogeneic PBSCT compared to BMT (Ottinger, *et al* 1996, Roberts, *et al* 1993, Talmadge, *et al* 1996), possibly due to the high content of B cells in PBSC allografts. Subsequently, the rise in B cells appears to be slower after allogeneic PBSCT than BMT.

B cell reconstitution in this study showed protracted B lymphopenia compared to other studies, probably due to the lymphocyte-depleting effect of alemtuzumab, but the rate of recovery of immunoglobulin levels was similar to that following myeloablative allogeneic transplantation. Neither stem cell source nor GVHD incidence affected recovery of B cell numbers in this cohort.

Following T-replete myeloablative transplantation, most circulating lymphocytes are of donor origin by 1 to 2 months, whereas a variable degree of mixed lymphoid chimerism is seen post T-cell-depleted myeloablative transplantation (Lapointe, *et al* 1996, Roux, *et al* 1992). All circulating B cells are of donor origin at several months to years after T-replete grafting (Korver, *et al* 1987). Interestingly, this concurs with the results of this study of T-depleted grafting, in which the majority of patients (16 out of 19) converted to full donor B lineage chimerism within 6 months of transplant, and 2 converted to full donor chimerism within 1 and 8 months of receiving DLI. Only one patient continued to show mixed B lineage chimerism after receiving 2 DLI's.

Serum immunoglobulin levels usually show an initial fall post myeloablative allogeneic transplantation followed by recovery within months (for IgM and IgG)

to years (for IgA) (Fujimaki, *et al* 2001, Velardi, *et al* 1988). In this study, approximately 60% of patients had normal IgM levels by 6 months post transplant, rising to 90% by 12 months. However normalisation of IgG and IgA levels was slower in this study, with subnormal levels seen more frequently in patients with GVHD and non-CMV viral infections. However, caution needs to be exercised when using immunoglobulin levels as surrogate markers of humoral immunity since post transplant immunoglobulins are frequently composed of autoantibodies or non-specific oligoclonal antibodies (not directed against post transplant infectious agents) (Gerritsen, *et al* 1996, Hebart, *et al* 1996). In addition, in this cohort of patients, persistent underlying disease may influence immunoglobulin levels post transplant. For a more complete analysis of B cell immunity, therefore, IgH CDR3 spectratyping was undertaken to study B cell repertoire in this study.

Early studies of V_H gene family usage after conventional allogeneic stem cell transplantation suggested that B cell reconstitution appeared to recapitulate fetal ontogeny (Fumoux, *et al* 1993, Storek, *et al* 1993). More recent studies have failed to confirm this, and suggest that the period of B cell immunodeficiency after stem cell transplantation may be due to factors other than a reversal to a fetal stage of development, such as absence of somatic hypermutation (due to a maturational arrest in B cell differentiation), delayed isotype-switching and clonal dominance (Gokmen, *et al* 1998, Raaphorst 1999, Suzuki, *et al* 1996). These studies later showed that early post-transplant usage of V_H segments by B cells is restricted, but beyond 6 months post transplant increasing diversity is seen, similar in extent to normal adults (Nasman and Lundkvist 1996, Storek, *et al* 1994).

The two V_H families selected for study in this patient group were designed to provide an overview of B cell reconstitution, by assessing usage of a well-represented (V_{H3}) and less well-represented (V_{H2}) V_H family, rather than the nature of recapitulation. In addition, it is important to note that the novel (fluorescent) method used in this study differs from other studies, which employed agarose gel electrophoresis to identify the spectratype profile. In contrast to previous studies, which used visual appearance of spectratype bands to assess B cell repertoire, a scoring system was used to analyse

spectratype data in this study. Irrespective of low B cell numbers there was improvement of spectratype complexity with time. This improvement was more pronounced in the less represented V_H2 family, where this was apparent by 6 months post transplant. The V_H3 family on the other hand showed complexity that was below that of normal controls throughout the follow up period. This latter finding is unexpected since the V_H3-expressing cells are more frequently occurring, and would therefore be expected to return to a Gaussian pattern more quickly.

In summary, CD19+ B cells are slow to recover following this nonmyeloablative preparative regimen, irrespective of majority conversion to full donor B lineage chimeric status. This finding is likely to be related to the marked lymphocyte-depleting effect of alemtuzumab. Normal immunoglobulin levels were found in half of the patients by 18 months although recovery appeared to be delayed by the presence of GVHD. Non-CMV viral infections were more common in patients with low levels of IgG and IgA. B cell repertoire increased with time following transplantation in terms of spectratype complexity in the IgG, IgA and IgM isotypes of the V_H2 and V_H3 families, although only the IgM isotype showed a tendency to become more Gaussian with time.

Chapter 7: Graft-versus-host disease, graft-versus-myeloma effect, donor lymphocyte infusions and effect on immune reconstitution

7.1 Introduction

Allogeneic stem cell transplantation was initially designed to deliver lethal doses of chemotherapy and radiotherapy in the treatment of malignant diseases. Myeloma is a disease that does show a dose-response relationship to chemotherapy and radiotherapy, so there is a rationale for dose-intensification strategies. However, although long-term remission is achievable (Corradini, *et al* 1996, Tricot, *et al* 1996a) allogeneic stem cell transplantation for myeloma has a high procedural mortality and late relapses continue to occur in survivors.

There is evidence in several diseases that high dose therapy does not eradicate the malignancy but the therapeutic benefit of allogeneic transplantation is largely related to an associated immune-mediated graft-versus-malignancy (GVM) effect (Baron and Storb 2004). Thus the nonmyeloablative regimens have been developed to reduce the dose intensity and toxicity of the conditioning regimen, whilst attempting to harness a GVM effect.

7.2 Graft-versus-host disease

GVHD is classically divided into 2 syndromes: that occurring within the first 100 days of transplant (acute GVHD) and that occurring later in the post-transplant period (chronic GVHD). In the setting of donor lymphocyte infusion (DLI) administration following the initial transplant procedure, there is an added facet to the interpretation of GVHD. Patients may develop *de novo* acute or chronic GVHD following DLI, regardless of any GVHD that they experienced following the initial transplant procedure.

7.2.1 Acute GVHD: pathophysiology

A number of interrelated processes are involved in the development of acute GVHD, which is thought to occur in 3 phases. In the first phase, conditioning of the patient, designed to ablate or cytoreduce the disease as well as induce acceptance of the donor graft by immunosuppression, results in host tissue damage and release of pro-inflammatory cytokines, including IL-1 and TNF- α . Total body irradiation (TBI) and high dose chemotherapy predictably results in damage to host tissues, which are also vulnerable to the effects of the

underlying disease, infections and previous therapies. In particular, TBI and certain intensive conditioning regimens such as cyclophosphamide and melphalan induce a potent combination of intestinal endothelial apoptosis and epithelial cell damage (clinically seen as mucositis), resulting in the entry of microbial immunostimulatory molecules into the systemic circulation. These molecules and the cytokines released, induce a cascade of activation events, including increased expression of adhesion molecules, co-stimulatory molecules and MHC molecules, which serves to amplify the second phase of the process (Reddy and Ferrara 2003). The toxicity of these conditioning approaches often outweighs the cytoreductive benefits against the disease and has led to an attempt to de-intensifying the conditioning therapy while placing greater emphasis on immunosuppression (reduced intensity conditioned transplants, RIT).

In the second phase of this 3-step model, exposed host antigens are presented by antigen presenting cells (APCs) in the form of an HLA-DR-peptide complex to the donor T cells. Although antigens may be presented by either host-derived APCs or donor-derived APCs, there is evidence for a predominance of the host-derived APC pathway in the pathogenesis of acute GVHD due to both minor and major histocompatibility mismatches (Reddy and Ferrara 2003). Host-derived dendritic cells are the most potent APCs in this process, being activated by inflammatory cytokines such as TNF- α and IL-1, microbial products such as LPS entering the circulation and cells that are undergoing necrosis as a result of conditioning therapy. This leads to donor T cell activation, proliferation and differentiation and further amplification of the immune response by enhanced cytokine secretion and expression of HLA.

T cell activation results in rapid intracellular biochemical events that result in transcription of genes encoding various cytokines and their receptors (Ho and Glimcher 2002). Th1 cytokines (section 4.1.5) are preferentially produced during the course of acute GVHD, with a critical role for IL-2 in the amplification of the immune response against alloantigens. IL-2 is produced by donor CD4+ cells, and has been the target of therapeutic strategies aimed at controlling GVHD, including ciclosporin and tacrolimus which inhibit IL-2 production and anti-IL-2 monoclonal antibodies (daclizumab) that target the IL-2 receptor (section 7.2.2).

Interferon- γ (IFN- γ) is another critical cytokine in the second phase of acute GVHD. IFN- γ is produced in large amounts by T cells, resulting in the upregulation of adhesion molecules, chemokines, MHC and associated antigen presenting molecules. IFN- γ both facilitates antigen presentation and influences the development of GVHD in the GI tract and skin as well as resulting in GVHD-mediated immunosuppression following transplant (Teshima and Ferrara 2002).

Although the pathogenesis of acute GVHD has been linked to Th1 polarisation of activated donor T cells (Ferrara 1994), the evidence for this is incomplete and contradictory. Whilst the Th1 phenotype appears to amplify the cytokine storm and correlate with acute GVHD in one study (Fowler and Gress 2000), early inducement into the Th1 phenotype by the administration of exogenous cytokines appears to attenuate GVHD in another (Reddy, *et al* 2001). Other studies have failed to show the beneficial effects of Th2 polarisation on acute GVHD (Murphy, *et al* 1998). Thus the so-called Th1/Th2 paradigm, may be an oversimplification of cytokine-related functions, which are interrelated and pleiotropic.

