IMMUNE RECONSTITUTION POST BONE MARROW TRANSPLANTATION

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BSc (Hons) (Medicine)

Submitted in fulfilment of the requirement for the Degree of MSc (Medicine)

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2000

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DECLARATION

I, **Glenda Mary Davison**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ACKNOWLEDGEMENTS

1. Professor Novitzky for his consistent support and encouragement. I would also like to thank him for his invaluable help with the statistical analysis.

2. Valda Thomas, for the cryopreservation of cells and performing the CFU-GM assays.

3. Sr. Louise Abrahams, for organising the collection of blood samples.

4. Sebastian Gelderbloem for his support and assistance in the laboratory.

5. My Father, Mr J.S. Bondietti for proof reading the manuscript.

6. My Husband and Son for their consistent support and encouragement .

7. The Haematology "transplant team" for ensuring that each transplant procedure is a success. Without the dedication of the entire team this study would not have been possible.

8. The National Cancer Association and Medical Research Council for the research grants which supported this project.

SUMMARY

Introduction and aims

Following bone marrow transplantation, the immune system remains suppressed for a period of time and the resulting immunocompromised state can predispose the patient to a number of clinically relevant infections. Recovery of the immune system can be affected by a number of factors, which include graft versus host disease, post transplant immunosuppression and a dysfunctional thymus. T-cell depletion of the graft by the CAMPATH I group of antibodies effectively prevents the development of graft versus host disease. However, this process has also been associated with a slower immune recovery and a higher incidence of relapse. The aims of this project were therefore: -

1) To document the immune reconstitution following T-cell depleted bone marrow and peripheral blood stem cell transplantation and to compare this with the recovery following autologous grafts.
2) To document the cell surface expression of CD95 in an attempt to comment on the role played by FAS mediated apoptosis in the post transplant immune deficiency.

Methodology

Seventy eight patients, receiving bone marrow transplants between 1995 and 1999 were studied. Nineteen of these received T-cell depleted allogeneic bone marrow, thirty two received T-cell depleted allogeneic peripheral blood stem cells and the remaining twenty seven were given autologous peripheral blood stem cell grafts. Preconditioning for those patients with malignancies consisted of total body irradiation of twelve Gray, 120mg/kg cyclophosphamide and four 1,5Gray fractions of total lymphoid irradiation. Patients with aplastic anaemia were conditioned with total lymphoid irradiation, consisting of twelve fractions of 150cGray and four daily doses of 50 mg /kg cyclophosphamide. Mobilisation of stem cells in the autologous grafts was with cyclophosphamide at a daily dose of 60 mg /kg, followed by 5μg/kg of granulocyte-colony stimulating factor daily. Similarly, donors of the patients receiving allogeneic peripheral blood stem cell transplants were mobilised with 5-10 μg of granulocyte-colony stimulating factor daily for five days. Thereafter the peripheral blood stem cells were harvested using a Cobe Spectra. Prior to infusion, 2,5 μg, per 10^6 mononuclear cells, of CAMPATH 1 was used *in vitro* to T-cell deplete the allogeneic grafts. No post transplant immunosuppression was administered. The various lymphoid populations were studied at one, two, three, six, twelve and eighteen months post infusion. Using direct immunofluorescent techniques the following surface antigens were studied: CD3, CD2, CD4, CD8, CD16, CD56 and CD19. In addition, the cell surface expression of CD45RO, CD45RA

and CD95 were investigated. Analysis took place on a Becton Dickinson FACS CALIBUR flow cytometer. Serum immunoglobulin levels were studied using standard nephelometry techniques.

Results

Natural killer cells achieved normal values, in all three modes of transplant, by one month. CD19+ B-cells steadily increased at the same rate in all three groups, and reached the normal range at twelve months post infusion. Serum IgG and IgM had recovered by one month, however IgA levels remained below the median of the normal controls throughout the study period. The total T-cell population, as measured by the antigens CD2 and CD3, recovered quickly in the autologous group, achieving normal values by one month. In contrast however, both allogeneic groups took longer, and attained normal values only at twelve months. The CD4+ helper T-cell subset was particularly slow to recover and remained abnormally low throughout. CD8+ cytotoxic T-cells recovered quickly, with many of the autologous patients having significantly increased numbers. These however, slowly decreased, reaching the normal range by eighteen months. This pattern of T-cell recovery gave rise to significantly decreased CD4:CD8 ratios in all three transplant groups. These remained abnormal throughout the study period. Analysis of the percentage of CD4+ helper T-cells expressing a naïve (CD45RA) or memory (CD45RO) phenotype revealed that, in all transplant types the majority of recovering helper T-cells expressed the antigen CD45RO. In addition, there was a significantly increased percentage of T-cells expressing FAS/CD95 as compared to that of normal adult controls.

Discussion and conclusion

We conclude, that CAMPATH mediated T-cell depletion is effective in preventing graft versus host disease. However, although both T-cell depleted allogeneic transplants achieved adequate haematological recovery and none have developed >grade II graft versus host disease, both groups took longer to normalise their T-cell populations when compared to the autologous transplant patients. The CD4+ helper subset was particularly affected and remained abnormal throughout. This prolonged recovery was probably due to a dysfunctional thymus and the intense T-cell depletion process, which removed the majority of residual T-cell progenitors prior to infusion. In addition, the increased expression of CD95 suggested a role for FAS mediated apoptosis in the prolonged immunodeficiency. Therapies, which include the re-infusion of graded numbers of T-cells, in order to replace the lost T-cell progenitors, and the administration of cytokines such as IL-7 need to be investigated.

PUBLICATIONS

Immune Reconstitution After Allogeneic Bone marrow Transplantation Depleted of T Cells

Davison, G. M., Novitzky, N., Kline, A., Thomas, V., Abrahams, L., Hale, G. & Waldmann, H.

Transplantation, Vol. 69, No. 7, April 2000

PRESENTATIONS

Immune Reconstitution post T-cell Depleted Allo-Geneic Bone Marrow transplantation

G.M. Davison, N. Novitzky, A. Kline, V. Thomas, L. Abrahams, G.Hale and H.Waldmann.

ISH-EHA Combined Haematology Congress, Amsterdam, The Netherlands, 4 –8 July 1998

Immune Reconstitution Following Autologous and Allogeneic (Allo) T-cell Depleted (TcD) Haematopoeitic Stem Cell Transplantation

G.M. Davison, N.Novitzky, V.Thomas, and I.Abrahams

XVth Meeting of the International Society of Haematology (African and European Division), Durban, South Africa, 18-23 September 1999

TABLE OF CONTENTS

ABBREVIATIONS

AICD	Activation induced Cell death
CD	Cluster of differentiation
CMV	Cytomegalovirus
FITC	Fluorescein isothiocynate
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
HIV	Human Immunodeficiency Virus
IL-1	Interleukin one
IL-2	Interleukin two
IL-3	Interleukin three
IL-6	Interleukin six
IL-7	Interleukin seven

IL-11	Interleukin eleven
IFN-γ	Interferon gamma
IMDM	Iscove's modified Dulbecco's culture medium
MHC	Major histocompatibility complex
NS	Not Significant
PBSCT	Peripheral blood stem cell transplantation
Pe	Phycoerythrin
Per-CP	Peridinin chlorophyll
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
TBI	Total body irradiation
TLI	Total lymphoid irradiation
TNF	Tumour necrosis factor

LIST OF TABLES AND ILLUSTRATIONS

1.0 INTRODUCTION AND AIMS

Bone marrow transplantation has become a standard form of therapy for many haematological malignancies, aplastic anaemia and congenital anaemia. The success of bone marrow transplantation is however threatened by infections, graft versus host disease (GVHD) and relapse of the original malignancy.

All of these are caused in part by a compromised immune system. Irrespective of the type of transplant, which includes autologous or allogeneic, following transplantation, all arms of the immune system remain suppressed for a period of time. In allogeneic bone marrow transplantation, the haemopoietic stem cells and lymphocytes derived from the donor are thought to reconstitute the immune system while in autologous transplantation the patients own cells are responsible for immune recovery. Immune recovery post bone marrow transplantation can be affected by a number of factors, which include GVHD, post transplant immunosuppression for both the prevention and treatment of GVHD and a dysfunctional thymus. The thymus, an essential organ involved in the development of T-cells can become dysfunctional due to a number of factors which include the natural ageing process, GVHD, previous chemotherapy, the intense preconditioning process and post transplant immunosuppression.

T-cell depletion of the graft effectively prevents the development of GVHD. The CAMPATH 1 group of antibodies are directed against the CD52 antigen and have been shown to deplete and kill the majority of T and B-cells in the presence of human complement (Hale et al, 1983). Clinical studies have shown that this effective method of T-cell depletion is paralleled by a clinically relevant decline in the incidence and severity of GVHD (Jacobs et al, 1994). However, T-cell depletion has also been associated with a slower immune recovery and a higher incidence of relapse post transplantation.

The immune recovery post allogeneic and autologous bone marrow transplantation has been previously studied (Small et al, 1990; Storek et al, 1993; Storek et al, 1995). Many of these investigators have demonstrated similarities between early neonatal immune ontogeny and the recovery of the immune system post bone marrow transplantation. Although this appears to be true for B-cell recovery, distinct differences have been demonstrated between early T-cell development and T-cell reconstitution post transplantation. Furthermore, the immune recovery post T-cell depletion is less clear due to the differing methodologies used to deplete the graft of T-cells.

Therefore, although immune reconstitution post bone marrow transplantation has been previously studied, many areas remain unclear. For instance, the true effect of T-cell depletion on immune recovery needs further study and, in addition, the mechanisms causing this prolonged immune deficiency require proper investigation.

The aims of this project were therefore to: -

1. Document the immune reconstitution following allogeneic T-cell depleted peripheral blood stem cell (PBSCT) and bone marrow transplants and to compare this with autologous grafts which were not T-cell depleted.

2. Document the cell surface expression of CD95/FAS, during the post transplant period, in an attempt to comment on the role played by FAS mediated apoptosis in the immune deficiency.

2.0 DISCUSSION OF THE LITERATURE

2.1 FACTORS INFLUENCING IMMUNE RECOVERY POST TRANSPLANTATION

2.1.1 The role of the thymus in T-cell recovery

The thymus is crucial to the development and maturation of T-cells. It is also well established that the generation of new, naïve (CD45RA+) T-lymphocytes depends on the function of the thymus (Mackall et al, 1993; Heitger et al, 1997).

T-cell precursors are produced in the bone marrow and home to the thymus where education takes place. Within the thymus the immature lymphoblastic T-cells proliferate. Firstly they undergo T-cell receptor gene rearrangement and then begin to express low levels of both CD8 and CD4. Secondly, the maturing cells undergo positive selection where those unable to recognise self- major histocompatibility complex (MHC) molecules will undergo apoptosis. Thereafter, the T-cells begin to express high levels of CD3 and are then subjected to a process of negative selection where they are presented to self- antigen. Those not recognising the self- antigen are released into the peripheral blood (Roitt, 1994). This process takes place within the thymic cortex and at the interface between the cortex and medulla. The thymic cortex comprises of a number of cells other than thymocytes. These include epithelial cells, macrophages, dendritic cells, fibroblasts and the extracellular matrix. The interaction between immature thymocytes with these cells is essential for the maturation and development of self tolerant T-cells (Weissman and Cooper, 1993).

It is well known that thymic atrophy begins soon after puberty and continues throughout life leading to a decrease in the production of new T-cells. (Fig 1) In addition, bone marrow transplant patients undergo an intensive pre-conditioning process, with the consequent effacement of all lymphoid tissues,

and therefore, following graft infusion, very little thymic tissue is available for T-cell development.

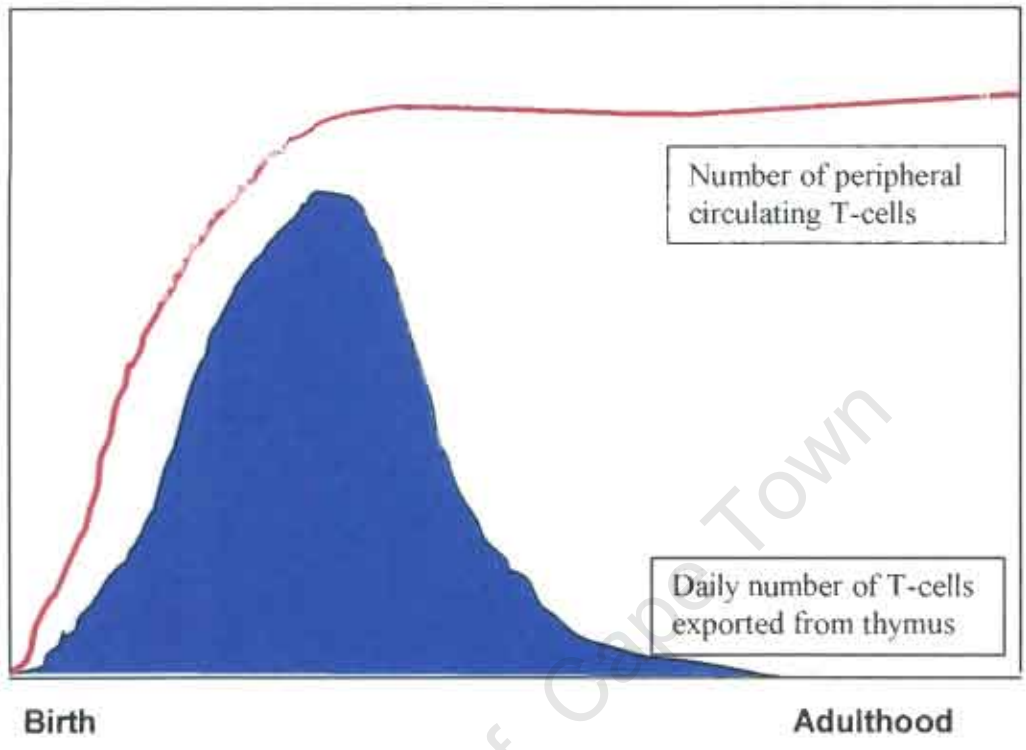


Fig 1: The production of new T-cells declines, as the thymus ceases to function, however, the number of circulating T-cells remains static. (Adapted from Immunobiology, Janeway and Travers, 3rd edition, Blackwell Scientific, 1994, 6.5)

In 1993 Crystal L. Mackall et al studied T-cell regeneration after bone marrow transplantation by comparing the T-cell reconstitution between thymectomized and thymus bearing mice. She concluded that the T-cells generated in the mice lacking a thymus were of the "memory" type, expressing low molecular weight CD45 isoforms (CD45RO) and the adhesion molecule CD44. These cells were probably generated from peripheral T-cell progenitors through a process of blastic transformation. Mice possessing a functioning thymus however, generated only "naïve" CD45RA+ /CD44- T-cells. It was concluded from these results that T-cell regeneration post transplantation, in patients lacking thymic

activity, is via peripheral memory T-cells (Mackall et al, 1993).

These investigations were again confirmed in 1997 when the essential role of the thymus was demonstrated in the reconstitution of naïve CD45RA+ helper T-cells in a thymectomised patient. Even after twenty four months, the patient was unable to produce CD45RA+/CD4+ helper T-cells. It was again theorised from this case study that the CD4+ T-cell population is reconstituted from peripheral memory T-cells (CD45RO+) (Heitger et al, 1997).

Studies in 1995 examined the reconstitution of T-cells post transplantation and compared it to neonates and normal adults. Important differences such as the increased number of CD4+ T-cells present in neonates compared to decreased numbers post transplantation were noted. It was concluded, that T-cell reconstitution after bone marrow transplantation in adults, does not resemble the development of T-cells in neonates. In addition, it was discovered that younger patients appear to recover their naïve CD4+ T-cell population quicker than older individuals. This was probably due to the described effects of residual thymic tissue. The development of naïve CD8+ cytotoxic T-cells, however, does not depend on age and therefore it was postulated that the CD8+ T-cell population is probably produced extrathymically in patients post bone marrow transplantation. This leads to these cells returning to normal numbers far sooner than their CD4 counter parts (Storek et al, 1995).

From the above evidence it can be concluded that the thymus is extremely important for the generation of new "naïve" T-cells. In it's absence residual peripheral T-cell progenitors play an important role in the reconstitution of T-cells, particularly CD4+ helper cells.

2.1.2 Graft versus host disease (GVHD)

GVHD remains one of the major problems, post bone marrow transplantation, and can affect fifty to seventy percent of patients receiving fully matched HLA grafts (Hale et al, 1983). GVHD is initiated by donor T-cells that recognise foreign major and minor histocompatibility (MHC) antigens of the host, leading to an inflammatory response and the inappropriate production of cytokines. This process eventually causes damage to host tissues, which include the skin, liver, lung, gastrointestinal tract and the immune system (Krenger and Ferrara, 1996).

Werner Krenger and James Ferrara have hypothesised that GVHD occurs in a three- step process, that begins with the intensive radiation and chemotherapy that patients receive during preconditioning. This results in damage to host tissues, the activation of macrophages and phagocytes, release of TNFα and IL-1 and consequently the further up-regulation of host MHC molecules. In the second step donor T-cells become activated. In acute GVHD stimulated T-helper 1 cells secrete IL-2 and IFNγ. These cytokines in turn activate cytotoxic T-cells, natural killer cells and mononuclear phagocytes. The third phase is characterised by nitric oxide production and the extensive damage to tissue and organs by activated macrophages. If, however, a T-helper 2 response is initiated the production of inflammatory cytokines is inhibited and a more chronic form of GVHD is observed (Krenger and Ferrara, 1996).

Acute GVHD results in damage to the skin, liver and intestine. The first organ to become affected is the skin. This begins as a pruritic maculopapular rash, which begins on the palms, soles and ears and eventually progresses to total body erythroderma. Gastrointestinal and liver damage occurs later. Gastrointestinal manifestations includes symptoms of anorexia, nausea, diarrhoea, abdominal pain and paralytic ileus, while liver dysfunction includes hyperbilirubinaemia and increased serum alkaline phosphatase and aminotransferase values (Ferrara and Deeg, 1991).

In order to quantify the severity of GVHD grading systems which include the clinical changes in the skin, intestinal tract and liver have been developed. GVHD with mild skin involvement is usually graded as I while patients with multi-organ involvement are graded as IV (Thomas et al, 1975).

Mild GVHD (grade I or II) is associated with little morbidity and no mortality, while higher grades (grade III and IV) are associated with high morbidity and mortality. Patients with grade IV GVHD usually have a mortality rate of one hundred percent (Ferrara and Deeg, 1991).

Chronic GVHD has been defined as a syndrome presenting more than one hundred days after transplantation. The incidence ranges from thirty to sixty percent and may be limited or extensive. Eighty percent of patients have skin changes, which include papulosquamous dermatitis, plaques, desquamation, dyspigmentation and vitiligo. Chronic cholestatic liver disease also occurs in eighty percent of patients, however it rarely progresses to cirrhosis. Unlike acute GVHD, gastrointestinal involvement is infrequent (Ferrara et al, 1991).

Both the chronic and acute form of GVHD, result in lymphoid death, with a consequent delay in immune recovery. This, of course, may predispose the patient to serious infections post transplantation (Lum, 1987).

Therefore, to control and treat GVHD immunosuppressive drugs such as cyclosporin A, methotrexate and corticosteroids have been prescribed. (Hale et al, 1998) However, these drugs can further downregulate the immune system and therefore alternative methods to prevent GVHD have been sought. Moreover, despite GVHD prophylaxis, thirty percent will still develop this syndrome.

Various methods of removing the T-cells from the graft have been attempted in order to prevent the development of GVHD. Agglutination by soy bean lectin

followed by sheep rosette formation has been used with some success (Reisner et al, 1981). The major disadvantage of this method is that it is cumbersome and can lead to the loss of stem cells (Hale et al, 1983).

Other methods include the anti T-cell antibody OKT3 which has been utilised both in solid organ and bone marrow transplantation and antibodies to the cytokine IL-2 and the αβ T-cell receptor. (Prentice et al, 1982; Herve et al, 1990; Neipp et al, 1999) Attempts have also been made to polarise donor T-cells towards the T-helper 2 phenotype and thereby prevent acute GVHD (Krenger and Ferrara, 1996). More recently the selective depletion of CD4+ T-cells prior to infusion has also been investigated (Nagler et al, 1998).

A further interesting concept was described by Xue-Zhong Yu et al, who proposed the use of agents blocking the function of the antigen CD28. CD28 is a co-stimulatory molecule present on T-cells and binds to B7-1 and B7-2, which are present on antigen presenting cells. The inhibition of this interaction could serve to downregulate T-cell activation and therefore decrease the severity of GVHD (Yu et al, 1998).

One of the most successful methods of T-cell depletion is the use of the CAMPATH 1 group of antibodies. The CAMPATH 1 group of antibodies are directed against the CD52 antigen which is expressed on the majority of human lymphocytes. CAMPATH 1, in the presence of human complement, has been shown to kill and deplete the majority of T-cells within the graft (Hale et al, 1983; Hale et al, 1998). This method of T-cell depletion has also been shown to prevent effectively the onset of clinical GVHD post transplantation (Jacobs et al, 1994). However, although T-cell depletion can prevent GVHD it has also been associated with rejection of the graft, relapse of the original malignancy and delayed immune recovery (Lowdell et al,1998; Hale and Waldman, 1994).

The high-dose chemotherapy and radiation contained within the pre-conditioning regimen is unlikely to kill all residual tumour cells. Therefore it is generally accepted that a second component associated with the graft itself must contribute to the anti-leukaemic effect seen in allogeneic bone marrow transplantation. This component, is mediated by immunocompetent donor lymphocytes contained within the graft, and is known as the graft versus leukaemic (GVL) effect (Porter and Antin, 1999).

Clinical evidence to support this include, the fact that donor marrow depleted of T-cells have a higher incidence of relapse, and more recently, donor lymphocyte infusions given to patients with relapsed chronic myeloid leukaemia have resulted in complete remissions (Lowdell et al, 1998, Dazzi and Goldman 1998).

Therefore, the balance between the prevention of GVHD, versus the preservation of the graft versus leukaemia (GVL) effect, needs further study, particularly in patients receiving transplants depleted of T-cells.

2.1.3 Mode of transplant

The type of transplant received can also influence the rate of immune reconstitution.

Autologous bone marrow transplantation describes a procedure whereby bone marrow or peripheral blood stem cells, from the patient, are harvested during the first complete remission. The harvest is then cryopreserved in liquid nitrogen and, after a myeloablative pre- conditioning regimen, re-infused. Autologous transplantation has many advantages. These include the absence of GVHD and the absence of the need to find a HLA – identical family donor. The major disadvantage is the risk of re-infusing residual leukaemic cells and therefore increasing the incidence of relapse post infusion. Because of this,

many researchers have investigated techniques of purging the graft of residual malignant cells (Gorin, 1998).

Patients receiving autologous transplants are reported to reconstitute their immune systems far quicker than those receiving both T-cell depleted and non T-cell depleted allogeneic grafts. The pattern of immune recovery, with a decreased CD4:CD8 ratio, is however similar (Roberts et al 1993; Verma and Mazumder, 1993). It is postulated that the increased rate of recovery is probably due to the fact that these individuals do not receive any immunosuppressive therapy and do not develop GVHD.

Allogeneic transplantation can be affected by the development of GVHD, which can further delay immune recovery. To prevent GVHD, T-cell depletion of the graft with a variety of methods have been attempted. The effect of T-cell depletion on immune recovery is however still controversial. Carolyn Keever et al in 1989 compared the immune reconstitution of patients receiving allogeneic transplants depleted of T-cells with those who had received conventional unmanipulated grafts. However, although prolonged functional deficiencies were discovered in the T-cell depleted group, she concluded that there was no difference in the rate or degree of immune recovery. Furthermore, there was no significant difference in the number of clinically relevant infections between the T-cell depleted and non T-cell depleted patients (Keever et al, 1989).

In contrast, others have published findings which suggest a far slower lymphoid recovery in T-cell depleted transplants. This is particularly relevant in the CD4+ T-cell subset (Lowdell et al, 1998). A possible reason for these discrepancies is the differing methods used for the T-cell depletion process.

PBSCT is rapidly becoming an attractive substitute to the traditional bone marrow transplant. Clinical results have shown that patients have an earlier haemopoietic response, lower morbidity and, in addition, there is increased

safety for the donor (Korbling, 1997;Lie and Bik To, 1997).

Haemopoietic stem cells circulate in low numbers within the peripheral blood. Using various methods in order to mobilize them into the circulation these early progenitor cells can be dramatically increased.

For example, during the recovery phase after intensive chemotherapy there is a fifty fold or more increase in circulating stem cells. The addition of growth factors such as G-CSF can increase the mobilization a further four to six times that of chemotherapy alone (Lie and Bik To, 1997).

The timing of the collection, after the commencement of growth factors, is however very important as, after reaching a peak at day five or seven, the progenitor cell level in the peripheral blood begins to fall. The PBSC collection is usually performed using a continuous flow apheresis machine and the number of CD34+ stem cells being collected is monitored throughout the procedure. The minimum number of stem cells needed for satisfactory engraftment is debatable. However, it is generally agreed that a count of 1-2 x 10^6/kg is adequate. The number of early progenitors can also be measured using the CFU-GM assay. The minimum number of CFU-GM's needed for engraftment is between 30 and 40 x10^4/kg (Lie and Bik To, 1997).

Allogeneic PBSC harvests contain increased numbers of T-cells compared to bone marrow harvests. This fact has raised fears of an increase in the incidence of GVHD. However, clinical evidence has shown that the incidence of GVHD has not been increased (Schmitz et al, 1996).

In addition, studies examining the immune recovery following intact PBSC grafts have also been encouraging. In autologous stem cell grafts, recovery of T-cells, B-cells and natural killer cells have all been enhanced and sustained. However, the pattern of recovery, with a decreased CD4:CD8 ratio, remained

the same (Rosillo et al, 1996).

Likewise, patients receiving allogeneic PBSCT have also been reported to have a more rapidly recovering immune system. In addition, proliferative responses to phytohaemagglutin (PHA) and other mitogens have been increased. These results are possibly explained by the increased numbers of lymphoid cells present in the graft compared to traditional bone marrow transplants (Ottinger et al, 1996 and Roberts et al, 1993).

However, not all investigators have reported similar results. Ashitara, in 1994, concluded that, apart from the B-cell population, immune recovery was extremely slow post PBSCT. Shenoy, after studying thirty patients, concluded that lymphoid recovery was seriously delayed. However, seventy two percent of these patients developed chronic GVHD which could have influenced the results (Ashihara et al,1994; Shenoy et al, 1999).

Therefore the true effect of peripheral blood stem cell transplantation on immune reconstitution still remains unclear and requires further investigation. In addition, the effects of T-cell depletion also need to be evaluated.

To summarise, it is clear from the above that the type of transplant received plays an important role in the rate of immune recovery post transplantation. Furthermore, the effects of T-cell depletion on this process remain controversial and further investigation is needed before conclusions can be made.

2.2 IMMUNE RECONSTITUTION POST BONE MARROW TRANSPLANTATION: IS IT A RECAPITULATION OF EARLY IMMUNE ONTOGENY?

Many investigators have detected similarities between immune reconstitution, post bone marrow transplantation, and that which occurs in early life (Small et al, 1990; Storek et al, 1993). It is therefore important to examine the current knowledge of early B and T-cell development and compare this with immune recovery post bone marrow transplantation.

2.2.1 Early B-cell ontogeny

Studies examining the lymphoid populations of normal children have revealed an increase in both the number of lymphocytes and consequently B-cells (Caldwell et al, 1991). Phenotypically, seventy five to ninety percent of these early B-cells are reported to express CD1c, CD38, CD5 and CD23. The CD5+ B-cells normally decrease with age and become only a small part of the B-cell repertoire in older children and adults (Small et al, 1990).

CD5+ B-lymphocytes are thought to represent a separate B-cell lineage and are synthesised within the liver (Roitt, 1994). They are a unique subset in the lymphoid repertoire in that they express surface IgM with little or no IgD. The CD5 molecule binds to another B-cell surface antigen namely CD72 which promotes B-cell - B-cell interaction. They do not require T-cell help and are thought to play a role in the primary immune response. The antibodies they produce are usually polyspecific and tend to bind common bacterial poly sacharides (Janeway and Travers, 1994).

Compared to their mature lymphoid counterparts, these early neonatal B-cells have been shown to be functionally deficient. Studies have demonstrated that they fail to produce IgG or IgA, even after activation and the provision of

stimulatory cytokines. The dominating immunoglobulin produced is IgM and yet even this is below normal adult levels. Normal immunoglobulin production takes approximately two years to achieve and follows a process in which IgG1 and IgG3 producing plasma cells are acquired early and the development of IgG2 and IgG4 much later (Andersson et al, 1981).

2.2.2 Recovery of B-cells post transplantation

Infections, characteristic of immunoglobulin deficiency, such as pneumococcus and other gram-positive bacteria are common after bone marrow transplantation (Velardi et al, 1988). Several investigators have documented the recovery of B-cells post transplantation and have found that within one to two months B-cell precursors begin appearing in the bone marrow. This is followed by the appearance of mature B-cells in the peripheral blood by four to eight months. This pattern was similar in both allogeneic and autologous transplantation (Leitenberg et al, 1994; Verma and Mazumder, 1993).

Analysis of the phenotype of the bone marrow precursors have revealed that the majority of the developing cells express CD10, CD20 and CD19. In the peripheral blood mature B-cells begin to appear within one to two months and the majority appear to express the antigens CD5 and CD23 (Leittenberg et al, 1994). As has been previously described this phenotypic pattern is characteristic during early life and therefore supports the theory that B-cell reconstitution follows a pattern similar to that of normal B-cell ontogeny. Interestingly, CD5+ B lymphocytes have been associated with autoimmune disorders and have been reported as the predominant B-cell in moth-eaten mice, a strain, which has widespread autoimmunity (I.Roitt, 1994). This fact could be important in the bone marrow transplant setting as Sherer in 1998 reported a number of incidences where auto immune diseases have occurred post transplantation (Sherer and Shoenfeld, 1998).

Another study examining the phenotype of recovering B-cells examined the expression of CD21, a mature B-cell marker. CD 21 is an ideal antigen for distinguishing foetal from adult B-lymphocytes as it is absent from foetal cells but present in older children and normal adults. The results indicated that although, post transplantation, the recovering B-cells had detectable CD21, expression was low and more in keeping with that seen in early B-cell ontogeny (Velardi et al, 1988).

Storek et al investigated the effect of GVHD on B-cell recovery when he compared patients with chronic GVHD to those who did not develop this complication. He concluded that B-cell recovery in patients without any evidence of GVHD parallels ontogeny. However, this is not the case when GVHD is present. B-cell expansion is limited and the resulting immunodeficiency is similar to patients that have X-linked hypogammaglobinaemia (Storek et al, 1993).

Proliferation of B-cells is however not paralleled by the normalisation of B-cell function. IgM levels recover within the first two months. However, IgG and IgA can remain below the normal range for up to three years post transplantation (Lum, 1987; Storek and Saxon, 1992). This again is similar to the pattern seen following birth.

In addition, numerous studies have shown that in the first three months, post transplantation, B-cells from patients do not produce antibody *in vitro* after stimulation with various activators and cytokines from normal T-cells (Ringden et al, 1979). By one year, antibody responses to recall antigens have returned to normal. In many cases however, this is only a primary type response rather than a secondary one (Ljungman et al, 1990).

Patients with chronic GVHD continue to have abnormal immunoglobulin synthesis and in addition to having impaired primary responses, fail to switch

from IgM production to IgG (Witherspoon et al, 1981; Witherspoon et al, 1982).

This pattern of immunoglobulin production is similar to that seen in early neonatal development and again supports the theory that B-cell development post bone marrow transplantation is a recapitulation of that seen in early life.

It is generally concluded therefore that there are many similarities between B-cell reconstitution post unfractionated bone marrow transplantation and early neonatal development. However, although B-cell numbers, in the peripheral blood recover within four to eight months post transplantation, functionally they can remain abnormal for many years.

2.2.3 Early T-cell ontogeny

The thymic stroma emerge early in embryonic development from the endodermal and ectodermal layers. The ectodermal layers give rise to epithelial cells in the thymic cortex while the endodermal layers develop into the epithelial component of the medulla. The human thymus is fully developed before birth and consists of both cortical and medullary tissue which together form the thymic stroma (Jane way and Travers, 1994).

T-cell production in the thymus increases significantly after birth, however, once puberty is reached, the thymus begins to shrink and convolute (Figure 1). The development of new T-cells declines, however the number of circulating T-cells remains static, suggesting that mature T-cells have the ability of replicating (Janeway and Travers, 1994).

The phenotype of the T-cells present in early neonates and childhood has been previously studied. Reports indicate that the majority of the T-cells display a naïve phenotype (CD45RA+) and that very few memory T-cells (CD45RO+) are present (Kotylo et al, 1990;Storek et al, 1995).

The initial dominant T- cells, in the embryo, express the γδ T-cell receptor and appears to lack diversity. Soon after birth, T-cells displaying the αβ receptor begin to appear and soon become dominant. The γδ T-cells, which are still being produced, are more heterogeneous and are found in the peripheral lymphoid tissues (Janeway and Travers, 1994).

γδ T-cells are a distinct lineage. They express surface CD3, however, they are not MHC restricted and therefore do not express either CD4 or CD8. γδ T-cells arise from the same early progenitors, as do their αβ counterparts, however after gene rearrangement has taken place they develop along a separate lineage. Interestingly, it has been shown in nude mice that γδ T-cells can develop in the absence of a functioning thymus. The function of these cells is not known, however they lack receptor diversity and as they are the first T-cells to be produced during embryonic development, it is thought that they play a role in a non-adaptive early line of host defence (Janeway and Travers, 1994).

CD4+ helper T-cells are usually normal or even slightly increased in early childhood giving rise to normal CD4:CD8 ratios of between 1.61 and 2.61 (Kotylo et al,1990; Landesberg et al,1988). Of interest is the increased appearance of CD4+/CD8+ or dual positive T-cells in the peripheral blood (Storek et al, 1992). These cells are usually present at the cortical stage of thymic development and probably indicate the appearance of immature T-cells in the peripheral blood.

The majority of neonatal T-lymphocytes appear to be functionally deficient. Studies examining the production of cytokines by cord blood T-cells have revealed that even after stimulation with phytohaemagglutin (PHA) and phorbol myristate acetate (PMA) the cells produce decreased levels of IL-2 and IL-6. In addition they are unable to provide the necessary help to B-cells in order for them to produce immunoglobulin. (Watson et al, 1990)

From the above information it is clear that the T-cells present in the early neonate differ from the normal adult. However does T-cell recovery post bone marrow transplantation parallel that which is seen in early life?

2.2.4 T-cell recovery post bone marrow transplantation

T-cells begin to reconstitute the peripheral blood in all allogeneic bone marrow transplant patients within three months. However, the pattern of subset reconstitution is abnormal. Unlike neonatal development, CD4+ helper T cells recover extremely slowly in comparison to their CD8+ counterparts. This pattern of recovery is similar in both autologous and allogeneic transplantation although allogeneic transplants take far longer (Roberts et al, 1993; Keever et al, 1989).

This pattern of recovery results in an inverse CD4:CD8 ratio, which can persist for over one year post transplantation. The abnormal ratio is thought to be the reason for the increased number of cell- mediated type of infections, which occur in the early period post transplantation. These include Cytomegalovirus (CMV), varicella zoster, Epstein Barr and Pneumocystis Carinii infections.

In vitro studies have examined both the CD4+ and CD8+ T-cells more closely and have discovered that post transplantation the number of naïve helper T-cells (CD4+ CD45RA+) are significantly lower than in normal adults or in neonates. In contrast, the percentage of CD4+ T-cells expressing the memory phenotype (CD45RO+), were increased (Storek et al, 1995).

Although CD8+ cytotoxic T-cells follow a similar pattern, levels of naïve CD8+ T-cells return to normal by the end of the first year post transplantation. This suggests, that CD8+ cells may follow a different proliferation pathway and unlike their CD4 counterparts are probably produced extrathymically (Cooley et al, 1991; Storek et al, 1995).

This pattern is in contrast to the early neonate, which possesses a fully functioning thymus, and in which the majority of the T-cells are naïve and express CD45RA.

Recent experiments in mice have demonstrated that the administration of IL-7 enhances the reconstitution of both T-cell numbers and function. IL-7 is secreted by both thymic and marrow stroma and is also known to have both proliferative and differentiative effects on T-lymphoid cells. This suggests that the lack of IL-7 could be one reason for the delayed recovery of T-cells in human bone marrow transplants. Further studies in humans need to be undertaken to evaluate this properly (Bolotin et al, 1996).

In older individuals, the majority of T-cells produced post transplantation surprisingly lack CD28 expression. This also differs from the neonate (Storek et al, 1995). It is postulated that this could enhance not only T-cell anergy but suppresser activity as well. Of interest is that when telomere length is lost and reaches 5kb, the cells are incapable of undergoing antigen stimulated proliferation and are more likely to undergo apoptosis. In addition, a correlation has been shown between reduced telomere length and lack of CD28 expression on T-cells (Parkman and Weinberg, 1997; De Boer and Noest, 1998). Recently, telomere shortening has been observed in patients post bone marrow transplantation. It was discovered in this study that both the neutrophils and T-cells had significant shortened telomeres (Wynn et al, 1999).

Research examining the ability of T-cells, and in particular CD4+ helper T-cells, to promote B-cell immunoglobulin production have shown that these cells failed to help and in fact in many cases suppressed this function (Lum, 1887, Witherspoon et al, 1981). Could this not be related to the lack of CD28 expression, which is essential for interaction with B-cells?

Another molecule responsible for the interaction between T and B cells is the CD40-ligand. This molecule is expressed on CD4+ T-lymphocytes and a small proportion of CD8+ T-cells. The CD40-ligand binds to CD40 which is present on B-cells and other antigen presenting cells. Interactions between these two molecules serve as a co-stimulatory signal to T-cells. In addition, B-cell proliferation, immunoglobulin production, class switching and the generation of memory B-cells are all dependant on interactions between these two molecules. The CD40-ligand also stimulates B-cells to up-regulate molecules such as B7-1 and B7-2 which bind to CD28. (Blazar et al, 1997) Therefore, inhibition of CD40 or it's ligand could also inhibit the function of CD4+ T-cells and the production of immunoglobulin.

The importance of the CD40-CD40 ligand pathway in the proliferation of CD4+ T-cells was demonstrated by a study which inhibited the expansion and function of these cells after treatment with an anti-CD40-ligand antibody. It was concluded from this study that the blocking of this pathway could help to prevent GVHD and also anti-donor-mediated-marrow allograft rejection which is mediated by CD4+T-lymphocytes (Blazar et al, 1997).

In addition, experiments with T-cells post transplantation revealed that they were unable to suppress B-cells infected with the Epstein Barr virus. This could explain the development of B-cell lymphomas in some transplant patients (Okos et al, 1983).

Further studies examining the proliferative responses of T-cells to different mitogens have revealed that these are abnormal in the early stages post transplantation. In conventional, unmanipulated allogeneic and autologous transplants these return to normal by six months to one and half years (Keever et al, 1989: Verma and Mazumder, 1993).

The increased or preferential development of $\gamma\delta$ T-cells in patients post bone

marrow transplantation has also been reported by a number of investigators (Villers et al, 1994; Bomberger et al, 1998). In normal adults the percentage of peripheral blood T-cells, which make up the γδ population is between 0,5 and 15. However, they appear to predominate in the intestinal epithelium and the skin (Roitt, 1994).

It is hypothesised that the origin of the increased γδ T-cells post transplantation is from an extrathymic site or an expansion of residual cells within the intestinal epithelium (Bomberger et al, 1998).

The exact role these cells play within the post transplant patient remains debatable and still requires investigation. However, it is thought that they could help to prevent the relapse of the original malignancy (Bomberger et al, 1998).

Of interest is the similarities between γδ T-cells and CD5+ B-cells. Both lymphocytes have a limited range of specificities, play a role in the non-adaptive immune response and predominate early in life. As both of these cells are reported to be increased in the early period post bone marrow transplantation, it can be hypothesised that they play a role in protecting the body while immune reconstitution takes place (Roitt, 1994).

It is clear therefore that although certain similarities exist between early T-cell ontogeny and T-cell recovery post bone marrow transplantation, many differences are also evident. Firstly, early neonates have a well -developed and functioning thymus while the majority of transplant patients do not. Secondly, CD4+ helper T-cells are often normal or increased in young infants and children while they can take up to one year or even longer to recover in the transplanted patient. Of interest however is the fact that dual positive (CD4+/CD8+) cells appear in both instances. Finally, the predominant T-cell in the neonate is of the naïve phenotype while, because of a dysfunctional thymus, the predominant cell in the transplant patient is of the memory type.

2.2.5 Natural Killer Cells

Natural killer cells are defined as large granular lymphocytes, which kill virally infected cells. Although studies have shown that they share a common stem cell with T-cells, they are not thymus dependent and develop within the bone marrow (Wang et al, 1997). In addition, they are not MHC restricted and are usually CD3 negative. Natural killer cells express CD56 and CD16 and also play a role in tumour surveillance (Janeway and Travers, 1994).

Studies performed on placental cord blood have shown that natural killer cells are present in numbers, which are similar to normal adults (Kotylo et al, 1990). Natural killer cells are also reported to be the first lymphoid cells to recover after bone marrow transplantation (Keever et al, 1989; Parrado et al, 1997; Roberts et al, 1993; Ashihara et al, 1994). Cells expressing CD56 and CD16 appear to peak at two to three months and then stabilise within the normal range. The majority of natural killer cells in the post transplant period exhibit a normal phenotype namely CD3-, CD56+, CD16+, however in some patients a different phenotype (CD3-, CD56+, CD16-) is displayed. This subtype, of natural killer cells has less natural killer activity and antibody mediated cellular cytotoxity (Verma and Mazumder, 1993). Studies examining the function of natural killer cells against K562 cell targets have revealed that functionally these cells return to normal within one month, post transplantation (Livnat et al, 1980).