Phase 3 of acute GVHD is termed the cellular and inflammatory phase. This is the efferent phase, referring to the processes that are followed through after initiation by the effector cells and cytokines. The effector cells are cytotoxic T cells (CTLs) and NK cells, which use a variety of mechanisms to lyse or kill cells, including the Fas/ Fas ligand and perforin/ granzyme pathways (Kagi, *et al* 1994, Russell and Ley 2002). The effector cytokines include TNF- α and IL-1, produced by monocytes and macrophages after stimulation by microbial products like LPS, which leak through the skin or intestinal mucosa as a result of the conditioning therapy or GVHD. TNF- α is crucial to the pathophysiology of gastrointestinal GVHD and is also an important effector cytokine in the skin and lymphoid organs (Hattori, *et al* 1998). Specific blockade of the TNF- α receptor by a monoclonal antibody is a potential clinical strategy in the treatment and prevention of acute GVHD (section 7.2.2). IL-1 is the other major proinflammatory cytokine that has important effector functions in this phase of acute GVHD, particularly in the spleen and skin (Abhyankar, *et al* 1993).

7.2.2 Acute GVHD: clinical features, grading and treatment

The clinical features of acute GVHD vary from a mild self-limiting condition requiring no treatment, to a severe and fatal disorder. The initial manifestation is usually a skin rash with or without a fever and influenza-like symptoms. The rash is usually found on the extensor surfaces of the limbs, the face and neck, and palms and soles. It may be localised or extensive, and may become confluent in more extensive cases, with the development of frank epidermolysis and bulla formation. If acute GVHD affects the gut, the most frequent manifestation is diarrhoea, accompanied by abdominal cramps, nausea and anorexia. The condition may progress to affect the whole gut with severe fluid, electrolyte and blood loss. Liver GVHD is usually the last to develop, typically beyond 40 days from transplant. It may be a manifestation of a progressive GVHD process or an isolated manifestation in the absence of or following the resolution of skin and gut GVHD. Pancytopenia and continued immune deficiency tend to reflect the severity of the process.

A firm diagnosis of acute GVHD can be difficult to make on clinical or histological grounds. No single feature is diagnostic, and usually the diagnosis is made on clinical grounds following exclusion of other possible explanations for the clinical findings. There are several systems for grading acute GVHD using clinical and histological criteria (Glucksberg, *et al* 1974, Thomas, *et al* 1975a, Thomas, *et al* 1975b). The Gluckberg system for clinical grading of acute GVHD is shown in Table 2.3.

Once established, the treatment of acute GVHD is determined by the severity of the condition. Asymptomatic patients may not need treatment. Patients with a localised rash often respond to topical steroid application. Systemic treatment is indicated if the patient is constitutionally ill with an extensive rash, or if involvement of the gut or liver is suspected. High dose methyl prednisolone at a dose of 2mg/kg/day is administered intravenously for 3-5 days, with a gradual dose reduction thereafter in the face of response, which occurs in the majority of patients. If acute GVHD persists in spite of high dose methyl prednisolone, it is regarded as refractory, and many patients have a poor prognosis. Fewer than 30% of patients with acute GVHD > grade III will survive, and many patients progress to chronic GVHD. Anti-thymocyte globulin (ATG) is commonly used as

first-line therapy for steroid-resistant acute GVHD. ATG is a polyclonal antibody whose primary target is surface antigens on T lymphocytes. The result of its use is the elimination of antigen-reactive T lymphocytes in the peripheral blood and alteration of T cell function. However, data on its efficacy are limited. In a study of 58 patients with steroid-resistant acute GVHD (Khoury, *et al* 2001), horse ATG was administered as first-line therapy, a median of 9 days (range, 3 to 39) after initiation of methyl prednisolone. Improvement was observed in 30% of patients treated with ATG. Skin disease was more likely to improve with ATG (79%), while progression of gut and liver acute GVHD was observed in 40% and 66% of patients, respectively. Despite initial improvement, 52 patients (90%) died a median of 40 days after ATG therapy from progressive acute GVHD and/or infection (74%), ARDS (15%), or relapse (11%). Only six patients (10%), three of whom had acute GVHD limited to the skin at the time ATG was administered, are long-term survivors. The study concluded that initial improvement of steroid-refractory acute GVHD occurs with ATG in a minority of patients, there are few long-term survivors and the treatment is associated with a high rate of major infective complications (Khoury, *et al* 2001). Furthermore ATG is associated with severe and prolonged lymphocyte depletion, but this is not associated with a higher efficacy in the treatment of GVHD.

Owing to the poor results of treating refractory acute GVHD with ATG, other immunomodulating agents have been tried in the treatment of acute GVHD. The administration of daclizumab, a humanized antibody that binds to the CD25 Tac receptor for IL-2, has recently been reported to produce response rates of 29% and 47%, respectively, using two different time schedules of antibody administration (Anasetti, *et al* 1994, Blaise, *et al* 1995). In another study (Willenbacher, *et al* 2001), 16 patients with steroid-resistant acute GVHD received daclizumab at a dose of 1 mg/kg on days 1-5 and once a week thereafter until day 28 or 1 mg/kg on days 1 and 2, followed by one dose per week thereafter for 28 days. Twelve patients suffered from grade III-IV acute GVHD and 4 patients from extensive chronic GVHD. Responses were observed in 9 patients (6 acute, 3 chronic GVHD). Fourteen out of 16 patients acquired infections during daclizumab treatment and 3 deaths were infection-related. Thus competitive inhibition of interleukin 2-dependent lymphocytes by

daclizumab demonstrates some beneficial effects in the treatment of graft-versus-host disease.

Another agent, infliximab, a chimeric human/mouse antibody that binds to soluble and membrane forms of TNF α has been used to treat refractory acute GVHD. In a study by Kobbe *et al* (Kobbe, *et al* 2001) 4 patients with grade III-IV steroid-refractory acute GVHD received the monoclonal antibody treatment. All patients had severe intestinal involvement in addition to skin and/or liver disease and had received treatment with high-dose steroids for a median of 11 days (range 5-17) in addition to Ciclosporin (4) and mycophenolate mofetil (MMF) (3). Infliximab (10 mg/kg) was given once a week until clinical improvement. In 3 out of 4 patients a complete resolution of diarrhoea and significant improvement of skin and liver disease were observed. Two patients are reported to be alive >200 days after therapy, of which one has limited chronic GVHD. Two patients died, one of progressive malignant disease without GVHD and one of refractory GVHD. Thus infliximab may have activity as single agent in the treatment of acute GVHD.

7.2.3 Acute GVHD in the present study

In the present study, 5 patients developed grade I-II acute skin GVHD post transplant, all of which responded to topical steroid therapy. No patient developed grade III/IV GVHD post transplant. Five patients developed grade II-IV acute GVHD following DLI, including 2 who had experienced grade I acute GVHD post transplant. With regard to stem cell source, 5 out of 8 unrelated donor/ bone marrow stem cell recipients experienced some form of GVHD compared to 2 out of 11 sibling donor/ peripheral blood stem cell recipients. Those who experienced up to grade II GVHD of the skin following transplant or DLI responded to topical steroid application. Two patients with grade III GVHD of the gut and liver following DLI responded to intravenous steroids, whilst 2 patients who developed grade IV GVHD of the liver following DLI died of liver failure despite treatment with high dose intravenous steroids and ATG. The third patient who developed grade IV GVHD of the skin and liver proved to be steroid-refractory, but responded fully to a combination of infliximab, daclizumab and reinstatement of ciclosporin therapy, and remains well and in a PR from his myeloma 1 year later.

7.2.4 Chronic GVHD: pathophysiology

The evidence that chronic GVHD is a disease of Th2-polarised activation is more consistent than that for Th1-polarisation in acute GVHD. There is however limited understanding of the pathophysiological processes leading to chronic GVHD because of the paucity of satisfactory animal models and studies in human subjects (Kansu 2004).

As discussed in section 4.1.1, the thymus plays a critical role in preventing the development of autoimmunity by generating T cells that are non-responsive to self antigens. T cells that express high affinity for self-peptides undergo programmed cell death (negative selection) within the thymus, such that >98% of these cells are never released in to the periphery. On the other hand, thymocytes with low affinity receptors for self-antigens undergo positive selection, survive and migrate out of the thymus into peripheral lymphoid organs, where they target foreign antigens.

Following transplantation, the development of chronic GVHD may result from the disruption of T cell 'education' by the thymus gland, which is invariably damaged by the conditioning therapy. As a result of this dysregulation of T cell development and concurrent loss of 'negative selection', autoreactive T cell clones may survive and ultimately cause release of autoreactive T cells in to the periphery. This results in an impairment of T cell homeostasis and self-tolerance, expansion of autoreactive T cells and promotion of autoimmunity. In addition, the polarisation of donor cells in a Th2 direction results in Th2-type cytokine release which causes polyclonal activation of B lymphocytes and autoantibody production against self-antigens. Organ-specific autoimmunity results from B cell hyperreactivity and production of autoantibodies with specificity against target organs such as joints, skin, eyes, liver & gut mucosa.