It is thought that the early increase in natural killer cells may play a role in defending the body against infection or tumour relapse while the immune system is still compromised.

2.2.6 Granulocyte and monocyte reconstitution post bone marrow transplantation

The first two weeks post transplantation are usually characterised by granulocytopaenia. However, after this initial period, polymorphs recover quickly, but can have functional abnormalities which include impaired chemotaxis, superoxide production and phagocytic activity (Sosa et al, 1980). In addition, in patients with GVHD, these abnormalities can linger for up to nine months (Verma and Mazumder, 1993). The reduced number and abnormal functional capabilities of granulocytes possibly explains the appearance of bacterial and fungal infections in the early post transplant period.

Monocytes appear in the peripheral blood early post transplantation. In addition to their rapid recovery, the functional capabilities of the monocytes also normalise. Cytokines (IL-1, IL-6, TNF), serum lysozyme production and antigen presentation, all achieve normality by six weeks post transplantation (Tsoi et al, 1984, Verma and Mazumder, 1993). However, although peripheral monocytes normalise quickly, abnormal chemotaxis, phagocytosis and cytotoxicity have been reported in alveolar macrophages. This could explain the increase of lung infections by cytomegalovirus and pneumocystis carinii, post transplantation (Verma and Mazumder, 1993).

2.3 APOPTOSIS AND FAS/CD95 EXPRESSION POST BONE MARROW TRANSPLANTATION

The term "apoptosis" refers to cell death arising from a pre-existing death programme that is encoded in the genome (Saikumar et al, 1999). This definition has led to apoptosis being referred to as "programmed cell death".

Apoptosis of the cell leads to distinct morphologic changes, which include cell shrinkage, nuclear condensation, membrane blebbing, fragmentation into membrane apoptotic bodies and eventual phagocytosis of the dead cell. The apoptotic process plays an important role in the removal of injured or unwanted cells. Examples of this are cells injured by genetic defects, ageing, disease or exposure to toxic agents (Saikumar et al, 1999).

The immune response is also regulated by the apoptotic removal of certain cell populations. Deleted clones include neutrophils during inflammatory responses and autoreactive T-cells within the thymus. During viral infections, cytotoxic T-cells induce infected cells to commit suicide. This they do by either inducing apoptosis via the FAS/FAS ligand pathway or by delivering granzyme B into the cell (Saikumar et al, 1999).

Apoptosis also plays a role in disease. Normally, cell proliferation and death are at equilibrium. However, during disease, abnormalities of apoptosis have been discovered. For example, deficiencies in apoptosis play an important role in the pathogenesis of autoimmunity and certain lymphoproliferative disorders. Increased apoptosis has also been implicated in diseases such as AIDS and neuro degenerative diseases (Donnenberg et al, 1995).

The decision to initiate apoptosis is made either by the removal of certain extra-cellular signals that are required for survival or by the receipt of a signal via one of the membrane receptors called "death receptors". These include the tumour

necrosis factor receptor and FAS or CD95 (Saikumar et al, 1999).

FAS or CD95 is a glycosylated 45KD type 1 transmembrane receptor. FAS binds to the FAS ligand and is responsible for the deletion of autoreactive T-cells and activation induced T-cell death (Brugnoni et al, 1999). FAS/CD95 contains a cytoplasmic domain of eighty amino acids. After binding with the FAS ligand, the FAS molecule forms a homotrimeric complex and binds certain intracellular adapter proteins forming the FAS associated death domain (FADD). This complex induces the activation of caspase 8 which subsequently sets in motion a series of events, which will lead to apoptosis of the cell (Saikumar et al, 1999).

In 1995, Donnenberg et al studied the rates of spontaneous lymphoid apoptosis in the peripheral blood of bone marrow transplant and HIV+ patients and compared them to normal adults. In both patient groups the rate of apoptosis was significantly increased. They hypothesised that this was due to the elevated rate of lymphopoiesis seen in both groups. In the bone marrow transplant patient, spontaneous apoptosis is an attempt by the immune system to regenerate a competent non-self reacting T-cell population. This theory was supported by the fact that as the immune system recovered the rate of spontaneous apoptosis decreased, eventually reaching normal levels (Donnenberg et al, 1995).

In 1999, D.Brugnoni et al took these studies a step further and not only examined the rate of spontaneous apoptosis but also the role of activation induced cell death and the role played by CD95/FAS. They discovered that T-cells in the post transplant period expressed high levels of CD95 and had reduced expression of Bcl 2, an inhibitor of apoptosis. It was hypothesised that it was the consequence of the multiple exposures to antigen in the post transplant period. This theory was supported by the fact that there was a correlation of activated T-cells with the rate of apoptosis. As the number of

normal T-cells recovered the rate of apoptosis decreased. In patients receiving T-cell depleted transplants, however, the lymphoid recovery was slower and it was hypothesised that the increased expression of CD95 and therefore activation induced cell death could play a role in this deficiency (Brugnoni et al, 1999).

A further study undertaken by Nadia Hebib, examined the rate of apoptosis in twenty- six non-T-cell depleted allogeneic bone marrow transplant patients. Lymphocytes were analysed, post infusion, for both spontaneous and anti-FAS induced apoptosis at various intervals between days forty-five and seven hundred and thirty. The lymphocytes demonstrated increased susceptibility to both types of apoptosis during the first year, and the rate of apoptosis was significantly correlated with lower levels of Bcl-2. Of interest is the fact that the pro-apoptotic protein BAX remained unchanged. By day seven hundred and thirty, when substantial numbers of naïve lymphocytes had appeared in the peripheral blood, the apoptotic phenotype had disappeared (Hebib et al, 1999).

These articles highlight the important role that both spontaneous and FAS induced apoptosis may play in the immune deficient period post bone marrow transplantation. Further study is however needed, particularly in those patients receiving T-cell depleted grafts.

2.4 THE WAY FORWARD

This review of the literature has shown that bone marrow transplantation patients experience a prolonged period of immune deficiency. The causes of this are multi-factorial and a better understanding of these mechanisms is required before attempts to solve the problem can be made. Future research will need to focus on ways of accelerating immune recovery post bone marrow transplantation.

Early attempts to improve immune reconstitution using thymic tissue transplantation failed and therefore other methods have been sought (Lum, 1987). Growth factors such as G-CSF and GM-CSF and intravenous immunoglobulins have proved encouraging and are currently being prescribed in many centres (Verma and Mazumder, 1993).

The use of cytokines, particularly IL-2, to enhance T-cell recovery has also been suggested. However, published reports have given conflicting results. Studies examining sublethally irradiated mice concluded that treatment with IL-6, IL-11 in combination with stem cell factor and IL-3, significantly improved the recovery of thymus, spleen and bone marrow cells (Frasca et al, 1999). Other studies have however concluded that cytokines and growth factors are only one of the mechanisms causing the immunodeficiency, and therefore their use is limited (Moller et al, 1997).

Research, examining mice treated with the cytokine IL-7 have however proved extremely encouraging. IL-7 is known to be produced by both thymic and marrow stroma and has important effects on both the proliferation and differentiation of immature T and B-cells. Initial experiments with transplanted mice have shown that those treated with IL-7 had a more rapid immune recovery than those who did not receive the cytokine. The authors suggested that IL-7 could have the same effect on thymopoiesis that G and GM-CSF have

on granulopoiesis (Bolitin et al, 1996).

Other approaches, which require further investigation, are the re-infusion of graded numbers of donor lymphocytes. Small et al attempted this approach. It was concluded from this study of twelve patients that small numbers of unirradiated donor lymphocytes is associated with the restoration of T-cell numbers. In addition, the infusion of antigen specific T-cells, generated *in vitro* against specific antigens such as CMV could help to treat some of the opportunistic infections experienced in the post transplant period (Small et al, 1999). This approach to the problem is encouraging, however it requires further investigation.

To conclude, although immune reconstitution post bone marrow transplantation is well studied many gaps still remain in the literature. Firstly, what is the true effect of T-cell depletion on immune recovery? And secondly, have all the mechanisms causing the prolonged immune deficiency been properly investigated? These questions need to be properly answered before further studies can be conducted in order to solve and enhance immune reconstitution post transplantation.

This project will attempt to add to the current knowledge and also to propose future directions of study.

3.0 FLOW CYTOMETRY

Flow cytometry is the measurement of particles in a flow system that has been designed to deliver particles, usually cells, in a single file past a point of measurement. A typical flow cytometer consists of a light source (usually a lazer), a flow cell, optical components to focus light of different colours onto the detectors, electronics to amplify and process the signals and a computer (Fig 2) (Ormerod, 1999).

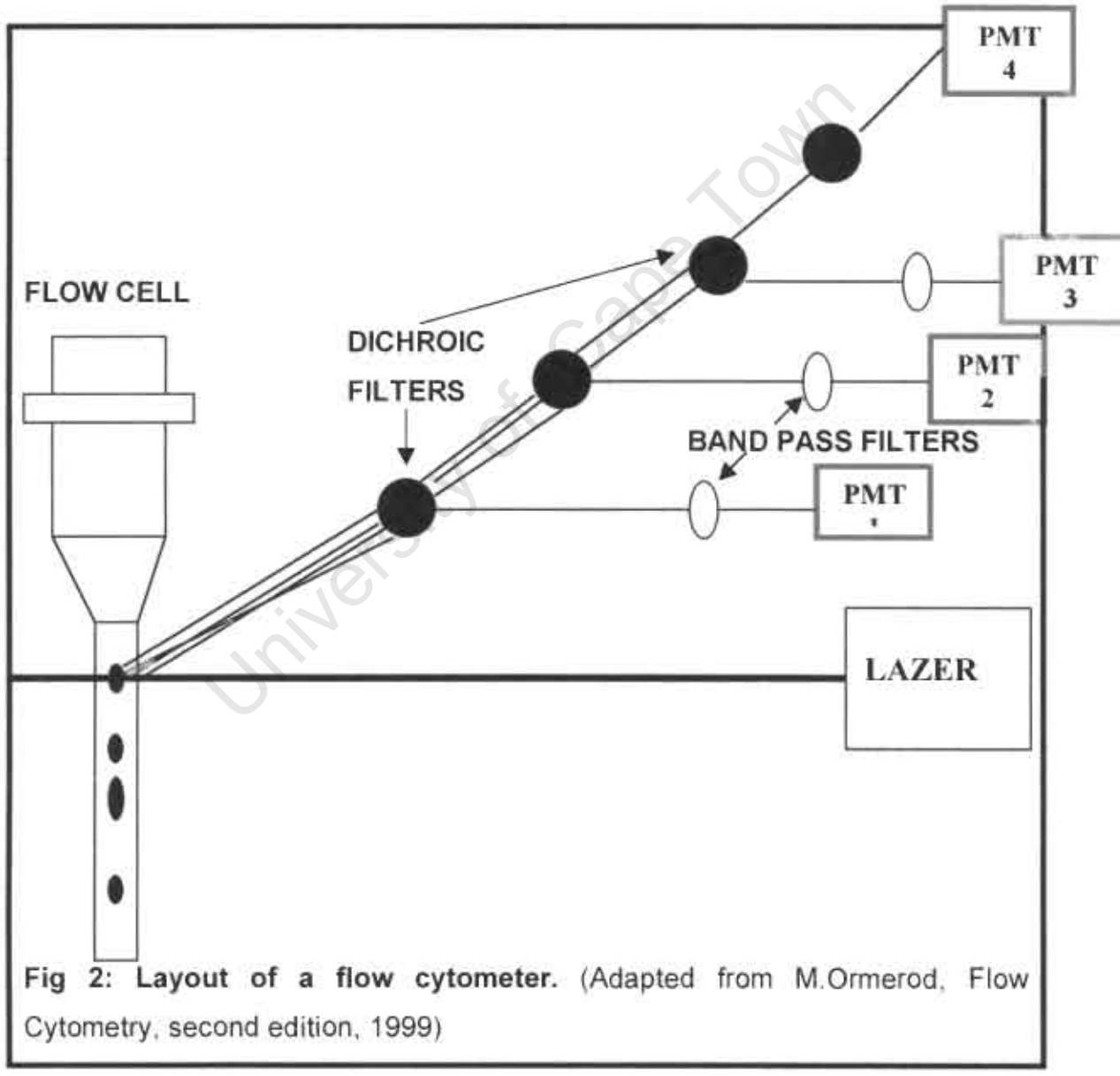


Fig 2: Layout of a flow cytometer. (Adapted from M.Ormerod, Flow Cytometry, second edition, 1999)

A cell or particle suspension is carried by the sample delivery system to the flow cell where it is injected into a rapidly flowing sheath stream. Within the flow cell, the sheath and sample stream undergo rapid acceleration. This process hydrodynamically focuses the cells to flow in single file past the light source. Each cell scatters light and emits fluorescence, if labelled with a fluorescent dye. Optical filters separate the different colours which are then measured by photosensitive detectors. The detectors are photomultiplier tubes which are used to measure side scatter and fluorescent signals. Photo-diodes are used for forward scatter signals. The signals from the photomultiplier tubes undergo analogue to digital conversion before being transmitted to the computer for analyses (Bauer et al, 1993).

The power of flow cytometry lies in its ability to measure several different parameters simultaneously. The most common parameters analysed are forward and side scatter, red, green and orange fluorescence. This technology can therefore be used to define and isolate various sub-populations of cells.

It is however important that the cells being studied are accurately selected on a dot plot of forward versus side scatter. Forward scatter signals are directly proportional to the size of the cell, whereas side scatter measures cell complexity such as cytoplasmic granularity and nuclear configuration (Tsieh Sun, 1993).

The most commonly analysed cell population are lymphocytes. However, monocytes, granulocytes and debris can often fall into the same gating area as the lymphocytes and, therefore a system of "back-gating" needs to be applied.

The Centre for Disease Control and Prevention (CDC) and The National Committee for Clinical Laboratory Standards (NCCLS) have published a set of guidelines for accurately analysing lymphocytes. One of the recommended strategies is the use of antibodies to CD45 (leucocyte common antigen) and

CD14 (monocytes). Lymphocytes are CD45 bright/CD14-, monocytes CD45+/CD14+ and granulocytes CD45 dim/CD14-. By applying this knowledge, lymphocytes can be accurately gated. (Fig 3) (CDC, 1997; NCCLS, 1998).

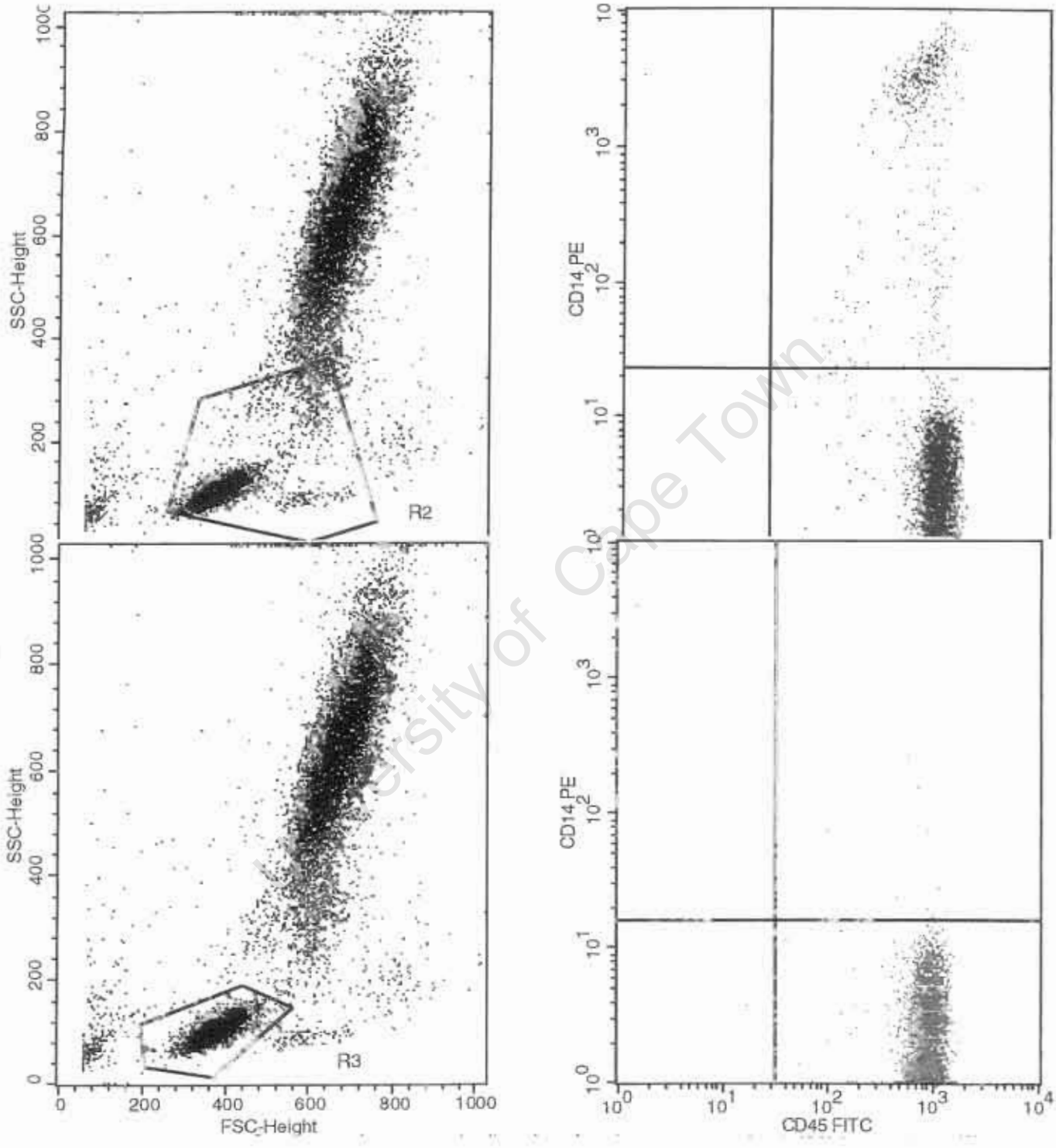


Fig 3: Exclusion of contaminating monocytes from lymphocyte gate using antibodies to CD14 and CD45

4.0 MATERIALS AND METHODS

4.1 Patient population

Seventy-eight patients, with haematological malignancies or aplastic anaemia, receiving bone marrow transplants, between June 1995 and December 1999, were studied. Nineteen of the patients received T-cell depleted allogeneic bone marrow transplants from HLA matched identical siblings. A further thirty-two individuals received allogeneic peripheral blood stem cells and the remaining twenty-seven patients were given autologous peripheral blood stem cells. The median age of the patient population was thirty-two years with a range of between six and sixty six.

Eleven individuals received adoptive immunotherapy by donor lymphocyte infusion for the treatment of relapse. These patients were excluded from the study once they had relapsed and the first donor lymphocyte infusion was administered.

4.2 Pre-conditioning

Patients with malignancies were conditioned with total body irradiation (TBI) delivered in fractions of two Gy, twice daily until a total of twelve Gy was reached. This was followed by 120mg/kg cyclophosphamide and four 1,5Gy fractions of total lymphoid irradiation. (TLI)

Those patients with aplastic anaemia were conditioned with TLI consisting of twelve fractions of 150 cGy. This was followed by four daily doses of 50 mg/kg cyclophosphamide.

4.3 Mobilisation of stem cells

Mobilisation of haemopoietic progenitors into the blood for the autologous grafts was with cylophosphamide at a daily dose of 60mg/kg followed by 5μg/kg of G-CSF daily for five to fifteen days. When the leucocyte count was > $7x10^9$/l, the number of circulating CD34+ stem cells were measured by flow cytometry. If the CD34 count was greater than 5 x 10^6, the mononuclear population was harvested using a Cobe Spectra (Cobe Laboratories Inc, Lakewood, Colorado). After harvesting, the cells were cryopreserved, in the presence of DMSO using a controlled rate freezer. For the stem cell rescue, once the patient had undergone preconditioning, the cells were thawed in a 37° waterbath and then rapidly infused intravenously.

Similarly, donors of the patients receiving allogeneic PBSC transplants were mobilised with the administration of 5 to 10 μg of G-CSF daily for five days. On the fifth day CD34+ cells were enumerated. If the number of circulating stem cells had reached 20 x 10^6/l, no further G-CSF was administered and the stem cells were harvested the following day, again using a Cobe Spectra. A double dose of G-CSF was given to the donor if the CD34+ cell count was below twenty. The following morning the number of CD34+ stem cells were again enumerated prior to collection.

In patients, receiving allogeneic bone marrow transplants, bone marrow was obtained from the donors under general anaesthesia. Multiple marrow aspirations were suspended into IMDM containing 10 units/ml of preservative free heparin (Nordisk, Johannesburg). The buffy coat was then collected using a Cobe Spectra.

4.3.1 T-cell Depletion of the graft

In patients receiving allogeneic transplants, to prevent graft versus host disease (GVHD) by T-cell depletion, 2,5 μg per 10^6 mononuclear cells of CAMPATH 1 was used, *ex vivo (*in the bag). The Campath antibodies were incubated with the cells at 20^0C for thirty minutes. Patients with aplastic anaemia received an additional nine daily intravenous infusions of 10μg of the CAMPATH antibody. No post transplant immunosuppression was administered.

4.4 Laboratory Techniques

4.4.1 CD34 enumeration by flow cytometry

For quantitative determinations of CD34+ cells, venous blood samples were collected into EDTA vacutainer tubes on day five of mobilisation. 100μl of whole blood was then incubated with 10μl of both FITC and Pe conjugated monoclonal antibodies to CD34 and CD45 (Becton Dickinson). After incubation for thirty minutes at 20°C, the cells were processed using FACSLYSE reagents (Becton Dickinson). Thereafter 150000 events were acquired using a FACS Calibur flow cytometer. Analysis of the CD34+ stem cells was performed using a multiparameter, sequential gating strategy as recommended by the ISHAGE guidelines (Sutherland et al, 1996).

Leucocyte values obtained from a Technikon H2 analyser (Terrytown, New Jersey) were used to calculate the absolute numbers with the following formula:

$$\frac{\text{Average total number of CD34+ events - isotypic control events}}{\text{Average total number of CD45+ events}} \times \text{WBC} \times \text{pack volume}$$

4.4.2 CFU-GM analysis

Bone marrow or peripheral blood progenitor cells at a concentration of $2x10^5$ mononuclear cells were added to a methyl cellulose solution containing twenty percent foetal calf serum, gentamyacin and 10μg rh GM-CSF. The cells and methyl cellulose solution were pipetted into cluster dishes (Nunc, England) and incubated at 37^0C for fourteen days. Thereafter, all colonies containing greater than forty cells were counted. The result was reported as CFU - GM x 10^4/kg using the following calculation.

$$\frac{\text{Colony count}}{\text{Plating concentration}} \text{ X mononuclear cells/kg}$$

4.4.3 Immunophenotyping of the blood

4.4.3.1 Analysis of lymphoid populations

Lymphoid populations were examined by collecting peripheral blood into EDTA vacutainer tubes at one, two, three, six, twelve and eighteen months post transplantation. In addition, heparinised peripheral blood was separated by standard ficoll density centrifugation and the mononuclear cells were cryopreserved at -70^{0}C for further analyses. Sibling donor lymphocyte blood values were used to calculate the normal ranges.

The various lymphoid populations were studied using direct immunofluorescent techniques with antibodies to the following surface antigens: CD3, CD2, CD4, CD8, CD19, CD16 and CD56 (Table 1). 100 µl of EDTA anticoagulated peripheral blood was incubated with 10µl of FITC or Pe conjugated antibody. After incubating the cells at room temperature for thirty minutes, the red cells were lysed using 2ml of diluted (1:10 with distilled water) Facslyse reagent (Becton Dickinson, Mountain View). Thereafter, the cells were washed twice and reconstituted with 0,5ml phosphate buffered saline. The lymphocytes were then analysed on a Becton Dickinson FACS CALIBUR flow cytometer.

To bitmap accurately the lymphocytes and exclude all contaminating monocytes, antibodies to the monocyte surface antigen CD14 and the leucocyte common antigen CD45 were utilised (Fig 2). In addition, fluorescent conjugated isotypic controls were employed to gate out all non-specific positivity.

5000 cells were then counted using a standard two-colour protocol. Analysis of the various lymphoid subsets was performed using Cell Quest software supplied by Becton Dickinson, Mountain View.

The absolute numbers of the various lymphoid subsets were calculated using the white cell and lymphocyte count obtained from a Technicon H2 automated cell counter. The lymphocyte count was confirmed with a manual differential.

Table 1: Antibodies used.

Antigen	Cellular Expression	Source
CD3	Mature T-cells	Becton Dickinson
CD2	T-cells, thymocytes, natural killer cells	Becton Dickinson
CD4	Helper T-cells, monocytes and macrophages	Becton Dickinson
CD8	Cytotoxic T-cells and natural killer cells	Becton Dickinson
CD19	B-cells	Becton Dickinson
CD16	Natural killer cells, Neutrophils	Becton Dickinson
CD56	Natural killer cells	Becton Dickinson
CD45RO	Majority of memory type T-cells, B-cells and monocytes	Becton Dickinson
CD45RA	Majority of naïve T-cells, B-cells and monocytes	Becton Dickinson
CD95/FAS	Variety of cells	Pharmingen

4.4.3.2 Analysis of memory and naïve T-cells

The presence of memory and naïve T-cells were examined using a three-colour protocol. Antibodies to CD45RO and CD45RA were incubated together with one of the following antibodies conjugated to Per CP, anti CD3 or CD4. Standard direct immunofluorescent techniques, as described above, were employed.

The number of each T-cell subset, expressing either the memory (CD45RO) or naïve (CD45RA) phenotype, was then obtained by calculating the percentage of cells expressing both CD45RO or RA and the relevant T-cell antigen. The absolute numbers of each subset was calculated using the lymphocyte count obtained from a Technicon H2 cell counter.

In order to establish clearly whether the majority of the CD4+ helper T-cell population were of the memory (CD45RO) or naïve (CD45RA) phenotype, the ratio between the CD4+ T-cells expressing CD45RO and CD45RA was calculated using the following calculation:

$$\frac{\text{CD4+/CD45RO+}}{\text{CD4+/CD45RA+}} = \text{Ratio}$$

4.4.3.3 Analysis of FAS/CD95 expression on T-cells

The percentage of T-cells expressing the CD95/FAS antigen was also determined using a three-colour protocol. An antibody to CD95 (Pharmingen) conjugated to PE was incubated with antibodies to CD3-FITC and CD4-PerCP. The percentage of each subset of T-cells expressing the CD95 antigen was analysed. In addition, the mean fluorescent intensity of CD95/FAS expression on the T-cell population was recorded. The mean fluorescent intensity of antigen expression is an indicator of the number of antigen receptors present on the cell surface.

4.4.4 Determination of serum immunoglobulins

Serum immunoglobulin levels were studied using standard nephelometry techniques. Patient's serum was incubated with specific antibodies to IgG, IgA and IgM. The resultant immune complexes were then measured with a nephelometer (Behringer Diagnostics, Marburg, Germany) and compared to the established normal ranges. The normal ranges in our laboratory are: -
IgG; 6,9 - 14g/l; IgA; 0,88 - 4,1 g/l and IgM; 0,34 - 2,1 g/l.

4.5 Statistical analysis

Medians and ranges were calculated for each parameter. The non parametric Mann-Whitney U test (2 tailed) was used to compare the values of a particular lymphoid subset of patients with the normal controls and between the autologous and allogeneic transplant groups.

5.0 RESULTS

This study sequentially analysed the lymphoid recovery of all transplant patients between 1995 and 1999. The methodologies of stem cell collection varied. Allogeneic bone marrow transplants were performed between 1995 and 1997 and PBSCT thereafter. Autologous PBSCT was established from 1995. All three groups of transplants had achieved adequate haematological recovery by six months post transplantation (Table 2).

5.1 Definition of the graft

5.1.1 Allogeneic Bone Marrow Cells

After the preconditioning process the patients receiving allogeneic bone marrow transplants were given a graft containing a median of 0,78 $x10^8$/kg (range: 0,28 - 2,09) mononuclear cells and 11,7 $x10^4$/kg (range: 2,05 -196,2) CFU-GM's (Table 3). The number of CD34+ cells was not quantitated, as this methodology had not yet been developed in our laboratory.

5.1.2 Allogeneic peripheral blood stem cell transplants

In contrast, the allogeneic peripheral blood stem cell harvests contained a median of 8,2 $x10^8$/kg (range: 1,68 - 16,9) mononuclear cells, 26,1 x 10^4/kg (range: 1,7 - 280) CFU-GM's and 1,0 x 10^6/kg (range: 0,5-5,09) CD34+ stem cells. Of interest, is the increased numbers of both mononuclear cells and CFU-GM's present in the peripheral blood stem cells harvests as compared to the traditional bone marrow grafts (Table 3).

5.1.3 Autologous transplants

After mobilisation, the autologous transplant patients achieved peripheral blood stem cell harvests containing the following numbers of mononuclear cells (median: 6,47 range: 1,74-16,9), CFU-GM's (median: 38,2 range: 0,08-148,6) and CD34+ stem cells (median: 2,4 range: 0,8-7,98) (Table 3).

Transplant type	White cell count ($x10^9$/l)	Platelets ($x10^{12}$/l)	Haemoglobin (g/dl)
Allogeneic bone marrow (N =19)	3.9 1 – 12.7	139 25 – 277	11.8 8.8 – 12.6
Allogeneic PBSC (N=32)	4.9 1.7 - 8.7	191 40 – 441	11.9 7.2 – 13.4
Autologous (N=27)	4.9 2.5 - 9.7	131 10- 386	12.2 6.6 – 14.2
Normal	4.0 – 11.0	150 – 400	M: 15.5 +/-2.5 F: 14.0 +/-2.5

Table 2: Median white cell, platelet and haemoglobin counts at six months post infusion

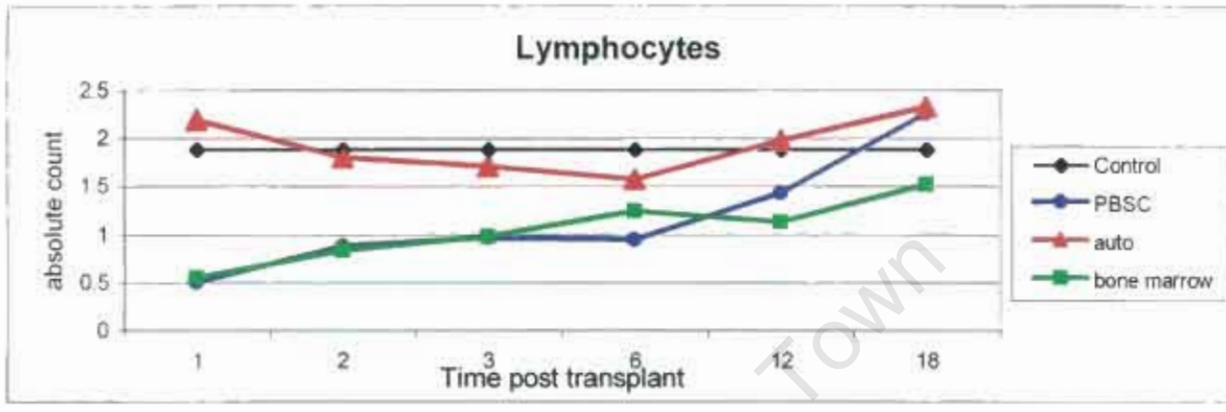
Transplant type	Mononuclear cells ($x10^8$/kg)	CD34+ stem cells ($x10^6$/kg)	CFU-GM's ($x10^4$/kg)
Allogeneic bone marrow (N =19)	0.78 (0.28-2.09)		11.7 (2.05-196.2)
Allogeneic PBSC (N=32)	8.2 (1.68-16.9)	1,0 (0.5-5.09)	26.1 (1.7-280)
Autologous (N=27)	6.47 (1.74-16.9)	2.4 (0.8-7.98)	38.2 (0.08148.6)

Table 3: Median numbers of harvested mononuclear, CD34+ stem cells and CFU-GM's infused

5.2 Reconstitution of lymphoid subpopulations

5.2.1 Lymphocytes and total T-cells

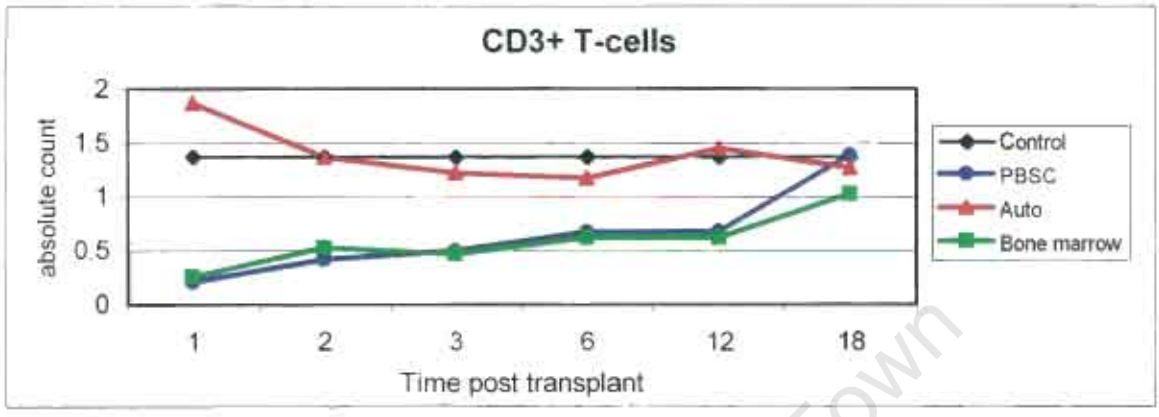
Figure 4 depicts the post transplant recovery of the lymphocytes in all three transplant types. Those patients receiving autologous stem cells normalised their lymphocytes by one month. In comparison, both types of allogeneic T-cell depleted transplants remained sub-normal until one year.



	1 month median Range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.54 0.18-2.46 *p=0.0003	0.82 0.2-2.79 *p=0.03	0.97 0.31-3.91 *p=0.02	1.23 0.26-2.94 *p=0.02	1.12 0.83-3.8 *p=0.19	1.51 0.8-3.12 *p=0.45
Allo PBSCT	0.49 0.2-1.8 *p=0.00005	0.87 0.4-2.01 *p=0.002	0.96 0.24-2.83 *p=0.0006	0.94 0.38-2.15 *p=0.005	1.42 0.23-2.37 *p=0.01	2.25 2.06-2.44 *p=0.34
Auto	2.18 0.35-5.75 *p=0.52	1.79 0.41-3.57 *p=0.93	1.7 0.32-3.88 *p=0.44	1.56 0.73-3.91 *p=0.76	1.97 0.69-2.61 *p=0.46	2.32 1.37-3.01 *p=0.04
Normal	1.88 1.35-2.41					
Allo PBSCT vs Auto	**p=0.0001	**p=0.01	**p=0.03	**p=0.02	**p=0.01	P=0.32

Fig 4: Total lymphocyte recovery post bone marrow transplantation. * P-value represents the significance in the difference of the values between the patients results and the control group. **p-value represents the significance in the difference between the allogeneic PBSCT and autologous transplant groups.

A similar pattern was observed when examining the total T-cell populations as measured by the antigens CD3 and CD2. Again a quicker recovery rate was observed in the autologous group, while both allogeneic transplant types achieved normal values only at eighteen months (Figure 5 and 6).

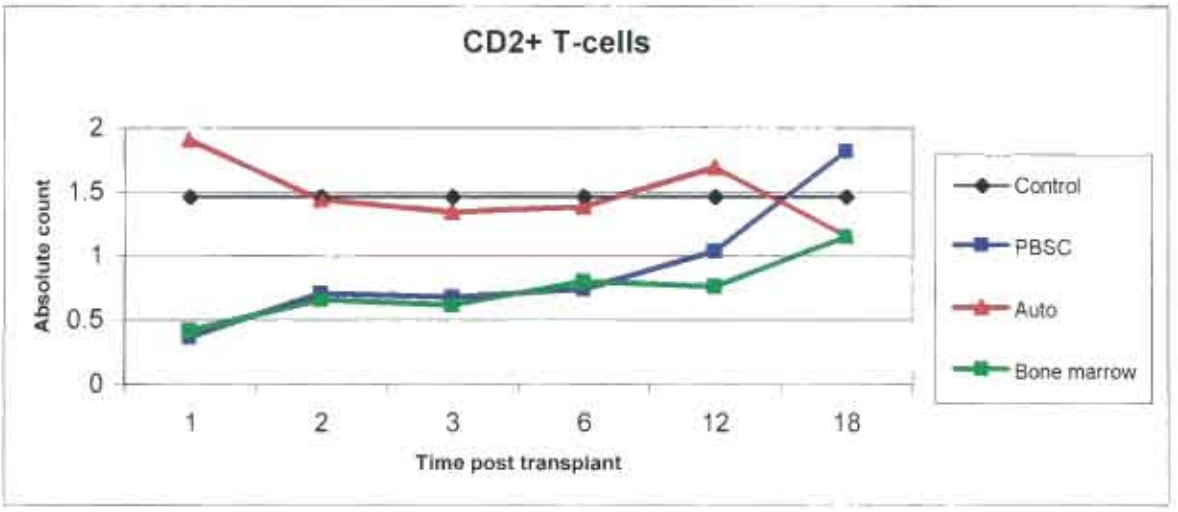


	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.25 0.02-1.84 *p=0.002	0.52 0.09-2.6 *p=0.01	0.46 0.09-3.73 *p=0.01	0.61 0.15-2.03 *p=0.005	0.61 0.46-2.95 *p=0.06	1.02 0.6-2.22 *p=0.19
Allo PBSCT	0.2 0.02-0.8 *p=0.00008	0.41 0.11-1.59 *p=0.0005	0.49 0.06-0.59 *p=0.00002	0.66 0.19-1.85 *p=0.002	0.67 0.13-1.34 *p=0.003	1.38 1.33-1.42 *p=0.81
Auto	1.86 0.06-6.24 *p=0.17	1.36 0.08-3.24 *p=0.52	1.21 0.15-5.41 *p=0.87	1.16 0.53-2.79 *p=1.0	1.44 0.48-1.65 *p=0.85	1.26 0.67-2.6 *p=0.74
Normal	1.37 0.92-1.82					
Allo PBSCT vs auto	**p=0.000002	**p=0.001	**p=0.03	**p=0.02	**p=0.01	**p=0.32

Fig 5: CD3+ T-cell recovery post transplantation.

*P-value= The significance of the difference between the patients result and the control group.

**P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.



	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.41 0.17-2.38 *p=0.003	0.65 0.18-2.63 *p=0.08	0.61 0.24-3.76 *p=0.06	0.79 0.32-2.47 *p=0.01	0.75 0.53-2.9 *p=0.07	1.14 0.67-2.24 *p=0.16
Allo PBSCT	0.36 0.13-1.36 *p=0.0001	0.70 0.23-1.89 *p=0.007	0.67 0.17-2.69 *p=0.002	0.73 0.23-1.99 *p=0.04	1.03 0.22-1.61 *p=0.02	1.81 1.61-2.01 *p=0.31
Auto	1.9 0.19-6.54 *p=0.19	1.43 0.17-3.36 *p=0.74	1.33 0.19-5.87 *p=0.93	1.37 0.56-3.2 *p=0.9	1.68 0.42-2.23 *p=0.51	1.15 0.84-2.35 *p=0.01
Normal	1.46 1.0-1.92					
Allo PBSCT vs Auto	**p=0.00005	**p=0.008	**P=0.01	**p=0.03	**p=0.01	**p=0.04

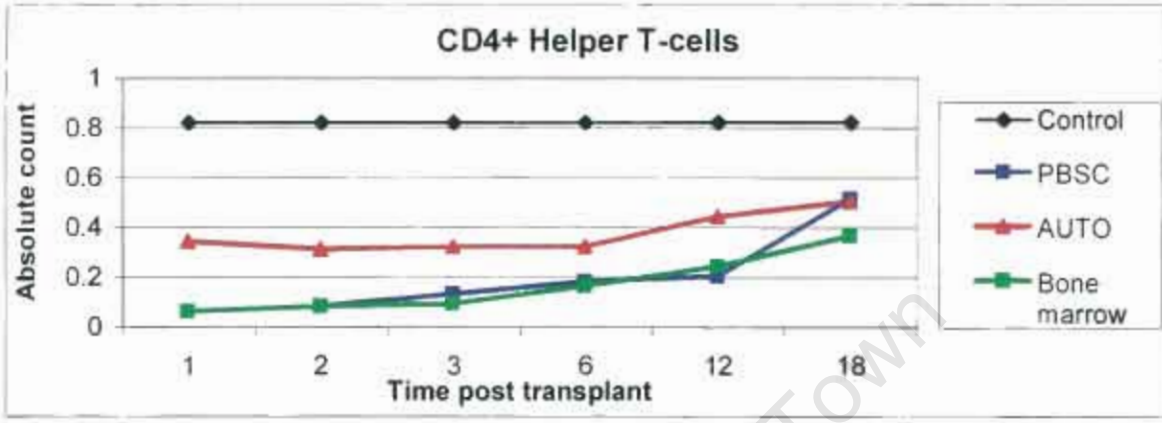
Fig 6: Total T-cell reconstitution as measured by anti CD2 .