The main pathological feature is the development of collagen deposition, sclerosis and atrophy of the dermis of the skin with resultant scaling erythroderma, depigmentation or hyperpigmentation, nail dystrophy and alopecia of varying degrees. The exocrine glands may be affected by a similar process, resulting in a sicca syndrome. In the GI tract, mucosal ulceration, lichen planus lesions, pancreatic insufficiency malabsorption and weight loss

may occur. Patients with chronic GVHD may develop a restrictive-obstructive bronchiolitis, recurrent chest infections, intrahepatic biliary obstruction, cirrhosis and liver failure. Musculoskeletal involvement may lead to restriction of joint movement, fascial sheath constriction and limb ischaemia. A particularly serious complication associated with chronic GVHD is immunodeficiency, leading to susceptibility to a wide range of opportunistic infections and frequently with a fatal outcome. Atrophy of the lymphoid system and hyposplenism are common. Antimicrobial prophylaxis against *Pneumocystis carinii*, CMV and *Pneumococcus pneumoniae* is crucial in the presence of ongoing chronic GVHD. The bone marrow may also be affected, with cytopenia due to decreased marrow function or autoimmune destruction of circulating blood cells. As shown in Table 2.4, chronic GVHD (Shulman, *et al* 1980) is classified as either limited or extensive. Apart from classifying chronic GVHD according to the extent of organ involvement, it can also be classified according to its pattern of onset: progressive chronic GVHD evolves without a hiatus from active acute GVHD, quiescent chronic GVHD evolves after a period of treated, responsive acute GVHD and *de novo* chronic GVHD arises in patients who never experienced acute GVHD at all.

7.2.5 Chronic GVHD: clinical features, grading and treatment

Although chronic GVHD is defined as GVHD occurring after 100 days of transplantation, clinical and histological features typical of chronic GVHD may occur as early as 30 days following transplantation, and may overlap with acute GVHD. Chronic GVHD may develop directly from acute GVHD or may follow a period of quiescence after acute GVHD, or indeed may occur in the absence of prior acute GVHD. Chronic GVHD typically occurs within 18 months of the transplant procedure, but may occasionally occur as late as 2 or years after transplantation. The clinical manifestations and treatment of chronic GVHD has been thoroughly reviewed (Ratanatharathorn, *et al* 2001) and frequently resemble autoimmune disorders associated with cellular and humoral defects of immunity. It frequently presents as a multiorgan process such as SLE, scleroderma or rheumatoid arthritis. Chronic GVHD occurs in 60-80% of long-term survivors of allogeneic stem cell transplantation, and its incidence is likely to rise due to the increasing availability of unrelated donors and the greater inclusion of older patients in nonmyeloablative transplant programmes. It may

be lethal in 20-40% of affected patients, despite aggressive drug therapy to curtail the process (Kansu and Sullivan 2000).

Risk factors for the development of chronic GVHD include a history of prior acute GVHD, HLA disparity between recipient and donor, the use of non T cell-depleted stem cells, male recipients of female donors, older age of recipient or donor, and the use of certain conditioning agents such as busulphan (Ochs, *et al* 1994). The effect of stem cell source (PB vs. BM) on the risk of developing chronic GVHD is controversial, with earlier studies suggesting a greater risk with PB source but others showing no difference (Bensinger, *et al* 2001).

The treatment of chronic GVHD is complicated by the diversity of organ involvement, the chronic nature of the illness and the haematological and immune dysfunction associated with the condition. Whilst patients with local chronic GVHD may not need specific treatment, those with extensive disease do require treatment to reduce the chance of progression to a more advanced or progressive form of the disease which can have irreversible effects on the structure of tissues and organs. Patients whose chronic GVHD progresses from prior acute GVHD which has never abated are more likely to receive steroid therapy and other immunosuppressive measures, but are less likely to respond to these measures. By contrast, those who develop chronic GVHD *de novo* or after an interval from resolved acute GVHD are more likely to respond to therapy with steroids, ciclosporin or tacrolimus. Numerous other agents have been explored, including thalidomide, hydroxychloroquine, extracorporeal phototherapy, UVB and PUVA. Whilst cutaneous involvement may respond to these measures, visceral chronic GVHD rarely does. Recently studies of infliximab have shown promise, with high response rates even for lung involvement (Couriel, *et al* 2004).

7.2.6 Chronic GVHD in the present study

In the present study, there were 3 patients affected by chronic GVHD, all to a local extent affecting the skin (Table 2.4). In two cases, the chronic GVHD occurred *de novo* following DLI. In both cases, topical steroid application was effective. One affected patient was aged 50 years and the other was 53 years, and one received PBSC from his sibling whereas the other received BMSC from

an unrelated donor. The second patient (Patient 12) went on to develop grade IV acute GVHD of the liver following his third DLI, which was delayed to allow his prior local chronic GVHD to fully settle. He subsequently responded to a combination of daclizumab and infliximab as discussed previously. In the third case, chronic GVHD ensued from acute GVHD following DLI, and has required multiple admissions to hospital to control flare-ups using high dose intravenous steroids. In all the patients in this study, there was a profound and persistent reduction of CD4+RA+ and CD4+RO+ cells up to 18 months of follow up. CD8+RA+ and RO+ cell recovery was somewhat superior in the patient group overall, but there was no patient-specific association between cell counts and the incidence of GVHD, acute or chronic. The lymphocyte counts of the 3 patients affected by chronic GVHD were no different from the rest of the group. Overall, the incidence of chronic GVHD was lower than that previously reported in allogeneic transplantation.

7.3 Prevention of GVHD

GVHD remains a major cause of morbidity and mortality after allogeneic stem cell transplantation. Pharmacological immunosuppression has been employed for many years in the preventive strategy against GVHD in the setting of myeloablative transplants. Methotrexate down-regulates T cells by inhibiting cellular proliferation; mycophenolate mofetil (MMF) inhibits purine synthesis; ciclosporin and tacrolimus suppress IL-2 secretion by blocking calcineurin activity; daclizumab reduces T cell responsiveness by blocking the IL-2 receptor (Willenbacher, *et al* 2001). In spite of these pharmacological approaches, moderate-to-severe acute GVHD occurs in 25-60% of matched related transplants and 45-70% of matched unrelated transplants (Gale, *et al* 1987). Acute GVHD also remains a significant complication of the reduced intensity approach (Giralt, *et al* 1997, Khouri, *et al* 1998).

The majority of allogeneic transplantation procedures use HLA-matched donors, so it is likely that minor histocompatibility antigens (mHA) contribute to the development of acute and chronic GVHD. These antigens are polymorphic proteins encoded in the genome that are presented to T cells in the context of HLA, with a consequent MCH-restricted immune response. Minor histocompatibility antigens that are ubiquitously expressed on all tissues are

thought to be associated with the pathogenesis of both GVHD and GVM, whereas mHA that are restricted to haematopoietic cells (such as HA-1, HA-2, HB-1 and BCL2A1) may be responsible for mediating GVM (Kircher, *et al* 2004, Marijt. *et al* 2003). Thus it should be possible to prevent GVHD by avoiding minor mismatches between donor and recipient (Mutis 2003).

7.3.1 T Cell depletion

Apart from pharmacological immunosuppression which results in blockade of T cell function, other approaches have been tried to minimise the incidence of GVHD. Pharmacological approaches have their limitations, both in terms of effectiveness and inherent toxicities, such as renal toxicity in the case of ciclosporin and mucositis in the case of methotrexate. Absolute T cell depletion as opposed to blockade of T cell function is an alternative and highly effective way of preventing GVHD, with the added benefit of reducing the need for excessive pharmacological immunosuppression and its inherent toxicities. This may be performed *ex vivo*, by depleting the graft of T cells before infusion, or *in vivo*, by administering T cell depleting agents as part of the conditioning therapy.

Ex vivo techniques have relied upon negative selection of T cells from the graft by physical separation (density gradient fractionation, soyabean lectin agglutination and E-rosette depletion and counterflow elutriation) or antibody-based purging using antithymocyte globulin (ATG) or monoclonal antibodies against target antigens on T cells (Ho and Soiffer 2001).

In the present study, T cell depletion was achieved *in vivo* by the administration of alemtuzumab at a dose of 20 mg/day for 5 days (section 2.2) together with fludarabine and melphalan as part of the conditioning regimen. Just 5 patients developed grade I-II acute skin GVHD post transplant, all of which responded to topical steroid therapy. No patient developed grade III/IV GVHD post transplant. We have previously reported in an overlapping group of patients, that this protocol results in a low incidence of GVHD, but at the expense of a durable response. Both of these factors are likely to be attributable to the alemtuzumab used in the conditioning schedule of the transplant (Peggs, *et al* 2003a). This is in contrast to the results of a study by a Spanish group, using an otherwise

received identical conditioning with fludarabine and melphalan but without alemtuzumab. They found that their use of ciclosporin and methotrexate as GVHD prophylaxis rather than alemtuzumab resulted in an acute GVHD rate of 45.1% (compared to 21.7% in the group of patients receiving an identical fludarabine/melphalan regimen but with alemtuzumab) and 66.7% rate of chronic GVHD compared to 5% in the alemtuzumab group (Perez-Simon, *et al* 2002). Interestingly, there was no significant difference between disease response, event free survival or overall survival between the 2 groups, but there was a significantly increased incidence of CMV reactivation in the alemtuzumab group (85% vs. 24%). However, patients in the alemtuzumab group did require DLI to achieve similar responses to those in the methotrexate group. These findings suggest that the degree of T cell depletion is an important consideration in the development of conditioning regimens for allogeneic transplantation from several points of view including risk of GVHD, risk of infection and disease response.