*P-value= The significance of the difference between the patients result and the control group.

**P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

5.2.2 CD4+ helper and CD8+ cytotoxic T-cells

The CD4+ T-cell population was slow to reach the normal range in all transplant types. The autologous patients did however recover at a faster rate than both of the allogeneic T-cell depleted groups. Both allogeneic groups maintained abnormally low CD4+ counts even at eighteen months post infusion (Figure 7).

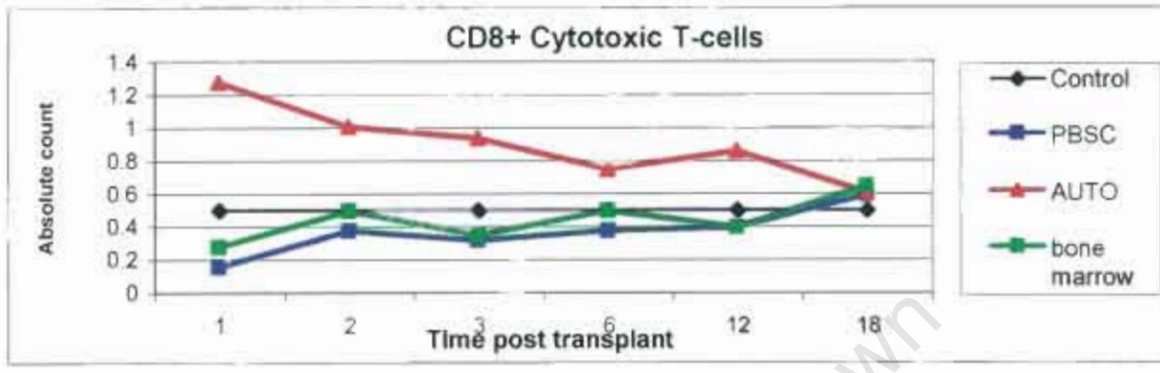


	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.06 0.008-0.19 *p=0.00003	0.08 0.01-0.89 *p=0.00003	0.09 0.04-1.61 *p=0.0006	0.16 0.06-0.71 *P=0.00002	0.24 0.17-0.42 *p=0.006	0.36 0.09-0.42 *p=0.0003
Allo PBSCT	0.06 0.02-0.16 *p=0.000006	0.08 0.03-0.76 *p=0.0002	0.13 0.03-0.6 *p=0.00002	0.18 0.06-0.31 *p=0.00001	0.2 0.02-0.49 *p=0.0002	0.51 0.27-0.75 *p=0.2
Auto	0.34 0.03-1.56 *p=0.002	0.31 0.05-0.99 *p-0.0005	0.32 0.03-1.69 *p=0.0004	0.32 0.15-1.18 *p=0.0003	0.44 0.19-0.81 *p=0.004	0.5 0.26-0.82 *p=0.97
Normal	0.82 0.55-1.09					
Allo PBSCT vs auto	**p=0.000007	**p=0.001	*p=0.0004	**p=0.00006	**p=0.01	**p=0.32

Fig 7: CD4+ helper T-cell recovery.

*P-value= The significance of the difference between the patients result and the control group. **P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

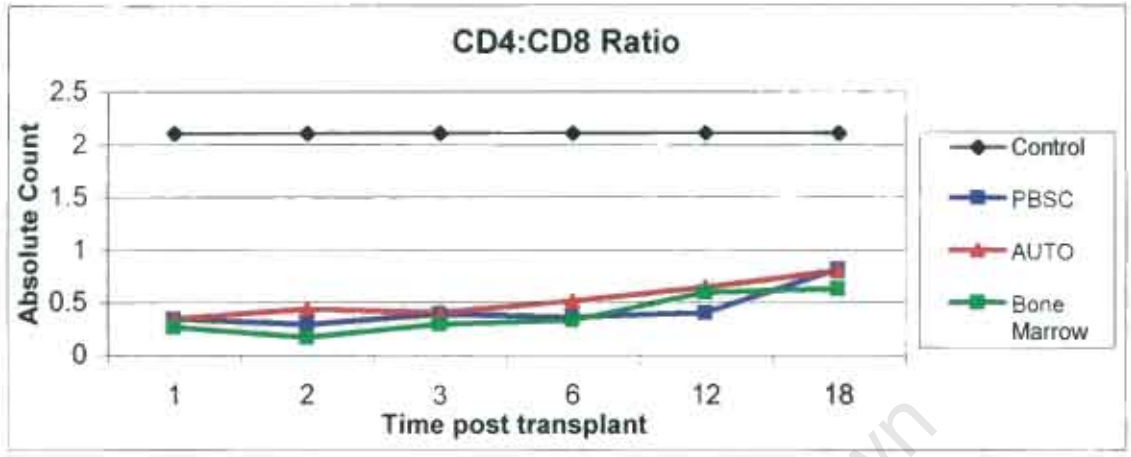
By contrast, the CD8+ cytotoxic T-cell subset had achieved normal levels, in all transplant types by eight weeks. An interesting observation was the significantly increased number of CD8+ T-cells in the autologous group. This however, was only in the early post transplant period and the values slowly decreased, reaching normal levels by eighteen months (Figure 8).



	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.27 0.03-1.58 *P=0.25	0.49 0.09-1.67 *p=0.38	0.34 0.07-2.18 *P=0.76	0.49 0.16-1.65 *P=0.70	0.39 0.26-1.9 *P=0.82	0.64 0.32-1.33 *p=0.23
Allo PBSCT	0.15 0.03-0.72 *p=0.003	0.37 0.12-0.95 *p=0.12	0.31 0.03-2.12 *p=0.50	0.37 0.12-1.52 *p=0.81	0.39 0.14-1.09 *p=0.17	0.59 0.52-0.66 *p=0.30
Auto	1.27 0.05-5.27 *p=0.001	1.0 0.07-2.61 *p=0.006	0.93 0.11-3.1 *p=0.05	0.74 0.26-2.55 *p=0.01	0.85 0.26-1.25 *p=0.009	0.6 0.39-1.96 *p=0.74
Normal	0.5 0.3-0.7					
Allo PBSCT vs Auto	**p=0.00002	**p=0.002	**p=0.01	**p=0.03	**p=0.008	**p=0.5

Fig 8: The recovery of CD8+ cytotoxic T-cells. * P-value= The significance of the difference between the patients result and the control group. **P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

This pattern of T-cell subset recovery gave rise to abnormal CD4:CD8 ratios in all three groups of transplant patients. These remained abnormal throughout the eighteen month study period (Figure 9).



	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.26 0.09-0.76 *p=0.0003	0.16 0.02-0.53 *P=0.000005	0.28 0.08-0.74 *p=0.00003	0.32 0.08-1.05 *p=0.0001	0.58 0.26-1.03 *p=0.0003	0.62 0.45-1.2 *p=0.0005
Allo PBSCT	0.34 0.1-4.4 *p=0.0001	0.28 0.16-0.35 *p=0.00005	0.38 0.06-1.47 *p=0.00006	0.35 0.15-1.22 *p=0.00001	0.39 0.13-1.22 *p=0.0004	0.8 0.24-1.45 *p=0.11
Auto	0.34 0.07-1.02 *p=0.000002	0.43 0.14-0.77 *p=0.000002	0.39 0.19-1.47 *p=0.00005	0.5 0.2-0.88 *p=0.000003	0.63 0.38-0.75 *p=0.00003	0.79 0.4-1.26 *p=0.002
Normal	2.1 1.61-2.61					
Allo PBSCT vs Auto	**p=0.81	**p=0.14	**p=0.13	**p=0.08	**p=0.43	**p=0.50

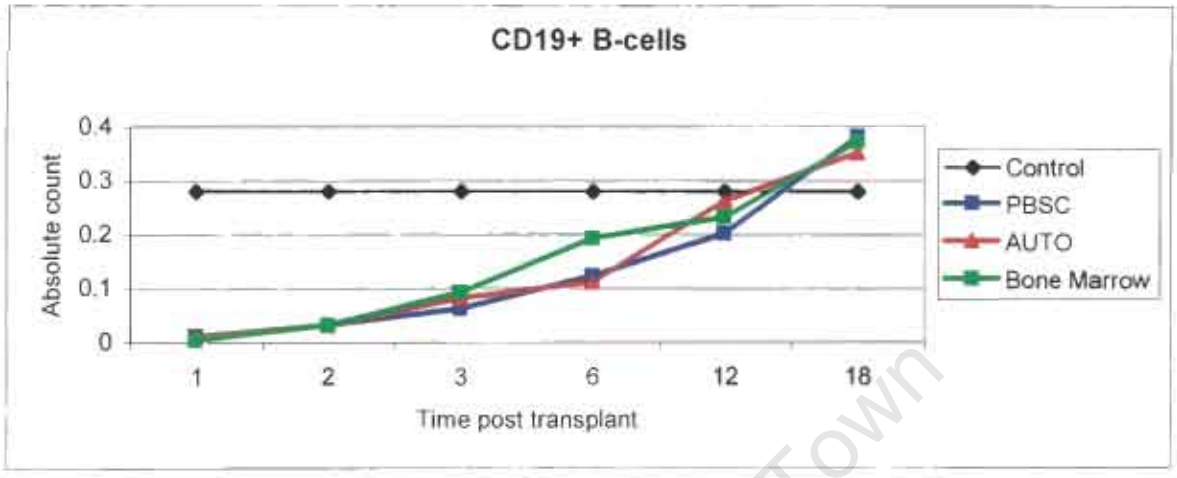
Fig 9: CD4:CD8 ratios in the post transplant period.

*P-value= The significance of the difference between the patients result and the control group.

** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

5.2.3 B-cell Recovery

B-cells as measured by the expression of the antigen CD19, recovered steadily in all transplant types, achieving normal values by twelve months. These values continued to rise throughout the eighteen month follow up period (Figure 10).

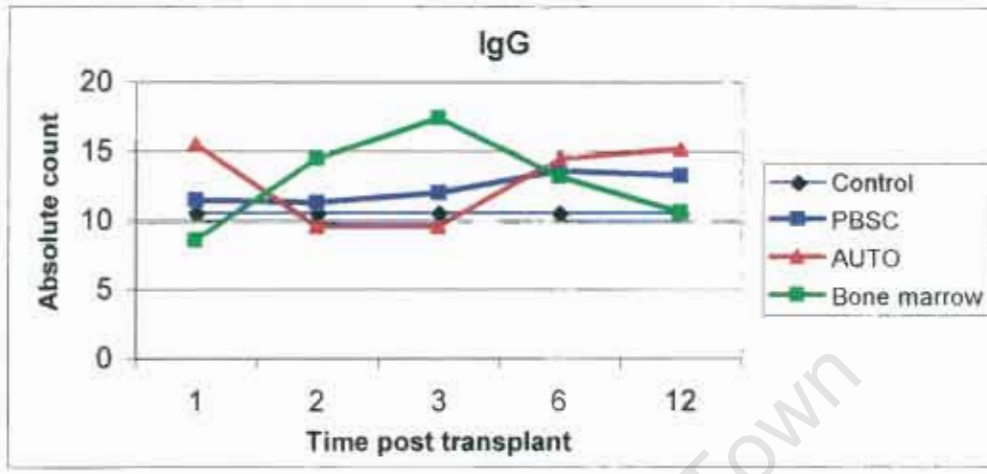


	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.002 0.0008-0.01 *p=0.000003	0.03 0.002-0.01 *p=0.00003	0.09 0.02-0.44 *p=0.02	0.19 0.01-0.64 *p=0.01	0.23 0.09-0.59 *p=0.61	0.37 0.04-0.54 *p=0.23
Allo PBSCT	0.01 0.0004-0.16 *p=0.00003	0.03 0.0007-0.32 *p=0.0008	0.06 0.01-0.41 *p=0.0002	0.12 0.002-0.74 *p=0.003	0.2 0.005-0.83 *p=0.33	0.38 0.31-0.44 *p=0.23
Auto	0.01 0.001-0.38 *p=0.00004	0.03 0.008-0.33 *p=0.0002	0.08 0.004-0.43 *p=0.002	0.11 0.01-0.59 *p=0.03	0.26 0.15-0.38 *p=0.91	0.35 0.22-0.9 *p=0.006
Normal	0.28 0.15-0.41					
Allo PBSCT vs Auto	**p=0.82	**p=0.98	**p=0.77	**P=0.47	**p=0.36	**p=0.18

Fig 10: CD19+ B-cell recovery post transplantation

*P-value= The significance of the difference between the patients result and the control group. ** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups

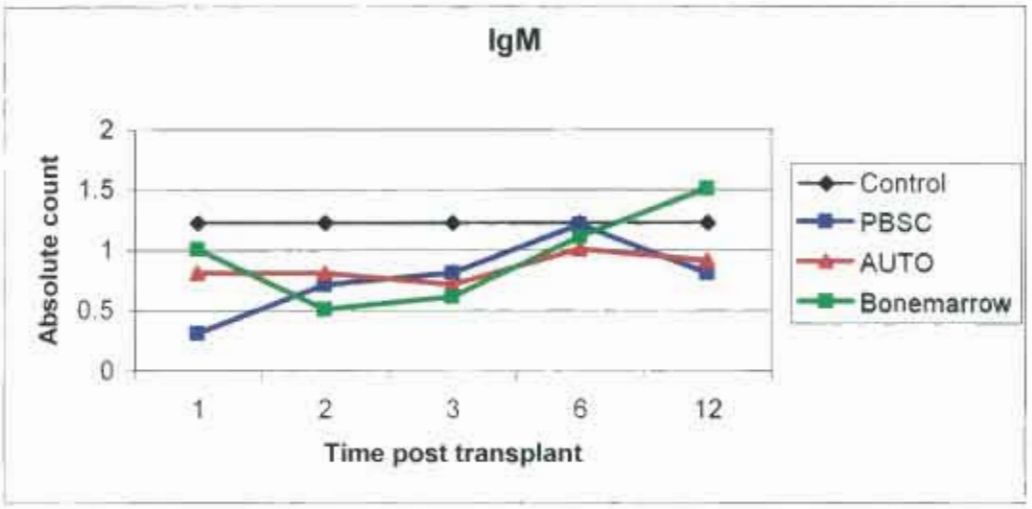
IgG and IgM serum levels rapidly rose in all patients and maintained normal levels throughout the study (Figure 11 and 12). In contrast however, although achieving low normal values, IgA remained persistently below the median level of the control group (Figure 13).



	1 month median range	2months median range	3 months median range	6 months median range	12 months median range
Allo bone marrow	8.5 6.9-18.6	14.4 8.3-24.1	17.4 6.7-20.1	13.1 7.6-19.3	10.5 7.6-20.4
Allo PBSCT	11.4 8.3-16.5	11.2 7.8-29.4	11.85 5.2-15.2	13.5 3.5-23.9	13.2 5.2-19.2
Auto	15.4 5.1-20.3	9.5 5.7-17.3	9.5 2.5-18.3	14.4 4.5-20.2	15.1 6.5-20.6
Normal	10.5 6.9-14.0				
Allo BMT vs auto			*p=0.05	*p=NS	*p=NS
Allo PBSCT vs Auto			**p=NS	**p=NS	**P=NS
Allo PBSCT vs BMT			***p=0.05	***p=NS	***P=NS

Fig 11 IgG recovery post transplantation. .

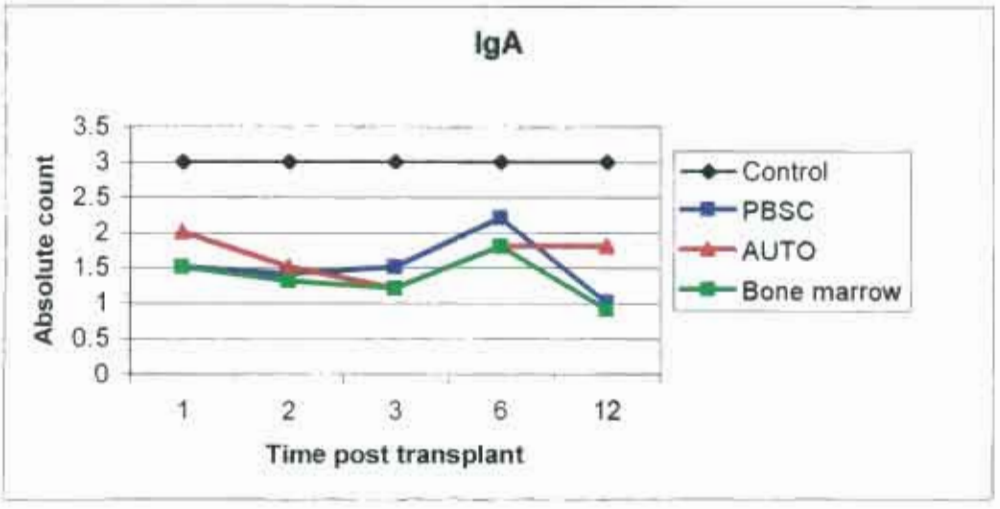
*P-value= The significance of the difference between the allogeneic BMT and the autologous transplant groups. ** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups. *** p-value= The significance difference between the Allogeneic PBSCT and BMT transplant groups.



	1 month median range	2months median range	3 months median range	6 months median range	12 months median range
Allo bone marrow	0.95 0.4-1.3	0.5 0.3-1.5	0.6 0.5-3.0	1.1 0.3-2.2	1.5 0.9-1.6
Allo PBSCT	0.3 0.2-0.7	0.7 0.1-1.1	0.8 0.2-1.3	1.2 0.6-2.9	0.8 0.6-1.5
Auto	0.8 0.3-9.9	0.8 0.2-6.3	0.7 0.2-1.6	1.0 0.2-2.8	0.9 0.5-1.4
Normal	1.22 0.34-2.1				
Allo BMT vs auto			*p=0.03	*p=NS	*p=NS
Allo PBSCT vs Auto			*p=NS	**p=NS	**p=NS
Allo PBSCT vs BMT			***p=0.01	***p=NS	***p=NS

Fig 12: IgM normalisation after transplantation. .

*P-value= The significance of the difference between the allogeneic BMT and the autologous transplant groups. ** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups. *** p-value= The significance difference between the Allogeneic PBSCT and BMT transplant groups.



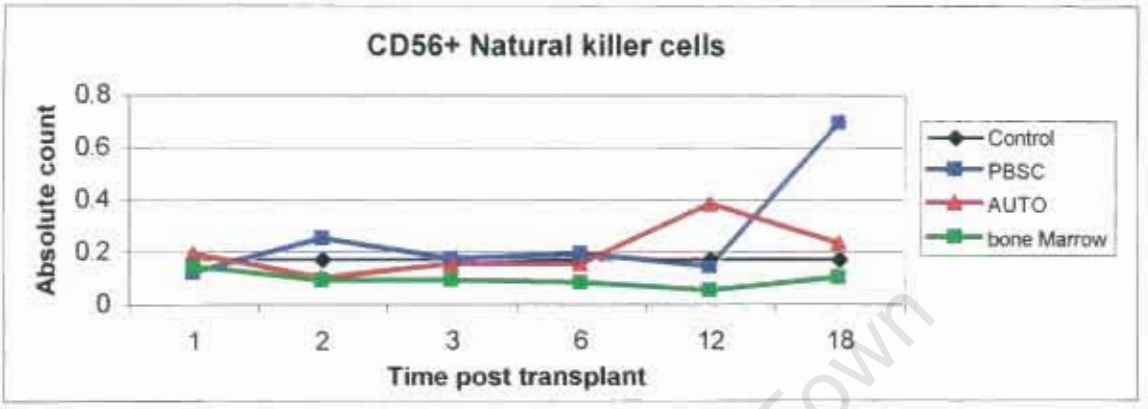
	1 month median range	2months median range	3 months median range	6 months median range	12 months median range
Allo bone marrow	1.5 0.5-3.2	1.3 0.3-2.5	1.2 0.3-2.7	1.8 0.3-4.5	0.9 0.4-1.4
Allo PBSCT	1.5 0.4-3.8	1.4 1.2-3.6	1.5 0.4-3.4	2.2 0.8-3.1	1.0 0.3-2.1
Auto	2.0 1.4-3.2	1.5 0.4-2.6	1.2 0.3-3.8	1.8 0.6-3.4	1.8 1.0-3.9
Normal	3.0 0.88-4.9				
Allo BMT vs auto			*p=NS	*p=NS	*p=NS
Allo PBSCT vs Auto			**p=NS	**p=NS	**p=NS
Allo PBSCT vs BMT			***p=NS	**p=NS	***p=NS

Fig 13: IgA recovery post bone marrow transplantation .

*P-value= The significance of the difference between the allogeneic BMT and the autologous transplant groups. ** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups. *** p-value= The significance difference between the Allogeneic PBSCT and BMT transplant groups.

5.2.4 Natural killer cells

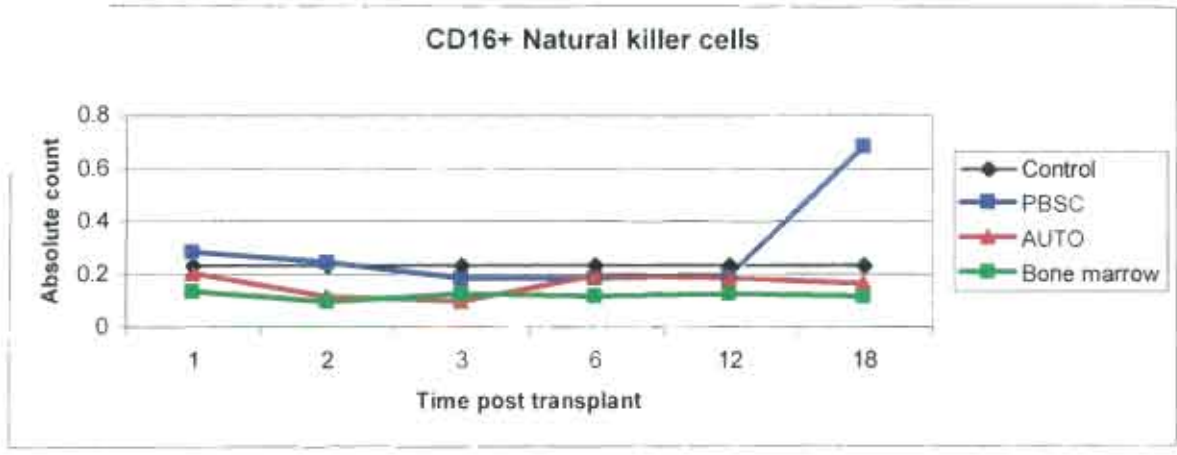
Natural killer cells were measured by using antibodies to the surface antigens CD56 and CD16.This subset of lymphocytes achieved normal levels by one month post infusion and these were maintained throughout the study as depicted by figures 14 and 15.



	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.14 0.04-0.51 *p=0.86	0.09 0.02-0.64 *p=0.96	0.09 0.005-0.36 *p=0.77	0.08 0.04-0.94 *p=0.37	0.05 0.02-0.13 *p=0.02	0.1 0.02-0.37 *p=0.54
Allo PBSCT	0.12 0.02-0.99 *p=0.73	0.25 0.11-0.34 *p=0.11	0.17 0.008-1.2 *p=0.78	0.19 0.005-0.75 *p=0.73	0.14 0.01-0.66 *p=0.87	0.69 0.36-1.02 *p=0.11
Auto	0.19 0.02-1.21 *p=0.50	0.1 0.01-0.64 *p=0.50	0.15 0.003-0.73 *p=0.66	0.15 0.04-0.94 *p=0.80	0.38 0.02-0.85 *p=0.38	0.23 0.03-0.85 *p=1.0
Normal	0.17 -1.5-3.8					
Allo PBSCT vs Auto	**p=0.54	**p=0.04	**p=0.30	**p=0.98	**p=0.30	**p=0.16

Fig 14: The normlisation of CD56+ natural killer cells.

*P-value= The significance of the difference between the patients result and the control group. ** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups



	1 month median range *p-value	2months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value	18 months median range *p-value
Allo bone marrow	0.13 0.04-0.28 *p=0.76	0.09 0.008-0.4 *p=0.10	0.12 0.03-0.5 *p=0.43	0.11 0.03-0.48 *p=0.16	0.12 0.03-0.24 *p=0.03	0.11 0.02-0.37 *p=0.12
Allo PBSCT	0.28 0.11-1.23 *p=0.54	0.24 0.12-0.99 *p=0.84	0.18 0.009-1.59 *p-0.79	0.18 0.05-0.72 *p=0.59	0.19 0.09-0.66 *p=0.85	0.68 0.34-1.01 *p=0.23
Auto	0.2 0.03-0.53 *p=0.32	0.11 0.03-0.29 *p=0.15	0.09 0.03-1.01 *p=0.25	0.19 0.02-0.38 *p=0.53	0.18 0.04-0.69 *p=0.34	0.16 0.05-0.33 *p=0.64
Normal	0.23 -0.01-0.87					
Allo PBSCT vs Auto	**p=0.25	**p=0.08	**p=0.24	**p=0.53	**p=0.43	**p=0.18

Fig 15: The recovery of CD16+ natural killer cells.

*P-value= The significance of the difference between the patients result and the control group.

** P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups

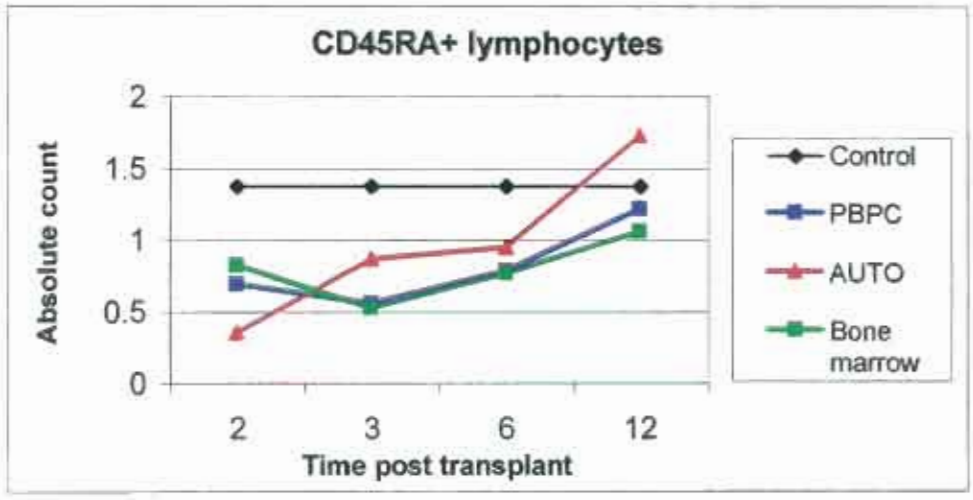
5.3 Analysis of memory and naïve lymphocytes

5.3.1 Total lymphoid population

Cells from patients belonging to each transplant group were cryopreserved for further analysis. After thawing, the total lymphoid populations were analysed for the presence of naïve (CD45RA+) and memory (CD45RO+) subsets.

Figure 16 shows that both T-cell depleted allogeneic transplant types achieved normal levels of CD45RA expression at six months. In addition, there was no significant difference between the two allogeneic groups. In comparison, the autologous patients reconstituted their naïve lymphocyte population more rapidly, reaching low normal levels by three months and equalling the median of the control group at twelve months.

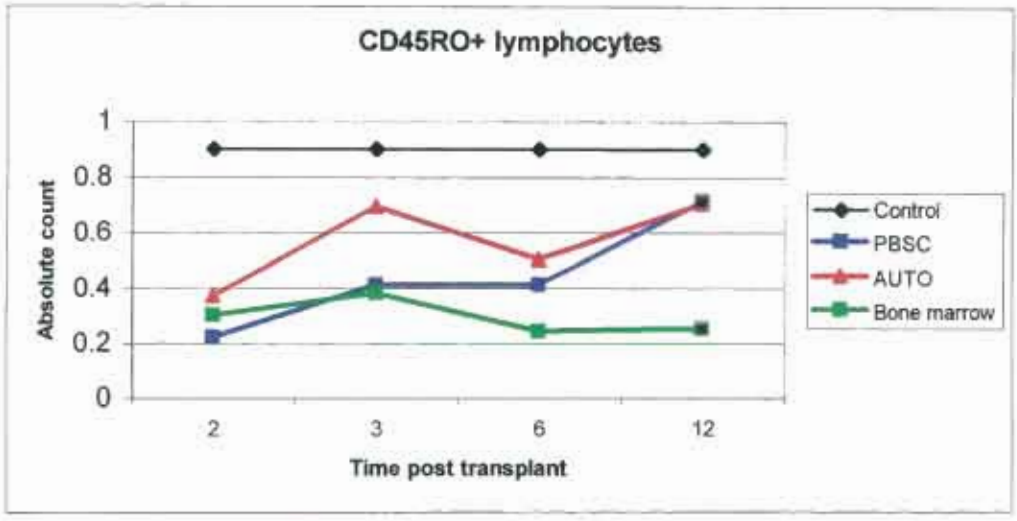
A similar pattern of recovery was observed when examining the expression of CD45RO on the total lymphoid population. The autologous group steadily recovered, achieving low normal values by twelve months, whereas both allogeneic T-cell depleted transplant groups remained abnormal throughout the twelve month follow up period (Figure 17).



	2 months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value
Allo Bone marrow	0.82 0,7-1,0	0.52 0.2-0.7	0.76 0.1-1.3	1.05 0.5-1.3
Allo PBSCT	0.69 0.3-1.5	0.55 0.1-1.2 p=0.00007	0.78 0.2-1.6 p=0.22	1.21 0.3-1.9 p=0.39
Auto transplant	0.35 0.2-1.6	0.86 0.3-2.9 p=0.39	0.94 0.46-2.9 p=0.22	1.72 0.52-2.0 p=0.39
Normal Controls	1.37 0.8-1.9	1.37 0.8-1.9	1.37 0.8-1.9	1.37 0.8-1.9

Fig 16: Recovery of the CD45RA subset in the total lymphoid population.

*P-value= The significance of the difference between the patients result and the control group.



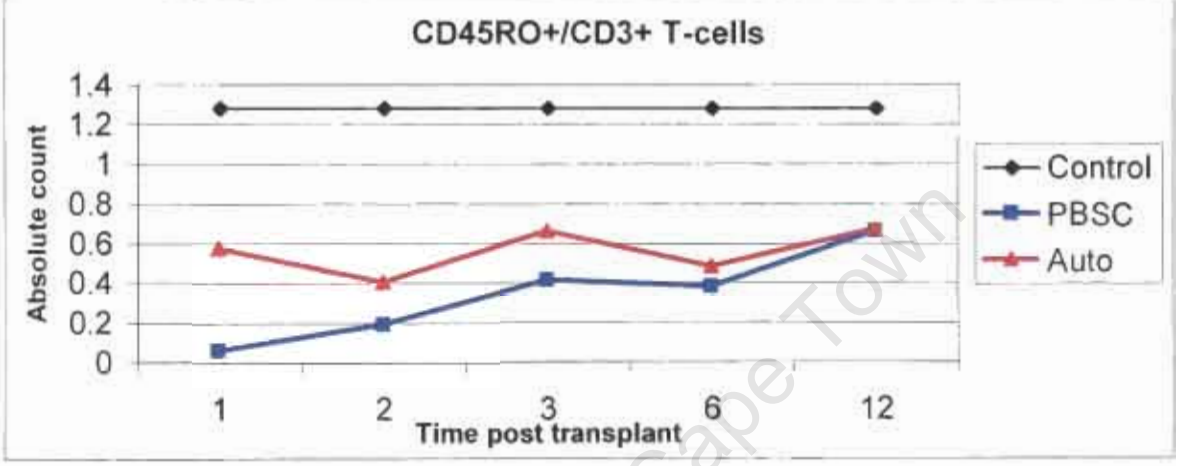
	2 months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value
Allo Bone marrow	0.3 0.2-0.4	0.4 0.01-0.8	0.2 0.07-0.6	0.25 0.05-0.99
Allo PBSCT	0.22 0.07-2.32	0.41 0.09-3.81 *p=0.009	0.41 0.12-1.46 *p=0.007	0.71 0.05-1.2 *p=0.15
Auto transplant	0.37 0.1-0.5	0.69 0.3-1.8 *p=0.55	0.5 0.1-1.27 *p=0.16	0.7 0.5-1.2 *p=0.24
Normal Controls	0.91 0.2-2.6	0.91 0.2-2.6	0.91 0.2-2.6	0.91 0.2-2.6

Fig 17 The recovery of the CD45RO+ lymphoid subset.

*P-value= The significance of the difference between the patients result and the control group.

5.3.2 Naïve and memory CD3+ T-cells

A further eleven autologous and twelve allogeneic PBSCT patients were analysed for the expression of both CD45RO and RA on the CD3+ T-cell population. As can be seen from Figure 18, both groups displayed low absolute numbers of memory (CD3+/CD45RO+) T-cells. The levels of these cells remained below the normal range throughout the twelve month follow up period.



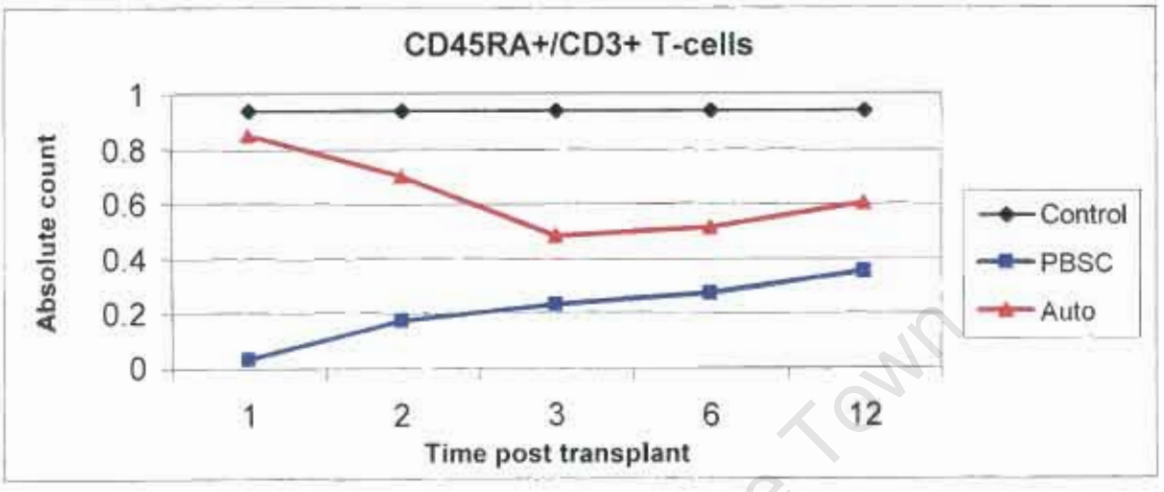
	1 month median range *p-value	2 months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value
Allo PBSCT	0.06 0.008-0.27	0.19 0.07-2.3	0.41 0.08-3.8 *p=0.006	0.38 0.1-1.43 *p=0.009	0.66 0.05-1.1 *p=0.05
Auto transplant	0.57 0.45-0.63	0.40 0.34-0.45	0.66 0.17-1.79 *p=0.04	0.48 0.14-1.57 *p=0.01	0.67 0.48-1.18 *p=0.02
Normal Controls	0.87 0.5-1.74				
Allo PBSCT vs Auto			**p =0.32	**p= 0.31	**p = 0.83

Fig 18: The recovery of CD45RO+/CD3+ T-cells.

*P-value= The significance of the difference between the patients result and the control group.

**P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

The naïve CD3+/CD45RA+ population steadily increased in both groups. The autologous patients achieved normality early post transplantation and maintained these levels throughout. However, although the allogeneic groups steadily increased, they did not achieve normal levels even at twelve months.



	1 month median range *p-value	2 months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value
Allo PBSCT	0.03 0.002-0.02	0.17 0.02-0.73	0.23 0.07-0.44 p=0.0007	0.27 0.04-0.92 p=0.005	0.35 0.06-0.57 p=0.04
Auto transplant	0.85 0.33-1.37	0.7 0.17-1.18	0.48 0.09-2.74 p=0.19	0.51 0.15-2.24 p=0.17	0.6 0.39-1.37 p=0.30
Normal Controls	0.87 0.5-1.74				
Allo PBSCT vs Auto			**P=0.06	**P=0.04	**P=0.20

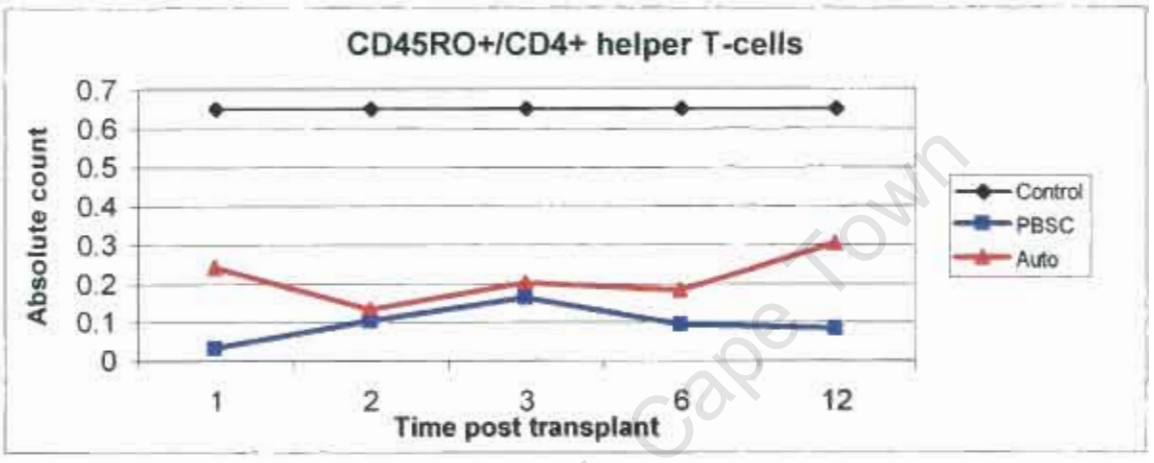
Fig 19: Naïve CD3+/CD45RA+ T-cell recovery.

*P-value= The significance of the difference between the patients result and the control group.

**P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

5.3.3 Naïve and memory CD4+ helper T-cells

Because the CD4+ helper T-cell subset was severely abnormal and took the longest to recover in all transplant types, it was decided to examine CD45RO and RA expression on these cells in more detail. Figures 20 and 21 graphically demonstrates the recovery of the absolute numbers of both memory and naive helper T-cells. Both populations remained below the normal range throughout the twelve month follow up period.

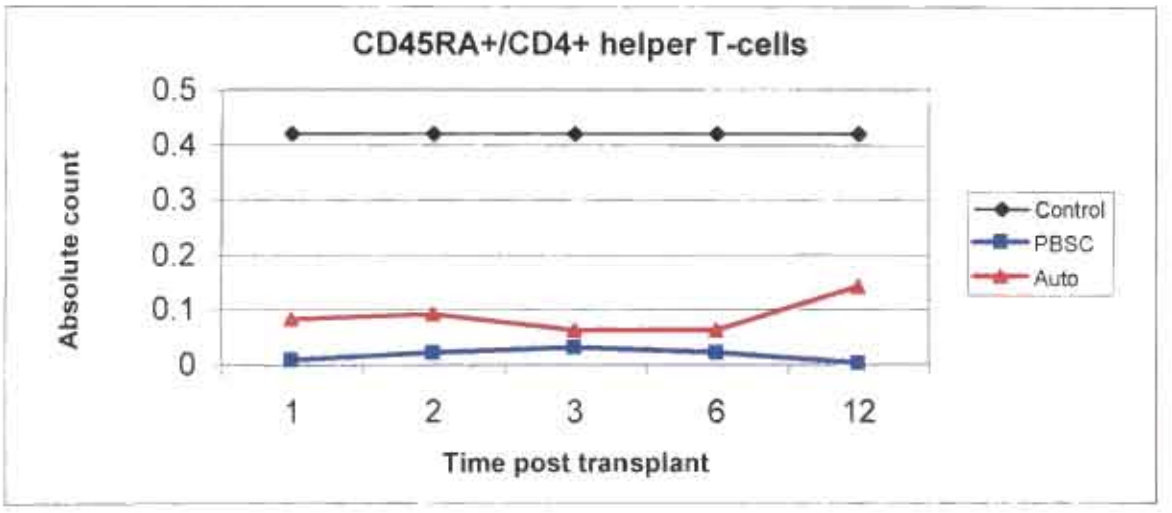


	1 month median range *p-value	2 months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value
Allo PBSCT	0.03 0.005-0.25	0.1 0.007-1.65	0.16 0.05-2.52 *p=0.004	0.09 0.03-0.45 *p=0.0004	0.08 0.03-0.1 *p=0.01
Auto transplant	0.24 0.13-0.25	0.13 0.01-0.24	0.2 0.05-0.38 *p=0.002	0.18 0.06-0.67 *p=0.005	0.3 0.14-0.71 *p=0.03
Normal Controls	0.51 0.1-1.01				
Allo PBSCT vs Auto			**p=0.76	**p=0.13	**p=0.02

Fig 20: CD45RO+/CD4+ T-helper cells during the first year post transplant.

*P-value= The significance of the difference between the patients result and the control group.