The precise degree of T cell depletion needed to prevent GVHD is not known, and is likely to vary between donor-recipient pairs, depending on the differences in minor antigen matching. On average, an unmodified marrow graft contains approximately 1 to 5×10^7 cells/kg of recipient body weight. The incidence of GVHD increases with T cell dose, as demonstrated in a study that showed a 45% incidence of GVHD in recipients who received a T cell dose of 1×10^6 cells/kg compared to a 22% incidence when the T cell dose was reduced to 0.5×10^6 cells/kg (Wagner, *et al* 1988), and abolition of GVHD when extensive T cell depletion was performed to a CD3⁺ cell dose of 3×10^4 cells/kg (Aversa, *et al* 1998). However, whilst a 3 to 4-log depletion of donor T cell almost completely eliminates GVHD, it is also associated with an increase in graft rejection, disease relapse (Horowitz, *et al* 1990) and delayed immune recovery with consequent increased infection rates (Chakraverty, *et al* 2001).

Thus, although fewer patients succumb to the effects of GVHD in T cell depleted transplants, this advantage is offset by a number of disadvantages, including an increase in graft failure, higher relapse rates, delayed immune reconstitution, increased risk of posttransplantation lymphoproliferative disease, and increased incidence of CMV reactivation (Ho and Soiffer 2001).

Prior to T cell depletion, graft failure was uncommon, occurring in up to 5% patients compared to reports of an incidence of 50-70% in T cell depleted transplants (Patterson, *et al* 1986). Graft failure can occur in 3 patterns: failure of initial engraftment, partial or full engraftment followed by graft rejection within a few weeks of transplant and delayed graft failure that happens months after the initial transplant. Early graft failure is thought to occur due to direct immunological rejection of the graft by host haematopoietic cells that have survived the conditioning process. This has been demonstrated by the finding of host T cells with donor-specific cytotoxicity in patients at the time of graft rejection (Voogt, *et al* 1990). Viral infections such as CMV or human herpes virus -6 (HHV-6) may also contribute to failure of the graft, possibly due to an increased incidence of viral infections following T cell depleted transplantation and the concurrent delayed immune reconstitution that occurs in this setting (Small, *et al* 1999). The exact pathophysiology of graft failure remains unclear. Mixed lymphoid and myeloid chimerism is more common after T cell depleted transplantation and may contribute to graft failure (Bertheas, *et al* 1991). In order to counteract the perceived threat by residual host T cells to causing graft rejection, early strategies focussed on dose intensification of the myeloablative conditioning, such as the inclusion of high doses of cytarabine, thiotepa and anthracyclines or total nodal irradiation. These strategies have led to a reduction in graft rejection, but at the expense of increased regimen-related toxicity (Kurusu, *et al* 1991, Schaap, *et al* 1997). Particularly relevant to the present study is the alternative strategy of using monoclonal antibodies such as ATG or CAMPATH-1 against host immune cells at the time of transplantation (also discussed in section 2.2). The results of early animal studies were later repeated in human subjects with the achievement of simultaneous host and donor T cell depletion by CAMPATH-1 antibodies that reduced the incidence of graft failure without compromising GVHD prophylaxis (Hale and Waldmann 1994). Importantly, there was no case of graft failure or rejection in the present study.

Other problems that arise following T cell depletion include delayed immune reconstitution and a consequent increase in opportunistic infections. This complication was prominent in the present study, but the increased incidence of viral infections did not translate into an increase in mortality (Section 2.4.1).

Also showing increased prevalence in the T cell depleted setting are the so-called post transplant lymphoproliferative disorders (PTLD), often EBV-driven that have been reported in up to 30% of T cell depleted transplant recipients (Shapiro, *et al* 1988). PTLDs are thought to arise from infected donor T cells that are co-transfused with the graft, but it may be possible for this condition to arise in recipients of EBV-seronegative donor grafts. When such a complication arises, clinically presenting as a lymphoma, DLI has been used to induce EBV-specific cytotoxicity against the PTLD cells (Papadopoulos, *et al* 1994). To date there have been no incidences of PTLDs in the patient group in the present study.

There has, however been a high incidence of disease progression/ relapse in the present study. This represents the other principal disadvantage of T cell depleted transplantation. In the present study, 16 patients (84%) have shown evidence of disease progression following transplant and/ or DLI after a median of 283 (range 153-895) days. This finding is in keeping with the higher incidence of disease relapse seen after T cell depleted transplants in other diseases, including acute myeloid and lymphoblastic leukaemia and chronic myeloid leukaemia (summarised in (Ho and Soiffer 2001).

Since these earlier studies, attempts have been made to refine the technique of T cell depletion by depleting only selective subsets of T cells in order to retain the benefits of T cell depletion while reducing the disadvantages. These approaches will be discussed further in Chapter 8.

7.4 Graft-versus malignancy effect

Confirmation of an immune-mediated GVM effect in myeloma patients has come from reports of the successful use of donor leukocyte infusions (DLI) to treat patients relapsing from allogeneic transplantation (Lokhorst, *et al* 1997, Tricot, *et al* 1996b, Verdonck, *et al* 1996). In order to achieve successful disease eradication without excessive toxicity, the goal is to induce a GVM effect in the absence of significant (>grade 2) GVHD. GVM frequently occurs coincidentally with GVHD, but the converse is also true. Many patients achieve a GVM response (i.e. disease remission due to DLI) without developing GVHD. This may be due to the different target antigens involved in each process, or the

greater sensitivity of malignant cells compared to normal tissues to a common immunological mechanism (Champlin, *et al* 2000). GVM may also be mediated by immune reactivity against specific haematopoietic targets such as minor histocompatibility antigens restricted to haematopoietic tissues (Bonnet, *et al* 1999, Clave, *et al* 1999, Mutis, *et al* 1999). Overexpressed or abnormally expressed cellular constituents could also serve as target antigens or malignancy-specific targets for GVM.

In the present study, a total of 10 patients experienced GVHD (Table 7.1).

Patient	GVHD post transplant	Disease status post transplant	GVHD post DLI	Best response after DLI	(Improved) disease response accompanied by GVHD?
3	No	MR	Acute Grade II & Limited Chronic	PR	YES (following DLI)
4	Acute Grade II	PR	No	Did not receive DLI	YES (following transplant)
8	No	PD	Acute Grade III	No Change	No
9	Acute Grade II	PR	No	No Change	YES (following transplant)
11	No	PR	Limited Chronic	CR	YES (following DLI)
12	No	NC	Limited Chronic (following DLI#1)	PR	YES (following DLI)
			Acute Grade IV (following DLI#2)	PR	YES (following DLI)
16	No	PD	Acute Grade IV	No Change	No
17	Acute Grade I	PD	Acute Grade IV	PR	YES (following DLI)
18	Acute Grade I	PD	Grade III	PR	YES (following transplant)
19	Acute Grade II	PR	No	Further PR	YES (following both)

Table 7.1. Association of GVHD with disease response following transplant or DLI.

Of the ten patients who experienced GVHD following transplant and/or DLI, 8 showed evidence of disease response accompanied by the presence of GVHD, whereas 2 patients showed no disease response despite evidence of GVHD.

Abbreviations: GVHD, graft-versus-host disease; DLI, donor lymphocyte infusions; CR, complete response; PR, partial response; MR, minor response; NC, no change; PD, progressive disease

Out of these 10 patients, 8 eventually demonstrated a disease response alongside clinical evidence of GVHD. These included 5 patients with GVHD following transplant and 3 patients who developed GVHD for the first time

following DLI in whom the disease response was improved by DLI as well as being associated with GVHD. These findings demonstrate that the GVM effect is frequently obtained at the expense of GVHD. Alternatively, the latter may occur without evidence of the former such as in 2 patients in this study who showed progressive disease following transplant in the absence of any GVHD, but went on to develop grade III or IV GVHD following DLI, without a demonstrable disease response. On the other hand, 2 patients in this study showed evidence of the GVM effect in the absence of clinical demonstrable GVHD (Table 7.2).

Patient	GVHD post transplant	Disease status post transplant	GVHD post DLI	Best response after DLI	Demonstrated clinical response?
1	N	PD	N	No Change	No
2	N	PD	N	No Change	No
5	N	MR	N	PR	Yes (Improved by DLI)
6	N	PD	N	PR	Yes (following DLI)
7	N	PD	N	No Change	No
10	N	No Change	N	No Change	No
13	N	PR	N	PD	Yes (following transplant)
14	N	MR	N	MR	Yes (following transplant)
15	N	PR	N	No Change	Yes (following transplant)

Table 7.2. Relationship of disease response to transplant and DLI in the absence of GVHD.

Five out of 9 patients who did not show clinical evidence of GVHD following transplant or DLI, nevertheless demonstrated disease response following transplant (n=3), DLI (n=1) or sequential response after both transplant and DLI (n=1). This included 2 patients who showed an improvement in response following DLI, after an initial MR (Patient 5) and PD (Patient 6). This demonstrates that a GVM effect may occur in the absence of GVHD.

Abbreviations: GVHD, graft-versus-host disease; DLI, donor lymphocyte infusions; CR, complete response; PR, partial response; MR, minor response; NC, no change; PD, progressive disease.

These 2 patients showed little or no clinical response to the conditioning procedure/ transplant, but went on to demonstrate a PR following DLI in the absence of GVHD. In 3 other patients, there was evidence of a disease response to transplantation in the absence of GVHD, but this response may in part be due to the cytoreductive effects of the conditioning therapy rather than a GVM effect post transplant. Thus for myeloma, there is no conclusive evidence that the occurrence of GVHD is predictive of an effective GVM response. This may be due to a failure to identify the appropriate effector T cell subsets that are involved in these different processes.

7.4.1 Improving allogeneic immune reconstitution to promote GVM

In patients with haematological malignancies, reconstitution of the allogeneic immune system following stem cell transplantation may be accompanied by the development of immunity to residual tumour cells that have not been eliminated by the conditioning therapy. Strategies to enhance and accelerate immune reconstitution and hence the GVM effect include rapid withdrawal of immunosuppression (Libura, *et al* 1999), the administration of cytokines (Collins, *et al* 1997, Kolb, *et al* 1993) and the administration of DLI; this latter method has been successful in the setting of myeloma, demonstrated by the high response rates achieved by the administration of DLI in patients with relapse (Lokhorst, *et al* 1997, Tricot, *et al* 1996b, Verdonck, *et al* 1996). In addition, the optimal intensity of conditioning therapy remains uncertain as does the optimal post-transplant immunosuppressive strategy. Excessive conditioning or immunosuppression may blunt a GVM effect as well as GVHD and result in higher relapse rates. The timing and dose of DLI also remain unclear and under investigation.

The objective of DLI administration is to enhance cellular immune function following allogeneic transplantation because cellular immunity is usually severely depressed for 6 months and takes up to 18 months to recover. The initial rise in absolute numbers of CD3⁺ T cells is not matched by the much slower pace of recovery of T cell receptor diversity, as assessed by TCR spectratyping (Bellucci, *et al* 2002) as confirmed in this study (Chapter 5, section 5.3). Following allogeneic transplantation, relapse occurs in host-

derived cells, whereas residual normal haematopoiesis and immunity remain donor-derived. Infused donor lymphocytes are therefore not subjected to rejection, but may in fact induce acute GVHD. Anti-tumour effectors proliferate *in vivo* following infusion and may eradicate residual malignant cells and host-derived haematopoietic cells once they reach a critical threshold level.

A recent study of the immunological effects of prophylactic DLI following myeloablative allogeneic stem cell transplantation for myeloma (Bellucci, *et al* 2002) demonstrated that planned infusion of CD4⁺ cells 6 months after transplant improved reconstitution of donor T cells, promoted complete donor chimerism and enhanced anti-myeloma activity. Similarly, a study of T cell depleted allogeneic transplantation for refractory myeloma demonstrated that subsequent DLI administration produced a disease response in 50% of patients, but also induced GVHD in 63% of the group (Huff, *et al* 2003). Our group has recently reported the results of the administration of dose-escalated DLI following RIT (in an overlapping group of patients with those in the current study) and demonstrated that 63% of patients with myeloma responded to DLI that were given for mixed chimerism, residual or progressive disease, (Peggs, *et al* 2004). GVHD did occur and was more common in the unrelated donor cohort and occurred at lower T cell doses than in the sibling donor cohort but neither the incidence of GVM nor GVHD were predictable on the basis of chimerism studies. In the present study, there was no demonstrable relationship between the incidence of GVHD whether acutely after transplant or following DLI administration and the level of NK cells or indeed any other T cell subset.

7.4.2 GVM mechanisms

An important reason for limited progress in successfully harnessing the GVM effect is an incomplete understanding of the mechanisms involved. It is useful to think of the GVM process as consisting of an afferent arm, in which host tumour antigens are presented to donor cells and an efferent arm in which donor effector cells induce immunological reactions against host tumour cells.

Within the afferent pathway, the target antigens of the GVM response remain poorly defined. The frequent co-existence of GVHD and GVM suggests that these targets may be shared by malignant cells and host tissues, particularly

the skin, gut and liver. Given the differences in incidence of GVHD and GVM however, it may be that although the target antigens are shared, the sensitivity to the effects of the effector mechanisms may be greater in tumour cells compared to visceral cells. The way that antigen is presented may also affect the eventual immune response. MHC class I and II molecules are involved in the presentation of cellular antigens to donor T cells. These cellular antigens include minor histocompatibility antigens that may be tissue restricted or widely distributed, normal protein sequences that are over-expressed or aberrantly expressed by malignant cells or unique tumour-specific peptides. There is evidence in the pathogenesis of GVHD that antigen presentation by host APCs as opposed to donor APCs is important (Shlomchik 2003) and in time similar evidence may emerge in the pathogenesis of GVM.

The relative contributions of different GVM effector populations, which are often interrelated, also remain poorly defined. Also, it is likely that any susceptibility to the GVM process will be influenced by the proliferation rate of the tumour as well as the phenotype of the tumour and its intrinsic ability to stimulate anti-tumour T cells (Barrett, *et al* 2003). Numerous studies in both murine models and human cell lines have identified a link between GVHD, GVM and virtually every T cell subset; CD4⁺, CD8⁺ and NK cells have all been implicated as mediators of GVM (Champlin, *et al* 1999). Even B cells have been recently implicated in the pathogenesis of tumour immunity, via antibody responses generated against tumour-associated antigens (Bellucci, *et al* 2005).

A recent study in a murine model has confirmed that the use of CD4⁺CD25⁺ T cells may facilitate donor engraftment in the absence of GVHD (Hanash and Levy 2005), following an earlier study that looked at the same subset (Edinger, *et al* 2003). CD25 represents the IL-2 receptor (IL-2R) α chain and when CD4⁺CD25⁺ T cells are selectively infused, they abrogate GVHD without affecting GVM, by suppressing the early expansion of alloreactive donor T cells and IL-2R α chain expression by these cells. The CD4⁺CD25⁺ subset (also termed regulatory T cells or T_{regs}) is thought to be essential for the induction and maintenance of tolerance to self antigen, for the prevention of autoimmune disease (Sakaguchi, *et al* 1995) and regulating the homeostasis of the peripheral T cell pool (Annacker, *et al* 2001, Murakami, *et al* 2002). With regard

to the CD8⁺ subset, antigen-specific CD8⁺ T cells have been reported to reach frequencies as high as 10% of the circulating T cell repertoire following DLI, and although a short-lived phenomenon is associated with tumour (in this case leukaemia) regression (Marijt, *et al* 2003).

NK cells are lymphocytes critical to host defence against infectious pathogens and malignant transformation through the release of cytokines as well as direct cell lysis. Earlier studies suggestive of useful anti-tumour activity of NK cells have been given more emphasis by a better understanding of NK cell biology in recent years (Farag, *et al* 2002). There is evidence of NK activity in the setting of myeloma, including a study which demonstrated that NK cells recognise and kill myeloma cells both in cell lines and fresh bone marrow samples from myeloma patients to varying degrees, but not CD34 positive stem cells or peripheral blood mononuclear cells, which were resistant under similar experimental conditions (Frohn, *et al* 2002).

7.5 Discussion

In conclusion, although there is evidence to support the use of adoptive cellular therapy following stem cell transplantation for myeloma, as yet, the correct immunological balance has not been achieved in favour of a GVM effect. The major challenge that remains is the separation of the GVHD effect from the GVM effect. A number of strategies have been employed, and their effectiveness is likely to be improved by a better understanding of the mechanisms involved. The balance may depend on cell dose or removing cell populations with a propensity to induce GVHD or the administration of suicidal (e.g. herpes virus thymidine kinase-transduced) lymphocytes, followed by killing the donor lymphocytes using ganciclovir. Also attempted has been the administration of cloned or selected cells with antitumour effects which may augment the GVM process without inducing GVHD. *Ex vivo* depletion of alloreactive T cells is another way of minimising GVHD whilst conserving a GVM effect. These and other approaches will be further explored in Chapter 8, which will discuss future directions to be considered in optimising this immunological balance.

Chapter 8: Conclusions and future directions

8.1 Introduction

The objective of this study was to document and investigate immunological recovery following allogeneic stem cell transplantation in the novel setting of reduced intensity conditioning and alemtuzumab-induced *in vivo* T cell depletion, and the role they play in the generation of a GVM effect. This was carried out in a group of patients with a disease that remains incurable for all but a small proportion of the few individuals who survive the rigours of myeloablative allogeneic transplantation. The apparent achievement of a long term remission in these patients with this otherwise fatal illness drives continued interest in the further development of allogeneic stem cell transplantation. The assumption is that the long-term suppression of the myeloma clone would result from an optimum balance of GVM and GVHD.

As previously mentioned, the rationale for the conditioning approach used in this study was to reduce toxicity by lowering the intensity of the preparative chemotherapy, and also to achieve a more controlled approach to the induction of a GVM effect, using T cell-depletion with subsequent, escalated DLI, whilst monitoring patients for evidence of GVHD. Indeed, within the first 100 days of transplant, mortality and morbidity in this cohort of patients was acceptable, given the older age group that underwent the procedure. In contrast to myeloablative regimens, despite the high incidence of viral infections and CMV reactivation, there was a no case of CMV disease and a low infection-related mortality in this study. In fact overall, in this patient group there was a surprisingly low incidence of opportunistic infections. This may be due in part to the concomitantly low incidence of GVHD in this group (approximately half of the patient group were affected by grades I-II GVHD) an effect also probably attributable to the T cell-depleting properties of alemtuzumab. Delayed T cell recovery following alemtuzumab-containing conditioning was found to be comparable to that of myeloablative protocols. B cell recovery was delayed even compared to myeloablative protocols, particularly in terms of CD19+ cell numbers, but B cell repertoire recovered well. The main disadvantage of this regimen was the high relapse rate seen in this patient group, suggesting that

the GVM effect was abrogated by the *in vivo* T cell depleting properties of alemtuzumab.