**P-value= The significance of the difference between the allogeneic PBSCT and autologous transplants.



	1 month median range *p-value	2 months median range *p-value	3 months median range *p-value	6 months median range *p-value	12 months median range *p-value
Allo PBSCT	0.007 0.0006-0.04	0.02 0.009-0.17	0.03 0.005-0.11 p=0.0006	0.02 0.004-0.06 p=0.0002	0.001 0.0004-0.12 p=0.01
Auto transplant	0.08 0.02-0.17	0.09 0.001-0.17	0.06 0.0007-0.17 p=0.0006	0.06 0.01-0.32 p=0.002	0.14 0.006-0.24 p=0.02
Normal Controls	0.38 0.3-1.38				
Allo PBSCT vs Auto			**P=0.34	**P=0.01	**P=0.07

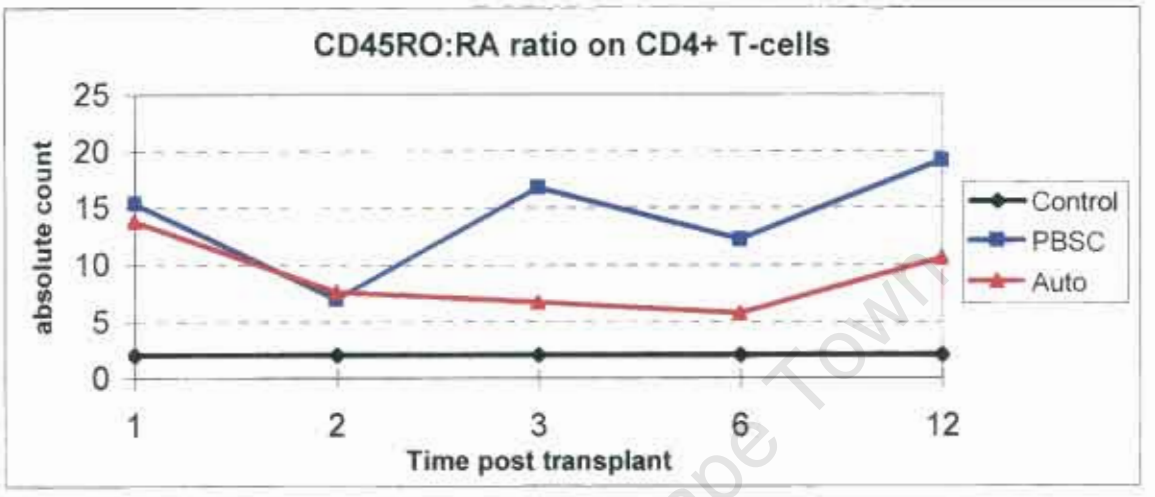
Fig 21: The recovery of CD4+/CD45RA+ helper T-cells.

*P-value= The significance of the difference between the patients result and the control group.

**P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

However, the calculation of absolute numbers could reflect the low numbers of CD4+ T-helper cells rather than any alteration in the memory and naïve populations. Therefore, it was decided to further analyse the expression of CD45RO and RA on these cells by calculating the ratio of CD4+/CD45RO+ to CD4+/CD45RA+ expressing cells. The results of this analysis revealed a

distinct alteration in the memory and naive CD4+ T-cell populations. The ratio of CD45RO:CD45RA expressing helper T-cells was increased and therefore suggested that the majority of the recovering CD4+ T-cells were of the memory phenotype. This phenomenon was evident in both autologous and allogeneic peripheral blood stem cell transplant patients (Figure 22).



	1 month mean range	2 months mean range	3 months mean range	6 months mean range	12 months mean range
Allo PBSCT	15.3 1.53-81.4	6.9 1.14-23.3	16.7 1.8-98.8	12.1 1.12-35.7	19.1 2.0-23.03
Auto transplant	13.7 1.4-37.7	7.5 1.14-13.8	6.6 2.5-22.4	5.6 0.93-24.7	10.5 6.58-41.6
Normal Controls	1.63 1.02-5.39				

Fig 22: The ratio of CD4+/CD45RO+ to CD4+/CD45RA+ expressing T-cells in the first 12 months post infusion.

Comparing the flow cytometric diagrams of a normal adult with a post transplantation patient again highlighted this phenomenon. This clearly demonstrated that the vast majority of the recovering CD4+ T-cells were of the memory phenotype (Figure 23).

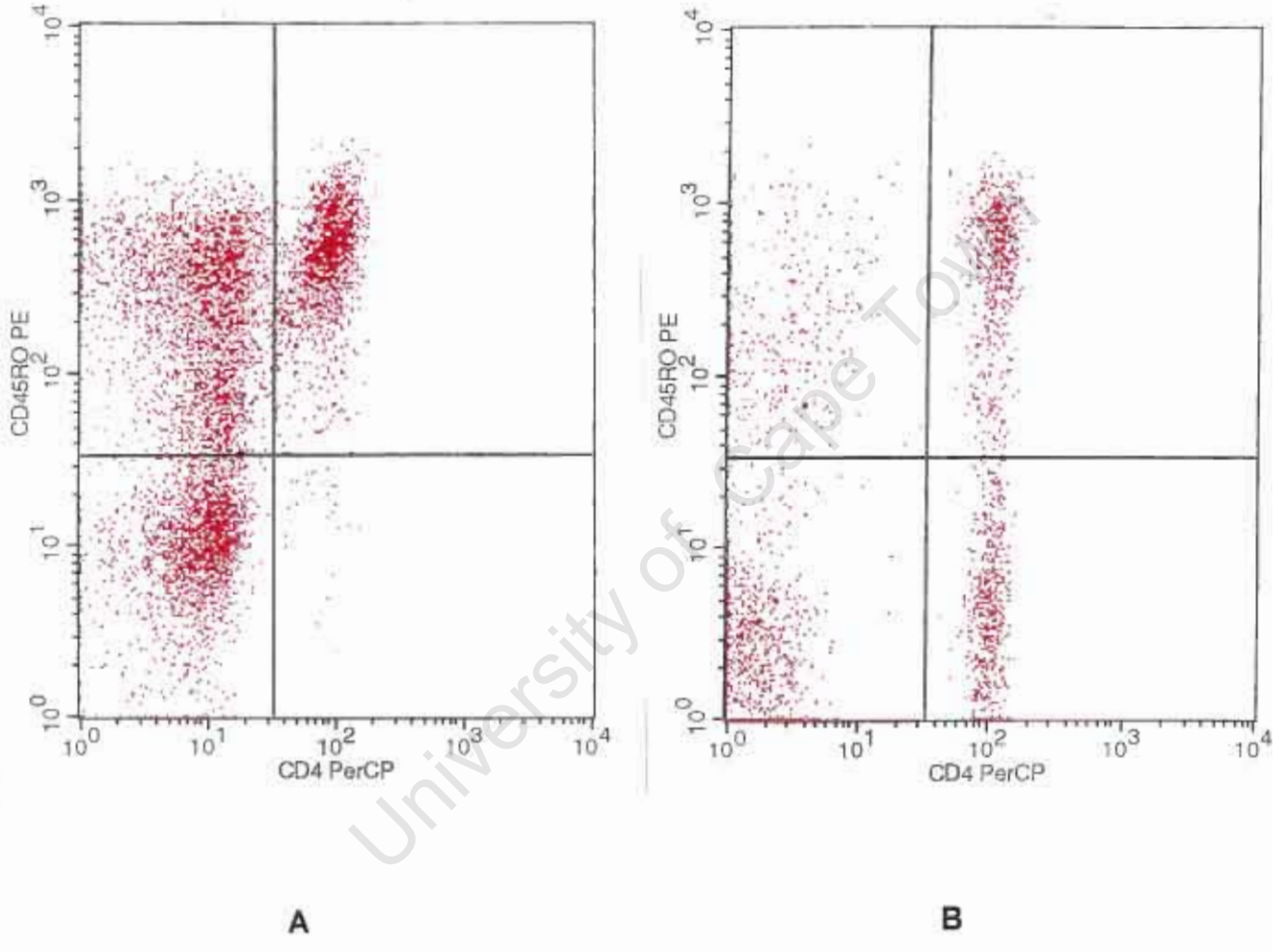


Fig 23: Flow cytometric dot plot demonstrating the increased numbers of CD4+ T-cells expressing the antigen CD45RO (A) as compared to a normal adult (B) at six months post transplantation.

5.4 Expression of FAS/CD95 on T-cells

5.4.1 FAS/CD95 expression on CD3+ T-cells

A further subset of autologous (n=5) and allogeneic (n=9) T-cell depleted transplant patients were analysed for surface expression of FAS/CD95.

The median percentage of T-cells expressing FAS/CD95 was significantly increased when compared to normal individuals. This is clearly demonstrated in Figure 24 which compares the flow cytometric dot plots of CD3/CD95 expression in a normal adult and a patient post allogeneic T-cell depleted peripheral blood stem transplantation. This increased expression was maintained throughout the twelve month study period (Figure 25).

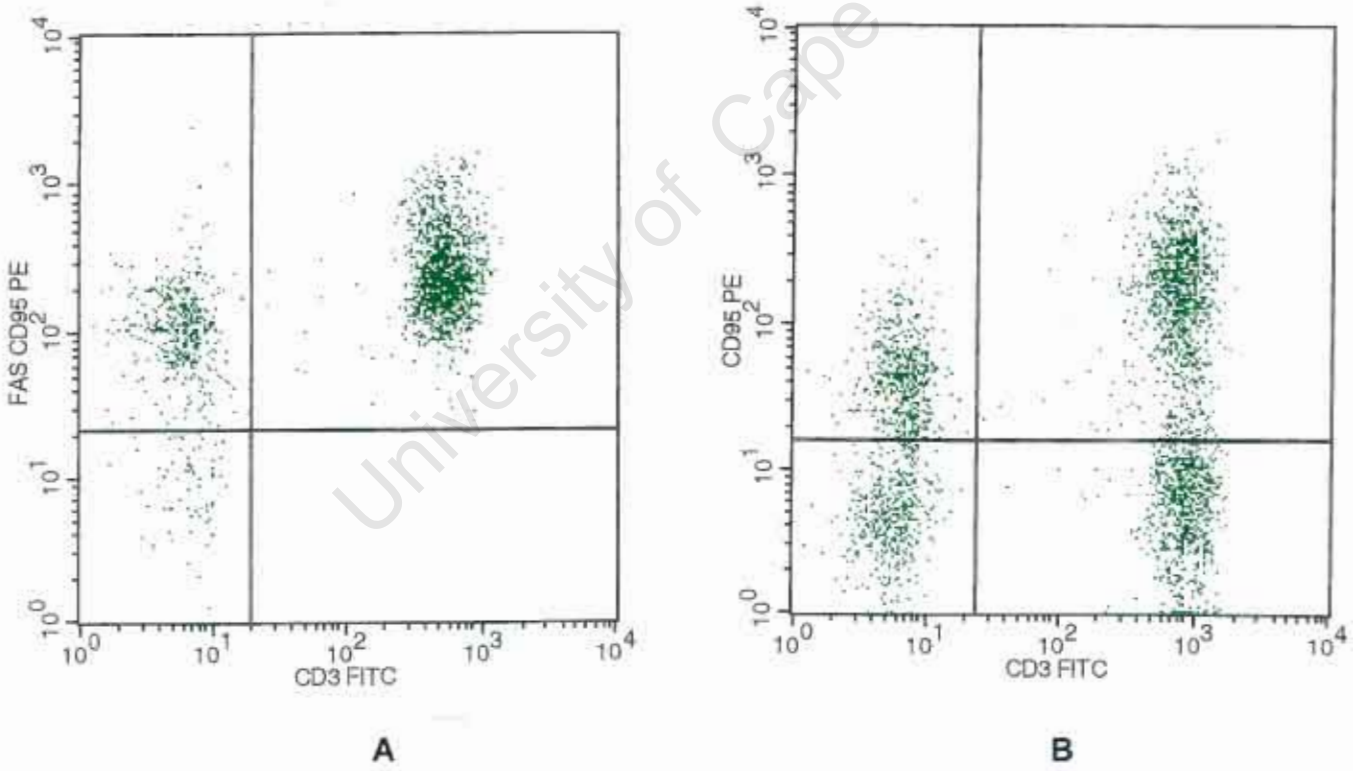
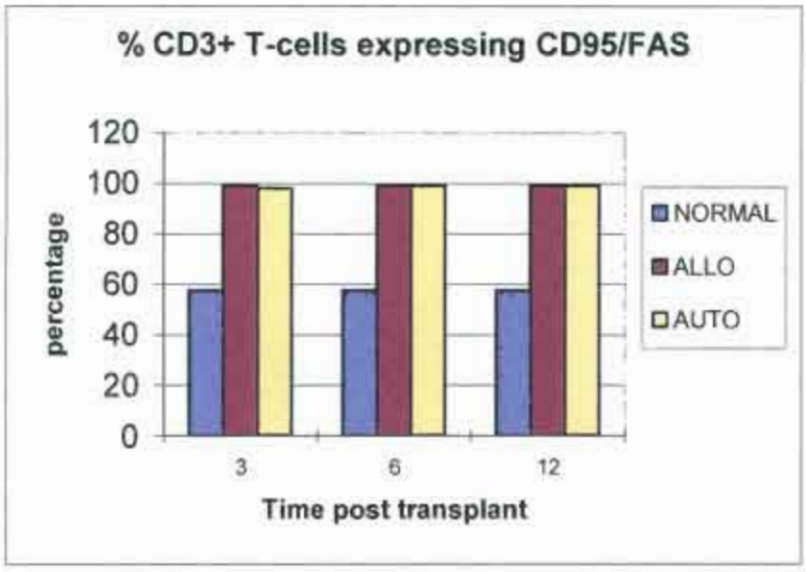


Fig 24: Flow cytometric dot plots demonstrating CD3+/CD95+ expression in an allogeneic T-cell depleted transplant patient (A) and a normal adult (B) at six months post transplantation.



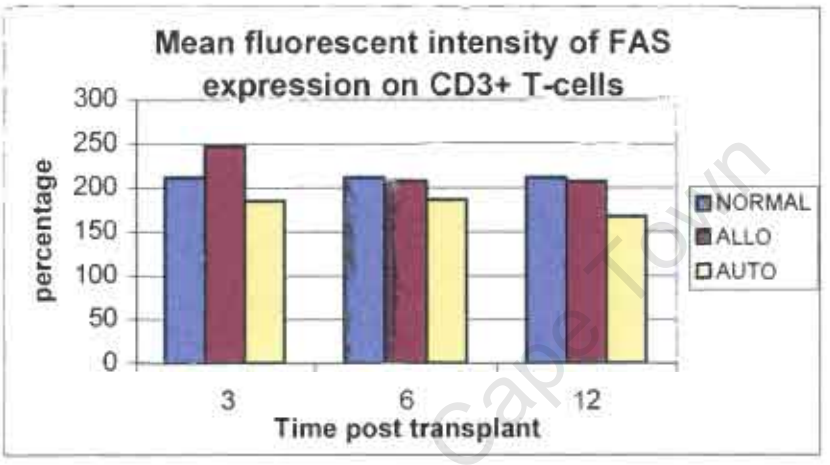
	3 months median range *p-value	6 months median range *p-value	12 months median range **p-value
Allo PBSCT	99 43-100 *p=0.001	99 89-100 *p=0.001	99 99-100 *p=0.04
Auto transplant	98 95-100 *p=0.003	99 35-99 *p=0.41	99 82-99 *p=0.006
Normal Controls	57.5 42-81		
Allo PBSCT vs Auto	**P=0.96	**P=0.90	**P=0.14

Fig 25: Percentage of CD3+ T-cells expressing CD95 at 3, 6 and 12 months post transplantation.

* P-value= The significance of the difference between the patients result and the control group.

**P-value= The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

The mean fluorescent intensity of antigen expression can be correlated with the number of receptors on the surface of the cell. In an attempt to determine whether post transplantation, CD3+ T-cells expressed an increased number of FAS/CD95 receptors, the mean fluorescent intensity of the CD3+/CD95+ T-cell population was analysed and compared to normal adults. The results of this analysis are shown in Figure 26. As can be seen from this graph there appeared to be no significant difference between normal adults and the two groups of transplant patients studied.



	3 months mean range *p-value	6 months mean range *p-value	12 months mean range *p-value
Allo PBSCT	247 101-465 *p=0.73	208 117-387 p=0.49	207 107-293 p=0.43
Auto transplant	185 102-424 *p=0.02	186 147-305 p=0.68	167 87-299 p=0.17
Normal Controls	211 175-287		
Allo PBSCT vs Auto	**p=0.66	**p=0.88	**p=0.24

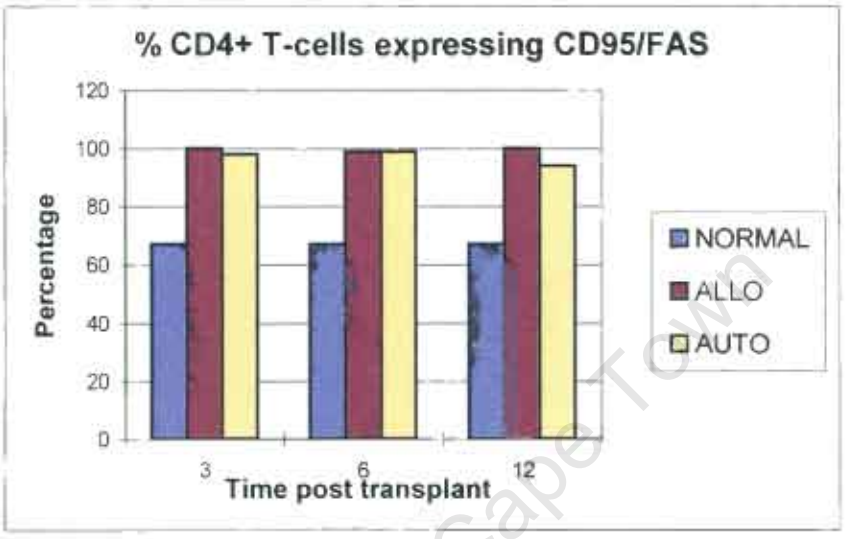
Fig 26: Mean fluorescent intensity of CD3+/CD95+ T-cells at 3, 6 and 12 months post transplantation.

*P-value= The significance of the difference between the patients result and the control group.

**P-value= The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

5.4.2 FAS/CD95 expression on CD4+ T-cells

A similar pattern of CD95 expression was observed when studying the CD4+ helper T-cell population (Figure 27). Once again, the percentage of CD4+ T-cells expressing FAS/CD95 was significantly increased in both transplant groups as compared to normal adults.



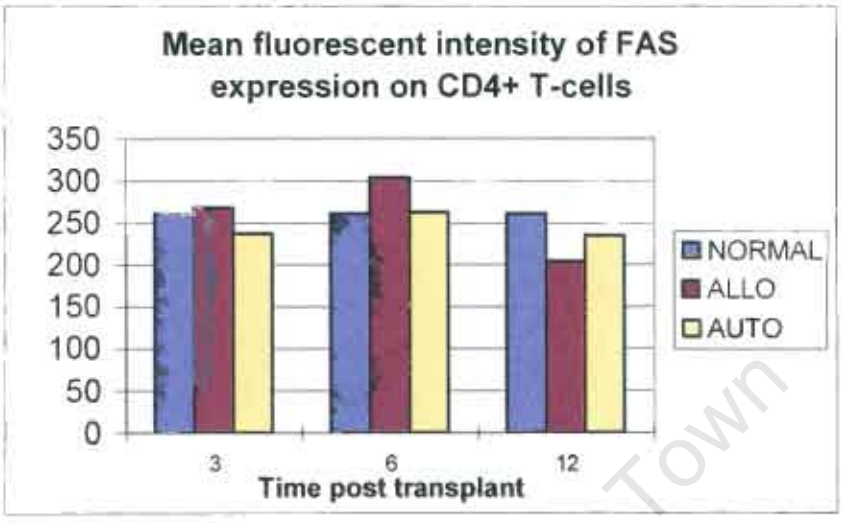
	3 months mean range *p-value	6 months mean range *p-value	12 months mean range *p-value
Allo PBSCT	100 71-100 *p=0.002	99 88-100 *p=0.009	99 99-100 *p=0.05
Auto transplant	98 97-100 *p=0.009	99 65-99 *p=0.30	94 67-100 *p=0.07
Normal Controls	67 54-81		
Allo PBSCT vs Auto	**p=0.66	**p=0.88	**p=0.24

Fig 27: Percentage of CD4+ T-cells expressing CD95 at 3, 6 and 12 months post transplantation.

*P-value= The significance of the difference between the patients result and the control group.

**P-value = The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

In addition, the mean fluorescent intensity of the CD4+/CD95+ T-cell population was analysed and compared to normal adult values. Like the CD3+ population no difference could be demonstrated between the transplant patients and normal adults (Figure 28).



	3 months mean range *p-value	6 months mean range *p-value	12 months mean range *p-value
Allo PBSCT	268 98-534 *p=0.21	304 118-420 *p=0.17	203 122-590 *p=1.0
Auto transplant	237 197-762 *p=0.60	262 232-352 *p=0.65	235 145-380 *p=0.81
Normal Controls	261 201-300		
Allo PBSCT vs Auto	**p=0.09	**p=0.46	**p=1.0

Fig 28: Mean fluorescent intensity of CD4+/CD95+ T-cells at 3, 6 and 12 months post transplantation.

*P-value= The significance of the difference between the patients result and the control group.