Numerous studies are ongoing to optimise the balance between the desired GVM effect and the unwanted GVHD effect that contributes significantly to patient mortality and morbidity following allogeneic transplantation. They involve different strategies, including variations in the approach to conditioning the patient prior to transplant to achieve an adequate cytotoxic effect against residual tumour cells with limited host toxicity, variations in the approach to T cell-depletion and GVHD-prophylaxis, manipulation of specific subsets of lymphocytes within the stem cell inoculum either *in vivo* or *in vitro*. In this chapter, some of these novel strategies will be discussed in more detail.

8.2 Conditioning regimens

Since the conclusion of the study which is the subject of this thesis, despite continued efforts worldwide, there has not been any single major advance in the development of more effective conditioning regimens for allogeneic stem cell transplantation in myeloma. There has been an accumulation of smaller, more subtle developments, which continue to add to the body of clinical evidence. Thus, myeloablative allogeneic transplantation is generally offered to patients up to the age of 45 years if there is a HLA-identical sibling donor and up to the age of 40 years if the donor is a matched unrelated one. Recent data have emerged about the superiority of melphalan and TBI as conditioning therapy for myeloablative transplants over cyclophosphamide and TBI, yielding CR rates of 65% and 47% respectively, no difference in non-relapse mortality, and relapse rates of 37% and 81% respectively at 5 years (Hunter, *et al* 2005). In other respects, data on the subject of other varieties of conditioning regimens remains inconclusive (Gahrton, *et al* 1995).

The current position of RIT in myeloma is far from certain. It has been shown to be a technically feasible procedure with acceptable non-relapse mortality rates even in patients with a higher incidence of co-morbidities. But the evidence for curative potential is not yet forthcoming with current approaches. The most promising approach to date is the tandem autologous transplant / RIT procedure, which aims to exert a GVM effect at a time when a state of minimal

residual disease has been achieved by the conditioning therapy for the initial autologous transplant. This would seem more likely to be successful given the limited success of using RIT as first-line consolidation or salvage therapy (Thomson and Peggs 2005). In one study of tandem autologous transplant /RIT, 54 patients received induction therapy followed by a melphalan 200 autograft and then a planned HLA-matched sibling RIT conditioned with 200 cGy TBI at a median of 62 days later (Maloney, *et al* 2003). Overall survival at a median of 552 days was 78% with a TRM of 17% and extensive chronic GVHD in 46%. Twenty five (52%) of the 48 patients who did not achieve CR following the autograft went on to achieve CR following the following the allograft, and the estimated progression free survival at 2 years is 55%. Another study of the tandem approach reported the outcome of a fludarabine, melphalan and ATG-conditioned RIT following induction therapy and a melphalan 200 autograft in a smaller number of patients (17). Complete remission was seen in 72% following the RIT and the estimated PFS at 2 years was 56% (Kroger, *et al* 2002). However, this approach in high risk patients seems less robust. An IFM study enrolled patients with poor risk features such as raised β_2 microglobulin and chromosome deletion (del (13) or (17)) by FISH analysis, to receive an ATG-containing RIT transplant following a melphalan autograft if they had an HLA-matched sibling donor or a second tandem autograft (Facon, *et al* 2001, Moreau 2003). No advantage of the autologous/RIT approach has been observed in follow up to date (the survival rate at 3 years is 50% in both groups), implying that inclusion of the RIT cannot, at least with the conditioning therapy that was used, salvage this poor risk group. This observation has been confirmed by another study showing a high risk of relapse (relapse rate of 77%; overall survival of 18% at 2 years) in the RIT setting in patients with the presence of chromosome 13q- (Kroger, *et al* 2004).

Interestingly, the Stanford group has recently published the results of a study of a novel approach to conditioning using total lymphoid irradiation (TLI, 10 doses of 80 cGy each over 11 days) plus ATG in 37 patients with lymphoid malignancy or acute leukaemia (Lowsky, *et al* 2005). This regimen was used in these subjects following evidence for a low GVHD rate in the preclinical murine setting, in which TLI was found to offer protection against acute GVHD by preferentially sparing host NK T cells, which became the main source of host IL-

4. As discussed in section 4.1.6, IL-4 downregulates IL-12 and interferon- γ (IFN- γ) production, thus favouring the development of a Th2 response and functions as an autocrine growth factor for differentiated Th2 cells; Th2 polarisation tends to protect against acute GVHD. In the reported clinical study, the investigators found a marked increase in the production of IL-4 by donor CD4+ cells after transplantation as opposed to IL-4 production by CD4+ cells in normal control subjects and a reduced incidence of GVHD (3%) compared to RIT conditioned with TBI or chemotherapy alone. This apparently abrogating effect on the incidence of GVHD was contributed to (particularly in the patients with lymphoid malignancy) by the direct anti-tumour effect of the TLI on residual tumour cells in the host's lymphoid tissues. TLI also has the advantage of causing less tissue damage than TBI and reducing the propensity towards the so-called cytokine storm that heralds the onset of acute GVHD.

Thus although advances such as these need to be developed further, attention is also being focussed on issues other than conditioning, in order to improve the outcome of allogeneic stem cell transplantation, and include some developments that have only become possible due to advances in molecular technology, as will be discussed below.

8.3 Disease-related characteristics: Recent advances in the identification and significance of prognostic factors

8.3.1 Influence of prognostic factors

Apart from age and stem cell source, the nature of the disease itself poses considerable challenges when deciding on treatment options. Myeloma is a disease with considerable heterogeneity in clinical outcome. Survival of patients can vary from a few months to more than a decade following diagnosis and treatment (Greipp. *et al* 2005). As well as the variation in myeloma related end-organ damage (such as renal failure and lytic disease) at presentation, the clinical course of the disease varies considerably from patient to patient. Staging systems such as the Durie-Salmon system link reduced survival with high tumour burden at diagnosis, but more recently, important advances have been made in determining the role of specific prognostic factors in the course of the disease (San Miguel and Garcia-Sanz 2005). With the advent of novel

therapies for myeloma, such as thalidomide, its analogue, lenalidomide and the proteasome inhibitor, bortezomib, the role of intensified treatment in the form of allogeneic stem cell transplantation as opposed to the use of these agents in the management of myeloma patients is under closer scrutiny than ever. These agents have shown significant activity against the disease (Jagannath 2005, Rajkumar 2005, Richardson, *et al* 2003) and have considerably lower toxicity. Thus their use may be more appropriate than stem cell transplantation in selected patients.

High serum levels of β_2 -microglobulin and C-reactive protein and low serum levels of albumin, atypical plasma cell morphology and high proliferative activity have long been recognised as having a negative impact on survival (Bataille, *et al* 1992, Greipp, *et al* 1993, Jacobson, *et al* 2003). In reality, however, only β_2 -microglobulin, C-reactive protein and serum albumin levels are routinely measured, and none of them are sufficiently discriminatory to dictate the intensity or selection of treatment. Certain cytogenetic abnormalities are also recognised prognostic factors at presentation. Deletions/monosomy of chromosome 13, non-hyperdiploidy and certain balanced translocations (which include the immunoglobulin heavy chain locus (IgH) at 14q32) such as t(4;14) and t(14;16) have a strong negative impact on prognosis (Fonseca, *et al* 2004). Only a limited number of centres routinely perform cytogenetic analysis in myeloma patients. One of the main reasons for this is the low likelihood of obtaining cells in metaphase from bone marrow samples owing to the low proliferative rate of plasma cells. With the advent of techniques such as interphase fluorescent *in situ* hybridisation (FISH), it has been possible to improve the yield of the genetic characteristics of the disease. Recently, further study has elucidated the relative importance of these cytogenetic abnormalities as studied by conventional cytogenetics, metaphase FISH and interphase FISH (Dewald, *et al* 2005). It was found that the poorest prognosis (median survival of 12.7 to 13.9 months) is associated with the 13q- abnormality, t(4;14), t(14;16), or 17p- when detected with metaphase analysis or t(4;14) or t(14;16) seen with interphase FISH. In contrast, patients with no abnormality on conventional cytogenetics, metaphase or interphase FISH, or t(11;14) without t(4;14), t(16;16), 17p- or 13q- were found to have median survivals of 45 months and 55.3 months respectively. Interestingly, patients who demonstrated 13q- or 17p-

without t(4;14) or t(14;16) on interphase FISH were found to have an intermediate median survival of 33.9 months. As these molecular techniques become more routinely available, it should become possible to carry out studies of therapy that is risk-stratified according to the prognostic markers identified at diagnosis. Thus patients with poor prognosis genetics, with the exception of those for whom targeted therapies, eg., FGFR3 inhibitors for those with t(4;14), are coming on line, could be offered allogeneic stem cell transplantation exploring new and experimental protocols.

8.3.2 Gene expression profiling and myeloma

Techniques such as gene expression profiling (GEP) are set to further advance the understanding of the influence of genetic abnormalities on the course of the disease. Already, results are emerging that demonstrate that this technique can distinguish between normal plasma cells and MGUS / myeloma plasma cells, although so far GEP has not been as successful in distinguishing between MGUS, which remains an indolent, pre-malignant condition in the majority of cases and myeloma (Kyle, *et al* 2002). This finding could be regarded as counter-intuitive, since myeloma is regarded as malignancy characterised by genomic instability that increases as the disease progresses. If MGUS already possesses the genetic hallmarks of malignancy, it is surprising that it rarely converts into overt myeloma, suggesting that other influences such as changes in the bone marrow microenvironment or failure of immune surveillance may play an important role in the progression of the disease. It is currently possible, in specialised laboratories, to identify IgH translocation status, chromosomal ploidy, deletion of chromosome 13 and other high-risk genetic signatures using a single GEP platform. Some investigators have begun to study such prognostic profiles in the setting of tandem autologous stem cell transplantation (Shaughnessy, *et al* 2005). Other investigators have proposed a risk-adapted therapy model, based on a comprehensive GEP-based prognostic classification system (Hideshima, *et al* 2004): patients with t(11;14) [cyclin D1] or t(6;14) [cyclin D3] are classified as translocation classification (TC) 1 and respond well to therapy; TC2 and TC3 express cyclin D2 due to an identifiable mechanism such as trisomy 11 or an unknown mechanism *and* lack IgH translocation and have an intermediate prognosis; patients in TC4 possess t(4;14) [FGFR3/MMSET] and express high levels of cyclin D2. TC5 patients have

t(14;16) [*c-maf*] and express cyclin D2. The patients who fall in to categories TC4 and TC5 have a shortened survival irrespective of treatment with standard or intensive therapy. As further data from such studies emerge, we may be able to assign specific therapies to different TC groups.

GEP also has the scope to predict the response of myeloma to specific drug treatments, by defining the genetic signatures of drug responsiveness and resistance. Preliminary results show that it is possible to predict the response to VAD, bortezomib, thalidomide and lenalidomid by segregating patients on the basis of the GEP of their disease (Barille-Nion, *et al* 2003, Barlogie, *et al* 2004, Shaughnessy and Barlogie 2003).

Thus an important direction for future study in the management of myeloma is the continued development of reliable and valid prognostic models and models of predicted drug efficacy based on GEP, in order to develop a stratified approach to the utility and order of application of different modalities of treatment. The use of novel therapies prior to or following stem cell transplantation could be planned on the basis of the genetic signature of the disease in individual patients. This approach will rely upon the development of large scale clinical trials to ensure the statistical significance of the various therapeutic subgroups that emerge.

8.4 Improving host and donor compatibility: the role of non-HLA encoded antigens

Apart from older patient age, the availability of an HLA-identical stem cell donor remains the principal limitation to offering allogeneic stem cell transplantation to myeloma patients. As discussed in Chapter 7, the incidence of GVHD following allogeneic transplantation is generally lower in sibling donor compared to unrelated donor transplants. Since the chance of having an HLA-identical sibling donor is 1 in 4 (assuming availability of a sibling), the current study also included patients who would receive stem cells from unrelated donors. Eleven patients received sibling donor-derived peripheral blood stem cells, whereas 8 received bone marrow stem cells from unrelated donors, and there was evidence of a higher incidence and severity of acute and chronic GVHD in the unrelated donor group. However, this finding was not statistically significant.

Even when an HLA-identical match is found using the state-of-the-art molecular techniques discussed in section 1.4.1, the incidence of GVHD and GVM remains unpredictable following allogeneic transplantation, making it difficult to accurately counsel patients and indeed to facilitate their decision-making about the risks of proceeding to transplant.

As previously discussed in Chapter 7, the triggering and promoting or ameliorating mechanisms for acute and chronic GVHD are complex and incompletely understood. Whether the immune response is triggered by an infective or inflammatory stimulus, the immune system exhibits a number of phenomena such as redundancy and pleiotropism, up/down-regulation of cellular and cytokines responses or internalisation of cell surface receptors and adhesion molecules following activation, that contribute to the complexity of the situation. However, continued immunological and molecular research is promoting a better appreciation of the various mechanisms involved. For example, although major HLA-compatibility remains the principal consideration when selecting stem cell transplant donors, the sequencing of the human genome has revealed that numerous non-HLA-encoded single nucleotide polymorphisms (SNPs) and minor histocompatibility antigens (mHag) are also of relevance in this setting (Dickinson, *et al* 2004).

This section will focus on non-major HLA genetic influences over the outcome of allogeneic stem cell transplantation. These include cytokine gene polymorphisms (CGPs), mHags and genes thought to be involved in the response to infection and drug metabolism.

8.4.1 Cytokine gene polymorphisms

Cytokines have long been known to be critical in the initiation and promotion of acute GVHD which occurs in between 30 and 80% of recipients of HLA-identical sibling grafts, despite the use of ever more sophisticated GVHD prophylaxis. T cells also play a crucial role, as outlined in section 7.2.1, but there is a greater understanding that cytokines have the capability to induce the pathophysiological changes associated with acute GVHD as well as other complications of allogeneic transplantation such as pneumonitis and veno-occlusive disease of the liver in their own right (Holler, *et al* 2000, Reddy and

Ferrara 2003). The amount of cytokine produced or the density of receptor expression in response to a stimulus can be influenced by polymorphisms that occur within or adjacent to the regulatory sequences of cytokine/ receptor genes. These polymorphisms are inherited and so there are low or high producers of cytokines in the normal population. These inherited differences have implications for recipients and donors involved in stem cell transplantation and are likely to contribute to the clinical outcome of the transplant. Important cytokine/ receptor genes in this respect include the TNF- α gene on chromosome 6, the TNF receptor (TNFR1 and TNFR2) on chromosome 12p13, the IL-10 gene on chromosome 1, the IL-6 gene on chromosome 7p21, the IFN- γ gene on chromosome 12q24, the IL-1 gene on chromosome 2, the TGF- β 1 gene on chromosome 19q13 and the TGF β -1 receptor gene on chromosome 3p22 (Dickinson. *et al* 2004). A variety of gene polymorphisms in the form of nucleotide substitutions, repeat microsatellites and variable number tandem repeats (VNTR) have been characterised in recipients and donors and linked to the incidence and severity of acute and chronic GVHD following allogeneic transplantation. In some cases, pro-inflammatory cytokine production (such as TNF α , IL-6 and IL-1) is up-regulated and in others, anti-inflammatory cytokines (such as IL-10) are down-regulated. However, the actual effect of these genetic differences may be ameliorated by certain associated influences such as polymorphisms in neighbouring genes or the dual properties of certain cytokines, such as IL-10 which is thought to have both pro- and anti-inflammatory properties. In addition, the possession of certain HLA antigens by recipient/ donor (such as HLA-A3, which is associated with higher risk of GVHD and HLA-DR1, which is associated with a lower incidence) may override the influence of CGPs. As well as this, the gene frequencies in different ethnic groups may account for differences in the influence of certain polymorphisms in different populations. Finally, interpretation of the influence of CGPs is also affected by the myriad of conditioning and GVHD prophylaxis regimens in use, as well as the disease type being studied.

Thus whilst the rationale for including CGPs in the recipient/ donor matching profile appears to be justifiable, further study is needed before these non-HLA encoded genes can be used to dictate clinical practice.

8.4.2 Minor Histocompatibility antigens

Since HLA-identical siblings are matched for major histocompatibility antigens, the GVHD that occurs in the setting of sibling donor transplantation must arise as a result of other mismatches. mHags are peptides derived from intracellular proteins of restricted polymorphisms encoded by autosomal or Y chromosomal genes and presented by HLA molecules. Five minor histocompatibility antigens, HA-1, 2, 3, 4, and 5, that are recognized by T cells in association with the major histocompatibility antigens HLA-A1 and A2 have been previously characterised (Goulmy, *et al* 1996). A mismatch of minor histocompatibility antigen HA-1 has been found to be associated with GVHD in adult recipients of allogeneic bone marrow from HLA-identical donors, raising the possibility that prospective HA-1 typing may improve donor selection and identify recipients who are at high risk for GVHD. More recently, HLA class II restricted mHags have also been characterised and associated with GVHD (Vogt, *et al* 2002). The contribution of donor/recipient sex disparity has also been found to be relevant, in that male recipients of female sibling grafts who received allogeneic transplants for haematological malignancies were found to have the highest incidence of GVHD and the lowest rate of relapse (Randolph, *et al* 2004). This effect was attributed to mHag encoded or regulated by genes in the Y chromosome. Further evidence for this effect has been forthcoming from another study that found that B cell as well as T cell responses are relevant in the setting of female to male allogeneic transplantation, with the demonstration of antibody responses to DBY, a model H-Y minor histocompatibility antigen (mHA) in a male patient with chronic GVHD (Zorn, *et al* 2004).

Tissue expression of some mHags, such as HA-1 and HA-2 is limited to haematopoietic cells, raising the possibility of targeting them immunotherapeutically using either *ex vivo* generated mHag HA-1 and HA-2-specific cytotoxic lymphocytes or DLI. This has been done by investigators who treated HA-1 and/or HA-2 positive acute leukaemia patients who relapsed following transplant with DLI from their HA-1 and/or HA-2 negative donors (Marijt, *et al* 2003). Subsequently, HA-1- and HA-2-specific CD8⁺ cells were found in the peripheral blood of the recipients, associated with complete donor chimerism and complete remission of the leukaemia.

It is hoped that further understanding of the role of mHags and other non-HLA encoded antigens in the GVHD/ GVM effect may in the future lead to a greater predictive power and a more sophisticated risk index for GVHD, to be used alongside the existing risk factors such as gender, age and CMV status.