**P-value= The significance of the difference between the allogeneic PBSCT and autologous transplant groups.

6.0 DISCUSSION

The purpose of this project was to document the immune recovery of bone marrow and peripheral blood stem cell transplants, that have been T-cell depleted with anti CD52 antibodies. We also attempted to correlate this pattern of recovery to a group of myeloablated patients who received autologous peripheral blood stem cells and who had been conditioned similarly.

The mechanisms contributing to the immune deficient state, in the post transplant period, are multi-factorial. In an attempt to establish the role played by CD95/FAS mediated apoptosis, we have recorded the cell surface expression of the CD95 antigen, in a subset of patients, during the first year post infusion.

The immune recovery of seventy eight patients post transplantation, over a period of eighteen months, has been documented. Both groups of T-cell depleted patients achieved adequate haematological recovery (Table 2) and none have developed > grade II GVHD (data not shown). However, although the pattern of immune recovery is similar in all three modes of transplant, both T-cell depleted groups took longer to normalise the T-cell populations and this ultimately may have resulted in a number of clinically relevant infections.

Natural killer cells are large granular lymphocytes, which kill virally infected cells and play a role in tumour surveillance. They express the antigens CD56 and CD16 and make up between one and twenty percent of the normal lymphoid population (Janeway and Travers, 1994).

In all three modes of transplant natural killer cells recovered rapidly and were the first lymphoid population to achieve normality in the early post transplant period (Figure14 and 15). These results confirm numerous other reports which document similar results (Keever et al, 1989; Roberts et al, 1993 ; Parrado et

al,1997 ; Ashihara et al, 1994). Thus, in the post transplant patient, the development of natural killer cells appears to parallel that seen in the neonate.

A reason for this rapid recovery could be that natural killer cells develop within the bone marrow and, although sharing a common precursor with T-cells, are not dependant on a functional thymus for their recovery (Bomberger et al, 1998).

The role of transplant donor natural killer cells have been investigated by studies carried out in grafted mice. Natural killer cells were activated with IL-2 and then infused with compatible bone marrow into a recipient. The mice receiving these activated natural killer cells had a more rapid haemopoietic engraftment and B-cell recovery. In addition, no GVHD was experienced. The authors suggested that natural killer cells could be useful in promoting engraftment and a more rapid immune recovery (Murphy et al, 1992). Furthermore, natural killer cells form part of the innate immune system and therefore these cells could be important in the defence of the body during the early post transplant period.

B-cells make up between five and fifteen percent of the normal lymphocyte population. They develop in the bone marrow and when activated produce immunoglobulin. In addition, they play a major role in the elimination of bacterial infections.

B-cells, as measured by the antigen CD19, recovered steadily achieving normal levels by twelve months (Figure 10). This observation is in agreement with other studies (Small et al, 1990; Keever et al, 1989, Leitenberg et al, 1994). Some investigators have however documented an improved B-cell recovery in autologous patients as compared to patients receiving allogeneic grafts (Roberts et al, 1993).

Analysing immunoglobulin production, post transplantation, revealed that serum IgM and IgG returned to normal by one month post infusion (Figure 11 and 12). However, IgA remained below the median of the normal controls through out the twelve-month study period (Figure13). This pattern of functional development has been previously documented and is similar to that seen in early B-cell ontogeny (Lum, 1987; Verma and Mazumder, 1993; Watson, 1990). Other investigators have documented the recovery of the different subtypes of IgG and have revealed deficiencies in both IgG2 and IgG4 up to eighteen months post infusion (Velardi et al, 1988). Since our study did not examine the various immunoglobulin subclasses, although the total IgG levels achieved normality, a subclass deficiency cannot be ruled out.

Although the allogeneic PBSCT's had a larger number of lymphocytes infused than that following bone marrow transplantation (Table 3) an improvement in B-cell recovery was not noted. CD19+ B-cells from all three transplant groups recovered at the same rate. This is in contrast with other reports, which have documented improved immune recovery post peripheral blood stem cell transplantation, particularly within the B-cell population (Roberts et al, 1993; Ottinger et al, 1996; Ashihara et al, 1994).

A possible explanation for this discrepancy is the fact that our allogeneic peripheral stem cell harvests were depleted of T-cells using CAMPATH while previous reports have described the recovery of patients receiving unmanipulated grafts. The CAMPATH group of antibodies binds to the CD52 antigen and has been shown to kill and deplete the majority of both T and B-cells (Hale et al, 1983).

T-cells are mononuclear leukocytes which form approximately sixty to eighty percent of the normal lymphocyte population. T-cell precursors are produced in the bone marrow and migrate to the thymus where development takes place.

The mature T-cell population can further be divided into two distinct sub-populations. The first subset, CD4+ helper T-cells, make up approximately two thirds of the total T-cell population and recognise antigen in association with MHC class II molecules. The second subset, CD8+ cytotoxic T-cells, can only recognise antigen presented in association with MHC class I. The normal ratio of CD4+ T-cells to CD8 is between 1.61 and 2.61.

Both allogeneic groups of patients recovered their T-cell populations slowly, reaching normal adult values at twelve months post transplantation (Figure 5 and 6).

The CD4+ helper T-cell subset was the most affected and remained abnormal throughout the study period (Figure 7). In contrast, the CD8+ cytotoxic T-cells rapidly achieved normal levels (Figure 8). These results gave rise to an extremely low CD4:CD8 ratio. This distinct pattern of recovery confirms numerous other publications (Lum, 1987; Lowdell et al, 1998; Roberts et al, 1993; Verma and Mazumder, 1993; Keever et al, 1989).

However, reports documenting the effects of T-cell depletion on the rate of T-cell recovery have been conflicting (Keever et al, 1989; Lowdell et al, 1998). This is probably due to the differing methodologies such as lectin soy - bean agglutination, sheep rosetting and CAMPATH antibodies used for this purpose. Our results confirm reports by Lowdell et al who studied immune reconstitution in a series of bone marrow patients. This group of transplants was depleted of T-cells using the CAMPATH group of antibodies (Lowdell et al, 1998).

Many reports in the literature document an improved immune recovery in patients receiving unfractionated allogeneic PBSC grafts (Roberts et al, 1993; Ottinger et al, 1996). In contrast, our investigation has shown that those patients receiving allogeneic PBSCT recovered their T-cell populations at the same rate as the traditional bone marrow transplants (Figure 5 and 6). The

main reason for this could be the intense T-cell depletion of the CAMPATH antibodies which have removed the majority of the lymphocytes from the graft prior to infusion.

The CD4+ helper T-cell population was slow to recover and did not achieve normal values even at eighteen months post infusion. Abnormalities in the development of T-cells and in particular CD4+ helper T-cells were further dissected by determining the cell surface expression of CD45RA (naïve phenotype) and CD45RO (memory phenotype). The results of this analysis revealed that the majority of the CD4+ T-cell population were of the memory phenotype (Figure 23).

This pattern of recovery is in contrast to that seen in the early neonate, in which the majority of the developing T-cells are of the naïve phenotype, expressing CD45RA (Kotylo et al, 1990; Landesberg et al, 1988). The main reason for this discrepancy is the fact that in early life the thymus is fully functional and therefore has the ability to produce immature, naïve T-cells. However, the post transplantation patient, possesses a dysfunctional thymus due to age and the preconditioning regime, and therefore the regeneration of new T-cells is dependant on extrathymic pathways. Studies have shown that in patients lacking thymic activity the regeneration of the CD4+ T-cell numbers are as an oligoclonal expansion of the residual memory T-cells (Mackall et al, 1993; Storek et al, 1995; Kennedy et al, 1992). The intense T-cell depletion by the CAMPATH antibodies further disrupts the process of recovery by removing the majority of residual progenitors and memory cells from the graft prior to infusion. The importance of the thymus in the regeneration of CD4+ helper T-cells is further illustrated by the fact that elderly transplant patients take far longer to recover this population of T-cells than younger individuals (Storek et al, 1995).

The median age of the patient population, studied in this project, was thirty two

with a range of between six and sixty six years. Of interest is that fact that age did not feature as a factor influencing the rate of immune recovery. (Correlation [gamma] analysis; p=NS)

A study undertaken in 1997 further demonstrated that in mice, not processing a functional thymus and who received T-cell depleted grafts, T-cell reconstitution occurred via extrathymic pathways. Possible sites include both the gut and the liver. Extrathymic development differs from thymic differentiation in that the cells undergo positive selection but not negative selection. Therefore, extra thymic T-cells could contain potentially auto reactive T-cells. The investigators of this study concluded that the presence or absence of a thymus and the composition of the graft (i.e. T-cell depleted or not T-cell depleted) determined the development pathway of the recovering T-lymphocytes. (Dulude et al, 1997)

In comparison to the CD4+ subset, CD8+ T-cells recovered extremely quickly resulting in a decreased CD4:CD8 ratio which persisted throughout the eighteen months (Figure 8 and 9).

CD8+ T-cell regeneration is not age dependant and therefore the proliferation of new CD8+ T-cells probably occurs extrathymically (Storek et al, 1995). Studies on nude mice have shown that CD8 reconstitution can occur in a number of sites other than the thymus. These include both the spleen and the lymph node. In lymph nodes the majority of the T-cells are mature and it is therefore suggested that antigen stimulation plays an important role in the regeneration of T-cells within this site. This process is particularly relevant to CD8+ T-cells, which are extremely efficient in expanding via this pathway. In addition, the initial antigen encounters, post bone marrow transplantation, are bacterial and are presented via MHC class I molecules which further stimulate CD8 proliferation (Kennedy et al, 1992, Mackall et al, 1997).

In comparison to the allogeneic transplant patients, pan T-cell recovery, within

the autologous group of patients was rapid and normal adult values were reached by one-month post infusion.

CD4+ helper T-cells, however, were slow to reach the normal range, and low normal levels were attained only at twelve months post transplantation. The slow normalisation of the CD4+ subset is probably because of a dysfunctional thymus due to ageing and the pre-conditioning regimen. In addition, the percentage of CD4+ T-cells expressing CD45RO (memory phenotype) was increased. Unlike the allogeneic group, however, the autologous grafts did not undergo T-cell depletion and this could account for the increased rate of normalisation.

The CD8+ subset recovered rapidly and in many cases values exceeded the normal range (Figure 8). This resulted in decreased CD4:CD8 ratios which persisted throughout the study period. The abnormally elevated CD8 values seen in this group of patients, could be explained by a study which compared the effects of G-CSF and GM-CSF on immune reconstitution in autologous transplant patients. The results were interesting, as they concluded that G-CSF contributed to a significantly faster recovery of the CD8+ population. They also confirmed that the regeneration of the CD8+ subset was via antigen stimulation (San Miguel et al, 1996).

Finally, a technical reason for the increased number of CD8+ T-cells, seen in all types of transplants, could be the inclusion of natural killer cells within the flow cytometric gate. Natural killer cells express the antigen CD8 (Roitt, 1994). Therefore, because natural killer cells are increased post transplantation, the inclusion of these cells within the gate could falsely contribute to the increased CD8 count. Our study eliminated this possibility as only cells expressing both CD3, a marker specific for T-cells, and CD8 were included.

Mackall et al, examined the rate of lymphoid recovery in a group of sixteen

patients who had received intense chemotherapy. Her results showed that natural killer cells, B-cells and CD8+ cells, rapidly achieved normal values during the post therapeutic period. However, the CD4+ T-cell subset took longer to recover and returned to normal only at twelve months (Mackall et al, 1997). The pattern and rate of lymphoid recovery described in this group of patients is similar to that seen in our autologous transplants. This suggests that lymphoid reconstitution, post autologous transplantation could parallel that which is observed post intensive chemotherapy.

B-cells, natural killer cells and T-cells all have different developmental pathways, and therefore differences in the rates of recovery is not unexpected. The pattern and rate of B-cell and natural killer cell recovery was similar in both the allogeneic and autologous groups of patients. However, although the pattern of recovery was similar, a distinct difference in the rate of T-cell reconstitution was observed between the two groups. Possible explanations for this difference could be the fact that autologous transplant patients do not develop GVHD and, in addition, T-cell depletion of the graft does not take place.

The reasons for the prolonged immune deficiency seen in the post transplant patient are multi-factorial. An increase in the rate of apoptosis has been proposed as a possible mechanism (Hebib et al, 1999; Brugnoni et al,1999).

Excessive apoptosis has been implicated in many diseases such as aplastic anaemia, HIV and degenerative neurological diseases. T-cells from patients with HIV are constantly exposed to foreign antigen, and instead of dividing and proliferating, many of them undergo activation induced cell death which is mediated by the antigen CD95. This process contributes to the decreasing CD4+ count seen in the terminal stages of the disease (Greene, 1993).

Recovery of T-cells, and in particular the CD4+ subset, is slow after bone marrow transplantation. In addition, like patients with HIV, the repopulating T-

cells are exposed to a variety of antigen and therefore undergo repeated activation. The question therefore arises: Does activation induced cell death play a role in prolonging the immunodeficiency seen in the post transplant patient?

In an attempt to evaluate this theory, a subset of both allogeneic and autologous patients was examined for CD95 expression. Our results have shown that within both the total CD3+ T-cell population and the CD4+ helper subset there was an increase in the percentage of cells expressing the FAS/CD95 antigen (Figure 25 and 27).

In addition to the percentage of cells expressing the antigen, the number of receptors on the cell surface were also analysed by recording the mean fluorescent intensity of expression and comparing this to normal controls (Figure 26 and 28). The mean fluorescent intensity of the patient groups was not significantly different to that of the control population.

It is therefore postulated that the elevated CD95 positivity is due to an increase in the number or percentage of T-cells expressing the antigen rather than an upregulation or increase of receptors on the cell surface. Our study of FAS/CD95 is in agreement with that of Brugnoni et al and suggests a role for CD95 induced apoptosis in the prolonged immunodeficient state that exists post bone marrow transplantation.

These results also confirm those of Hebib et al. The results of this investigation demonstrated that the proliferation of the memory (CD45RO+) T-cell population was accompanied by an increase in cells expressing CD95. This was also associated with a decrease in the expression of Bcl-2, an inhibitor of apoptosis. As the number of naïve cells (CD45RA+) returned to normal the apoptotic phenotype disappeared (Hebib et al, 1999).

In addition to a possible role in prolonging the immunodeficient state, FAS mediated apoptosis has been implicated in the pathogenesis of GVHD. Experiments analysing the expression of both FAS and FAS ligand on the donor T-cells of mice, with acute GVHD, revealed that both molecules are significantly upregulated. Furthermore, the increased expression was associated with the activation of donor CD8+ T-cells and IFN-γ production. In contrast, the expression of FAS and FAS ligand was decreased in chronic GVHD, leading to the impaired elimination of auto reactive B-cells and consequently persistent auto-antibody production (Shustov et al, 1998).

Further studies have analysed the expression of FAS in the recipient and it's role in the development of GVHD. This was achieved by comparing the outcome of the disease in FAS deficient B6-*lpr* mice with a control group of B6 mice. The results of these experiments revealed that, although the B6-*lpr* mice had very little hepatic GVHD, all other specific GVHD organs such as the skin, intestines and thymus were severely affected. In addition, the FAS deficient mice had a higher morbidity and mortality compared with the control group. It was concluded that FAS expression in the recipient is required for GVHD of the liver, but not for the development of GVHD in other organs (Van den Brink et al, 2000).

The above evidence suggests that FAS mediated apoptosis could play a significant role in both the immunodeficient state post transplantation and also in the development of GVHD. Therefore, therapies which prevent apoptotic death may need to be investigated.

Experiments with the antagonistic M3 anti-FAS monoclonal antibody however, failed to prevent the increased spontaneous death of the T-cells in vitro. However, it was postulated that the post transplant T-cells are probably the targets of FAS-ligand expressing cells (Hebib et al, 1999).

Other possible therapies include the up-regulation of the anti-apoptotic protein Bcl-2 or the provision of signals which rescue the cell from apoptotic death. Cytokines such as interferon-β and IL-2 have both been associated with the rescue of T-cells from apoptosis. Cytokine therapy could therefore play a vital role in preventing apoptotic death during the post transplant period (Pilling et al, 1999; Adachi et al, 1996). However, further study using lymphocytes from bone marrow transplant patients is needed.

T-cell depletion has been shown to prevent effectively the onset of GVHD, however it is also associated with prolonged immune recovery and relapse of the original malignancy. This is particularly evident in patients with chronic myeloid leukaemia (Porter et al, 1998). In our group of T-cell depleted transplants, seven (36.8 %) of the allogeneic bone marrow and nine (28.1%) of the PBSCT recipients had relapsed at the time of this study. Nine of these individuals had chronic myeloid leukaemia.

The challenge therefore remains to prevent GVHD yet preserve the GVL effect. Donor lymphocyte infusions or adoptive immunotherapy post transplantation have been attempted to treat and prevent the relapse of CML. These have produced encouraging results (Barret and Van Rhee, 1997).

Adoptive immunotherapy has also been used, with some success, in the treatment of other tumours. Lymphoproliferative disorders can develop post solid organ transplants and also post bone marrow transplantation, particularly those depleted of T-cells. These lymphoproliferative disorders are often associated with the Epstein-Barr-virus. Initial studies examining the role of donor lymphocyte infusions in these tumours have also been successful (Haque et al, 1999).

The potential therapeutic use of donor lymphocyte infusions has again been highlighted in a case report. S.Cesaro et al reported a case of therapy - related

secondary acute myeloid leukaemia treated with bone marrow transplantation. Three months after transplantation the patient relapsed and was treated with donor lymphocyte infusions and the chemotherapeutic drug etoposide. A complete remission was achieved with no further treatment being required. However, the patient remained in remission for only seven months and then relapsed with the original malignancy (Cesaro et al ,1999).

The above evidence clearly suggests that the infusion of donor lymphocytes has a distinct role to play in the treatment of relapsed malignancy post transplantation. However, could this approach be used to assist and improve immune recovery?

In patients suffering from clinical immunosuppression following T-cell depletion, the infusion of donor lymphocytes, post transplantation, could serve to replace the lost memory T-cells. Small et al who studied the response to donor lymphocyte infusions in a series of twelve patients investigated this approach. In addition, antigen specific T-cells were generated in vitro against defined targets prior to infusion. The results of this investigation were encouraging as a significant improvement in lymphoid recovery was observed (Small et al, 1999). This research paves the way for further studies in this area.

The risk of initiating GVHD, with the infusion of donor lymphocytes, has led to the development of adoptive immunotherapy using leukaemic specific T-cells. Research in this area has focused on the dendritic cell. Dendritic cells are professional antigen presenting cells and in chronic myeloid leukaemia have been shown to express a protein associated with the BCR/ABL gene product. These cells can be generated successfully *in vitro*, from mononuclear cells obtained from patients with chronic myeloid leukaemia by culturing them in medium containing GM-CSF, IL-4 and TNF-α. The cultured dendritic cells can then, by presenting leukaemia specific antigen, stimulate donor and autologous T-cells to mount a cytotoxic response specific for Philadelphia Chromosome

positive CML cells (Choudhury et al,1997 ; Eibl et al,1997). The results of this research have been encouraging and could serve to improve further the outcome of chronic myeloid leukaemia post transplantation.

This project and others have highlighted the importance of the thymus in immune reconstitution post bone marrow transplantation. Attempts at using thymic tissue transplants have not been successful and therefore attention has focused on the use of cytokines as another possible solution.

Inappropriate cytokine production is not the only cause of the immunodeficiency post transplantation, however, research on the use of IL-7 in transplanted mice have shown that treatment with this cytokine could induce a more rapid recovery of the T-cell population. The result of these experiments warrants further investigation in a clinical setting (Bolitin et al, 1996).

7.0 CONCLUSIONS

In conclusion therefore, this project has shown that, although the CAMPATH group of antibodies are effective in preventing GVHD, the T-cell depletion process leads to a delay in immune recovery which is particularly relevant to the CD4+ helper T-cell subset. The resulting immunocompromised state may lead to a number of clinically relevant infections and disease relapse in the post transplant period. In addition, the increased lymphocyte count observed in the PBSC harvests did not serve to improve the rate of recovery as has been previously reported (Ottinger et al, 1996; Roberts et al 1993).

We have also investigated the role played by FAS/CD95 induced apoptosis in the resulting immunodeficiency and have found that CD95 is expressed on the majority of the recovering T-cells. This implies that FAS mediated apoptosis could also play a role in delaying their normalisation.

The causes of the prolonged immunodeficiency seen in the post transplant period are therefore multi-factorial and the solution probably lies in a combination of therapies. These could include: -

- The re-infusion of graded numbers of donor lymphocytes which could serve to replace the memory T-cells that were lost during the T-cell depletion process.
- The treatment with specific cytokines, such as IL-7, which stimulate thymopoiesis.
- Therapies which interfere and prevent apoptosis, such as anti-FAS antibodies and specific cytokines.

Finally, future research also needs to focus on further defining extra thymic pathways of T-cell development, as these sites could have particular relevance to patients receiving T-cell depleted allogeneic grafts.

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