8.5 Depletion of specific lymphocyte subsets

Mature donor T cells that are co-transfused with the stem cell graft have both beneficial and negative effects. Their presence in the inoculum facilitates stem cell engraftment, contributes to the protection of the recipient from opportunistic infections as well as the all-important GVM effect. However, these cells also induce immune-mediated tissue damage in the recipient, resulting in GVHD. Depletion of this donor T cell population from the graft has not resulted in an improvement in overall survival, due to the resultant increase in opportunistic infections, increased graft rejection rates and higher frequencies of disease relapse (Champlin, *et al* 2000).

Thus attention has switched to strategies that might enhance the GVM effect by removing selected T cell populations with a propensity to induce GVHD from the inoculum. For example CD8-depletion may allow GVM-polarised effectors to play a more central role in the post transplant period (Champlin, *et al* 1990). An alternative approach is to use suicidal (herpes virus thymidine kinase-transduced) lymphocytes which can be killed using ganciclovir, thus ameliorating the GVHD process (Bonini, *et al* 1997). Also attempted has been the administration of cloned or selected cells with antitumour effects which may augment the GVM process without inducing GVHD, provided these effectors are devoid of anti-host activity (Rooney, *et al* 1995). *Ex vivo* depletion of alloreactive T cells is another way of minimising GVHD whilst hopefully conserving a GVM effect (Cavazzana-Calvo, *et al* 1994). Alloreactive T cells can be stimulated in a mixed lymphocyte culture (MLC) and then be removed by treating the cells with an immunotoxin that targets activation antigens such as the IL-2 receptor. The remaining cells retain reactivity against infectious agents and possibly residual malignancy, whilst having a reduced potential to induce GVHD. So far, none of these strategies has proved singularly reliable or practical enough to be widely applicable to allogeneic transplantation for myeloma or indeed other haematological malignancies.

8.5.1 CD4+CD25+ Regulatory T cells: modulators of immune tolerance and alloreactivity

A particular subset of T cells, termed regulatory T cells (T_{reg}) has become the focus of renewed interest in the setting of allogeneic transplantation (Edinger, *et al* 2003). These cells are CD4+ cells that constitutively express the α -chain of the IL-2 receptor (CD25). They play an important role in the maintenance of self-tolerance, control of autoimmunity, regulation of T cell homeostasis, modulation of the overall immune response against infectious agents and tumour cells (Bluestone and Abbas 2003) and allogeneic grafts (Waldmann, *et al* 2004). They are thymus-derived cells that preferentially express molecules such as CTLA-4, glucocorticoid-induced TNF receptor family-related gene (GITR) and forkhead/winged helix transcriptional regulator (*Foxp3*) and make up 5-10% of the T cell pool in peripheral blood and lymphoid organs (Hoffmann, *et al* 2005). This group of cells is also distinct from its CD4+CD25- counterparts in functional terms: their most characteristic features are anergy and an inherent suppressive activity. Their anergic state (impaired proliferative response to standard T cell stimuli) is not, however absolute: they require antigen-specific activation via their TCR in order to gain their suppressive function; once activated, they suppress the proliferation and cytokine production of co-cultured CD4+ and CD8+ that have been activated non-specifically by antigen.

The ability of T_{reg} cells to suppress the proliferative response of conventional CD4+ cells was first demonstrated in a mixed lymphocyte reaction, following stimulation by MHC-mismatched MNCs. These findings prompted further studies of GVHD prevention in animal models, which showed that transplantation of purified donor T_{reg} cells into completely or partially mismatched recipients did not induce signs of GVHD even when large numbers of T cells were used. As well as this, neither residual host T_{reg} cells in the setting of RIT nor donor T_{reg} within the graft interfered with stem cell engraftment, but instead facilitated immune reconstitution and the development of full donor chimerism.

8.5.2 The use of CD4+CD25+ cells in the clinical setting

The replication of results seen in the murine setting in humans has proved difficult. Human stem cell grafts vary in composition with respect to numbers and function of CD4+ and CD8+ cells and furthermore, the co-expression of CD4 and CD25 may be insufficient to reliably identify the T_{reg} subset in humans. This was a finding of Stanzani *et al*, who quantitated the co-expression of CD25 on CD4+ and CD8+ cells in 60 donor grafts infused into sibling recipients and examined the incidence of GVHD (Stanzani, *et al* 2004). They found that the incidence of GVHD was significantly higher in recipients of grafts that contained higher numbers of CD4+ cells co-expressing CD25 than those who did not. It is clear that currently accepted markers of T_{reg} cells, which are also expressed on activated T cells do not correctly identify the subset of interest. The other possibility that needs to be considered is that there may be functional differences in the identified subset, perhaps owing to the activation of these cells during the mobilisation or collection process, the current method of choice for stem cell procurement.

Some investigators have examined *Foxp3*, a key regulatory gene required for the development and function of T_{reg} cells as a potentially more-specific marker of T_{reg} cells, but to date have not found a difference in the relative *Foxp3* mRNA expression between controls and transplant recipients, regardless of GVHD status, time following transplantation or degree of lymphopenia (Meignin, *et al* 2005).

The other problem is the isolation of sufficient numbers of antigen-specific and functional T_{reg} cells to make cell-based therapy feasible. Current methods used for expansion have proved inefficient, the expanded cells lose their inhibitory potential over time, or the degree of purification needed to overcome the co-expansion of CD4+CD25- cells *ex vivo* remains beyond reach (Hoffmann, *et al* 2004). The use of rapamycin in the culture system may be a way of preferentially expanding CD4+CD25+ cells, as demonstrated more recently (Battaglia, *et al* 2005).

Finally, monitoring T_{reg} survival and function in humans post transplant will also be hampered by the lack of standardised identification markers and functional

assay systems. It also remains to be seen whether the clinical setting of HLA-matched transplantation will be rigorous enough to induce the induction of T_{reg} cell suppressive function in the same way that it has done so in mis-matched murine models.

8.6 Conclusions

Continued systematic study of these and other potential strategies is needed to further the development of the optimum immunological balance between GVHD and GVM following allogeneic stem cell transplantation. Given the complexity of the mechanisms involved, it is unlikely that a one size-fits-all approach will work, but rather patient/donor and myeloma-specific characteristics will need to be taken into account to develop a tailored approach to myeloma therapy that incorporates appropriate use of immunological and novel therapies. A more rational therapeutic aim might be to achieve long-term disease control rather than a cure *per se*.

APPENDIX 1 RESPONSE CRITERIA (EBMT 1998)

Complete Response (CR)

Requires all of the following:

- Absence of the original monoclonal paraprotein in serum and urine by routine electrophoresis and by immunofixation, maintained for a minimum of 6 weeks. The presence of oligoclonal bands consistent with oligoclonal immune reconstitution does not exclude CR.
- <5% plasma cells in a bone marrow aspirate and also on trephine bone biopsy, if biopsy is performed. If absence of monoclonal protein is sustained for 6 weeks it is not necessary to repeat the bone marrow unless the patient had non-secretory myeloma
- No increase in size or number of lytic bone lesions on radiological investigations, if performed (development of a compression fracture does not exclude response)
- Disappearance of soft tissue plasmacytomas.

Patients in whom some, but not all, the criteria for CR are fulfilled are classified as PR, providing the remaining criteria satisfy the requirements for PR.

Partial Response (PR)

Requires all of the following:

- >50% reduction in the levels of the serum monoclonal paraprotein, maintained for a minimum of 6 weeks
- Reduction in 24 hour urinary light chain excretion either by >90% or to <200mg/24 hours, maintained for a minimum of 6 weeks.
- For patients with non-secretory myeloma only: >50% reduction in plasma cells in a bone marrow aspirate and on trephine biopsy, if biopsy is performed, maintained for a minimum of 6 weeks.
- 50% reduction in the size of soft tissue plasmacytomas (by radiology or clinical examination).
- No increase in size or number of lytic bone lesion on radiological investigations, if performed (development of a compression fracture does not exclude response)

Patients in whom some, but not all, of the criteria for a PR are fulfilled are classified as MR, provided the remaining criteria satisfy the requirements for MR.

Minimal Response (MR)

Requires all of the following:

- 25-49% reduction in the level of the serum monoclonal paraprotein maintained for a minimum of 6 weeks
- 50-89% reduction in 24-hour urinary light chain excretion, which still exceeds 200mg/24 hours, maintained for a minimum of 6 weeks.

For patients with non-secretory myeloma only:

- 25-49% reduction in plasma cells in a bone marrow aspirate and on trephine biopsy, if biopsy performed, maintained for a minimum of 6 weeks
- 25-49% reduction in the size of soft tissue plasmacytomas (by radiology or clinical examination)

No increase in the size or number of lytic bone lesions, lesions on radiological investigations, if performed (development of a compression fracture does not exclude response).

No Change (NC)

- Not meeting the criteria of either minimal response or progressive disease

Plateau

- Stable values (within 25% above or below value at time response is assessed) maintained for at least 3 months

Progressive Disease (PD)

Require one or more of the following:

- >25% increase in the level of the serum monoclonal paraprotein, which must also be an absolute increase of at least 5g/L and confirmed by at least one repeated investigation.
- >25% increase in 24 hour urinary light chain excretion, which must also be an absolute increase of at least 200mg/24 hours and confirmed by at least one repeated investigation.

- >25% increase in plasma cells in a bone marrow aspirate or on a trephine biopsy, which must also be an absolute increase of at least 10%.
- Definite increase in the size of existing bone lesions or soft tissue plasmacytomas (development of a compression fracture does not exclude continued response and may not indicate progression)
- Development of hypercalcaemia (corrected serum Ca>11.5mg/dL or 2.8mmol/L) not attributable to any other cause

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